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Original Research

Rubimaillin decreases the viability of human ovarian cancer cells via mitochondria-dependent apoptosis

Zhang Siwei[1], Wu Zhen[2], Zhou Zhi[3], Hu Xuguang[2], Li Yousheng[1*]

[1] Department of Traditional Chinese Medicine, Shenzhen People's Hospital (Second Clinical Medical College of Jinan University, First Affiliated Hospital of Southern University of Science and Technology), NO. 1017 Dongmen North Road, Luohu District, Shenzhen, Guangdong Province, China, 518020

[2] School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, NO. 280 Daxuecheng Outer Ring East Road, Guangzhou, Guangdong Province, China, 510006

[3] Microbiology Laboratory, Shenzhen Institute for Drug Control, No. 28 Gaoxin Middle Second Road, Nanshan District, Shenzhen City, Guangdong Province, China, 518057

[*]**Correspondence to:** ymdf83@163.com

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Abstract: This study was aimed to investigate the anti-tumor activity of rubimaillin *in vitro*, and the mechanism involved. The inhibitory effect of rubimaillin on cell proliferation was determined with MTT assay. Apoptosis was assayed using AV/PI double staining, while the mitochondrial membrane potential of SKOV-3 cells was determined with Rhodamine 123 (Rh123) staining. Western blot assay was used to determine the effect of rubimaillin on the expressions of Bcl-2, Bax, PARP, cleaved PARP, caspase3, cleaved caspase3, and other apoptosis-related proteins in SKOV-3 cells. Rubimaillin inhibited the growth of SKOV-3 cells in a concentration-dependent manner and induced apoptosis of tumor cells through the mitochondrial apoptosis pathway. These results indicate that rubimaillin is a potential anti-ovarian cancer drug.

Key words: Rubimaillin; Ovarian cancer; Mitochondria-dependent apoptosis.

Introduction

Ovarian cancer is ranked as the third most common malignant tumor in the female reproductive system, but the mortality rate is far higher than that of any other gynecological malignancy (1). The high mortality of ovarian cancer is due to its insidious onset, inconspicuous early symptoms, and lack of effective methods for early diagnosis. The 5-year survival of 60 – 70 % of patients diagnosed at the late stage is only 30 % (2). The traditional chemotherapeutic strategies for ovarian cancer are mainly cytotoxic drugs, the mechanism of which depends on the differences between tumor cells and normal cells in terms of growth, repair and death kinetics, although these drugs often lack specificity (3). Therefore, the selective killing of tumor cells and reduction in damage to normal tissue pose serious challenges to malignant tumor chemotherapy.

Traditional Chinese medicine plays an important role in cancer treatment because of its advantages such as naturalness of their components, low side effects, multiple drug targets, wide therapeutic directions and lack of drug resistance (4). Previous studies have shown that traditional Chinese medicine improves the immune function of cancer patients, reduces the side effects of radiotherapy and chemotherapy, improves the tolerance of cancer patients to radiotherapy and chemotherapy, contributes to the recovery of cancer patients, and improves the effect of tumor treatment. The combination of traditional Chinese medicine and other cancer treatment methods plays a significant role in cancer treatment practice. The curative effect of the combination treatment significantly improves the survival and quality of life of cancer patients, prolongs their survival time, reduces side effects, and increases the effect of cancer treatment. Therefore, recent studies on the anti-tumor effects of effective drug components and structural modifiers extracted from traditional Chinese medicine, and their mechanisms of action, have attracted much attention.

Rubidiaceae is a plant of the genus of *Rubidiaceae* in the *Rubidiaceae* family. It is widely distributed, from Africa to tropical Asia, India, China, Japan and Australia. As a medicinal plant, it is recorded in Shennong Herbal. Its roots and stems are used for medicinal purposes. It is one of the traditional Chinese medicines in China, and it is officially listed in the Chinese Pharmacopoeia. *Rubidiaceae* is used for hemostasis, meridian circulation, stopping cough and *dispelling phlegm.* In India, the plant is used to treat rheumatism, menstrual pain and urinary system diseases. There are 24 species of *Rubidiaceae* in China, with Henan as one of the key producing areas. The main chemical constituents of *Rubidiaceae* are complex, and the medicinally active components are numerous. It contains water-soluble cyclohexopeptide, fat-soluble anthraquinone and its

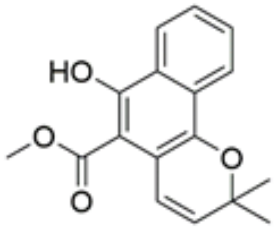

Figure 1. Structure of rubimaillin.

glycosides; reductive naphthoquinone and glycosides, polysaccharides, terpenes, trace elements, alizarin and munjistin (5). It has been reported that *Rubidiaceae* has a significant effect on tumor therapy, and that cyclohexyl peptide compounds exert inhibitory effect on leukemia and ascites cancer. Rubimaillin (Rub) is a major phytochemical compound isolated from *R. cordifolia* (Figure 1) (6 - 8). Pharmacological studies have shown that Rub exhibits a variety of beneficial biological characteristics such as anti-tumor (9), anti-mutagenesis (10), as well as anti-leukemia, anti-inflammatory and anti-allergic properties (11).

In the present study, the *in vitro* anti-tumor effect of rubimaillin on cell growth, cell proliferation and apoptosis, and the underlying mechanisms were investigated in human epithelial ovarian cancer cell line SKOV-3. Olaparib was used as positive control drug.

Materials and Methods

Experimental reagents

Rubimaillin (> 98 % pure) was product of Shanghai Taitan Technology Co., Ltd.; trypsin, MTT, RIPA lysate, sample buffer, BCA protein concentration assay kit and all secondary antibodies were purchased from Beyotime Biotechnology Co., Ltd. Phosphate buffer and dimethyl sulfoxide were purchased from Chongqing Haiyun Biotechnology Co., Ltd.; sodium dodecyl sulfate (SDS), concentrated hydrochloric acid, Tween 20, sodium chloride, Tris buffer, acrylamide and ammonium persulfate were purchased from Shanghai Sangon Co., Ltd.; luminous solution was product of Beijing YingGeEn Biotechnology Co., Ltd.; skim milk powder was purchased from Inner Mongolia Yili Industrial Group Co., Ltd., while Bcl-2, Bax, PARP, Cleaved PARP, Caspase3, Cleaved Caspase3 and β-actin antibodies were products of Cell Signaling Technology Company (USA).

Cells and cell culture

Human epithelial ovarian cancer cell line SKOV-3 was purchased from Shanghai Jitaikesai Biotechnology Co., Ltd., while RPMI1640 medium and fetal bovine serum were purchased from Hyclone Company (USA). The SKOV-3 cells were inoculated in a 25-mL glass culture flask and cultured on RPMI1640 medium containing 10 % fetal bovine serum and 1 % penicillin/ streptomycin mixture at 37 ℃ in an atmosphere of 5 % CO_2 and saturated humidity. When the cells attained 80 % growth, cells in the logarithmic growth period were taken and used for the study.

Determination of cell proliferation using MTT assay

The SKOV-3 cells in logarithmic growth phase were collected via trypsin digestion method. After cell count, the cell concentration was adjusted to 5×10^4 to 8×10^4 cells/ mL with complete cell culture medium containing 10 % serum and 1 % penicillin / streptomycin mixture. Then, the cells were inoculated in a 96-well plate (100 µL single cell suspension per well) and cultured in a saturated humidity incubator containing 5 % CO_2 at 37 ℃. On the next day when the cells were adhered to the walls of the well plates, the culture medium was replaced with drug (Rub or Olaparib) in a final volume of 100 µL. The concentrations of Rub and Olaparib were 5, 10, 20, 40, and 80 µM, with 3 multiple wells for each drug concentration. After incubation at 37 ℃ and 5 % CO_2 atmosphere for 48 h, each well was replaced with 100 µL of 5 mg/mL MTT. Then, the cells were cultured under the same conditions for 4 h, and the supernatant was discarded, followed by addition of 100 µL of DMSO to each well. After further incubation for 10 min at 37 ℃, the absorbance of each well was measured at 570 nm in a multifunctional enzyme standard instrument. The percentage cell survival was calculated, and the IC_{50} was computed using software GraphPad 7.0 (12).

$$Cell\ survival\ (\%) = \frac{Absorbance\ value\ of\ the\ experimental\ group \times 100}{Absorbance\ value\ of\ the\ control\ group}$$

Determination of apoptosis with flow cytometry

The SKOV-3 cells in logarithmic phase and in good growth state were used. After cell count, 4000 cells were inoculated into 96-well plates and cultured overnight in an incubator at 37 ℃ and 5 % CO_2 atmosphere. Three replicate culture well plates were used. After 24 h, the medium was replaced with 100 µL of fresh medium containing different concentrations of Rub. The concentrations of Rub were 5, 10 and 20 µM (used separately for the 3 replicates). The well plates were incubated under the same conditions as before for 12 h. After 12 hours, the supernatant was discarded. Then, 5 µL of AnnexinV-FITC was added, with gentle mixing, followed by incubation for 15 min at room temperature in the dark. Thereafter, 10 µL of PI dye solution was added, with gentle mixing, and the well plates were placed on ice bath for 5 min. Dye fluorescence was stimulated with FACScan flow cytometry (Becton Dickenson), and the second channel mean fluorescence intensity (PI fluorescence intensity) represented the late apoptosis of cells.

Comparison of mitochondrial membrane potential with Rh123 fluorescence intensity

Rhodamine 123 is a cationic fluorescent dye that can penetrate cell membrane, and it is a mitochondrial transmembrane potential indicator. In normal cells, it relies on mitochondrial transmembrane potential to enter the mitochondrial matrix, and the fluorescence intensity decreases or disappears. However, during apoptosis, the integrity of mitochondrial membrane is compromised, and the permeability of mitochondrial membrane is opened, resulting in the breakdown of mitochondrial transmembrane potential and the release Rh123 by mitochondria. This results in a strong yellow-green fluorescence, and the greater the fluorescence intensity, the lower the mitochondrial membrane potential (13). The SKOV-3 cells in logarithmic growth stage were collected and inoculated in 6 well plates (2 ml cell suspension per well) after adjusting the cell concentration. After 12

h, the medium was replaced with 2mL of Rub at final concentrations of 5, 10 and 20 μM, and the well plates were incubated for 48 h. After digesting with 0.25 % trypsin, the digestion was terminated with complete culture medium. The digest was centrifuged and the cells were collected in a 15-ml centrifuge tube while the supernatant was discarded. The cells were washed once with pre-cooled PBS buffer and the supernatant was discarded after centrifugation. The cells were resuspended in 500 μL of 5 μg/mL Rh123 and stained for 20 min at 37 ℃ in the dark. After centrifugation, the supernatant was discarded, and the cells diluted with PBS buffer to a density of about 5000 cells/100 μL. The cells were then inoculated in 96-well plates, with 100 μL cell suspensions in each well. The fluorescence intensity of Rh123 in each well was measured with a multifunctional enzyme standard instrument (Thermo Scientific PerkinElme EnSpirer) at excitation and emission wavelengths of 507 and 530 nm, respectively.

Determination of protein expression by western blotting

The SKOV-3 cells were incubated with different concentrations of RUB for 48 h, after which the cells were subjected to lysis and protein extraction. The protein concentration was determined using bicinchoninic protein assay kits in line with manufacturer's protocol. The protein was subjected to 10 % SDS-PAGE, and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was immersed in TBST sealing solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1 % Tween 20) containing 5 % skimmed milk powder, and placed on a shaker to seal at room temperature for 1 h. Then, primary antibody and the internal reference (1:500 dilution) were added to the protein (1:1000 dilution). After adding the primary antibody, the protein was incubated at room temperature for 15 min in a shaking bed, and thereafter incubated in a 4 ℃ refrigerator overnight. The membrane was rinsed thrice with BST at room temperature for 10 min. Then, the secondary antibody was added, and after incubating at room temperature for 2 h, TBST was used to rinse the membrane thrice at room temperature for 10 min. Thereafter, chemiluminescence reaction was carried out. The membrane was scanned using image scanner, and the gray value of image strip was analyzed with Quantity One software.

Statistical analysis

Data were analyzed with GraphPad Prism 7.0 statistical software. Measurement data are expressed as mean±standard deviation (x(—);x±s). Single-factor analysis of variance was used for multi-group comparison, while *q*-test was used for pairwise comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Inhibitory effect of rubimaillin on proliferation of SKOV-3 cells

There was no obvious inhibitory effect of rubimaillin on the proliferation of non-cancer cells (Figure 2 A). However, Olaparib and rubimaillin inhibited the proliferation of SKOV-3 tumor cells and decreased the percentage survival of SKOV-3 cells in a concentration-dependent fashion (Figure 2 B).

Rubimaillin induced apoptosis in SKOV-3 cells

As shown in Figure 3, rubimaillin induced apoptosis of SKOV-3 cells in a dose-dependent manner. Compared with the control group, apoptosis of SKOV-3 cells increased after 12 h of rubimaillin treatment. Furthermore, with increase in rubimaillin concentration, apoptosis was gradually enhanced.

Rub decreased mitochondrial membrane potential in SKOV-3 cells

The results in Figure 4 show that rubimaillin increased the average fluorescence intensity of Rh123 in a dose-dependent manner, implying a reduction in the mitochondrial membrane potential of SKOV-3 cells. Relative to the control group, mean fluorescence intensity of Rh123 in the cytoplasm of SKOV-3 was increased after treatment with different concentrations of rubimaillin, which indicated that rubimaillin decreased the mitochondrial membrane potential of the tumor cells. Furthermore, this effect was concentration-dependent. These results are shown in Figure 4.

Rubimaillin induced SKOV-3 apoptosis by inducing cleavage of caspase-3 and PARP1

The results of Western blotting showed that RUB

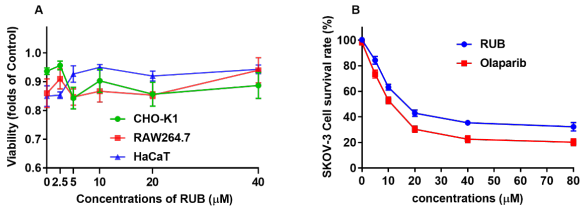

Figure 2. (A) Effect of rubimaillin on levels of RAW 264.7, CHO-K1 and HaCaT as indices of non-cancer cell viability. (B) Effects of olaparib and rubimaillin on % cell viability of SKOV-3 cells.

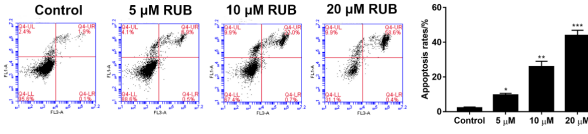

Figure 3. Effect of Rub on apoptosis in S KOV-3 cell line. Values are presented as mean ± S.E. * $p < 0.05$, compared to control group.

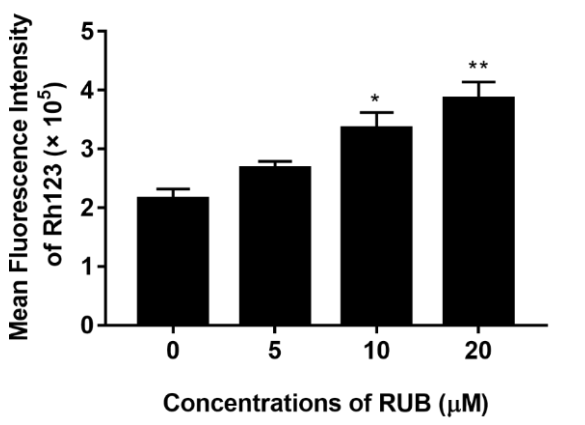

Figure 4. Effect of Rub on mitochondrial membrane potential of SKOV-3 cells. *$p < 0.05$, **$p < 0.01$, compared with control group.

regulated the expressions of apoptosis-related proteins in the Bcl-2 and caspase3 families. In the caspase3 apoptotic protein family, RUB inhibited expression of activated caspase3 cascade. It up-regulated the expressions of cleaved-PARP and cleaved-caspase3 protein in SKOV-3 cells in a concentration-dependent manner. In the Bcl-2 apoptotic protein family, RUB significantly dose-dependently down-regulated the relative expression of the anti-apoptotic protein Bcl-2, and up-regulated the relative expression of the pro-apoptotic protein Bax in SKOV-3 cells (Figure 5).

Discussion

Studies have shown that rubimaillin exerts antitumor activity *in vitro* (14). During apoptosis, Bcl-2 and the caspase family are activated. Apoptosis activation is associated with the death receptor pathway, endoplasmic reticulum pathway and mitochondrial pathway (15). The Bcl-2 family and caspase family are key factors involved in apoptosis. In the present study, the molecular mechanism underlying the *in vitro* anti-tumor effect of rubimaillin was studied from three perspectives: apoptosis, mitochondrial membrane potential and relative protein expressions. Results from flow cytometric analysis showed that the percentage apoptosis of SKOV-3 cells increased with increase in rubimaillin concentration. In Rh123 assay, the average fluorescence intensity of Rh123 in SKOV-3 cells was increased by rubimaillin in a concentration-dependent manner, implying that rubimaillin decreased the mitochondrial membrane potential of SKOV-3 cells. There is usually a negative correlation between the degree of apoptosis and mitochondrial membrane potential (16). The release of cytochrome C, which is a typical feature of mitochondrial pathway activation, is closely related to increase in outer mitochondrial membrane permeability and change in membrane potential (17). Western blot results showed that rubimaillin increased the expressions of Bax, cleaved-caspase3 and cleaved-PARP protein, and decreased the expression of Bcl-2 while promoting apoptosis of SKOV-3 cells through decreased mitochondrial membrane potential. These results are consistent with previous results on AV/PI apoptosis.

Apoptosis occurs through endogenous and exogenous pathways. The endogenous pathway, also known as the mitochondrial pathway, is mediated by the Bcl-2 protein family which regulates the permeability of the mitochondria outer membrane (18). When AKT is activated, Bcl-2 of the mitochondrial pathway is activated. The Bcl-2 protein contains BH4 domain and TM anchored in the mitochondrial outer membrane, and it blocks the release of cytochrome C from mitochondria, thereby preventing the activation of caspase apoptotic protein in many cells. Therefore, the mechanism of rubimaillin-induced apoptosis may be associated with distortion of the balance between Bax and Bcl-2, resulting in inability of Bcl-2 (located outside mitochondria) to prevent the release of cytochrome C from mitochondria to cytoplasm. This leads to decrease in mitochondrial membrane potential and initiation of endogenous apoptotic pathway, activation of caspase3 and PARP proteins, and irreversible apoptosis of cells (19, 20).

These results suggest that rubimaillin reduces the activity of human ovarian cancer SKOV-3 cells through mitochondria-dependent cell apoptosis. Thus, rubimaillin is a potential anti-ovarian cancer drug.

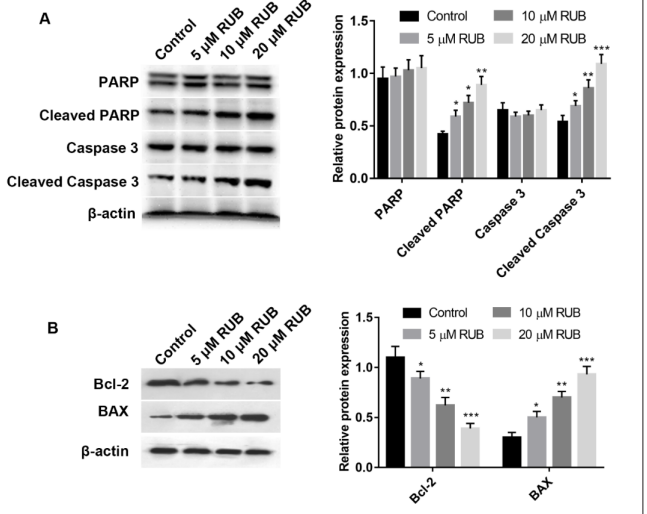

Figure 5. (A) Effect of Rub on caspase3 protein expression. . (B) Effect of Rub on Bcl-2 protein expression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$compared with control group.

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Conflict of Interest

There are no conflict of interest in this study.

Author's contribution

All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its content The study was conceived and designed by Li Yousheng; Zhang Siwei, Wu Zhen, Zhou Zhi, Hu Xuguang, Li Yousheng collected and analysed the data; Zhang Siwei wrote the text and all authors have read and approved the text prior to publication.

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