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Mechanism of the reversal effect of mifepristone on drug resistance of the human cervical cancer cell line HeLa/MMC

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ABSTRACT. We examined the ability of mifepristone to reverse the *in vitro* drug resistance of human cervical cancer cells resistant to mitomycin-C (HeLa/MMC) cells and investigated the mechanism of this effect. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to detect the drug resistance of HeLa/MMC cells and the reversed drug resistance *in vitro*. Expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and glucosylceramide synthase (GCS) were measured in HeLa and HeLa/MMC cells. The resistance index of HeLa/MMC cells on MMC was reduced from 5.02 to 1.46 after 10 μg/mL mifepristone exposure. A combination of mifepristone upregulated the Bax/Bcl-2 protein expression ratio and apoptosis in HeLa/MMC cells. GCS expression was significantly higher in HeLa/MMC cells than in HeLa cells ($P < 0.01$), but distinctly declined in both cell lines after mifepristone application ($P < 0.01$). Mifepristone reversed the resistance of HeLa/MMC cells to MMC *in vitro*; the overexpression of the GCS gene and the increased expression of apoptosis-related protein Bcl-2 may play important roles

in the formation of multidrug resistance in cervical cancer.

Key words: Mifepristone; Glucosylceramide synthase gene; Multidrug resistance; Apoptosis-related protein

INTRODUCTION

Cervical cancer is the most common malignant tumor in the female genital tract and one of the most important diseases threatening the lives and health statuses of women. Since 1999, the treatment of cervical cancer has involved comprehensive treatment that combines surgery and radiotherapy with chemotherapy. Neoadjuvant chemotherapy reduces the gross tumor volume, extends the 5-year survival rate, and decreases the recurrence rate, and has thus attracted extensive attention for various studies (Hamed et al., 2012; Lai et al., 2013; Okazawa et al., 2013). However, the multidrug resistance (MDR) of tumor cells to chemotherapy influences the clinical application and efficacy of treatments.

MDR is a term used to describe the ability of drug-resistant tumors to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents, and is a crucial determinant in the failure of clinical chemotherapy (Kantarjian et al., 2001). MDR shows complex mechanisms and is likely correlated with high expression of MDR-associated proteins and P-glycoprotein, a plasma membrane ATP-binding cassette transporter, as well as abnormalities in drug-metabolizing enzymes and rate-limiting enzymes in protein synthesis (Baguley, 2010). Mitomycin-C (MMC), a broad-spectrum antitumor antibiotic, has been applied in chemotherapy for gastric, colorectal, bladder, and cervical cancers; however, MDR has limited its clinical application (Zalipsky et al., 2007).

Mifepristone, as a progesterone/glucocorticoid receptor antagonist, was originally and widely used to terminate early pregnancy in clinical settings. Mifepristone was also found to increase the sensitivity of chemotherapies for gastric cancer, breast cancer, endometrial cancer, and leukemia (Gaddy et al., 2004; Li et al., 2004; Check et al., 2007; Navo et al., 2008). Its mechanisms may include: 1) blocking ceramide glycosylation and promoting cell apoptosis; 2) reducing exocytosis of MDR-associated proteins and P-glycoprotein while elevating the intracellular drug concentration; 3) enhancing the DNA repair capacity and changing the activity of topoisomerase II and the expression of tumor suppressor genes such as p53.

In this study, mifepristone was applied to human cervical cancer cells (HeLa) and the MMC resistant cell subline HeLa/MMC *in vitro* to verify its reverse effect and conduct a preliminary investigation of its mechanisms by detecting changes in the expression of B-cell lymphoma 2 (Bcl-2) and glucosylceramide synthase (GCS).

MATERIAL AND METHODS

Cell lines

The parental HeLa and HeLa/MMC cell line were provided by the Scientific Research Center in Zhongnan Hospital of Wuhan University. HeLa and HeLa/MMC cells were cultured in RPMI-1640 medium and RPMI-1640 medium containing 6.0 ng/mL 18 nM MMC, respectively. A stock solution of MMC (Zhejiang Hisun Pharmaceutical Co., Ltd.; Zhejiang, China)

at a concentration of 0.4 mg/mL was prepared in RPMI-1640 medium, sterilized by filtration, and stored at -20°C.

Reverse effect of mifepristone on drug resistance

Crude mifepristone (a gift from Hubei Gedian Humanwell Pharmaceutical Co., Ltd.; Hubei, China) was prepared as a 0.4 mg/mL stock solution with absolute ethanol, and diluted to a working concentration with RPMI-1640 medium; the final concentration of absolute ethanol was ≤0.2% (v/v). This solution was filter-sterilized.

HeLa and HeLa/MMC cells in logarithmic phase were seeded on a 96-well plate with 2 x 10^4 cells in each well, and were cultured at 37°C for 24 h. Different MMC and/or mifepristone concentrations were incubated with the cells in RPMI-1640 medium containing 5% fetal bovine serum for 72 h.

The drug treatment scheme included the following: 1) MMC group (0.00625, 0.0125, 0.025, 0.05, and 0.1 μg/mL); 2) mifepristone group (10 and 20 μg/mL); 3) MMC plus mifepristone group; 4) control group with equal amounts culture medium; 5) blank group. Each group was replicated in 3 wells at a final volume of 200 μL. The culture medium was removed after 72 h, and 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added to each well (Wuhan Cyber Biological Products Co., Ltd., Wuhan, China). After 4 h of incubation, 100 μL dimethylsulfoxide was added and the mixture was oscillated until all of the crystals had been dissolved. An enzyme-linked immunosorbent assay reader was used to detect optical density (OD) values at 490 nm for each well. The cell proliferation inhibition rate was calculated as follows: inhibition rate = (1 - OD value in the test group / OD value in the control group) x 100%. The half maximal inhibitory concentration (IC_{50}) was calculated from the dose-response curve. The resistance index (RI) was calculated according to the following formula: $RI = IC_{50a} / IC_{50b}$ (a = HeLa/MMC cells, b = HeLa cells).

Immunohistochemistry (IHC)

HeLa and HeLa/MMC cells were seeded on 6-well plates with 2 x 10^4 cells in each 1-mL well. The drug treatment scheme was as follows: 1) blank group; 2) 10 μg/mL mifepristone group; 3) 0.00625 μg/mL MMC group; 4) MMC plus mifepristone group. Each group was replicated in 2 wells with two 1-cm disinfected cover glass pieces in each well. The culture medium was removed after 72 h, followed by glass washing, fixation, serum blocking, and incubation with Bax and Bcl-2 primary antibodies (Wuhan Cyber Biological Products Co., Ltd.) and goat anti-mouse IgG secondary antibodies. The glass was washed with phosphate-buffered saline and incubated with streptavidin-biotin complex, followed by incubation with diaminobenzidine color development; hematoxylin counterstaining and mounting were performed before observation.

Reverse transcription polymerase chain reaction (RT-PCR)

Mifepristone and/or MMC were respectively applied to HeLa and HeLa/MMC cells in logarithmic phase for 48 h, and the drug treatment scheme was the same as that used in the IHC experiment. Total RNA from cultured cells was extracted using the TRIzoL method (Invitrogen;

Carlsbad, CA, USA) and RNA purity was analyzed using an ultraviolet spectrophotometer.

The Primer 5.0 software was used to design primers based on the gene sequences of *GCS* and *β-actin*, which were synthesized by Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China). The sequence of the *GCS* gene forward primer was 5'-GATACGCTTACTGACATGGTGA-3'; the sequence of the *GCS* reverse primer was 5'-GAAACCAGTTACATTGGCAGAG-3'; the size of the *GCS* gene products was 162 bp. The sequence of the *β-actin* forward primer was 5'-AGCGAGCATCCCCCAAAGTT-3'; the sequence of the *β-actin* reverse primer was 5'-GGGCACGAAGGCTCATCATT-3'; the size of the *β-actin* gene product was 284 bp. A One-step RT-PCR Kit (Fermentas; Vilnius, Lithuania) was used to amplify the *GCS* and *β-actin* genes. The procedure for amplification of cDNA included 28 cycles, each of which was as follows: 90°C for 30 s → *GCS* 50°C/*β-actin* 54°C for 30 s → 72°C for 25 s. Products were visualized by 1.5% agarose gel electrophoresis with constant voltage, and images were acquired and analyzed using a gel imaging analysis system.

Statistical analysis

Data reported as means ± SE , and multiple groups in the same cell lines were compared using one-way analysis of variance; the Student *t*-test was utilized to compare groups with the same treatment in different cell lines and the group combined with mifepristone in the same cell lines before and after medication. $P < 0.05$ was considered to be significant.

RESULTS

Mifepristone reversed drug resistance

Figure 1 shows that the survival rate of the 2 cell lines was reduced with increasing MMC concentration, and that the survival rate of HeLa/MMC cells was higher than that of HeLa cells at all MMC concentrations ($P < 0.01$). The growth inhibitory effects of 20 and 10 μg/mL mifepristone were 8.67 and 1.55% for HeLa/MMC cells and 33.69 and 6.18% for HeLa cells, respectively. In the subsequent experiment that reversed drug resistance of HeLa/MMC, treatment of mifepristone alone at low concentration (10 μg/mL) was selected as a drug treatment factor owing to its low cell growth inhibitory effect.

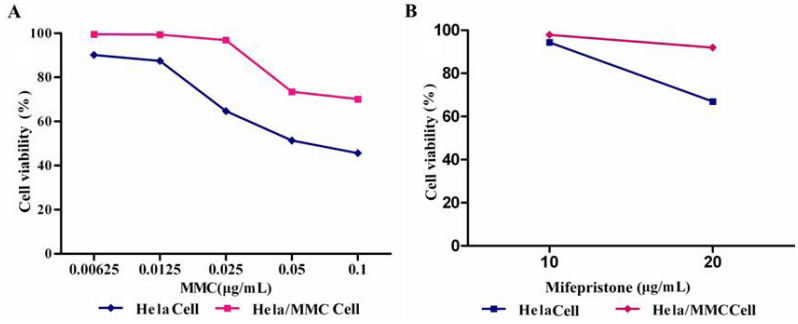

Figure 1. Cell growth inhibitory effects of MMC and mifepristone on HeLa and HeLa/MMC cells. **A.** Cell growth inhibitory effect of MMC; **B.** cell growth inhibitory effect of mifepristone.

Table 1 shows that the IC_{50} values of HeLa/MMC and HeLa cells with MMC were 0.324 μg/mL and 0.064 μg/mL, respectively, and the RI of HeLa/MMC cells against MMC was 5.02. The RI of HeLa/MMC cells was reduced to 1.46 after treatment with mifepristone. Drug resistance of HeLa/MMC cells markedly reversed after application of 0.00625 μg/mL MMC with mifepristone. Therefore, subsequent experiments used 0.00625 μg/mL MMC as a processing factor.

Table 1. IC_{50} of HeLa and HeLa/MMC cells before and after combined application of 10 μg/mL mifepristone (N = 4, means ± SE).

Cell line	MMC (μg/mL)	MMC with mifepristone (μg/mL)
HeLa/MMC	0.324 ± 0.002	0.095 ± 0.001**
HeLa	0.064 ± 0.001	0.063 ± 0.002

In the HeLa/MMC cell line, cell viability was significantly inhibited in the combination group compared with the MMC group, **P < 0.01.

Bcl-2 and Bax expression

Tables 2 and 3 show that Bax protein expression was higher and Bcl-2 protein expression was lower in HeLa cells than in HeLa/MMC cells for both the control and MMC groups (P < 0.05). After combination treatment with mifepristone, HeLa/MMC cells expressed increased amounts of Bax protein and decreased amounts of Bcl-2; the ratio of Bax/Bcl-2 was elevated, indicating that a combination of MMC and mifepristone treatment enhanced cellular Bax expression and the Bax/Bcl-2 ratio, thereby reversing drug resistance and promoting cell apoptosis.

Table 2. Expression of Bax protein in HeLa and HeLa/MMC cells from each group (N = 5, means ± SE).

Group	HeLa (OD value)	HeLa/MMC (OD value)
Control group	0.31 ± 0.01	0.28 ± 0.03*
MMC group	0.41 ± 0.04	0.34 ± 0.03*
Mifepristone group	0.36 ± 0.03	0.32 ± 0.01
Combination group	0.55 ± 0.02	0.57 ± 0.01▲

Compared with the HeLa cell line, expression of Bax protein was lower in the HeLa/MMC cell line in the control and MMC groups, *P < 0.05. In the HeLa/MMC cell line, compared with the MMC group, the expression of Bax was significantly increased, ▲P < 0.05.

Table 3. Expression of Bcl-2 protein in HeLa and HeLa/MMC cells from each group (N = 5, means ± SE).

Group	HeLa (OD value)	HeLa/MMC (OD value)
Control group	0.33 ± 0.01	0.38 ± 0.02*
MMC group	0.28 ± 0.03	0.33 ± 0.01*
Mifepristone group	0.35 ± 0.01	0.38 ± 0.01
Combination group	0.25 ± 0.02	0.27 ± 0.01▲

Compared with HeLa cells, expression of Bcl-2 was higher in the HeLa/MMC cell line for the control and MMC groups, *P < 0.05. In the HeLa/MMC cell line, combination use of mifepristone and MMC inhibited expression of Bcl-2 compared with the MMC group, ▲P < 0.05.

GCS expression

As shown in Figure 2 and Table 4, GCS expression was significantly higher in HeLa/

MMC cells than in HeLa cells in both the control and MMC groups ($P < 0.01$); only mifepristone showed no obvious effect on GCS expression in the 2 cell lines ($P > 0.05$); individual application of MMC upregulated GCS expression by 1.55 times compared with that of the control group in the HeLa/MMC cell line ($P < 0.01$); a combination of MMC and mifepristone treatment distinctly reduced GCS expression in both cell lines ($P < 0.01$). These results indicate that combination treatment with MMC and mifepristone dramatically inhibited GCS expression in parental and resistant cell lines and may affect MMC function.

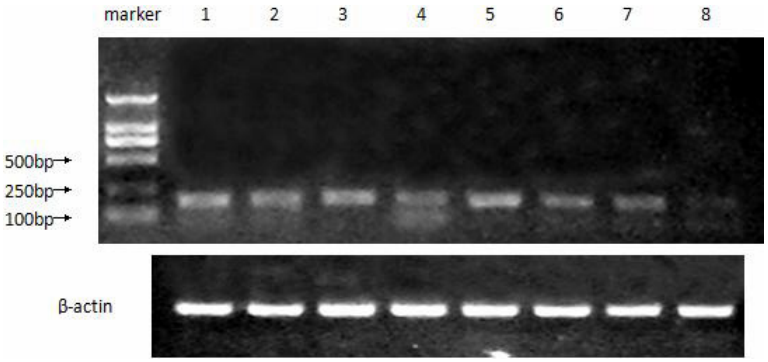

Figure 2. Expression of GCS in HeLa and HeLa/MMC cells and the effects of mifepristone and MMC on GCS expression. *Lane 1* = HeLa/MMC, control group; *lane 2* = HeLa, control group; *lane 3* = HeLa/MMC, mifepristone group; *lane 4* = HeLa, mifepristone group; *lane 5* = HeLa/MMC, MMC group; *lane 6* = HeLa, MMC group; *lane 7* = HeLa/MMC, combination group; *lane 8* = HeLa, combination group.

Table 4. Semi-quantitative RT-PCR results of GCS expression in HeLa and HeLa/MMC cells of each group (N = 4, means ± SE).

Cell line	Control group	Mifepristone group	MMC group	Combination group
HeLa/MMC	0.36 ± 0.04**	0.32 ± 0.07*	0.54 ± 0.1**/#	0.24 ± 0.02**#
HeLa	0.24 ± 0.03	0.21 ± 0.03	0.16 ± 0.01	0.04 ± 0.01#

Compared with the HeLa cell line, GCS expression was increased in the HeLa/MMC cell line, *$P < 0.05$, **$P < 0.01$. GCS expression was significantly inhibited in the combination treatment group in both cell lines compared with other groups, MMC stimulated GCS expression in the HeLa/MMC cell line, #$P < 0.01$.

DISCUSSION

Mifepristone selectively inhibits the formation and development of many tumor types and plays an anti-tumor role as an antagonist through progesterone and glucocorticoid receptors (Kacinski et al., 2001; Luo et al., 2009; Ligr et al., 2012). In this study, 10 μg/mL mifepristone and MMC treatment at different concentrations was applied to HeLa/MMC cells; mifepristone alone had no significant inhibitory effect on the development of HeLa/MMC cells, but both the inhibition rate and drug sensitivity of the cells increased after combination treatment with MMC, indicating that other than anti-tumor activity, mifepristone reverses the drug resistance of tumor cells to some extent. It has been reported that mifepristone can also reverse the drug resistance of tumor cell lines from lung cancer, breast cancer, thymic tumors, ovarian cancer, prostate cancer, stomach cancer, and leukemia, among others, which confirms

its role as a resistance modifier in reversing the drug resistance of tumor cells (Payen et al., 1999; Check et al., 2010).

The genes encoding Bcl-2 and Bax regulate cell apoptosis, and Bcl-2 protein encoded by the *Bcl-2* gene resists cell apoptosis; Bax shows the opposite effect, which is to promote apoptosis. According to previous studies, overexpression of the *Bcl-2* gene and related proteins is important in tumorigenesis and multiple drug resistance. Schneider et al. (1998) found that mifepristone inhibits the growth of KLE cells and endometrial cancer cells, as well as reduces the expression of the *Bax* gene. In this study, mifepristone was found to enhance Bax protein expression and reduce Bcl-2 protein expression in HeLa/MMC cells after combination treatment of MMC with mifepristone. This treatment inhibited the proliferation of drug-resistant cervical cancer cells and promoted cell apoptosis. A sensitization effect of mifepristone on renal carcinoma Caki cells to TRAIL-induced apoptosis was found by Min et al. (2012). This effect was independent of mifepristone's antiglucocorticoid and antiprogesterone roles, and one of these roles downregulated *Bcl-2*.

Lucci et al. (1999) reported that mifepristone could block ceramide glycosylation, reversing the resistance of the human breast cancer cell MCF-7-AdrR to adriamycin. A series of studies showed that mifepristone reverses the resistance of human breast cancer MCF-7-AdrR cells to doxorubicin and the resistance of COC (1)/DDP cells to cisplatin (Hu et al., 2008; Liu and Wang, 2008). In this study, we found that mifepristone alone could not significantly affect the expression of GCS in HeLa cells and HeLa/MMC cells ($P > 0.05$), but combination of mifepristone and MMC treatment significantly reduced the GCS expression level ($P < 0.01$) while cell sensitivity to MMC increased. We hypothesized that the inhibitory effect of mifepristone on GCS expression in HeLa/MMC cells required the co-existence of MMC, and that this combination reversed the drug resistance to MMC by inhibiting GCS expression. This also supported our hypothesis that the mifepristone effect was independent of its antiprogesterone role and directly influenced the MDR mechanism.

Ceramide was negatively correlated with Bcl-2 expression, indicating that a reduction in ceramide results in upregulation of *Bcl-2* expression, whereas GCS decreases the amount of ceramide through ceramide glycosylation, influencing the signaling pathways of cell apoptosis/anti-apoptosis and causing drug resistance. Mifepristone induces cell apoptosis by downregulating Bcl-2 expression and elevating transforming growth factor-beta 1 (TGF-β1) expression in LNCaP cells (a prostate cancer cell line) (Check et al., 2010). Silencing of the *GCS* gene in human ovarian cancer cells enhances the sensitivity of p53 mutant cells to doxorubicin and induces the expression of p53-responsive genes, including *Bax* (Liu et al., 2011). In our study, HeLa/MMC cells overexpressed GCS and enhanced Bcl-2 protein expression. Combination treatment with mifepristone and MMC markedly inhibited GCS expression and simultaneously reduced Bcl-2 protein expression in HeLa/MMC cells, indicating that mifepristone regulated the expression of *Bcl-2* and *Bax* as well as *GCS*. This data is in agreement with another study conducted by Zhang et al. (2012), who found that doxorubicin significantly upregulated the expression of GCS in estrogen receptor alpha (ERα)-positive MCF-7 cells. When the Sp1 site of the *GCS* promoter or ERα was inhibited, this upregulation of *GCS* by doxorubicin was also inhibited. The cascade sequence on the HeLa/MMC cells by mifepristone requires further investigation.

In conclusion, GCS overexpression, enhanced Bcl-2 protein expression, and decreased Bax protein expression may play crucial roles in the development of MDR in cervical cancer

cells. Mifepristone can reverse the drug resistance of HeLa/MMC cells *in vitro*, which is likely associated with downregulation of *Bcl-2* expression and inhibition of *GCS* expression. However, the detailed cascade correlation was not evaluated in this study and should be further examined.

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