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# Mechanism of betulinic acid inhibition of collagen biosynthesis in human endometrial adenocarcinoma cells

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Collagen as a ligand for integrin receptors plays important role in the integrin – dependent regulation of cellular metabolism. Since betulinic acid (BA) evokes anticancer activity, its effect on collagen biosynthesis was studied in cultured endometrial adenocarcinoma cells. Confluent cells were treated with different concentrations of BA for 24 hours. It was found that BA inhibit collagen biosynthesis ([$^3$H] proline incorporation assay). The mechanism of this phenomenon was found at the level of insulin-like growth factor-I receptor (IGF-IR) and $\alpha_2$ integrin signalling (Western immunoblot analysis). The expressions of IGF-I receptor and $\alpha_2$ integrin subunit as well as integrin activated focal adhesion kinase (FAK) were decreased in the cells treated with BA. It was accompanied by a parallel decrease in the expression of Sos protein and phosphorylated MAP-kinases ($ERK_1$, $ERK_2$) and up – regulation of NF-κB. The data suggest that BA-dependent inhibition of collagen biosynthesis in cultured human endometrial adenocarcinoma cells undergoes through $\alpha_2$ integrin and IGF-IR signaling that activate NF-κB, potent inhibitor of collagen gene expression.

*Key words: betulinic acid; $\alpha_2\beta_1$ integrin and IGF-I receptors; collagen biosynthesis; human endometrial adenocarcinoma cells.*

Betulinic acid (3beta-Hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic lupane-type triterpene of natural origin isolated from various plants. BA is known to evoke antiinflamatory [1], immunomodulatory [2] antiviral [3], and antineoplastic activities [4]. Betulinic acid (BA) is cytotoxic against several human neoplastic cell lines [5]. There is evidence that BA is effective against lung, ovarian, cervical, head and neck carcinomas. Some data suggest that BA induces apoptosis in cells independently of their p53 status [6]. The mechanism of BA-induced apoptosis may originate at the level of mitochondrial-mediated pathway, generation of reactive oxygen species, inhibition of topoisomerase I, activation of the MAP kinase cascade, inhibition of angiogenesis, and modulation of pro-growth transcriptional activators and aminopeptidase N activity [6].

One of the consequences of neoplastic transformation is an aberration in the biosynthesis of some proteins of the extracellular matrix (ECM), mainly fibronectin and type I collagen [7]. Collagen is not only essential for the maintainance of tissue architecture and integrity. It has an important role in interacting with cell surface receptors. It is known that the interaction between cells and the ECM proteins, can regulate cellular gene expression, differentiation and cell growth [8, 9] and can play an important role in tumorigenicity and invasiveness [10]. It has been shown that most normal and neoplastic cells recognize many extracellular proteins by specific cell surface receptors, called integrins. They are thought to be important for tumor cell attachement, migration, proliferation, progression and survival [11]. For example the main collagen receptor, composed of $\alpha_2\beta_1$ integrin subunits, has an important role in the formation of tumor metastasis [12].

Some studies suggest that, $\beta_1$ integrin is particularly important in signal transduction [8,13]. Therefore, any changes in quantity, structure and distribution of collagens and integrins would be likely to alter metabolism and function of cells.

It has been postulated that the accumulating matrix components are produced by host mesenchymal cells but not by the tumor cells [14]. Tumor cells have been shown to secrete a variety of cytokines and growth factors [15].One of them is insulin-like growth factor – IGF-I, a small, mitogenic peptide, that stimulate growth, collagen biosynthesis, wound healing and many other processes [16]. The mechanism of IGF-I action is multifunctional. For instance, stimulation of collagen biosynthesis undergoes through induction of collagen gene expression [17] and prolidase activity [18], the enzyme

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that play important role in post-transcriptional regulation of proline-containing proteins.

IGF-I, acting predominantly through the IGF-I receptor [19], has been demonstrated to stimulate proliferation and metastasis of breast cancer cells [20] and to prevent them from undergoing apoptosis [21]. The MAP-kinase (ERK1 and ERK2) pathway induced by activated IGF-I receptor is considered to play an important role in carcinogenesis and tumor progression.

IGF-I receptor signaling involves mostly the same proteins and kinases as the $\beta_1$-integrin transduction pathway, except for the participation of FAK kinase and Src protein [21].

The present study was undertaken to evaluate the effect of BA on expressions of $\alpha_2\beta_1$ integrin receptor, IGF-IR and several signaling proteins (FAK, Grb2, Sos, phosphorylated ERK1/2), prolidase activity and expression, expression of NF-κB transcription factor and collagen biosynthesis in human endometrial Ishikawa cells.

## Materials and methods

Alkaline phosphatase-labeled anti–mouse IgG, anti-rabbit IgG and anti-goat IgG antibodies, bacterial collagenase, betulinic acid, DMSO (dimethyl sulfoxide), Fast BCIP/NBT reagent, L-glycyl-proline, L-proline, monoclonal (rabbit) anti-FAK antibody, monoclonal (mouse) anti-IGF-IR antibody, monoclonal (mouse) anti-phosphorylated MAPK antibody, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 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 μm), sodium dodecylsulphate (SDS), polyacrylamide, molecular weight standards and Coomassie Briliant Blue R-250 were received from Bio-Rad Laboratories, USA. L-5[$^3$H] proline (28 Ci/mmol) was purchased from Amersham, UK. Monoclonal (mouse) anti-$\beta_1$, polyclonal (rabbit) anti-$\alpha_2$-integrin, polyclonal (rabbit) NF-κB antibodies, polyclonal (goat) anti-β-actin antibody were the products of Santa Cruz Biotechnology Inc., USA. Monoclonal (mouse) anti-Sos and anti-Grb 2 antibodies were obtained from Becton, Dickinson Co., USA. Polyclonal anti-human prolidase antibody was donated by Dr. James Phang (NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA).

*Tissue culture***.** All studies were performed on human endometrial adenocarcinoma (Ishikawa cell line). The cells were maintained in growth medium (DMEM supplemented with 10 % fetal bovine serum (FBS), 2 mmol/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin) at 37$^0$C in a 5 % $CO_2$ incubator. Cells were counted in hemocytometer and cultured at 1 x $10^5$ cells per well in 2 ml of growth medium in 6 well plates (Costar). Cells reached confluence at day 6 and in most cases such cells were used for assays. Cells were used in the 8th to 14th passages. Confluent cells were treated with different concentrations of betulinic acid (20, 10, 5, 2.5, 1.25, 0.635 μg/mL) in growth medium. Betulinic acid was dissolved before use at 4 mg/mL in DMSO and diluted in DMEM containing 10% FBS. The highest final concentration of DMSO was 0.25%. To control for the effects of DMSO, we adjusted the final concentration of DMSO in the medium to 0.25% (v/v) in cell cultures.

*Cell viability assay***.** The assay was performed according to the method of Carmichael [22] using 3-(4,5-di-methylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were cultured for 24h with various concentrations of BA in six-well plates, washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37$^0$C. The medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability in the presence of BA was calculated as a percent of control cells.

*Collagen production***.** Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells in growth medium with butyrate for the last 24 h with 5[$^3$H] proline (5 μCi/ml, 28 Ci/mM) as described previously [23]. Incorporation of tracer into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky [24]. Results are shown as combined values for cell plus medium fractions.

*SDS-PAGE***.** Slab SDS/PAGE was used, according to the method of Laemmli [25], by using 10% SDS-polyacrylamide gel. Protein concentration was measured by the method of Lowry [26].

*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 transfered to 0.2 μm pore-sized nitrocellulose at 100 mA for 1 hour by using a LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with: monoclonal anti-$\beta_1$, polyclonal anti-$\alpha_2$-integrin and polyclonal NF-κB antibodies at concentration 1:1000; polyclonal antibody against β- actin at concentration 1:3,000; monoclonal anti-FAK antibody at concentration 1:1,000; monoclonal anti-SOS and anti-Grb 2 antibodies at concentration 1:5,000; monoclonal antibodies against phosphorylated-MAPK protein at concentration 1:5,000; monoclonal anti-IGF-IR antibody at concentration 1:1,000 in 5% dried milk in TBS-T (20 mmol/l Tris-HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20) for 1 hour. In order to analyze $\beta_1$ integrin subunit, FAK, Sos and GRB-2 proteins, IGF-IR and phosphorylated MAP kinases second antibody-alkaline phosphatase conjugated, anti-mouse IgG (whole molecule) was added at concentration 1:7,500 in TBS-T; in order to analyze $\alpha_2$-integrin subunit and NF-κB anti-rabbit IgG (whole molecule) alkaline phosphatase conjugated was added at concentration 1:5,000, and in order to analyze β-actin second antibody-alkaline phosphatase conjugated, anti-goat IgG (whole molecule) was added at

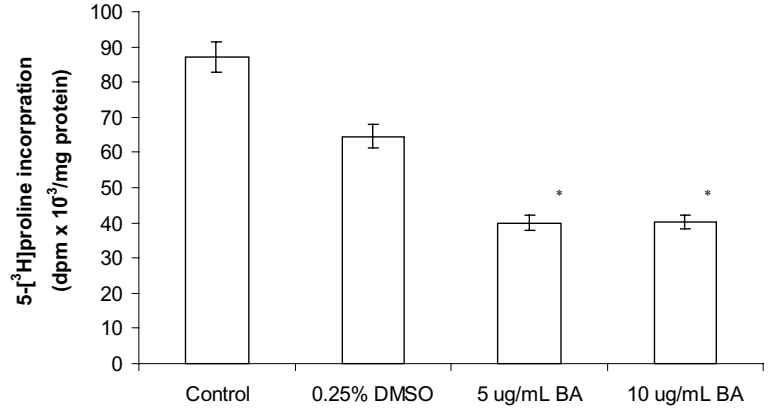


**Figure 1. Collagen biosynthesis measured as 5[$^3$H] proline incorporation into proteins susceptible to the action of bacterial collagenase in confluent human endometrial adenocarcinoma cells incubated for 24 h in the medium containing 0.25% DMSO and different concentrations of betulinic acid (BA). The results present the mean values from 6 assays ± S.D. * P<0.01**

concentration 1:5,000 in TBS-T and incubated for 30 min slowly shaking. Then nitrocellulose was washed with TBS-T (5 x 5 min) and submitted to Sigma-Fast BCIP/NBT reagent. The intensity of the bands was quantified by densitometric analysis using apparatus for gel documentation Syngen UVI-KS400 I (CO, USA) with digital densitometry (in arbitral units).

*Statistical Analysis***.** In all experiments, the mean values for three independent experiments done in duplicates ± standard deviation (S.D.) were calculated, unless otherwise indicated.

The results were submitted to statistical analysis using the Student's t-test, accepting $P < 0.05$ as significant.

## Results

Since BA is known as a cytotoxic agent against cancer cell lines [27], the cytotoxicity assay for BA in cultured human endometrial Ishikawa cells was performed. To exclude the possible DMSO-induced cytotoxic effects on studied cells, the parallel control experiments were done. Cell viability was measured by the method of Carmichael et al. [22] using tetrazolinum salt. The viability of cells incubated for 24 hours with indicated concentrations of BA is presented in Table 1. BA at the concentration of 10 μg/mL (0.25% DMSO) led to the decrease in cell viability by 50%, compared to controls (Table 1).

The effect of BA on collagen biosynthesis was measured in confluent Ishikawa cells, treated with BA for 24h. As can be seen on Fig. 1, 24h incubation of cells with BA contributed to decrease in collagen biosynthesis. At 5 and 10 μg/mL of BA about 50% decrease in collagen biosynthesis was observed. It was accompanied by decrease in prolidase expression (Fig. 2 A).

Collagen biosynthesis and prolidase activity was previously shown to be regulated by $\beta_1$-integrin-dependent signaling [28]. Although, no differences were observed in $\beta_1$ integrin receptor expression (Fig. 2 B) there was distinct decrease in the $\alpha_2$ integrin receptor (Fig. 2 C) after 24h

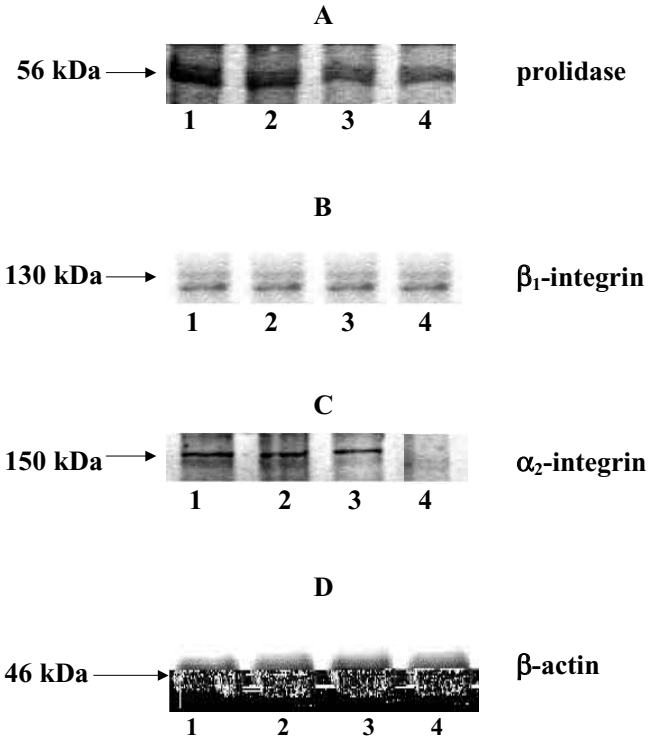


**Figure 2. Western blot analysis for prolidase (A), $\beta_1$-integrin receptor (B), $\alpha_2$-integrin receptor (C) in control human endometrial adenocarcinoma cells (lane 1) and cultured in the medium containing 0.25% DMSO (lane 2), 5 μg/mL of BA (lane 3) and 10 μg/mL of BA (lane 4). The mean values of 6 pooled cell homogenate extracts are presented. The intensity of the bands was quantified by densitometric analysis. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading (D).**

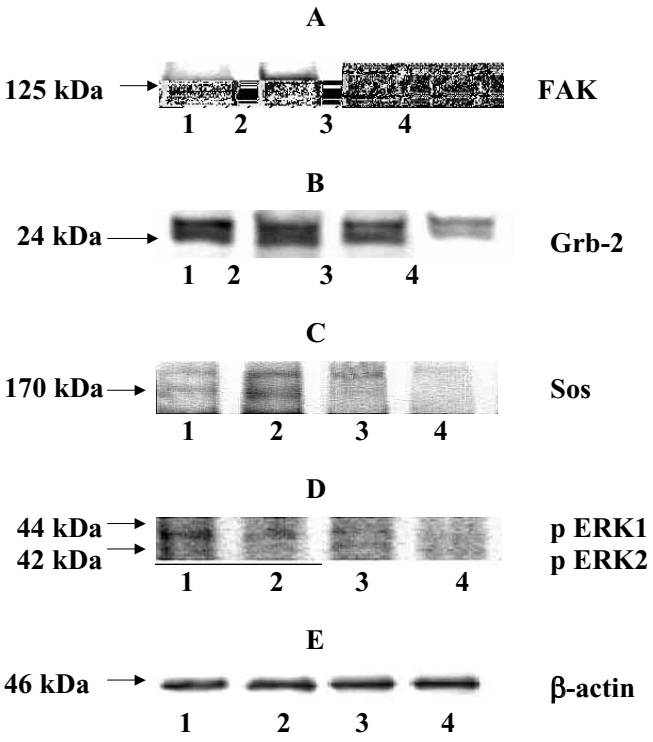


**Figure 3. Western blot analysis for FAK (A), Grb-2 (B), Sos (C), MAP kinases ERK1 and ERK2 (D) in control human endometrial adenocarcinoma (lane 1) and cultured in the medium containing 0.25% DMSO (lane 2), 5 μg/mL of BA (lane 3) and 10 μg/mL of BA (lane 4). The mean values of 6 pooled cell homogenate extracts are presented. The intensity of the bands was quantified by densitometric analysis. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading (E).**

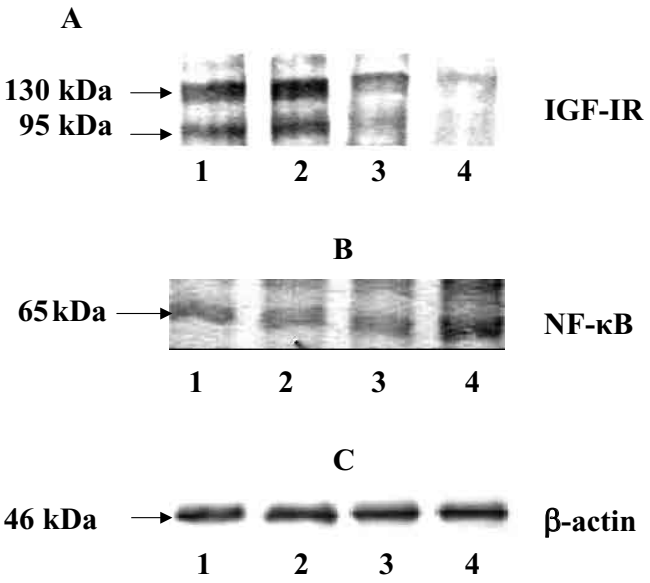


**Figure 4. Western blot analysis for IGF receptor (A) and NF-κB (B) in control human endometrial adenocarcinoma cells (lane 1) and cultured in the medium containing 0.25% DMSO (lane 2), 5 μg/mL of BA (lane 3) and 10 μg/mL of BA (lane 4). The mean values of 6 pooled cell homogenate extracts are presented. The intensity of the bands was quantified by densitometric analysis. The arrows indicate the molecular mass of standards. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading (C).**

incubation of human endometrial Ishikawa cells with 10 μg/mL BA.

Similarly, in BA-treated cells we observed distinct decrease in the expression of FAK (Fig. 3 A), GRB-2 (Fig. 3B), Sos (Fig. 3 C) and phosphorylated MAP kinases, ERK1, ERK2 (Fig. 3 D). Since phosphorylation of MAP kinases ERK1 and ERK2 is due to IGF-I receptor signaling [29] and collagen biosynthesis is regulated by insulin-like growth facμŽr-I (IGF-I) receptor [30] the expression of this receptor in the cells treated for 24 hours with BA was measured. As shown on Fig. 4 A, in BA-treated cells distinct decrease in the IGF-I receptor expression (by about 200%), compared to control cells was found. Simultunaously, we found increase in the expression of NF-κB, the known inhibitor of collagen gene expression [31]. It suggests that ability of BA to induce decrease of collagen biosynthesis may involve inhibition of IGF-I receptor expression and signaling and activation of NF-κB.

**Table 1. Viability of confluent human endometrial adenocarcinoma (Ishikawa cell line) incubated for 24 h with different concentrations of betulinic acid (BA). Slight cytotoxic effect of DMSO (0.25%) was substracted from control values and viability of cells was considered as 100%.**

| Concentration (μg/ml BA) | Viability of cells (% of control) |
|---|---|
| 0 | 100 |
| 635.635 | 98 ± 2 |
| 25.25 | 95 ± 2 |
| 5.5 | 90 ± 3 |
| 5.0 | 55 ± 6 |
| 10.0 | 50 ± 4 |
| 20.0 | 66 ± 7 |

## Discussion

Betulinic acid is a very promising new chemotherapeutic agent for the treatment of cancer, because of its selective cytotoxicity against tumor cells and favorable therapeutic index, even at doses up to 500 mg/kg body weight [5].

The data presented here show that betulinic acid is active in vitro against human endometrial adenocarcinoma cells (Ishikawa cell line). BA exerted cytotoxic activity at $IC_{50}$ of 10 μg/mL. BA at the same concentration also evoked inhibitory effect on collagen biosynthesis and prolidase expression. Prolidase plays an important role in collagen metabolism [E.C. 3.4.13.9]. This is

a cytosolic enzyme that splits imidodipeptides with C-terminal proline [32]. Therefore prolidase supports proline for collagen biosynthesis and may be a rate-limiting factor in the regulation of collagen production at post-transcriptional level [18].

Collagen is known as a ligand for $\alpha_2\beta_1$ integrin. Previously, it has been shown that $\beta_1$-integrin receptor is involved in signaling, which regulates collagen biosynthesis and prolidase activity [28]. Therefore, we considered $\beta_1$ integrin as a potential target in BA-induced modulation of the above processes. However, no differences were observed in $\beta_1$ integrin subunit receptor expression. The expression of beta-1 integrins is known to be reduced in endometrial adenocarcinoma cells [33].

It is known that, integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the major cellular receptors for collagen, and collagens bind to these integrins at the alpha subunit [34]. We demonstrated that BA-dependent decrease in collagen production was accompanied by decrease in $\alpha_2$ integrin, as well as FAK expression. Therefore, we suggest that BA-dependent effect on collagen biosynthesis may be related to the $\alpha_2$ integrin signaling.

Down stream signaling of integrin receptor may be amplified by IGF-IR signaling sharing some proteins of the signaling pathway. Stimulated IGF-I receptor induces interaction of several signaling proteins, such as Grb2, Src and Shc. This interaction allows activating further cascade of signaling pathway through Sos, Ras and Raf proteins and subsequently, two MAP kinases: $ERK_1$ and $ERK_2$ [35]. The end point of this phenomenon is induction of transcription factor that up-regulates collagen gene expression. Deregulation of the signaling cascade may contribute to the impairement of collagen synthesis.

We supposed that the effect of BA on collagen production may be also related to the alterations in signaling pathway generated by insulin-like growth factor I receptor (IGF-IR), that is known to regulate collagen synthesis [30]. In fact the data presented here show that BA-dependent decrease in collagen biosynthesis is accompanied by decrease in the expression of IGF-IR. Decrease in the expression of IGF-IR contributed to the decrease in the expressions of Sos-protein and p-ERK1/2. It is known, that blocking ERK 1/2 phosphorylation by selective inhibitor of ERK1/2 significantly reduced the proliferation of Ishikawa cells [36]. In addition our data showed that BA-dependent decrease in collagen biosynthesis is accompanied by increase in the expression of NF-κB, the known inhibitor of collagen gene expression [31].

The results of present study suggest that BA may exert antineoplastic effect through inhibition of cell division, collagen biosynthesis, $\alpha_2$ integrin, FAK, IGF-IR, Sos-protein, phosphorylated ERK1/2 expressions and stimulation of NF- κB expression. In view of this data, it seems that the inhibition of collagen biosynthesis caused by betulinic acid may be mostly a consequence of the disruption of the IGF-I receptor-mediated MAPK (pERK1/2) associated signaling pathway, in which NF- κB stimulation is responsible for inhibition of collagen gene expression.

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