PDK1/AKT signaling is involved in the anti-tumor effect of gramine and cisplatin chemoresistance in ovarian cancer cells

HAIYAN WANG, RUIMING ZHENG, BUJUN WANG, SUMIAO HONG and HONGXIA CHEN

Department of Obstetrics and Gynecology, Pingyang People's Hospital, Wenzhou, Zhejiang 325400, P.R. China

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Abstract. Ovarian cancer (OC) remains one of the most lethal gynecologic malignancies, with limited therapeutic options and poor long-term prognosis. Gramine, an indole alkaloid predominantly derived from the rhizomes of *Arundo donax* L., has demonstrated antitumor activity in various cancer types; however, its effects and mechanistic basis in OC have not been systematically investigated. In the present study, the biological activity of gramine in OC cells were evaluated using cell counting kit-8, colony formation and EdU assays for proliferation; TUNEL staining for apoptosis; and wound healing and Transwell assays for migration and invasion. Western blotting was performed to examine underlying molecular changes. The present results showed that gramine significantly suppressed OC cell proliferation, migration and invasion, while promoting cell apoptosis. Mechanistically, gramine reduced PDK1 protein levels, and enforced PDK1 overexpression largely reversed the suppressive effects of gramine on malignant phenotypes. Consistently, PDK1 restoration also attenuated gramine-induced downregulation of phosphorylated AKT. Furthermore, gramine enhanced the sensitivity of OC cells to cisplatin, an effect associated with PDK1/AKT signaling inhibition. Collectively, these findings suggest that gramine inhibits malignant progression and attenuates cisplatin resistance in OC cells, at least in part, through blockade of the PDK1/AKT pathway. Although limited to *in vitro* models, this work provides preliminary evidence supporting further exploration of gramine as a potential research tool or lead compound in OC.

Introduction

Ovarian cancer (OC) is a lethal malignancy of the female reproductive system, characterized by insidious onset, pronounced aggressiveness and poor prognosis. It ranks among the leading causes of cancer-related mortality in women worldwide. Currently, the standard therapeutic regimens for OC include surgery, platinum-based chemotherapy combined with paclitaxel (PTX), radiotherapy, and targeted therapy (1). Although the initial response rate to first-line treatment is relatively high, most patients with advanced OC ultimately experience disease recurrence, metastasis and acquired chemoresistance. Consequently, the 5-year survival rate for patients with OC remains constrained at 30-40% (1). Therefore, there remains a need to identify novel and effective therapeutic targets for OC and to expedite the development of anti-OC drugs.

Traditional Chinese medicine has demonstrated notable efficacy in alleviating clinical symptoms of OC, mitigating the toxicity and side effects associated with radiotherapy and chemotherapy and improving the quality of life of affected patients (2-4). Gramine, also known as 3-(N, N-dimethylaminomethyl) indole (5), is primarily extracted from the rhizomes of *Arundo donax* L., a perennial grass species belonging to the genus *Arundo* within the family Poaceae. Gramine features a distinctive eterocyclic skeleton and exhibits a broad spectrum of biological activities, including antiviral, antioxidant, antitumor and neuroprotective effects, thereby indicating considerable promise in terms of druggability and therapeutic potential (6). Several structural analogs of gramine, including the marketed drugs sumatriptan and rizatriptan, have been successfully developed for clinical use. In the context of antitumor research, structural optimization based on gramine scaffold has yielded derivatives with notable activity against gastric cancer. Among these, the derivative designated '16 h' has been shown not only to induce cell apoptosis and arrest the cell cycle at the G2/M phase, but also to suppress metastatic and proliferative capabilities of cancer cells (7). In oral squamous cell carcinoma, gramine was reported to repress angiogenesis and provoked apoptosis through downregulation of TGF-β signaling (8). In addition, gramine has been shown to attenuate EGFR-mediated inflammation and cell proliferation by regulating NF-κB and STAT3 signaling pathways (9). These findings collectively indicate that gramine possesses considerable antitumor potential. However, the pharmacological effects and molecular mechanism of gramine on OC have not yet been elucidated.

PDK1 is an upstream serine/threonine protein kinase that regulates protein kinase B (PKB/C-AKT). PDK1 phosphorylates the T-loop region of AGC family kinases, thereby regulating cell proliferation, invasive metastasis

Correspondence to: Dr Haiyan Wang, Department of Obstetrics and Gynecology, Pingyang People's Hospital, 555 Kunao Avenue, Kunyang Town, Pingyang, Wenzhou, Zhejiang 325400, P.R. China
E-mail: 517229621@qq.com

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and apoptosis (10). PDK1 drives OC malignancy through multiple mechanisms, positioning it as a potential prognostic marker and therapeutic target (11-13). Additionally, PDK1 is associated with chemoresistance in OC cells (13). These data indicate that interfering with PDK1 signaling pathway can provide potential value for the treatment of OC. The present study aimed to explore the anti-tumor effects of gramine in OC cells and clarify the underlying mechanism involving the PDK1 signaling pathway.

Materials and methods

Cell culture and drugs. The human OC cell lines OV-90 and SK-OV-3, and normal ovarian epithelial IOSE80 cells were purchased form Zhong Qiao Xin Zhou Biotechnology Co., Ltd. Cisplatin-resistant SK-OV-3 cells (SK-OV-3-R) were obtained from Zhejiang Meisen Cell Technology Co Ltd. All cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Beijing Solarbio Science & Technology Co., Ltd.), and cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Gramine (HY-N0166, purity: 99.80%), Cisplatin (HY-17394, purity: 99.30%) and dimethyl sulfoxide (DMSO; HY-Y0320C, purity: 99.98%) were purchased from MedChemExpress. DMSO was used as the vehicle, and the final concentration of DMSO in all cell culture systems was maintained <0.1% (v/v) to exclude solvent cytotoxicity.

Establishment of PDK-stably transfected cells. Recombinant lentiviruses encoding 3-phosphoinositide-dependent protein kinase-1 (PDK1) (backbone: pCDH-CMV-MCS-EF1α-Puro) and empty vector control were purchased from Shanghai GenePharma Co., Ltd. Lentiviral particles were generated via the third-generation lentiviral packaging system in 293T cells (American Type Culture Collection). For transfection in 10-cm culture dishes, the mass ratio of transfer plasmid:packaging plasmid (psPAX2):envelope plasmid (pMD2.G) was 4:3:1, with a total plasmid amount of 8 μg per dish. Lentivirus-containing supernatant was collected after transfection. Target cells were infected with PDK1-overexpressing lentivirus (PDK1-OV) or empty vector lentivirus (EV) at a multiplicity of infection of 10 and incubated at 37˚C for 48 h. After transduction, the cells were cultured for 8 days before subsequent experimentation. Stable transfectants were then selected and maintained using 2 μg/ml puromycin (P9620; MilliporeSigma).

Cell counting kit-8 (CCK-8). Cell viability was evaluated using the CCK-8 kit (Beyotime Biotechnology). Cells plated in 96-well plates were treated with various concentrations of gramine (12.5, 25, 50, 100 or 200 μM) or/and cisplatin (5, 10, 20, 40 or 80 μM) at 37˚C for 24 h. Subsequently, the culture medium was substituted with 10% CCK-8 solution prepared in 10% FBS-supplemented medium, and the plates were incubated at 37˚C for 3 h. The optical density was then measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Each group contained 6 replicate wells, and the assay was repeated 3 times biologically.

Colony formation assay. Cells ($5x10^2$) plated in 12-well plates were allowed to grow for 5 days, followed by treatment with gramine for another 3 days. Thereafter, the colonies were fixed and stained with 0.1% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) dissolved in methanol at room temperature for 30 min. Images were captured under a light microscope. Colonies containing >50 cells were quantified using ImageJ software version 1.8.0 (National Institutes of Health). A total of 3 biological replicates were performed, and each group had 3 parallel plates.

5-ethynyl-2'-deoxyuridine (EdU) and TdT-mediated dUTP nick-end labeling (TUNEL) staining assays. For the EdU assay, cells grown on 12-well glass slides were treated with gramine at 37˚C for 24 h, followed by incubation with 10 μM EdU at 37˚C for 3 h. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 at room temperature, and stained using the Click Reaction Mixture (Beyotime Biotechnology) according to the manufacturer's protocol. For the TUNEL assay, cells were fixed and permeabilized as aforementioned and subsequently incubated with the TUNEL reaction mixture at 37˚C (Beyotime Biotechnology). Nuclei were dyed with DAPI (Beijing Solarbio Science & Technology Co., Ltd.). Fluorescent images were visualized using a fluorescence microscope (Zeiss GmbH). The percentage of EdU-positive cells was quantified using ImageJ software. For each sample, at least 3 randomly selected microscopic fields were analyzed, with 3 biological replicates for each group.

Wound healing assay. Confluent cell monolayers were scratched using a 200 μl pipette tip to create a uniform wound gap. Cells were then cultured in serum-free medium and treated with gramine for 24 h. Wound images were imaged at 0 and 24 h using an inverted microscope (Nikon Corporation). The wound areas were measured using ImageJ software, and the wound closure rate was calculated by the formula: Wound closure rate (%)=(Area $_{0\,h}$-Area $_{24\,h}$)/Area $_{0\,h}$ x100. For each sample, at least 3 randomly selected microscopic fields were analyzed, with 3 biological replicates for each group.

Transwell invasion assay. Transwell inserts (8-μm pore size, Corning, Inc.) were coated with 0.2% Matrigel (Corning, Inc.) and incubated at 37˚C for 2 h to allow gel solidification. Cells ($5x10^4$) suspended in 200 μl of serum-free medium were introduced to the upper chamber, and 600 μl of complete medium containing 10% FBS was placed in the lower chamber as a chemoattractant. After overnight incubation to allow cell attachment, gramine was added to the upper chamber and the cells were incubated at 37˚C for 24 h. Non-invading cells on the upper surface of the membrane were carefully erased using cotton swabs. Cells that had invaded through the membrane and adhered to the lower surface were fixed with 4% paraformaldehyde at room temperature for 30 min, followed by staining with 0.1% crystal violet (Beyotime Biotechnology) at room temperature for 15 min. Images were acquired using a light microscope (Zeiss GmbH), and the numbers of invaded cells were quantified using ImageJ software. For each sample, at least 3 randomly selected microscopic fields were analyzed, with 3 biological replicates for each group.

Western blotting. Cells treated with gramine for 24 h were lysed in RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with PMSF (Beijing Solarbio Science & Technology Co., Ltd.) and phosphatase inhibitors (Bimake). Protein quantification was performed via the BCA assay, and identical quantities of protein (20 μg) were loaded into each lane for western blot analysis. Protein samples were then separated by 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies at 4˚C overnight, followed by incubation with anti-rabbit (1:5,000; SA00001-2; Proteintech Group, Inc.) or anti-mouse (1:5,000; SA00001-1; Proteintech Group, Inc.) secondary antibodies for 1 h at room temperature. The primary antibodies used were as follows: Ki67 (1:2,000; 28074-1-AP; Proteintech Group, Inc.), Bax (1:20,000; 50599-2-Ig; Proteintech Group, Inc.), Bcl-2 (1:5,000; 68103-1-Ig; Proteintech Group, Inc.), PARP1 (1:1,000; 13371-1-AP; Proteintech Group, Inc.), cleaved PAPR1 (1:5,000; 60555-1-Ig; Proteintech Group, Inc.), N-cadherin (1:20,000; 22018-1-AP; Proteintech Group, Inc.), E-cadherin (1:20,000; 20874-1-AP; Proteintech Group, Inc.), vimentin (1:20,000; 10366-1-AP; Proteintech Group, Inc.), GAPDH (1:50,000; 60004-1-Ig; Proteintech Group, Inc.), PDK1 (1:1,000; A0834; Abclonal Biotech Co., Ltd.), AKT (1:1,000; 4691; Cell Signaling Technology, Inc.) and p-AKT (Thr308) (1:1,000; 9275; Cell Signaling Technology, Inc.). Protein bands were visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.) with Amersham Imager 680 (GE Healthcare) in auto-exposure mode. Band densities were quantified with ImageJ: p-AKT (Thr308) signals were normalized to total AKT, and all other protein bands were normalized to GAPDH. A total of 3 independent biological replicates were performed.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad Prism software version 8.3.1 (Dotmatics). Normality of the data were first assessed using the Shapiro-Wilk test. Comparisons between two groups were conducted using two-tailed Student's t-test, while comparisons among multiple groups were tested by one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate statistically significant difference.

Results

Gramine suppressed cell proliferation and provoked apoptosis in OC cells. Fig. 1A depicts the chemical structure of gramine. The biological effects of gramine were evaluated in two OC cell lines (SK-OV-3 and OV-90) and normal ovarian epithelial IOSE80 cells. As shown in Fig. 1B, gramine triggered a dose-dependent suppression of cell viability across all three cell lines, with half-maximal inhibitory concentration (IC_{50}) values of 78.63 μM (SK-OV-3), 117.2 μM (OV-90) and 393.6 μM (IOSE80). The markedly lower IC_{50} of gramine in OC cells compared with IOSE80 cells suggest a degree of selective cytotoxicity against malignant ovarian cells. Consistent with the viability data, gramine dose-dependently diminished the colony-forming capacity in OC cells (Fig. 1C). Based on these results and its minimal cytotoxicity toward normal ovarian epithelial cells, concentrations of 50 and 100 μM gramine were selected for the subsequent investigations. Additionally, the proportion of EdU-positive cells (Fig. 1D) and the expression level of Ki67 protein (Fig. 1E) were notably decreased following gramine administration. Next, it was explored whether gramine induced apoptotic cell death in OC cells. As shown in Fig. 2A, an increased number of TUNEL-positive cells was observed in gramine-treated cells. Concurrently, elevated ratios of BAX/Bcl-2 and cleaved PARP1/PAPR1 were detected after gramine exposure (Fig. 2B). These data suggest that gramine inhibits OC cell proliferation and promotes apoptosis.

Gramine inhibited cell migration and invasion in OC cells. Then it was assessed whether gramine affects the metastatic potential of OC cells. As shown in Fig. 3A and B, wound healing and Transwell invasion assays revealed that gramine led to reduced migration and invasion capacities, respectively. Western blot analysis further demonstrated that gramine downregulated the expressions of mesenchymal markers N-cadherin and vimentin, while concomitantly upregulating the epithelial biomarker E-cadherin (Fig. 3C). These findings suggest that gramine attenuates the invasive capacity of OC cells.

PDK1 overexpression impaired the suppressive effects of gramine on OC cell growth. Given the vital role of PDK1 in the tumorigenesis of OC, the present study sought to investigate whether gramine impedes ovarian cancer cell growth by suppressing PDK1. Western blot analysis revealed that gramine exposure for 24 h markedly reduced PDK1 protein levels in OC cells in a concentration-dependent manner (Fig. 4A). To further clarify the functional role of PDK1 in gramine-mediated cellular responses, lentiviral transfection was performed to generate stable PDK1-overexpressing OC cell lines for subsequent rescue experiments (Fig. 4B). Functional assays validated that PDK1 overexpression effectively counteracted the suppressive effects of gramine on OC cell proliferation. Specifically, following gramine treatment, cells with increased PDK1 expression exhibited higher cellular viability (Fig. 4C), enhanced colony-forming capacity (Fig. 4D) and elevated Ki67 levels (Fig. 4E) compared with control cells. Furthermore, PDK1 overexpression partially mitigated the pro-apoptotic property of gramine. Although the proportion of TUNEL-positive cells did not reach statistical significance, ectopic PDK1 expression tended to abrogate gramine-induced apoptosis (Fig. 5A). Consistently, the increased Bax/Bcl-2 ratio induced by gramine was also rescued upon PDK1 overexpression (Fig. 5B). These data collectively suggest that gramine-mediated suppression of OC cell growth is associated with PDK1 downregulation.

PDK1/AKT signaling was involved in anti-migration and invasion effects of gramine in OC cells. Ectopic overexpression of PDK1 potentiated the migratory and invasive capacities of OC cells (Fig. 6A and B). Consistent with this phenotypic change, PDK1 overexpression also upregulated N-cadherin while downregulating E-cadherin protein levels (Fig. 6C). Notably, the inhibitory effects of gramine on cell migration

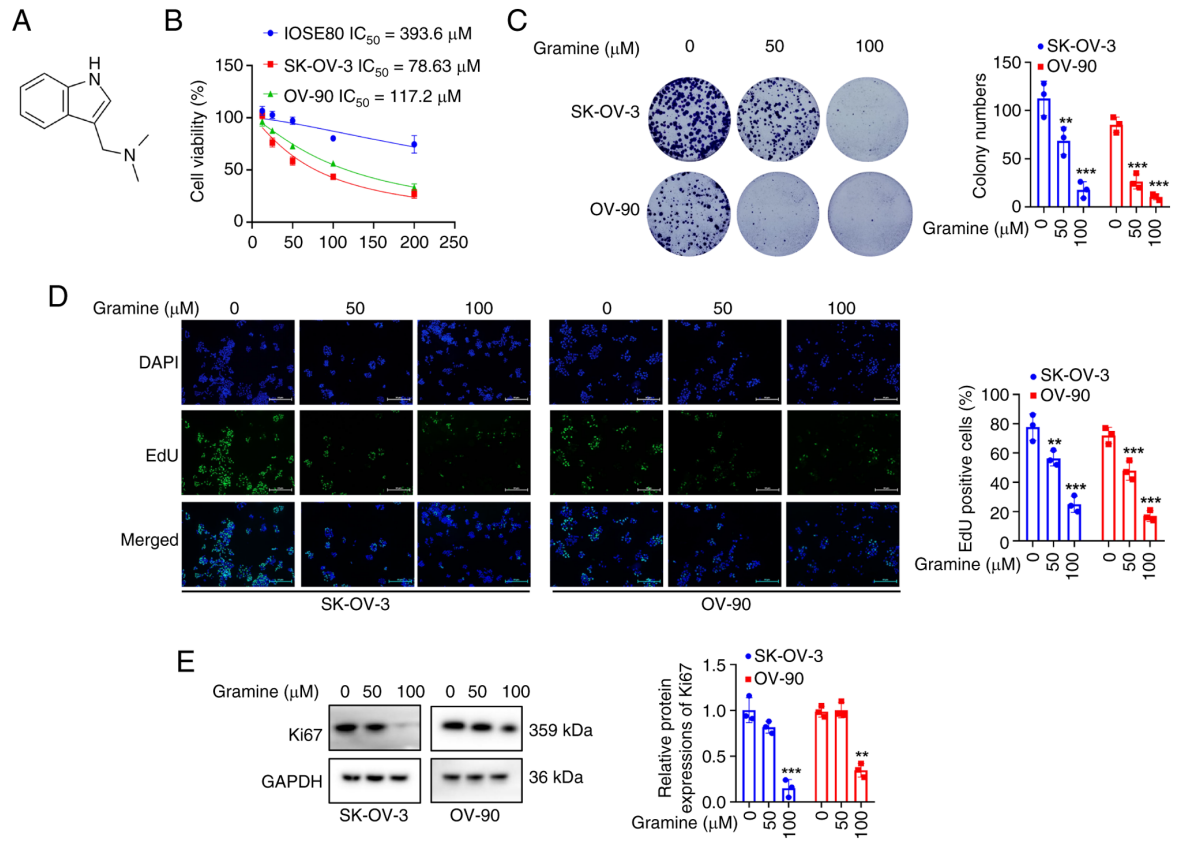

Figure 1. Gramine suppressed cell proliferation in ovarian cancer cells. (A) The chemical structure of gramine. (B) Cell counting kit-8 assay detecting the effect of gramine on cell viability. SK-OV-3 and OV-90 cells were incubated with gramine (12.5, 25, 50, 100, 200 μM) for 24 h. (C) Clone formation assay assessing the effect of gramine on clone formation ability. SK-OV-3 and OV-90 cells were incubated with gramine (50, 100 μM) for 72 h. (D) EdU assay measuring the effect of gramine on the cell proliferation. Scale bar, 200 μm. (E) Western blotting assay testing the effect of gramine on Ki67 expressions. SK-OV-3 and OV-90 cells were incubated with gramine (50, 100 μM) for 24 h. **P<0.01, ***P<0.001 vs. the untreated control. Data represent the mean ± SD of three independent experiments.

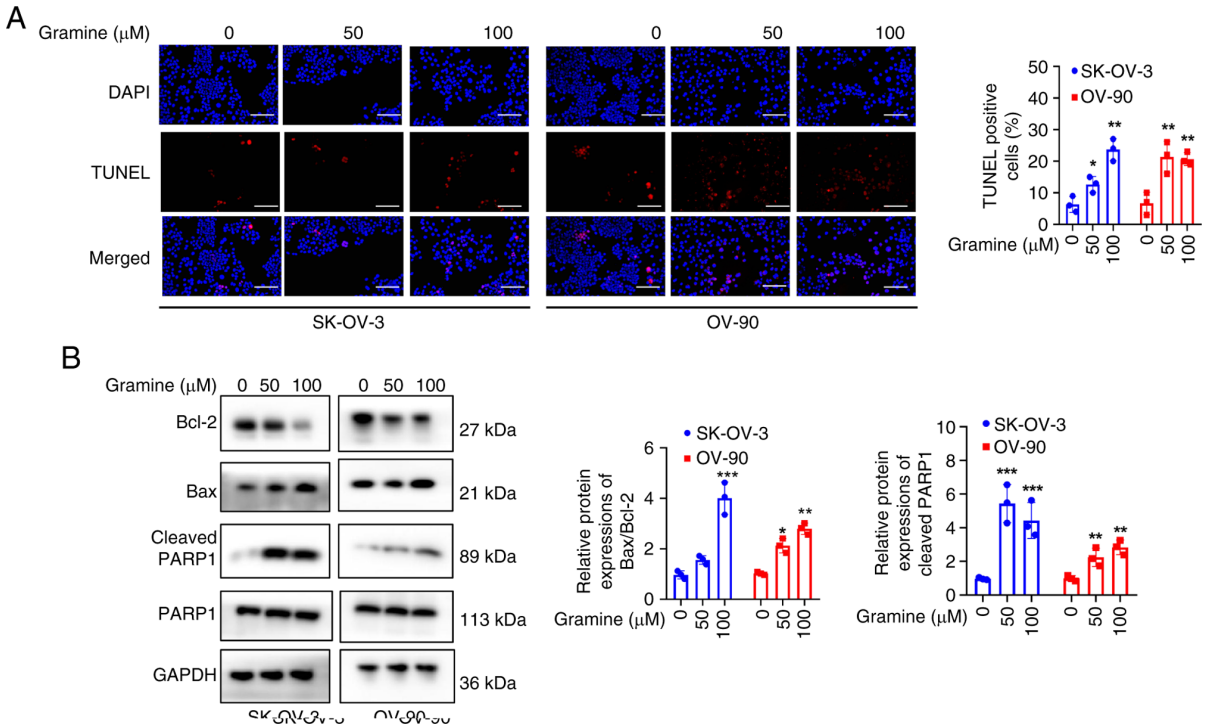

Figure 2. Gramine induced apoptosis in OC cells. (A) TUNEL assay evaluating the effect of gramine on cell apoptosis. Scale bar, 200 μm. (B) Western blotting assay determining the effect of gramine on Bax, Bcl2, PARP1 and cleaved PARP1 expressions. SK-OV-3 and OV-90 cells were incubated with gramine (50,100 μM) for 24 h. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control. Data represent the mean ± SD of three independent experiments.

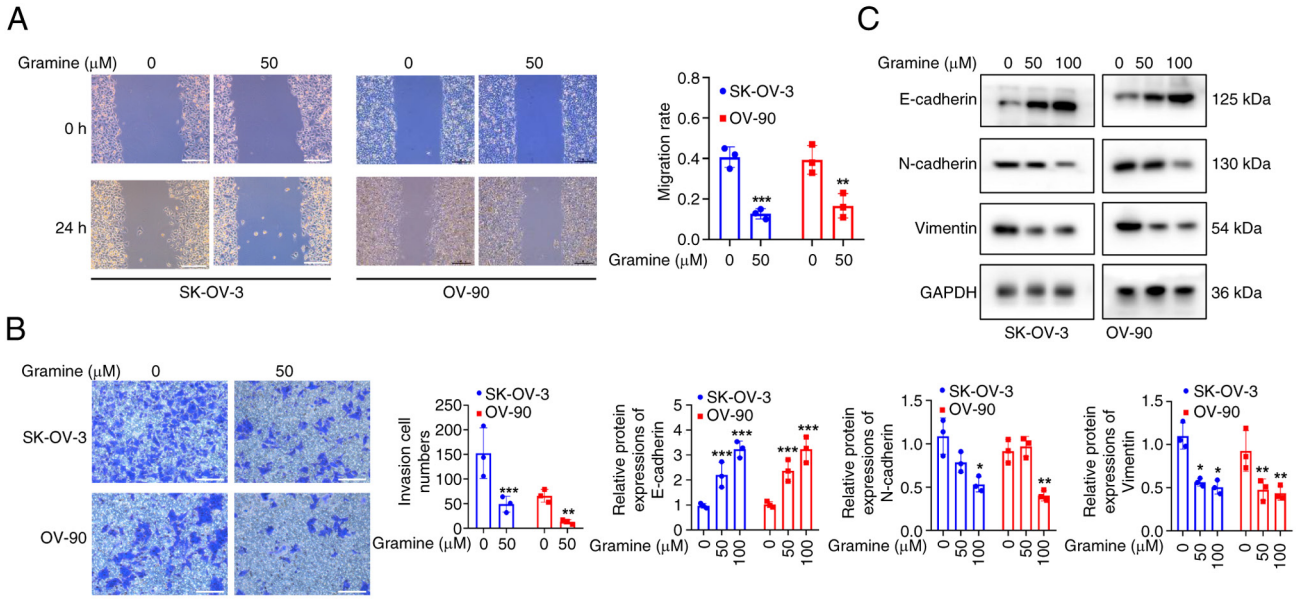

Figure 3. Gramine inhibited cell migration and invasion in OC cells. (A) Wound healing and (B) transwell invasion assays detecting the effect of gramine on the abilities of cell migration and invasion respectively. Scale bar, 200 μm. SK-OV-3 and OV-90 cells were incubated with gramine (50 μM) for 24 h. (C) Western blotting assay determining the effect of gramine on N-cadherin and vimentin expressions. SK-OV-3 and OV-90 cells were incubated with gramine (50, 100 μM) for 24 h. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control. Data represent the mean ± SD of three independent experiments.

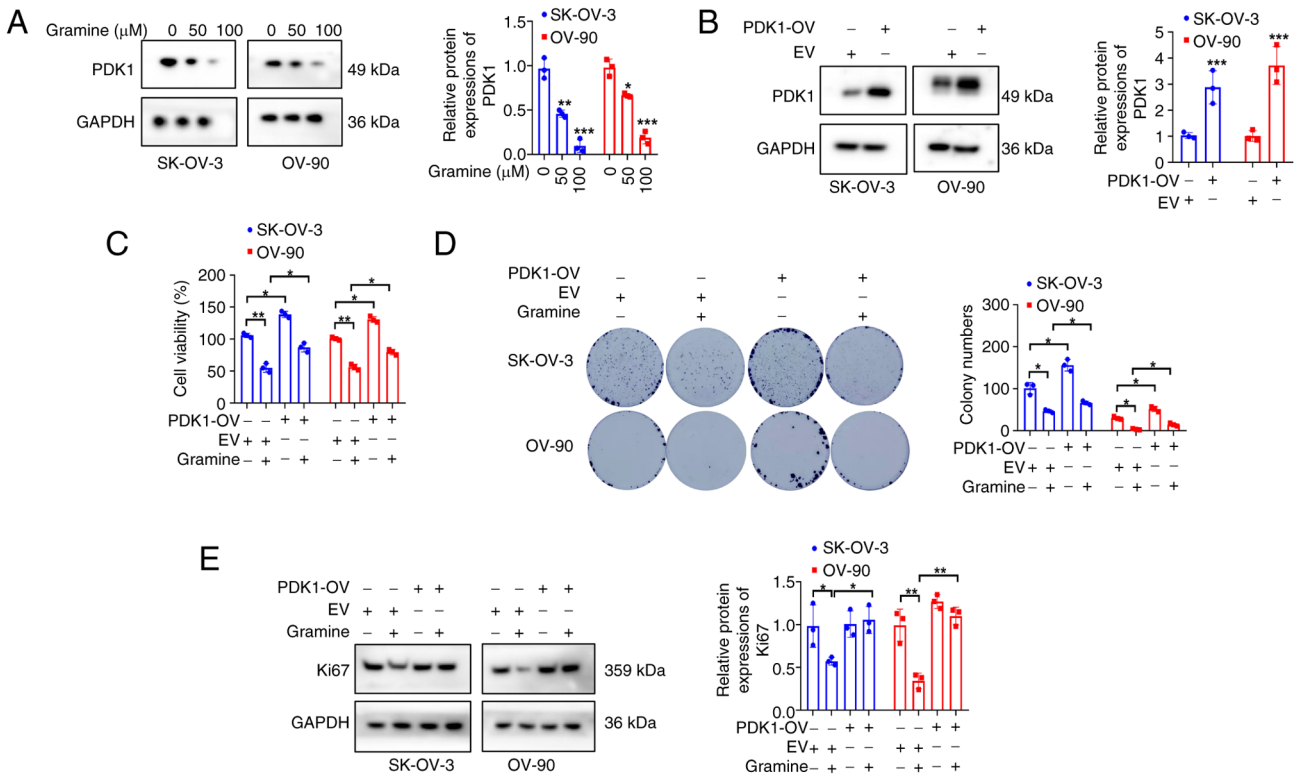

Figure 4. PDK1 overexpression impaired the anti-proliferation effect of gramine on OC cells. (A) Western blotting assay determining the effect of gramine on PDK1 expressions. SK-OV-3 and OV-90 cells were incubated with gramine (50, 100 μM) for 24 h. *P<0.05, **P<0.01, ***P<0.001 vs. the untreated control. (B) Western blotting assay assessing the transfection efficiency of PDK1 overexpression lentivirus. ***P<0.001 vs. the EV control. (C) Cell counting kit-8 assay detecting the effect of PDK1 overexpression on cell viability. (D) Clone formation assay assessing the effect of PDK1 overexpression on colony formation ability. (E) Western blotting assay examining the effect of PDK1 overexpression on Ki67 expression. *P<0.05, **P<0.01 vs. the EV control or gramine-treated EV group (C-E). Data represent the mean ± SD of three independent experiments. EV, empty vector; OV, overexpression.

and invasion were substantially abrogated following PDK1 overexpression (Fig. 6A and B). Similarly, PDK1 enrichment counteracted gramine-mediated N-cadherin reduction and E-cadherin elevation (Fig. 6C). In addition, PDK1 overexpression also effectively restored the level of p-AKT (Thr308), which was suppressed by gramine treatment (Fig. 6C). Together,

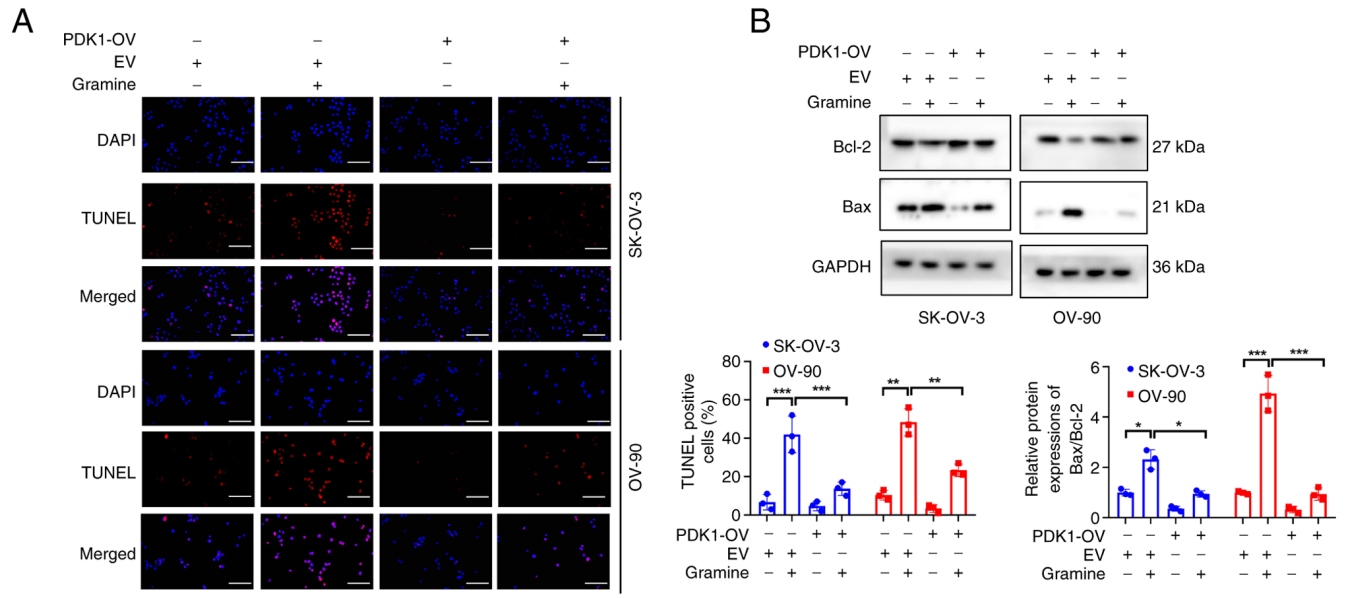

Figure 5. PDK1 overexpression impaired the pro-apoptotic effect of gramine on ovarian cancer cells. (A) TUNEL assay evaluating the effect of PDK1 overexpression on cell apoptosis. Scale bar, 200 μm. (B) Western blotting assay determining the effect of PDK1 overexpression on Bax and Bcl2 expressions. PDK1 overexpressing SK-OV-3 and OV-90 cells were treated with gramine (100 μM) for 24 h. *P<0.05, **P<0.01, ***P<0.001 vs. the EV control or gramine-treated EV group. Data represent the mean ± SD of three independent experiments. EV, empty vector; OV, overexpression.

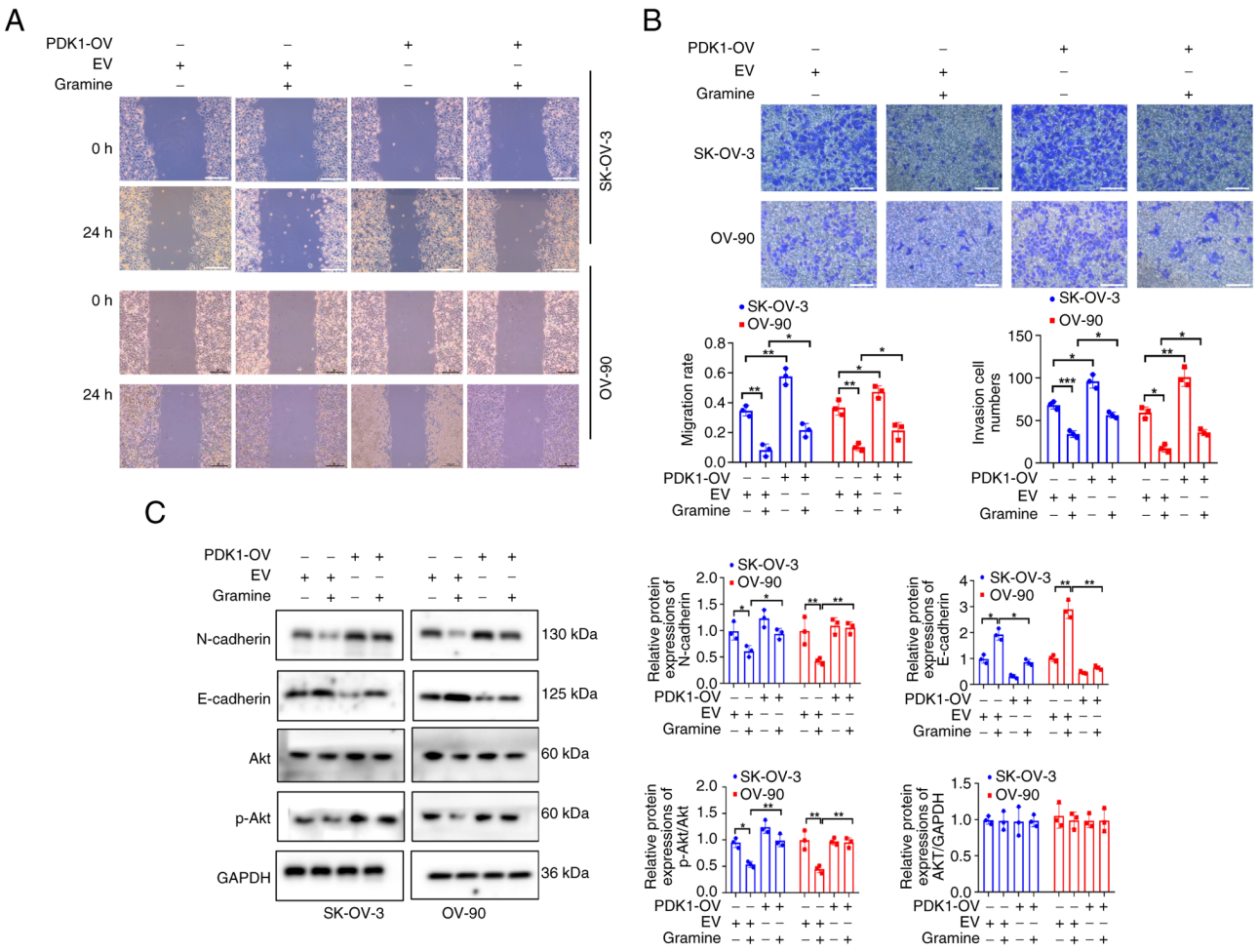

Figure 6. PDK1/AKT signaling pathway was involved in the anti-migratory and anti-invasive effects of gramine in ovarian cancer cells. (A) Wound healing and (B) transwell invasion assays detecting the effect of PDK1 overexpression on the abilities of cell migration and invasion respectively. Scale bar, 200 μm. (C) Western blotting assay determining the effect of PDK1 overexpression on N-cadherin, vimentin and p-AKT (Thr308) expressions. PDK1 overexpressing SK-OV-3 and OV-90 cells were incubated with gramine (50 μM) for 24 h. *P<0.05, **P<0.01, ***P<0.001 vs. the EV control or gramine-treated EV group. Data represent the mean ± SD of three independent experiments. EV, empty vector; OV, overexpression.

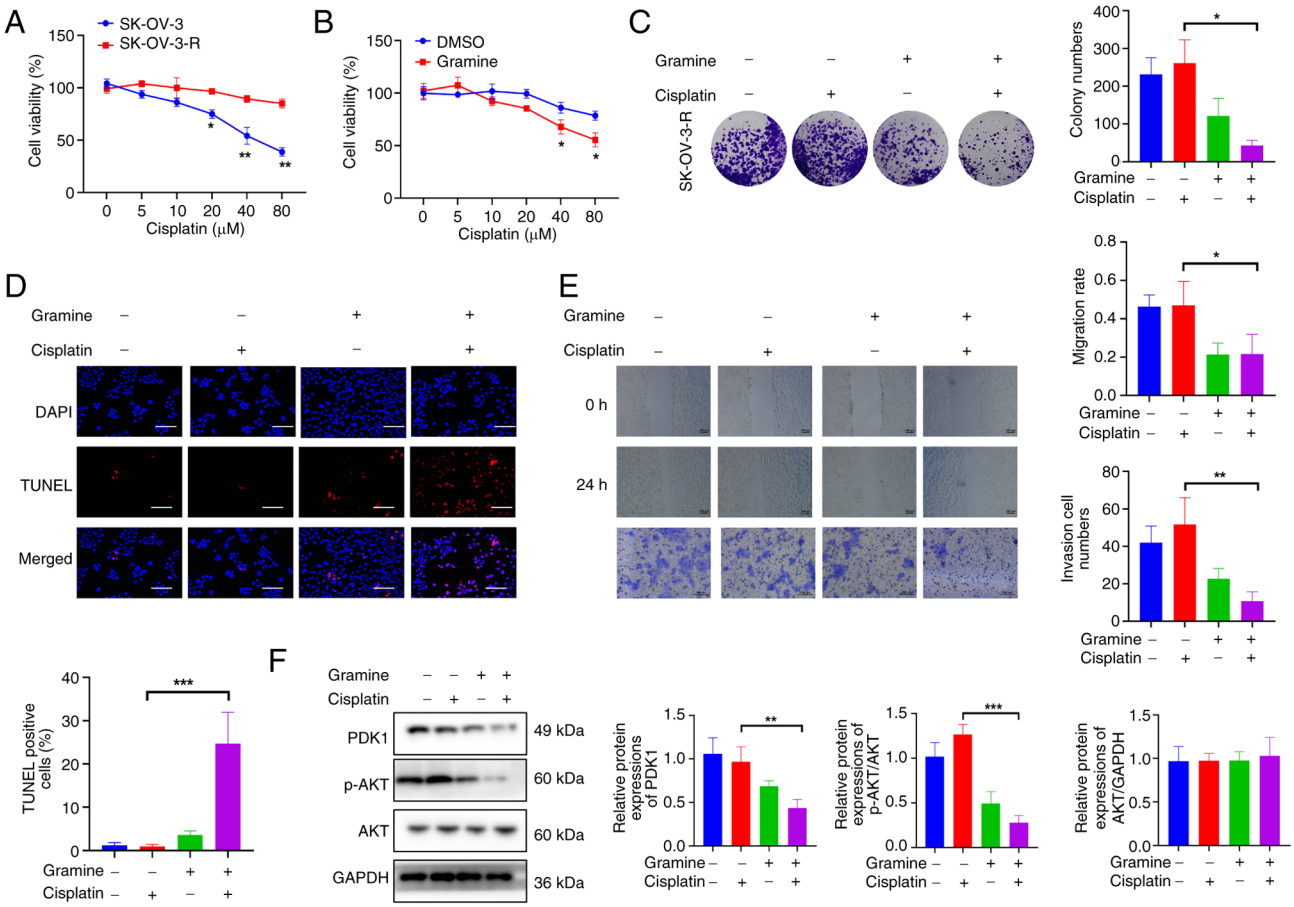

Figure 7. Gramine downregulates PDK1/AKT linked to cisplatin sensitization of OC cells. (A) CCK-8 assay detecting the effect of cisplatin on cell viability. SK-OV-3 and SK-OV-3-R cells were incubated with cisplatin (5, 10, 20, 40, 80 μM) for 24 h. *P<0.05, **P<0.01 vs. the untreated control. (B) CCK-8 assay detecting the effect of gramine and cisplatin on cell viability. SK-OV-3-R cells were incubated with gramine (50 μM) and cisplatin (5, 10, 20, 40, 80 μM) for 24 h. *P<0.05 vs. the DMSO group at the identical cisplatin concentration. (C) Clone formation assay assessing the effect of gramine and cisplatin on colony formation ability. (D) TUNEL assay evaluating the effect of gramine and cisplatin on cell apoptosis. Scale bar, 200 μm. (E) Wound healing and transwell invasion assays detecting the effect of gramine and cisplatin on the abilities of cell migration and invasion respectively. Scale bar, 200 μm. (F) Western blotting assay determining the effect of gramine and cisplatin on the expressions of PDK1 and p-AKT (Thr308). SK-OV-3-R cells were incubated with gramine (50 μM) and cisplatin (80 μM) for 24 h. *P<0.05, **P<0.01, ***P<0.001 vs. the cisplatin group (C-F). Data represent the mean ± SD of three independent experiments. CCK-8, cell counting kit-8.

these data suggest that PDK1/AKT cascade contributes to the anti-tumor activity of gramine in OC cells.

Gramine downregulates PDK1/AKT and is associated with enhanced cisplatin sensitivity in OC cells. It was explored whether gramine could alleviate cisplatin resistance in OC cells. As depicted in Fig. 7A, SK-OV-3-R cells exhibited significant tolerance to cisplatin compared with parental SK-OV-3 cells, even at the maximum tested concentration of 80 μM, confirming successful establishment of the cisplatin-resistant cell model. Functional assays revealed that combined treatment with gramine (50 μM) and cisplatin significantly enhanced the sensitivity of SK-OV-3-R cells to cisplatin, as evidenced by reduced cell viability and colony-forming potential (Fig. 7B and C). Moreover, the co-treatment group presented an increased proportion of TUNEL-positive cells and impaired migration and invasion capacities (Fig. 7D and E). Mechanistically, western blot results demonstrated that combined application of gramine and cisplatin induced a more pronounced reduction in PDK1 and p-AKT (Thr308) proteins abundance compared with the cisplatin monotherapy (Fig. 7F). These data suggest that gramine may increase cisplatin responsiveness in SK-OV-3-R cells, an effect associated with reduced PDK1/AKT signaling.

Discussion

In the present study, it was found that gramine reduced PDK1 protein levels in a dose-dependent manner, however, whether this reflects direct molecular interaction or an indirect consequence remains unresolved. Based on current evidence, the interpretation that PDK1 downregulation is more likely an indirect result of cellular stress rather than direct binding and inhibition. In the present study, biophysical binding assays were not performed to confirm a physical interaction between gramine and PDK1. In addition, the relatively high micromolar concentrations required for observable effects are consistent with a pleiotropic stress response (14). Furthermore, the incomplete rescue by PDK1 overexpression implies that gramine may perturb more upstream signaling nodes (such as PI3K activity or PTEN function) (15,16) or induce broader cellular stress (oxidative or endoplasmic reticulum stress), which

secondarily reduces PDK1 expression (17). Definitive clarification will require reverse transcription-quantitative PCR analysis of *PDK1* mRNA, assessment of ubiquitin-proteasome degradation and direct binding studies.

Among the various cellular phenotypes elicited by gramine, PDK1 overexpression exerted the most pronounced rescue effect on cell proliferation and colony formation, where it partially but consistently reversed gramine-induced growth inhibition. By contrast, the suppression of cell migration and invasion was weakly and incompletely restored by PDK1 overexpression. This disparity suggests that the anti-migratory and anti-invasive activities of gramine are independent of the PDK1/AKT axis, whereas its impact on proliferation is more tightly coupled to this pathway. In addition, proliferation-blocking controls were not included in the present study and time-matched viability tests aligned with the wound-healing incubation period were not performed. Therefore, anti-proliferative effects cannot be fully separated from the specific migratory inhibition in the current dataset. Notably, none of the phenotypes were fully reversed by PDK1 overexpression, indicating that gramine does not act solely through PDK1 inhibition and that additional targets or parallel pathways are involved.

Full AKT activation requires both PDK1-mediated phosphorylation at Thr308 and mTORC2-mediated phosphorylation at Ser473 (18,19). The present data showed that gramine markedly inhibits AKT phosphorylation, but it cannot yet be concluded that this inhibition occurs exclusively through PDK1. While PDK1 is the primary kinase for AKT-Thr308, changes in PI3K activity, activation of AKT phosphatases (such as PP2A or PHLPP) (20) or perturbation of mTORC2 function could equally affect AKT phosphorylation (21). Moreover, published evidence suggests that gramine may influence multiple kinases or receptor pathways (9,22). Therefore, the present conclusion should be cautiously framed as 'gramine suppresses the PDK1/AKT signaling axis', leaving the question of whether AKT inhibition is strictly PDK1-dependent as an open issue. Future cross-validation using selective PI3K inhibitors or AKT phosphatase modulators will help delineate the specificity of this effect.

Cisplatin-based chemotherapy is the first-line treatment for OC, while its resistance markedly limits the efficacy of chemotherapy in OC (23). In the present study, cisplatin-resistant OC cells were used to preliminarily observe that gramine enhances cisplatin sensitivity, associating this effect with PDK1 downregulation and AKT inactivation. However, this conclusion remains preliminary and insufficiently validated. The major limitations of the present study include: i) The absence of quantitative synergy metrics, such as combination index or isobologram analysis, preventing determination of whether the effect is synergistic, additive or merely additive; ii) a lack of PDK1 rescue experiments in resistant cells, which are essential to confirm that sensitization is indeed mediated by PDK1 inhibition; and iii) failure to compare the magnitude of PDK1/AKT suppression between sensitive and resistant cells, or to exclude alternative mechanisms such as modulation of drug efflux pumps (such asABCB1) (24) or apoptosis regulators (such as p53) (25). Therefore, the current data only support that the combination of gramine and cisplatin warrants further investigation, and fall far short of establishing a definitive sensitization mechanism. Future work should systematically address these gaps, including evaluation in animal models and primary cells.

In addition, further limitations must be acknowledged to contextualize the present *in vitro* findings. First, all functional and mechanistic experiments were conducted solely in OC cell lines, with no *in vivo* animal models or clinical specimen validation to assess the activity of gramine under physiological conditions. Second, there is a lack of biophysical binding or target engagement experiments, so it the direct molecular interaction between gramine and PDK1 cannot be confirmed to distinguish direct inhibition from indirect stress-triggered PDK1 downregulation. Third, partial rescue by PDK1 overexpression prevents full establishment of a causal PDK1/AKT-dependent mechanism for the anti-tumor activity of gramine, leaving PDK1-independent pathways uncharacterized. Fourth, the present evaluation of cisplatin responsiveness lacks quantitative synergy metrics, IC_{50} shift data and PDK1 rescue validation in resistant cells, which are necessary to confirm gramine-mediated cisplatin sensitization. Lastly, the relatively high micromolar concentrations of gramine used to produce cellular phenotypes raise concerns about potential non-specific off-target cytotoxicity. Future work will address all these experimental gaps to further validate and expand our preliminary observations.

In summary, gramine effectively inhibits OC cell proliferation, migration and invasion *in vitro*, and shows preliminary potential to sensitize cells to cisplatin, with these effects being at least partially attributable to suppression of the PDK1/AKT pathway. The present findings provide a valuable starting point for deeper investigation but establishing gramine as a credible PDK1-targeting candidate or a clinically applicable sensitizer will require a substantially stronger body of *in vitro* and *in vivo* evidence.

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Availability of data and materials

Data generated in the present study may be requested from the corresponding author.

Authors' contributions

HW, conceptualized the study, performed the literature search and experiments, and wrote the original draft. RZ and BW performed the analysis and interpretation of data, and reviewed and edited the manuscript. SH and HC performed the analysis and interpretation of data, and supervised the study. HW and RZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no that they have no competing interests.

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