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ORIGINAL RESEARCH

Magnoflorine inhibits cisplatin-induced protective autophagy by down-regulating HMGB1 and increases drug sensitivity in drug-resistant ovarian cancer cells

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Abstract

Ovarian cancer (OC) is a prevalent type of gynecologic malignancy, and to improve its treatment effectiveness, it is essential to identify new and more potent therapeutic drugs. Magnoflorine is a fundamental chlorine alkaloid with various pharmacological properties, including anti-diabetic and anti-inflammatory effects. However, whether Magnoflorine can inhibit the protective autophagy induced by cisplatin by modulating (high-mobility group B1) HMGB1 requires further investigation. This study aims to assess the impact of Magnoflorine on cisplatin resistance in OC. Our findings demonstrate that Magnoflorine enhances the sensitivity of OC cells to cisplatin. Additionally, it promotes apoptosis in cisplatin-resistant OC cells and simultaneously inhibits cisplatin-induced protective autophagy. Mechanistically, Magnoflorine reduces HMGB1 expression in cisplatin-resistant OC cells. Collectively, these results suggest that Magnoflorine has potential as a therapeutic agent for cisplatin-resistant OC in future clinical applications.

Keywords

Ovarian cancer; Cisplatin; Apoptosis; Autophagy; HMGB1

1. Introduction

Ovarian cancer (OC) is a prevalent gynecologic malignancy [1], and its main form of treatment is cisplatin-based chemotherapy [2]. Ovarian cancer is a malignant tumor of ovarian tumor, which refers to the malignant tumor growing on the ovary, of which 90%~95% is the primary cancer of the ovary, and the other 5%~10% is the primary cancer of other parts of the ovary. Early diagnosis of ovarian cancer is difficult due to the lack of specific symptoms, and the lack of effective screening. Most patients have advanced stage disease at the time of diagnosis [1, 2]. However, resistance of OC cells to cisplatin has resulted in a 5-year survival rate of approximately 30% for OC patients with advanced stage disease [2]. In addition, platinum resistance has emerged as a significant obstacle in the successful treatment of OC, and its underlying mechanisms remain unclear [3]. Autophagy represents the primary pathway responsible for the degradation of non-functional proteins and organelles [4, 5]. Numerous studies have highlighted the close association between autophagy and cancer, with its impact on the sensitivity of tumor cells to chemotherapy drugs is well-documented [6–8]. Thus, inhibiting cisplatin-induced protective autophagy in OC presents a potentially promising strategy against drug resistance [9]. However, to further enhance the therapeutic outcomes for OC patients, it remains important to clearly understand its underlying molecular mechanisms and develop novel and more efficacious targeted therapeutic agents.

Magnoflorine is a fundamental chlorine alkaloid that can be extracted from various plant families, including Magnoliaceae, Berberaceae, Papaveraceae and Aspartaceae, and has been found to exhibit diverse pharmacological activities, including anti-diabetic and anti-inflammatory properties [10, 11], as well as anti-tumor effects [12, 13]. For instance, Magnoflorine has been demonstrated to enhance the sensitivity of breast cancer cells to doxorubicin (DOX) by inducing apoptosis and autophagy through the protein kinase B (AKT)/mechanistic target of Rapamycin (mTOR) and p38 signaling pathways [10]. It has also been found to impede the progression of osteosarcoma by inhibiting the HMGB1/nuclear factor kappa-B (NF-κB) pathway, thereby suppressing the malignant characteristics of these cells and increasing their sensitivity to cisplatin [14]. HMGB1, a highly conserved nuclear protein, plays a crucial role in promoting autophagy and inhibiting apoptosis in tumor cells, thus contributing to chemotherapy resistance [14]. Previous studies have widely confirmed the important role of HMGB1 in the progression of a variety of tumors. On the other hand, multiple drugs affect tumor progression through this protein [14]. However, whether Magnoflorine has any influence on the progression of OC remains unclear.

This study was performed to elucidate the impact of Magnoflorine on cisplatin resistance in OC, and we thought Magnoflorine could be a promising therapeutic agent for treating OC.

2. Materials and methods

2.1 Cell culture

All cell lines, including A2780 and SKOV3, were obtained from ATCC (VA, USA). The cells were cultured in 100 mm dishes using 10 mL Dulbecco's modified eagls medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS). Magnoflorine (Sigma, USA) was administered into A2780 and SKOV3 cells at a concentration of 40 μM for 24 hours. To establish cisplatin-resistant cell lines, A2780 and SKOV3 cells were continuously cultured with cisplatin (Sigma, MA, USA) for six months at concentrations ranging from 0.06 mg/L to 0.6 mg/L. These cisplatin-resistant cell lines were denoted as A2780/Diaminedichloroplatinum (DDP) and SKOV3/DDP.

2.2 Cell viability

Cell viability was assessed using a cell counting kit-8 (CCK-8) kit (C0078, Beyotime, Beijing, China). Briefly, approximately 1000 cells per well were seeded into 96-well plates and incubated with 100 μL of CCK-8 solution for 1.5 hours. The absorbance was then measured at 450 nm using a microplate reader (ER504, Thermo, Waltham, MA, USA).

2.3 Western blot assay

Proteins (20 μg) isolated from 1×10^6 cells per sample were transferred onto a polyvinylidene fluoride (PVDF) membrane and subsequently incubated with primary antibodies (all purchased from Abcam) overnight at 4 °C. The primary antibodies and their respective dilutions were as follows: anti-Bax (1:1000, ab32503), anti-Bcl-2 (1:1000, ab182858), anti-LC3 (1:1000, ab192890), anti-p62 (1:1000, ab109012), anti-Beclin1 (1:1000, ab302669), anti-HMGB1 (1:1000, ab18256), anti-p65 (1:500, ab32536), anti-p-p65 (S536, 1:500, ab76302), anti-IκBα (1:1000, sc-203, Santa Cruz, California, USA), anti-p-IκBα (Tyr42, 1:500, sc-101714, Santa Cruz, California, USA), and anti-β-actin (1:1000, ab8226). Then, after incubation with a secondary antibody (1:1000, #31430 and #31460, CST, MA, USA) conjugated with horseradish peroxidase (HRP), the membrane was visualized, and protein bands were detected using an enhanced chemiluminescence detection reagent (#38553, Pierce; Thermo Fisher Scientific, Inc.).

2.4 Colony formation assay

A2780/Diaminedichloroplatinum (DDP) and SKOV3/DDP cells were cultured at a density of 5×10^3 cells per well in 6-well plates and incubated for two weeks. Then, the cells were fixed with 4% paraformaldehyde for 25 minutes at room temperature, stained with 2% crystal violet solution for 25 minutes at room temperature, and assessed under light microscopy.

2.5 Cell apoptosis assay

A2780/DDP and SKOV3/DDP cells (1×10^6 cells/well) were fixed using 70% ethanol at −20 °C for 2 hours. Then, these cells were stained with propidium iodide (PI) and fluorescein isothiocyanate isomer I (FITC) Annexin V at 4 °C, and the levels of apoptosis were quantified using a FACSCalibur flow cytometer with the CellQuest Pro 5.1 software (BD Biosciences, Inc., Franklin Lake, NJ, USA). Cells displaying Annexin V+/PI+ staining were located in the upper right quadrant (UR), while the early apoptotic cells, characterized by Annexin V+/PI− staining, were found in the lower right quadrant (LR), which together constituted the total apoptotic cells.

2.6 Immunofluorescent staining

The cells (1×10^6 cells/well) were initially treated with a blocking solution comprising 4% paraformaldehyde (PFA) in 5% bovine serum albumin (BSA). Next, they were incubated with a primary antibody against LC3B (anti-LC3, 1:1000, ab192890, Abcam, Cambridge, UK), and after three rounds of thorough rinsing, they were incubated with Alexa 488 secondary antibodies (ab150077, abcam, Cambridge, UK). The coverslips were then examined under microscopy, and subsequent image analysis was performed using ImageJ 9.0.

2.7 Statistical analysis

Data analysis was conducted using GraphPad Prism 8.0 (La Jolla, CA, USA). Each experiment was repeated three times. Statistical significance between two groups was assessed using Student's *t*-test, and for multiple comparisons, one-way analysis of variance (ANOVA) was performed, followed by Tukey's *post hoc* test. The results are shown as mean $\pm$ standard deviation (SD). $p < 0.05$ was considered statistically significant.

3. Results

3.1 Magnoflorine increases the sensitivity of OC cells to cisplatin

Cisplatin resistance was induced in A2780 and SKOV3 cells by exposing them to escalating concentrations of cisplatin (ranging from 0.06 mg/L to 0.6 mg/L) and designated as A2780/Diaminedichloroplatinum (DDP) and SKOV3/DDP cells. The half-maximal inhibitory concentration (IC50) levels of cisplatin following DDP treatment in A2780 and SKOV3 cells revealed that DDP increased the IC50 of cisplatin (Fig. 1a). Additionally, colony formation assays showed that DDP contributed to the proliferation of A2780 and SKOV3 cells (Fig. 1b). The molecular formula of Magnoflorine is shown in Fig. 1c. Importantly, the IC50 values for A2780 and SKOV3 cells were 25.73 and 22.11 μg/mL, respectively, and substantially decreased to 9.51 and 4.16 μg/mL upon treatment with Magnoflorine at a concentration of 40 μM in combination with cisplatin (Fig. 1d). Furthermore, treatment with Magnoflorine significantly reduced the number of colonies when incubated with Magnoflorine in both A2780/DDP and SKOV3/DDP cells, as confirmed by CCK-8 assays (Fig. 1e). These results indicate that Magnoflorine could enhance the sensitivity of OC cells to cisplatin.

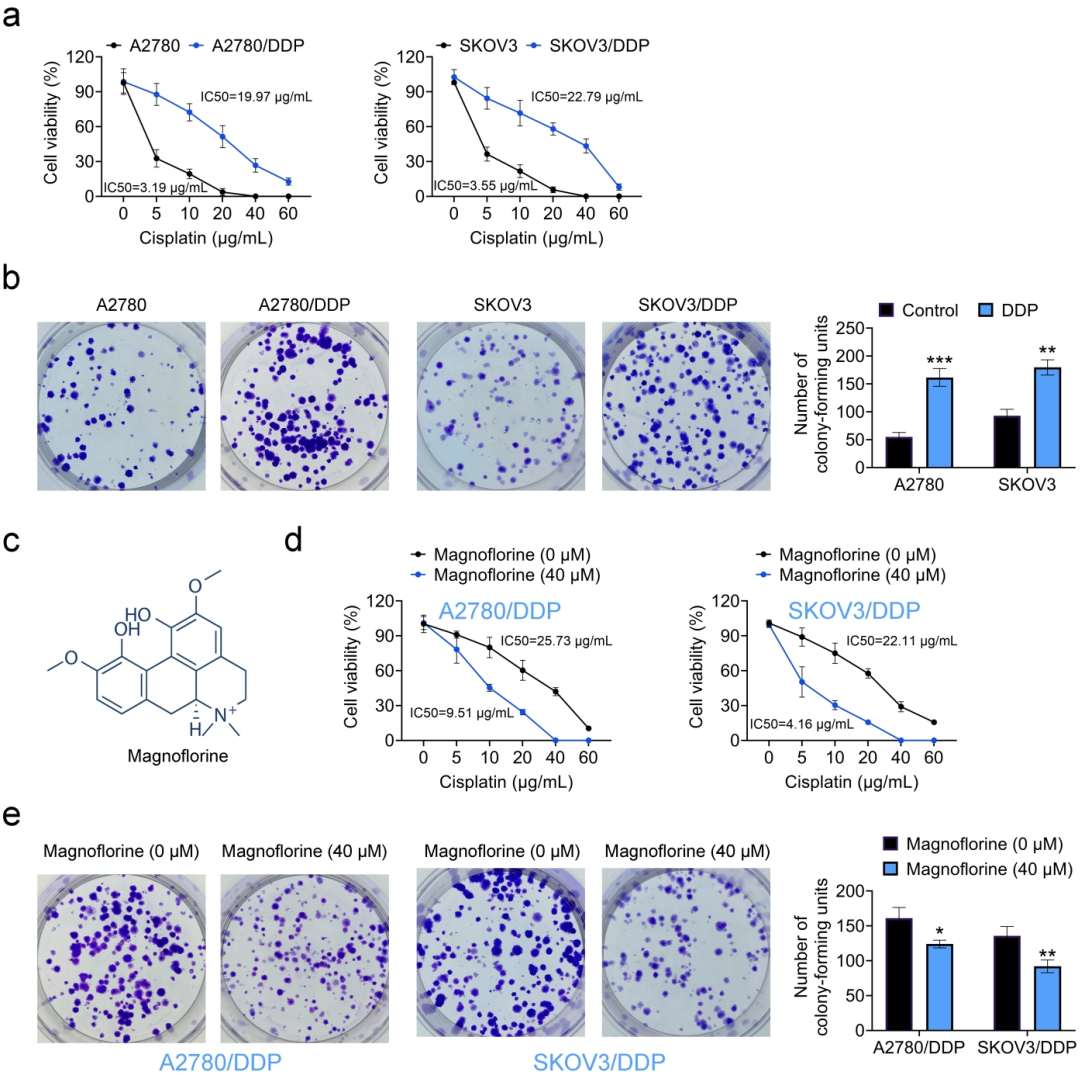

FIGURE 1. Magnoflorine increases ovarian cancer cell sensitivity to cisplatin. (a) CCK-8 assay results of the IC50 value of Cisplatin in A2780, SKOV3, A2780/DDP and SKOV3/DDP cells. The experiment was repeated three times. (b) Colony formation assays demonstrate the viability of A2780, SKOV3, A2780/DDP and SKOV3/DDP cells following the indicated treatments. The experiment was repeated three times. (c) The molecular formula of Magnoflorine. (d) CCK-8 assays reveal the IC50 value of cisplatin in A2780/DDP and SKOV3/DDP cells after treatment with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. (e) Colony formation assays show the colony numbers of A2780/DDP and SKOV3/DDP cells upon treatment with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. Data are presented as mean $\pm$ SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. DDP: Diaminedichloroplatinum; IC: inhibitory concentration.

3.2 Magnoflorine promotes apoptosis of cisplatin-resistant OC cells

To elucidate the biological functions of Magnoflorine in cisplatin-resistant OC cells, fluorescence-activated cell sorting (FCM) assays were conducted to assess its impact on apoptosis in A2780/DDP and SKOV3/DDP cells. The results showed that treatment with Magnoflorine led to a significant increase in the percentage of apoptotic cells in both A2780/DDP and SKOV3/DDP cells (Fig. 2a). Immunoblot assays revealed that Magnoflorine treatment suppressed the expression of Bcl-2 in A2780/DDP and SKOV3/DDP cells while increasing the expression of Bax, cleaved caspase-3 and cleaved PARP, further promoting apoptosis (Fig. 2b). Thus, Magnoflorine could induce apoptosis in cisplatin-resistant OC cells.

3.3 Magnoflorine inhibits cisplatin-induced protective autophagy

As autophagy plays a pivotal role in cisplatin resistance in cancers, we subsequently investigated the potential impact of Magnoflorine on autophagy in cisplatin-resistant OC cells. Immunoblot results indicated that Magnoflorine treatment led to a reduction in the ratio of LC3II/LC3I in A2780/DDP and SKOV3/DDP cells, suggesting the inhibition of autophagy (Fig. 3a). Additionally, the protein levels of the autophagy marker Beclin1 was decreased following Magnoflorine treatment, further supporting autophagy inhibition (Fig. 3b). Furthermore, the expression of the autophagy marker p62 was increased following Magnoflorine treatment, confirming the inhibition of autophagy (Fig. 3b). Immunostaining assays provided additional evidence, showing a decrease in the ex-

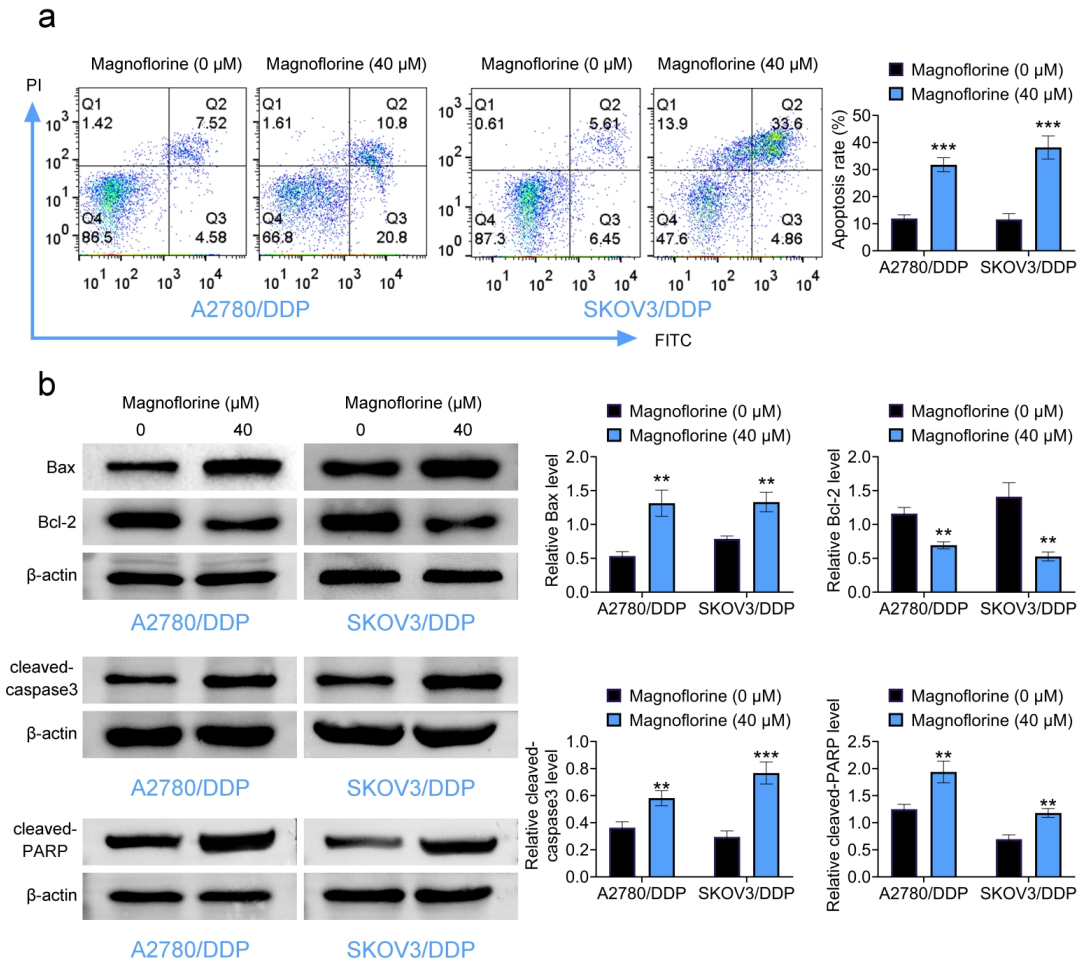

FIGURE 2. Magnoflorine promotes apoptosis of cisplatin-resistant ovarian cancer cells. (a) Fluorescence-activated cell sorting (FCM) assays illustrate the apoptosis of A2780/DDP and SKOV3/DDP cells following treatment with Magnoflorine at concentrations of 0 and 40 μM. The percentage of apoptotic cells is quantified. The experiment was repeated three times. (b) Immunoblot assays depict the expression levels of Bax, Bcl-2, cleaved caspase-3, and cleaved PARP in A2780/DDP and SKOV3/DDP cells treated with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. Data are presented as mean $\pm$ SD. $^{**}p < 0.01$, $^{***}p < 0.001$. DDP: Diaminedichloroplatinum; PI: Propyl iodide; FITC: fluorescein isothiocyanate isomer I; Bcl: B cell leukemia; PARP: poly (ADP-ribose) polymerase.

pression of LC3B in A2780/DDP and SKOV3/DDP cells following Magnoflorine treatment (Fig. 3c). Conversely, DDP treatment increased the ratio of LC3II/LC3I, indicative of the promotion of autophagy (Fig. 3d). Collectively, Magnoflorine could inhibit cisplatin-induced protective autophagy.

3.4 Magnoflorine down-regulates HMGB1 in cisplatin-resistant OC cells

To elucidate the mechanism underlying Magnoflorine's inhibition of cisplatin resistance in OC cells, we assessed the expression levels of HMGB1 in A2780/DDP and SKOV3/DDP cells using immunoblot assays and observed that Magnoflorine treatment led to a reduction in HMGB1 expression (Fig. 4a). Next, we investigated the impact of Magnoflorine on this pathway. The results indicated that Magnoflorine treatment reduced the phosphorylation levels of p65 and IκBα in both A2780/DDP and SKOV3/DDP cells (Fig. 4b), suggesting that Magnoflorine positively modulates the NF-κB pathway through HMGB1.

4. Discussion

The mortality of OC is the highest among gynecological malignancies [1] as it is commonly diagnosed at an advanced stage [15], and for which surgical intervention plus platinum combined with paclitaxel is the standard first-line treatment [3]. However, late-stage diagnosis and the development of drug resistance to chemotherapy are the primary factors contributing to the unfavorable prognosis [2]. While first-line treatments for OC often yield a high response rate and clinical remission [2], approximately 70% of OC cases experience recurrence, and tumors eventually acquire strong resistance to platinum-based chemotherapy [2]. Abnormal DNA damage repair function, including nucleotide excision repair abnormalities, base mismatch repair abnormalities, DNA double-strand break damage repair abnormalities and cross-damage repair abnormalities, represent the main mechanisms of cisplatin resistance [2]. Therefore, there is an urgent and pressing need to investigate the molecular factors associated with cisplatin resistance and develop novel targeted therapies for OC. In this study, we

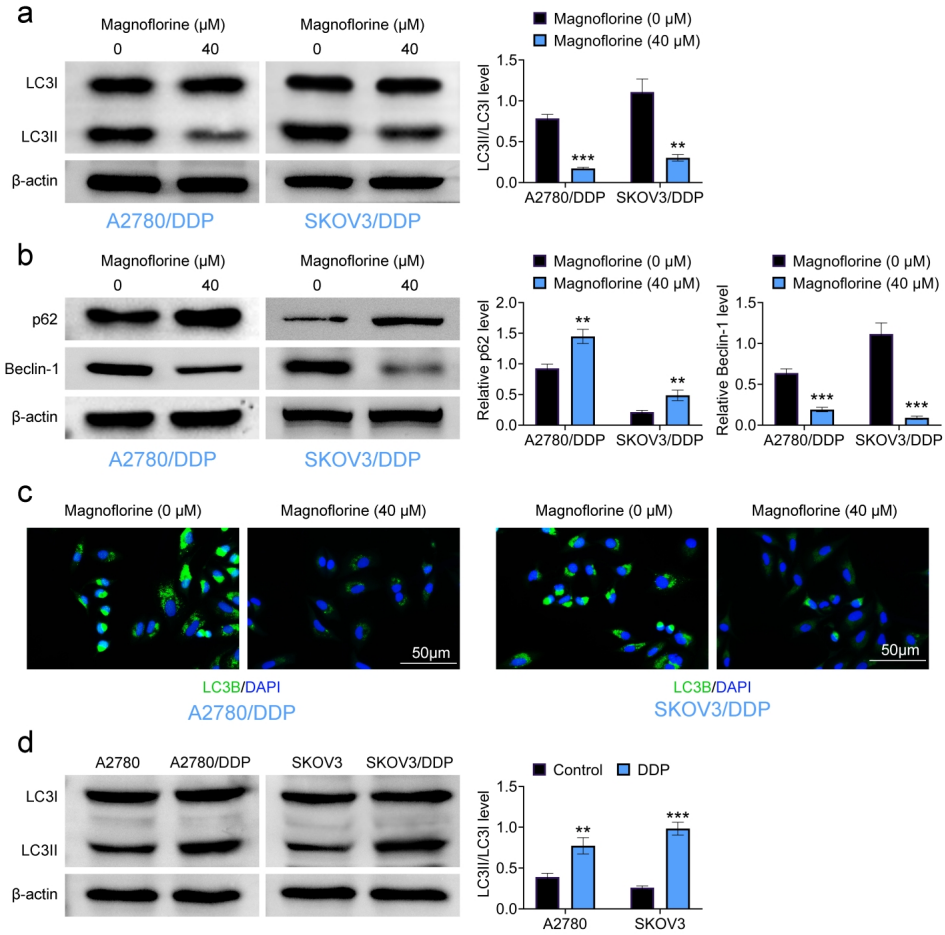

FIGURE 3. Magnoflorine inhibits cisplatin-induced protective autophagy. (a) Immunoblot assays depict the expression of LC3 in A2780/DDP and SKOV3/DDP cells treated with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. (b) Immunoblot assays illustrate the expression of Beclin1 and p62 in A2780/DDP and SKOV3/DDP cells following treatment with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. (c) Immunostaining assays show the expression of LC3B in A2780/DDP and SKOV3/DDP cells upon treatment with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. (d) Immunoblot assays reveal the expression of LC3 in A2780 and SKOV3 cells treated with DDP. The experiment was repeated three times. Data are presented as mean $\pm$ SD. $^{**}p < 0.01$, $^{***}p < 0.001$. DDP: Diaminedichloroplatinum; LC: Microtubule-associated protein 1A/1B-light chain; DAPI: 4,6-diamidino-2-phenylindole.

showed the inhibitory effects of Magnoflorine on cisplatin-induced protective autophagy in OC, suggesting its potential as a therapeutic agent for OC.

Magnoflorine has been reported to possess diverse properties, including anxiolytic, anti-tumor, and anti-inflammatory activities [16]. It has been shown to ameliorate collagen-stimulated arthritis by inhibiting the inflammatory response through the NF-κB/mitogen-activated protein kinase (MAPK) pathways [17]. Additionally, it has been demonstrated that it can improve cognitive deficits and mitigate the pathology associated with Alzheimer's disease by modulating the c-Jun N-terminal kinase (JNK) pathway [16]. Another study indicated that Magnoflorine can attenuate neuronal injury induced by cerebral ischemia through autophagy [18]. In breast cancer, Magnoflorine has been found to enhance sensitivity to DOX by inducing apoptosis and autophagy through the AKT/mTOR and p38 signaling pathways [10]. Similarly, our findings in this study reveal that Magnoflorine could suppress OC progression by modulating autophagy. Its anti-tumor properties have been widely documented, as evidenced by its ability to significantly enhance the anti-proliferative effects of DOX in breast cancer cells [13]. Importantly, a previous study reported that Magnoflorine effectively suppressed the malignant characteristics of osteosarcoma cells and enhanced their sensitivity to cisplatin through the HMGB1/NF-κB pathway [14]. In parallel, our findings in this study also demonstrate that Magnoflorine could impede the growth of drug-resistant OC cells, induce apoptosis, enhance the drug sensitivity of drug-resistant OC cells, and suppress cisplatin-induced protective autophagy.

Autophagy serves as a survival mechanism for OC cells in an environment characterized by continuous nutrient deprivation, shielding cancer cells from stressors like chemotherapy and eventually fostering drug resistance [19, 20]. Our discovery that Magnoflorine could hinder cisplatin-induced protective autophagy suggests a potential strategy against drug resistance. However, the precise molecular mechanisms underlying this

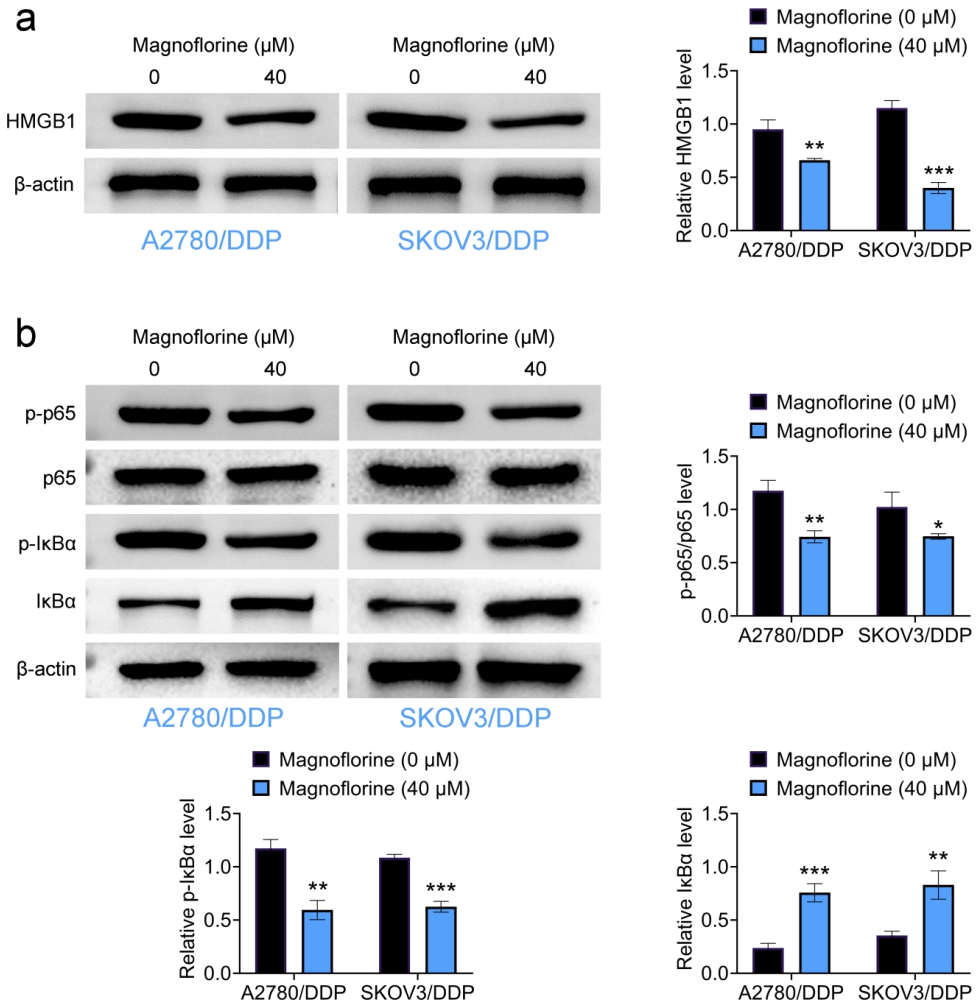

FIGURE 4. Magnoflorine down-regulates HMGB1 levels in cisplatin-resistant ovarian cancer cells. (a) Immunoblot assays showing the expression of HMGB1 in A2780/DDP and SKOV3/DDP cells treated with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. (b) Immunoblot assays illustrate the expression and phosphorylation levels of p65 and IκBα in A2780/DDP and SKOV3/DDP cells following treatment with Magnoflorine at concentrations of 0 and 40 μM. The experiment was repeated three times. Data are presented as mean $\pm$ SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DDP: Diaminedichloroplatinum; HMGB: high mobility group protein B.

process warrant further investigation.

Through FCM and Immunoblot assays, we noticed Magnoflorine promotes apoptosis of cisplatin-resistant ovarian cancer cells. Chemotherapy drugs mainly induce tumor cell apoptosis. Therefore, the sensitivity of chemotherapeutic drugs is mainly determined by whether they can promote the apoptosis caused by them. Therefore, we believe that Magnoflorine plays a key role in it. Given the pivotal role of Bax/Bcl-2 in regulating both caspase-dependent and caspase-independent apoptosis through the mitochondrial pathway, the drugs used in our present study were also found to contribute to the reduction of inflammation [20]. Consequently, we assessed the expression of inflammatory factors, such as tumor necrosis factor (TNF)-α, and apoptotic mediators, including P50, and the results further support our findings.

HMGB1 is involved in various physiological and pathological processes, such as the promotion of DNA damage repair within the nucleus, the sensing of nucleic acids leading to the induction of innate immune responses and autophagy in the cytoplasm, as well as the binding to proteins and the stimulation of immune receptors in the extracellular environment. Moreover, HMGB1 can be considered a broad cellular stress sensor that creates a balance between cell death and survival responses, which are crucial for maintaining cell homeostasis and tissue integrity [14]. In this study, we demonstrated that Magnoflorine down-regulates HMGB1, resulting in the inhibition of OC resistance, indicating that Magnoflorine may modulate cell death and survival processes in OC by impacting HMGB1.

The anti-tumor activities of Magnoflorine have been widely indicated. For example, Magnoflorine suppressed the growth of several types of tumor cells, such as lung, breast, glioma, and rhabdomyosarcoma cancer cells, suggesting that it could serve as a drug in these tumors [10]. The role and mechanism of Magnoflorine are still different in different tumors [21]. The role and mechanism of Magnoflorine in OC that we have discovered are still worthy of further study.

5. Conclusions

In conclusion, our findings indicate that Magnoflorine may effectively inhibit cisplatin-induced protective autophagy by suppressing HMGB1, leading to an enhanced drug sensitivity in drug-resistant OC cells. Thus, Magnoflorine could be a potential therapeutic agent for OC, particularly for cisplatin-resistant therapy.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

YLZ—designed the study and carried them out; prepared the manuscript for publication and reviewed the draft of the manuscript. YLZ, XH, LF and QRZ—supervised the data collection, analyzed the data, interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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