

# Combined therapy with disintegrin and melphalan as a new strategy in inhibition of endometrial cancer cell line (Ishikawa) growth

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**Abstract:** Endometrial cancer is one of the most frequently diagnosed cancer in females with prevalence of 22 in 100,000 women. The etiology of the cancer remains unclear. Despite significant progress towards understanding the patho-mechanism of the disease, effective treatment is still lacking. The results of the study suggest that combined treatment of Ishikawa cells for 24 h with disintegrin and then for 24 h with melphalan severely inhibits important biological functions of the cells. We showed that such strategy have a potent cytotoxic effect. The mechanism of process undergoes probably through inhibition of integrin – dependent signaling. In this study we shown down regulation of Shc and FAK proteins in cells treated with echistatin and melphalan. It suggests that signaling pathways that involve Shc and FAK participation may represent target for anti-neoplastic strategy. The functional significance of the combined treatment of Ishikwa cells with echistatin and melphalan was found at the level of collagen biosynthesis. Decreased biosynthesis of collagen in extracellular matrix may suppress cell growth and induce apoptosis. The treatment with echistatin and melphalan also showed decreased expression of IGF receptor in comparison to the cells treated with both compounds separately. The data presented suggest that combined therapy with disintegrin – echistatin and alkylating drug – melphalan may represent a new approach to more effective and safe cancer therapy.

**Key words:** echistatin, melphalan, Ishikwa cell line,  $\beta_1$ -integrin, cytotoxicity, collagen.

## Introduction

Endometrial cancer is one of the most frequently diagnosed cancer in females with prevalence of 22 in 100,000 women [1]. According to NIH report in USA in 2008 developed approximately 35000 new cases of the disease and 7500 women died. Most cases occur in women at the age over 60. The etiology of the cancer remains unclear. Despite significant progress towards understanding the patho-mechanism of the disease, effective treatment is still lacking.

Chemotherapy is indicated as frontline treatment for endometrial cancer. Combination of Anthracyclines, platinum compounds, and taxanes produces higher response rates, although combination regimens have not always improved survival rate. Doxorubicin plus cisplatin has been accepted as the Gynecologic Oncology Group standard regimen based on phase

III clinical data. Hormonal therapy is beneficial only in specified group of patients [2].

From previous experiments it is known, that echistatin an inhibitor of  $\beta_1$ -integrin receptor contributes to inhibition of collagen biosynthesis and decrease in the expression of FAK, SOS-protein and phosphorylated MAP-kinases, ERK1 and ERK2 [3]. Stimulated  $\beta_1$ -integrin receptor induces autophosphorylation of non-receptor protein kinase FAK [4], which is then capable of interacting with adaptor-proteins, such as Grb2, through Src and Shc proteins. This interaction allows activating further cascade of signaling pathway through Sos, Ras and Raf proteins [5] and subsequently, two MAP kinases: ERK<sub>1</sub> and ERK<sub>2</sub> [6]. The end point of this cascade is induction of transcription factor(s) that regulate(s) gene expression of integrins, proteinases and many proteins involved in regulation of cell growth and differentiation [7].

Another potent stimulator of cell growth and collagen biosynthesis is insulin like growth factor I [8]. IGF-I interacting with its receptor stimulates proliferation, enhances metastasis [9], prevents apoptosis [10]. IGF receptor signaling pathway involves the same proteins

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as integrin receptor signaling cascade, except Src and FAK proteins [11].

Melphalan represents one of nitrogen mustard derivatives, the group of earliest and effective antitumor drugs used in cancer therapy [12]. It acts through formation of interstrand cross-links in major groove of DNA [13]. Melphalan also inhibits collagen biosynthesis in breast cancer cell lines [14] and was also proposed as a component of combined therapy in metastatic or recurrent endometrial carcinoma [15].

The present study was undertaken to evaluate the effect of combined treatment of disintegrin, echistatin and melphalan on collagen biosynthesis, expressions of  $\beta_1$ -integrin and IGF-I receptors and some signaling proteins as Shc protein and FAK-kinases as well as on cytotoxicity in endometrial cancer (Ishikawa) cells.

## Materials and methods

**Materials.** Anti-Goat IgG antibody, Anti-Mouse IgG antibody, Fast BCIP/NBT reagent, Nonidet P-40, were provided by Sigma Corp., USA., as were most other chemicals and buffers used. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in cell culture were products of Gibco, USA. Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc., USA. Nitrocellulose membrane (0,2  $\mu$ m), sodium dodecylsulphate (SDS), polyacrylamide, molecular weight standards were received from Bio-Rad Laboratories, USA. L-5[ $^3$ H] proline (28 Ci/mmol) was purchased from Amersham, UK. Monoclonal (mouse) anti-IGFR, anti Shc, anti-FAK antibodies were obtained from Becton Dickinson Co., USA. Monoclonal (mouse) anti- $\beta_1$ -integrin antibody was obtained from ICN Biomedicals Inc., USA.

**Tissue culture.** All studies were performed on Ishikawa cell line purchased in American Type Culture Collection, Rockville, MD. The cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS), 2 mmol/l glutamine, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin at 37°C in a 5% CO<sub>2</sub> incubator.

**Cytotoxicity assay.** Toxicity of evaluated compounds was determined by method of Plumb *et al.* [16]. Ishikawa cells were maintained as described above. After 48 h incubation of the cells with drugs the medium was discarded and the cells were rinsed three times with phosphate buffered saline (PBS). Then the cells were incubated for 4 h in 2 ml of PBS with 50  $\mu$ l of MTT (5mg/ml). Medium was removed from the wells, and the cells were lysed in 200  $\mu$ l of DMSO with 20  $\mu$ l of Sorensen's buffer (0.1 M glycine with 0.1 M NaCl, pH 10.5). The absorbance was measured at 570 nm.

**Collagen production.** Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells in growth medium with evaluated compounds for 48 h with 5[ $^3$ H]-proline (5  $\mu$ Ci/ml, 28 Ci/mM) as described by Peterkofsky *et al.* [17].

**SDS-PAGE.** Slab SDS/PAGE was used, according to the method of Laemmli [18]. Samples of cell supernatants (25-50  $\mu$ g of protein) were incubated for 10 min. at 100°C in 62.5 mmol/l Tris-HCl, pH 6.8, containing 2.0% (w/v) SDS, 5% (v/v)  $\beta$ -mercaptoethanol, 10% (v/v) glycerol and 0.001% bromophenol blue. Samples were electrophoresed on a Tris-Glycine gels at 100 V per gel for 30 minutes at room temperature.

**Western immunoblot analysis.** After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2  $\mu$ m pore-

sized nitrocellulose at 100 mA for 90 minutes using a BioRad electrophoresis unit according to the method described in the manual accompanying the unit. The membrane was blocked with 5% dried milk in TBS-T for 1 hour in room temperature, slowly shaking. Then the nitrocellulose was incubated with monoclonal antibody against  $\beta_1$ -integrin, FAK, Shc or IGFR at concentration 1:5,000 overnight. After the incubation, nitrocellulose was washed with TBS-T (1 x 15 min and 2 x 10 min) intensively shaking. In order to analyze the proteins second alkaline phosphatase labeled antibodies against mouse's Fc IgG was added at concentration 1:2,500 in TBS-T and incubated for 1 hour slowly shaking. Then the membrane was extensively washed with TBS-T (5 x 10 min) and submitted to BCIP/NBT western blotting detection system.

**Statistical analysis.** In all experiments, the mean values for three independent experiments  $\pm$  standard deviation were calculated. The results were submitted to statistical analysis using Student's t-test, accepting  $p < 0.05$  as significant.

## Results

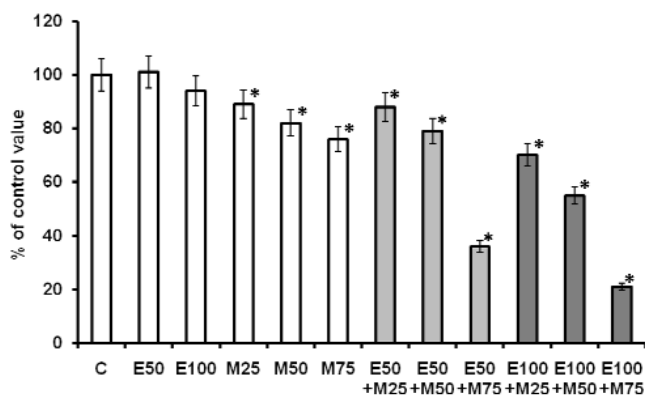
To evaluate cytotoxic effect of echistatin and melphalan, the viability of Ishikawa cells was measured by the method of Plumb *et al.* [16]. Incubation of the cells for 24 h with disintegrin at concentrations 50 and 100 ng/ml of medium had no significant effect on the cell viability (Fig. 1). Melphalan at concentrations 25, 50 and 75  $\mu$ M induced decreased of the cell viability to 89, 82 and 76% of control value, respectively. Incubation of Ishikawa cells with 50 ng/ml or 100 ng/ml of echistatin for 24 h and then incubated for additional 24 h with 25, 50 or 75  $\mu$ M melphalan significantly decreased the cell viability (Fig. 1). Different order of added substances produced much lower effects (not shown).

Similar effect was observed in respect to collagen biosynthesis. The inhibition of the protein biosynthesis was the most effective when the cells were treated with disintegrin for 24 h and then with melphalan for 24 h (Fig. 2).

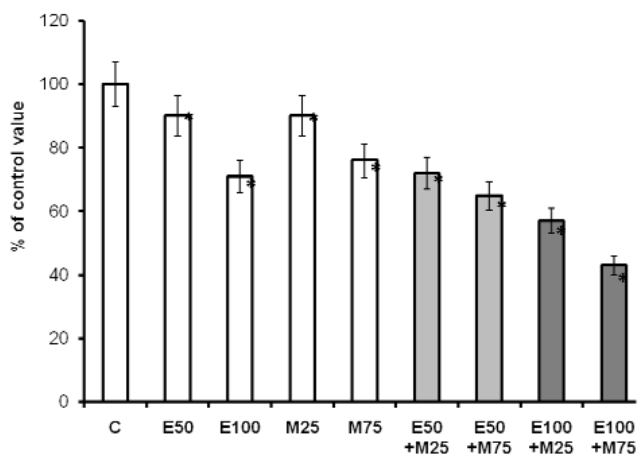
Collagen biosynthesis is regulated by the signal induced by  $\beta_1$ -integrin receptor. Expression of the receptor was evaluated by western immunoblot analysis. Expression of the  $\beta_1$ -integrin was not changed in Ishikawa cell line treated with echistatin, melphalan or both compounds (Fig. 3).

Expression of proteins involved in signaling pathway induced by stimulated  $\beta_1$ -integrin receptor was also evaluated. (Fig. 4) Treatment of the cells with echistatin for 24 hours and then with melphalan, induced decrease in the expression of Shc and phosphorylated FAK-proteins as compared to control cells or the cells treated with both compounds alone (Fig. 4).

Since IGF-I receptor plays important role in regulation of mitogenesis, apoptosis and collagen biosynthesis the expression of the receptor in Ishikawa cell line treated with echistatin and melphalan was estimated. As shown on Fig. 5. the expression of the receptor was decreased in the cells treated with echistatin for 24 h and



**Fig. 1.** Viability of Ishikawa cells treated with various concentrations of echistatin (E) and melphalan (M). The cells were incubated for 48 h with echistatin at concentrations 50 and 100 ng/ml condition medium or melphalan at concentrations 25, 50 and 75  $\mu$ M. Another set of the cells was treated with 50 ng/ml or 100 ng of echistatin for 24 hours then melphalan was added to achieve concentrations 25, 50 and 75  $\mu$ M, incubation continued for 24 hours. Mean values from three independent experiments done in triplicates  $\pm$  standard deviation are presented. \* $p < 0.05$ .

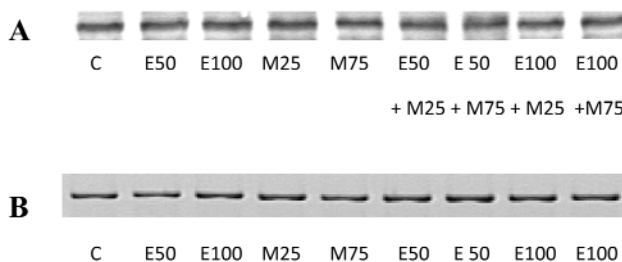


**Fig. 2.** Collagen production, measured by 5-[ $^3$ H]-proline incorporation into proteins susceptible to the action of bacterial collagenase in Ishikawa cell culture. The cells were incubated for 48 h with echistatin at concentrations 50 and 100 ng/ml condition medium or melphalan at concentrations 25, 50 and 75  $\mu$ M. Another set of the cells was treated with 50 ng/ml or 100 ng of echistatin for 24 hours then melphalan was added to achieve concentrations 25, 50 and 75  $\mu$ M, incubation continued for 24 hours. Mean values from three independent experiments done in triplicates  $\pm$  standard deviation are presented. \* $p < 0.05$ .

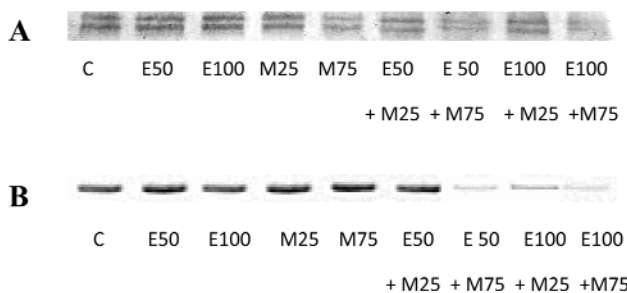
than with melphalan for additional 24 h. Treatment of the cells with respective compound alone had no effect on the IGF receptor protein expression.

### Discussion

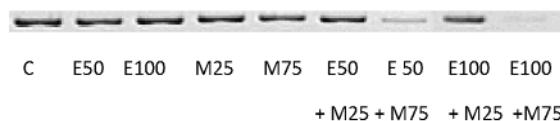
The integrins family are responsible for adhesion of cells to extracellular matrix components as well as to



**Fig. 3.** Western immunoblot analysis for  $\beta_1$ -integrin receptor (A) in Ishikawa cells treated with echistatin (E) and melphalan (M).  $\beta$ -actin serves as a loading control (B). The result represent mean from three independent experiments. Samples used to the experiments consisted 50  $\mu$ g of protein from pooled cell extracts.



**Fig. 4.** Western immunoblot analysis for Shc protein (A), FAK (B) in Ishikawa cells treated with echistatin (E) and melphalan (M) as described in Materials and methods section. The result represent mean from three independent experiments. Samples used to the experiments consisted 50  $\mu$ g of protein from pooled cell extracts.



**Fig. 5.** Western immunoblot analysis for IGF receptor in Ishikawa cells treated with echistatin (E) and melphalan (M) as described in Materials and methods section. The result represent mean from three independent experiments. Samples used to the experiments consisted 50  $\mu$ g of protein from pooled cell extracts.

other cells [19]. The interaction affects cytoskeleton organization, lipid metabolism, kinase activation, gene expression [20], cell cycle progression [21] and metastasis [22].  $\beta_1$  and  $\beta_3$  integrins play an important role in tumor invasion [23].

Cell migration, invasion, matrix degradation, proliferation, and angiogenesis are all mediated by integrins and integrin signaling [24,25].

Ishikawa cells, similarly as endometrial, epithelial cells express integrins  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ ,  $\alpha_6\beta_4$  [26].  $\alpha_2\beta_1$  integrin receptor is the main receptor for collagen and is involved in regulation of collagen production [27].

Melphalan is one of the nitrogen mustard derivatives, that represent the group of earliest and effective

antitumor drugs used in cancer therapy [12,13], acting through induction of interstrand cross-links in major groove of DNA [12,13]. Melphalan is used in therapy of advanced cancer of the ovaries, multiple myeloma, malignant melanoma, childhood neuroblastoma, soft tissue sarcoma. Side effects of melphalan include toxicity on blood cells, platelets, lung and liver and may contribute to nausea, diarrhea, mouth ulcers, hair loss, skin rashes and leukaemia. It is extremely important to keep doses of anticancer drug as low as possible to avoid serious side effects.

Echistatin and other disintegrins have been extensively studied in the past. It is now established that disintegrins are effective agents in limiting tumor growth and spread [28,29]. Disintegrins interacting with several integrins block various integrin-mediated processes involved in tumor growth, metastasis and angiogenesis [30].

The results of the study suggest that combined treatment of Ishikawa cells with echistatin and melphalan severely inhibits important biological functions of the cells. We showed that such strategy have a potent cytotoxic effect. The mechanism of the process undergoes probably through inhibition of integrin – dependent signaling. In this study we shown down regulation of Shc and FAK proteins in cells treated with echistatin and melphalan. It suggests that signaling pathways that involve Shc and FAK participation may represent target for antineoplastic strategy.

The functional significance of the combined treatment of Ishikwa cells with echistatin and melphalan was found at the level of collagen biosynthesis. Decreased biosynthesis of collagen in extracellular matrix on one hand may facilitate motility and invasion of neoplastic cells (31), but on another may suppress cell growth and induce apoptosis [32].

Important role in induction of those processes may play also IGF-I receptor. The cells treated for 24 h with disintegrin and then for 24 h with melphalan showed decreased expression of IGF receptor in comparison to the cells treated with both compounds separately. IGF-R is involved in regulation of cellular growth and transformation [33]. Therefore inhibition of the receptor expression may represent approach to the inhibition of tumor growth. Blockade of the receptor (34) or down regulation of its expression [35] reduces cancer proliferation and induces apoptosis. Decreased expression of the receptor by echistatin and melphalan could explain decreased biosynthesis of collagen in the cells, since IGF-I is a most potent inducer of collagen biosynthesis [36].

Finally, echistatin (that by itself is only slightly cytotoxic for Ishikawa cells) strongly potentiate cytotoxic effect of melphalan on this cells.

The data presented suggest that combined therapy with disintegrin – echistatin and alkylating drug –

mephalan may represent a new approach to more effective and safe endometrial cancer therapy.

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