Dimethylenastron suppresses human pancreatic cancer cell migration and invasion in vitro via allosteric inhibition of mitotic kinesin Eg5

Xiao-dong SUN1, #, Xing-juan SHI1, #, Xiao-ou SUN1, #, You-guang LUO1, Xiao-jing WU1, Chang-fu YAO1, Hai-yang YU1, Deng-wen LI1, Min LIU2, *, Jun ZHOU1, *

1Department of Genetics and Cell Biology, Tianjin Key Laboratory of Protein Science, College of Life Sciences, Nankai University, Tianjin 300071, China; 2Department of Biochemistry, Key Laboratory of Immune Microenvironment and Disease of the Ministry of Education, Basic Medical College, Tianjin Medical University, Tianjin 300070, China

Aim: The mitotic kinesin Eg5 plays a critical role in bipolar spindle assembly, and its inhibitors have shown impressive anticancer activity in preclinical studies. This study was undertaken to investigate the effect of dimethylenastron, a specific inhibitor of Eg5, on the migration and invasion of pancreatic cancer cells.

Methods: Human pancreatic cancer cell lines PANC1, EPP85, BxPC3, CFPAC1, and AsPAC1 were used. Eg5 expression was examined using immunofluorescence microscopy. Cell migration and invasion were analyzed with wound healing and transwell assays. Cell proliferation was examined using sulforhodamine B and MTT assays. The binding of dimethylenastron to Eg5 was analyzed with a molecular modeling study, and the ADP release rate was examined with the MANT-ADP reagent.

Results: Eg5 expression was 9–16-fold up-regulated in the 5 pancreatic cancer cell lines. Treatment of PANC1 pancreatic cancer cells with dimethylenastron (3 and 10 μmol/L) for 24 h suppressed the migratory ability of the cancer cells in a concentration-dependent manner. The invasion ability of the cancer cells was also reduced by the treatment. However, treatment of PANC1 cells with dimethylenastron (3 and 10 μmol/L) for 24 h had no detectable effect on their proliferation, which was inhibited when the cancer cells were treated with the drug for 72 h. Molecular modeling study showed that dimethylenastron could allosterically inhibit the motor domain ATPase of Eg5 by decreasing the rate of ADP release.

Conclusion: Dimethylenastron inhibits the migration and invasion of PANC1 pancreatic cancer cells, independent of suppressing the cell proliferation. The findings provide a novel insight into the mechanisms of targeting Eg5 for pancreatic cancer chemotherapy.

Keywords: kinesin; Eg5; dimethylenastron; pancreatic cancer; cell migration; cell invasion; cell proliferation; molecular modeling

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Introduction

Eg5 (also known as kinesin-5, kinesin spindle protein, or Kif11) is encoded by the KIF-11 gene located at chromosome 10q24.1. As a member of the BimC family of kinesin-related proteins, Eg5 is a microtubule-dependent motor protein and plays a crucial role in the assembly and maintenance of the bipolar spindle, by hydrolysis of ATP to generate outward forces and push apart anti-parallel microtubules[1, 2]. In addition, accumulating evidence indicates that Eg5 is highly expressed in cancer cell lines and tumor samples[3, 4]. It has been reported that the overexpression of Eg5 leads to abnormal spindle formation, genomic instability, and the development of a broad spectrum of cancers[3, 4].

Eg5 has been demonstrated as an effective target for cancer treatment. Antisense oligonucleotides against Eg5 has been shown to reduce the growth of tumors in xenograft models[5]. In blast crisis chronic myeloid leukemia and prostate cancer cells, in which Eg5 is highly expressed, inhibition of Eg5 causes cell cycle arrest and significantly suppresses cell proliferation[5, 6]. Over the last decade, the effects of various Eg5 inhibitors on the proliferation of cancer cells have been investigated, and the mechanisms of action of several Eg5 inhibitors have been studied[7–11]. Dimethylenastron is a cell-permeable quinazoline-thione compound that acts as a potent inhibitor of Eg5[12]. We have demonstrated previously that dimethylenastron inhibits pancreatic tumor growth by suppressing cell
pancreatic cancer is a highly malignant neoplasm of the pancreas and the fourth leading cause of cancer-related deaths worldwide. The prognosis of this disease is poor, with fewer than 5% of those diagnosed still alive five years after diagnosis. The high mortality rate of pancreatic cancer results mainly from the delay in diagnosis and the high rate of metastasis, of which abnormal cancer cell motility is an essential component. It remains elusive whether Eg5 inhibitors affect cancer cell motility, despite the intensive studies of the mechanisms of action of this group of compounds. In this study, we provide the first evidence that dimethylenastron allosterically inhibits Eg5 activity and reduces the migration and invasion of pancreatic cancer cells, independent of its inhibitory effect on pancreatic cancer cell proliferation.

Materials and methods

Materials
Dimethylenastron was purchased from Calbiochem. Sulforhodamine B, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma–Aldrich, and the mouse monoclonal antibody against Eg5 was from Abcam. The rhodamine-conjugated anti-mouse secondary antibody was obtained from Amersham Biosciences.

Cell culture
PANC1, EPP85, BxPC3, CFPAC1, and AsPC1 human pancreatic cancer cells were cultured in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂. Normal pancreatic epithelial cells were grown as primary cultures as described previously.

In vitro cell proliferation assay
Cells grown in 96-well plates were treated with gradient concentrations of dimethylenastron for 24 or 72 h. Sulforhodamine B and MTT assays were then performed as described previously. The percentage of cell proliferation as a function of drug concentration was plotted.

Immunofluorescence microscopy
Cells grown on glass coverslips were fixed with cold (-20°C) methanol for 8 min and then washed with phosphate-buffered saline (PBS). Nonspecific sites were blocked by incubating with 2% bovine serum albumin diluted with PBS for 20 min at room temperature. Cells were incubated with mouse monoclonal anti-Eg5 antibody (1:100 dilution) for 2 h and then with rhodamine-conjugated anti-mouse secondary antibody for 2 h, followed by staining with DAPI for 5 min. Coverslips were mounted with 90% glycerol in PBS and examined with a Zeiss fluorescence microscope. Images of interphase cells were taken, and 300 cells were analyzed. Eg5 expression in cells was quantified by measuring the fluorescence intensity of Eg5 in a humidified atmosphere with 5% CO₂. Normal pancreatic epithelial cells were grown as primary cultures as described previously.

Measurement of ADP release rate
The effects of dimethylenastron on the ADP release rate of the Eg5 motor domain were examined with the MANT-ADP reagent (Molecular Probes), by measuring the changes in MANT-ADP fluorescence as described previously. In brief, purified Eg5 motor domain was incubated with the MANT-ADP racemate at a 1:1 stoichiometry. The Eg5-MANT-ADP complex was then mixed with 1 mmol/L MgATP in the presence or absence of preformed microtubules. The decrease in MANT-ADP fluorescence was then examined over time at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The observed exponential rate constant was then fit to a hyperbola to obtain the ADP release rate.

Preparation of microtubules
For the preparation of microtubules, 10 µmol/L microtubule
associated protein-free tubulin (cytoskeleton) was incubated at 35°C for 30 min in a buffer containing 80 mmol/L Na-PIPES (pH 6.9), 1 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L GTP, and 5% glycerol.

**Statistical analysis**

Student’s t test was used to determine statistical significance. P values less than 0.05 were considered significant, and P values less than 0.01 were considered highly significant. Experiments were repeated 3 times, and error bars represent standard deviations between experiments.

**Results**

**Eg5 expression is up-regulated in pancreatic cancer cell lines**

To investigate the potential of Eg5 as a target for pancreatic cancer treatment, we examined its expression in several well-characterized pancreatic cancer cell lines, including BxPC3, PANC1, EPP85, CFPAC1, and AsPAC1, and compared to Eg5 expression in normal pancreatic epithelial cells. Consistent with previous studies[4], we found that Eg5 was highly expressed in all the pancreatic cancer cell lines examined (Figure 1A–1B). Interestingly, while only a small portion of Eg5 was localized in the nucleus in normal cells, a significant increase in its nuclear localization was observed in pancreatic cancer cells (Figure 1C).

**Inhibition of Eg5 activity suppresses pancreatic cancer cell migration**

The overexpression of Eg5 in pancreatic cancer cells prompted us to examine whether the inhibition of Eg5 activity affects the motility of pancreatic cancer cells. PANC1 cells were used for the analysis of cell motility because of their well-characterized genetic background, growth conditions, and migratory ability[21]. Serum-starved PANC1 cells grown as confluent monolayers were treated with the Eg5 inhibitor dimethylastron and mechanically scratched to create the wound. Culture medium containing 10% serum was then added to allow wound healing, and phase-contrast images of the wound were taken 24 h later to examine the extent of wound closure as a measure of cell migration. As shown in Figure 2A–2B, dimethylastron inhibited pancreatic cancer cell migration in a concentration-dependent manner, as evidenced by compromised wound closure in response to dimethylastron treatment. The inhibitory effect of dimethylastron on cell migration was more evident when culture medium containing 5% serum was used for wound healing (Figure 2C).

**Dimethylastron inhibits pancreatic cancer cell invasion**

We then performed transwell assays to evaluate the effect of inhibition of Eg5 activity on pancreatic cancer cell invasion. PANC1 cells were treated with dimethylastron and loaded to the transwell chambers (the upper surface of the transwell filters was coated with matrigel). After 24 h, cells migrated to the underside of the transwell filters were stained with crystal violet solution and imaged. As shown in Figure 3A–3B, inhibition of Eg5 activity by dimethylastron significantly suppressed the ability of pancreatic cancer cells to invade to the underside of the transwell filters. Similar results were achieved when the upper surface of the transwell filters was coated with fibronectin (Figure 3C).

**Dimethylastron inhibits pancreatic cancer cell proliferation in a concentration-dependent manner**

We then examined whether the reduced migration and invasion of pancreatic cancer cells in response to dimethylastron results from its effect on cell proliferation. To investigate this possibility, sulforhodamine B and MTT-based cell proliferation assays were performed. As shown in Figure 4A, both sulforhodamine B and MTT assays revealed that at concentrations 1 to 10 μmol/L, dimethylastron did not obviously affect PANC1 cell proliferation when cells were treated for 24 h. In contrast, 1 to 10 μmol/L dimethylastron could significantly inhibit cell proliferation when cells were treated for 72 h (Figure 4B). These results thus demonstrate that the inhibitory
effects of dimethylenastron on pancreatic cancer cell migration and invasion are independent of its effect on cell proliferation.

**Dimethylenastron allosterically inhibits the motor domain ATPase of Eg5**

To gain more mechanistic insight into how dimethylenastron inhibits Eg5 activity, we analyzed their interaction by molecular modeling. Dimethylenastron was docked onto the coordinates obtained from the crystal structure of Eg5, using standard DOCK methodology, and the lowest-energy Eg5/dimethylenastron interaction model was presented (Figure 5A). Detailed analysis revealed that dimethylenastron binds to the motor domain of Eg5 in a pocket close to the ATP/ADP binding pocket on Eg5; there exist a loop (E116-R119) and an α-helix (G110-F113) between the two binding pockets (Figure 5B). This model suggests that in the absence of ATP/ADP, dimethylenastron does not bind to Eg5 stably. Following ATP binding and hydrolysis to ADP, the ADP molecule pushes the aforementioned loop and α-helix to the dimethylenastron pocket and promotes the interaction between Eg5 and dimethylenastron.

Analysis of the ADP release rate has been useful for studying the effect of Eg5 inhibitors on its activity towards ATP hydrolysis. To investigate whether dimethylenastron inhibits Eg5 activity by affecting ADP release, we analyzed the ADP release rate of Eg5 motor domain with the MANT-ADP reagent, a fluorescent analog of ADP that displays increased fluorescence when bound to Eg5. As shown in Figure 5C, dimethylenastron significantly inhibited the basal rate of ADP release (in the absence of microtubules). In the presence of microtubules, dimethylenastron inhibited the rate of ADP release to a similar extent. These data thus suggest that dimethylenastron allosterically inhibits the motor domain ATPase of Eg5 by decreasing ADP release, independently of the binding of Eg5 to microtubules.

**Discussion**

The incidence of pancreatic cancer has been increasing at an alarming rate on a global scale over the past two decades. In spite of recent improvement in patient survival, the treatment of pancreatic cancer is still difficult. Our previous studies have shown that the Eg5 inhibitor dimethylenastron inhibits the growth of pancreatic tumor both in vitro and in the mouse model. In the present study, our results show that dimethylenastron inhibits pancreatic cancer cell migration and invasion. These findings thus provide novel mechanistic insight into the anticancer effect of dimethylenastron, suggesting the potential of using this agent for pancreatic cancer treatment. Given that the ability to rapidly metastasize is the most deadly aspect of pancreatic cancer and that tumor metastasis results
mainly from cancer cell migration and invasion\textsuperscript{[14]}, the use of dimethylenastron for pancreatic cancer management might decrease cancer metastasis and improve patient survival. Our study has also demonstrated that the inhibitory effects of dimethylenastron on pancreatic cancer cell migration and invasion are independent of its effect on cell proliferation. At present, the molecular mechanism of how dimethylenastron decreases cancer cell motility remains elusive. It has been shown in a previous study that knockdown of myosin II in fibroblasts could enhance cell migration and that the increase in cell motility by knockdown of myosin II could be rescued by the inhibition of Eg5 activity\textsuperscript{[22]}. Therefore, it would be important to investigate whether the inhibition of Eg5 activity in pancreatic cancer cells by dimethylenastron could trigger the myosin II-associated signaling pathway.

The effectiveness of most chemotherapeutic agents has been impaired by various side effects. In this study, by molecular modeling and analysis of ADP release rate, we find that dimethylenastron allosterically inhibits the motor domain ATPase of Eg5, independent of the Eg5/microtubule interaction. Although the relationship between the allosteric inhibition of Eg5 activity and the inhibition of migration and invasion of pancreatic cancer cells remains unclear, our data suggest that dimethylenastron might have few (if any) side effects as a chemotherapeutic agent, because it would not affect the normal physiological functions of Eg5 that require its interaction with microtubules.

Another important question is how Eg5 expression is regulated in normal cells and how it is highly expressed in pancreatic cancer cells. In our previous study, we presented evidence that cellular Eg5 level is down-regulated by parkin, an E3 ubiquitin ligase well known for its role in Parkinson’s disease. Interestingly, parkin represses Eg5 gene transcription by Hsp70 monoubiquitination-dependent inactivation of the c-Jun NH\textsubscript{2}-terminal kinase pathway and blocking c-Jun binding to the activator protein 1 site in the Eg5 promoter, instead of triggering Eg5 protein degradation through the ubiquitin-proteasome pathway\textsuperscript{[23]}. In addition, the Bcr-Abl tyrosine kinase has been reported to regulate Eg5, although the molecular details remain unclear\textsuperscript{[6]}. Considering that both parkin and Bcr-Abl have been implicated in tumorigenesis, it would be interesting to examine whether abnormalities in these proteins play a role in triggering Eg5 overexpression in pancreatic cancer cells.

Figure 4. Dimethylenastron inhibits the proliferation of pancreatic cancer cells in a concentration-dependent manner. PANC1 cells were treated with gradient concentrations of dimethylenastron for 24 h (A) or 72 h (B). The percentage of cell proliferation was measured by sulforhodamine B (SRB)- or MTT-based cell proliferation assays. \textsuperscript{a}P<0.05 (SRB data versus MTT data).

Figure 5. Dimethylenastron allosterically inhibits Eg5 activity by decreasing the rate of ADP release. (A) Schematic models showing the structure of Eg5 bound with ADP (upper) and dimethylenastron (lower). (B) Details of important interactions of ADP (left) and dimethylenastron (right) with the motor domain of Eg5, with red depicting oxygen, blue depicting nitrogen, yellow showing carbon, and grey representing hydrogen. Amino acid residues of Eg5 involved in the interaction with ADP and dimethylenastron are labeled. (C) Effects of dimethylenastron on the ADP release rate of Eg5 motor domain in the presence or absence of microtubules. Mean±SD. n=3. \textsuperscript{a}P<0.01 vs control.
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Author contribution
Min LIU and Jun ZHOU designed research; Xiao-dong SUN, Xing-juan SHI, Xiao-ou SUN, Xiao-jing WU, Chang-fu YAO, and Hai-yang YU performed research; Deng-wen LI and Min LIU contributed new reagents or analytic tools; You-guang LUO and Jun ZHOU wrote the paper.

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