Lipid Atlas of Keratinocytes and Betulin Effects on its Lipidome Profiled by Comprehensive UHPLC–MS/MS with Data Independent Acquisition Using Targeted Data Processing

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Betulin is a pentacyclic triterpene with demonstrated healing properties in mid-dermal wounds. A few earlier studies have provided insights into the wound healing effects on the molecular level. However, there are still questions left on the molecular targets of betulin. Therefore, a pharmacolipidomics analysis of betulin is undertaken in human immortalized keratinocytes to monitor alterations in the lipid profiles induced by treatment with betulin. For this purpose, lipid extracts of keratinocytes treated with betulin and untreated controls are comprehensively analyzed by an untargeted UHPLC–ESI–QTOF-MS/MS lipidomics profiling workflow using data-independent acquisition. Targeted data processing allows the identification of 611 lipid species from 21 different lipid classes. Statistical analysis of the identified lipids shows significant changes in 440 lipid species that can be described as downregulation of cholesterylesters and triacylglycerides and upregulation of glycerophospholipids, sphingolipids, and diacylglycerides. Additionally, some other signals corresponding to triterpenes are found in the betulin group and suggested that betulin is incorporated (in the membrane) and metabolized in keratinocytes.

1. Introduction

Betulin, a pentacyclic triterpene, was shown to exert a variety of different biological effects including anticancer, antiinflammatory, antifungal, or antiviral activities. Interestingly, in 2016, a birch bark preparation (Episalvan), which contains betulin to around 80%, has been approved by the European Medicines Agency (EMA) for the treatment of partial-thickness skin wounds. Studies on the molecular level with betulin have been conducted that explain the clinically proven wound healing effects. Betulin influences the inflammatory and the new tissue formation phase in the wound healing process. It enhances the migration of keratinocytes and stimulates their differentiation. However, it has not yet been clarified whether or in which way the lipophilic betulin may act on cell membranes, permeate membranes to enter the cell, or have an impact on the lipid profile of the cell. Up to now, it has only been proven with erythrocytes that betulin can replace cholesterol in the membranes leading to alterations of their membrane shapes.

In the case of skin, two of its most important barrier functions are the barrier to the movement of water and electrolytes and the barrier against invasive and toxic microbes. Lipids are considered important players in the maintenance of these two functions, and ceramides, cholesterol, and free fatty acids are particularly enriched in stratum corneum (SC), the outer layer of the epidermis, where keratinocytes show their last step of differentiation.

Specific studies on murine keratinocytes, normal human keratinocytes or immortalized human keratinocytes showed a correlation between their lipid content and their state of differentiation. Analysis of lipid classes content by densitometry after High performance TLC and fatty acid profiling by GC after derivatization were performed for that purpose. More recently, lipidomics studies in keratinocytes have been performed to elucidate the effect of some factors such as narrow band ultraviolet B, allergen and irritant compounds, dioxin, radical generator, and a glycolytic inhibitor. From a methodological perspective, lipidomics profiling of biological samples is nowadays achieved by either targeted or untargeted approaches. Untargeted assays are based on...
high-resolution MS using direct infusion with FT-ICR- or Orbitrap-MS (shotgun lipidomics)\[^{[24,25]}\] or employing UHPLC–MS/MS (with QTOF or Qorbitrap) in data-dependent acquisition (DDA) or data-independent acquisition (DIA) mode. UHPLC–MS/MS with DIA\[^{[26]}\] offers the possibility to acquire MS and MS/MS data comprehensively over the entire chromatogram and access all samples. It allows uncompromised retrospective data processing so that the measurements represent a digital map of the lipid phenotype of the biological sample. Data processing of untargeted lipidomics data can be quite elaborate and a number of distinct procedures have been proposed for DIA data including software tools such as MS-DIAL\[^{[27]}\] or Lipid-Pro\[^{[28]}\] which allow automated identification by matching in silico or experimental databases besides some general tools to process lipidomics data. Typical approaches for metabolite identification include a first filtering step based on matching of a precursor ion \(m/z\) and a second step based on similarity scores with MS/MS databases.\[^{[29]}\] Also, scores for isotopic pattern similarity and retention time (in case of LC–MS) have been implemented as part of some identification algorithms.\[^{[27]}\]

In this work, we employ a targeted data processing approach on untargeted lipidomics data generated by DIA with sequential window acquisition of all theoretical fragment ion mass spectra (SWATH) in order to determine significant changes in human immortalized keratinocytes after treatment with betulin. For identification of lipid species, we performed an approach that combines the analysis of specific fragments for each lipid class, the matching of retention times for identification in positive and negative mode and the analysis of retention time for a particular species within the retention time pattern of the whole lipid class.

2. Experimental Section

2.1. Materials

Methanol (MeOH), acetonitrile (ACN), and isopropanol (IPA), all LC–MS quality and formic acid (98%) were purchased from Roth (Karlsruhe, Germany). Ammonium formate was obtained from Sigma–Aldrich (Steinheim, Germany). Water was purified by in-house Elga purification system (High Wycombe, United Kingdom).

Isotopically labeled internal standards (ILIS) were purchased from Avanti Polar Lipids (Alabama, USA) as a ready to use mixture: SPLASH Lipidmix solution (see Table A1, Supporting Information for more information about ILIS). Odd-chained lipid standards LPC 17:1 and PC 17:0-20:4 were obtained from Avanti Polar Lipids. Lupeol, betulinic acid, and erythrodiol standards were obtained from Sigma–Aldrich.

Keratinocyte serum-free growth medium, supplements (recombinant human epidermal growth factor rhEGF, bovine pituitary extract BPE), 0.05% trypsin/EDTA (w/v) were bought from Thermo Fischer Scientific (Waltham, MA, USA); penicillin–streptomycin was obtained from Roche (Mannheim, Germany). Betulin was a gift from Birken AG, Niefern-Öschelbronn, Germany. A 10 mM stock solution of betulin was prepared in DMSO.

2.2. Cell Culture and Lipid Extraction

Human immortalized keratinocytes were kindly provided from Prof. Dr. L. Bruckner-Tuderman, Department of Dermatology, Medical Center, University of Freiburg. Human immortalized keratinocytes were cultivated in Keratinocyte SFM supplemented with rhEGF, BPE, and 1% v/v penicillin/streptomycin at 5% CO\(_2\) and 37 °C. Cells were split when they reached a confluence of 80%. Passage 3 or 4 was used. Cells were plated in 20 Petri dishes (10 cm\(^2\), \(1 \times 10^6\) cells per dish) and cultivated for 4 days in 10 mL of the above mentioned medium for adherence. Ten dishes were incubated with 1.95 µM betulin (10 µL of the 1.95 mM stock solution in DMSO) for 8 h prior to removal of the medium and the remaining ones were used as control. Control samples were treated with 10 µL DMSO. The concentration of 1.95 µM of betulin was used, as this concentration showed effects in previous experiments on the molecular wound healing effect.\[^{[30]}\] On day 4, cells were incubated with 3 mL of trypsin (0.05%) at 37 °C. After 5 min, cells were washed by adding 7 mL medium. The suspension was transferred into 15 mL falcon tubes and centrifuged for 5 min at 4 °C and 1200 rpm, respectively. The supernatant was withdrawn and the remaining cell pellets were washed and then frozen at −20 °C in the falcon tubes until extraction.

Lipid extraction was performed with 1 million cells using IPA:H\(_2\)O (90:10 v/v) as described previously\[^{[30]}\] (see details of extraction protocol in Supporting Information A1).

2.3. LC–MS Measurement

Analyses were performed by Agilent 1290 Series UHPLC instrument (Agilent, Waldbronn, Germany) coupled to Sciex TriplLetOF 5600+ MS (Sciex, Concord, Ontario, Canada) with duospray source and Pal HTC-XS autosampler from CTC (Zwingen, Switzerland). Positive and negative ESI ionization were used in separate LC–MS runs with the same chromatographic separation conditions as described previously\[^{[27,30]}\]. Briefly: Acquity UPLC CSH C18 (130 Å, 1.7 µm, 2.1 mm × 100 mm) column was utilized with Acquity UPLC CSH C18 VanGuard pre-column (130 Å, 1.7 µm, 2.1 mm × 5 mm) (Waters, Eschborn, Germany). The mobile phase was composed of 10 mM ammonium formate and 0.1% formic acid dissolved in 60:40 ACN:H\(_2\)O (v/v) (A) and 90:10 (v/v) IPA:ACN (B). Further details on LC gradient elution

Significance Statement

In this study, we performed a comprehensive description of the lipidome of human immortalized keratinocytes and its changes after the treatment with betulin. Data processing was based on a target list of lipids. Our identification approach combines information from precursor and product ions obtained from analyses in both polarities under same chromatographic conditions and from elution patterns in RP-LC of each lipid class. Cholesterol esters (CEs) with very long fatty acid chains (up to 36 carbons) were detected and may serve as depot to support the synthesis of ceramides that play an important role in the skin barrier function. Significant changes of CEs (downregulated) upon betulin treatment as many other lipid classes are described. Results also suggest that betulin is incorporated and metabolized in the keratinocytes.
conditions and MS parameters can be found in Supporting Information A2.

MS/MS data were obtained by DIA using SWATH\textsuperscript{[31,32]} Q1 window sizes (see Table A3, Supporting Information) were optimized with SwathTuner software\textsuperscript{[33]} based on a quality control (QC) sample.

2.4. Lipid Identification

A lipid list was prepared and the target lipids were first searched and identified in a QC sample (pool of an aliquot of all study samples and injected after every fifth sample). Lipid classes were analyzed one by one. Notation of identified lipid species and fragment ions was done according to detailed rules proposed by Liebisch et al.\textsuperscript{[34]} and Pauling et al.\textsuperscript{[35]} Thus, lipids were annotated at the “lipid species level” (e.g., PC 34:2) or “molecular lipid species level” (e.g., PC 16:1-18:1) depending on analyzed fragments, which were of three different types: lipid class-selective fragment (LCF) (e.g., PC 18:1(+C3H6O2)), intermediate molecular lipid species-selective fragment (iMLF; e.g., -PE O- (141)), and molecular lipid species-selective fragments (MLFs; e.g., FA 18:1(+C3H6O2)), whose definitions given by Pauling et al.\textsuperscript{[35]} are copied in Supporting Information A3 (note, no distinction between alkyl ether (O-) and alk-1-enyl ether (P-) lipids was made in this work). For identification (see example in Figure 1), a set of characteristic ions for each lipid class was selected and their m/z values were obtained from LipidBlast database.\textsuperscript{[36,37]} Each set consisted of the most intense adduct ion in the TOF-MS scan as well as the most intensive LCF or iMLF in the corresponding SWATH experiment, in both ESI(+) and ESI(–) (see list of fragments for each lipid class in Table A4, Supporting Information). Extracted ion chromatograms (EICs) (of selected ions for each lipid) were compared by using PeakView (Sciex) in order to determine whether the lipid was present in the QC sample. For some of the lipid classes, also EICs of MLFs were analyzed to identify fatty acyl chains in the lipid species. Additionally, peak spotting maps of retention time versus precursor ion m/z of each identified lipid were generated for each lipid class.

2.5. Lipid Comparison and Quantitation

Precursor and fragment ions of identified lipids were analyzed through the whole set of samples with MultiQuant 3.0 (Sciex, for integration parameters, see Table A5, Supporting Information). Peak apex intensities were exported for statistical analysis. Intensities were normalized with corresponding intensities (from TOF-MS scan) of standard PC 17:0-20:4, which was added with the resuspension solvent after extraction and prior to LC–MS measurement. Concentrations for each lipid species were estimated based on their normalized intensity and the response factor of ILIS from the corresponding lipid class. For those lipid classes with no internal standard added before extraction, a standard addition curve was prepared with QC sample. Response factors to estimate concentrations were obtained from the slope of standard addition curves (see Table A6, Supporting Information).

Reported concentrations of a lipid species are an average of its corresponding estimated concentrations from ESI (+) and ESI (–), and from precursor and fragment ion traces.

2.6. Statistics

Normalized intensities of precursor and product ions from positive and negative mode were combined and hypothesis testing was done in R statistical language (https://cran.r-project.org) using the nonparametric Mann–Whitney U-test (obtaining a p-value for each studied trace) and correction for multiple hypothesis testing adjusting a false discovery rate of < 0.05 according to the approach proposed by Storey.\textsuperscript{[38]} (results reported as q-values).

3. Results and Discussion

3.1. Lipid Identification

Identification of lipid species in QC samples was performed by analyzing EICs of specific precursor and product ions for each lipid class in TOF-MS and SWATH, acquired in ESI(+)- and ESI(–) using the same LC separation. Figure 1 shows an example comprising the following steps for identification of lipid species.

Table A4, Supporting Information, summarizes the list of precursor and fragment (product) ions selected for the identification of lipids from each lipid class. For each lipid class, the most abundant precursor ion in ESI-TOF-MS, positive and negative polarity, and the most intense LCF or iMLF in SWATH (positive or negative or both) were employed to identify all the lipids at the “lipid species level”. In total, 611 lipids from 21 lipid classes were identified at this level in QC samples and their distribution is shown in Figure A1, Supporting Information.

EICs of precursor ions and LCF or iMLF for species of the same lipid class with equal number of carbons and different number of unsaturations (e.g., PE O-36:1 and PE O-36:2, see Figure 1C) show that their chromatographic separation is achieved by the employed LC method.

Additional analysis of MLF allowed us to identify around 70% of the lipids at the “molecular lipid species level” (see Table B1, Supporting Information) but also showed that in most cases, a (partial) co-elution of lipid species (isomers) with the same number of carbons and unsaturations is occurring. For example, analysis of possible MLFs “FA xy(+C3H6O2)” for PE O-36:2 showed the co-elution of PE O-18:1-18:1, PE O-16:1-20:1, and PE O-18:2-18:0. Therefore, we decided to keep the annotation of lipids that were identified at the “molecular lipid species level,” but quantitative comparison was done at the “lipid species level” because of the better S/N obtained from EICs of precursor ions, LCFs, and iMLFs.

In those cases, in which lipid species of the same class with the same number of carbons and unsaturations were chromatographically separated, individual species were analyzed and annotated with an extra Arabic number (e.g., PE O-36:4 (1) at 6.07 min, PE O-36:4 (2) at 5.91 min, and PE O-36:4 (3) at 5.79 min, see Figure 1C).

From Figure A2, Supporting Information, the distribution of MS data (precursor ions, LCFs and iMLFs) from different polarities and acquisition modes that were used for identification of
Figure 1. Scheme for lipid identification process in the case of Ether-linked phosphatidylethanolamines (EtherPEs). Briefly, A) a specific set of fragments for each lipid class was selected, B) m/z values from selected ions were obtained from LipidBlast database, C) EICs of selected ions were obtained and compared in corresponding TOF-MS–ESI (–) (top), TOF-MS–ESI (+) (middle), and SWATH (MS/MS) ESI (+) (bottom) experiments to determine which lipids are present in the sample, D) spotting maps for precursor ions of lipids identified, from the same lipid class, with ESI (+), and E) ESI (–) were plotted to check for possible misidentifications, db: amount of double bonds.
3.2. Presence of Long Fatty Acyl Chains

A first glance into the identified lipid species, especially those ones with only one fatty acyl chain, allows a good overview of the fatty acyl composition present in the studied sample. In the case of CE, it was interesting to observe the presence of an extended range of fatty acyl chains from CE 16:1 to CE 36:7. Figure A5, Supporting Information show the distribution in terms of intensity for CEs identified in keratinocytes. CEs with long chain fatty acids have been suggested as a depot for long chain fatty acids in human secretions. This function may also apply for keratinocytes. Here, long fatty acyl chains in CE could be important for the synthesis of Cers and HexCers with long fatty acyl side chains, which play an important role in the skin barrier.

3.3. Concentration of Lipid Species

ILIS from different lipid classes that were added to each sample before lipid extraction were used to estimate concentrations of lipid species in control samples, which are reported in Table B1, Supporting Information. The total sum for each lipid class was calculated as micrograms of each lipid class per million of cells. Figure 2 shows the mass percentage of each lipid class with respect to the total amount of lipid. A comparison to previously reported estimations on lipid concentrations in keratinocytes cannot be done in a straightforward manner because of methodological differences such as extraction protocol, analytical technique for detection, or lipid classes included in each particular study. However, most of the estimated percentages for the lipid classes content seem to be similar and only the percentage of CEs obtained in our study (22%) seems to be considerably higher than previously reported values, which were <10% in all the cases.
3.4. Comparison of Lipid Classes After Betulin Treatment

Characteristic precursor and fragment ion (LCFs and iMLFs) intensities from TOF-MS and SWATH experiments, respectively, were compared for each lipid species from betulin (B1 to B10) and control samples (C1 to C10). The coefficients of variation of the signal intensities for each of these ion traces (MS and MS/MS) both in control and betulin-treated groups including measurement and biological variance was in average around 15% (see Figure A6 and Table A7, Supporting Information). The mere measurement variance was significantly less and close to 100% of the features showed a CV < 30%.

Nonparametric Mann–Whitney U-test and a correction for false discovery rate were employed to determine statistically significant differences. Table B1, Supporting Information shows the values of the fold change (FC) between betulin versus control group and corresponding q-values for each monitored signal (1294 in total, see Table B4, Supporting Information) of all 611 identified lipids. In general, similar FCs and q-values were obtained for the same lipid species regardless whether they were analyzed on precursor or fragment ion level in ESI (+) or ESI (−) being indicative for the good assay specificity. Out of 611 identified lipids, 440 turned out to be significantly different (q < 0.05). Relative intensities (as Z-scores) of significantly changed lipids are shown in Figure 3. It can be observed that even when some differences are present between samples of the same group (B1–B4 compared to B5–B10), significant changes in the lipid profile of keratinocytes occur after treatment with betulin in certain
The upregulation of ceramides is an important part of the intercellular lipid lamellae forming the barrier functions of the skin. Here, upregulation of Cers is pronounced (<0.67 for most of them while in the case of TGs the FC is between 0.67 and 1.0). On the other side of the volcano plot, it is possible to see that the rest of studied lipid classes show a clear upregulation and few species from some of these lipid classes are more significantly upregulated (FC > 1.5, see Table B3, Supporting Information). Upregulation of acyl carnitines (AC) can be highlighted here considering that four of them are included in the ten most upregulated species with an FC > 2.0.

Interestingly, most of the upregulated lipid classes are components of phospholipids and sphingolipids that are essential structural membrane constituents and play an important role in barrier functions of the skin. Here, upregulation of Cers (e.g., Cer d18:0/24:0, Cer d18:0/24:1) with long-chain fatty acids by betulin serve special interest. These lipids are known to be synthesized intracellularly by keratinocytes. They are transported in specialized vesicles to the stratum corneum where they are synthesized intracellularly by keratinocytes. They are transported by betulin serve special interest. These lipids are known to be components of phospholipids and sphingolipids that are essential structural membrane constituents and play an important role in barrier functions of the skin. Here, upregulation of Cers (e.g., Cer d18:0/24:0, Cer d18:0/24:1) with long-chain fatty acids by betulin serve special interest. These lipids are known to be synthesized intracellularly by keratinocytes. They are transported in specialized vesicles to the stratum corneum where they are an important part of the intercellular lipid lamellae forming the water permeability barrier. The upregulation of ceramides with long chain fatty acids by betulin may contribute to explain the formation of the skin barrier shown in the ex vivo porcine wound healing model. The downregulation of (long chain acyl) CEs may be an indication for a metabolic remodeling for the above purpose. Our comprehensive study about the influence of betulin on the lipidome of keratinocytes may also support new fields of application, such as treatment of atopic dermatitis, as it was reported that this skin disease is combined with a decrease of long chain fatty acid Cers.

**3.5. Betulin and Betulin-Related Metabolites in Treated Keratinocytes**

In addition to the identified lipid species, some other signals were independently found to be differently abundant in the two sample groups, betulin-treated and control. For example, betulin and some related compounds were detected in the lipid extracts of the betulin-treated samples although the betulin solution was removed after incubation and the keratinocytes washed before analysis. m/z-Values and retention times of these peaks are summarized in Table A8, Supporting Information. Combined information for addsucts eluted at the same retention time and in different polarities allowed the determination of five molecular formulas of compounds, present in the extracts, corresponding to triterpenes.

In order to determine if these signals originate from the betulin solution used for treatment of keratinocytes, standard solutions of betulin and other triterpenes (see chemical structures in Figure A7, Supporting Information) were analyzed by the same method (see Table A9, Supporting Information).

Figure A8, Supporting Information shows intensities of analyzed signals in samples treated with betulin, control samples, and standard solution of betulin. As shown, two compounds, which were found in samples treated with betulin, were present in the betulin standard solution and were identified as betulin (eluted a 2.39 min) and betulinic acid (eluted at 2.64 min). This means that betulin and the impurity betulinic acid, either remained absorbed to the surface of keratinocytes despite washing steps after treatment or more likely that betulin was incorporated into the lipid membrane of the keratinocytes and extracted with the organic solvents, which is worthwhile to be further investigated. The other three compounds, which have an increased intensity in treated samples compared to the control ones and are not present in the standard solution of betulin, were annotated as “betulin isomer,” “betulin loss of O,” and “betulinic acid loss of CH2.” These compounds could correspond to metabolites of betulin produced in the keratinocytes. Future experiments should be undertaken to clarify in which way these metabolites arise.

**4. Concluding Remarks**

The presented untargeted lipidomics approach with targeted data processing enables a detailed characterization of the lipidome in keratinocytes and can detect significant differences after “drug” treatment, shown by the natural product betulin. In total, 611 lipids were identified at the “lipid species level” and 440 of them showed to be significantly changed. Changes can be described in terms of lipid classes. Thus, CEs and TGs are significantly downregulated in betulin treated samples and glycerophospholipids, sphingolipids, and diacylglycerides are upregulated.

Additionally, the presence of betulin in extracts of keratinocytes (previously treated with betulin and washed) indicate that betulin may be incorporated into the membrane of keratinocytes. The presence of other triterpenes in betulin treated...
samples and absent in control samples could mean that betulin is metabolized in the keratinocytes.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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