ORIGINAL ARTICLE

Roflumilast enhances cisplatin-sensitivity and reverses cisplatin-resistance of ovarian cancer cells via cAMP/PKA/CREB-FtMt signalling axis

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Abstract

Objective: We previously demonstrated the roflumilast inhibited cell proliferation and increased cell apoptosis in ovarian cancer. In this study, we aimed to investigate the roles of roflumilast in development of cisplatin (DDP)-sensitive and -resistant ovarian cancer.

Methods: OVCAR3 and SKOV3 were selected and the corresponding DDP-resistant cells were constructed. Cell viability, proliferation, apoptosis, cycle were performed. Expression cAMP, PKA, CREB, phosphorylation of CREB and FtMt were detected. The roles of roflumilast in development of DDP-sensitive and -resistant ovarian cancer were confirmed by xenograft model.

Results: Roflumilast + DDP inhibited cell proliferation, and induced cell apoptosis and G0/G1 arrest in OVCAR3 and SKOV3 cells, roflumilast induced expression of FtMt, the activity of cAMP and PKA and phosphorylation of CREB in ovarian cancer cells and the above-effect were inhibited by H89. Downregulation of CREB inhibited the roflumilast-increased DDP sensitivity of ovarian cancer cells, and the roflumilast-induced FtMt expression and phosphorylation of CREB. Also, roflumilast reversed cisplatin-resistance, and induced expression of FtMt and activation of cAMP/PKA/CREB in DDP-resistant ovarian cancer cells. Similarly, treated with H89 or downregulation of CREB inhibited the changes induced by roflumilast. In vivo, roflumilast inhibited the development of SKOV3 or SKOV3-DDP-R xenograft models.

Conclusions: Roflumilast enhanced DDP sensitivity and reversed the DDP resistance of ovarian cancer cells via activation of cAMP/PKA/CREB pathway and upregulation of the downstream FtMt expression, which has great promise in clinical treatment.

1 | INTRODUCTION

The incidence of ovarian cancer is ranked third in female genital malignant tumours.^{1,2} But the mortality rate of ovarian cancer is ranked first in female genital malignant tumours, which seriously threatens the life and health of women.³⁻⁵ Despite

extensive cytoreductive surgery and cisplatin (DDP)-based post-operative chemotherapy in ovarian cancer patients, the annual survival rate of ovarian cancer patients is still hovering at 20%-30%, and the 10-year survival rate of ovarian cancer patients is only 4%-20%.^{6,7} There is lack of specific clinical symptoms and corresponding methods for early diagnosis of ovarian cancer, so that the majority of patients with ovarian cancer at the time of treatment has been in advanced stages. When ovarian

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cancer patients were treated with DDP-based chemotherapy, about 15%-25% patients were present primary drug resistance and among them about 80% patients were eventually developed to secondary DDP resistance.⁸⁻¹¹ Therefore, drug resistance can directly affect the efficacy of chemotherapy and the prognosis of ovarian cancer patients. At present, exploring the mechanism of drug resistance in ovarian cancer and developing new drug-resistant reversing agent have become a hot spot in the field of oncology worldwide.

Cyclic adenosine monophosphate (cAMP) could activate protein kinase A (PKA) and cyclic AMP response element-binding protein (CREB), playing important roles in gene regulation, cell migration, cell proliferation, cell apoptosis and mitochondrial homeostasis.¹²⁻¹⁴ Phosphodiesterases (PDEs) are responsible for hydrolyzation of cAMP to its inactive 5'-monophosphate.¹⁵ In recent years, several scholars have begun to explore whether inhibition of PDE4 could play an anti-tumor effect.¹⁶⁻²¹ Our previous study investigated the role of PDE4 inhibitor roflumilast in the treatment of ovarian cancer.²² After administration with roflumilast, the proliferations of ovarian cancer cell lines OVCAR3 and SKOV3 were significantly inhibited, and the cell apoptosis in those cells were significantly increased.²² Roflumilast activated the cAMP/PKA/CREB pathway and upregulated the mitochondrial ferritin (FtMt) level in OVCAR3 and SKOV3 cells.²² Overexpression of FtMt in OVCAR3 and SKOV3 cells induced cell apoptosis and inhibited tumour development.²² The PKA inhibitor H89 suppressed the expression of FtMt and restored the roflumilast-inhibited cell proliferation and -induced cell apoptosis, indicating the PKA pathway involved in the roflumilast-inhibited cell proliferation and -induced-cell apoptosis.²² In addition, downregulation of CREB significantly inhibited the expression of FtMt, and levels of PKA activity and phosphorylation of CREB.²² Those findings suggested that roflumilast promoted cell proliferation and inhibited cell apoptosis in ovarian cancer through upregulation of FtMt via cAMP/PKA/CREB pathway. DDP is a basic medicine for combined chemotherapy of ovarian cancer and plays an important role in the comprehensive treatment of ovarian cancer. However, the roles of roflumilast in DDP sensitivity and DDP resistance of ovarian cancer cells are still unclear.

In this study, we aimed to study the effect of PDE4 inhibitor roflumilast on the proliferation, apoptosis and cell-cycle progression ovarian cancer cell lines OVCAR3 and SKOV3 cells that were treated with DDP. Then, DDP-resistant ovarian cancer cells including OVCAR3-DDP-R and SKOV3-DDP-R were constructed. The effect of roflumilast on the proliferation, apoptosis and cell cycle progression of DDP-resistant ovarian cancer cells were investigated. The changes in FtMt expression and cAMP/PKA/CREB pathway were also investigated. Functional analysis of chemoresistance-related genes will not only help to elucidate the molecular mechanism of chemoresistance in ovarian cancer, but will also help to find new targets for drug screening and prognosis for ovarian cancer treatment.

2 | MATERIAL AND METHODS

2.1 | Cell culture

This study was performed on two ovarian cancer cell lines, OVCAR3 and SKOV3 (Cell bank of the Chinese academy of sciences, Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% foetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 1% streptomycin and 1% ampicillin in 5% CO₂ at 37°C. DDP-resistant ovarian cancer cells were produced by treatment with DDP.

2.2 | Cell transfection

Cells were transfected with shRNA pGenesil-CREB or shRNA negative control (NC) in 12-well plates for the indicated time. The shRNA vectors were synthesized by GenePharma, Shanghai, China. Transfection was performed with 1 ng/mL shRNA, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3 | Roflumilast and H89 treatments

Cells were treated with the PDE4 inhibitor roflumilast (15 µM) (Sigma, MO, USA) and/or the PKA inhibitor H89 (10 µM) (Sigma, St. Louis, MO, USA) for the indicated times and at the indicated concentrations. Cell proliferation, apoptosis, cell cycle, and tumour formation assays were then performed.

2.4 | Cell proliferation assays

After treatment, cell proliferation was detected by cell counting kit-8 (CCK-8; Kumamoto, Japan), EDU staining (Roche, USA), and colon formation assays. For the CCK-8 assay, cells were seeded in 96-well plates. The CCK-8 test solution was added 4 h before the end of treatment. The absorbance was measured at 450 nm using a microplate reader. For the EDU assay, cells were fixed with 4% paraformaldehyde (PFA) for 30 min and then stained with EDU and DAPI. The images were captured with FV10i fluorescence microscopy (Olympus, Shinjuku-ku, Tokyo, Japan). For colon formation assay, cells were seeded in 6-well plates at 500 cells/well and exposed to the indicated treatment. After 13 days, cells were fixed with 4% PFA and stained with 0.1% crystal violet.

2.5 | Flow cytometry assay

After treatment, cell apoptosis and cell cycle were detected by flow cytometry (BD FACS Calibur, USA). To assess apoptosis, 1×10⁵ cells were stained with an annexin V and propidium iodide (PI, eBioscience) mixture. For cell cycle, 6×10⁴ cells were stained with 10 µg/mL RNase (Thermo, Grand Island, New York, USA) and 50 µg/mL PI solution in the dark for 30 min. Then, the distribution of cell cycle phase was analysed.

TABLE 1 Primers used in this study

ID	Sequence (5'-3')	Product length (bp)
GAPDH F	TGTTTCGTCATGGGTGTGAAC	154
GAPDH R	ATGGCATGGACTGTGGTCAT	
Lnc-CCAT1 F	CCTACGCATACCTCTGCTTCT	138
Lnc-CCAT1 R	GATTGCTCCTGTTTCCCTTT	

2.6 | Hoechst staining assay

After treatment, cells were fixed with 4% PFA, and stained with 0.1 µg/mL Hoechst 33342 (Sigma, MO, USA). The changes in nuclear morphology were visualized, using FV10i fluorescence microscopy (Olympus, Shinjuku-ku, Tokyo, Japan) with a 365-nm filter.

2.7 | ELISA detection of cAMP and PKA activities

After treatment, the levels of cAMP and PKA activities were detected, using competitive ELISA kits (Invitrogen, Carlsbad, CA, USA). The cAMP ELISA kit (Cat. EMSCAMPL) and PKA ELISA kit (Cat. EIAPKA) were used in this study according to the manufacturer's instructions.

2.8 | RNA isolation and real time-quantitative PCR (RT-qPCR)

After treatment, total RNAs from cells were isolated using Trizol reagent (Invitrogen) according to the standard protocol. RT-qPCR was performed using the SYBR Premix Ex Taq™ (Takara, Shiga, Japan) and Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers used in this study are listed in Table 1. The tests were performed in triplicate. Gene expression was normalized to GAPDH expression, and calculated using the method of $2^{-\Delta\Delta C_t}$.

2.9 | Immunofluorescence

After treatments, cells were fixed with 4% PFA in PBS for 15 min and permeabilized with 100% methanol for 10 min at -20°C. After blocking with 2% goat serum for 60 min, cells were incubated with the primary antibody against FtMt (#ab66111, Abcam, Cambridge, Massachusetts, USA) overnight at 4°C. After incubation with fluorochrome-conjugated secondary antibody for 1-2 h at room temperature in the dark, the nucleus was stained using DAPI. The cells were visualized by LSM 510.²³

2.10 | Xenograft model

The animal experiments were approved by the Animal Care and Use Committee of Nanfang Hospital, Southern Medical University. A total of 2×10^6 SKOV3 or SKOV3-DDP-R cells were injected subcutaneously into 6-week-old male BALB/c nude mice. Tumour diameters were measured using a digital caliper and calculated using the formula: volume = $\pi/6(\text{width})^2 \times \text{length}$. When tumours were grown to 100-150 mm³, the mice were randomly divided into control,

DDP, and DDP + roflumilast groups (for SKOV3), and control and roflumilast groups (for SKOV3-DDP-R). For roflumilast and/or DDP treatment, 5 mg/kg roflumilast and/or 2 mg/kg DDP was injected intraperitoneally (i.p.) into mice five times, 3 days apart. After the last treatment, the mice were sacrificed and the xenograft tumours were harvested for subsequent experiments.

2.11 | Immunohistochemistry

The tumour tissues were paraffin embedded and sectioned to 2-µm thickness. After heating at 60°C for 30 min, the sections were deparaffinized with xylene for 5 min and rehydrated in graded concentrations of ethanol. Antigen retrieval was performed and then the slides were blocked with 3% H₂O₂ for 30 min and 3% BSA for 1 h. Then, sections were incubated with primary antibody against ki-67 (Abcam, Cambridge, Massachusetts, USA), p-CREB (Cell Signaling Tech, MA, USA) and FtMt (Abcam, USA) at 4°C overnight. After washing, horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed Laboratories Inc., San Francisco, California) and DAB chromogen EnVision Detection System kit (DAKO, Produktionsvej, Glostrup, Denmark) were used for detection. Hematoxylin was used for nuclear staining.

2.12 | Western blotting

Proteins were extracted from cells or tissues using RIPA lysis buffer and quantified by Pierce BCA Protein Assay Kit (Thermo, USA). The proteins were separated by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. After blocking with 5% skim milk, the membranes were incubated with primary antibodies against GAPDH (#ab8245, Abcam, USA), FtMt (#ab124889, Abcam, USA), CREB (#ab32096, Abcam, USA), and p-CREB (#9198, Cell Signalling Technology, USA) overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibody (#BS12478, #BS13278, Bioworld, China) for 1 h at room temperature, and the blots were visualized, using the ECL-Plus reagent (Millipore, Billerica, MA, USA).

2.13 | Statistical analysis

Data were performed in triplicate and presented as mean ± standard deviation. The analysis was performed with SPSS 16.0 using student *t* tests for two groups, and one-way ANOVA with SNK for multiple group comparisons. *P* < .05 was considered to be statistically significant.

3 | RESULTS

3.1 | Roflumilast increased the DDP sensitivity of ovarian cancer cells

In both OVCAR3 (Figure 1A) and SKOV3 cells (Figure 1B), administration of roflumilast for 24 h increased the inhibitor rate in a dose-dependent manner. The calculated IC₅₀ values of OVCAR3 and SKOV3 cells after DDP treatment were 2.936 and 3.574 µM, respectively. After roflumilast + DDP treatment, the calculated IC₅₀ values

FIGURE 1 Effect of roflumilast on DDP sensitivity in ovarian cancer cells. OVCAR3 and SKOV3 cells were exposed to different concentrations of roflumilast. Inhibition rates in A, OVCAR3 and B, SKOV3 cells were determined. Then, after administration of DDP (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 $\mu\text{g}/\text{mL}$) or DDP (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 $\mu\text{g}/\text{mL}$) + roflumilast (15 μM), flow cytometry and Hoechst assays were performed. Apoptosis in C, OVCAR3 and D, SKOV3 cells was evaluated. Hoechst staining was performed in E, OVCAR3 and F, SKOV3 cells. Cell cycle distribution was determined in G, OVCAR3 and H, SKOV3 cells

decreased to 1.61 and 1.315 μM , respectively. DDP increased the apoptosis rates in OVCAR3 (Figure 1C) and SKOV3 (Figure 1D) cells. After administration of roflumilast + DDP, the apoptosis rates were significantly increased, compared with DDP. Hoechst assay showed that roflumilast significantly increased apoptosis in OVCAR3 and SKOV3 cells in presence of DDP (Figure 1E,F). The cell cycle was then assessed, and the results showed that roflumilast increased the DDP-induced G0/G1 arrest in OVCAR3 and SKOV3 cells (Figure 1G,H).

3.2 | Roflumilast induced expression of FtMt and increased the activity of cAMP/PKA/CREB in ovarian cancer cells

We found that roflumilast significantly increased the DDP-induced expression of FtMt, as assessed by levels of both mRNA (Figure 2A,B) and protein (Figure 2C–F) in OVCAR3 and SKOV3 cells.

Given that PDE4 could hydrolyse and inactivate cAMP, it was expected that roflumilast, an inhibitor of PDE4, elevated the level of cAMP in OVCAR3 cells (Figure 3A). Also, roflumilast increased the activity of PKA, a cAMP effector in OVCAR3 cells (Figure 3B). Moreover, roflumilast induced the phosphorylation of CREB in both OVCAR3 (Figure 3C) and SKOV3 cells (Figure 3D).

3.3 | H89 inhibited roflumilast-mediated increase in DDP sensitivity of ovarian cancer cells

H89 reversed roflumilast inhibition of cell viability to a level similar to that seen with DDP treatment in both OVCAR3 (Figure 4A) and SKOV3 (Figure 4B) cells. Also, H89 reversed the roflumilast inhibition of cell proliferation to a level similar to that seen with DDP treatment in both cells (Figures 4C,D). Both flow cytometry and

FIGURE 2 Effect of roflumilast on expression of FtMt. OVCAR3 and SKOV3 cells were treated with DDP (1 $\mu\text{g/mL}$) or DDP (1 $\mu\text{g/mL}$) + roflumilast (15 μM) for 24 h. The expression of FtMt in mRNA and protein levels in OVCAR3 and SKOV3 cells was assessed by A–B, qRT-PCR, C–D, Western blotting, and E–F, immunofluorescence, respectively. ** $P < .01$, *** $P < .001$ vs control; ## $P < .01$ vs DDP

Hoechst assays showed that H89 significantly inhibited roflumilast-induced cell apoptosis (Figures 4E–H). Interestingly, H89 reduced roflumilast-induced G0/G1 arrest in both cells (Figure 4I,J).

H89 inhibited the DDP + roflumilast-induced increase in FtMt mRNA in both OVCAR3 and SKOV3 cells (Figure 5A,B). Consistent results in protein levels were observed by immunostaining and Western blotting (Figure 5C–F). H89 also inhibited the DDP + roflumilast-induced phosphorylation of CREB in both cells (Figure 5E,F). In addition, H89 inhibited the DDP + roflumilast-induced PKA activity (Figure 5G).

3.4 | Downregulation of CREB inhibited the roflumilast-mediated increase in DDP sensitivity of ovarian cancer cells

Downregulation of CREB reversed roflumilast inhibition of cell viability to a level similar to DDP treatment in OVCAR3 (Figure 6A) and SKOV3 (Figure 6B) cells. Roflumilast inhibition of cell proliferation was reversed by CREB shRNA to levels similar to DDP treatment in OVCAR3 (Figure 6C) and SKOV3 (Figure 6D) cells. Downregulation of CREB inhibited roflumilast-induced cell

FIGURE 3 Effect of roflumilast on activity of cAMP/PKA/CREB. After administration with DDP (1 μ g/mL) or DDP (1 μ g/mL) + roflumilast (15 μ M) for 24 h, the activity of A, cAMP and B, PKA were detected by ELISA in OVCAR3 cells. The expression of CREB and phosphorylation of CREB were detected by Western blotting in both C, OVCAR3 and D, SKOV3 cells. ** P < .01, *** P < .001 vs control; ### P < .01 vs DDP

apoptosis (Figure 6E–H) and roflumilast-induced G0/G1 arrest in both cells (Figure 6I,J).

Expression of FtMt mRNA in OVCAR3 and SKOV3 cells was significantly inhibited by CREB shRNA, compared with DDP + roflumilast (Figures 7A,B). Downregulation of CREB inhibited DDP + roflumilast-induced FtMt expression and phosphorylation of CREB (Figure 7C–F).

3.5 | Roflumilast inhibited the growth of ovarian cancer cell xenograft tumour and induced the expression of FtMt and phosphorylation of CREB

To investigate the anti-tumour effects of roflumilast in vivo, SKOV3 xenograft models were established and treated with roflumilast (5 mg/kg). Roflumilast inhibited tumour growth and restricted the tumour volume (Figure 8A,B). Ki-67 staining showed that roflumilast inhibited cell proliferation (Figure 8C). TUNEL assay showed that roflumilast induced cell apoptosis (Figure 8C). Roflumilast induced expression of FtMt and phosphorylation of CREB (Figure 8C,D).

3.6 | Roflumilast reversed cisplatin resistance of DDP-resistant ovarian cancer cells

DDP increased inhibitor rates in OVCAR3-DDP-R and SKOV3-DDP-R cells in a dose-dependent manner. The calculated IC₅₀

values of DDP in OVCAR3-DDP-R and SKOV3-DDP-R were 18.58 and 35.33 μ M respectively (Figure 9A,B). Cells were treated with various concentrations of roflumilast, and cell viability was assessed (Figure 9C,D). Roflumilast increased cell viability in OVCAR3-DDP-R and SKOV3-DDP-R cells in a dose-dependent manner. Roflumilast (15 μ M) also increased cell viability in OVCAR3-DDP-R and SKOV3-DDP-R cells in a time-dependent manner (Figure 9E,F). The inhibitor rates were increased in a dose-dependent manner (Figure 9G,H). The calculated IC₅₀ values of roflumilast in OVCAR3-DDP-R and SKOV3-DDP-R were 10.07 and 15.44 μ M, respectively (Figure 9G,H). After treatment with roflumilast, cell proliferation and colony formation in OVCAR3-DDP-R and SKOV3-DDP-R cells were significantly inhibited (Figure 9I–K). Roflumilast induced cell apoptosis in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 9L,M). In addition, roflumilast induced G0/G1 arrest in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 9N).

3.7 | Roflumilast induced expression of FtMt and increased the activity of cAMP/PKA/CREB in DDP-resistant ovarian cancer cells

Roflumilast induced FtMt mRNA expression in both cells (Figure 10A,B), FtMt protein expression in both cells (Figure 10C–D), activities of cAMP and PKA (Figure 10E,F). In addition, the phosphorylation of CREB was significantly increased by roflumilast in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 10G).

FIGURE 4 Effect of PKA on DDP sensitivity of ovarian cancer cells. Cells were treated with H89 before administration of DDP and roflumilast. A, Viability of OVCAR3 cells. B, Viability of SKOV3 cells. C, Edu staining in OVCAR3 cells. D, Edu staining in SKOV3 cells. E, Apoptosis in OVCAR3 cells. F, Apoptosis in SKOV3 cells. G, Hoechst staining in OVCAR3 cells. H, Hoechst staining in SKOV3 cells. I, Cell cycle in OVCAR3 cells. J, Cell cycle in SKOV3 cells

3.8 | H89 inhibited the roflumilast reversal of cisplatin resistance in DDP-resistant ovarian cancer cells

H89 significantly reversed roflumilast inhibition of cell viability in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 11A,B). H89 reversed roflumilast-inhibition of cell proliferation in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 11C), roflumilast

induction of cell apoptosis in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 11D,E), and roflumilast inhibition of colon formation in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 11F). In addition, H89 reversed roflumilast-mediated cell arrest in G0/G1 phase in OVCAR3-DDP-R and SKOV3-DDP-R cells (Figure 11G).

H89 inhibited roflumilast-induced FtMt expression (Figure 11H,I), phosphorylation of CREB (Figure 11I), and PKA activity (Figure 11J) in OVCAR3-DDP-R and SKOV3-DDP-R cells.

FIGURE 5 Expression of FtMt and p-CERB, and PKA activity in cells treated with H89. Cells were treated with H89 before administration of DDP and roflumilast. A, FtMt mRNA in OVCAR3 cells. B, FtMt mRNA in SKOV3 cells. C, Staining of FtMt in OVCAR3 cells. D, Staining of FtMt in SKOV3 cells. E, Western blotting of FtMt and p-CERB in OVCAR3 cells. F, Western blotting of FtMt and p-CERB in SKOV3 cells. G, PKA activity in SKOV3 cells. ** $P < .01$, *** $P < .001$ vs control; ## $P < .01$ vs DDP. %% $P < .01$ vs DDP + roflumilast

FIGURE 6 Effect of CREB shRNA on cell proliferation, cell apoptosis, and cell cycle. Cells were transfected with CREB shRNA and then treated with DDP and roflumilast. Viability of A, OVCAR3 and B, SKOV3 cells. Edu staining in C, OVCAR3 and D, SKOV3 cells. Apoptosis in E, OVCAR3 and F, SKOV3 cells. Hoechst staining in G, OVCAR3 and H, SKOV3 cells. Cell cycle in I, OVCAR3 and J, SKOV3 cells

FIGURE 7 Effect of CREB shRNA on expression of FtMt and p-CERB in cells treated with DDP and roflumilast. Cells were transfected with CREB shRNA and then treated with DDP and roflumilast. FtMt mRNA in A, OVCAR3 and B, SKOV3 cells. Staining of FtMt in C, OVCAR3 and D, SKOV3 cells. Western blotting of FtMt and p-CERB in E, OVCAR3 and F, SKOV3 cells. ** $P < .01$, *** $P < .001$ vs control; ## $P < .01$ vs DDP. %% $P < .01$ vs DDP + roflumilast

3.9 | Downregulation of CREB inhibited roflumilast reversal of cisplatin resistance in DDP-resistant ovarian cancer cells

Downregulation of CREB reversed roflumilast inhibition of cell viability (Figure 12A,B), roflumilast inhibition of cell proliferation (Figure 12C), roflumilast induction of cell apoptosis (Figure 12D,E), roflumilast inhibition of colon formation (Figure 12F), and roflumilast-induced G0/G1 arrest (Figure 12G) in OVCAR3-DDP-R and SKOV3-DDP-R cells.

Roflumilast-induced expression of FtMt in OVCAR3-DDP-R and SKOV3-DDP-R cells was significantly inhibited by CREB shRNA

(Figures 12H,I). Downregulation of CREB inhibited roflumilast-induced phosphorylation of CREB (Figure 12I).

3.10 | Roflumilast inhibited the growth and induced the expression of FtMt and phosphorylation of CREB in a DDP-resistant ovarian cancer cell xenograft model

The effects of roflumilast on DDP resistance in ovarian cancer cells was investigated in vivo. SKOV3-DDP-R xenograft models were established and treated with roflumilast (5 mg/kg). Roflumilast

FIGURE 8 The anti-tumour effects of roflumilast in vivo. A total of 2×10^6 SKOV3 cells were subcutaneously injected in rear flank of nude mice (4 per group) and 5 mg/kg roflumilast or PBS was administered (i.p.) in mice. A, Tumour. B, The mean tumour size was measured in mm³. C, HE and TUNEL staining, and IHC staining of Ki-67, FtMt, CREB, and p-CREB were determined. D, Western blots of FtMt, CREB, and p-CREB

inhibited tumour growth and restricted the tumour volume (Figure 13 A,B). Roflumilast induced levels of cAMP and PKA (Figure 13C,D). Ki-67 staining showed that roflumilast inhibited cell proliferation (Figure 13E), and TUNEL assay showed that roflumilast induced cell apoptosis (Figure 13E). Roflumilast induced the expression of FtMt and phosphorylation of CREB (Figure 13 E,F).

4 | DISCUSSION

Ovarian cancer is one of the malignant tumours of the female reproductive system. Its incidence has risen in recent years.^{1,2} Ovarian cancer is a highly lethal malignancy that is prone to blood and lymph node metastasis and local infiltration and easily develops resistance to chemotherapeutic drugs. The prognosis for ovarian cancer patients is poor, with a 5-year survival rate of only 25%-30%.³⁻⁵ The mechanism of resistance to chemotherapeutic drugs in ovarian cancer has become a hot area of research in recent years.^{6,7}

Roflumilast is a PDE4 inhibitor that has been shown to enhance glucocorticoid-mediated apoptosis via upregulation of mRNA levels of glucocorticoid receptor in B cell chronic lymphocytic leukaemia cells.²⁴ However, in breast cancer cells, PDE4 inhibitor suppresses cell migration with little effect on cell proliferation.²⁵ In non-small cell lung cancer, roflumilast is used as an orally administered drug for the treatment of inflammatory conditions of the lungs. The combination of DDP with roflumilast has been shown

to induce satisfactory apoptosis in lung cancer.²¹ It was implicated that the anti-tumour effect of roflumilast might depend on cell type. Consistent with findings in non-small-cell lung cancer, roflumilast induced cell apoptosis and inhibited proliferation of ovarian cancer cells via upregulation of FtMt.²² It was suggested that roflumilast could be used to treat ovarian cancer. However, roflumilast has many side effects in lung cancer treatment. Although the side effects in ovary cancer are unclear, alterations in the structure of the drug or improvement in synthetic analogues may decrease side effects.²⁶

Upregulation of FtMt has been hypothesized as a possible mechanism for roflumilast-induced cell apoptosis. In this study, we found that roflumilast induced cell apoptosis and inhibited proliferation of OVC cells via upregulation of FtMt. FtMt is an H-ferritin-like protein in mitochondria. It is encoded by an intronless gene mapped to chromosome 5q23.1 and involved in modulating cellular iron metabolism and oxidative stress.²⁷⁻³⁰ FtMt dysregulation has been reported in erythroid cells from patients with sideroblastic anaemia.³¹ FtMt was upregulated after ABCB7 silencing in normal progenitors during erythroid differentiation.³² FtMt was decreased in nervous system tumour tissues, including neuroblastoma and neurospongoma,³³ and overexpression of FtMt suppresses the proliferation of neuroblastoma cells.³³ In this study, roflumilast enhanced cisplatin sensitivity in OVCAR3 and SKOV3 cells, and reversed cisplatin resistance in DDP-resistant ovarian cancer cells. The role of roflumilast in cisplatin sensitivity and cisplatin resistance in ovarian cancer cells was confirmed in a xenograft model. Therefore, upregulation of

FIGURE 9 Effect of roflumilast on cisplatin resistance of DDP-resistant ovarian cancer cells. OVCAR3-DDP-R and SKOV3-DDP-R were constructed. After treatment with DDP, the inhibitor rate of A, OVCAR3-DDP-R and B, SKOV3-DDP-R cells was detected. C and D, Viability of OVCAR3-DDP-R and SKOV3-DDP-R after treatment with indicated concentration of roflumilast. E–F, Cell viabilities of OVCAR3-DDP-R and SKOV3-DDP-R after treatment with roflumilast for the indicated time. G–H, Inhibitor rate in OVCAR3-DDP-R and SKOV3-DDP-R cells after treatment with roflumilast at the indicated concentration. The cells were treated with roflumilast (15 μ M) for 24 h. I–J, Edu staining in OVCAR3-DDP-R and SKOV3-DDP-R cells. K, Colon formation in OVCAR3-DDP-R and SKOV3-DDP-R cells. L, Apoptosis in OVCAR3-DDP-R and SKOV3-DDP-R cells. M, Hoechst staining in OVCAR3-DDP-R and SKOV3-DDP-R cells. N, Cell cycle in OVCAR3-DDP-R and SKOV3-DDP-R cells

FIGURE 10 Effect of roflumilast on expression of FtMt and activity of cAMP/PKA/CREB in DDP-resistant ovarian cancer cells. OVCAR3-DDP-R and SKOV3-DDP-R were treated with roflumilast. Then, A and B, FtMt mRNA expression in OVCAR3-DDP-R and SKOV3-DDP-R cells was detected by RT-qPCR. C, FtMt protein expression in OVCAR3-DDP-R and SKOV3-DDP-R cells was detected by Western blotting. D, Staining of FtMt in OVCAR3-DDP-R and SKOV3-DDP-R cells was detected. E and F, Activities of cAMP and PKA in SKOV3-DDP-R cells were detected. G, Phosphorylation of CREB in OVCAR3-DDP-R and SKOV3-DDP-R cells was detected

FIGURE 11 Effect of PKA on roflumilast-mediated reversal of cisplatin-resistance in DDP-resistant ovarian cancer cells. OVCAR3-DDP-R and SKOV3-DDP-R cells were treated with H89 before administration of roflumilast. A, Viability of OVCAR3-DDP-R cells. B, Viability of SKOV3-DDP-R cells. C, Edu staining in OVCAR3-DDP-R and SKOV3-DDP-R cells. D, Apoptosis in OVCAR3-DDP-R and SKOV3-DDP-R cells. E, Hoechst staining in OVCAR3-DDP-R and SKOV3-DDP-R cells. F, Colony formation of OVCAR3-DDP-R and SKOV3-DDP-R cells. G, Cell cycle in OVCAR3-DDP-R and SKOV3-DDP-R cells. H, Staining of FtMt in OVCAR3-DDP-R and SKOV3-DDP-R cells. I, Western blotting of FtMt and p-CERB in OVCAR3-DDP-R and SKOV3-DDP-R cells. J, PKA activity in OVCAR3-DDP-R and SKOV3-DDP-R cells. ** $P < .01$, *** $P < .001$ vs control; ## $P < .01$ vs roflumilast

FtMt might be involved in both roflumilast-mediated enhancement of cisplatin sensitivity and roflumilast-mediated reversal of cisplatin resistance in ovarian cancer. It is still unclear why FtMt has different

roles. Additional research will be needed to understand details of the mechanism of roflumilast enhancement of cisplatin sensitivity and roflumilast reversal of cisplatin resistance in ovarian cancer. Studies

FIGURE 12 Effect of CREB shRNA on cell proliferation, cell apoptosis, cell cycle, expression of FtMt and p-CERB in DDP-resistant ovarian cancer cells. A, Viability of OVCAR3-DDP-R and B, SKOV3-DDP-R cells. C, Edu staining. D, Apoptosis was detected by flow cytometry. E, Hoechst staining. F, Colon formation. G, Cell cycle. H, Staining of FtMt. I, Western blotting of FtMt and p-CERB in OVCAR3-DDP-R and SKOV3-DDP-R cells

will also be needed to identify the molecules that differentiate DDP-sensitive from DDP-resistant ovarian cells. The details in mechanism should be studied in the future.

cAMP is an important second messenger involved in signalling cascades. The cAMP analogs such as 8-Cl-cAMP significantly abrogated proliferation and induced cell apoptosis in medullary thyroid cancer cells.³⁴ PDEs can hydrolyse cAMP,¹⁵ which in turn can activate PKA and CREB.¹²⁻¹⁴ Roflumilast treatment significantly induced cAMP levels in a murine lung cancer model.¹⁷ Our previous study suggested that roflumilast promoted cell proliferation and inhibited cell apoptosis in ovarian cancer through upregulation of FtMt via cAMP/PKA/CREB pathway.²² Both FtMt and cAMP regulated CREB.^{22,35,36} cAMP-mediated inhibition of JNK activation

antagonized UV-induced apoptosis in CREB-dependent manner.³⁷ This study showed that roflumilast significantly enhanced cAMP levels in DDP-sensitive cells and in DDP-resistant cells. The increased levels of cAMP could activate cAMP-dependent PKA, which phosphorylates many target proteins including CREB.^{35,36} However, the relationship between FtMt and cAMP is unclear.

The level of CREB expression in ovarian cancer is related to tumour stage and prognosis of the ovarian cancer patient.³⁸ With an increase in staging, CREB expression increased significantly, indicating that CREB plays an important role in the progress of ovarian cancer.³⁸ High CREB expression is associated with poor prognosis.³⁹ In mucinous ovarian cancer, the most common mutation is in K-ras, and activating mutant K-ras will lead to CREB phosphorylation, resulting

FIGURE 13 The effects of roflumilast on DDP resistance in ovarian cancer in vivo. A total of 2×10^6 SKOV3-DDP-R cells were subcutaneously injected in the rear flank of nude mice (4 per group) and 5 mg/kg roflumilast or PBS was administered (i.p.) to mice. A, Tumour. B, The mean tumour size measured in mm³. C and D, Activities of cAMP and PKA in SKOV3-DDP-R cells were detected. E, HE and TUNEL staining, and IHC staining of Ki-67, FtMt, CREB, and p-CREB were determined. F, WB results of FtMt, CREB, and p-CREB

in enhanced CREB activity.⁴⁰ CREB plays an important role in the development of ovarian cancer. The PKA inhibitor H89 inhibited CREB activity in both cisplatin-sensitivity and cisplatin-resistance

of ovarian cancer cells. Furthermore, knockdown of CREB by CREB shRNA confirmed the CREB mediated the upregulation of FtMt and was involved in roflumilast enhancement of cisplatin sensitivity and

roflumilast reversal of cisplatin resistance in ovarian cancer cells. Thus, the activity of PKA and phosphorylation of CREB were upstream of FtMt.

In conclusion, roflumilast enhanced DDP sensitivity in DDP-sensitive ovarian cancer cells, and reversed DDP resistance in DDP-resistant ovarian cancer cells via activation of the cAMP/PKA/CREB pathway and upregulation of downstream FtMt expression. These findings suggest that the PDE4 inhibitor roflumilast may have therapeutic potential as an anti-tumour treatment or may help overcome clinical drug resistance in ovarian cancer. However, which molecular decides the difference between DDP-sensitive and DDP-resistant ovarian cells need to be studied in the future.

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CONFLICT OF INTEREST

No competing financial interests exist.

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