Differential effects of Helenalin, an anti-inflammatory sesquiterpene lactone, on the proteome, metabolome and the oxidative stress response in several immune cell types

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ABSTRACT

Extracts of *Arnica* spp. are traditionally used due to their anti-inflammatory effects for the topical treatment of e.g. haematoma or muscle distortions. One of the main active compounds is Helenalin, a sesquiterpene lactone that can be found in various Asteraceae. However, immunotoxic effects of the compound are only poorly analysed.
In this study, a 2D gel electrophoresis based proteomic approach together with a membrane based proteomic assay, metabolomics and the detection of intracellular reactive oxygen species (iROS) were used to investigate potential immunotoxic properties of Helenalin on the human immune cell lines Jurkat and THP-1 and on human peripheral blood mononuclear cells (PBMC). The study revealed a dose-dependent cytotoxicity towards both tested cell lines and the PBMC. However, the cell lines were less sensitive to the Helenalin treatment than the PBMC. The proteomic assays showed strong effects on the carbohydrate metabolism and the protein folding in THP-1 cells but only weak impact on Jurkat cells. Metabolomic studies as well as iROS detection in THP-1 cells verified the results of the proteomic analysis.
In summary, the approaches used in this study were able to identify target pathways of Helenalin especially in THP-1 monocytes and thus enable a risk assessment of the substance.

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1. Introduction

Arnica spp., belonging to the family of Asteraceae, are traditionally used due to their anti-inflammatory effects for the topical treatment of haematoma, muscle distortions or inflammation because of insect bites. Further fields of applications are e.g. local muscular pain or inflammations of the mucosa. Arnica preparations are mainly sold as creams, gels, oils and alcoholic extracts. However, treatment with Arnica extracts can cause allergic contact dermatitis. The main active compounds responsible for the biological effects of *Arnica* are sesquiterpene lactones, besides flavonoids and triterpenes.

One sesquiterpene lactone thereby is Helenalin (HEL). Next to its sesquiterpene lactone structure, HEL has an α,β-unsaturated carbonyl system and an α-methylene-γ-butyrolactone ring structure (Fig. 1) (Dirsch et al., 2001). These structural features allow HEL to react via the Michael addition with proteins, especially their thiol groups, resulting in their alkylation. This reaction with proteins e. g. with transcription factors affects the proper function of the cell and, thereby, influences the cell functionality. Especially the transcription factor NFκB is a target of sesquiterpene lactones. With the inhibition of NFκB the transcription of inflammatory cytokines is inhibited resulting in decreased recruitment of T- and B-cells (Dirsch et al., 2001; Chadwick et al., 2013; Kupchan et al., 1971; Lyß et al., 1998; Lawrence, 2009). Further effects on cell functionality were investigated in some in vitro studies detecting apoptosis induction, the formation of intracellular reactive oxygen species (iROS) and impacts on the cell proliferation (Berges et al., 2009; Lim et al., 2012; Jang et al., 2013). Furthermore, Helenalin seems to inhibit the activity of the telomerase and is therefore a potential anti-cancer agent (Huang et al., 2005).

Until now, there exist no comprehensive studies of proteomic changes after treatment of immune cells with HEL. Proteomics in toxicology research (Toxicoproteomics) enables the identification of
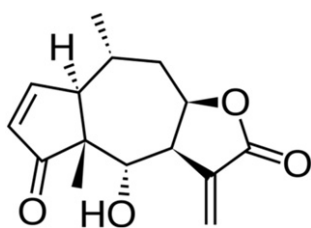
Fig. 1. Structural formula of the sesquiterpene lactone Helenalin.

toxicological mechanisms and allows therefore a fast risk assessment of substances without the use of animal experiments (Ge et al., 2009; Bandara and Kennedy, 2002; Wetmore and Merrick, 2004; Benninghoff, 2006). The use of animals for risk assessment raises ethical concerns, is time and cost consuming and has only a low predictability for humans. The 3Rs, dealing with the *Refinement*, *Reduction* and *Replacement* of animal experiments are therefore a possibility to omit these problems (Tannenbaum and Bennett, 2015; Jennings, 2015). Until now, several in vitro methods, especially for skin sensitization analysis (OECD, 2014), are implemented in international test guidelines as the OECD guidelines for the testing of chemicals. But a full replacement of animal experiments is up to now not feasible.

In this study a monocyte cell line (THP-1) and a T cell line (Jurkat) were used for proteomic analysis using a 2D gel electrophoresis based approach after exposure to HEL with a subtoxic concentration (IC$_{10}$). The anti-inflammatory properties as well as the possible evocation of contact dermatitis by HEL led to the assumption that T cells can be influenced by HEL. Furthermore, THP-1 cells were used. For testing skin sensitizing capacities this cell line is often applied (Lambrechts et al., 2009; Ade et al., 2009; Roggen, 2014; Corti et al., 2015). In addition to proteomic analyses, the induction of intracellular reactive oxygen species (iROS) in THP-1 monocytes and Jurkat T cells due to HEL treatment with IC$_{10}$ and changes in the lactate secretion of THP-1 cells were examined. Next to the investigations using cell lines, the cytotoxicity against human peripheral blood mononuclear cells (PBMC) and their cytokine production after HEL treatment were analysed.

These comprehensive in vitro investigations shall allow a risk assessment of HEL after low-dose exposure to human immune cells and enables the identification of target proteins or mechanisms of action of the substance. In addition, knowledge about the influence on the functionality of human immune cells is gained.

2. Materials and methods

2.1. Reagents and materials

Cell culture medium RPMI 1640 (+glutamine) as well as cell culture water, PBS and fetal calf serum (FCS) were from Sigma Aldrich (Taufkirchen, Germany). Furthermore, Tulipalin A, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), boric acid, citric acid, CM-H$_2$DCFDA, Dithiotreitol (DTT), DMSO, EDTA, Etoposide, Histopaque®-1077, Jodacetamide, Lipopolysaccharides from *Pseudomonas aeruginosa* 10 (LPS), Ninhydrin, Thiourea, Bromophenol blue, Penicillin/Streptomycin, Sodium hydroxide and Trypan blue were also supplied from Sigma Aldrich.

For 2D gel electrophoresis non-linear IPG-strips (18 cm, pH 3–10), 2D HPE™ large gel (12.5%) Kits and Serva purple stain were purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Urea was obtained from Merck (Darmstadt, Germany). PMSF, Tris and CHAPS were purchased from Carl Roth (Karlsruhe, Germany). Pharmalyte (3–19) was acquired from GE Healthcare (Little Chalfont, UK). Trypsin was from Promega (Madison, WI, USA).

2.2. Cell culture

Human leukemic Jurkat T cells and human leukemic monocytes (THP-1) (purchased from DSMZ, Braunschweig, Germany) were grown with a density of 0.3×10^6 cells/ml in a humidified atmosphere (37 °C, 5% CO$_2$) in RPMI 1640 supplemented with 1% v/v antibiotics (Penicillin/Streptomycin) and 8% v/v or 10% v/v heat inactivated fetal calf serum, respectively. Twice a week, cells were subcultured and the morphology was checked.

As tested by PCR, the cell lines were free of mycoplasmas. When subcultured, cell viability and cell number were assessed using Luna™ automated cell counter (Logos Biosystems) via trypan blue exclusion.

2.3. Isolation of human PBMC

PBMC were isolated from human buffy coat. One part buffy coat was diluted with three parts PBS (without Ca^{2+}, Mg^{2+}). Afterwards, 15 ml Histopaque®-1077 were overlaid with 25 ml of the diluted cell suspension and centrifuged without brake for 15 min at room temperature ($1700 \times g$). Subsequently, the PBMC layer was transferred to a new 50 ml-centrifugal tube, washed with 10 ml PBS and centrifuged for 6 min at $630 \times g$. The obtained cell pellet was then washed three times with PBS (7.5 ml) and centrifuged at $140 \times g$ for 6 min. After isolation, the PBMC were cultured in RPMI1640 supplemented with 1% v/v antibiotics (Penicillin/Streptomycin) and 10% v/v heat inactivated fetal calf serum with a cell density of 0.3×10^6 cells/ml in a humidified atmosphere (37 °C, 5% CO$_2$) for the test duration. For the analysis of the cytokine release and its changes due to compound treatment, PBMC were stimulated with 100 ng ml^{-1} LPS for 24 h before compound-treatment.

2.4. MTT assay

Determination of cell viability decrease due to HEL treatment was performed using the MTT assay.

The cells were seeded in flat bottom 96-well plates with a density of 4×10^5 cells/ml. Afterwards the cells were exposed to HEL in decreasing concentrations starting at 6.00 μM up to 0.09 μM. After incubation for 72 h under humidified conditions (5% CO$_2$, 37 °C), MTT (final concentration 0.5 mg/ml) was added and the cells further incubated for 3 h for the reduction of MTT to the formazan. For cell lysis and formazan solubilization, 200 μl DMSO per well were added. After shaking of the microtiterplate for 15 min, absorbance was measured at 550 nm and 620 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Germany). Each plate also contained untreated, solvent-treated (negative control) and Etoposide-treated (2.5 μM, positive control) cells.

2.5. Treatment with Helenalin (HEL)

The stock solution of HEL was made using ethanol (EtOH) as solvent. Working solutions freshly prepared before use were made with RPMI 1640. The cells were seeded in T150 cell culture flasks (0.3×10^6 cells/ml) and the substance was added in a concentration causing a cell growth inhibition of 10% (IC$_{10}$) or 50% (IC$_{50}$), respectively, compared to growth of untreated cells as determined by MTT assay. THP-1 cells were treated with 1.08 μM (IC$_{50}$) or 0.52 μM (IC$_{10}$) HEL, respectively. Jurkat cells were treated with 1.16 μM (IC$_{50}$) or 0.51 μM (IC$_{10}$) and PBMC were treated with 0.16 μM (IC$_{10}$). The treatment was performed in three independent experiments.

For proteomic as well as metabolomic and functional analysis, treated cells were cultured for 72 h. Intracellular reactive Oxygen species were determined after 24 h of HEL treatment in THP-1 and Jurkat T cells. For measurement of cytokine release, PBMC were stimulated with 100 ng/ml LPS before treatment with HEL for 24 h with the IC$_{10}$.