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Lecanoric acid mediates anti-proliferative effects by an M phase arrest in colon cancer cells

Luise A. Roser [a,b], Pelin Erkoc [b,c], Rebecca Ingelfinger [b,c], Marina Henke [a,b], Thomas Ulshöfer [a,b], Ann-Kathrin Schneider [a], Volker Laux [a], Gerd Geisslinger [a,b,d], Imke Schmitt [b,e,f], Robert Fürst [b,c,1], Susanne Schiffmann [a,1,*]

[a] Fraunhofer Institute for Translational Medicine and Pharmacology ITMP, Theodor-Stern-Kai 7, 60596 Frankfurt, Germany
[b] LOEWE Center for Translational Biodiversity Genomics (LOEWE-TBG), Frankfurt, Germany
[c] Institute of Pharmaceutical Biology, Faculty of Biochemistry, Chemistry and Pharmacy, Goethe University Frankfurt, Frankfurt, Germany
[d] Pharmazentrum Frankfurt/ZAFES, Department of Clinical Pharmacology, Goethe-University Hospital Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany
[e] Senckenberg Biodiversity and Climate Research Centre (SBiK-F), Senckenberganlage 25, D-60325 Frankfurt, Germany
[f] Institute of Ecology, Evolution, and Diversity, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt, Germany

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ABSTRACT

Lichen extracts containing, among other compounds, depsides such as evernic acid, atranorin, and lecanoric acid possess anti-proliferative effects. We aimed to identify lichen metabolites that are responsible for the observed anti-proliferative effects. We performed cytotoxicity, cell colony, cell cycle and apoptosis assays in various cell lines or primary immune cells. We analyzed several cell cycle proteins and apoptosis-related proteins to gain insights into the underlying mechanism. All depsides reduced the viability of the tested cell lines (HCT-116, HEK293T, HeLa, NIH3T3, RAW246.7) in a cell line-dependent manner with lecanoric acid being the most effective. Atranorin did not influence the cell cycle or colony formation in HCT-116 cells, but induced apoptosis in HCT-116 cells. Evernic acid showed no anti-proliferative effects. Lecanoric acid inhibited cell colony formation already at 0.03 μg/ml in HCT-116 cells and induced a G2 cell cycle block in several cell lines. Moreover, lecanoric acid arrested the cell cycle, presumably in the M phase, since expression of cyclin B1 and phosphorylated histone H3 was upregulated, whereas the inactive cyclin-dependent kinase 1 (CDK1) was reduced in HCT-116 cells. Most importantly, cell death induced by lecanoric acid was more prominent in cancer cells than in primary human immune and endothelial cells. In conclusion, lecanoric acid seems to mediate its anti-proliferative effects via arrest of cells in the M phase. Our data suggest lecanoric acid may be a potential new candidate for anti-cancer therapy, because it has anti-proliferative effects on cancer cell lines, and does not affect primary immune cells.

1. Introduction

The identification of natural product-based drugs has revolutionized medicine. Paclitaxel derived from *Taxus brevifolia* and vinblastine from *Catharanthus roseus* for cancer medication are widely known examples of natural products used in modern medicine [1]. In 2018, 16% of drugs approved by the U.S. Food and Drug Administration were based on natural products or their derivatives, thereby underlining the relevance of natural products to current drug discovery [2]. Possible sources for natural products include lichen-forming fungi, representing fungi which form symbiotic relationships with green algae or cyanobacteria. For more than 100 years lichens have been known as versatile producers of secondary metabolites, and today about 1000 metabolites have been identified [3,4]. Many biological properties of lichen secondary

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; cdc25C, cell division cycle 25C; CDK, cyclin-dependent kinase; GADD45A, Growth Arrest, and DNA Damage 45A; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; pH3, phosphor Histon H3; SEM, standard error of the mean.

* Corresponding author.
E-mail address: susanne.schiffmann@itmp.fraunhofer.de (S. Schiffmann).
[1] contributed equally

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metabolites have been described, including pharmaceutically valuable activities, such as anti-proliferative/cytotoxic [5], antibiotic [6], anti-mycobacterial [7], antiviral [8], or anti-inflammatory [9] effects. However, lichens are slow-growing, more or less uncultivable, and not readily accessible to genetic manipulation and biotechnological approaches. Therefore, the pharmaceutical potential of lichenized fungi has not been fully explored.

Secondary metabolites are classically defined as compounds that, in contrast to products of primary metabolism, are exclusively synthesized by a definite species or group of plants or fungi [10]. Lichen secondary metabolites are low molecular weight substances, mainly produced by the mycobiont [11]. In a previous study, we described the anti-proliferative effects of extracts of the lichens *Evernia prunastri*, *Pseudevernia furfuracea,* and *Flavoparmelia caperata* [12]. Certain depsides are present as major metabolites in the extracts of these species. To identify the secondary lichen metabolites that are potentially responsible for the observed effects of the lichen extracts, we analyzed the depsides atranorin, evernic acid, and lecanoric acid using various test systems relevant to cancer.

Anti-proliferative effects can be mediated by apoptosis induction or cell cycle arrest, among other mechanisms. A main characteristic of apoptosis is the activation of caspases leading to the degradation of proteins and chromosomal DNA, to cytomorphological changes, and finally to the formation of apoptotic bodies [13]. Furthermore, the transition of the cell cycle phases (G1, S, G2, M) is regulated by checkpoint proteins, including cyclin-dependent kinases (CDKs) complexed with specific subunits called cyclins, whose levels oscillate during the cell cycle. For the transition of the G2 phase to mitosis, upregulation of cyclin A/B and active CDK1 are prerequisites. CDK1 binds to cyclin B1 forming the cyclin B1/CDK1 complex, whose enzymatic activity is responsible for the initiation of mitosis. However, this complex formation is not sufficient to regulate the initiation of mitosis. Dephosphorylation of CDK1 at the tyrosine-15 site through cell division cycle (cdc) 25C phosphatase is a crucial event for activation of this complex, while CDK1 phosphorylation at tyrosine-15 induces its inactivation [14].

The aim of the present study was to investigate whether the depsides atranorin, evernic acid, and lecanoric acid show pharmacological potential as a cancer medication. For that purpose, the secondary metabolites were analyzed using cellular assays to assess their effects on viability, apoptosis induction, cell colony formation, and the cell cycle.

2. Materials and methods

2.1. Cells and reagents

HCT-116 and HEK293T were purchased from DSMZ GmbH (Braunschweig, Germany). RAW246.7, NIH3T3 and HeLa were obtained from ATCC (Virginia, USA). Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords according to Jaffe et al. [15]. HCT116 cells were cultivated in McCoy's 5A (modified) medium, HEK293T in DMEM medium, NIH3T3 cells in DMEM GlutaMAX and HeLa as well as RAW264.7 cells in RPMI medium. These media contained 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Primary HUVECs were cultured in EC growth medium (ECGM) (PELO-Biotech, Martinsried, Germany) supplemented with 10% FCS (Biochrom AG), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (PAN-Biotech, Aidenbach, Germany), and a supplement mixture (PELOBiotech). HUVECs were used for experimental purposes exclusively in passage three. All cells were cultured at 37 °C in a 5% CO_2 atmosphere. Atranorin was purchased from Biozol (Eching Germany). Lecanoric acid and evernic acid were purchased from Ambinter c/o Greenpharma, (Orléans, France). Lichen metabolites were dissolved in DMSO and further diluted in media (maximal DMSO concentration during experiments 0.3%).

2.2. Cell viability/metabolic activity assay

The cell viability assay was performed as previously described [12]. Briefly, to determine the cell viability the Orangu™ assay (Cell Guidance Systems Ltd, Cambridge, UK) was used. 2×10^5 HCT-116, 2×10^5 HEK293T, 0.5×10^5 HeLa, 0.5×10^5 NIH3T3, 2×10^5 RAW246.7 or 1×10^5 peripheral blood mononuclear cells (PBMCs) were seeded in 96-well plates. Different concentrations (0, 0.3, 3, 30 μg/ml) of evernic acid, lecanoric acid, atranorin, or vehicle (DMSO) were added. After 24 h of incubation at 37 °C and 5% CO_2, 10 μl of Orangu™ cell counting solution was added and incubated for 60 min (cell lines) or 4 h (PBMCs). After incubation, absorbance was measured at a wavelength of 450 nm with a reference at 650 nm at an EnSpire® 2300 Multimode Plate Reader (Perkin Elmer, Lübeck, Germany). To calculate cell viability in the Orangu™ assay, the absorbance of vehicle-treated cells was set to 100%, and the lichen metabolites treated samples were correlated to them.

To analyze the influence of lichen metabolites on the metabolic activity of HUVECs, a CellTiter-Blue cell viability assay (Promega GmbH, Mannheim, Germany) was performed. Briefly, confluent HUVECs were treated with the indicated concentrations of lichen metabolites or DMSO as vehicle control for 24 h. Four hours before the end of the treatment, CellTiter-Blue reagent containing resazurin was added to the cells in a ratio of 1:10. Resazurin is reduced into the fluorescent dye resorufin by viable cells. The metabolic activity was determined by fluorescence measurements (ex: 579 nm, em: 584 nm) using a microplate reader (Tecan Infinite F200 Pro; Tecan, Männedorf, Switzerland).

2.3. Cell colony assay

1000 HCT-116 cells were seeded per well in a 6-well plate and incubated for at least 5 h. Cells were treated with increasing concentrations atranorin (0.3, 3 μg/ml), evernic acid (0.3, 3 μg/ml), lecanoric acid (0.003, 0.03, 0.3 μg/ml) or vehicle (DMSO) for 8 days. Cells were washed with PBS, fixed with a mixture of 6% glutaraldehyde and 0.5% cresyl violet acetate for 30 min. The glutaraldehyde/cresyl violet mixture was carefully removed and cells were rinsed with tap water. Images were taken from each well and the area of the cell colonies per well was determined using the ImageJ software (https://imagej.nih.gov/ij/). For this, images of the individual wells were cut out of the image of the 6-well plate, and the areas of the colonies determined with the multi-point tool. The colony area for vehicle samples was set to 100%, and the lichen metabolite treated samples were related to the vehicle sample.

2.4. Apoptosis assay

The apoptosis assay was performed on HCT-116 cells as previously described [12]. Briefly, 2×10^5 HCT-116 cells were seeded in a black poly-D-lysin coated 96-well plate, cultured for 24 h, and subjected to the different lichen metabolites (0, 0.3, 3, 30 μg/ml) for 24 h. For apoptosis detection, cells were incubated with CellEvent Caspase 3/7 Green Detection Reagent (1:1000) (Thermo Fisher Scientific, Massachusetts, USA) for 90 min. Then, DRAQ5 (1:250) (BioLegend, California, USA) was added and incubated for an additional 30 min at room temperature. Each well was examined with the ImageXpress Micro Confocal High Content Imaging System (Molecular Devices, California, USA). The number of apoptotic cells was determined using the MetaXpress 6 Software (Molecular Devices, California, USA).

Apoptosis of HUVECs was detected according to the method of Nicoletti et al. [16]. Cells were treated as indicated and were incubated overnight in the dark in a PBS solution containing propidium iodide (PI) (50 μg/ml; MilliporeSigma, Darmstadt, Germany), sodium citrate (0.1%; Carl Roth, Karlsruhe, Germany), and Triton X-100 (0.1%; MilliporeSigma) at 4 °C. The percentage of cells with sub-diploidic DNA content was measured using a FACSVerse flow cytometer (BD Biosciences, Heidelberg, Germany).

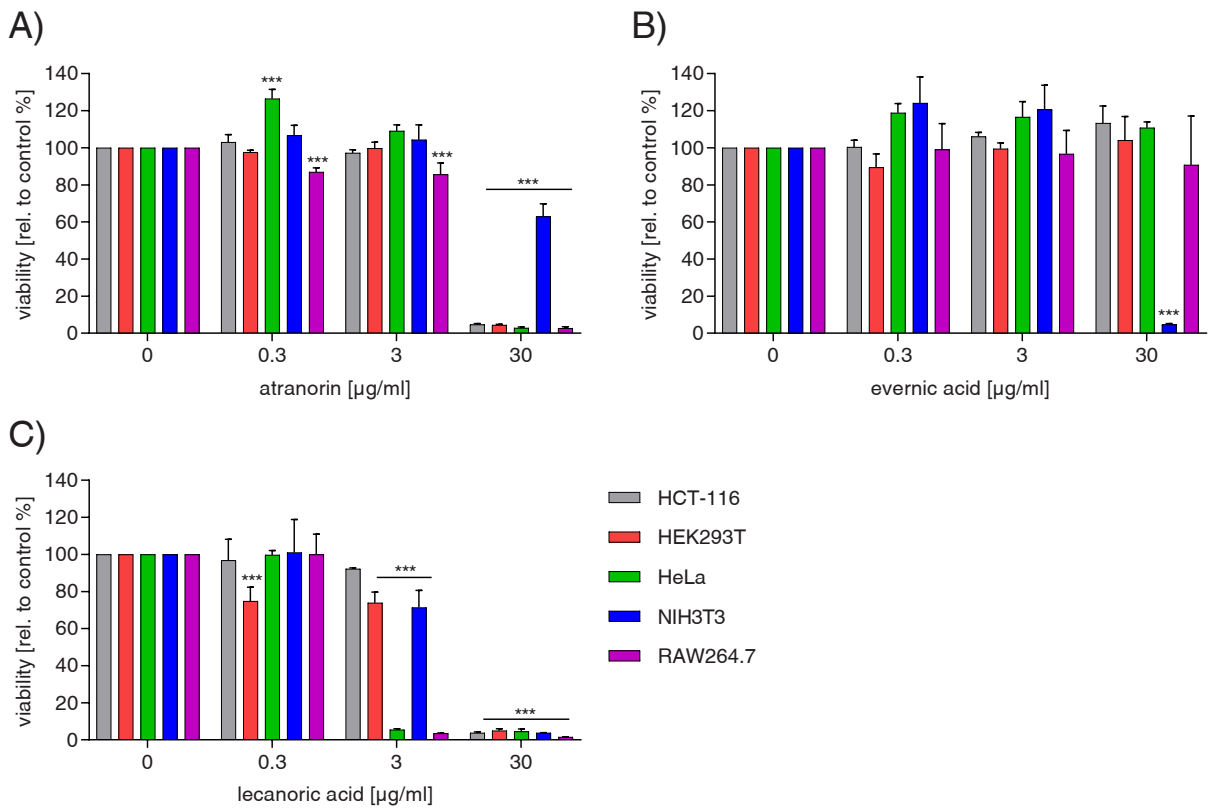

Fig. 1. Effect of depsides on cell viability. HCT-116, HEK293T, HeLa, NIH3T3 and RAW264.7 cells were treated with depsides (atranorin, evernic acid, lecanoric acid) or vehicle at the indicated concentrations for 24 h. Viability was determined with the Orangu™ proliferation assay. The absorbance of depside treated samples was related to vehicle treated samples to obtain the fold induction. The experiment was carried out in three biological and three technical replicates. The mean of three technical replicates is shown and used for statistical analysis (two-way ANOVA with Dunnett's multiple comparisons test). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistical significant differences between depside treated and vehicle treated samples.

2.5. Cell cycle analysis

Cell cycle analysis was performed as previously described[12]. Briefly, 2×10^5 HCT-116 were seeded in a 96-well cell culture plate and cultured for 24 h. Cells were treated with the different lichen metabolites (0, 0.3, 3, and 30 µg/ml) for 24 h. Afterwards, cells were harvested, suspended in 200 µl sample buffer (1 g/l glucose in PBS without Ca^{2+} and Mg^{2+}), mixed, centrifuged (200 g, 4 min, 4 °C) and the supernatant was discarded. This step was repeated once. The cells were fixed with 150 µl of ice-cold 70% ethanol overnight (> 18 h) at 4 °C. The cell pellet was washed with sample buffer, resuspended in 100 µl staining buffer (20 µg/ml PI and 0.2 mg/ml RNase in sample buffer), and incubated for 40 min at room temperature. Samples were measured within 24 h in a MACSQuant Analyzer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell cycle distribution was determined using FlowJo V10.5.3 software.

2.6. Determination of protein expression

HCT-116 cells were incubated with increasing concentrations (0, 0.3, 3, 30 µg/ml) of evernic acid, lecanoric acid, or vehicle (DMSO) for 24 h. Cells were harvested, cell pellets were homogenized and sonicated in RIPA-buffer (25 mM Tris-HCl (pH 7.6), 1% Sodium deoxycholate, 0.1% SDS, 1% IPEGAL, 150 mM NaCl, Roche cOmplete™ Mini tablets (Sigma Aldrich, Schnelldrof, Germany)). The homogenate was centrifuged, the supernatants were collected and stored at − 80 °C. Protein concentrations were assessed using the bicinchoninic acid assay. 60 µg of total protein extract were separated electrophoretically by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes (Amersham Life Science, Freiburg, Germany). Membranes were blocked in 5% non-fat dry milk in tris-buffered saline (TBS) supplemented with 0.05% Tween 20 and incubated with the respective primary antibody directed against cyclin A2 (1:1000), cyclin B1 (1:1000), cyclin E2 (1:1000), pH3 (1:1000), pCDK1 (1:1000), CDK1 (1:1000), p21 (1:1000), pWee1 (1:1000), Myt1 (1:1000), GADD45A (1:1000), pcdc25C (1:1000), cdc25C (1:1000) overnight at 4 °C and ß-actin (1:5000) for 2 h at room temperature. All antibodies were diluted in 5% BSA in 0.1% Tween 20 in TBS. Membranes were washed three times with 0.05% Tween 20 in TBS and then incubated with an anti-rabbit AF680 or an anti-mouse AF488 in 5% BSA in 0.1% Tween 20 in TBS. Membranes were washed three times with 0.05% Tween 20 in TBS and were analyzed on ChemiDoc™ MP Imaging System from Bio-Rad Laboratories (Hercules, USA). The mouse monoclonal anti-β-actin was purchased from Sigma Aldrich (Schnelldorf, Germany), the cell cycle antibodies from Cell Signaling Technology (Danvers, USA), and the secondary antibodies (anti-rabbit AF680; anti-mouse AF488) from Thermo Scientific (Schwerte, Germany).

2.7. Peripheral blood mononuclear cells isolation

PBMCs were isolated as previously described [17]. Briefly, they were isolated from buffy coats using a density gradient. For this, 25 ml of blood from healthy donors (German Red Cross, Frankfurt, Germany) were mixed with the same amount of Hank's balanced salt solution (Thermo Fisher Scientific, Oberhausen, Germany) and were layered over 15 ml of Biocoll (Merck, Darmstadt, Germany) in Sep-Mate™-50 tubes (Stemcell Technologies, Cologne, Germany). Tubes were centrifuged (1200 g, 10 min, RT), and human PBMCs were collected from the

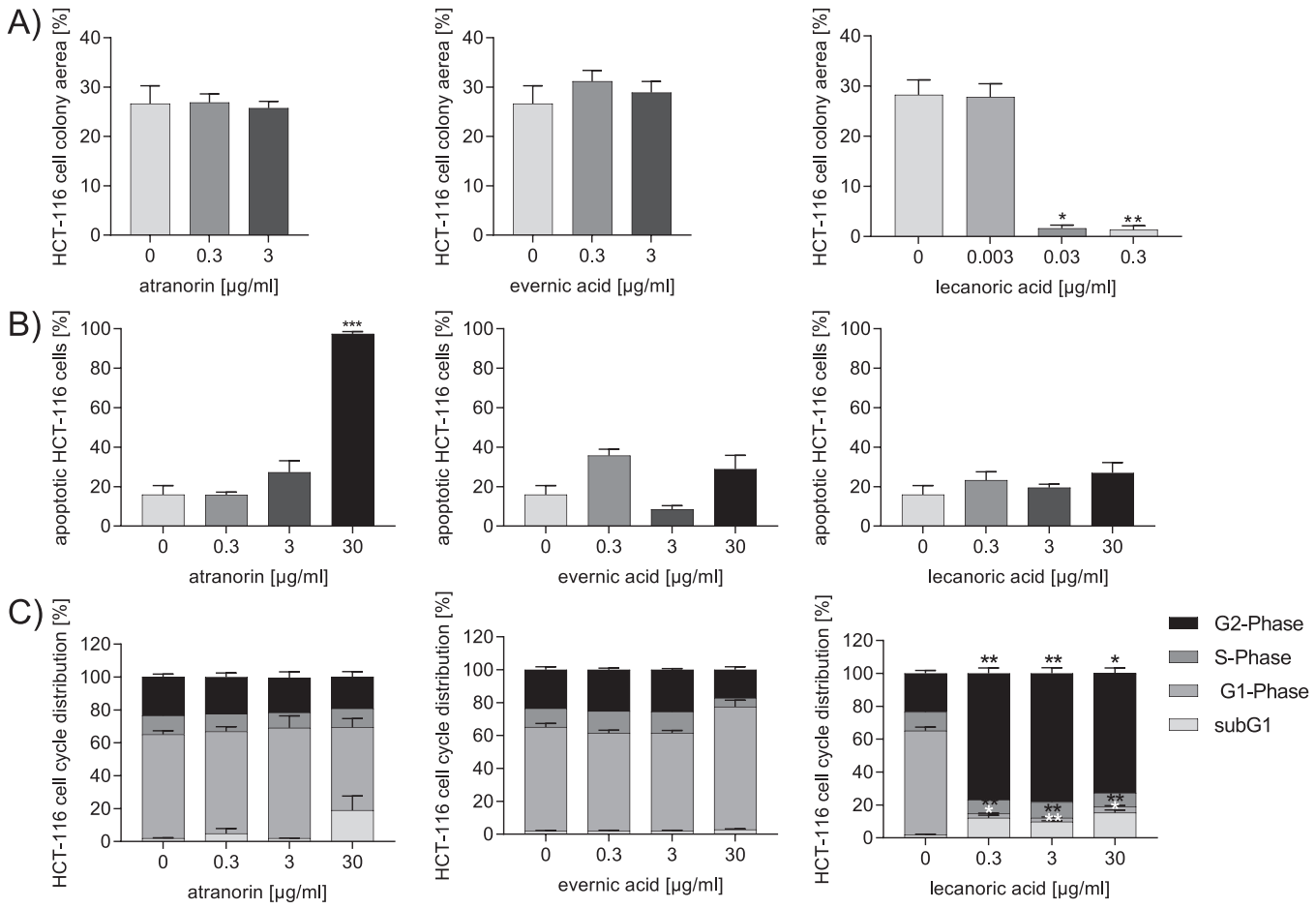

Fig. 2. Effect of depsides on cell colony formation, apoptosis and cell cycle distribution. HCT-116 cells were treated with depsides (atranorin, evernic acid, lecanoric acid) or vehicle at the indicated concentrations for 8 days (A) or 24 h (B/C). A) The cell colonies were stained with crystal violet, images were taken and the area of the colonies determined by Image J software. B) Apoptotic cells stained with caspase3/7 antibody were related to all cells stained with the nuclei marker DRAQ5. Number of apoptotic cells and total cell number was calculated with the MetaXpress 6 Software. C) DNA content of the cells was measured by flow cytometry. Cell cycle distribution was determined with FlowJo 10.5.3 software. The experiments were performed in three biological (A-C) and three technical replicates (B/C). Two-way ANOVA with Dunnett`s multiple comparisons test was used to analyze statistical differences between depside treated and vehicle treated samples. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistical significant differences between depside treated and vehicle treated samples.

interphase, washed with 2 mM EDTA/PBS four times, and counted using a MACSQuant® Analyzer 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.8. Statistics

For all calculations and for the creation of graphs, GraphPad Prism 8 (GraphPad, San Diego, USA) was used. Results are presented as mean ± standard error of the mean (SEM). The numbers of independently performed experiments (n) are stated in the corresponding figure captions, and at least three technical replicates were used for the experiments. The data were analyzed with one-way or two-way ANOVA and with Dunnett's multiple comparisons and Tukey's post hoc tests. $p < 0.05$ was considered the threshold for significance.

3. Results

3.1. Lichen metabolites impaired HCT-116 cell viability

Previous results from our group revealed that lichen extracts reduce the viability of the colon cancer epithelial cell line HCT-116 [12]. To identify the secondary metabolites that are responsible for this effect, the depsides atranorin, evernic acid, and lecanoric acid were investigated. HCT-116 cells treated with increasing concentrations of atranorin, evernic acid, and lecanoric acid for 24 h were analyzed with a formazan-based viability and proliferation assay. Thirty µg/ml atranorin and 30 µg/ml lecanoric acid significantly reduced the viability of HCT-116 cells, whereas evernic acid did not affect HCT-116 cell viability (Fig. 1). To investigate whether the observed cytotoxicity is cell type-specific, the viability assay was also performed on a human embryonal kidney (HEK293T), a human cervical epithelial cancer (HeLa), a murine fibroblast (NIH3T3), and a murine leukemic macrophage (RAW264.7) cell line. Thirty µg/ml evernic acid reduced viability only in NIH3T3 cells, whereas 30 µg/ml atranorin reduced viability in all cell lines, the NIH3T3 cell line being the least responsive (Fig. 1). All the tested cell lines were responsive to low concentrations of lecanoric acid (0.3 µg/ml for HEK293 and 3 µg/ml for HeLa, NIH3T3, and RAW264.7) (Fig. 1). These data identify lecanoric acid as the most effective secondary metabolite and indicate that the effects of depsides on viability are cell-type dependent.

3.2. Lecanoric acid prevented colony formation

To confirm the effects in the viability assay, we performed a cell colony assay. The areas of HCT-116 cell colonies treated for 8 days with the secondary lichen metabolites were evaluated. Evernic acid and atranorin did not affect cell colony formation. However, lecanoric acid significantly reduced the formation of cell colonies already at 0.03 µg/

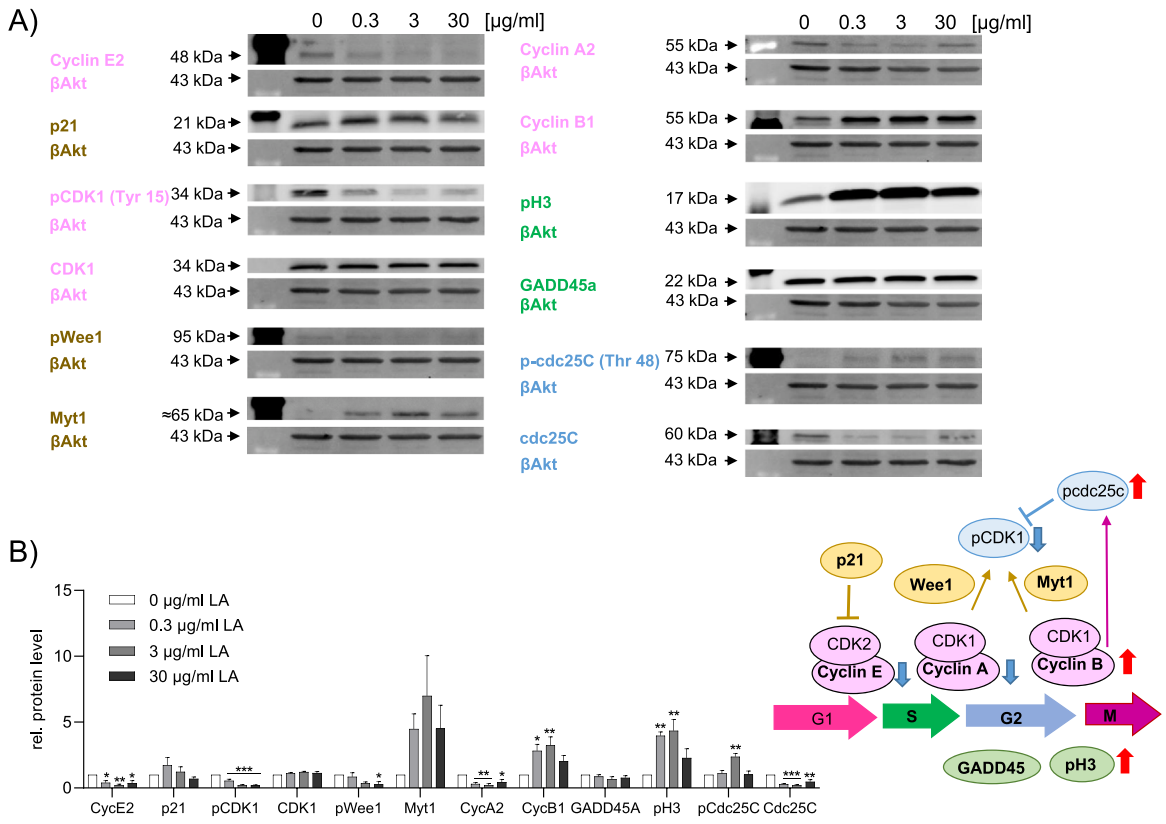

Fig. 3. Effect of lecanoric acid on cell cycle proteins. HCT-116 cells were incubated with lecanoric acid or vehicle at the indicated concentrations for 24 h. A) The protein expression was determined using western blot technology. B) The optical densitometric analysis was carried out with Image Lab software (Bio-Rad Laboratories, Hercules, USA). The protein expression was normalized to β-actin. The protein expression of depside treated samples was related to vehicle treated samples to obtain the fold induction. The experiment was performed in three to five biological replicates. One-way ANOVA with Dunnett`s multiple comparisons test was used to analyze statistical differences between depside treated and vehicle treated samples. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistical significant differences between lecanoric acid and vehicle treated samples.

ml (Fig. 2A). These data confirm that lecanoric acid is the most effective of the tested secondary metabolites.

3.3. Atranorin induced apoptosis

To elucidate the mechanism underlying the reduced HCT-116 cell viability mediated by the lichen metabolites, an apoptosis assay was performed. Caspase 3/7 activation – a hallmark of apoptosis induction – was determined by immunocytochemistry in HCT-116 cells treated with increasing concentrations of the depsides for 24 h. Evernic acid and lecanoric acid did not induce apoptosis, whereas atranorin at 30 µg/ml significantly induced apoptosis in HCT-116 cells (Fig. 2B).

3.4. Lecanoric acid increased the cell number in the G2 phase

Besides apoptosis, reduced cell viability can be mediated by cell cycle arrest. Therefore, we investigated whether the lichen metabolites influence cell distribution during different cell cycle phases. The cell cycle distribution of HCT-116 cells treated with increasing concentrations of the depsides for 24 h was determined by flow cytometry. For atranorin and evernic acid, no effects were observed on the cell cycle distribution in HCT-116 cells. Most interestingly, lecanoric acid at the low concentration of 0.3 µg/ml significantly increased the cell number in the G2 phase, accompanied by a reduction of cells in the G1 and S phase (Fig. 2C). Cells in the subG1 phase, which represent cells undergoing late-stage apoptosis, also increased slightly. Since the influence of the secondary lichen metabolites on viability was cell type-dependent, we also investigated the effects of the depsides on the cell cycle distribution in other cell lines (HeLa, NIH3T3, RAW264.7). Already at 3 µg/ml, atranorin increased the number of HeLa cells in the subG1 phase confirming that atranorin induces apoptosis. Evernic acid had little impact on the cell cycle distribution in HeLa, NIH3T3, and RAW246.7 cells. Lecanoric acid at 0.3 µg/ml increased the cell number in the G2 and subG1 phases in HeLa cells and at 3 µg/ml in NIH3T3 cells. In RAW246.7 cells, lecanoric acid increased cell number in the subG1 phase accompanied by a decrease of cells in the G1 and S phases (Supplemental Fig. 1). These data indicate that atranorin does not have a significant effect on the cell cycle distribution. Evernic acid may have little effect, and lecanoric acid arrests HCT-116, NIH3T3, and HeLa cells in the G2 phase.

3.5. Lecanoric acid regulated expression levels of cell cycle proteins

Next, we evaluated whether lecanoric acid and evernic acid regulate cell cycle proteins in HCT-116 cells. We did not investigate the effect of atranorin on cell cycle proteins because no effect on the cell cycle distribution was observed with this compound. Several cell cycle proteins were investigated by western blotting, including cyclin E, which is required to overcome the G1/S checkpoint to commence DNA replication, and p21, a cyclin-dependent kinase inhibitor that interacts with the cyclin E-CDK2-complex and thereby, stops the transition from G1 to S phase [18,19]. Interestingly, lecanoric acid significantly reduced the expression of cyclin E but not of p21, whereas evernic acid had no effect (Fig. 3, Supplemental Fig. 2).

For the transition of the S to the G2 phase, CDK1 and cyclin A are required [20]. The kinases Myt1 and Wee1 can inactivate CDK1 by phosphorylation at positions Tyr14 and Tyr15 [21,22]. The phosphatase pcdc25C (active form) dephosphorylates pCDK1 at Tyr15 and thereby

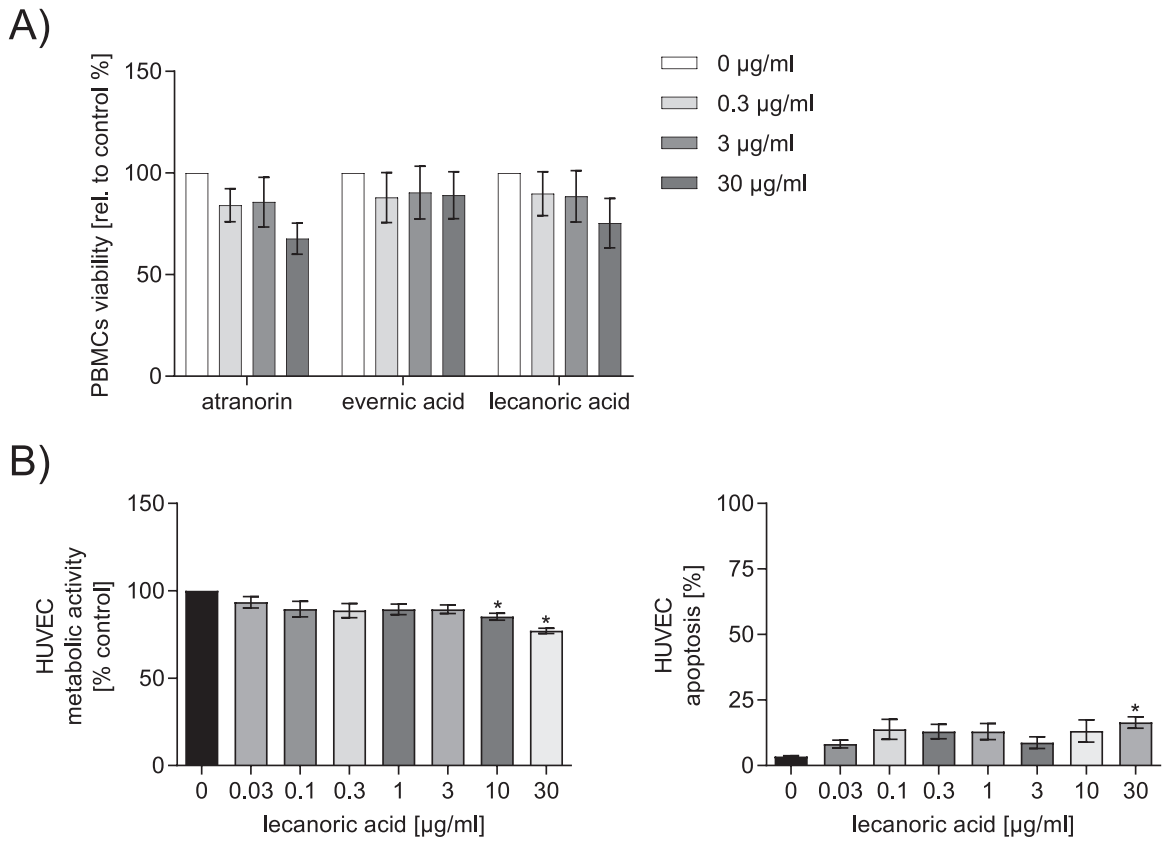

Fig. 4. Effect of depsides on PBMC and endothelial cell viability. (A) PBMCs were treated with depsides (atranorin, evernic acid, lecanoric acid) or vehicle at the indicated concentrations for 24 h. Their viability was determined with the Orangu™ proliferation assay. The absorbance of depside treated samples was related to vehicle treated samples to obtain the percent induction. The experiment was carried out with PBMCs from four different donors. Two-way ANOVA with Dunnett's multiple comparisons test was used to analyze statistically significant differences between depside treated and vehicle treated samples. (B) In viability assays of endothelial cells, HUVECs were grown to confluence and treated with lecanoric acid or vehicle (DMSO) as control for 24 h. Metabolic activity was quantified with the CellTiter Blue assay (left), and the number of sub-diploidic cells was quantified to assess apoptosis (right). The experiment was performed in three biological replicates. One-way ANOVA with Dunnett`s multiple comparisons test was used to analyze statistical differences between depside treated and vehicle treated samples. *$p \leq 0.05$ versus control.

activates CDK1 [23]. Lecanoric acid reduced the expression of cyclin A, pCDK1 and pWee1 (inactive form), whereas Myt1 expression was not significantly influenced. Moreover, lecanoric acid significantly reduced the level of cdc25C and increased pcdc25C expression (Fig. 3). Evernic acid only induced pWee1 protein (Supplemental Fig. 2).

Traversing the G2/M checkpoint to initiate mitosis requires CDK1 as well as cyclin B and cyclin A [24]. Phosphorylation of histone H3 (pH3) is correlated with chromosome condensation during mitosis [25]. Moreover, we detected GADD45A as a marker for apoptosis [26]. Lecanoric acid induced the expression of cyclin B1 and pH3, but did not affect GADD45A (Fig. 3). With evernic acid, we observed no effect on cell cycle proteins involved in the G2/M transition (Supplemental Fig. 2). These data reveal that lecanoric acid arrests cells in the M phase rather than in the G2 phase.

3.6. Lecanoric acid did not influence the viability of primary peripheral immune and endothelial cells

A common side effect of anti-cancer drugs is to influence the viability of healthy cells, including immune cells. This is an unfortunate side effect, since functional cytotoxic innate and adaptive immune cells can aid in controlling cancer development [27]. Therefore, we investigated whether lichen secondary metabolites also exert cytotoxic effects on peripheral blood mononuclear cells (PBMCs).

Treatment of PBMCs with lecanoric acid, evernic acid, and atranorin for 24 h did not significantly reduce cell viability (Fig. 4A). In addition, we tested the effects of these compounds on the viability of primary human umbilical vein endothelial cells (HUVECs). After 24 h treatment with evernic acid and atranorin, the viability of HUVECs was not reduced (Supplementary Figure 3). However, lecanoric acid at the highest concentration (30 µg/ml), caused a 20% reduction in viability through effects on metabolic activity and an increase in apoptosis (Fig. 4B).

4. Discussion

The lichen depsides, evernic acid, lecanoric acid and atranorin were characterized for their pharmacological potential in the treatment of cancer (Fig. 5). Atranorin reduced cell viability in various cancer cell lines possibly by induction of apoptosis. Evernic acid only reduced NIH3T3 cell viability. Lecanoric acid is the most promising of the investigated depsides because it decreased viability in all investigated cell lines, sometimes at concentrations of 3 µg/ml and higher, and it reduced cell colony formation in HCT-116 cells already at 0.03 µg/ml. The underlying mechanism could be an arrest of cells in the M phase since lecanoric acid downregulated inactive CDK1 and upregulated cyclin B1, indicating progression to the M phase [24]. The observation that pH3, which is important for chromatin condensation [25], is also upregulated by lecanoric acid, reinforces the hypothesis of an M phase arrest. Lecanoric acid does not seem to generate genotoxic stress, as GADD45A protein levels [26] were not increased in HCT-116 cells. Most importantly, lecanoric acid did not reduce cell viability in primary

	Cell type	*Atranorin*	*Evernic acid*	*Lecanoric acid*
Cell viability reduction < 25%	HCT-116	30 µg/ml		30 µg/ml
	HEK293T	30 µg/ml		30 µg/ml
	HeLa	30 µg/ml		3 µg/ml
	NIH3T3	30 µg/ml	30 µg/ml	30 µg/ml
	RAW264.7	30 µg/ml		3 µg/ml
	PBMCs			
	HUVEC			
Cell colony assay	HCT-116			0.03 µg/ml
Apoptosis < 25 %	HCT-116	30 µg/ml		
	HUVEC			
Cell cycle assay	HCT-116			G2-Block 0.3 µg/ml
	HeLa	Sub G1 30 µg/ml		G2 block 0.3 µg/ml
	NIH3T3			G2 block 3 µg/ml
	RAW264.7			Sub G1 3 µg/ml

Fig. 5. Summary of the effects of the depsides in the various anti-proliferative test systems.

immune cells and had only minor cytotoxic effects on primary endothelial cells.

In a previous study in HCT-116 cells, we observed anti-proliferative effects of lichen extracts from *Flavoparmelia caperata*, but not for lichen extracts containing lecanoric acid, atranorin, or evernic acid such as *Umbilicaria pustulata*, *Platismatia glauca*, and *Evernia prunastri* [12]. In extracts of *Umbilicaria* species, lecanoric acid is presumably a degradation product of gyrophoric acid [28]. Gyrophoric acid showed no anti-proliferative effects in HCT-116 cells (data not shown). At 30 µg/ml (94 µM) lecanoric acid and 30 µg/ml (80 µM) atranorin, we observed a reduction of HCT-116 cell viability to about 5%. Paluszczak et al. found minor effects of lecanoric acid in HCT-116 cells, and 100 µM atranorin reduced HCT-116 cell viability to about 40% [29]. Lecanoric acid showed an IC_{50} value above 50 µg/ml in other cell lines (MCF-7, Vero, Hep-2) [30]. This raises the questions as to why effects produced by lichen extracts differ from the effects of their isolated metabolites and why the effects of lecanoric acid observed by Paluszczak et al. [29] and Bogo et al. [30] differ from our results. Possible reasons for the differences between extracts and isolated metabolites could be that the effects of the different secondary metabolites cancel each other out. The differences between our findings with lecanoric acid and the results of others may be due to varied isolation procedures for the metabolite. The isolation processes differed in terms of the lichens studied (e.g., Paluszczak et al. [29] extracted lecanoric acid from *H. salaris*, Bogo et al. and Ambinter - our provider of lecanoric acid - from *Parmotrema tinctorum*), the purification method (recristallization [30], thin layer chromatography [29]) and solvent (acetone [29], ether [30]). For the latter we could already show that the solvent affects the biochemical property of the lichen extracts [12]. Thus to sum up, the differences in the detected pharmacological potential of lecanoric acids is possibly due to the divergent purities of the metabolites, resulting from different isolation procedures.

Our cell cycle data revealed a G2 cell cycle arrest with lecanoric acid. However, cell cycle analysis relying solely on the quantification of DNA content does not allow discrimination between cells in the G2 and M phase, therefore additional markers are required to facilitate this distinction [31]. Assessment of cell cycle protein expression in HCT-116 cells indicates that lecanoric acid induced an M block rather than a G2 block. The downregulation of inactive pCDK1 and upregulation of pH3 suggests progression to the M phase and condensation of chromatin. The reduced expression level of pCDK1 correlates with the increase in the phosphatase pcdc25C which dephosphorylates pCDK1 [23]. In addition, cyclin B1 levels were upregulated, suggesting an intact CDK1/cyclinB1 complex that was not tagged for degradation by the anaphase-promoting complex [32]. The reduced levels of cyclin E and cyclin A may be a consequence of the arrest in the M phase. Published data confirm that lecanoric acid arrests cells in the M phase rather than in the G2 phase, since molecules that solely induce a G2 cell cycle arrest only show partially similar effects on cell cycle protein expression to those of lecanoric acid. Curcumin, a known inducer of G2 cell cycle arrest, reduces cyclin B1 and inhibits the cdc25C phosphatase which activates CDK1 by dephosphorylation at Tyr14 and Tyr15, leading to deactivation of CDK1 [33]. Urolithin induced a G2 cell cycle arrest by increasing cyclin B1 and increasing deactivated CDK1 (Tyr 15-phosphorylated) [34]. Myricetin increased the p53/p21 cascade and increased inactive CDK1 (Tyr 15-phosphorylated), accompanied by decreased active Cdc2 and cyclin B1 protein levels in HepG2 cells, leading to G2 cell cycle arrest [35]. In summary, G2 arrest is characterized by a non-functioning cyclin B/CDK1 complex either as a result of a decreased level of cyclin B or by inactivation of CDK1.

Supporting the findings at the protein level, lecanoric acid incubation led to morphological changes in HCT-116 cells (Supplemental Fig. 3). While unstimulated cells appeared flat, cells treated with lecanoric acid acquired a round shape, which is characteristic of

adherent cells undergoing mitosis. Also, the observed detachment from the cell culture plate has been described in a model for active cell rounding during mitosis [36].

A polyketide synthase gene associated with lecanoric acid biosynthesis has recently been identified in the lichenized fungus *Pseudevernia furfuracea* through heterologous expression in *Saccharomyces cerevisiae* [37]. The gene consists of 2115 amino acids and has the following domain architecture: SAT-KS-MAT-PT-ACP-ACP-TE (starter unit acyl transferase-SAT, ketosynthase-KS, malonyl-CoA:acyl-carrier protein transacylase-MAT, product template domain-PT, acyl carrier protein-ACP, thioesterase-TE). The authors artificially synthesized the PKS gene using an optimized *S. cerevisiae* codon. They report a peak titer of 364 mg/l lecanoric acid after 7 days of incubation of the yeast expression system. This study shows great promise for the biotechnological production of lecanoric acid, and possibly other lichen secondary metabolites, on larger scales.

5. Conclusion

In conclusion, we investigated the potential of three typical and phylogenetically widely distributed lichen depsides (evernic acid, lecanoric acid, atranorin) for use as new chemotherapeutic drug candidates. We identified lecanoric acid as a promising candidate due to its mode of action as a cell cycle inhibitor and because of its low cytotoxic effects on primary immune and endothelial cells. In general, the idea to exploit cell cycle inhibitors as anti-cancer drugs is widely employed with the so-called CDK inhibitors. The first cell cycle kinase inhibitor, palbociclib which inhibits CDK4/6, is already approved for the treatment of breast cancer [38]. Taken together, lecanoric acid represents an interesting compound that exerts valuable pharmacological actions *in vitro* in the context of cancer formation and development processes. As an additional plus, lecanoric acid can be biotechnologically synthesized [37]. Our findings warrant further investigation of this promising natural product in a preclinical setting.

Ethics approval and consent to participate

Buffy Coats were obtained from the “Deutsche Blutspendedienst”.

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CRediT authorship contribution statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Luise A. Roser, Pelin Erkok, Rebecca Ingelfinger, Thomas Ulshöfer, Marina Henke and Ann-Kathrin Schneider. The first draft of the manuscript was written by Luise A. Roser, Robert Fürst, Gerd Geisslinger, Volker Laux, Imke Schmitt, Susanne Schiffmann and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare no competing interests.

Data availability

Data available upon request.

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Consent for publication

All authors have consented for a publication.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112734.

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