



IL-6 INDUCTION BY BETULINIC ACID AND ITS PROLINE ETHYL ESTER SALT

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ABSTRACT:

Purpose: This study aims to compare the effects of betulinic acid (BA) and its proline ethyl ester ([ProOEt][BA]) on cell viability and IL-6 production by peripheral blood mononuclear cells (PBMCs).

Materials/Methods: PBMC were isolated from 10 mL of whole blood donated from healthy volunteers. PBMC were stimulated with 1 µg/mL Lipopolysaccharide (LPS), BA or [ProOEt][BA] at final concentrations of 2.5, 5, 10 and 20 µM. Cell viability was assessed using the MTT assay. IL-6 concentration in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) after 24 hours of incubation.

Results: Cell viability of PBMCs decreased in a dose-dependent manner upon stimulation with either BA or [ProOEt][BA]. A significant reduction in cell viability was observed with 10 and 20 µM concentrations of [ProOEt][BA] compared to BA after 24 hours of incubation. The lower concentrations of both compounds (2.5 and 5 µM) had comparable effects on cell viability. The estimated IC₅₀ values after 24 hours of PBMC stimulation were approximately equal for both BA (8.42 µM) and [ProOEt][BA] (8.86 µM).

A dose-dependent effect on IL-6 production was also observed in PBMCs after *in vitro* stimulation with BA or [ProOEt][BA]. At the lowest concentration (2.5 µM), both BA and [ProOEt][BA] caused a more than five-fold decrease in IL-6 production compared to LPS-treated cells (523.25 pg/mL and 581.90 pg/mL vs. 2994.50 pg/mL, respectively).

Conclusion: [ProOEt][BA] demonstrated similar activity to BA on PBMCs. A dose-dependent reduction in PBMC viability and IL-6 production was observed after *in vitro* stimulation with either BA or [ProOEt][BA].

Keywords: Betulinic acid salts, peripheral blood mononuclear cells, cytokine,

INTRODUCTION

In recent years, natural compounds have gained attention as effective therapeutic agents. Betulinic acid (BA) is a naturally occurring pentacyclic triterpenoid widely distributed in the bark of birch trees (*Betula* species). It has attracted considerable scientific attention due to its diverse pharmacological properties. It was reported that BA exhibits anti-inflammatory, anti-HIV, antimalarial, and anticancer activities [1, 2]. There are numerous reports on the biological activities of betulin and BA and their synthetic derivatives, which have been very recently reviewed by Amiri et al. [3]. Despite its promising bioactivity, the clinical application of BA is still limited mainly by its poor hydrophilic properties and bioavailability.

To address these limitations, various chemical modifications have been explored, including the formation of ionic liquids (ILs) with amino acid-based cations [4]. Recently, 15 new ILs of BA were synthesized and characterized both structurally and physicochemically. One of these compounds was the proline ethyl ester salt of BA ([ProOEt][BA]), but its biological activities in *in vitro* system of normal/healthy immune cells remains unexplored [5].

Interleukin-6 (IL-6) is a multifunctional cytokine involved in immune regulation and inflammation. Depending on the context, IL-6 can exert both pro-inflammatory and anti-inflammatory effects and plays a significant role in innate, B-cell-, and T-cell mediated adaptive immune responses. Respectively, therapeutic modulation of the IL-6 axis has led to the approval of multiple therapeutic agents for several autoimmune disorders [6, 7]. IL-6 production is a common marker of immune cell activation, particularly in response to lipopolysaccharide (LPS) stimulation in peripheral blood mononuclear cells (PBMCs). Investigating how novel derivatives of BA affect IL-6 expression could

provide valuable insight into their immunomodulatory potential.

The objective of this study was to examine the effect of [ProOEt][BA] on healthy PBMC viability and IL-6 production following *in vitro* stimulation. By analyzing the dose-dependent response of PBMCs, we aim to assess whether ionic modification alters the cytotoxic and immunomodulatory properties of BA. The rationale for selecting this ionic liquid is based on our earlier observations in cancer cell lines [5, 8]. Among a series of amino acid ethyl-ester (AAE) betulinates, [ProOEt][BA] was one of the most cytotoxic compounds, exhibiting 1.5-fold and 5-fold higher cytotoxicity than BA in hormone-dependent breast cancer cells (MCF-7) and colon cancer cells (HT-29), respectively. In addition, it was eight times more selective for hormone-dependent over hormone-independent breast cancer cells. Analysis of preliminary differential scanning calorimetry data of MCF-7 cells treated with BA or their AAE salts showed that only cells exposed to [ProOEt][BA] exhibited an upshift in the low-temperature transition, associated with membrane components, indicating a pronounced alteration in membrane-protein interactions (unpublished data).

MATERIALS AND METHODS:

Isolation and in vitro cultivation of PBMCs

PBMCs were isolated from 10 mL of whole blood donated from ten healthy volunteers by density gradient centrifugation, using Histopaque-1077 (Sigma-Aldrich, Germany). The resulting data represent at least six independent experiments for each cell culture condition. PBMCs (1×10^6 cells/mL) were cultured in RPMI-1640 medium supplemented with 20 mM HEPES, L-Glutamine, and 10% heat-inactivated fetal calf serum (FCS). PBMCs were stimulated with 1 μ g/mL Lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich, Germany), BA and an ionic liquid containing a cation ethyl ester of the amino acid proline [ProOEt] and an anion BA [ProOEt][BA], at final concentrations of 2.5 μ M; 5 μ M; 10 μ M and 20 μ M. The stock solutions of BA and [ProOEt][BA] were diluted in Dimethyl sulfoxide (DMSO) such that the final solvent concentration in the cell cultures did not exceed 0.5% and appropriate vehicle controls were included. The [ProOEt][BA] was synthesized as described by Ossowicz-Rupniewska et al. [5]. PBMC cultures were incubated at 37°C for 24 hours.

Ethical considerations

This study was approved by the ethics committee of the Medical Faculty, Trakia University, Bulgaria (Decision number No.23/10.03.2023). Informed consent was obtained from all participants in accordance with the ethical standards of the Helsinki Declaration.

MTT Assay

Cell viability was assessed using the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), a colorimetric reduction assay. A total of 10 μ L MTT stock solution (5 mg/mL, Sigma-Aldrich, Germany) was added to 100 μ L of cell culture and incubated for 3 hours at 37°C. Following incubation, an equal volume of 0.1N HCl in absolute isopropanol was added. Absorbance was measured at 570 nm using a Multiskan EX (Thermo Scientific, USA). Results are presented as percentages relative to non-treated cells. Each experiment was conducted in triplicate.

Half-maximal inhibitory concentration (IC₅₀) values were determined using a four-parameter logistic regression model with Quest Graph IC₅₀ Calculator from AAT Bioquest, Inc. (<https://www.aatbio.com/tools/ic50-calculator>).

Cytokine quantification

According to the manufacturer's instructions, IL-6 concentration in culture supernatants was quantified after 24 hours using a commercially available ELISA kit (Elabscience, USA). The detection range was 1.56-100.00 pg/mL with a sensitivity of 0.94 pg/mL. The optical density was measured using a Multiskan EX (Thermo Fisher Scientific Inc., USA). Cytokine levels were expressed as picograms per milliliter (pg/mL) based on standard curves generated using the kit-provided standards, multiplied by the dilution factor.

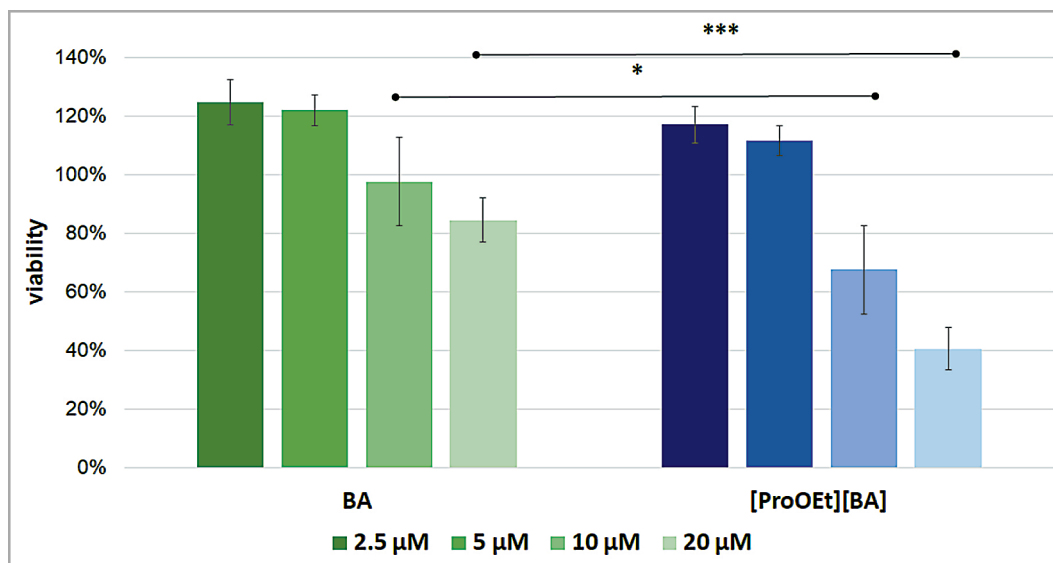
Statistical analysis

The data were analyzed using Statistica version 12 (StatSoft Inc., Tulsa, OK, USA). Distribution normality was assessed with the Shapiro-Wilk test. For non-normally distributed variables, comparisons between groups were performed using the Mann-Whitney U test. For normally distributed variables, comparisons were made using Student's t-test. The p-value less than 0.05 was considered significant.

RESULTS:

The results of the cell viability assay are presented in Figure 1.

Fig. 1. Cell viability of PBMCs after 24 hours of incubation with Betulinic acid (BA) or its proline ethyl ester ([ProOEt][BA]) at various concentrations. * $p < 0.05$ and ** $p < 0.01$, compared with BA-treated cells at the same concentration.



Cell viability decreased in a dose-dependent manner following stimulation with either BA or [ProOEt][BA]. At lower concentrations, both compounds had a comparable effect on PBMC viability. However, at higher concentrations (10 μM and 20 μM), a significant reduction in cell viability was observed. Notably, stimulation with 10 μM [ProOEt][BA] led to a greater reduction in PBMC viability compared to 10 μM BA ($p = 0.042$), with an even more pronounced effect observed at 20 μM ($p = 0.0009$).

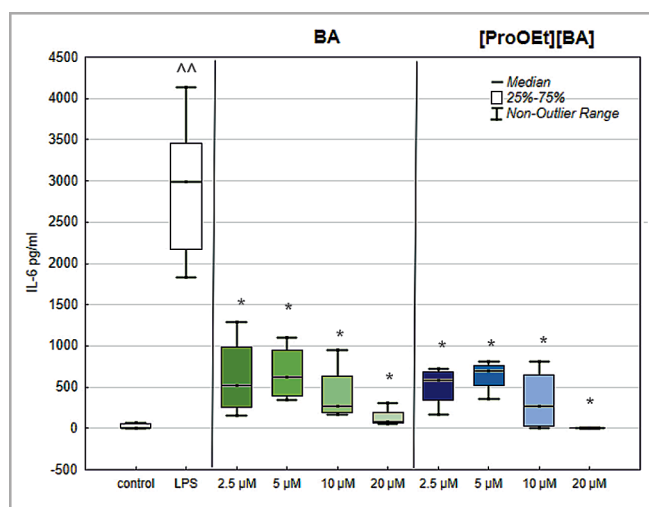
The estimated IC_{50} values after 24 hours of PBMC stimulation were approximately equal for both BA (8.42 μM) and [ProOEt][BA] (8.86 μM).

Treatment of PBMCs with BA or [ProOEt][BA] induces IL-6 production at all studied concentrations (Fig. 2).

Even at the lowest concentration (2.5 μM), both BA (median: 523.25; IQR: 260.2-725.2 pg/mL) and [ProOEt][BA] (median: 581.9; IQR: 342.6-684.2 pg/mL) caused a more than five-fold decrease of IL-6 production compared to LPS-treated cells (median: 2994.5; IQR: 2164.7-3451.7 pg/mL; $p = 0.01$).

In addition, a dose-dependent effect on IL-6 production was observed in PBMCs after *in vitro* stimulation with BA or [ProOEt][BA]. Higher concentration of BA and [ProOEt][BA] further reduced the IL-6 production, with the most pronounced effect observed at the closest concentration to half maximal inhibitory concentration - 10 μM or higher. At the highest concentration (20 μM), [ProOEt][BA] induced IL-6 levels similar to untreated cells, and significantly lower than BA at the same concentration.

Fig. 2. IL-6 production of PBMCs after 24 hours of incubation with Betulinic acid (BA) or its proline ethyl ester ([ProOEt][BA]) at various concentrations. * $p < 0.05$ compared with LPS-treated cells, ^^ $p < 0.01$ compared with non-treated cell cultures (controls).



There were no significant differences between the induced IL-6 of BA and [ProOEt][BA] at the same concentration.

DISCUSSION:

In this study, we investigated the effects of BA and its proline ethyl ester salt - [ProOEt][BA] on the viability and cytokine production of human PBMCs. Both compounds showed a dose-dependent reduction in cell viability, with [ProOEt][BA] demonstrating slightly higher cy-

toxicity at the 10 μM and 20 μM concentrations. Despite this, the estimated IC_{50} values were comparable, indicating that ionic modification did not substantially alter the half-maximal inhibitory potential of BA. Previous studies have reported IC_{50} values for [ProOEt][BA] of 7.6 μM against MCF-7 cells and 3.82 μM against HT-29 cells [5, 8], supporting its retained anticancer activity. Based on the above, we assume that this modification of BA did not significantly affect its cytotoxicity and anticancer activities.

The IL-6 secretion profile further confirmed the immunosuppressive properties of both compounds. Notably, both BA and [ProOEt][BA] caused a more than five-fold decrease in IL-6 production at the lowest tested concentration (2.5 μM) compared to LPS-stimulated PBMCs. This reduction was dose-dependent, with higher concentrations showing stronger suppression. Interestingly, [ProOEt][BA] at 20 μM reduced IL-6 levels to those observed in unstimulated cells, suggesting a potent suppressive effect on immune activation. However, this result should be interpreted with caution, since PBMC viability was reduced significantly at 20 μM [ProOEt][BA] compared to BA. These findings align with previous studies reporting anti-inflammatory and immunosuppressive actions of BA [3, 9]. BA is able to modulate a number of key mediators, including cytokine production of IL-1 β , IL-6, IL-12, and TNF, both *in vitro* and in different disease models. These biological activities were related mainly to the inhibition of the NF- κB pathway [10, 11].

Several BA derivatives showed promising anti-inflammatory activity, including BA5, a semi-synthetic amide derivative of BA [12], a heterocyclic ring-fused BA

derivative, SH479 [13], and a series of betulinic acid-azaprostanoïd hybrids [14]. In our study, a lack of statistically significant differences in IL-6 production between BA and [ProOEt][BA] at equivalent concentrations was observed. Our data suggest that ionic modification of BA with the nonpolar proteinogenic amino acid proline does not alter its biological activities, and the anti-inflammatory mechanisms of action may remain similar.

Limitations of this study include the small number of donors and the use of a single cytokine (IL-6) as a readout. Future studies should include a broader cytokine panel, multiple time points, and different cell types.

CONCLUSION:

In conclusion, the [ProOEt][BA] salt demonstrated similar activity to BA on PBMCs. A dose-dependent reduction in PBMC viability and IL-6 production was observed after *in vitro* stimulation with either BA or [ProOEt][BA].

Abbreviations:

BA - betulinic acid
ELISA - enzyme-linked immunosorbent assay
 IC_{50} - Half-maximal inhibitory concentration
IL-6 - Interleukin-6
LPS - Lipopolysaccharide
PBMC - peripheral blood mononuclear cells
[ProOEt][BA] - proline ethyl ester betulinate

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