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Authors  Silvie Rimpelová, Michal Jurášek, Lucie Peterková, Jiří Bejček, Vojtěch Spiwok, Miloš Majdl, Michal Jirásko, Miloš Buděšínský, Juraj Harmatha, Eva Kmoníčková, Pavel Drašar and Tomáš Ruml

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ORCID® iDs  Silvie Rimpelová - https://orcid.org/0000-0002-3008-1396; Michal Jurášek - https://orcid.org/0000-0002-5069-9716; Michal Jirásko - https://orcid.org/0000-0002-4874-7723; Juraj Harmatha - https://orcid.org/0000-0003-4385-0841; Pavel Drašar - https://orcid.org/0000-0003-0093-7007
Archangelolide: Sesquiterpene lactone with immunobiological potential

from Laserpitium archangelica

Silvie Rimpelová¹*, Michal Jurášek², Lucie Peterková¹, Jiří Bejček¹, Vojtěch Spiwok¹, Miloš Majdl², Michal Jirásko³, Miloš Buděšínský⁴, Juraj Harmatha⁴, Eva Kmoníčková³⁵, Pavel Drašar²*, Tomáš Ruml¹*

¹Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague, Technická 5, 166 28, Prague 6, Czech Republic

²Department of Chemistry of Natural Compounds, University of Chemistry and Technology Prague, Technická 5, 166 28, Prague 6, Czech Republic

³Charles University in Prague, Faculty of Medicine in Pilsen, 301 66 Pilsen, Czech Republic

⁴Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo náměstí 2, 166 10 Prague 6, Czech Republic

⁵Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, v.v.i., 14220 Prague 4, Czech Republic

*Corresponding authors: silvie.rimpelova@vscht.cz

pavel.drasar@vscht.cz

tomas.ruml@vscht.cz
ABSTRACT

Sesquiterpene lactones are secondary plant metabolites with sundry biological effects. In plants, they are synthesized mainly for their pesticidal, antimicrobial and other effects. Two such compounds, archangelolide and trilobolide of the guaianolide type, are structurally similar to the well-known and clinically tested lactone thapsigargin. Here we present the development of a facile method for isolation of these two sesquiterpene lactones from the seeds of *Laserpitium archangelica* Wulfen using supercritical CO₂ extraction. Furthermore, not much has been reported on biological activity of archangelolide. We prepared its fluorescent derivative based on a dansyl moiety using azide-alkyne Huisgen cycloaddition. We showed that dansyl-archangelolide localized in endoplasmic reticulum of living cells similarly to trilobolide; nevertheless, localization in mitochondria was also detected. This led us to study the anticancer potential of archangelolide. Interestingly, we found that neither archangelolide nor its dansyl conjugate did exhibit cytotoxic effect in contrast to its structurally closely related counterparts trilobolide and thapsigargin. We explain this observation by a molecular dynamics simulation, in which, in contrast to trilobolide, archangelolide did not bind into the sarco/endoplasmic reticular calcium ATPase cavity utilized by thapsigargin. Last, but not least, archangelolide exhibited anti-inflammatory activity, which makes it promising compound for medicinal purposes.

KEYWORDS

Archangelolide; Anti-inflammatory properties; Dansyl fluorescent conjugate; Sarco/endoplasmic reticulum calcium ATPase, Sesquiterpene lactone; Trilobolide analogue
**ABBREVIATIONS**

4-DMAP, 4-(N,N-dimethylamino)pyridine; AcOEt, ethyl acetate; DCC, dicyclohexylcarbodiimide; Dns, dansyl; DMAP, 4-dimethylaminopyridine; DTB, 8-O-(dodecanoyl-8-O-debutanoyltribololid); Et$_3$N, triethylamine; IL-6, interleukin 6; IL-1β, interleukin 1β; INF-γ, interferon gamma; PME, particle mesh Ewald technique; TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; TEA, triethylamine; THF, tetrahydrofuran; TLC, thin layer chromatography; TNF-α, tumor necrosis factor alpha; SCE, supercritical CO$_2$ extraction; SL, sesquiterpene lactones.

This article uses semitrivial terpene nomenclature and hence the numbering of compounds atoms may differ from these obtained by using IUPAC names.
1. INTRODUCTION

Sesquiterpene lactones (SLs) have been already for some time in the spotlight due to the plethora of biological effects they elicit. Various SLs show anticancer, antimicrobial, antioxidant, antiprotozoal, antiviral and immuno-biological activities (reviewed in [1, 2]). Two SLs remarkable for their immuno-biological potential are trilobolide and archangelolide, depicted in Fig. 1. Trilobolide exhibits strong induction of nitric oxide (NO) in eukaryotic cells, which in turn evokes synthesis of IL-6, INF-γ and TNF-α. Furthermore, trilobolide has very similar structure to the well-described SL thapsigargin and was found cytotoxic [3] due to acting by the same mechanism in cells [4]. It was, however, found that trilobolide may be modified in such a way that its cytotoxicity is reduced while the immuno-biological properties are retained [5]. Contrary to that, archangelolide inhibits NO production and synthesis of IL-1β and INF-γ [6]. Although the structure of archangelolide was already elucidated in the 1970s [7], the information on its cytotoxicity is very limited [6] and its intracellular localization or mechanism of action are unknown. Therefore, based on the structural similarity between archangelolide and trilobolide, we suspected that new interesting and relevant information on this biologically largely undescribed SL could be identified.

In order to study the action of these commercially unavailable compounds, efficient isolation must be performed in order to gain sufficient amount of pure SLs. Even though the presence of several immune-active SLs, including trilobolide and archangelolide, was previously reported in *Laser trilobum* (L.) Borkh and the method of their isolation from petroleum ether extracts of plant roots [6] was described, we aimed to develop a facile method for isolation of these SLs from the seeds of *Laserpitium archangelica* Wulfen using supercritical CO₂ extraction. (SCE). In our experience, any part of the plant may be used for archangelolide extraction, however, the roots and seeds give much higher yields than other
parts. Utilization of SCE gives improved efficiency of the extraction and shortens the work-up in contrast with classical methods.

By the above-mentioned methodology, we obtained sufficient amounts of these compounds for further work including synthetic modifications using azide-alkyne Huisgen cycloaddition. We previously showed the preparation of fluorescent trilobolide conjugates [3] which retained the activity of parental compounds and proved to be useful for live-cell imaging. In this article, we present a similar approach for archangelolide. Using cancerous, non-transformed, and, also primary cell lines, we evaluated the viability of cells treated with archangelolide and its fluorescent dansyl conjugate which we then used to study its intracellular localization. Finally, we also determined the anti-inflammatory activity of archangelolide using rat macrophages.

Figure 1. The structure of sesquiterpene lactones archangelolide and trilobolide

2. MATERIALS AND METHODS

2.1 Source of the natural material

Fresh seeds of Laserpitium archangelica Wulfen were provided by the Farmacognostic garden in Poznan (Mazowiecka Street) of the Medical University in Poznan (Poland). The plant was identified by a research specialists of the Department of Medicinal Plants of the same
University, where a voucher specimen was deposited. A voucher specimen has also been deposited at the Department of Natural Compounds at the Faculty of Food and Biochemical Technology, University of Chemistry and Technology, Prague (CZ). Trilobolide was obtained from SciTech spol. s r. o. Prague (CZ).

2.2 Synthesis

The synthesis is depicted in Scheme 1. Compound 1 represents archangelolide.

![Scheme 1](image)

**Scheme 1. Reagents and conditions:** a) MeOH, TEA, 48 h, yield 32%; b) i. 5-azidopentanoic acid, DCC, DCM, 90 min, rt; ii. 4-DMAP, DCM, 8 h, rt, 86%; c) dansylated propargylamine, CuI, TBTA, THF, MW, 70 °C, 2 h, yield 64%.

2.2.1 11-Deacetyl archangelolide (3)

Archangelolide (500 mg, 0.91 mmol) was dissolved in freshly distilled MeOH (10 mL) and Et₃N (2.5 mL) was being added for 1 h. The mixture was stirred for 48 h at room temperature; then, the solvents were removed under reduced pressure and the product was chromatographed twice (hexanes-AcOEt, gradient 5/1→3/1) to obtain 11-deacetylarchangelolide (148 mg, 0.29
mmol) in 32% yield. The unreacted archangelolide was recovered with R_F(1)=0.6; R_F(3)=0.4 in hexanes-AcOEt, 3/1. For analytical data, see Supporting information, Sections 1.2 – 1.4.

2.2.2 Synthesis of 11-azidovaleroyl-11-deacetyl archangelolide (4)

5-Azidopentanoic acid (80 mg, 0.56 mmol) was dissolved in dry CH_2Cl_2 (3 mL), then DCC (58 mg, 0.28 mmol) was added and the mixture was stirred 90 min. at room temperature after which the DCU was filtered out. 11α-deacetylarchangelolide (60 mg, 0.12 mmol) and DMAP (18 mg, 0.14 mmol) were dissolved in CH_2Cl_2 (3 mL) and freshly prepared anhydride of 5-azidopentanoic acid was added to the stirred solution. The mixture was stirred at room temperature for 8 h, after which it was filtered and the solvents were removed under reduced pressure. The residue was chromatographed twice (toluene-Et_2O, 10/1) to obtain the final product (66 mg, 0.1 mmol) in 86% yield with R_F=0.75 in hexanes-AcOEt, 3/1. For analytical data, see Supporting information, Sections 1.2 – 1.4.

2.2.3 Synthesis of 11-(5-(4-((5-(dimethylamino)naphthalene-1-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)pentanoyl archangelolide (5)

To a solution of azidovalerate 4 (40 mg, 0.063 mmol) and 5-(dimethylamino)-N-(prop-2-yn-1-yl)naphthalene-1-sulfonamide (20 mg, 0.07 mmol) in THF (3 mL), CuI (50 µL of 1 M water solution) and TBTA (3 mg, 5.6 µmol) were added. The mixture was placed into a microwave reactor and irradiated for 2 h at 70°C. Then, the solvent was evaporated under reduced pressure and the residue was chromatographed (hexanes-AcOEt, 1/1). The obtained product 5 was re-chromatographed twice to obtain pure product (37 mg, 0.04 mmol) as a slightly yellowish solid in 64% yield with R_F=0.25 in hexanes-AcOEt, 1/1. For analytical data, see Supporting information, Sections 1.2 – 1.4.
2.3 Computational studies

2.3.1 Simulation of molecular dynamics of archangelolide and sarco/endoplasmic reticular calcium ATPase

For docking and following simulations, a structure of sarco/endoplasmic reticular calcium ATPase (SERCA) protein bound to 8-O-(dodecanoyl-8-O-debutanoyl)trilobolide [DTB] with 3NAL code was obtained from Protein Data Bank [8]. Archangelolide structure was drawn using MarvinSketch 6.006 software (ChemAxon Ltd.), structure of trilobolide was obtained by editing the DTB structure.

First, we computed General Amber Force Field parameters for molecules of trilobolide and archangelolide in Antechamber software. Charges were calculated by restrained electrostatic potential method (RESP) based on a wave function calculated using quantum chemistry at the HF/6-31G*/HF/6-31G* level. Archangelolide molecule was manually docked into the SERCA binding site for thapsigargin using UCSF CHIMERA 1.10.2 (University of California, San Francisco) software by two approaches. In the first one, the molecule was docked so that the spatial orientation of the central seven-membered ring reached the best agreement with the orientation of DTB. In the other approach, the docked molecule was rotated over by 180°. The following simulations proceeded with the same parameters independently of each other.

The computed complex was transformed into a box with the size of 10.309 × 10.111 × 15.063 nm with periodical boundary condition and centered. For simulations of energy minimization and molecular dynamics, Gromacs-4.5.5 software was used. The first part of the simulations occurred in vacuum, the other then in water. In order to keep the solution neutral, 24 molecules of water were randomly replaced by sodium ions. The system was minimized and equilibrated by series of restrained simulations (2.02 ns in total).
2.3.2 Simulation of SERCA in a phospholipid bilayer

In order to build a complex of SERCA protein with phospholipid membrane, we used UCFS Chimera 1.10.2 software (University of California, San Francisco). The membrane composition was as follows: 10% phosphatidylserine, 30% phosphatidylethanolamine and 60% phosphatidylcholine. The membrane itself was created using CHARMM-GUI c40b1 software. Then, phospholipids at any distance greater than 0.35 nm from the enzyme were removed. The system was simulated in a periodic box of the sized of 15.65 × 15.65 × 18.00 nm. The system was minimized and equilibrated by series of restrained simulations (3.02 ns in total).

2.4 Biological assays

2.4.1 Cell lines and their cultivation

In this study, the following human cancer cell lines were used: LNCaP and PC-3 (prostate carcinoma), U-2 OS (osteosarcoma), MCF-7 (breast carcinoma), HT-29 (colon carcinoma), MIA PaCa-2 (pancreatic carcinoma), A549 (lung carcinoma); one transformed human cell line HEK 293T (embryonic kidney cells); one mouse cell line C2C12 (myoblasts) and a primary cell line MRC-5 (lung fibroblasts). The cell lines were purchased from American Type Culture Collection (ATCC, Manassas, USA) and from Sigma-Aldrich, USA. Unless otherwise specified, cells were cultured in medium (Thermo Fisher, USA) recommended by ATCC with stable glutamine dipeptide and supplemented with 10% fetal bovine serum (FBS; Thermo Fisher, USA). Cells were maintained at exponential phase of growth at 37 °C in humidified atmosphere with 5% CO₂.

2.4.2 Cell viability assay

Viability of cells treated with the tested compounds was determined using WST-1 (Sigma-Aldrich, USA) assay by spectrophotometric detection (450 nm, reference 630 nm) as described
in [10]. Briefly, WST-1 assay is based on reduction of a tetrazolium salt on soluble formazan in metabolically active cells. The measured absorbance is directly proportional to the number of metabolically active cells.

For this assay, cells were seeded into individual wells of 96-well plates (5,000 cells per well) in 100 µL of cell culture medium supplemented with 10% FBS. The cells were incubated overnight (16 h) under standard cultivation conditions. Then, 100 µL of fresh media was added with the tested compounds (final concentration 0-50 µM) and, the cells were incubated for another 24, 48 and 72 h. Next, the medium was removed and the cells were incubated with 5 µL of WST-1 dissolved in 100 µL of high glucose DMEM without phenol red with 10% FBS for 2 h. Then, the absorbance was measured. Cells incubated with a vehicle (DMSO) in medium were used as control. All experiments were done in quadruplicates. The data were analyzed in Microsoft Excel; the deviations were calculated as standard error of the mean (SEM).

For primary peritoneal cells (described in Section 3.3.6), a slightly modified procedure was used: the number of 1∙10^5 cells per well was cultivated overnight in triplicates, treated with the tested compounds and incubated for another 24 h. The amount of WST-1 was 10 µL per 100 µL of media and the incubation took 3 h. The results are expressed as the percentage of cytotoxicity relative to 100 % of dead cells treated with 1 % Triton X-100.

### 2.4.3 Cell uptake study

U- OS and MRC-5 cells (1∙10^5.well⁻¹) were seeded on 35 mm glass bottom dishes for live-cell imaging (MatTek Corporation, USA) and left to adhere for 24 h. Then, the cells were washed with phosphate buffered saline (PBS) and incubated with compound 5 or dansyl fluorophore (0.1 – 2 µM) dissolved in FluoroBrite DMEM medium (Thermo Fisher, USA) for 0.5 – 2.5 h. After the incubation period, the cells were washed with PBS and fresh FluoroBrite DMEM was added.
2.4.4 Determination of localization in cell organelles

U-2 OS and MRC-5 cells were seeded and influenced by the tested compounds as described in Section 2.4.3. To assess the intracellular localization of compound 5 in MRC-5 cells, an endoplasmic reticulum marker ER-Tracker™ Red (120 nM, 30 min.; ThermoFisher Scientific, USA) and a mitochondria-specific dye (70 nM, 30 min.; UCT Prague, CZ) from [11] were used. In U-2 OS cells, the endoplasmic reticulum was visualized by transfection with a plasmid DNA coding mCherry-ER (0.5 µg) using Fugene HD (Promega, USA; DNA : Fugene HD = 1 : 3).

2.4.5 Fluorescence microscopy

The intracellular localization of compound 5 was studied by real-time live-cell fluorescence microscopy using an inverse fluorescence microscope Olympus IX-81 operated by xCellence software (Olympus, Japan) and equipped with a high-stability 150 W xenon arc burner and EM-CCD camera C9100-02 (Hamamatsu, Germany). A 60× oil immersion objective (Olympus, Japan) with the numerical aperture of 1.4 was used. All images were deconvolved and background corrected by xCellence software.

2.4.6 Animals and cells

For isolation of resident peritoneal macrophages, female Wistar rats weighing 175–185 g (VELAZ, Czech Republic) were used. They were kept in plastic cages under standard conditions, i.e. a temperature of 22 ± 2 °C, 12/12 h of light/dark cycle, relative humidity (50 ± 10 %) and standard pelleted diet and water were provided ad libitum. Procedures were approved by Institutional protocol MSMT-15894/2013-310. Rats were sacrificed by cervical dislocation. The resident peritoneal macrophages of individual rats were collected by a lavage using sterile saline. The cells were washed, resuspended and seeded into 96-well round-bottom
microplates (2·10^5 cells per well) in complete RPMI-1640 medium containing 10 % heat-
inactivated FBS, 2 mM L-glutamine, 50 µg·mL^{-1} gentamicin, and 5·10^{-5} M 2-mercaptoethanol
(all Sigma-Aldrich, St. Louis, MO, USA). The treated or untreated cell (DMSO as a vehicle)
were cultured for 24 h and plates were maintained at 37 °C, 5 % CO2 in humidified Heraeus
incubator. Stock solutions of compound 1 and 5 were prepared 100 mM in DMSO. For the
cultivation of macrophages and assessment of toxicity and NO level, the majority of chemicals
was purchased from Sigma-Aldrich and Merck-Sigma, USA.

2.4.7 Nitrite oxide production in primary rat macrophages

To evaluate immunomodulatory activity of archangelolide and its derivatives, 100 pg·mL^{-1}
of lipopolysaccharide (LPS) was applied to cells in appropriate wells. After 24 h, the
concentration of nitrites was detected in individual supernatants (50 µL) incubated 10 min. at
ambient temperature with an aliquot of the Griess reagent (1 % sulphanilamide / 0.1 %
naphthylendiamine / 2.5 % H₃PO₄). The absorbance was recorded at 540 nm using a microplate
spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance
to concentration (µM) of nitrite.

2.4.8 Cytokine assay

Analogically to NO detection, supernatants of all samples were analyzed for cytokine
content after 24 h. The concentration of secreted TNF-α was determined by ELISA assay (R&D
Systems, Abingdon, UK) following manufacturer protocol.

2.4.9 Statistical analysis

Analysis of variance (ANOVA) and graphical presentation of data were done using
GraphPad Prism 6.05, San Diego, CA.
3. RESULTS AND DISCUSSION

3.1 L. archangelica metabolite isolation and identification

For isolation of major metabolites of L. archangelica, we used 100 g of fine ground seeds (Fig. 2) and the method of supercritical CO\textsubscript{2} extraction, which was carried out under pressure of 40 MPa at 40 °C until the extract substances passed from the extracted material (when several subsequent fractions were not adding any addition to the weight to the extract). The obtained yellowish extract (42.5 g) was stored in fridge over 72 h during which the crystals were formed. These were collected by filtration over a frit and washed with hexane obtaining 7.9 g of the matter which was analyzed by TLC and LRMS analyses. We observed that the matter was composed in particular of two terpene type compounds – trilobolide and archangelolide, and also of β-sitosterol. Using TLC, we found the mother liquor contained minimal quantity of trilobolide and archangelolide, but was abundant in β-sitosterol. Therefore, the obtained matter was further re-dissolved in methylene chloride and coated on silica. The purification by column chromatography using a toluene-diethyl ether gradient system (0 % → 10 % of ether) yielded 2.7 g of archangelolide and 1.2 g of trilobolide. The identity of these two SLs was verified by NMR, HRMS-ESI and IR analyses (in Supporting information, Section 1). The melting points of the compounds were also in accordance with previously reported data. Archangelolide was crystalized from the mixture of diethyl ether–hexanes (m.p. 108–111 °C) [12] and trilobolide from the mixture of toluene-Et\textsubscript{2}O (m.p. 190–192 °C) [6].
3.2 Derivatization of archangelolide with a dansyl probe

In order to evaluate intracellular localization and the fate of archangelolide in living cell, we synthesized a blue emitting derivative employing a dansyl label. This fluorophore is convenient not only for its small size but also for its membrane permeability. Moreover, it was successfully used in other studies of visualizing other natural compounds [13-15].

The synthesis (Scheme 1) of a dansyl-labeled archangelolide (5) started with mild solvolysis of archangelolide by triethylamine in methanol. Surprisingly, the only product which we obtained after 48 h of treatment was 11-deacetyl archangelolide (3), only in 32 % yield. The quaternary 11α-hydroxy group was acylated by freshly prepared 5-azidopentanoic anhydride in presence of 4-DMAP at room temperature. Finally, the azidopentanoate (4) was successively introduced into a click reaction with fluorescent 5-(dimethylamino)-N-(prop-2-yn-1-yl)naphthalene-1-sulfonamide (synthesized according to [16]) using CuI and TBTA [17] catalysis. All of the synthesized compounds were thoroughly described by NMR (Supporting information, Table S1.), IR, optical rotation (Supporting information, Section 1.2) and HRMS (Supporting information, Section 1.3). All compounds were re-purified on a short silica column prior to testing and afterwards lyophilized from tert-butanol. The substances were analyzed by
HPLC proving the purity of $\geq 95\%$ (Supporting information, Section 1.4). Fluorescent properties of compound 5 were determined in PBS and methanol, the emission maxima corresponded to 484 and 519 nm in these two solvents, respectively (Supporting information, Fig. S10).

3.3 Intracellular localization of archangelolide-dansyl conjugate

In this study we aimed to evaluate biological effects of a poorly described natural compound archangelolide and its fluorescent dansyl conjugate (5) in cancer, primary and immune cells. First, we analyzed the rate of cell uptake of compound 5 in U-2 OS (Fig. 3) and MRC-5 (Supporting information, Fig. S5) cells. In both cell lines, compound 5 localized already after 30 min., nevertheless, the fluorescence intensity increased during 2 h of incubation. In order to identify the intracellular localization of compound 5, co-localization experiments using endoplasmic reticulum and mitochondrial markers were performed. As it is apparent from Fig. 4 and Fig. S6 (Supporting information), compound 5 localized in endoplasmic reticulum, which is in agreement with the site of localization of another SL, a SERCA inhibitor, trilobolide [3]. However, we observed partial co-localization also in mitochondria, in which SERCA is not present.
Fig. 3. Intracellular localization of archangelolide-dansyl (5) in human cells from osteosarcoma (U-2 OS). Fluorescence microscopy of living cells treated with 1 µM concentration of compound 5 for 90 min.

Fig. 4. Co-localization of archangelolide-dansyl (5) with a marker of endoplasmic reticulum (top row) and with a mitochondrial marker (bottom row) in human cells from osteosarcoma (U-2 OS). Fluorescence microscopy of living cells treated with 1 µM concentration of compound 5 for 90 min. and pDNA coding mCherry-ER or a mitochondria-specific dye from [11] (10 min.).

3.4 Impact of archangelolide on cell viability

In order to reveal whether archangelolide is as potent compound as thapsigargin or trilobolide in terms of inhibition of cancer cell proliferation, we evaluated its cytotoxicity in a number of cell lines originating from various tumors: prostate, osteosarcoma, breast, colon, pancreas and lung. Cells were treated with archangelolide and its derivatives up to the final concentration of 50 µM for 24, 48 and 72 h. Surprisingly, archangelolide exhibited almost no cytotoxic effect upon these conditions (see Supporting information, Table S2-S5). The IC₅₀
were in upper micromolar and up to the tested concentration of 50 µM reached only for HEK 293T and LNCaP after 72 h of treatment. Moreover, we also did not detect archangelolide toxicity in primary and transformed cell lines. Based on these differences from thapsigargin and trilobolide, it seems that this SL does not act as SERCA inhibitor. Therefore, we proceeded to confirm this hypothesis by a molecular dynamics simulation study.

3.5 Molecular dynamics simulation of archangelolide with SERCA

The binding cavity for thapsigargin and trilobolide in SERCA protein lies in the transmembrane domain between the helices 3, 5 and 7. Comparing the polarity of these compounds with archangelolide based on the formation of hydrogen bonds, the most polar compound capable of forming the highest number of hydrogen bonds is thapsigargin, the least polar archangelolide (for details, see Supporting information, Table S6). Nevertheless, the complex of SERCA with thapsigargin is preferentially stabilized by hydrophobic interactions, and all three mentioned SLs are strongly hydrophobic molecules. We therefore assumed that trilobolide and archangelolide, which can form even fewer hydrogen bonds than thapsigargin, may interact with SERCA similarly to thapsigargin. However, this is clearly not the case as represented by our cytotoxicity evaluation using multiple cell lines.

In order to elucidate why archangelolide does not exhibit the same cytotoxicity as its structural counterparts thapsigargin and trilobolide, the potent SERCA inhibitors, archangelolide manual docking into SERCA followed by MD simulation (1-10 ns) was performed. For this simulation, four different complexes of SERCA and SL were chosen: i) archangelolide molecule positioned correspondingly to the orientation of DTB in the SERCA binding cavity (simulation 1); ii) archangelolide rotated by 180° (simulation 2); iii) trilobolide (for comparison) was used as a ligand with the orientation equal to DTB (simulation 3); iv)
SERCA positioned in phospholipid membrane to reflect the fact that it is a transmembrane protein, with trilobolide as a ligand (simulation 4).

The first round of simulations (1-10 ns) of archangelolide docking into SERCA cavity with archangelolide constrained as described (simulation 1) showed the presence of hydrophobic interactions of ligand side chains with Phe256, Val263, Ile829 and the hydrophobic part of Gln259 amino acid residues. Gln259 was the main residue involved in hydrophilic interaction. Interestingly, during the simulations in water environment, archangelolide had the tendency to escape from the SERCA cavity.

In simulation 2 of archangelolide rotated by 180° with constrained positions (10 ns), interactions with Phe256 and hydrophobic parts of Glu255 and Gln259 were detected. When the simulation was performed without constrained positions, archangelolide escaped from the SERCA protein cavity.

After first simulation of trilobolide (simulation 3) in non-water environment, hydrogen bonds of trilobolide with Glu255 and Gln259 residues, and α amino group of Ile829 were obvious. The hydrophobic interactions were directed preferentially to Phe256, Val263, Leu260 and Ile829 residues and a hydrophobic part of Lys252. In contrast to simulation 1 with archangelolide, there was no indication of trilobolide escaping from the SERCA cavity.

During simulations with SERCA positioned in phospholipid membrane (Simulation 4; 1 ns), the free space between individual phospholipids in cell membrane was filled. SERCA remained stable and tightly embedded in the membrane and did not have any tendency to escape from it. Trilobolide interacted similarly to simulation 3, in which it remained in the thapsigargin SERCA cavity for the duration of the simulation.
Fig. 5. Cartoon representation of sarco/endoplasmic reticulum $\text{Ca}^{2+}$ ATPase binding pocket with A, C) archangelolide or B, D) trilobolide after molecular dynamic simulations. Depicted are also amino acid residues in 5 Å range of respective ligand. The images were done by VMD software, version 1.9.2.
Fig. 6. Molecular surface representation of sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase binding pocket with A) archangelolide and B) trilobolide after molecular dynamic simulations. SURF function and probe size 1.4 Å were used. SURF function was written by Amitabh Varshney in University of North Carolina. The images were done by VMD software, version 1.9.2.

When archangelolide was positioned coordinately as trilobolide and DTB during simulations, it evinced the tendency to escape from the SERCA cavity, which was significantly pronounced in the case of archangelolide rotated by 180°. Therefore, we are convinced that especially the orientation and positions of the side chains of archangelolide are very important for its affinity to SERCA or the lack thereof. Winther et al. [8] reported that the dissociation constant of thapsigargin each lacking one of the four thapsigargin side chains (e.g., 2-deoctanoyl-4,5-dihydrothapsigargin or 8-O-(dodecanoyl-8-O-debutanoyl)trilobolide) augments in the following order: O-2 deoctanoyl < O-8 debutanoyl < O-10 deacetyl < O-3 deangeloyl. The individual side chains are depicted in Fig. 7.
Fig. 7. Structural formulae of a) thapsigargin, b) trilobolide, and c) archangelolide. Red parts show structural moieties of thapsigargin and its derivatives contributing to SERCA binding affinity (according to [8]): A) O-2 octanoyl, B) O-8 butanoyl, C) O-10 acetyl, D) O-3 angeloyl.

Considering the inhibition potency of SERCA by thapsigargin and trilobolide [4, 19] we are not convinced that the missing structural moiety O-2-octanoyl (see Fig. 7, A) in the case of trilobolide and archangelolide has a significant influence on its affinity to SERCA. Furthermore, the O-8 butanoyl and O-10 acetyl moieties (Fig. 7, B and C) are not significantly distinct in archangelolide (presence of a methyl group in the O-8 butanoyl side chain) from those of thapsigargin and trilobolide. Contrary to that, the O-3 angeloyl moiety (Fig. 7, D) is positioned at C3 in thapsigargin/trilobolide, whereas in archangelolide it is at C2. An O-3 deangeloyl thapsigargin derivative was previously prepared by Winther et al. (2010), who reported that 500-600x higher concentrations of this derivative than of thapsigargin were needed to achieve 50% inhibition of SERCA, which was the highest of all the derivatives lacking a single side chain each. [8] Taking their result as well as our simulations into consideration, we found that archangelolide is unlikely to act as a SERCA inhibitor and, also, it seems that the O-3 angeloyl moiety might play a rather significant role in the affinity of thapsigargin/trilobolide to SERCA.

3.6 Immuno-biological properties of archangelolide
SLs, natural compounds predominantly isolated from species Asteraceae and Apiaceae represent a rich source of small molecules with a potential for a pharmacological effect. Indeed, some of them have reached clinical applications as antimalarial (artemisinin; [20]) or antitumor (thapsigargin; [21]) agents or have been experimentally investigated with an increasing rate (see PubMed results by year).

To assess the immuno-biological potential of archangelolide, first, its cytotoxicity had to be determined. Therefore, we measured its impact on the viability of rat peritoneal macrophages after 24-h treatment. No cytotoxicity was detected at tested concentrations of 4 and 40 µM (Fig. 8) which is in accordance with results of [6], in which archangelolide and 2-deangeoylarchangelolide did not exhibit any cytotoxic effect. On the other hand, compound 5 partially decreased viability of rat peritoneal macrophages to 67 % (*P < 0.05) at 40 µM concentration. It seems that this phenomenon was not related to the fluorescent dye of compound 5, since dansyl itself did not change the viability of rat macrophages at both concentrations. The decrease in cell viability to 75 % (statistically not significant) was also recorded when rat primary macrophages were incubated with 1,000 pg·mL⁻¹ LPS + 40 µM compound 5, however, no toxicity was present for the combination of LPS + dansyl at the same concentration.
**Fig. 8.** Viability of rat peritoneal cells treated with archangelolide (1), dansyl-archangelolide (5) and dansyl itself. Compounds were applied at 4 µM and 40 µM concentrations and cells were cultured for 24 h. WST-1 assay was used for viability evaluation. The results are expressed as percentage of untreated control ± SEM of n = 6-8 values from 2 independent experiments. Statistical significance: *P < 0.05, compound Arch4 is statistically different from untreated cells.

Immunomodulatory [22] and anti-inflammatory [23] effects of many SLs are well-documented. In this study, we investigated the ability of archangelolide and compound 5 to modulate NO and cytokine TNF-α (tumor necrosis factor alpha). We found that when rat primary macrophages were stimulated by 1,000 pg·mL⁻¹ of LPS, a dose-dependent decrease in NO production was present in cells treated also with archangelolide in comparison to LPS-treated cells (Fig. 9). The decrease in NO was significant (*P < 0.01) for 40 µM concentration of archangelolide. The inhibitory effect on NO production was more pronounced in cells treated
by compound 5, with statistics **P < 0.001 for both, 10 and 40 µM concentrations. It is possible that the decline in NO at 40 µM concentration of compound 5 was at least partially induced by reduced cell viability. On the other hand, dansyl alone did not lower NO production.

**Fig. 9.** NO production in primary rat macrophages. The cells were treated with archangelolide (1) and dansyl-archangelolide (5) at the concentration range of 0.1 - 40 µM for 24 h with or without lipopolysaccharide (LPS, 1,000 pg·mL\(^{-1}\)) or with 40 µM dansyl itself. The results represent the mean ± SEM of 3 independent experiments, n = 6. Statistical significance: * P < 0.01, **P < 0.001, compounds are statistically different from LPS-treated cells.

In contrast to other SLs such as thapsigargin [24] or trilobolide [25] and its derivatives [26], archangelolide did not exhibit immuno-stimulatory activity, which was confirmed in this study. On the other hand, a slight to moderate effect on the decrease in NO production is clear and
corresponds to the result of [6]. Newly, we present the biological activity of a semi-synthetic conjugate of archangelolide as inhibitor of NO production in very low micromolar concentration.

Furthermore, the anti-inflammatory activity of compounds usually demonstrated by inhibitory activity against TNF-α secretion has never been explored for archangelolide. Therefore, in this study, we examined the effect of archangelolide and compound 5 on TNF-α secretion in rat primary macrophages (Fig. 10). We found a mild decrease in TNF-α secretion in LPS-treated cells for both compounds. We did not detect any effect of dansyl or dansyl + LPS (data not shown) on cytokine levels. Previously, weak anti-inflammatory activity was also found for cytokine IL-6 and negligible inhibition of IL-1β was detected for archangelolide and 2-deangeolylarchangelolide [6], whereas strong anti-inflammatory effect was found for another SL, laserolide [6]. Laserolide is a germacrane-type SL which has a ten-membered ring adjoining the five-membered lactone ring whereas archangelolide belongs to the guaiane type. In guaianolides, a bond is present between C1 and C5 of the ten-membered ring, creating a seven-membered and five-membered ring. Nevertheless, the arrangement of the five-membered lactone ring to the ten-membered part of the molecule with regards to adjoining substituents is similar in both archangelolide and laserolide. This implies that the arrangement of the five-membered ring in a SL molecule towards the rest of the molecule may be related to its anti-inflammatory activity.
4. CONCLUSION

In summary, we developed a method for the isolation of two related SLs trilobolide and archangelolide from the seeds of *Laserpitium archangelica*, Wulfen. Further, we extended the knowledge on the latter one, so far almost undescribed, and revealed that despite a high degree of structural similarity with two cytotoxic SLs trilobolide and thapsigargin, it did exhibit cytotoxicity neither in the tested cancer cell lines nor in primary cells. However, archangelolide exhibited anti-inflammatory activity, which was demonstrated by a decrease in NO production
and TNF-α secretion in rat primary macrophages, which makes it an interesting SL for further study of its immuno-biological activity.

To explain the reason for different cytotoxicity of archangelolide compared to trilobolide and thapsigargin, we prepared its fluorescent derivative for live-cell imaging studies, which determined its intracellular localization in endoplasmic reticulum and mitochondria of both cancer and normal cells. This led us to examine the binding of archangelolide to SERCA, the target of trilobolide and thapsigargin, by a docking study. Indeed, the results strongly argue that archangelolide is biologically very distinct from the other two SLs, since it does not bind and probably also not inhibit SERCA. Finally, archangelolide exhibited anti-inflammatory activity which was demonstrated by a decrease in NO production and TNF-α secretion in rat primary macrophages, which makes it an interesting SL for further study of its immuno-biological activity.

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5. REFERENCES


