

## DIFFERENTIAL EFFECT OF BETULIN AND BETULINIC ACID ON CYTOKINE PRODUCTION IN HUMAN WHOLE BLOOD CELL CULTURES

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*Differential effect of betulin and betulinic acid on cytokine production in human whole blood cell cultures. B. ZDZISIŃSKA, W. RZESKI, R. PADUCH, A. SZUSTER-CIESIELSKA, J. KACZOR, K. WEJKSZA, M. KANDEFER-SZERSZEŃ. Pol. J. Pharmacol., 2003, 55, 235–238.*

Betulin and betulinic acid, plant-derived triterpenoid compounds, have been described to possess anti-inflammatory activity. In this paper, we examine the ability of both compounds to induce and modulate cytokine production in human whole blood cell cultures. The results indicate that betulin is a modest TNF- $\alpha$  inducer and also an enhancer of mitogen-induced TNF- $\alpha$  production. In contrast to betulin, betulinic acid is a modulator of cytokine production by Th1/Th2 cell subpopulations which slightly enhances IL-10 formation and inhibits IFN- $\gamma$  production, reducing IFN- $\gamma$ /IL-10 ratio from 3.6 to 2.6.

**Key words:** *betulin, betulinic acid, TNF- $\alpha$ , IFN- $\gamma$ , IL-10*

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*Abbreviations:* DMSO – dimethyl sulfoxide, IFN- $\gamma$  – interferon gamma, IL-10 – interleukin 10, LPS – lipopolysaccharide, PBC – peripheral blood cells, PHA – phytohemagglutinin,  $R_f$  – retention factor, TNF- $\alpha$  – tumor necrosis factor

## INTRODUCTION

Triterpenoids represent a very large class of plant secondary metabolites, which have been demonstrated to exhibit a variety of biological activities. The reported biological effects of triterpenoids include cytotoxic, anti-tumor, anti-inflammatory and antiviral activities [6, 10, 11, 14, 15, 18]. The anti-inflammatory activity of triterpenoids is connected with their interaction with several cellular and extracellular proteins. Betulin and betulinic acid have been shown as potent phospholipase A2 inhibitors [1], while ursolic and oleanolic acids have been shown to inhibit lipoxygenase and cyclooxygenase activity [7]. Moreover, they inhibit leukocyte elastase or serine proteases such as trypsin and chymotrypsin [9, 16]. The anti-inflammatory activity of oleanolic and ursolic acid and some of their derivatives is also related to their influence on proinflammatory cytokine production. In mouse macrophages stimulated with IFN- $\gamma$ , they are highly active inhibitors of IL-1 and TNF- $\alpha$  production, and of inducible nitric oxide synthase and cyclooxygenase 2 (COX 2) activities [12, 13]. However, when resting mouse macrophages are treated with oleanolic acid or ursolic acid, these compounds stimulate inducible nitric oxide synthase activity as well as TNF- $\alpha$  expression through NF-kappa B transactivation [3, 17]. It was shown that at least some triterpenoid compounds exert immunoregulatory activity.

In order to extend our knowledge concerning immunoregulatory properties of triterpenoids, the activity of betulin and betulinic acid as cytokine inducers has been examined in human whole blood cell cultures. Moreover, their activity as modulators of production of proinflammatory cytokines, such as TNF- $\alpha$  and IFN- $\gamma$  as well as anti-inflammatory cytokines such as IL-10 has also been investigated in mitogen-stimulated blood cell cultures.

## MATERIALS and METHODS

### Materials

Betulin (> 98% purity) was obtained from Stanisław Piela, Sylveco, Jasionka, Poland. Standard

betulin (> 97%) had been obtained from Sigma Aldrich (St. Louis MO, USA). Other chemicals such as betulinic acid (> 90%), PHA and LPS from *E. coli* 0111:B4 were also obtained from Sigma. The betulin obtained from Sylveco was analyzed by thin-layer chromatography on a Kieselgel 60 (Merck, Darmstadt, Germany) plate 100 × 200 mm with mobile phase composed of chloroform/ethyl acetate (10:1) and compared with that obtained from Sigma Aldrich.  $R_f$  (0.46) was identical for both betulin preparations. For further experiments betulin from Sylveco was used.

### Cytokine induction and assay

Peripheral blood cells (PBC) of healthy blood donors were cultivated using whole blood [8]. Briefly, heparinized blood (10 donors) was diluted at a 1:10 ratio with RPMI-1640 medium supplemented with antibiotics (100 U/ml of penicillin and 100  $\mu$ g/ml of streptomycin) and distributed into wells in plastic 24 – well plates (1 ml of diluted blood per well). Betulin and betulinic acid were dissolved in dimethyl sulfoxide (DMSO) and added directly to the culture media. Control cells were treated only with solvents, the final concentration of which never exceeded 0.1%, and this concentration did not show any effect on the assay system. In order to examine the influence of betulin and betulinic acid on mitogen-induced cytokine production, blood suspensions were stimulated with a mixture of PHA (Sigma, St. Louis MO, USA) and LPS from *E. coli* 0111:B4 (Sigma) at final concentrations of 10  $\mu$ g/ml and 2  $\mu$ g/ml, respectively, and at the moment of induction, they were treated with betulin or betulinic acid. The samples were incubated at 37°C in 5% CO<sub>2</sub> in air. Supernatants were collected 24 h after the induction, centrifuged and stored at –20°C before cytokine assay (no longer than 3 weeks).

Concentrations of IL-10, TNF- $\alpha$  and IFN- $\gamma$  were measured using ELISA kits from Endogen, Woburn, MA, USA according to the manufacturer's instruction. Each concentration was measured in duplicate. The limit of detection for IL-10 was < 3 pg/ml, for TNF- $\alpha$  < 5 pg/ml and IFN- $\gamma$  < 2 pg/ml. The values of cytokine concentrations below the limit of detection were reported as the value of 0 for analytical purposes.

### Statistical analysis

All experiments were repeated at least three times. The Student's *t*-test was used to assess the statistical significance of the differences. A confidence level of  $p < 0.05$  was considered significant.

## RESULTS and DISCUSSION

To our knowledge, the effect of betulin and betulinic acid on cytokine production in human blood leukocytes has not previously been investigated; therefore, we used them as cytokine inducers or cytokine production modifiers in whole blood cell cultures.

Our findings indicated that betulin could induce a low but detectable level of TNF- $\alpha$  in human leukocytes, however, it was ineffective in the induction of other pro-inflammatory cytokines such as IFN- $\gamma$  and anti-inflammatory IL-10 (Tab. 1). In contrast to betulin, betulinic acid was ineffective in cytokine induction.

Table 1. Cytokine induction by betulin and betulinic acid in whole blood cell cultures

Compound $\mu\text{g/ml}$	Cytokine pg/ml		
	TNF- $\alpha$	IFN- $\gamma$	IL-10
–	1.18 $\pm$ 1.0	1.36 $\pm$ 0.4	0
Betulin			
10	7.56 $\pm$ 5.7*	1.0 $\pm$ 0.4	0
5	5.4 $\pm$ 4.7	1.2 $\pm$ 0.7	0
Betulinic acid			
10	1.42 $\pm$ 0.5	1.4 $\pm$ 0.5	0
5	1.04 $\pm$ 1.0	1.7 $\pm$ 0.8	0

\* Statistically significant in comparison with the control at  $p < 0.05$

When blood cells were stimulated with mitogens (PHA + LPS), betulin added to cell cultures significantly, in a dose-dependent manner, enhanced TNF- $\alpha$  production (Fig. 1) but not other examined cytokines such as IFN- $\gamma$  or IL-10. These results suggest that secretion of IFN- $\gamma$ , IL-10 and TNF- $\alpha$  may be regulated by different mechanisms, or that various types of leukocytes present in whole blood differ in their sensitivity to betulin. It is known that monocytes are the main source of TNF- $\alpha$  in whole blood induced by mitogens, while IFN- $\gamma$  and IL-10 are produced by Th lymphocytes. It is likely that monocytes, but not lymphocytes, are the cells sensitive to betulin. Another possibility is that betulin, similarly to oleanolic acid [3], may act *via* NF-kappaB transactivation. Gene products whose expression is regulated by NF-kappaB proteins are known to include TNF- $\alpha$  and other proinflammatory cytokines such as IL-6, IL-8 and lymphotoxin

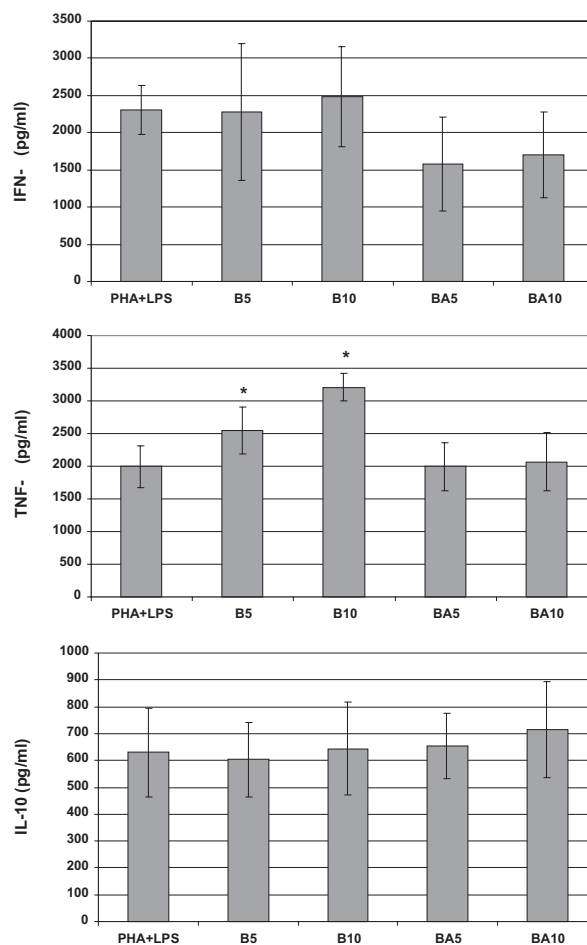


Fig. 1. The influence of betulin (B) and betulinic acid (BA) at concentrations of 5  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$  on cytokine production in PHA + LPS-stimulated blood cell cultures. \* Statistically significantly different in comparison with the control (PHA + LPS)

but not IL-10 and IFN- $\gamma$  [5]. It should be noted, however, that another pentacyclic triterpene, lupeol, at concentrations in the range between 10–100  $\mu\text{M}$  inhibited LPS-induced TNF- $\alpha$  and IL-1 $\beta$  production [4]. Lupeol is structurally related to betulin, so it is difficult to speculate why it exhibited opposite activity. More experiments are necessary to explain the structure/function relationship of triterpenoids.

In contrast to betulin, which was an inducer and an enhancer of TNF- $\alpha$ , its oxidized form, betulinic acid, did not influence TNF- $\alpha$  production in our experiments, but it slightly inhibited IFN- $\gamma$  and enhanced IL-10 production. The differences were not statistically significant in comparison to the control (not treated with betulinic acid) because of high standard deviation values. However, when IFN- $\gamma$ /IL-10 ratio was calculated, betulinic acid at both used concentrations reduced this ratio significantly

from 3.6 to 2.6, which indicates that in the presence of this compound stimulatory signals produced by immune cells decreased with the parallel increase in the inhibitory ones.

A major step in understanding the modulation of cell-mediated immunity and the inflammatory response system was the identification of two mutually exclusive populations of T helper (Th) cells, i.e. Th1, which produce IFN- $\gamma$  and IL-2, and Th2, which produce IL-4 and IL-10. The latter is a negative immunoregulatory cytokine, which potentially suppresses Th1 functions such as the production of pro-inflammatory cytokines [2]. As betulinic acid, except for being an inhibitor of phospholipase A2 [1], seems to be a modulator of the ability of Th1/Th2 cells to produce cytokines, its influence on the inflammatory reactions in the organism may be very complex. The extracts from *Betula alba* (*Betulaeae*) leaves and bark which are known to be among the plants richer in betulin and betulinic acid are often used in phytopharmacology as diuretic, antimycotic and anti-inflammatory remedies. Therefore, our results indicating the influence of both triterpenoids on cytokine production are very important for understanding the mechanism of their action. Experiments concerning the influence of betulin and betulinic acid on cytokine network are in progress.

## REFERENCES

- Bernard P., Scior T., Didier B., Hibert M., Berthon J.Y.: Ethnopharmacology and bioinformatic combination for leads discovery: application to phospholipase A2 inhibitors. *Phytochemistry*, 2001, 58, 865–874.
- Cavaillon J.M.: *Les Cytokines*, Ed. Cavaillon J.M., Masson, Paris, 1996, 184–199.
- Choi C.Y., You H.J., Jeong H.G.: Nitric oxide and tumor necrosis factor- $\alpha$  production by oleanolic acid via nuclear factor- $\kappa$ B activation in macrophages. *Biochem. Biophys. Res. Commun.*, 2001, 288, 49–55.
- Fernandez M.A., Delas Heras B., Garcia M.D., Saenz M.T., Villar A.: New insights into the mechanism of action of anti-inflammatory triterpene lupeol. *J. Pharm. Pharmacol.*, 2001, 53, 1533–1539.
- Foo S.Y., Nolan G.P.: NF- $\kappa$ B to the rescue. RELs, apoptosis and cellular transformation. *Trends Genet.*, 1999, 15, 229–235.
- Fulda S., Debatin K.M.: Betulinic acid induces apoptosis through a direct effect on mitochondria in neuroectodermal tumors. *Med. Pediat. Oncol.*, 2000, 35, 616–618.
- Giner-Larza E.M., Manez S., Recio M.C., Giner R.M., Prieto J.M., Cerda-Nicolas M., Rios J.L.: Oleanonic acid, a 3-oxotriterpene from *Pistacia*, inhibits leukotriene synthesis and has anti-inflammatory activity. *Eur. J. Pharmacol.*, 2001, 428, 137–143.
- Kirchner H., Kleinicke C., Diegel W.: A whole blood technique for testing production of human interferon by leukocytes. *Immunol. Methods*, 1982, 48, 213–219.
- Rajic A., Akihisa T., Ukiya M., Yasakawa K., Sandeman R.M., Chandler D.S., Polya G.M.: Inhibition of trypsin and chymotrypsin by anti-inflammatory triterpenoids from *Compositae* flowers. *Planta Med.*, 2001, 67, 599–604.
- Recio M.C., Giner R.M., Manez S., Gueho J., Julien H.R., Hostettmann K., Rios J.L.: Investigations on the steroidal anti-inflammatory activity of triterpenoids from *Diospyros leucomelas*. *Planta Med.*, 1995, 61, 9–12.
- Ryu S.Y., Oak M.H., Yoon S.K., Cho D.I., Yoo G.S., Kim T.S., Kim K.M.: Anti-allergic and anti-inflammatory triterpenes from herb of *Prunella vulgaris*. *Planta Med.*, 2000, 66, 358–360.
- Suh N., Honda T., Finlay H.J., Barchowsky A., Williams C., Benoit N.E., Xie Q.W., Nathan C., Gribble G.W., Sporn M.B.: Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res.*, 1998, 58, 717–723.
- Suh N., Wang Y., Honda T., Gribble G.W., Dmitrovsky E., Hickey W.F., Maue R.A., Place A.E., Porter D.M., Spinella M.J., Williams C.R., Wu G., Dannenberg A.J., Flanders K.C., Letterio J.J., Mangelsdorf D.J., Nathan C.F., Nguyen L., Porter W.W., Ren R.F., Roberts A.B., Roche N.S., Subbaramaiah K., Sporn M.B.: A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res.*, 1999, 59, 336–341.
- Sun I.C., Wang H.K., Kashiwada Y., Shen J.K., Co-sentino L.M., Chen C.H., Yang L.M., Lee K.H.: Anti-AIDS agents. 34. Synthesis and structure-activity relationships of betulin derivatives as anti-HIV agents. *J. Med. Chem.*, 1998, 41, 4648–4657.
- Wang B.H., Polya G.M.: Selective inhibition of cyclic AMP-dependent protein kinase by amphiphilic triterpenoids and related compounds. *Phytochemistry*, 1996, 41, 55–63.
- Ying Q.L., Rinerart A.R., Simon S.R., Cheronis J.C.: Inhibition of human leukocyte elastase by ursolic acid. Evidence for a binding site for pentacyclic triterpenoids. *Biochem J.*, 1991, 277, 521–526.
- You H.J., Choi C.Y., Kim J.Y., Park S.J., Hahm K.S., Jeong H.G.: Ursolic acid enhances nitric oxide and tumor necrosis factor- $\alpha$  production via nuclear factor  $\kappa$ B activation in the resting macrophages. *FEBS Lett.*, 2001, 509, 156–160.
- Zuco V., Supino R., Righetti S.C., Cleris L., Marchesi E., Gambacorti-Pascerini C., Formelli F.: Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Lett.*, 2002, 175, 17–25.

Received: November 5, 2002; in revised form: February 10, 2003.