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# *In Vitro* Cytotoxicity Assessment of Betulinic Acid Organic Salts on Triple-Negative Breast Cancer Cells

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**Abstract:** The conversion of betulinic acid (BA) to organic salts is a strategic approach to modulate its physicochemical properties and biological activity. In our previous study, we demonstrated the enhanced cytotoxicity of certain amino acid ethyl ester betulinates ([AAOEt][BA]) compared to BA against hormone-dependent breast cancer cells (MCF-7). In this study, we extended our investigation to evaluate the cytotoxic response and thermodynamic properties of hormone-independent breast cancer cells (MDA-MB-231) following 72 h of treatment with the same series of betulinates. Our data reveal a lower cytotoxic response in MDA-MB-231 cells, indicated by higher half-maximal inhibitory concentration ($IC_{50}$) values, which ranged between 31 and 109 μM. Differential scanning calorimetry analysis supported these findings, showing negligible changes in the thermodynamic parameters of the treated MDA-MB-231 cells. However, consistent with our previous observations, [LysOEt][BA]$_2$, exhibited the highest cytotoxicity and induced the most pronounced morphological alterations in the cancer cells. Overall, our results suggest that MDA-MB-231 cells are less sensitive to [AAOEt][BA] compared to MCF-7 cells, likely due to their distinct phenotypic and genotypic profiles and differences in oncogenic signalling pathways. Nonetheless, the fact that [LysOEt][BA]$_2$ enhances the cytotoxic activity of BA even in hormone-independent breast cancer cells underscores its therapeutic potential and warrants further investigation, particularly in the context of adjuvant breast cancer therapy.

**Keywords:** betulinic acid; ionic liquids; triple-negative breast cancer; cytotoxicity; differential scanning calorimetry

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Academic Editor: David R. Wallace

Received: 20 September 2024
Revised: 16 December 2024
Accepted: 30 December 2024
Published: 2 January 2025

**Citation:** Apostolova, S.; Georgieva, I.; Ossowicz-Rupniewska, P.; Klebeko, J.; Todinova, S.; Tzoneva, R.; Guncheva, M. *In Vitro* Cytotoxicity Assessment of Betulinic Acid Organic Salts on Triple-Negative Breast Cancer Cells. *Sci* **2025**, *7*, 2. https://doi.org/10.3390/sci7010002

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## 1. Introduction

Breast cancer, one of the leading causes of death in women worldwide, is a highly heterogeneous disease. Based on gene expression profiling, it is currently classified into four subtypes: luminal A, luminal B, HER2-positive, and triple-negative. The luminal subtypes are positive for estrogen (ER+) and progesterone (PR+) receptors, whereas HER2-positive and triple-negative breast cancer (TNBC) do not express hormone receptors. The genetic diversity of breast cancer correlates with different responses to treatment, requiring specific targeted therapies based on molecular profiling. There is currently no effective treatment for TNBC, which is the most malignant subtype with a high propensity to metastasize [1,2].

In recent years, investigations regarding the utilization of natural products derived from distinct plant species have tremendously increased in experimental breast cancer therapy due to their potential to affect multiple targets. The latter allows simultaneous inhibition of several signalling pathways associated with proliferation, migration, and invasiveness, enhancing the treatment efficacy [3].

Pentacyclic triterpenoids are a group of plant secondary metabolites consisting of a 30-carbon skeleton organized into five six-membered rings or four six-membered rings and one five-membered ring, which exhibit a wide range of biological activities [4]. In terms of their antitumour activities, they act as activators of programmed cell death in various types of cancer cells by affecting multiple signalling pathways, as demonstrated by numerous in vitro and in vivo studies [5,6]. Betulinic acid (BA) is a lupane-type pentacyclic triterpenoid, which is well-studied for its inhibition of cancer cell proliferation and tumour suppression [7], making it a promising anticancer drug candidate. It exhibits strong anti-breast cancer activity through various mechanisms, including topoisomerase inhibition, induction of reactive oxygen species (ROS), apoptosis via the mitochondrial pathway, and inhibition of the NF-kB signalling pathway, sp transcription factors, and estrogen receptors. BA also shows anti-angiogenic and antimetastatic effects by inhibiting vascular endothelial growth factors and receptors and matrix metalloproteinases 2 and 9 (MMP-2, MMP-9), respectively [8,9]. Furthermore, it has demonstrated synergistic effects with other chemotherapeutics and chemosensitizers in taxol-resistant breast cancer cells [9,10].

Unfortunately, many natural compounds that show great pharmaceutical potential in vitro and in vivo fail in further investigations due to low intestinal absorption and high levels of clearance, resulting in poor bioavailability. The low oral bioavailability is one of the main reasons associated with clinical development failure of approximately 90% of potential drug candidates [11]. If the pharmacokinetic profile is poor, it may be difficult to achieve the dose profile that is required for therapeutic efficacy. Drug–drug interactions and the presence of active metabolites are additional reasons for failure. Therefore, the effective prediction of pharmacokinetic effects is of vital importance in the selection of drug candidates for development. In this respect, the main challenge in using BA as a pharmaceutical is its extremely low water solubility, which is approximately 0.02 μg/mL [12]. This low solubility also leads to inconsistencies in the results of in vitro assays reported in the literature. Moreover, due to its poor water solubility and permeability, it is not easy to prepare oral drug BA-containing formulations [13]. Currently, the only FDA (Food and Drug Administration)-approved drug containing betulinic acid is Filsuvez®, which is used for topical treatment of the rare skin disease Epidermolysis Bullosa [14].

In recent years, scientists have focused on improving the therapeutic effectiveness of drug formulations by enhancing their aqueous solubility through various strategies. For instance, several studies have demonstrated that BA oil-in-water nanoemulsions exhibit high loading efficiencies and increased bioavailability for BA. Moreover, they have shown a slight increase in anti-angiogenic and antitumour activity compared to the free compound [15,16]. Another approach to increase the bioavailability and activity of BA is its inclusion in various particle formulations. For instance, BA spray-dried mucoadhesive microparticle formulations have been reported to show a 3.9-fold increase in the plasma concentration of BA and improved permeability through the gastrointestinal tract [17]. Similarly, a nucleoside analogue of BA (gemcitabine), co-delivered in poly(lactic-co-glycolic acid) (PLGA)-polyethylene glycol (PEG) nanocarriers, demonstrated enhanced activity in an Ehrlich solid tumour model compared to the free drugs [18]. Other attempts to include BA in biopolymer microspheres based on chitosan or microbial cyclic glucan showed only minor effects on loading efficiency for BA (up to 10%) [19,20].

The use of ionic liquids (ILs) in drug formulations offers an innovative approach to overcoming challenges in drug development, such as poor solubility and stability in biological fluids, while enhancing bioavailability. This strategy can also prevent polymorphic conversion, reduce systemic toxicity, and enable new routes of drug administration [21,22]. ILs are organic lipophilic salts capable of interacting with highly hydrophobic drug molecules, thereby improving their poor solubility and facilitating drug passage across cell membranes [23].

Numerous studies have demonstrated that IL formulations combined with various drugs effectively enhance solubility [24]. Different types of ILs, including those based on imidazoles, piperidines, morpholines, choline, amino acids, and fatty acids, have been successfully used in drug crystal engineering and the preparation of drug vesicles such as nanoemulsions, micelles, hydrates, and solvates for oral, injectable, or topical delivery [25,26]. Additionally, converting active pharmaceutical ingredients into salts can improve drug delivery efficacy and pharmacokinetics [25,26]. For example, cholinium-, chloride-, and imidazolium-based ILs and salts have effectively increased the solubility of hydrophobic drugs like the non-steroidal anti-inflammatory drug ibuprofen [27,28]. Solubility-boosting properties of ILs have also been demonstrated in vivo, where choline- and geranic acid-based organic salts of the hydrophobic anticancer drug sorafenib significantly enhanced solubility and oral absorption in rat models compared to the parent drug [29].

Despite over 3000 research papers highlighting the biological activity of BA and showing its potential, particularly in breast cancer treatment, the number of studies on novel BA-based formulations remains surprisingly low. Several studies have shown that the solubility and cytotoxicity of BA can be enhanced through single-site derivatization at positions C-3 or C-19 [30,31]. For example, the introduction of a propargylpyrrolidine moiety at C-3, or a 3,5-dinitrophenylhydrazine moiety at C-3 or C-19, resulted in a 2-fold decrease in the half-maximal inhibitory concentration ($IC_{50}$) against hormone-dependent breast cancer cells (MCF-7) compared to parent BA [30]. Notably, adding a benzenesulfonyl group at C-3 of BA reduced the $IC_{50}$ against TNBC cells (MDA-MB-231) by 18-fold [32]. In contrast, skeletal modifications to BA negatively impacted cytotoxicity. Only a few reports have explored improving the solubility and activity of BA by converting it to ILs. BA-ILs containing choline or benzalkonium cation have been the most extensively studied and show enhanced solubility in aqueous media, with a 3- to 5-fold increase in cytotoxicity against MCF-7 cells [33–36].

Recently, we reported the synthesis and characterization of 15 amino acid ethyl ester salts of betulinic acid ([AAOEt][BA]) [37]. We found that formulations containing polar or charged amino acid cations exhibited improved water solubility compared to BA. Furthermore, some of these salts, particularly $[LysOEt][BA]_2$, with enhanced aqueous solubility, showed remarkable cytotoxic effects on hormone-dependent MCF-7 breast cancer cells, compared to BA alone. This was further confirmed by a loss in heat capacity. Therefore, this study aims to expand the research on the anti-proliferative activity of the same IL formulations on MDA-MB-231, as an in vitro representative of the triple-negative subtype of breast cancer.

## 2. Materials and Methods

### 2.1. Materials

Betulinic acid (BA) (≥98%), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and Dulbecco's modified Eagle's medium (DMEM) were purchased from (Sigma-Aldrich, Steinheim, Germany). 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-lysine ethyl ester bis(betulinate) [LysOEt][BA]$_2$; L-tryptophan ethyl ester betulinate [TrpOEt][BA]; L-tyrosine ethyl ester betulinate [TyrOEt][BA] were synthesized as described in [37].

The MycoStripTM mycoplasma detection kit was purchased from InvivoGen (Toulouse, France).

### *2.2. Methods*

#### 2.2.1. Cell Culturing

The triple-negative human breast cancer cells MDA-MB-231 were a gift from Prof. Martin Berger, DKFZ, Heidelberg, Germany. Cells were cultured with DMEM supplemented with 10% fetal bovine serum, L-glutamine (2 mM) and penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL). The cells were maintained in a thermostat at 37 °C and 5% $CO_2$ and were passaged every 2–3 days. Cells were routinely tested for mycoplasma by a MycoStripTM mycoplasma detection kit. Only MDA-MB-231 cells free of mycoplasma contamination were used in all experiments performed.

#### 2.2.2. Viability Assay

The cell viability was analyzed colorimetrically by an MTT assay, measuring metabolic activity and assessing cell proliferation and cytotoxicity [38]. MDA-MB-231 cells were seeded at a density of $5 \times 10^3$ cells/mL in 96-well plates. The cells were incubated for 24 h and then were treated with varying concentrations of [AAOEt][BA] (5 µM, 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM, and 120 µM). The cytotoxicity was followed for a period of 72 h. Cells treated only with dimethylsulphoxide at a concentration below 1% were used as a negative control. After 72 h treatment, MTT solution with a concentration of 5 mg/mL diluted in PBS 1× was directly added to the wells of 96-well plates and cells were incubated for 3 h in a thermostat. After incubation, the liquid was aspirated and the obtained formazan crystals were dissolved by adding 100 µL of 5% formic acid in iso-propanol. Then, the absorbance was measured immediately at a wavelength of 570 nm by a microplate reader (Tecan Infinite F200 PRO, Mannedorf, Switzerland). Cell viability (%) was estimated as a percentage of the control using the following formula:

$$\% \text{ cell viability} = \text{OD treated cells/OD control cells} \times 100$$

The values of half maximal inhibitory concentration (IC50) were calculated by a non-linear regression analysis using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

#### 2.2.3. Cell Morphology Assessment by Phase Contrast Microscopy

Cell morphology of treated MDA-MB-231 cells with the most effective ionic liquids was performed by inverted phase contrast microscopy (MEIJI, CHUO-KU, Japan) with camera Optikam B1 Digital (Optica, Ponteranica, Italy).

#### 2.2.4. Differential Scanning Calorimetry (DSC) Experiment

MDA-MB-231 cells were seeded in T-150 culture flasks at a density of $2 \times 10^6$ cells per flask and grown in complete DMEM for 24 h at 37 °C and 5% $CO_2$. The cancer cells were exposed to BA or [LysOEt][BA]$_2$ at concentrations corresponding to the estimated $IC_{50}$ for 72 h. The cancer cells were then trypsinized and collected by centrifugation, together

with the dead cells floating in the culture medium. The cancer cells were thoughtfully washed with PBS (pH 7.4). For comparison, a DSC experiment was also carried out with non-treated MDA-MB-231. The DSC measurements required about $2 \times 10^7 - 4 \times 10^7$ cells in PBS.

The DSC measurements were performed using a DASM4 (Privalov, BioPribor, Moscow, Russia) built-in, high-sensitivity calorimeter. The measuring cell volume was 0.47 mL to prevent any degassing of the solution, and a constant pressure of 2 atm was applied to the cells. The heating scan of the samples was carried out at a scan rate of 1.0 °C/min in the range of 20 °C to 120 °C. DSC thermograms provide a heat capacity curve as a function of temperature. The sample heating scans showing the thermal denaturation profiles of the native samples were preceded by a baseline run with buffer-filled cells. Each sample solution was reheated after it had cooled down from the first scan. Transitions observed in the first heating were found to be irreversible since they were not observed in the second heating runs. Raw DSC data were corrected by subtracting the second scan from the first one. Corrected calorimetric profiles were normalized to the DNA concentration (determined spectrophotometrically using the extinction coefficient of 0.020 $(\mu g/mL)^{-1}$ $cm^{-1}$ for double-stranded DNA at 260 nm [39]. The obtained calorimetric data were analyzed by the Origin Pro 2018 software package, and the main thermodynamic parameters: transition temperature (Tm), excess heat capacity ($c_{Pex}$) of successive thermal transitions, and total calorimetric enthalpy (ΔH) were determined.

## 3. Results and Discussion

### *3.1. Cell Viability of MDA-MB-231 Cells Treated with [AAOEt][BA]*

In our recent work, we have shown that some of [AAOEt][BA] exhibit enhanced cytotoxic activity against hormone-dependent MCF-7 breast cancer cells compared to BA [37]. In this study, we further investigated the cytotoxic effects of [AAOEt][BA] on breast cancer, focusing on the highly invasive and hormone-independent MDA-MB-231 cell line. We hypothesize that the ethyl ester modification of AAs (cations) may increase their lipophilicity, thereby facilitating their uptake by breast cancer cells. The increased intracellular concentration of AA may affect the metabolic pathways that cancer cells rely on growth and survival and potentially exert a synergistic antiproliferative effect with BA. All tested [AAOEt][BA] show a dose-dependent decrease in cell proliferation. The ILs with non-polar cations and negatively charged hydrophobic amino acids generally have a cytotoxicity similar to or lower than the parent BA. However, the salts with polar and positively charged hydrophilic AAs showed a slightly higher cytotoxic effect on the TNBC cells. This observation is likely related to the negatively charged cell membrane of MDA-MB-231 cells, which can engage in favourable electrostatic interactions with the positively charged ILs, enhancing their internalization and cytotoxic potential [40].

As shown in Table 1, the estimated IC50 values of the [AAOEt][BA] formulations are between 31 and 109 μM, and most are comparable to that of BA. For BA, the results are consistent with those reported by other authors [41].

As expected, this cell line is less sensitive to the tested ILs than MCF-7 cells, whose IC50 values ranged from 4.8 to 25.7 μM [37]. Indeed, the most prominent anti-proliferative effect on MDA-MB-231 cells was detected after exposure to the polar and positively charged IL ([LysOEt][BA]2 compared to BA). These data are similar to our previous results obtained in MCF-7, although the effect was less pronounced in the TNBC cells. However, in addition to the net positive charge of the ionic liquid, the increased cytotoxicity must also be due to the increased net amount of BA in this IL.

**Table 1.** IC50 values of BA and [AAOEt][BA] in MDA-MB-231 cells.

| Compound | $IC_{50}$ (μM); 72 h |
|---|---|
| [BA] | 42.96 ± 0.56 |
| [ValOEt][BA] | 45.12 ± 5.74 |
| [ProOEt][BA] | 61.53 ± 3.65 * |
| [ThrOEt][BA] | 109.1 ± 13.59 * |
| [LeuOEt][BA] | 49.34 ± 4.63 |
| [IleOEt][BA] | 52.30 ± 4.32 |
| [$Asp(OEt)_2$][BA] | 46.10 ± 11.75 |
| [MetOEt][BA] | 66.21 ± 1.08 ** |
| [AlaOEt][BA] | 41.23 ± 5.62 |
| [CysOEt][BA] | 39.50 ± 0.36 * |
| [SerOEt][BA] | 36.47 ± 15.70 |
| [PheOEt][BA] | 54.82 ± 7.07 |
| [LysOEt][BA] | 37.56 ± 6.64 |
| [LysOEt][$BA]_2$ | 31.95 ± 0.75 ** |
| [TrpOEt][BA] | 37.93 ± 6.36 |
| [TyrOEt][BA] | 54.01 ± 6.85 |

Values represent the mean ± SD from six repeats of three experiments. * $p < 0.05$ and ** $p < 0.01$ compared to BA. Statistical analysis was performed using an unpaired Student's t-test in GraphPad Prism ver. 5.

### *3.2. Morphological Alterations of MDA-MB-231 Cells Treated with [AAOEt][BA]*

We analyzed the morphology of MDA-MB-231 cells after exposure to lower (20 μM), around $IC_{50}$, and above IC50 concentrations of BA or [AAOEt][BA] for 72 h by inverted light microscopy. As shown in the microscopic images (Figure 1), treatment with 20 μM BA and IL formulations with the pronounced cytotoxic effect ([LysOEt][BA] and [LysOEt][$BA]_2$) did not induce significant morphological changes in MDA-MB-231 cells compared to BA. Additionally, no significant changes in cell density were observed in any treated cells compared to the control.

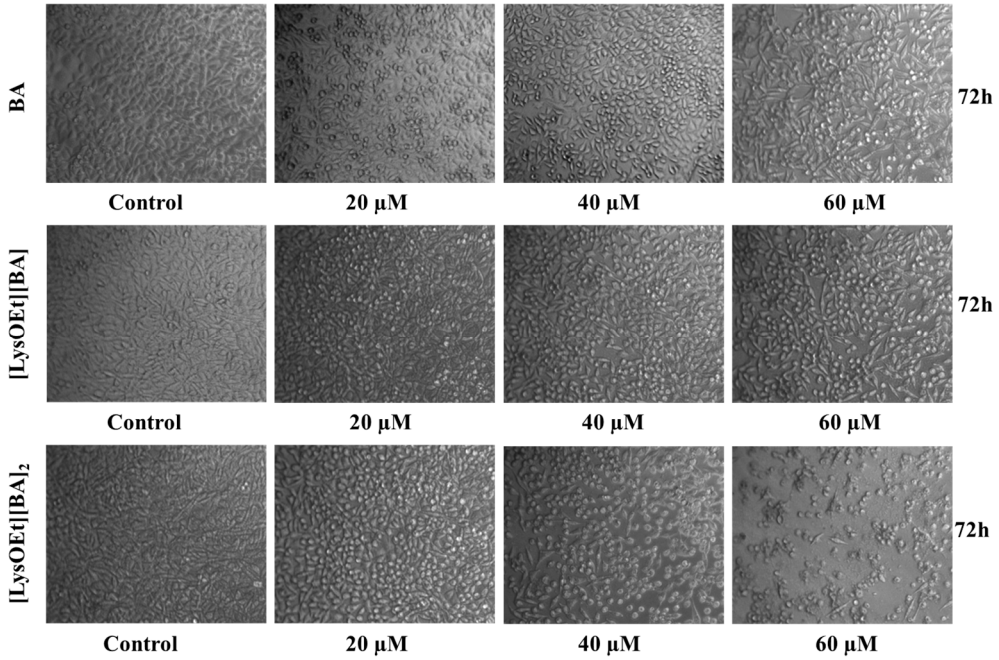



**Figure 1.** Phase contrast images of MDA-MB231 exposed to BA, [LysOEt][BA], and [LysOEt][$BA]_2$ with 20 μM, 40 μM, and 60 μM concentrations for 72 h. Images were taken at 10× magnification.

At concentrations close to the IC50 of the compounds, only the cell culture treated with [LysOEt][$BA]_2$ showed a large number of rounded cells, suggesting apoptotic cell

death. Increasing the concentration above IC50 (60 μM) exacerbated this process only in the $[LysOEt][BA]_2$ treatment (Figure 1), whereas in the [LysOEt][BA] and BA treatments, the cells retained their typical spreading shape, although their number decreased, indicating inhibited cell proliferation.

In summary, we observed that the MDA-MB-231 cell line is less sensitive to the tested ILs than MCF-7 cells, for which the IC50 values ranged from 4.8 to 25.7 μM [37]. The lower response of the MDA-MB-231 cell line to treatment with natural products (including BA) compared to the hormone-dependent cell line (MCF-7) can be attributed to several factors such as higher expression of drug efflux pumps, lack of estrogen and progesterone receptors, mutational profiles, and metabolic differences [42]. For example, one of the major targets of BA is estrogen receptor signalling, specifically estrogen receptor alpha (ERα), which mediates the proliferative capacity of MCF-7 cells [8,43]. In this context, it is not surprising that the hormone-independent breast cancer cell line MDA-MB-231 is less sensitive to BA treatments. In addition, the observed differences in cytotoxicity could also be attributed to the different status in the two cell lines of p53 tumour suppressor protein, which is also a target of BA [8,44]. The impaired function of p53 in MDA-MB-231 leads to an increase in genomic instability and abnormal mitosis, promoting tumour cell survival and escape from the apoptosis cascade [45], which may explain why BA is unable to induce p53-mediated G1 arrest in these cells [8]. Another known target of BA in cancer cells is aerobic glycolysis by altering Cav-1/NF-κB/c-Myc signalling in MCF-7 and MDA-MB-231 breast cancer cells [46]. The authors found a stronger inhibitory effect of BA on glycolysis in MCF-7 compared to MDA-MB-231. The reduced effect of BA in MDA-MB-231 may be related to the increased level of glycolysis found in this highly invasive cell line, which is associated with impaired mitochondrial function, suppression of apoptosis, and correlates with tumour progression and a high rate of metastasis due to upregulation of genes involved in angiogenesis [47,48].

All these findings support our previous and present study data and suggest that BA exerts a more potent cytotoxic effect in MCF-7 due to its effect on multiple molecular targets, specifically the estrogen receptor and p53 signalling. In contrast, in MDA-MB-231 cells, BA may target alternative signalling pathways. The latter may be one of the possible explanations for the observed lower sensitivity of the hormone-negative breast cancer cells to BA and [AAOEt][BA].

### *3.3. Calorimetric Analysis of MDA-MB-231 Cells Treated with [AAOEt][BA]*

In parallel, we investigated the effect of BA and $[LysOEt][BA]_2$, the most cytotoxic compound, on the thermal denaturation of MDA-MB-231 cells by differential scanning calorimetry (DSC). DSC can provide preliminary information on possible interactions between the cell components (DNA, lipid membrane, cytoplasmic or nuclear matrix proteins, etc.) and the compound [49] and can be potentially used for monitoring the side effects of cancer therapy [50]. Figure 2 displays the DSC thermograms of non-treated MDA-MB-231 cells and cells exposed to BA and $[LysOEt][BA]_2$ for 72 h, at concentrations corresponding to $IC_{50}$ values.

The DSC profile of non-treated MDA-MB-231 cells shows five endothermic thermal transitions in the temperature range of 30–110 °C (designated as $T_1$ to $T_5$) and three shoulders ($Sh_1$, $Sh_2$, and $Sh_3$) (Figure 2). The DSC curves of the treated cells have similar profiles; however, the first shoulder ($Sh_1$) is missing in the thermogram of MDA-MB-231 treated with $[LysOEt][BA]_2$. The transition temperatures ($T_m$) and the excess heat capacities for the main peaks are listed in Table 2. The calorimetric enthalpy of the overall cell curve ($\Delta H_{cal}$) and those estimated for the low- ($\Delta H_L$) and high- ($\Delta H_H$) temperature regions were also determined. Typically, the low-temperature ($L_T$) peaks (up to 72 °C) are attributed

to the thermal denaturation of non-chromosomal cellular components, whereas the high-temperature ($H_T$) peaks (above 72 °C) refer to the nuclear structures [49]. Interestingly, we did not observe any significant shifts in the temperature maxima in the DSC curves of the treated cells compared to those of non-treated cells.

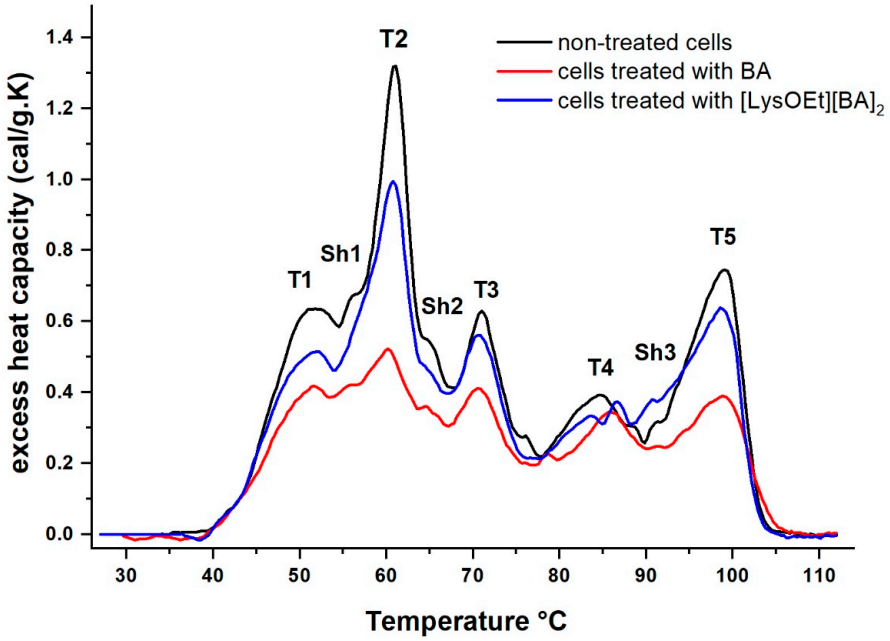



**Figure 2.** DSC curves of non-treated MDA-MB-231 cells (black) (control) and MDA-MB-231 cells treated for 72 h with BA (red) or $[LysOEt][BA]_2$ (blue). The successive transitions in the control curve are denoted by T1–T5 and the shoulders by Sh1, Sh2, and Sh3. Representative curves from each condition were obtained from two independent experiments.

**Table 2.** Thermodynamic characteristics determined from the analysis of DSC scans of non-treated MDA-MB-231 cells and cells treated with BA or $[LysOEt][BA]_2$ for 72 h.

| Thermodynamic Parameters | Non-Treated | Treated with BA | Treated with $[LysOEt][BA]_2$ |
|---|---|---|---|
| $T_{m1}$ (°C) | 51.7 | 51.7 | 51.9 |
| $c_P^{T1}$ (cal/g·K) | 0.64 | 0.42 | 0.51 |
| $Sh_{m1}$ (°C) | 56.0 | 55.6 | - |
| $c_P^{Sh1}$ (cal/g·K) | 0.68 | 0.42 | - |
| $T_{m2}$ (°C) | 61.1 | 60.4 | 60.8 |
| $c_P^{T2}$ (cal/g·K) | 1.32 | 0.52 | 0.99 |
| $Sh_{m2}$ (°C) | 65.0 | 64.6 | 64.8 |
| $c_P^{Sh2}$ (cal/g·K) | 0.54 | 0.36 | 0.47 |
| $T_{m3}$ (°C) | 71.0 | 70.5 | 70.6 |
| $c_P^{T3}$ (cal/g·K) | 0.63 | 0.41 | 0.56 |
| $T_{m4}$ (°C) | 84.6 | 86.7 | 86.0 |
| $c_P^{T4}$ (cal/g·K) | 0.39 | 0.37 | 0.35 |
| $Sh_{m3}$ (°C) | 91.3 | 91.3 | 90.7 |
| $c_P^{Sh3}$ (cal/g·K) | 0.32 | 0.25 | 0.38 |
| $T_{m5}$ (°C) | 98.9 | 98.9 | 98.6 |
| $c_P^{T5}$ (cal/g·K) | 0.75 | 0.39 | 0.64 |
| $\Delta H_{total}$ (cal/g) | 23.2 | 13.0 | 19.2 |
| $\Delta H_L$ (cal/g) | 16.2 | 9.1 | 12.6 |
| $\Delta H_H$ (cal/g) | 7.0 | 3.9 | 6.6 |

On the other hand, we found that treatment with BA or [LysOEt] salt resulted in a remarkable reduction in the excess heat capacity ($c_{Pex}$) of the transition and, consequently, in the total calorimetric enthalpies of the thermogram (Table 2). Although the treatment with [LysOEt][BA]$_2$ displayed the highest cytotoxic effect on MDA-MB-231 cells, it had little influence on the thermograms from a thermodynamic perspective. However, it should be noted that $\Delta H_L$ for this compound is diminished by more than 20% versus that of untreated cells. For BA-treated cells, the enthalpy change is dropped by more than 40% for both the low- and high-temperature regions compared to the respective values of untreated cells. In particular, the endotherms in the low-temperature region (L) decreased sharply, mostly related to the highly cooperative transition with Tm at ~61 °C and that at ~99 °C in the high-temperature range (H) (Figure 2).

The observed effect may be associated with changes in the cellular structure and metabolism. For example, apoptotic cells are characterized by morphological alterations, including chromatin condensation and cellular shrinkage, which can decrease enthalpy change [51]. On the other hand, a decrease in the enthalpy change may be due to a reduction in cell proliferation, characterized by diminished metabolic activity and changes in the structural dynamics of cellular components, including proteins and membranes [52]. Recent studies have confirmed that some anticancer drugs, including those obtained from medicinal plants, conventional chemotherapeutics, etc., can alter the thermal properties of the membrane lipid bilayer, including the phase transition enthalpy and/or the lipid domains organization, fluidity, or elasticity of cell membranes [53–55]. Compared to the effect observed in our previous study for MCF-7 cells, the lower impact of BA and the tested ILs on the thermodynamic profile of MDA-MB-231 cells is consistent with their lower cytotoxicity. Although [LysOEt][BA]$_2$ exhibited slightly higher cytotoxicity than BA, the effect is probably not high enough and, therefore, changes in the stability of biomolecules may be difficult to detect.

Multiple factors of metabolic, genetic, and epigenetic origin contribute to the multidrug resistance of MDA-MB-231 cells, making them less responsive to treatment regimens [56]. A widely explored strategy to overcome this resistance in TNBC cells is to increase their sensitivity using natural products that target multiple oncogenic pathways [3,5,57,58]. An example of such a natural product would be BA in an ionic liquid formulation. Our findings suggest that L-lysine esters allow conjugation with double amounts of BA, enhance BA activity, and improve its aqueous solubility, which is probably the reason for the observed higher cytotoxic effect of [LysOEt][BA]$_2$ towards MDA-MB-231 cells. The increased activity of this salt could also be explained by electrostatic interactions of the charged AA cation with the phospholipid membrane of the cells, causing membrane deformations or sensing of membrane potentials, effects widely studied in model systems regarding the interactions of cells with charged AAs [59]. Consequently, the cell membrane damage disturbs homeostasis by significantly increasing intracellular calcium concentrations, activating proteolysis, and inducing osmotic stress. All these molecular events at the end lead to cell death [60]. Unfortunately, the $IC_{50}$ of [LysOEt][BA]$_2$ for normal human mammary epithelial cells, found in our previous study [37], was lower than $IC_{50}$ of MDA-MB-231 cells. Therefore, these findings could restrain the use of [LysOEt][BA]$_2$ in further potential treatment of TNBC, due to possible toxic effects to normal cells. However, BA is found to sensitize breast cancer cells to several chemotherapeutics [5,58]. Hence, the response of normal and triple negative breast cancer cells to a low-dose combination of [LysOEt][BA]$_2$ and other anticancer drugs is worth further analysis.

## 4. Conclusions

In conclusion, our results indicate that the hormone-independent breast cancer cells, MDA-MB-231, are less sensitive to the amino acid ethyl ester betulinates [AAOEt][BA], compared to the hormone-dependent breast cancer cells (MCF-7) obtained in our previous study. These results are also confirmed by DSC analysis showing a lower impact of BA and [AAOEt][BA] on the MDA-MB-231 thermodynamic profile. These findings suggest that BA and its IL formulations provoke differential responses in breast cancer cell subtypes due to their phenotypic and genotypic differences, resulting in the activation of distinct oncogenic pathways. However, similar to our previous work, the IL formulation based on the polar and positively charged lysine, $[LysOEt][BA]_2$, enhances the sensitivity of MDA-MB-231 to BA and exhibits a higher cytotoxicity. The latter is probably due to the increased activity of the double amount of BA and its improved water solubility, allowing intracellular distribution of the active compound. The effect might also be promoted by disruption of cell membranes as a result of their interactions with the charged amino acid. Further studies investigating the sensitizing effect of ILs on the basis of AAs and BA on hormone-independent breast cancer cells to other therapeutic drugs would be of great importance.

**Author Contributions:** Conceptualization, P.O.-R. and M.G.; methodology, S.A., R.T. and S.T.; formal analysis, S.A., I.G., J.K. and S.T.; investigation, S.A., I.G., P.O.-R., R.T. and M.G.; writing—original draft preparation, M.G.; writing—review and editing, S.A., P.O.-R., R.T., S.T. and M.G.; visualization, S.A. and S.T.; project administration, M.G.; funding acquisition, M.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Bulgarian National Science Fund, grant number KP-06-H69/2.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The original contributions presented in this study are included in the article.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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