SGI-1776, an imidazo pyridazine compound, inhibits the proliferation of ovarian cancer cells by inactivating Pim-1

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ABSTRACT

Objective: To investigate the antitumor effect of SGI-1776 on human ovarian cancer HO-8910 cells and its molecular mechanism.

Methods: HO-8910 cells were cultured in vitro, and the proliferation inhibitory effects of SGI-1776 were determined by MTT assay and colony formation assay. The effect of SGI-1776 on the distribution of cell cycle phase was observed by flow cytometry with propidium iodide (PI) staining. The inhibition rate of migration and invasion were valued by transwell cell assay. Multiple molecular techniques, such as ELISA, Western blot, siRNA and cDNA transfection were used to explore the molecular mechanism.

Results: SGI-1776 presented dramatic anti-tumor activity against HO-8910 cells in vitro, inhibited the cells proliferation and colony formation, and attenuated the migration and invasion in a dose-dependent manner, accompanied by cell cycle arrest in G1 phase. SGI-1776 caused the proliferation inhibition with concomitant decrease in Pim-1 kinase activity, down-regulated the expression of Pim-1 protein and its downstream genes, such as CDK6, pCDK6, CDK4, pCDK4, CDK2 and pCDK2, and increased the expression of P21 and P27. Down-regulation expression of Pim-1 by siRNA followed SGI-1776 treatment resulted in enhanced cell proliferation inhibition rate and attenuated migration/invasion. Up-regulation of Pim-1 by cDNA transfection attenuated SGI-1776-induced cell proliferation inhibition and its migration/invasion.

Conclusion: Pim-1 mediates the biological effect of SGI-1776 in human ovarian cancer HO-8910 cells, suggesting Pim-1 might be a novel target for human ovarian cancer.

KEY WORDS

ovarian cancer; SGI-1776; proliferation; migration; invasion; Pim-1
Ovarian cancer is one of the most common causes of death from all cancers among women and the leading cause of death from malignancies [1]. This high mortality rate is associated with difficulties in diagnosis of the early stages of the disease and because of a high rate of recurrence. Although 80% of cancers initially respond to chemotherapy, the majority ultimately reoccurs with less than 15% remaining in remission [2]. Therefore, the need for new drugs for the prevention and treatment of ovarian cancer is urgent.

Ovarian cancer has been shown to have an activated Pim-1 signaling pathway, and it belongs to a family of evolutionarily conserved transcriptional regulators that are characterized by the presence of an ATP-binding pocket, an active site, a kinase domain, and lack regulatory domains making them constitutively active [3]. The expression of provirus integration site for Moloney murine leukemia virus kinase 1 (Pim-1), is mediated by the Janus-activated kinase/signal transducers and activators of transcription (STAT) signaling pathway [4]. Pim-1 regulates cell-cycle progression by directly phosphorylating cyclin-dependent kinase inhibitor (CDKI), including P21 and P27 and so on [3]. Studies by Mahalingam et al [4] have shown that SGI-1776 may inhibit Pim-1 activation in renal cancer cells. Accordingly, we hypothesized that SGI-1776 may target the inactivation of Pim-1, which could represent a promising strategy for ovarian cancer therapy.

In the present study, we investigated whether SGI-1776 inhibited the proliferation, migration and invasion of ovarian cancer HO-8910 cells and could be attributed to the inhibition of Pim-1 expression. We found that SGI-1776 decreased Pim-1 activity and downregulated the Pim-1 protein expression and its downstream genes, including CDK6, pCDK6, CDK4, pCDK4, CDK2 and pCDK2, resulted in the proliferation, migration and invasion inhibition in ovarian cancer HO-8910 cells. These results provided supportive evidence that Pim-1 is a legitimate target in ovarian cancer and that the targeted inactivation of Pim-1, SGI-1776 as shown here, would be highly relevant for designing novel strategies for the prevention of tumor progression and/or treatment of ovarian cancer.

1 Materials and methods

1.1 Cell culture and experimental reagents

The human ovarian cancer HO-8910 cells were purchased from China Centre for Type Culture Collection (CCTCC, Wuhan, China) and were maintained in DMEM medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% FBS (Boster Wuhan Biological Technology Ltd., Wuhan, China), 4 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, and incubated at 37 °C in a humidified atmosphere of 5% CO₂. SGI-1776 was kindly provided by Super-Gen Inc. (Dublin, CA, USA). Primary antibodies for Pim-1, CDK6, pCDK6,
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1.4 Flow cytometry (FCM) analysis using propidium iodide staining

Cells were seeded at a density of 5×10^6 cells/well in 100 mL culture flasks for 24 h and then treated with the medium containing various concentrations of the testing agents and 10% FBS for 24 h. Then the cells were harvested and washed by cold PBS twice, fixed in 70% ethanol at 4 °C and stained in propidium iodide (PI) in darkness for distribution of cell cycle phase was performed by flow cytometry as previously described [6].

1.5 Transwell cell migration and invasion assay

Cells were seeded at a density of 2.5×10^5 in the upper well of each transwell chamber. Conditioned culture medium and 300 µL DMEM containing 10% FBS were placed in the lower compartment of the chemotaxis chamber as a source of chemoattractants. The cells were incubated for 24 h at 37 °C with 5% CO₂. The cells that had migrated and invaded were fixed with methanol and stained with hexamethylpararosaniline (GenMed). Using light microscopy, at least 4 random fields were selected and the cells in each field were counted. Subsequently, the cells were eluted in 600 µL 33% acetic acid for 10 min and the optical density (OD) of the final cells through the matrigel was determined at 570 nm. HO-8910 cells proliferation in standard medium were set as the control group [10].

1.6 ELISA

Anti-Pim-1 protein was diluted with carbonate buffer (0.05 mol/L, pH=9.6) into 10 mg/L concentration, and 0.1 mL solution was added into each reacting hole of ploystyrene board, then incubated in 4 °C overnight, and then removed the solution and washed in PBS three times, at last blocked using 1% BSA/PBST at room temperature, and the Pim-1 activity of HO-8910 cells exposed to different concentration of SGI-1776 (2.5, 5 and 10 µmol/L) was detected using HTScan® Pim-1 kinase assay kit (Cell Signaling Technology) that peptide substrate of Pim-1 was biotinylated recombined utilizing colorimetric method to detect Pim-1 kinase activity. Pim-1 kinase activity of the normal saline (NS) group was regarded as 100%, and the relative activity of Pim-1 kinase of different concentration SGI-1776 groups were got through comparing with NS group. All operations were according to the instruction manual [11].

1.7 Plasmids and transfections

Pim-1 siRNA and siRNA controls were obtained from Santa Cruz Biotechnology. The Pim-1 cDNA plasmid was purchased from OriGene Technologies Inc. (Rockville, MD, USA). Human ovarian cancer HO-8910 cells were cultured at a density of 1×10⁶ cells/well in a 6-well plate, and transfected with 20 µg Pim-1 siRNA, and cDNA respectively using Lipofectamine 2000 (Invitrogen) as described by Yang et al. [12]. At 24 h post-transfection, G418 was added to the culture medium (400 µg/mL). The cells
were collected at 10 days post-transfection, when the majority of the cultured cells had died. The remaining cells were diluted to 1 cell/10 µL and 10 µL cells was added into the 96-well plate with G418. The cells were identified by fluorescence and the positive clones were transferred into a 6-well plate. Following amplification for 3 days, the cells were collected for Western blot.

1.8 Western blot

Western blot was carried out as previously described\(^{13}\). Anti-Pim-1, anti-CDK6, anti-pCDK6, anti-CDK4, anti-pCDK4, anti-CDK2, anti-pCDK2, Anti-P27, anti-P21 and GAPDH were used as primary antibodies. Cells were lysed in a lysis buffer by incubating for 20 min at 4 °C. The protein concentration was determined by using the Bio-Rad assay system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Total proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA, USA). The signals were detected using an ECL advance Western blot analysis system (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA).

1.9 Statistical analysis

The database was set up with the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA) for analysis. Data are presented as the mean±standard deviation (x±s). The means of multiple groups were compared with one-way ANOVA, after the equal check of variance, and the comparisons among the means were performed using the LSD method. Statistical comparison was also performed with two-tailed t-test when appropriate. \(P<0.05\) was considered as statistically significant.

2 Results

2.1 Effects of SGI-1776 on cell proliferation inhibition and cell cycle arrest of ovarian cancer cells

First, we examined the effect of SGI-1776 on cell proliferation viability of HO-8910 cells using MTT assay. SGI-1776 showed the significant activity against HO-8910 cells in a dose-dependent manner in vitro, the inhibiting effect of SGI-1776 (5 µmol/L) was roughly equivalent to DDP (5 µmol/L), and IC\(_{50}\) of SGI-1776 treated for 48 h was (5.2±0.6) Μmol/L for HO-8910 cells (Figure 1A). Furthermore, the inhibiting effect of SGI-1776 was sharply increased from 1.25 Μmol/L to 20 Μmol/L in vitro (Figure 1B). In order to confirm our results, we tested the effects of SGI-1776 on cell proliferation viability by clonogenic assay. HO-8910 cells exposed to different concentration of SGI-1776 showed in a significant inhibition of colony formation in a dose-dependent manner compared to control (Figure 1C and 1D). Overall, the results from MTT assay and clonogenic assay suggest that SGI-1776 inhibited the proliferation of HO-8910 cells in vitro. Additionally, to assess whether the loss of cell viability could be due to the induction of cell cycle arrest partly, we evaluated the effects of SGI-1776 treatment on the distribution in the cell cycle phase using FCM after PI staining. As shown in Figure 1E and 1F, in HO-8910 cell line, SGI-1776 treatment caused a significant accumulation of cells in the G1 phase and a marked decreased in the S/G\(_2\)/M phase when compared with control cells. These results provided convincing data by showing that SGI-1776 induced the proliferation inhibition and accumulation of cell cycle in G1 phase in HO-8910 cells.

2.2 Effects of SGI-1776 on cell migration and invasion ability of ovarian cancer cells

Transwell cell assay was performed to evaluate the migration and invasion activity of HO-8910 cells, morphological migration and invasive features of HO-8910 cells in the differently conditioned media are shown in Figure 2A and 2C. SGI-1776 inhibited the migration and invasion of HO-8910 cells in a dose-dependent manner compared with control groups, and the inhibiting migration and invasion rate of 5 µmol/L SGI-1776 was approximately equal to 5 µmol/L DDP (Figure 2B and 2D). These results suggested that SGI-1776 inhibited the migratory and invasive activity of HO-8910 cells in a dose-dependent manner in vitro.

2.3 Effect of SGI-1776 on the activity of Pim-1 kinase in ovarian cancer cells

The effect of SGI-1776 on the activity of Pim-1 kinase in ovarian cancer cells was determined using HTScan\(^{\text{®}}\) Pim-1 kinase assay kit. The results showed that Pim-1 kinase activity of HO-8910 cells exposed to different concentration SGI-1776 (2.5, 5 and 10 µmol/L) was decreased in a dose-dependent manner, and the Pim-1 kinase activity were (45.8±3.42)%, (30.7±6.09)% and (23.6±2.96)% compared with the NS groups (assume the activity is 100%) respectively, and there was significantly difference among groups (\(P<0.05\)).
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2.4 Effect of SGI-1776 on the expression of Pim-1 and its downstream target genes in ovarian cancer cells

To determine the expression of these proteins, we used Western blot analysis and found that SGI-1776 inhibited the expression of Pim-1, CDK6, pCDK6, CDK4, pCDK4, CDK2 and pCDK2, and increased P27 and P21 at the protein levels in HO-8910 cells (Figure 3).
Figure 3 Western blot showing the effect of SGI-1776 on the proteins expression of Pim-1 and its downstream target gene in HO-8910 cells. Data was the relative gray value. *P<0.05 vs the NS group; **P<0.01 vs the 5 µmol/L DDP group.

2.5 Effect of down-regulation of Pim-1 expression by siRNA on SGI-1776 induced proliferation inhibition and migration and invasion in HO-8910 cell

Down-regulation of Pim-1 by siRNA transfection showed less expression of Pim-1 protein in HO-8910 cells, as confirmed by Western blot (Figure 4A). Furthermore, we found that the down-regulation of Pim-1 expression by SGI-1776 significantly inhibited cell viability (Figure 4B), arrested cell in G1 phase (Figure 4C), and inhibited the migration and invasion (Figure 4D and 4E). In addition, SGI-1776 plus Pim-1 siRNA inhibited cell proliferation, migration and invasion, and induced G1 phase accumulation to a greater degree compared with SGI-1776 alone (Figure 4B–4E). These results provide some molecular evidence suggesting the SGI-1776 induced inhibition of cell proliferation, migration and invasion are mediated via the inactivation of Pim-1 in HO-8910 cells.

2.6 Effect of over-expression of Pim-1 by cDNA on SGI-1776 induced proliferation inhibition and migration and invasion in HO-8910 cells

Up-regulation of Pim-1 by cDNA transfection showed an over-expression of Pim-1 protein in HO-8910 cells,
SGI-1776, an imidazo pyridazine compound, inhibits the proliferation of ovarian cancer cells by inactivating Pim-1 (XIE Jing, et al.). The results showed that over-expression of Pim-1 rescued SGI-1776 induced cell viability inhibition, migration and invasion, and cell cycle G₁ phase accumulation to a certain degree (Figure 4B–4E). These results provided mechanistic evidence suggesting that SGI-1776 inhibited cell proliferation, migration and invasion were partly due to the inactivation of Pim-1 signaling pathway in HO-8910 cells.

![Figure 4](image)

**Figure 4** Effect of Pim-1 siRNA, Pim-1 cDNA transfection, and Pim-1 siRNA/ Pim-1 cDNA transfection combining with SGI-1776 on HO-8910 cells. A: Western blot showing the proliferation, migration and invasion; B: MTT assay showing inhibition of cell proliferation; C: Flow cytometry showing induction of cell cycle G₁ accumulation; D: Transwell cell assay showing inhibition rate of migration; E: Transwell cell assay showing inhibition rate of invasion. Data was the relative gray value. *P<0.05, **P<0.01 vs the NS group; †P<0.05 vs the 5 µmol/L SGI-1776 group.

### 3 Discussion

A variety of studies have shown the over-expressed Pim-1 in human cancer cells and tissues, including ovarian cancer cells and so on. Pim-1 gene play an important role in tumor cells formation, proliferation, differentiation, apoptosis, and inhibiting Pim-1 gene could suppress cancer cells proliferation and promote them apoptosis, which suggests that the inactivation of Pim-1 may play an important role in cancer therapy. SGI-1776, an imidazo
pyridazine compound initially found through virtual screening, which has been shown to have inhibitory activity against Pim-1, Pim-2, Pim-3 kinase, and induced tumor cells apoptosis and inhibited cancer cell proliferation, has already entered phase I of clinical trials\(^5\)\(^-\)\(^6\), but the effects and mechanism of SGI-1776 on ovarian cancer has not been reported in public. Thus, in the present study, we investigated whether SGI-1776 induced inhibition of ovarian cancer HO-8910 cells viability and its molecular mechanisms. In our study, SGI-1776 elicited a dramatic inhibitory effect on the proliferation of HO-8910 cells as shown by MTT assay, clonogenic assay, transwell cell migration and invasion assay, accompanied by decrease of Pim-1 kinase activity and down-regulation of Pim-1 protein expression. Our results suggested that Pim-1 as a target of SGI-1776 in HO-8910 cells because Pim-1 is known to induce oncogenesis and its down-regulation causes inhibition of cell proliferation. Undeniably, we found that down-regulation of Pim-1 by siRNA together with SGI-1776 treatment inhibited cell proliferation to a greater degree in HO-8910 cells compared to SGI-1776 treatment alone. Up-regulation of Pim-1 by cDNA transfection showed over-expression of Pim-1 protein in HO-8910 cells, and the over-expression of Pim-1 rescued SGI-1776 induced cell viability inhibition, migration, invasion, and cell cycle G1 phase to a certain degree compared to SGI-1776 treatment alone. In view of these findings, SGI-1776 not only inhibited proliferation, migration and invasion of the normal ovarian cancer HO-8910 cells but also had the same effects on HO-8910 cells transfected siRNA and cDNA, we strongly believe that the inactivation of Pim-1 by SGI-1776 resulted in the down-regulation of its target genes, which are believed to be mechanistically linked with inhibition of tumor cells proliferation, migration and invasion by treatment with SGI-1776.

In summary, we presented experimental evidence that strongly supported the role of SGI-1776 as an antitumor agent mediated through inactivation of Pim-1 signaling pathway. Further in depth investigations are necessary in order to identify whether SGI-1776 could regulate the expression level of Pim-1 mRNA and how to affect the promoter gene transcription. It is also tempting to speculate that the inactivation of Pim-1 combining with the treatment of ovarian cancer cells with conventional agents could be a useful strategy toward better treatment.

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参考文献

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