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Short Communication

Anti-colon cancer activity of amino acid ester betulinates: Apoptosis induction and *IL1B* gene expression in PBMCs by the lead compound

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Abstract

Betulinic acid (BA) is a natural pentacyclic triterpenoid with significant pharmaceutical potential. We found that converting BA into amino acid ethyl ester salts alters its cytotoxic effect on colorectal adenocarcinoma cells (HT-29). Among these salts, the estimated half-maximal inhibitory concentration (IC_{50}) at 72 h varies by up to fifteenfold. The proline ethyl ester [ProOEt] salt, the most active compound, exhibited an IC_{50} value of 3.8 μM, which is 4.8 times lower than that of the parent BA. We observed that exposure to BA or [ProOEt][BA] induced apoptosis in HT-29 cells. Additionally, the proline-based salt led to a higher level of *IL1B* gene expression in PBMCs at a lower stimulation concentration.

Keywords

betulinic acid salts, colon cancer, peripheral blood mononuclear cells, immunomodulation

Introduction

Betulinic acid (BA) is a pentacyclic triterpenoid, a natural secondary plant metabolite showing immunomodulatory, anti-inflammatory, antioxidant, anti-diabetes, antiviral, and some other activities (Oliveira-Costa et al. 2024). Remarkably, BA also exhibits excellent cytotoxicity against melanoma, glioblastoma, lung, breast, colon, and prostate cancer, as well as some hematological malignancies (Zhang et al. 2016). Typically, its anticancer activity involves multiple molecular targets (Jiang et al. 2021). In addition, it has been reported that BA is an effective chemosensitizer of drug-resistant breast and colon cancer cells (Jung et al. 2007; Cai et al. 2018). Despite its significant pharmaceutical potential, there are currently no approved anti-cancer drugs based on BA due to its low solubility in water and low bioavailability. Moreover, some variations in the results of in vitro assays reported in the literature are attributed to BA low solubility. In recent years, researchers have been exploring various strategies to enhance solubility, stability,

and activity or propose different routes of administration. For example, modifications at the C3 position of the lupine-heterocycle, resulting in the substitution of the secondary hydroxyl group with fragments containing peptides, epoxides, a trifluoromethyl group, or incorporation of alkynol, secondary amine, or ester group improved selectivity, anti-inflammatory activity, anticancer activity, etc. (Rárová et al. 2022). The delivery of BA in innovative formulations such as oil-in-water nanoemulsions, spray-dried mucoadhesive microparticles, liposomes, or polymers is another promising strategy to improve the safety profile of BA, its absorption in the gastrointestinal tract, and its bioavailability (Serain et al. 2021). In the last decade, there has been growing research interest in the application of ionic liquids (ILs) as drug delivery systems (Jadhav et al. 2021). ILs are salts composed of bulky asymmetric organic cations and organic or inorganic cations, which may include active-pharmaceutical ingredients (Jadhav et al. 2021). In our recent study, we demonstrated that the conversion of betulinic acid into amino acid ethyl ester salts ([AAOEt][BA]) only slightly improved its water solubility in some cases (Ossowicz-Rupniewska et al. 2024). However, within the series, we observed up to a fivefold difference in the cytotoxic effect against hormone-dependent breast cancer cells (MCF-7) (Ossowicz-Rupniewska et al. 2024). The promising results motivated us to evaluate what will be the effect of BA-based ILs on colorectal adenocarcinoma (HT-29) cells. Colorectal cancer is the third most common type of cancer in the world, with around 2 million new cases being diagnosed each year, and yet its cure is challenging (Roshandel et al. 2024). In addition, for the IL that showed the highest cytotoxic effect [ProOEt][BA], we evaluated the apoptosis-inducing potential on HT-29 and its effect on IL1B gene expression after a six-hour stimulation of peripheral blood mononuclear cells (PBMC) isolated from the blood of healthy volunteers.

Materials and methods

Materials

Betulinic acid (BA) (≥98%) and 3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich. The tested [AAOEt][BA], namely L-alanine ethyl ester betulinate [AlaOEt][BA]; L-valine ethyl ester betulinate [ValOEt][BA; L-proline ethyl ester betulinate [ProOEt][BA]; L-threonine ethyl ester betulinate [ThrOEt][BA]; L-leucine ethyl ester betulinate [LeuOEt][BA]; L-isoleucine ethyl ester betulinate [IleOEt][BA]; L-aspartic acid ethyl ester betulinate [Asp(OEt)2][BA]; L-methionine ethyl ester betulinate [MetOEt][BA]; L-cysteine ethyl ester betulinate [CysOEt][BA]; L-serine ethyl ester betulinate [SerOEt][BA]; L-phenylalanine ethyl ester betulinate [PheOEt][BA]; L-lysine ethyl ester betulinate [LysOEt][BA]; L-tryptophan ethyl ester betulinate [TrpOEt][BA]; L-tyrosine ethyl ester betulinate [TyrOEt][BA] were synthesized as described in (Ossowicz-Rupniewska et al. 2024).

Methods

Cell culturing and treatment

The human colorectal adenocarcinoma cells (HT-29) (American Type Culture Collection (Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose containing 2 mM L-glutamine and 1 mM sodium pyruvate (VWR) and supplemented with 10% fetal bovine serum (FBS) (Gibco) and 100 U/mL penicillin-100 µg/mL streptomycin (VWR) at 37 °C in a humidified 5% CO_2-incubator (ESCO Life Science). Confluent cell monolayers were trypsinized with 0.05% porcine trypsin containing 0.02% ethylenediaminetetraacetic acid (Biowest). Cells were used in the exponential phase.

For the cytotoxicity assay, HT-29 cells were seeded in a sterile 96-well plate at 1×10^4 cells/well and incubated for 24 h at 37 °C and 5% CO_2 for obtaining adherent cell cultures and good cell spreading. Then, they were incubated with 0–100 µM of BA or the tested ILs for 72 h. The MTT assay was used to assess the cytotoxicity of the compounds (Mosmann 1983). Non-treated cells were used as control, and their viability was taken for 100%. The half-maximal inhibitory concentration (IC_{50}) values were determined by non-linear regression analysis using GraphPad Prism version 5.0 (GraphPad Software, USA) from three independent experiments.

For the cytomorphological studies, HT-29 cells were grown on sterile cover glasses placed on the bottom of 24-well plates (2.0×10^5 cells/well) for 24 h in a CO_2 incubator to form a cell monolayer. The cells were then incubated for 24 h with BA or [ProOEt][BA] at a concentration corresponding to the estimated IC50. Samples were double stained with acridine orange (AO, 10 µg/mL in PBS) and ethidium bromide (EtBr, 10 µg/mL in PBS) or 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/mL) and monitored on a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany).

Isolation and in vitro cultivation of PBMC

PBMC were isolated from 10 mL of whole blood donated from five healthy volunteers by density gradient centrifugation using Histopaque-1077 (Sigma).

PBMC (1×10^6 cells/mL; 2 mL) were cultured in RPMI-1640 medium modified with 20 mM HEPES and L-glutamine, containing 5% FBS. The IC_{50} values for 72 h treatment of PBMC with BA and [ProOEt][BA] were assessed using the MTT assay as described for HT-29 (Mosmann 1983).

Gene expression assay

We evaluated the effect of a six-hour stimulation with BA or [ProOEt][BA] at concentrations of 5 µM and 10 µM on *IL1B* gene expression in PBMCs. PBMC cells stimulated with 1 µg/mL lipopolysaccharide (LPS) from *Escherichia coli* (Merck) were used as a control. Total RNA was isolated using TRI Reagent solution (Applied Biosystems) following the manufacturer's instructions. The concentrations of the isolated RNA samples were determined

spectrophotometrically using a BioDrop μLite+ (Biochrom Ltd.). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamer primers by incubating for 5 min at 25 °C followed by 60 min at 42 °C on a GeneAmp PCR System 9700 (Applied Biosystems). Quantitative real-time polymerase chain reaction (qPCR) was performed on a 7500 Real-Time PCR System (Applied Biosystems) using TaqMan pre-designed inventoried primers and probes for *IL1B* (Hs01555410_m1) and two reference genes, β2-microglobulin (Hs00187842_m1) and GAPDH (Hs02758991_g1) (Thermo Scientific). The thermocycling conditions were as follows: an initial incubation for 10 minutes at 95 °C, followed by 40 cycles consisting of denaturation for 15 seconds at 95 °C and annealing/extension for 1 minute at 60 °C. qPCR data were collected using Sequence Detection System (SDS) software, version 2.3. The data were normalized to reference genes, and relative quantification was performed using the ΔCt method. The results are presented as fold changes of the target genes compared to the calibrator (non-treated cells).

Results and discussion

The preliminary screening showed that converting BA into AAOEt salts modulates its cytotoxicity against HT-29 cells. The IC_{50} (72 h) value obtained for the parent BA agrees with those reported in the literature for HT-29 and other colon cancer cells (Silva et al. 2019). As shown in Table 1, the estimated values of IC50 (72 h) varied more than 15-fold within the series. Interestingly, we did not observe a correlation between the cytotoxicity of the BA salt derivatives with the polarity or hydrophobicity/hydrophilicity of the cation.

Table 1. IC_{50} values (72 h) of BA and [AAOEt][BA] on HT-29 and PBMC cells.

Compound	IC50, μM	Compound	IC50, μM
BA	18.49±0.99 (9.68±0.97 for PBMC)		
[ValOEt][BA]	17.89±1.03	[AlaOEt][BA]	40.14±1.60
[ProOEt][BA]	3.82±0.74*** (7.84±0.55 for PBMC)	[CysOEt][BA]	42.87±1.63
[ThrOEt][BA]	13.46±0.84	[SerOEt][BA]	29.39±1.47
[LeuOEt][BA]	21.30±0.63*	[PheOEt][BA]	57.75±1.76
[IleOEt][BA]	26.90±1.02*	[LysOEt][BA]	11.94±1.08***
[Asp(OEt)2][BA	20.41±0.77	[TrpOEt][BA]	54.60±1.73*
[MetOEt][BA]	12.90±0.76	[TyrOEt][BA]	40.90±1.62

Each determined IC_{50} value of [AAOEt][BA] was compared to the IC_{50} of BA using one-way ANOVA. * $p < 0.05$ and *** $p < 0.001$; (n = 3).

Compared to BA, [ProOEt][BA] is more than 4.8 times more cytotoxic to HT-29 cells but less toxic to PBMC cells after prolonged treatment of 72 h. In particular, ProOEt is considered to have very low toxicity, with an IC50 of 50 mM against murine fibroblasts (L929 cells) (Moshikur et al. 2018). Interestingly, the IL based on the positively charged AA cation (Lys) is twice as toxic to HT-29 as that based on the negatively charged AA cation (Asp). Most likely, the penetration of the lysine-based salt through the HT-29 cell membrane is facilitated by favorable electrostatic interactions between the compound and the negatively charged cell surface (Le et al. 2018).

To analyze the mechanisms underlying the detected suppressive effects on the colorectal cancer carcinoma cell viability, the alterations in the cellular and nuclear morphology induced by BA or [ProOEt][BA] were examined by fluorescent microscopy of AO/EtBr- and DAPI-stained HT-29 cells (Fig. 1).

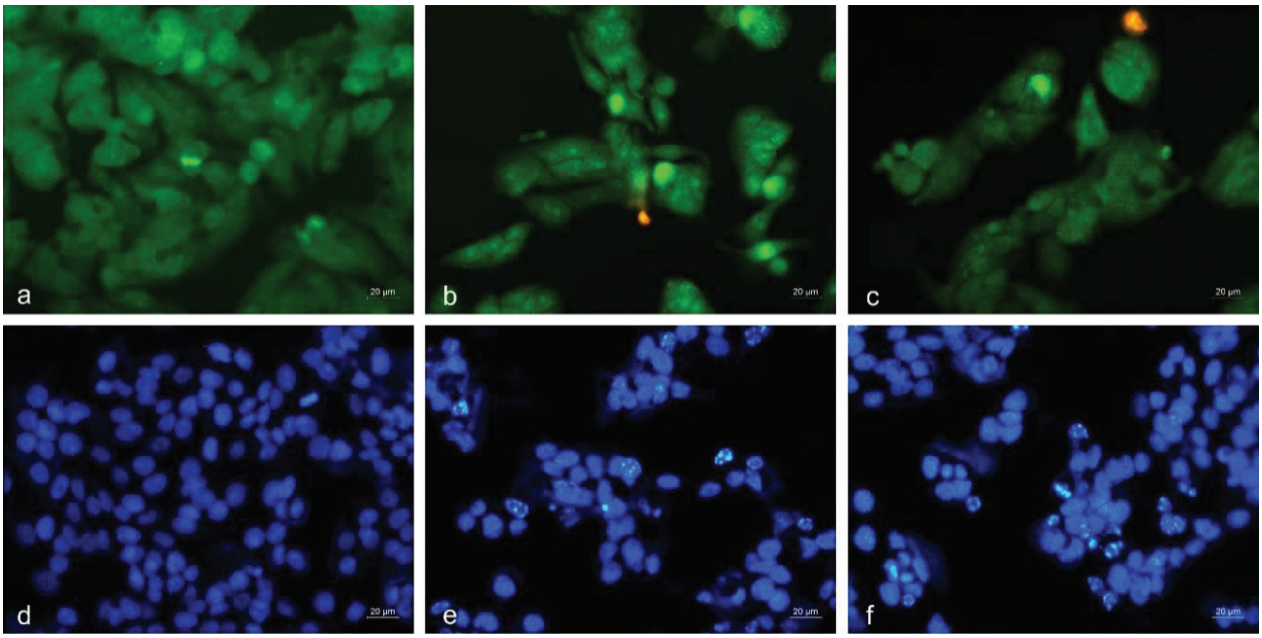

Figure 1. Fluorescence microscopy images of human colorectal adenocarcinoma cells (HT-29). **Upper panel**: Acridine Orange (AO)/Ethidium Bromide (EtBr) double staining; **Lower panel**: DAPI staining. (a, d) Untreated HT-29 cells; (b, e) HT-29 cells treated with 18.5 μM BA; (c, f) HT-29 cells treated with 3.8 μM [ProOEt][BA].

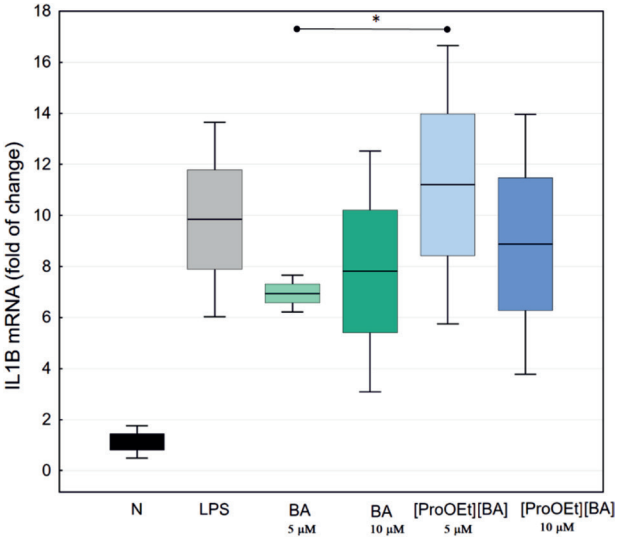

Figure 2. The effect of 5 μM and 10 μM BA or [ProOEt][BA] on the *IL1B* gene expression of PBMC after stimulation for 6 h. The results are presented as the mean ± SE and 1.96*SE of a fold of change in the *IL1B* compared to the calibrator (non-treated cells–N) after normalization to the reference genes *B2M* and *GAPDH*. *$p < 0.05$.

Both tested compounds applied at IC_{50} concentrations (18.5 μM and 3.8 μM, respectively) induced a marked reduction of cancer cell proliferation as evidenced by the decreased monolayer confluency and the reduced number of mitotic cells (Fig. 1b, c, e, f) compared to the control (Fig. 1a, d). Moreover, AO/EtBr staining revealed the presence of numerous early apoptotic cells with intense green nuclear fluorescence and membrane blebbing as well as a single red-stained late apoptotic or dead cell in the HT-29 cell cultures exposed to BA and [ProOEt][BA] (Fig. 1b, c). Fluorescent staining of the treated cells with the DNA-binding dye DAPI allowed the visualization of cells with condensed chromatin and fragmented nuclei, which are hallmarks of apoptotic cell death (Fig. 1e, f).

Next, we analyzed the *IL1B* mRNA levels in PBMCs stimulated with BA or [ProOEt][BA] at concentrations of 5 μM and 10 μM, considering the estimated IC_{50}. LPS, BA, and [ProOEt][BA] all enhanced *IL1B* expression in PBMCs under baseline activation conditions (Fig. 2).

Although BA induced a lower *IL1B* mRNA level compared to LPS, the difference was not statistically significant. In contrast, the *IL1B* gene expression was significantly lower after stimulation with 5 μM BA compared to 5 μM [ProOEt][BA] ($p = 0.03$). No significant difference was observed between 10 μM BA and 10 μM [ProOEt][BA] in *IL1B* expression in healthy control PBMCs. Several studies have reported that BA inhibits LPS-induced pro-inflammatory cytokine production, including TNF-α, IL-6, and IL-1β, in both *in vitro* and *in vivo* models of inflammatory diseases (Oliveira-Costa et al. 2024). Although our current data may seem contradictory, a plausible explanation could be the immune context and the type of cells used. BA and BA-based ILs, as natural immunomodulators, might increase *IL1B* expression in PBMCs under baseline activation conditions, in contrast to inflammatory conditions. Such context-dependent and cell-specific effects have been previously reported for another natural immunomodulator, resveratrol. Resveratrol was found to enhance *IL1B* expression in peripheral blood lymphocytes but had opposite effects in macrophages (Malaguarnera 2019). The specific outcome of treatment with BA and the BA-based IL likely depends on the concentration and the initial state of the PBMCs. Based on our preliminary data, we may assume that [ProOEt][BA] has a higher potency to induce IL-1β than BA at lower concentrations in healthy control PBMCs.

Conclusion

The cytotoxic effect of BA against HT-29 cells can be enhanced by converting it into amino acid salts. Compared to BA, [ProOEt][BA] shows greater cytotoxicity against HT-29 cells and lower toxicity to PBMCs, while also showing greater potency in inducing *IL1B* at lower concentrations in healthy control PBMCs. Further investigation is warranted to assess the immune-mediated anti-cancer potential of the salt.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that experiments on humans or human tissues were performed for the present study.

Informed consent from the humans, donors or donors' representatives: Medical Faculty, Trakia University, Stara Zagora.

The authors declared that no experiments on animals were performed for the present study.

Use of commercially available immortalised human and animal cell lines: American Type Culture Collection (Rockville, MD, USA).

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Author contributions

Conceptualization, L.M, M. G, ; methodology, L.V., A.Georg., P. O, M. G.; formal analysis, L.M, A.G, N.D, B.G, A. Georg., P.O, J.K, M.G.; data curation, all co-authors.; writing—L.M., A. Georg., M.G.; visualization, L.M., A. Georg. All authors have read and agreed to the published version of the manuscript

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Data availability

All of the data that support the findings of this study are available in the main text.

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