



Morusin induces paraptosis-like cell death through mitochondrial calcium overload and dysfunction in epithelial ovarian cancer

Jing Xue, Rui Li, Xinrui Zhao, Congcong Ma, Xin Lv, Lidong Liu, Peishu Liu*

Department of Obstetrics and Gynecology, Qilu Hospital of Shandong University, 107 West Wenhua Road, Jinan, 250012, Shandong Province, People's Republic of China

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ABSTRACT

Epithelial ovarian cancer (EOC) is the leading cause of death among all gynecological cancers. Morusin, a prenylated flavonoid extracted from the root bark of *Morus australis*, has been reported to exhibit anti-tumor activity against various human cancers except EOC. In the present study, we explored the potential anti-cancer activity of morusin against EOC *in vitro* and *in vivo* and possible underlying mechanisms for the first time. We first found that morusin effectively inhibited EOC cell proliferation and survival *in vitro* and suppressed tumor growth *in vivo*. Then we observed that treatment of EOC cells with morusin resulted in paraptosis-like cell death, a novel mode of non-apoptotic programmed cell death that is characterized by extensive cytoplasmic vacuolation due to dilation of the endoplasmic reticulum (ER) and mitochondria and lack of apoptotic hallmarks. In addition, we discovered that morusin induced obvious increase in mitochondrial Ca^{2+} levels, accumulation of ER stress markers, generation of reactive oxygen species (ROS), and loss of mitochondrial membrane potential ($\Delta\psi_m$) in EOC cells. Furthermore, pretreatment with 4, 4'-diisothiocyanostilbene-2, 2'-disulfonic acid (DIDS), a chemical inhibitor of voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane, effectively inhibited mitochondrial Ca^{2+} influx, cytoplasmic vacuolation and cell death induced by morusin in EOC cells. Moreover, DIDS pretreatment also suppressed morusin-induced accumulation of ER stress markers, ROS production and depletion of $\Delta\psi_m$. Consistently, tumor xenograft assays showed that co-treatment with DIDS partially reversed the inhibitory effects of morusin on tumor growth *in vivo* and inhibited the increased levels of ER stress markers induced by morusin in tumor tissues. Collectively, our results suggest that VDAC-mediated Ca^{2+} influx into mitochondria and subsequent mitochondrial Ca^{2+} overload contribute to mitochondrial swelling and dysfunction, leading to morusin-induced paraptosis-like cell death in EOC. This study may provide alternative therapeutic strategies for EOC exhibiting resistance to apoptosis.

1. Introduction

Epithelial ovarian cancer (EOC) is the leading cause of gynecological cancer-associated death worldwide [1–3]. Due to dormant clinical symptoms and lack of effective biomarkers for early detection, most patients were diagnosed at the advanced stages with a poor prognosis [3,4]. Despite advances in therapies over the past decades, the five-year survival rate of EOC remains below 45% [5]. Surgery and chemotherapy are conventional therapeutic strategies for EOC, but most women present with advanced disease will develop recurrence and even chemoresistance [3]. Therefore, identifying novel effective agents

and developing alternative therapeutic strategies are crucial for improving EOC outcomes.

Resistance to apoptosis is one of the most common mechanisms of chemoresistance in EOC [6]. Thus, investigating alternative non-apoptotic cell death pathways may contribute to the development of new therapies. Besides apoptosis and autophagic cell death, other types of non-canonical programmed cell death have been studied such as necroptosis, mitotic catastrophe, anoikis, ferroptosis, methuosis, and paraptosis [7,8]. Paraptosis is a type of non-apoptotic programmed cell death that has been discovered in recent years. It is characterized by extensive cytoplasmic vacuolation due to the dilation of the

Abbreviations: EOC, epithelial ovarian cancer; VDAC, voltage-dependent anion channel; DIDS, 4, 4'-diisothiocyanostilbene-2, 2'-disulfonic acid; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; $\Delta\psi_m$, mitochondrial membrane potential; EdU, 5-ethynyl-2'-deoxyuridine; TNF- α , tumor necrosis factor-alpha; CHX, cycloheximide; 3-MA, 3-Methyladenine; CQ, chloroquine; EBSS, Earle's Balanced Salt Solution; CTSS, Cathepsin B; CTSD, Cathepsin D; MAMs, mitochondria-associated ER membranes; $[\text{Ca}^{2+}]_c$, cytosolic Ca^{2+} concentration; $[\text{Ca}^{2+}]_m$, mitochondrial Ca^{2+} concentration; TBST, Tris-Buffered Saline with Tween-20; HBSS, Hank's Balanced Salt Solution; BAPTA, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; DCFH-DA, 2',7'-Dichlorodihydrofluorescein diacetate; PBS, Phosphate-Buffered Saline; JC-1, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidazocarbocyanine iodide

* Corresponding author.

E-mail addresses: 201620468@mail.sdu.edu.cn (J. Xue), 201620441@mail.sdu.edu.cn (R. Li), 201520430@mail.sdu.edu.cn (X. Zhao), 201514035@mail.sdu.edu.cn (C. Ma), 201514034@mail.sdu.edu.cn (X. Lv), 201613769@mail.sdu.edu.cn (L. Liu), Peishuli@126.com (P. Liu).

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endoplasmic reticulum (ER) and/or mitochondria [7–15]. Paraptosis lacks the hallmarks of apoptosis such as pyknosis, formation of apoptotic bodies, DNA fragmentation, and caspase activation [8,9,12–16]. However, the molecular mechanisms underlying paraptosis have not been fully understood yet. It has been reported that paraptosis is associated with activation of mitogen-activated protein kinases (MAPKs) and can be inhibited by AIP-1/Alix and a protein synthesis inhibitor called cycloheximide [14–18]. In addition, ER stress, reactive oxygen species (ROS) production and perturbation of intracellular calcium homeostasis have been demonstrated to play a role in paraptosis induced by different stimuli [11–15,17–28]. Since cell death modes accompanied with the typical morphology of paraptosis may involve different biochemical and physiological mechanisms, they are commonly called paraptosis-like cell death [10].

Recent studies have revealed that multifarious natural compounds present anti-cancer effects by inducing paraptosis or paraptosis-like cell death [7,29,30]. Morusin is a prenylated flavonoid isolated from the root bark of *Morus australis* [31,32], which has been reported to possess various biological activities such as anti-inflammatory, antioxidant, and antibacterial activities [33–37]. Moreover, previous reports have proved that morusin exhibit anti-tumor effects against different types of cancer *in vitro* and *in vivo*, including colorectal cancer, cervical cancer, hepatocarcinoma, breast cancer, prostate cancer, glioblastoma, pancreatic cancer, and gastric cancer [38–50]. These studies have demonstrated that morusin can cause apoptotic cell death in various human cancer cells through inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and signal transducer and activator of transcription 3 (STAT3) pathways [38–41,46–48], induce adipogenic differentiation and lipoapoptosis mediated by peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT-enhancer binding protein beta (C/EBP β) in breast cancer and glioblastoma [43,44], lead to cell cycle arrest by down-regulating c-Myc in gastric cancer [50], and enhance the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in glioblastoma cells *via* modulating expression of death receptor 5 (DR5) and epidermal growth factor receptor (EGFR) [45]. However, whether morusin has potential anti-cancer activity against EOC and possible mechanisms underlying the activity have never been explored before. In the present study, we investigated the growth inhibitory effects of morusin on EOC *in vitro* and *in vivo* for the first time. Here we propose that morusin induces paraptosis-like cell death through mitochondrial Ca²⁺ overload and subsequent mitochondrial dysfunction in EOC.

2. Materials and methods

2.1. Cell culture

The A2780 cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). The SKOV-3 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HO-8910 cell line was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). SKOV-3 cells were maintained in McCoy's 5A medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). A2780 and HO-8910 cells were cultured in RPMI-1640 medium (Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). All cell lines were cultured in the incubator with 5% CO₂ at 37 °C.

2.2. Reagents

Morusin was purchased from ChemFaces (Wuhan, China). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4, 4'-diisothiocyanostilbene-2, 2'-disulfonic acid (DIDS) and N-acetyl-L-cysteine (NAC) were obtained from Sigma-

Aldrich (St. Louis, MO, USA). Necrostatin-1, z-VAD-fmk, cycloheximide (CHX), 3-Methyladenine (3-MA) and chloroquine (CQ) were purchased from MedChem Express (Monmouth Junction, NJ, USA). Tumor necrosis factor- α (TNF- α) was bought from Proteintech Group (Rosemont, IL, USA). ER-Tracker Red and Rhod-2 AM were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Crystal violet staining solution, Mito-Tracker Green, Fluo-4 AM, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1), 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) and RIPA lysis buffer were purchased from Beyotime Biotechnology (Shanghai, China). The following antibodies were used in this study: anti-GAPDH, anti-caspase-9, anti-caspase-3, anti-PARP, anti-LC3B, anti-p62, anti-Cathepsin B, anti-IRE1 α , anti-Phospho-eIF2 α , HRP-linked anti-rabbit IgG, and HRP-linked anti-mouse IgG antibodies (Cell Signaling Technology, Danvers, MA, USA); anti-BiP, anti-CHOP, anti-Alix and anti-Cathepsin D antibodies (Abcam, Cambridge, MA, USA).

2.3. MTT assay

The cell viability of human epithelial ovarian cancer cells was measured by MTT assay. Briefly, A2780, SKOV-3 and HO-8910 cells (5×10^3 cells/well) were seeded in 96-well plates. After incubation overnight, the cells were subsequently treated with the indicated concentrations of different agents for the indicated hours. Then MTT solution (10 μ L, 5 mg/ml) was added into each culture well. The supernatant was discarded after incubation at 37 °C for 4 h, and 100 μ L DMSO was added into each well to dissolve formazan. The absorbance was measured by a microplate reader at a wavelength of 490 nm, and the percentage of cell viability was calculated compared to the control. For each measuring point, quadruplicate cultures were prepared and at least three independent experiments were carried out.

2.4. Colony formation assay

Colony formation assay was performed to investigate the long-term inhibitory effect of morusin on EOC cell proliferation. A2780, SKOV-3 and HO-8910 cells (500 cells/well) were seeded and incubated in six-well plates overnight. Subsequently, the cells were treated with various concentrations of morusin (0, 10, 20, 30 μ M) for 24 h and then incubated in normal medium for the following 11 days at 37 °C. Afterwards, cells were fixed in 4% paraformaldehyde solution for 15 min and stained with 1% crystal violet staining solution for 30 min. Colonies (> 50 cells) were counted under an optical microscope. Three independent experiments were performed.

2.5. Western blot analysis

EOC cells after the indicated treatments were collected, lysed with RIPA lysis buffer, and then centrifuged at 10000 g for 15 min. Subsequently, the supernatants were separated, mixed with loading buffer ($5 \times$), and boiled for 5 min. Then equal amounts of total proteins from each sample were separated by 12% SDS-PAGE and transferred to PVDF membranes. Afterwards, PVDF membranes were blocked in 5% non-fat milk at room temperature for 1 h and then incubated with the indicated primary antibodies (1:1000) overnight at 4 °C. Then the PVDF membranes were washed with Tris-Buffered Saline with Tween-20 (TBST) and incubated with HRP-linked anti-rabbit or anti-mouse IgG secondary antibodies (1:2000) at room temperature for 1 h. Antibody-bound proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA) and visualized by ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The gray values were analyzed by ImageJ software. Three independent experiments were performed for statistical analysis.

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