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Synergistic effects on pregnancy-termination activity of DL111-IT in combination with mifepristone[1]

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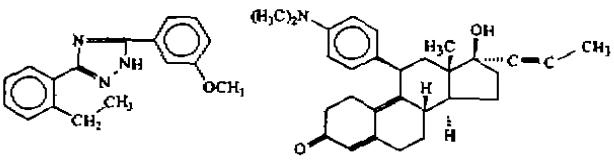
KEY WORDS mifepristone; DL111-IT; decidua; progesterone receptors; fetus; cultured cells; drug synergism

AIM: To clarify the role of DL111-IT when combined with mifepristone (Mif) on termination of early pregnancy **METHODS**: Progesterone receptors (PR) was measured using radioligand assay (RA), and peroxidase-antiperoxidase (PAP) immuno-histochemistry. Decidual cells (DC) were estimated using cell culture and histological examination (HE) (including fetus). ***RESULTS***: DL111-IT 100 mg·kg^{-1} *im*, Mif 5 mg·kg^{-1} ig, DL111-IT 16 mg·kg^{-1} im + Mif 1.5 mg·kg^{-1} ig and tea seed oil (TSO) 2 mL·kg^{-1} im on d 7 of pregnancy in rats, DC and fetus of treated groups were found to be degenerated at 24 h after treatment, at 48 h after treatment, PAP labeling index (%) of uterus PR of 4 groups were 22 ± 4, 18.7 ± 2.9, 10.3 ± 1.2, 52 ± 15, respectively. Rats im DL111-IT 100 mg·kg^{-1} 24 h after treatment, the quantity of PR decreased by 47 % *vs* that of TSO. The affinity of PR with Mif and progesterone did not change. Cultured human DC were exposed to DL111-IT and Mif 0 - 50 mg·L^{-1}, alone or combinatively, for 24 h. LD_{50} (mg·L^{-1}) were: DL111-IT 18.1 (15.1 - 21.4), Mif 25.0 (23.1 - 26 9), DL111-IT 5.0 plus Mif 3.5 (1.8 - 6.5) or Mif 5.0 plus DL111-IT 4.6 (1.1 - 7.3), respectively. **CONCLUSION**: DL111-IT enhanced action of Mif on DC, reduced quantity of PR, so the 2 drugs had the synergistic action in termination of early pregnancy.

Mifepristone (RU486, Mif) is a progesterone (P) antagonist that acts by binding the P receptor (PR) and preventing P-induced mRNA transcription[1]. Mif terminates 95 % of early pregnancies when administered with a prostaglandin, but the combination had several problems to be solved[2]. DL111-IT [3-(2-ethyl phenyl)-5-(3-methoxy phenyl)-1H-1, 2, 4 triazol] is a nonhormonal contragestative agent[3]. Mif in combination with DL111-IT increased the rate of complete abortion, enhanced the efficacy, decreased the effective dose, and reduced the side effects in mice, rats, hamsters, guinea pigs, and monkeys (in press). The present study was conducted to determine the synergistic action of the combination of these 2 drugs on PR, decidua, and fetus.



DL111-IT **Mifepristone**

MATERIALS AND METHODS

Drugs and Reagents Mif, DL111-IT, and injectable tea seed oil (TSO) were manufactured by Zhejiang Xianju Pharmaceutical Co. [^{3}H] Promegestone ([^{3}H] Pro, 156.1 TBq·mol^{-1}) was purchased from New England Nuclear. FD (F_{12} + Duibecco's modified Eagls's medium) culture medium, Pro, trypsin, 3, 3-diaminobenzidine tetrahydrochloride (DAB) were from Sigma. Rabbit anti-rat monoclonal PR antibody (Ⅰ Ab) (diluted 1:50), goat anti-rabbit immuno-globulin G antiserum (Ⅱ Ab) (diluted 1:50), rabbit PAP complex (Ⅲ Ab) (diluted 1:200), and bovine serum albumin (BSA) were purchased from Sigma Chemicol Co.

Unpregnancy test in rat Sprague-Dawley rats (♀, n = 80, 210 - 250 g; ♂, n = 40, 300 - 340 g) were provided by the Shanghai Institute of Materia Medica or Zhejiang Medical University Animal Center) were mated and pregnancy timed from the occurrence of sperm in the vaginal smear (d 1).

Ethical human decidual cells were obtained from the healthy pregnant woman (6 - 9 wk) by vacuum aspiration of

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decidua, and distributed by Shanghai Ruijing Hospital. Rats were kept in 14-h light – 10-h dark period, 22 ℃, and 60 % humidity. On d 7, rats were randomly divided into 4 groups: A) DL111-IT 100 mg·kg^{-1} im, B) Mif 5 mg·kg^{-1} ig, C) DL111-IT 16 mg·kg^{-1} im + Mif 1.5 mg·kg^{-1} ig and D) TSO 2 mL·kg^{-1} im. [^{3}H]Pro binding assay was only used in groups A and D.

Histological examination Rats were decapitated at 24 h and 48 h after treatment. Whole utero-conceptus complex were rapidly dissected out, fixed in 10 % neutral buffered formalin, embedded in paraffin, and stained with hematoxylin-eosine.

PAP immunohistochemistry[4] After dewaxed in xylene and taken to water via graded EtOH, sections (48 h after treated) were incubated with 3 % (v/v) H_2O_2 in methanol for 30 min, 0.05 % (w/v) trypsin and $CaCl_2$, pH 7.4, for 20 min, 2 % (w/v) bovine serum albumin (BSA) for 20 min, Ⅰ Ab at 4 ℃ for 10 h, Ⅱ Ab at 25 ℃ for 60 min, Ⅲ Ab at 25 ℃ for 60 min, and visualized by 0.04 % (w/v) DAB added 0.2 mL 3 % (v/v) H_2O_2 for 5-15 min. Sections were stained with hematoxylin, the labeling index (LI %) was calculated[5]. The labeling density was evaluated by the shade of the labeled cells.

[^{3}H]Pro binding assay Rats on d 8 of pregnancy were killed at 24 h after im DL111-IT or TSO. Cytosolic PR were determined in the uterus according to following process[6]: tissues were homogenized in TEMGD buffer (containing Tris-HCl 20 mmol·L^{-1}, edetic acid 3 mmol·L^{-1}, dithiothreitol 1 mmol·L^{-1}, glycerol 30 %, $MgCl_2$ 3 mmol·L^{-1}, pH 7.4). Cytosol was obtained by centrifuging the homogenate at 13 000 × g at 0 – 4 ℃ for 60 min, then 0.2 mL of cytosol was incubated with [^{3}H]Pro (0.01 – 1.89 nmol·L^{-1} 0.1 mL) for 18 h at 0 – 4 ℃ in the presence or the absence of unlabeled promegestone (1000 times *vs* [^{3}H]Pro, 0.1 mL). Bound radioactivity was separated by dextran coated charcoal (DCC) absorption. Competition studies were made by incubating cytosol with [^{3}H]Pro 0.80 nmol·L^{-1} and various doses of Mif (1.86 – 186 nmol·L^{-1}), DL111-IT (5.99 – 429 μmol·L^{-1}), progesterone (9.11 – 456 nmol·L^{-1}) for 18 h at 4 ℃. the PR concentrations were calculated by Scatchard analysis, and PR were measured by *logit* analysis. Proteins were measured by the method of Lowry[7].

Cell culture Human decidual cells were dispersed[8] and planted 2 – 3 × 10^5 cells/well in 0.5 mL FD medium, and supplemented with 10 % BSA, penicillin 25 kU·L^{-1}, streptomycin 25 g·L^{-1}. DL111-IT 5 – 50 mg·L^{-1}, Mif 10 – 50 mg·L^{-1}, DL111-IT 0 – 5 mg·L^{-1} + Mif 0 – 5 mg·L^{-1}, were also added respectively after 24-h incubation. All treatments were performed in 4 wells. Besides, 4 wells received media or vehicle (Me_2SO) as control group. Cells were cultured at 37 ℃ in 5 % CO_2. Cell viability was assessed by trypan blue dye exclusion.

Statistic Results were expressed as $\bar{x} \pm s$ and compared by t test. LD_{50} of cultured cells was calculated as Bliss. Number of rat uteri was 72, and samples of human decidua were 31.

RESULTS

Uterus, decidual cells, and fetus Edema, degeneration and solvate of the decidual cells were seen with nuclei indefinite. But no vascular, endometrial, or myometrial damages were found. Fetus was surrounded by RBC and inflammatory cells and absorbed. The group C was injured most (Fig 1).

Uterus PR A decrease of [^{3}H]Pro binding was found at 24 h, the steroid binding in treated rats was 48 % lower than that in group D. The Scatchard analysis of data suggested that the affinity was unchanged. The K_d of DL111-IT with PR was very low (Tab 1).

Tab 1. Binding of [^{3}H] promegestone to pregnant rat uterine PR, im DL111-IT 100 mg·kg^{-1} and TSO 2 mL·kg^{-1}, 24 h after treatment. $n = 4$, $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* TSO.

	B_{max}, pmol/g protein	K_d, nmol·L^{-1}
TSO	244 ± 38	0.72 ± 0.20
DL111-IT	127 ± 27^c	0.88 ± 0.24^a

Competitive inhibition binding also revealed that the affinity of PR (group A) with Mif, progesterone, DL111-IT was unchanged *vs* group D (Tab 2).

Tab 2. IC_{50} of 3 compounds for PR (im TSO 2 mL·kg^{-1} and DL111-IT 100 mg·kg^{-1}, 24 h after treatment) in pregnant rat uterus. $n = 4$, $\bar{x} \pm s$. $^aP > 0.05$ *vs* TSO.

Compound	TSO	DL111-IT
Progesterone, nmol·L^{-1}	38 ± 11	42 ± 6^a
Mifepristone, nmol·L^{-1}	19 ± 5	24 ± 7^a
DL111-IT, μmol·L^{-1}	46 ± 17	38 ± 8^a

A dramatically decreasing of LI in PAP immunohistochemistry was found at 48 h. LI of group C was the lowest, and the density of labeling was also the lightest among the 4 groups. LI of group A or B decreased *vs* group D (Tab 3).