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EK-6136 (3-methyl-4-(*O*-methyl-oximino)-1-phenylpyrazolin-5-one): A novel Cdc25B inhibitor with antiproliferative activity

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Abstract

Cdc25B is a dual specific phosphatase, which plays a pivotal role in the activation of cell-cycle-dependent kinase 1 (Cdk1). A novel Cdc25B inhibitor, EK-6136, was identified by high throughput screening (HTS) using compounds from Korea Chemical Bank and examined for its biological effects. EK-6136 inhibited Cdc25B with an IC_{50} of 6.4±1.5 μM. EK-6136 showed selectivity against several phosphatases including PTP-1B, CD45, Cdc25A, PP1, VHR and Yop. In the inhibition kinetic study, EK-6136 displayed a mixed inhibition pattern with a K_i value of 7.8±1.2 μM. Consistent with in vitro results, EK-6136 inhibited the proliferation of MCF-7 (human breast carcinoma), HT-29 (human colorectal adenocarcinoma) and A549 (lung carcinoma) cells with increased Cdk-1 phosphorylation. Herein, we propose that EK-6136 is an active HTS hit as a Cdc25B inhibitor with antiproliferative activity, and can be used for the design of more potent and selective antiproliferative agents.

Keywords: Cdc25B; HTS (high throughput screening); Selectivity; MCF-7; Antiproliferation; Cdk1 (cell-cycle-dependent kinase 1)

1. Introduction

The mammalian cell cycle is controlled by the activities of various cyclin-dependent kinase (Cdk)/cyclin complexes. Of Cdks, Cdk1 is known to be involved in G2–M transition, and the activity of Cdk1 can be regulated by association with cyclin B and by Cdc25B phosphatase. Cdc25B removes inhibitory phosphate group from Thr 14 and Tyr 15 residues of Cdk1, leading to activation of Cdk1 and allowing cell cycle entry into mitosis (Malumbres and Barbacid, 2001).

In human cells, three Cdc25 phosphatases have been found: Cdc25A, Cdc25B and Cdc25C. Three phosphatases share approximately 40% to 50% amino acid identity and are differentially expressed in cell division cycle (Eckstein, 2000). Accumulating evidence suggests that inappropriate regulation of Cdc25A and Cdc25B may be involved in a number of human cancers. First, Cdc25A and Cdc25B are transcriptional targets of *c-myc* oncogene, and have oncogenic properties in cooperation with either Ha-*ras* or *Rb*1 (Galaktionov et al., 1996). Secondly, the overexpression of Cdc25A and Cdc25B has been observed in some cancer tissues and cell lines (Gasparotto et al., 1997; Hernandez et al., 1998; Wu et al., 1998). Thirdly, the transgenic mice with Cdc25B overexpression produced mammary gland hyperplasia, suggesting that overexpression of Cdc25B is related with the initiation of mammary tumorigenesis (Ma et al., 1999).

Based upon these observations, Cdc25B phosphatase inhibitors should prove to be useful for certain types of cancer chemotherapy. Using Cdc25B antisense oligonucleotides, it was shown that Cdc25B inhibition has persistent antiproliferative effects in asynchronous HeLa cells (Garner-Hamrick and Fisher, 1998). Although a few compounds have been introduced as Cdc25B inhibitors including dnacins, tetrahydroisoquinolines, nocardiones, vitamin K analogues and tetronic acid derivatives, they are relatively nonselective and weak inhibitors with IC_{50} values of 10–100 μM (Ham et al., 1998; Otani et al., 2000; Tamura et al., 2000; Sodeoka et al., 2001). Recently, more potent quinone derivatives have been identified as Cdc25 inhibitors (Pu et al., 2002; Sohn et al., 2003), while a few natural product derivatives such as cholesterol derivative, HP-19 and modified dysidiolide have been reported to more effectively

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inhibit Cdc25A, and to cause inhibition of cancer cell proliferation (Peng et al., 2001; Takahashi et al., 2000).

With the aim of discovering novel Cdc25B inhibitors, we carried out HTS for Cdc25B using compounds from Korea Chemical Bank and identified EK-6136 as a novel Cdc25B inhibitor. EK-6136 exhibited moderate selectivity against several phosphatases and marked antiproliferative activity in various cancer cell lines.

2. Materials and methods

2.1. Materials

All chemicals were purchased from commercial suppliers: Tris, HEPES, dimethylsulfoxide (DMSO), dithiothreitol (DTT), EDTA, NaCl, $MnCl_2$, KCl, $CaCl_2$, $MgSO_4$, Na_2HPO_4, bovine serum albumin (BSA), $NaHCO_3$ and protein phosphatase 1 (PP1) (Sigma-Aldrich Co.); isopropyl β-D-thiogalactoside (IPTG) and protein phosphatase 2 (PP2A) (Promega Co.); fluorescein diphosphate (FDP) (Molecular Probe, Inc.); phosphate-buffered saline (PBS), DMEM, MEM and fetal bovine serum (Gibco BRL Inc.); leukocyte antigen-related phosphatase (LAR), vaccinia H1-related protein tyrosine phosphatase (VHR) and Yersinia outer protein tyrosine phosphatase (Yop) (Biomol, Inc.). The catalytic domains of cluster of differentiation 45 phosphatase (CD45; residues 641–1268), Cdc25A (residues 336–523) and protein tyrosine phosphatase-1B (PTP-1B; residues 1–322) were expressed in *Escherichia coli* and purified by glutathione sepharose column (CD45, Cdc25A) or by Ni-chelated column chromatography (PTP-1B).

2.2. Cdc25B HTS and enzyme assay

The catalytic domain corresponding to amino acid 378–566 was inserted in pGEX-4T vector and expressed as a glutathione-*S*-transferase (GST) fusion protein in *E. coli* BL21. The GST–Cdc25B fusion protein was purified by glutathione sepharose column as previously described (Gottlin et al., 1996). Cdc25B HTS was done by using compounds from Korea Chemical Bank. Briefly, enzyme assay was carried out in a final volume of 200 μl on 96-well plates. To each well were added 20 μM FDP and 0.2 μg of Cdc25B diluted in 30 mM Tris buffer (pH 8.5) containing 75 mM NaCl, 0.67 mM EDTA and 1 mM DTT with or without compounds (5% DMSO). Following incubation at room temperature for 1 h, fluorescence released by enzyme catalysis was measured at 485 nm (excitation) and 538 nm (emission) using fluorometer (HT Synergy, BioTek).

2.3. PTP isozyme assay of EK-6136

For CD45 enzyme assay, the enzyme (0.05 μg/well) was incubated in a buffer containing 30 mM Tris–HCl (pH 7.0), 75 mM NaCl, 0.67 mM EDTA, 1 mM DTT and 20 μM FDP with or without the inhibitor. For PTP-1B, the enzyme (0.1 μg/well) was incubated in a buffer containing 30 mM Tris (pH 8.0), 75 mM NaCl, 0.67 mM EDTA, 1 mM DTT and 20 μM FDP. For PP1, the enzyme (0.2 μg/well) was incubated in a buffer containing 25 mM Tris–HCl (pH 7.5), 5 mM DTT, 0.5 mM $MnCl_2$ and 35 μM FDP. For Cdc25A, the enzyme (1.0 μg/well) was incubated in a buffer containing 30 mM Tris–HCl (pH 8.5), 75 mM NaCl, 0.67 mM EDTA, 1 mM DTT and 20 μM FDP. For Yop, the enzyme (5 ng/well) was incubated in a buffer containing 50 mM sodium citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, 1 mM DTT and 20 μM FDP. For VHR, the enzyme (0.1 μg/well) was incubated in a buffer containing 50 mM HEPES (pH 7.2), 1 mM EDTA, 1 mM DTT, 0.05% NP-40 and 20 μM FDP. Incubation was continued for 30 min (CD45 and PP1) or 60 min (Cdc25A, PTP-1B, Yop and VHR) at room temperature, and the fluorescence released from FDP was measured at 485 nm (excitation) and 538 nm (emission).

2.4. Inhibition kinetics

Initial rates at five different inhibitor concentrations (0, 2, 5, 10 and 20 μM) were measured. For each inhibitor concentration, measurements were performed at four different concentrations of FDP (2, 5, 10 and 20 μM) in a buffer containing 50 mM Tris (pH 7.5), 125 mM NaCl and 1 mM DTT. The inhibition pattern was evaluated and K_i was determined using a direct curve-fitting program, Prism 3.0 (Graphpad Software, Inc.).

2.5. Antiproliferative effect of EK-6136

MCF-7, HT-29 and A549 cancer cells were used to determine the antiproliferative activity of EK-6136. MCF-7 cells were plated in 96-well microtiter plates at a density of 2000 cells/well in RPMI-1640 culture medium containing 10% fetal bovine serum and 10 μg/ml insulin. HT-29 and A549 cell lines were cultured in DMEM containing 10% fetal bovine serum and plated in 96-well microtiter plates at 2000 cells/well. After 24 h incubation, 100 μl of media containing different concentrations of EK-6136 was added. Cell numbers were determined by 0.4% sulforhodamine in 1% acetic acid after 48 h incubation at 37 °C as previously described (Glaab et al., 2001).

2.6. Western blot analysis

MCF-7 cells (2×10^5 cells/well) were treated with EK-6136 (10 μM) in 6-well plates for 4 h. The cells were washed, scraped and lysed in lysis buffer. Supernatants were obtained after centrifugation at 14,000×*g* for 10 min. Protein concentration of supernatants was determined using Bio-Rad DC protein assay kit. After sodium dodecyl sulfate (SDS)-polyarylamide gel electrophoresis (PAGE; 50 μg protein in each well) and transfer to nitrocellulose membrane, Western blotting was performed with anti phospho-Cdk1 (tyrosine 15) antibody (Sigma). Protein bands were detected with enhanced chemiluminescence (ECL) plus (Amersham) and images were digitalized by using Doc-It system (UVP). The density of graph was calculated by using Prism 3.0 software (Graphpad Software, Inc.).

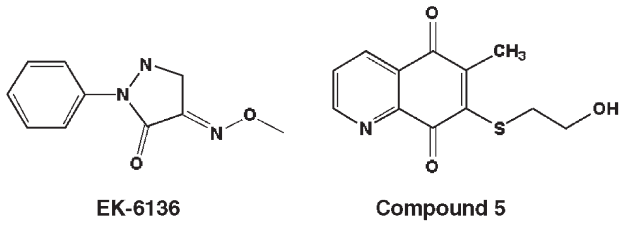

Fig. 1. Chemical structures of EK-6136 and compound 5.

2.7. Cell cycle analysis

MCF-7 cells (2×10^5 cells/well) were incubated in complete medium with EK-6136 (10 μM) in 6-well plates for 12 h. The cells were fixed in 90% ethanol, and stained with propidium iodide (30 μg/ml) in PBS containing 1% BSA and RNase A (0.25 mg/ ml). Data were collected on Cytomics F500 (Beckman) and analyzed using Cytomics RXP analysis software (Beckman).

2.8. Statistical analysis

Data are represented as means±S.E.M. of three separate experiments performed in triplicates. The significance of difference from the respective control for each experiment was assessed by ANOVA model equation in Prism 3.0 software, and $p<0.05$ was considered as statistically significant.

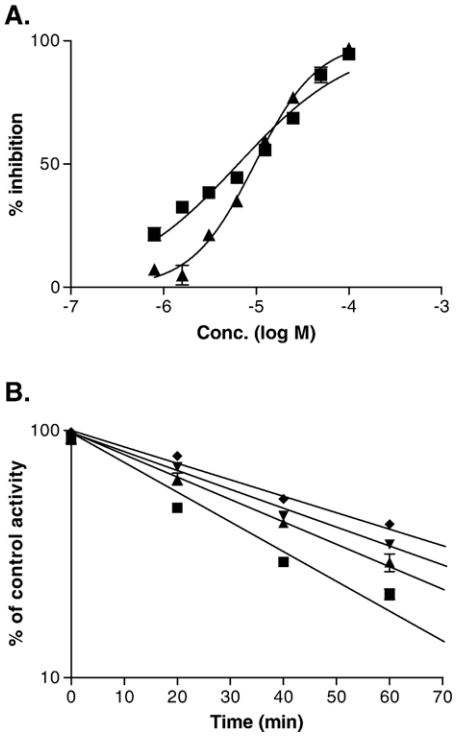

Fig. 2. Inhibition of recombinant human Cdc25B by EK-6136. (A) Human recombinant Cdc25B was incubated with 0–20 μM of the compound for 60 min and IC_{50} was determined by Prism 3.0 (Graphpad Software, Inc.). The filled squares (■) and triangles (▲) indicate EK-6136 and compound 5, respectively. (B) Cdc25B was incubated with EK-6136 with the indicated times and the remaining activity was determined as described in Materials and methods. Symbols: (■) 5 μM, (▲) 10 μM, (▼) 20 μM, (♦) 40 μM. The data shown are the means±S.E.M. of three independent experiments done in triplicates.

3. Results

3.1. Concentration- and time-dependent inhibition of Cdc25B by EK-6136

To discover novel scaffolds for Cdc25B inhibitors, a chemical library of approximately 40,000 compounds (Korea Chemical Bank) was screened using HTS techniques. Of compounds tested, 104 compounds inhibited the enzyme more than 80% at 25 μM: the most active had an IC_{50} value of 1.55 μM. Cdc25B hit rate was 0.26%. Among five novel scaffolds discovered, a pyrazolone derivative, EK-6136, was chosen for further evaluation, based on the patentability and the suitability for analogue synthesis. The structure of EK-6136 was shown in Fig. 1.

As shown in Fig. 2A, EK-6136 inhibited Cdc25B in a concentration-dependent manner and its IC_{50} value was determined to be 6.4±1.5 μM. As a reference compound, a menadione derivative synthesized in our laboratory was used for comparative purpose. A menadione derivative (compound 5 in Fig. 1) was previously reported as a potent Cdc25B inhibitor with an IC_{50} value of 3.8 μM (Tamura et al., 2000), and it inhibited Cdc25B with an IC_{50} of 9.7±2.3 μM in our experimental condition. In addition, the inhibition of Cdc25B activity by EK-6136 displayed a time-dependent pattern as shown by Fig. 2B.

3.2. Selectivity of EK-6136 against phosphatases

To examine the selectivity profile of EK-6136 in vitro, we evaluated the effect of EK-6136 on various phosphatases. As shown in Table 1, the inhibitory potency of EK-6136 against several phosphatases was weak with IC_{50}s>50–100 μM. On the other hand, EK-6136 showed modest inhibitory activity against PTP-1B and Cdc25A, their IC_{50}s being four-fold and seven-fold less potent than that of Cdc25B, respectively.

3.3. Inhibition kinetics of EK-6136

Because HTS hits can inhibit the enzyme via nonspecific mechanisms, we tested the inhibition kinetics of EK-6136 with FDP as a substrate to determine whether the compound inhibits the enzyme by interacting at the active site of the enzyme. EK-

Table 1
Isozyme selectivity of EK-6136

Isozyme	IC_{50} (μM)
Cdc25B	6.4±1.5
Cdc25A	49±2.5
PTP-1B	28.4±2.7
CD45	>100
VHR	>100
YOP	>100
PP1	>50

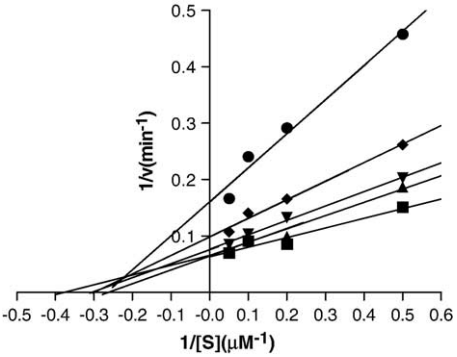

Fig. 3. Kinetic analysis of Cdc25B inhibition by EK-6136. Initial rates of Cdc25B inhibition by EK-6136 were determined with different inhibitor concentrations in the presence of different concentrations of FDP as described in Material and methods. Symbols: (■) 0 μM, (▲) 1 μM, (▼) 5 μM, (♦) 10 μM, (●) 20 μM of EK-6136. Results are expressed as a Lineweaver-Burk plot and K_i was determined by using Prism 3.0 (Graphpad Software, Inc.).

6136 inhibited Cdc25B in a mixed inhibition pattern under our experimental condition (Fig. 3). K_i value was estimated to be 7.8±1.2 μM.

3.4. Antiproliferative activity against human cancer cell lines

The cell growth and viability were measured by using sulforhodamine method, which detects only living cells by binding to basic amino acids of proteins in the cells (Glaab et al., 2001). Preliminary experiments showed that MCF-7, HT-29 and A549 cells expressed considerable amounts of Cdc25B, as determined by Western blot (results not shown).

EK-6136 inhibited the proliferation of MCF-7 cells to 60% of the control at 10 μM. When MCF-7 cells were treated with various concentrations of EK-6136, cell proliferation was suppressed in a concentration-dependent manner with an EC_{50} of 7.2±1.0 μM (Fig. 4). In addition, EK-6136 showed growth inhibitory EC_{50} values of 8.4±1.0 μM and 7.7±1.1 μM in HT-29 and A549 cells, respectively (Fig. 4). On the other hand, EK-6136 did not show any cytotoxicity in normal cell lines (Chang liver cells and WI-38 fibroblasts) up to 40 μM (results not shown), at which it exhibited complete inhibition of cancer cell proliferation.

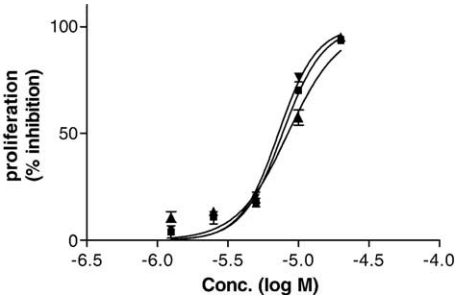

Fig. 4. Antiproliferative activity of EK-6136 in three types of cancer cell lines. Cell proliferation was determined by sulforhodamine after 48 h treatment with various concentrations of EK-6136 (1.25, 2.5, 5, 10 and 20 μM). Symbols: (■) A549, (▲) HT-29, (▼) MCF-7. The data shown are the means±S.E.M. of three independent experiments done in triplicates.

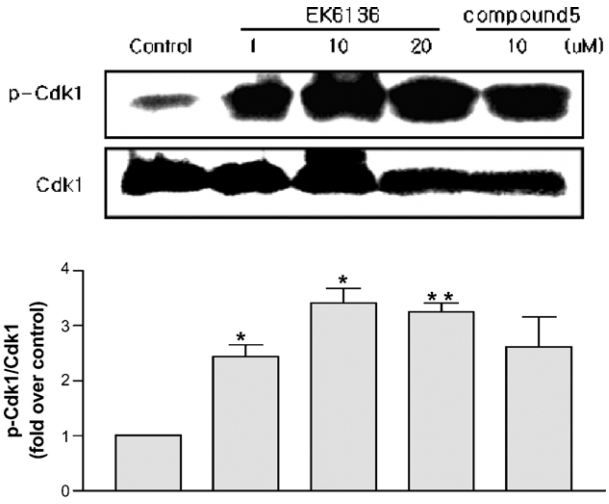

Fig. 5. Increased phosphorylation of Cdk1 by EK-6136. MCF-7 cells were treated with different concentrations of EK-6136 for 4 h. Then, the cells were lysed and processed for Western blot analysis using antibody against tyrosine 15 phosphorylated Cdk1 and actin. The densitometric analysis was carried out, and the data shown are the means±S.E.M. of three independent experiments done in triplicates. *P<0.05 vs. control, **P<0.01 vs. control.

Compounds 5 (10 μM) inhibited the proliferation of cancer cell lines with similar potency to EK-6136 (approximately 50% inhibition), but showed cytotoxicities at 20 μM in normal cell lines (Chang liver cells, 53% inhibition; WI-38 fibroblasts, 35% inhibition).

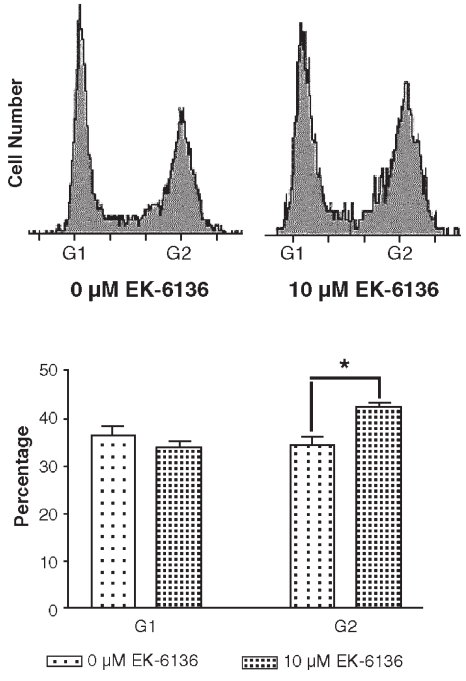

Fig. 6. Effect of EK-6136 on cell cycle progress. Exponentially growing MCF-7 cells were treated with 10 μM of EK-6136 for 12 h. The cells were harvested and subjected to flow cytometry analysis after propidium iodide staining. Data were collected on Cytomics 500 and analyzed using Cytomics analysis software. The data shown are the means±S.E.M. of three independent experiments done in triplicates. *P<0.05 vs. control (no EK-6136).

3.5. EK-6136 increases the phosphorylation of Cdk1

To verify that antiproliferative effect of EK-6136 is mediated by Cdc25B inhibition in MCF-7 cells, we investigated whether EK-6136 modulates tyrosine phosphorylation status of the Cdk1, a natural substrate of Cdc25B. MCF-7 cells (2×10^5 cells) were treated with EK-6136 (1, 10 and 20 μM) for 4 h at 37 °C. The cells were lysed and Western blot was carried out by using antibody specific for the tyrosine 15-phosphorylated form of the Cdk1. As shown in Fig. 5, EK-6136 induced the accumulation of the tyrosine phosphorylation form of Cdk1 in a concentration-dependent manner. The phosphorylation of Cdk1 was saturated at 10 μM of EK-6136, consistent with the inhibitory activity of the compound on cancer cell proliferation. These results suggest that EK-6136 effectively inhibits the activity of Cdc25B, with increased phosphorylation of Cdk1 in MCF-7 cells, thus leading to the suppression of cell proliferation.

3.6. EK-6136 inhibits cell cycle progression

To investigate the effect of EK-6136 on the cell cycle pattern of MCF-7 cells, the cells (2×10^5 cells) were treated with 10 μM EK-6136 for 12 h at 37 °C. Then cells were fixed and analyzed for cell cycle status by flow cytometry after propidium iodide staining. As shown in Fig. 6, EK-6136 (10 μM) treatment resulted in the accumulation of the cells on G2 phase (10% increase compared with DMSO-treated cells).

4. Discussion

The cell division cycle is constituted of two major phases, the S phase and the M phase, separated by G1 and G2 phases. A major mechanism controlling the cell division cycle is known to be protein phosphorylation at serine, threonine and tyrosine residues (Malumbres and Barbacid, 2001). While protein kinases especially cyclin-dependent kinases (Cdks) play essential roles in cell cycle progress, dual specific phosphatases such as Cdc25 are also important in cell proliferation by regulating Cdk activities. Cdc25 removes inhibitory phosphates on threonine and tyrosine residues in Cdks, thus activating the kinases. These consequently result in the activation of G2/M transition of the cell cycle (Bulavin et al., 2002).

Consistent with the important function of Cdc25 in cell proliferation, several reports have shown the correlation between the level of Cdc25 mRNA or protein with tumor development. In head and neck squamous carcinomas, 55% of the cases showed overexpression of Cdc25B mRNA levels (Gasparotto et al., 1997). Similarly, Cdc25B was overexpressed in 56% of the non-Hodgkin's lymphomas, where c-myc oncogene was also overexpressed (Hernandez et al., 1998). In addition, overexpression of Cdc25B was detected in human breast cancers, gastric carcinomas and non-small cell lung carcinomas (Wu et al., 1998; Eckstein, 2000). Although Cdc25A is also oncogenic and overexpressed in some tumors, the enzyme exhibits more general mechanism of action than Cdc25B, interacting Cdk2/cyclin A, Cdc2/cyclin B and Cdk2/cyclin E (Bartek and Lukas, 2001). Therefore, Cdc25B appears to be a better therapeutic target for the development of anticancer agents.

A few reports have introduced Cdc25B inhibitors, but mostly they are originated from natural products, and show relatively weak activities against Cdc enzymes (Otani et al., 2000; Tamura et al., 2000). Recently, small molecule inhibitors of Cdc25 were reported, which were discovered by HTS from NCI diversity set. In the report, NSC663284, a quinolinedione derivative, with potent inhibition of Cdc25B (IC_{50} = 206 nM) did not distinguish Cdc25 subtypes although it showed marked selectivity against VHR and PTP-1B. Similar series of the compound arrested cells in G2 phase and decreased Cdk1 kinase activity (Lazo et al., 2001, 2002; Pu et al., 2002). On the other hand, indolyldihydroxyquinones exhibited differential activity against Cdc25A and Cdc25B, where 10-fold difference was observed using catalytic domains of the enzymes (Sohn et al., 2003). BN82002, a novel and nonselective inhibitor against Cdc25A, B and C, has shown the suppressive effects on growth rate of human tumor xenografts in athymic nude mice, reinforcing the interests of Cdc inhibitors as anticancer agents (Brezak et al., 2004).

In the present study, we discovered a novel structure, a pyrazolone derivative, as a Cdc25B inhibitor. Among several hits, EK-6136 was chosen for hit validation, and tested for biological activities such as inhibition kinetics, phosphatase selectivity and cell proliferation. EK-6136 inhibited Cdc25B with an IC_{50} of 6.4 ± 1.5 μM. In the inhibition kinetic study, EK-6136 displayed mixed inhibition pattern, suggesting that EK-6136 binds to both free enzyme and the enzyme–substrate complex or binds to the adjacent site to the active site such as anionic binding sites. The inhibition of Cdc25B by EK-6136 was reversed by washing out the inhibitor using Sephadex G-25 column (data not shown), suggesting that EK-6136 is less likely to induce covalent binding to the active site of Cdc25B.

The development of phosphatase inhibitors has been hampered partly due to the difficulty in conferring high selectivity against related phosphatases. EK-6136 inhibited Cdc25B activity with fair selectivity except PTP-1B, which was inhibited by the compound to a moderate extent. Since the crystal structure of Cdc25B differs from that of PTP-1B, it would be possible to confer further selectivity of the compound against PTP-1B (Reynolds et al., 1999). Furthermore, the additional binding site near the active site can be utilized for the design of compounds to enhance the affinity as well as the selectivity against other phosphatases.

In the cell proliferation assay, EK-6136 showed antiproliferative activity against MCF-7 (human breast cancer), A549 (human lung cancer) and HT-29 (human colon cancer) in a concentration-dependent manner. The inhibitory effect of EK-6136 on cell proliferation suggests that the compound would be able to penetrate cell membrane, and active as a Cdc25B inhibitor inside the cells. In addition, the antiproliferative activity of EK-6136 was associated with the increase in Cdk1 phosphorylation and G2–M arrest of the cell cycle, further supporting that suppressive effects of EK-6136 on cell proliferation are mediated by Cdc25B inhibition.

Taken together, these results provided the evidence that EK-6136 can be a lead compound of Cdc25B inhibitor for further

optimization to develop anticancer agents. Cocrystal structure between Cdc25B and EK-6136 as well as in vivo efficacy of the compound remains to be determined.

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