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# Antitumor effect of mifepristone on human endometrial stromal cell line

## Antitumorski efekat mifepristona na stromalnu ćelijsku liniju humanog endometrijuma

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### Abstract

**Background/Aim.** The main cause for development of endometrial hyperplasia is unopposed effect of estrogen on endometrial cells. The aim of our study was to investigate and compare cytotoxic and apoptotic effects of mifepristone on human endometrial stromal cell line for the first time. Both percentage of cytotoxic and apoptotic cells were determined after 24 h treatment with different doses of mifepristone. **Methods.** The percentage of cytotoxic cell was evaluated by viability assay while the percentage of apoptotic cells was determined using flow cytometry. Determination of apoptotic effects was confirmed using immunofluorescence method determining expression and localization of active Bax and Bcl-2 proteins. **Results.** Our results indicated that mifepristone induced cytotoxic and apoptotic effect on human endometrial stromal cell line (ThESC) through changes in expression level of Bcl-2 and active Bax proteins. **Conclusion.** Cytotoxic and pro-apoptotic effects of mifepristone on human endometrial stromal cell line *in vitro* was investigated in this study for the first time. It is crucial to point out that mifepristone expressed both cytotoxic and pro-apoptotic effect on ThESC cell line. Our results may contribute to determination of localization and expression level of the crucial proteins involved in apoptosis in ThESC cell line after the treatment with the lowest cytotoxic doses of mifepristone.

**Key words:**
**endometrial hyperplasia; mifepristone; apoptosis; cell death; carcinoma.**

### Apstrakt

**Uvod/Cilj.** Glavni uzrok razvoja hiperplazije endometrijuma je neoponirani efekat estrogena na endometrijalne ćelije. Cilj naše studije bio je da se prvi put istraže i uporede citotoksični i apoptotični efekti mifepristona na stromalnoj liniji humanog endometrijuma. Procenat citotoksičnih i apoptotičnih ćelija je određivan nakon tretmana različitim dozama mifepristona u periodu od 24 časa. **Metode.** Procenat citotoksičnih ćelija bio je procenjen korišćenjem testa za ispitivanje vijabilnosti ćelija, dok je procenat apoptotičnih ćelija bio određen korišćenjem protočne citometrije. Utvrđivanje apoptotičnog efekta je potvrđeno određivanjem ekspresije i lokalizacije aktivnog Bax proteina i Bcl-2 proteina. **Rezultati.** Naši rezultati su pokazali da mifepriston ispoljava citotoksični i apoptotični efekat na humanu endometrijalnu ćelijsku liniju (ThESC) putem promene nivoa ekspresije aktivnog Bax i Bcl-2 proteina. **Zaključak.** U ovoj studiji smo prvi put ispitali citotoksični i pro-apoptotični efekat mifepristona na humanu stromalnu ćelijsku liniju endometrijuma *in vitro.* Od suštinskog značaja je nalaz da je mifepriston ispoljio i citotoksični i pro-apoptotični efekat na ThESC ćelijsku liniju. Naši rezulatati ukazuju na moguć nivo lokalizacije i ekspresije ključnih proteina apoptoze u ThESC ćelijama nakon tretmana sa najnižim citotoksičnim dozama mifepristona.

**Ključne reči:**
**endometrijum, hiperplazija; mifepriston; apoptoza; ćelija, smrt; karcinom.**

## Introduction

Different types of morphological and physiological endometrial cell disorders may lead to development of endometrial diseases. One of them is presented in a form of endometrial hyperplasia. Uterine cells changes morfologicaly and physiologicaly during endometrial hyperplasia. These changes are represented as excessive glandular and stromal endo

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metrial cells proliferation associated with different degrees of cellular atypia and morphological abnormalities [1, 2]. These changes include cystic dilatation of endometrial glands without secretion, anovulatory cycles and presence of abnormal bleeding [3]. As a result of ongoing cell changes during endometrial hyperplasia, endometrium switches from normal towards tumorous tissue [2]. In women under the age of 30, endometrial hyperplasia is rare; however in age group of 50–54 years, increasing of incidence has been recorded. During aging, incidence of atypical endometrial hyperplasia with cytological changes increases as a prelude to a precancerous condition [4]. Treatment of endometrial hyperplasia is restricted to two types of approach. First treatment type is represented in a form of progestin (hormone) therapy, while second type of treatment is represented in surgical approach (hysterectomy) [5]. Progestin therapy approach is oriented towards one of the main problems leading to the development of endometrial hyperplasia – lack of progesterone component; while operative approach constitutes the last used technique considering complete removal of uterus and ovary. Mifepristone (RU-486, RU-38486) belongs to the class of progesterone and glucocorticoid receptor antagonist [6, 7]. This drug has various applications in different types of diseases. It has been showed that mifepristone can be suitable for patients with hyperglycemia as a secondary disorder in the Cushing's syndrome [8], it induces miscarriage and it is used to oppose proliferative effect of estrogen on endometrium, treatment of endometriosis, leiomyoma, breast cancer, and meningioma [9, 10]. Application of low doses of mifepristone express antiproliferative effects in various cancer of reproductive and non-reproductive organs [7, 9, 11, 12]. These data were confirmed in *in vitro* study performed by Goyenche et al. [13]. In this study, mifepristone exhibited inhibitory effect on ovarian cancer cell (SK-OV-3, Caov-3, OV2008, and IGROV-1) growth *in vitro*. In research that was conducted by Li et al. [12] it was shown that mifepristone induced apoptosis in Ishikawa cell lines through Bax translocation and caspase 3 activation; Some authors also confirmed the role of mifepristone on apoptosis induction in endometrial cancer cell lines (Hec-1A, KLE, and RL95-2) that involved changes in Bax and Bcl-2 regulation [11].

The aim of our study was to investigate the effects of single application of mifepristone on induction of apoptosis using human endometrial stromal cell line (ThESC cell line).

## Methods

### *Cell line*

In our study we used human endometrial stromal cell line (ThESC cell line – ATCC® CRL-4003) obtained from an adult woman with myoma. In our experiment, the passage of ThESC cells 8–10 was used and this cell line was telomerase reverse transcriptase (hTERT) immortalized. Cells were grown in complete Dulbeceo's Modified Eagle Mwedia (DMEM) medium containing glucose 4.5 g/L, 2% L-glutamine (2 mM), 1% penicilyn/streptomycin, 1% of non-essential amino acids, 1% of insulin transferrin supplement and 10% fetal bovine serum (FBS) in control environment at 37C° and 5% $CO_2$. The cells were divided into control group and cells treated with different doses of mifepristone (10, 20, 40, 60 and 80 μM) during 24 hour period. Cells were seeded in 96 well plates and treatment with investigated drugs was performed after reaching 85% of cell confluence.

### *MTT assay*

Using a viability assay (MTT assay) cytotoxic effects of mifepristone was evaluated. MTT assay is based on the possibility of the metabolically active cell to perform the reduction of yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide) by the action of dehydrogenase enzymes in order to generate reducing equivalents such as $NADH+H^+$ and $NADPH+H^+$. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. Both control and experimental cells were resuspended in complete medium ($1.8 \times 10^4$ cells/200 μL medium), seeded in 96 well micro titer plate and treated with investigated drugs. After 24 h period, supernatant was extracted and cells were incubated with MTT solution (5 mg/mL MTT dissolved in PBS) for 4 h (37°C, 5% $CO_2$). After incubation period for 4 hours, MTT solution was removed and cells were resuspended with 200 μL DMSO (Sigma Chemical, ST. Lois, Mo.) per well and incubated for 30 min on a shaker at room temperature. The absorbance was measured at wavelength 595 nm (multimode micro plate detector, Zenith 3100).

### *Apoptosis detection – flow cytometry*

Type of cell death induced with investigated substances was determined with Annexin V-FITC/PI staining using flow cytometry. ThESC cells were seeded in 24-well plate ($1 \times 10^5$ cells/well) and treated with different doses of mifepristone during 24 h period. At the end of incubation period cells were collected and washed 3 times with PBS, resuspended in 100 μL of ice-cold 1× binding buffer, stained with 10 μL of Annexin V-FITC and 20 μL of PI and incubated in the dark for 15 min at +4ºC. Finally, 400 μL binding buffer was added and the cells were analyzed by flow cytometer Cytomics FC500 (Beckman Coulter, USA). Data were analyzed using Flowing Software 2 and presented by bar charts.

### *Immunofluorescence assay*

Following the determination of cytotoxic effect of investigated drugs, in our next experiment we treated the cells with the lowest cytotoxic doses of investigated substances. In order evaluate both the expression level and localization of two key members of Bcl-2 protein family, with pro- and anti-apoptotic activity [14], Bcl-2 and Bax, respectively, we used the immunofluorescence method. Incubation of the treated cells (1 h period; 1:50 dilution) was performed with different anti-rabbit primary antibodies: Bax (N20, sc-493, Santa Cruz Biotech. Inc), Bcl-2 (DC21, sc-783, Santa Cruz Biotech. Inc) and β-actin (A5316, Sigma Aldrich, Germany). Following the primary antibodies incubation, cells were washed three times in 1xPBS and incubated in the dark with specific secondary fluo-

rescent antibodies conjugated with Alexa 488 (11001, Invitrogen, USA) and Alexa 594 (gift from Dr Ljubica Ivanišević, Ottawa, Canada) (1:100) for 30 minutes. Cells were visualized by fluorescence microscopy at 100 × and 400 × magnifications on Olympus microscope (model BX51). In order to quantify fluorescence intensity of used specific primary and secondary antibodies, we used noncommercial software ImageJ 1.51j version. These values of fluorescence intensity obtained using ImageJ were transferred to Excel and used to produce bar charts that represented the intensity of fluorescence for specific antibodies which were used in these experiments.

*Statistical analysis*

All values were expressed as mean ± standard deviation (SD). Each experiment was performed in triplicate and conducted on every sample as described earlier. Commercial SPSS version 24.0 was used for statistical analysis. Statistical evaluation was performed using Student's *t*-test for paired observations, or one-way ANOVA depending on data distribution. *P* values that were less than 0.05 ($p \leq 0.05$) were considered as significant.

## Results

Results of cytotoxic effects of different doses of mifepristone on ThESC cell line during 24 h period using MTT test, indicated that mifepristone expressed dose dependent cytotoxic effect on ThESC cell line in comparison to untreated cells (Figure 1). These results indicated that application of mifepristone in the highest dose (80 µM) caused cytotoxic effect in 72.72% loss viable of cells, while this percentage after 10 µM mifepristone treatment cytotoxic effect on the cells was 20.14 % (cytotoxicity was 3.61 times lower compared to cytotoxicity that was induced in the case of 8 times higher dose of mifepristone).

In order to evaluate the type of the cell death in mifepristone ThESC treated cells we used Annexin FITC/PI staining (Figure 2). Given the fact that higher doses of mife-

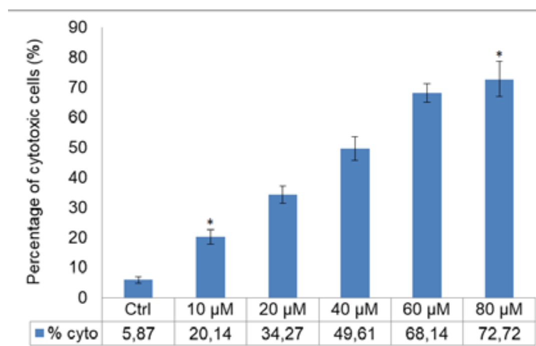


**Fig. 1 – Cytotoxic effect of different mifepristone doses on human endometrial stromal cell line (ThESC) cells during 24 h period (Ctrl – control).**

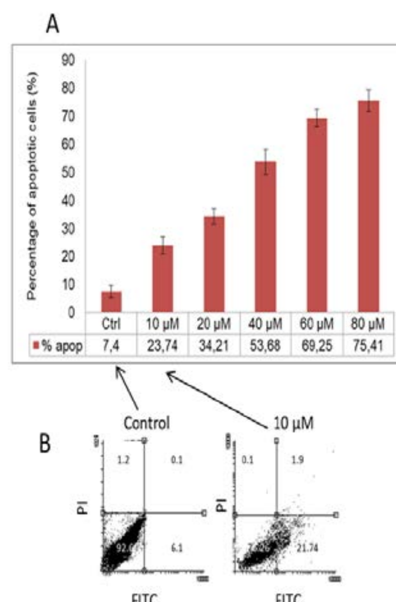


**Fig. 2 – A) Human endometrial stromal cells (ThESC) were treated with different concentrations of mifepristone (10, 20, 40, 60, and 80 µM) for 24 h, cell apoptosis was analyzed by Annexin V FITC/PI staining (bar chart);**
**B) Mifepristone promoted ThESC cells apoptosis in a dose-dependent manner (Dot plot analysis: upper left quadrant – necrotic cells, upper right – late stage apoptotic cells, lower right – early stage apoptotic cells and lower left – live cells).**
**Bar charts analysis was given for control and cells treated with 10 µM mifepristone.**

pristone induced statistically significant percentage of apoptotic cells (from 34.21% for 20 μM to 75.41% for 80 μM), higher doses of mifepristone (from 20 to 80 μM) ($p \leq 0.05$) were excluded from our further experiments.

Fluorescence-activated cell sorting (FACS) analysis showed that ThESC treatment with mifepristone (10 μM) resulted in 3-fold increase of apoptotic (23.74%) ThESC cells compared to control (7.4%) after 24 h period. The percentage of treated cells in early and late stage of apoptosis were 21.74% and 1.9%, respectively while in control cells percentage of early apoptotic cells was 4 times lower (6.1%). Results of Annexin V FITC/PI staining were in direct correlation with the result obtained by MTT test. Based on the results obtained with MTT assay and FACS analysis, in our next experiment we applied the immunofluorescence method in order to determine the mechanism of apoptosis induced with mifepristone. Our first step in determination of the apoptotic mechanism was to investigate and compare the level of expression of the anti-apoptotic Bcl-2 protein in treated and control cells. Immunofluorescence assay revealed the highest level of Bcl-2 expression of 96.1% in untreated, control cells (Figure 3). However, expression level of Bcl-2 protein in mifepristone treated cells (50.4%) was significantly lower with 1.9-fold decrease compared to the control (96.1%). Decreased level of the Bcl-2 protein expression after mifepristone treatment led us to investigate the effects of mifepristone treatment on the expression level of N terminal, active, mitochondrial Bax protein.

These data we used in order to show that changes in level of the Bax protein expression after mifepristone treatment directly induced apoptosis due to ongoing changes affecting mitochondrial membrane integrity. Our data confirmed these findings (Figure 3). The expression level of active Bax protein was lowest in the case of control cells (2.3%) while in the case of mifepristone treatment, expression level of Bax protein was statistically significantly higher compared to the control cells (29.9%) (Figure 3). Eleven-fold increase in expression level of Bax protein in mifepristone treated cells was statistically significant compared to the control cells. Our overall results indicate that mifepristone induced apoptosis in ThESC with the same mechanism involving inner, mitochondrial pathway.

**Discussion**

The benign changes in uterus that arise as the effect of prolonged and unopposed estrogen stimulation are dangerous for several reasons. First of all, extremely proliferated endometrial tissue derived as a result of estrogen effect on endometrium, cannot be adequately nourished by blood vessels, ultimately resulting in endometrial shedding followed by prolonged and irregular discharge that is not relevant to menstrual cycle [15]. Second, accumulation of replication/transcriptional errors, high frequencies of chromosomal aberrations and gene rearrangements and amplifications results in formation of the tumor [16]. These changes affecting endometrial cells are accompanied by others factors such as: obesity, inadequate hormonal balance, genetics, age, life style, early age menarche [17] lead to infertility and imminent onset of endometrial cancer. Treatment approaches for endometrial cancer include surgery and different types of drugs. One of those drugs is mifepristone which is registered for termination of early pregnancy. However, different data are still arising from new studies indicating mifepristone to be applicable for other diseases. Besides its original approval, mifepristone inhibited growth of cancer cells of reproductive and non-reproductive origin regardless of progesterone receptor expression [18]. In these experiments mifepristone induced maximum cell growth inhibition of MDA-MB-231 and MCF-7 breast cancer cell line at dose of 5 μM (90%), while in the case of higher dose (40 μM), cell growth inhibition percentage for both cell lines was 25%. However, when mifepristone was administered in ovarian cancer cell line, results indicated that higher doses of mifepristone (40 μM) induced cell growth inhibition of OVCAR-3 and SK-OV-3 cell line for 40% and 15%, respectively [19]. These data indicate that cancer cell growth inhibition of mifepristone directly depends on the cell line type. In the treatment of endometrial

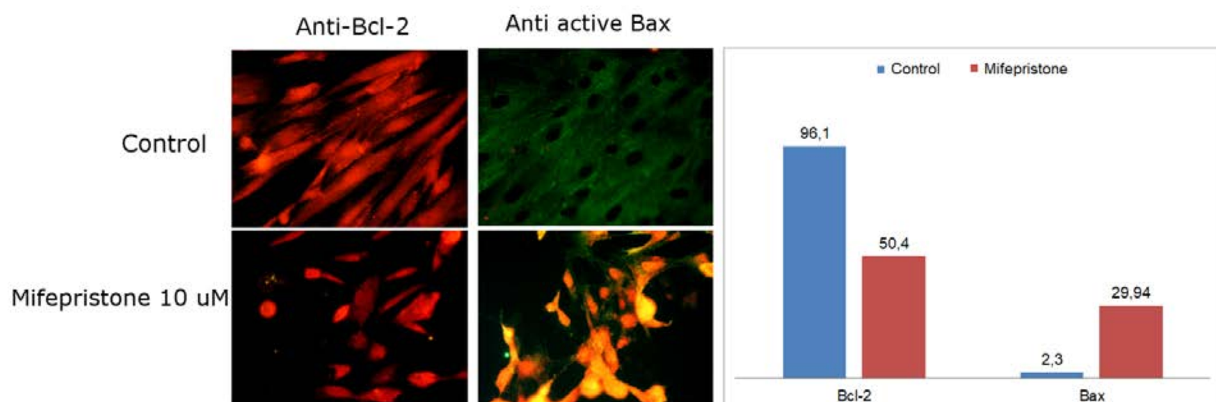


**Fig. 3 – Expression of Bcl-2 and active Bax protein in human endometrial stromal cell line (ThESC) cells after treatment by mifepristone during 24 h period. Expression level of Bcl-2 protein in control cells was statistically higher compared to the cells treated with mifepristone (down-regulation of Bcl-2 protein in mifepristone treated cells). Active Bax is mainly localized in cytosol compared to mifepristone treated cells which show redistribution of active Bax to the mitochondria. Intensity of Bcl-2 and active Bax was calculated using ImageJ software (active Bax = 100 – mean of measured fluorescence).**

hyperplasia, mifepristone openness' effects of estrogen on endometrial tissue. In studies conducted both on Ishikawa and human endometrial cell line (HEC1A) it was showed that mifepristone inhibited cell growth and induced apoptosis through caspase-3 activation [19–21]. This study indicated that $IC_{50}$ values of mifepristone for HEC-1-A and Ishikawa were 16 and 19 μg/mL. Our results correspond to the previously published data [19–21], mifepristone applied in 10 μM during 24 h period caused down regulation of Bcl-2 and overexpression of active Bax protein in mifepristone treated ThESC cells, indicating direct involvement of mitochondrial apoptotic pathway. In our experiment we obtained similar results. Mifepristone expressed cytotoxic and apoptotic effect on human endometrial stromal cell line during a 24 h period. However, our findings indicated that mifepristone applied at a dose of 10 μM caused cytotoxic effect on 20.14% of the investigated cell population. When we compared the applied doses of mifepristone to the percentage of cytotoxic cells, results indicated that highest mifepristone dose resulted in nearly 2-fold increase in the percentage of cytotoxic cells compared to the effect that was induced with the lowest investigated dose of mifepristone. Our data are in correlation with the result that was obtained in different studies [12, 22]. Adequate dose of mifepristone compared to its cytotoxic effect on ThESC cell line was 10 μM. In study conducted by Ørbo et al. [21], researchers investigated effects of different doses of mifepristone on growth inhibition and apoptosis induction on Ishikawa cell line. Their results indicate that mifepristone caused cell cycle arrest in G1/G0 phase and induced apoptosis. In experiments conducted on Ishikawa, EM42, KLE, RL95-2 and HEC-1-A cell line, results also indicate that mifepristone promote apoptosis by overexpressing Bax and down-regulating Bcl-2 protein [12]. Along with C terminal mouth of Bax which is responsible for channel formation, N terminal mouth of Bax affects the permeability of the outer mitochondrial membrane and preserves its apoptotic capacity [22–24]. Translocation of active Bax towards outer mitochondrial membrane followed with pore formation, causes release of cytochrome c, formation of apoptosome and cleavage of executioner caspases. This type of pathway is called inner apoptotic pathway. Our results showed that mifepristone causes down-regulation of Bcl-2 protein and overexpression of active Bax toward outer mitochondrial membrane, thus indicating that mifepristone apoptotic mechanism of action in ThESC cells is orchestrated via inner, mitochondrial pathway.

## Conclusion

Cytotoxic and pro-apoptotic effects of mifepristone on human endometrial stromal cell line *in vitro* was investigated in this study for the first time. Our results showed that mifepristone treatment statistically and significantly changed the expression levels of analyzed proteins involved in apoptosis, in opposite manner. It is important to point out that mifepristone expressed both cytotoxic and apoptotic effect on ThESC cell line. Our results indicate to a possible localization and expression level of the crucial proteins involved in apoptosis in ThESC cell line after the treatment with the lowest cytotoxic doses of mifepristone.

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## Conflict of interest

Authors wish to declare that there is no conflict of interest.

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