Online Supplementary Information to Scharinger et al.

Leoligin, the Major Lignan from Edelweiss, Inhibits 3-hydroxy-3-methyl-glutaryl-CoA Reductase and Reduces Cholesterol Levels in ApoE -/- mice

Short title: Leoligin – a new cholesterol-lowering drug

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1. MATERIAL and METHODS

1.1 Leoligin quantification in mouse serum

Leoligin (100 mM in DMSO) was generously provided by Dr. Stefan Schwaiger, Institute for Pharmacy, University of Innsbruck, and the Leoligin derivative LT-RO1 (100mM in DMSO), which was used as internal standard (IS) was synthesized and provided by the Institute of Applied Synthetic Chemistry of the Vienna University of Technology (Vienna, Austria). Control mouse serum, methanol, and water (both solvents LC-MS grade) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Ethyl acetate (analytical grade) was obtained from VWR (Vienna, Austria). 1000 µM stock solutions of Leoligin and LT-RO1 were prepared in methanol and stored at 4 °C. The Leoligin stock solution was diluted with water to provide working solutions in the range of 1 µM-100 µM. For LT-RO1 a 50 µM working solution was prepared in the same way. Serum samples for calibration (6 concentration levels) were prepared by adding 50µl of respective Leoligin working solution and 50 µl of IS working solution to 50 µl of mouse serum (Sigma-Aldrich) resulting in final concentrations of 1, 5, 10, 25, 50 and 100 µM.

Sample preparation prior to gas chromatography-mass spectrometry (GC-MS) analysis was performed by supported liquid extraction (SLE) as follows: 50 µl serum samples were mixed with 50 µl IS working solution and 250 µl water. The mixture was then loaded onto an Isolute SLE + 400 µl array well (Biotage, Uppsala, Sweden). SLE was performed according to the manufacturer’s recommendations. Elution was carried out in two repeated steps of 700 µl ethyl acetate each. The eluate was evaporated to dryness under a gentle stream of nitrogen at 60°C. The residue was re-suspended in 100 µl ethyl acetate. After a waiting time of 30 minutes the sample was transferred to a screw vial with micro-insert and again evaporated to dryness under a gentle stream of nitrogen at 60°C. Prior to GC-MS analysis the residue was re-suspended in 25 µl ethyl acetate.

GC-MS analysis was performed on a GC (6890, Agilent, Waldbronn, Germany) hyphenated to a single-quadrupole-mass spectrometer (5973 MSD, Agilent, Waldbronn, Germany). 1 µl
sample volume was injected into the GC in splitless mode (purge time 2 minutes, purge flow 40 ml/min) via a CTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separation was achieved using a DB-XLB capillary column (25m x 0.25 mm, 0.25 µm, Agilent, Waldbronn, Germany) and helium as carrier gas at a constant flowrate of 1.7 ml/min. A temperature program started from 80°C (hold time 1 minute) to 340°C (hold time 6 minutes) with a heating ramp of 40°C/min. The injector temperature was set to 300°C. A single tapered liner (4 mm), packed with deactivated glass wool, was used. The mass spectrometer was operated in single ion monitoring mode. Three ions (1 quantifier and 2 qualifier) were monitored for Leoligin (m/z 151, 470 and 370) and for the IS LT-RO1 (m/z 279, 310 and 410), respectively. The MS method was divided in two acquisition windows: window one starting after a solvent delay time of 3 minutes until 9.5 minutes monitored the three ions of the IS. The second window starting from 9.5 minutes monitored the preselected Leoligin ions. Ion source and quadrupole temperature were 230°C and 150°C, respectively. Data acquisition and analysis were performed on a personal computer with Agilent Chemstation software (version E.01.01.335). The method was linear for leoligin concentrations ranging from 1 to 100 µM and the LOD was determined at 0.5 µM.

1.2. Leoligin quantification in mouse liver tissue

Following excision, liver samples were immediately snap frozen in liquid nitrogen and stored at -20°C until further use. For the standards, mouse liver tissue was placed in sterile 2 ml polystyrene tubes (Eppendorf, Hamburg, Germany) on ice, followed by adding 100 µl of the internal standard solution per 100 mg of tissue. Then, the fourfold volume of Chloroform (Merck, Darmstadt, Germany) was added to the tissue samples. Consequently, a 5 mm diameter hardened steel ball (Retsch, Haan, Germany) was added into the tubes, and tubes were placed in a Mixer Mill MM400 (Retsch, Haan, Germany), followed by breaking up of tissue pieces by applying a vibrational frequency of 30 Hz for 3 minutes. In the next step all solid components were separated by a centrifugation step (10 min/20,800 x g/4°C;
Eppendorf, Hamburg, Germany). The aqueous and interphase were collected and transferred into a new polystyrene tube. Probes were stored at -20°C until further use. Analysis was performed on a GC (6890, Agilent, Waldbronn, Germany) hyphenated to a single-quadrupole-mass spectrometer (5973 MSD, Agilent, Waldbronn, Germany). 1 µl sample volume was injected into the GC in splitless mode (purge time 2 min, purge flow 40 ml/min) via a CTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separation was achieved using a DB-XLB capillary column (25 m × 0.25 mm, 0.25 µm, Agilent, Waldbronn, Germany) and helium as carrier gas at a constant flowrate of 1.7 ml/min. A temperature program started from 80°C (hold time 1 min) to 340°C (hold time 6 min) with a heating ramp of 40 °C/min. The injector temperature was set to 300°C. A single tapered liner (4 mm), packed with deactivated glass wool, was used. The mass spectrometer was operated in single ion monitoring mode. Two ions (1 quantifier and 1 qualifier) were monitored for Leoligin (m/z 151 and 370) and for the IS Methoxyleoligin (m/z 151 and 195). MS acquisition started after a solvent delay time of 4 minutes. Ion source and quadrupole temperature were 230°C and 150°C, respectively. Data acquisition and analysis were performed on a personal computer with Agilent Chemstation software (version E.01.01.335). The method was linear for leoligin concentrations ranging from 5 to 100 µM and the LOD was determined at 1 µM.

1.3 Immunofluorescent staining of atherosclerotic plaques in the aortic arch
Aortic arches with atherosclerotic plaques were cut frozen (5µm) and then fixed in ice-cold acetone. After washing with TBST and permeabilisation with 0.2% Triton X-100 in TBS, blocking of unspecific binding sites was performed using 1% BSA and 10% goat serum in TBS for 30 minutes. Thereafter, slides were incubated with an anti-von Willebrand Factor antibody (ab6994, Abcam, USA) for 1 hour, followed by incubation with an Alexa Flour 546 goat anti-rabbit antibody (A11035, Life Technologies, USA) for 1 hour. A second blocking step was performed using 1% BSA and 10% rabbit serum in TBS for 30 minutes. Then, slides were incubated with an anti-alpha- smooth muscle actin antibody (ab66133, Abcam,
USA) for 1 hour and an Alexa Flour 488 goat anti-rabbit antibody (A11034, Life Technologies, USA) for 1 hour. Cell nuclei were stained with TO-PRO-3 iodide (T3605, Thermo Fisher Scientific, USA)

Slides were then mounted in ProLong Gold antifade reagent (P36930, Life Technologies, USA). Image acquisition was conducted using a LSM 510 Meta attached to an Axioplan 2 imaging MOT using ZEN software 2008 (Zeiss, Germany). Quantification of alpha-smooth muscle actin positive area in the plaque cap was performed using Adobe Photoshop CS4 for Windows (Adobe System, USA). Alpha-smooth muscle actin positive area was calculated in pixel and then related to the pixel area of the plaque.

1.4 HDL uptake measurement

HDL uptake assays were carried out essentially as described. [1] Briefly, HEK293 cells were incubated with medium B (DMEM without glutamine containing 0.2% fatty acid-free BSA (ICN, Aurora, OH)) containing 10 µg/well [3H]HDL for 5 h in the absence (total uptake) or presence of a 20-fold excess of unlabelled HDL (nonspecific binding). Cells were washed three times with buffer A (50 mM Tris, 0.9% NaCl, and 0.2% serum albumin, pH 7.4) followed by three washes with buffer B (50 mM Tris and 0.9% NaCl, pH 7.4) and solubilized in 0.1 N NaOH for 20 min at room temperature before protein and radioactivity quantitation.

1.5 Cholesterol efflux measurement

Cholesterol efflux capacity was essentially measured as described previously. [2, 3] J774 cells, derived from a murine macrophage cell line were plated and radiolabelled with 2 µCi of [3H]-cholesterol per millilitre for 24 hours followed by incubation with 0.3 mM cAMP (C3912, Sigma-Aldrich) for another 24 hours to upregulate ABCA1. Subsequently, efflux medium containing 25 µg/ml HDL-cholesterol was added for 4 hours. All steps were performed in the presence of 2 µg/ml acyl–coenzyme A:cholesterol acyltransferase inhibitor (Sc-215839A, Santa-Cruz Biotechnology). Liquid scintillation counting was used to quantify the efflux of radioactive cholesterol from the cells. Percent efflux was calculated by the following formula:
[(microcuries of $^{3}$H-cholesterol in medium containing 25 µg/ml HDL-cholesterol – microcuries of $^{3}$H-cholesterol in HDL-free medium) / microcuries of $^{3}$H-cholesterol in cells extracted before the efflux step] × 100.

1.6 Hepatic protein extraction, Western blotting and immunodetection

For protein extraction, frozen liver samples of each mouse were cut into small pieces on ice and then suspended in RIPA-buffer (containing 50 mM TrisCl, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitors). Samples were sonicated (Ultrasonic Processor UP200St, Hielscher, Germany) and the suspension was centrifuged at 10,000 rpm for 10 minutes at 4°C (Microfuge 22R Centrifuge, Beckmann Coulter, USA). After separation of insoluble parts, protein concentrations were determined using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (23225, Thermo Scientific Fisher, USA) according to the manufacturers’ instructions. Thereafter proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and blotted onto nitrocellulose membranes. Subsequent to blocking of unspecific binding sites with phosphate buffered saline without calcium and magnesium (PBS/-) containing 3% non-fat diet milk powder and 0.05% Tween-20, immunostaining was performed for ATP-binding cassette transporter A1 (ABCA-1; mouse anti-ABCA-1 antibody; ab18180, Abcam, USA), scavenger receptor class B member 1 (SR-B1; rabbit anti-SR-B1 antibody; NB400-104H, Novus Biologicals, USA) and LDL-receptor (LDL-R; rabbit anti-LDL-R antibody; 3939-100, BioVision Inc., USA). Following 3 washing steps in PBS/- with 0.05% Tween-20, membranes were incubated with either horse anti-mouse horseradish peroxidase (HRP)-linked antibody (7076, Cell Signaling Technology, USA) or goat anti-rabbit HRP-linked antibody (32460, Thermo Fisher Scientific, USA). After 3 washing steps, chemiluminescent detection was performed using either Super Signal West Femto Maximum Sensitivity Substrate (34096, Thermo Fisher Scientific, USA) or Super Signal West Pico Chemiluminescent Substrate (34011, Thermo Fisher Scientific, USA), followed by exposure
of x-ray films (34091, Thermo Fisher Scientific, USA). Densitometric analysis of Western blot images was performed using ImageJ software.

1.7 Feed consumption

Feed consumption was measured once a week over 16 weeks. The weight difference of feed between two measurements was assumed to be the consumption of one individual cage. Then, the consumption was divided by the number of mice in this cage. Consumptions per week were then added together.
2. RESULTS

2.1 Linearity, limit of quantification, and limit of detection of Leoligin

Calibration curves were obtained by analysing spiked serum samples at six concentration levels from 1 µM to 100 µM. Linearity was evaluated by plotting the relative response (area analyte / area IS) against the analytes concentration. The correlation coefficients were greater 0.99. The limit of quantification (LOQ) and LOD were estimated on the basis of signal-to-noise-ratios of m/z 151 (quantifier ion of Leoligin). The LOQ was found to be 1 µM with an S/N of > 10. The LOD with S/N > 3 was estimated to 0.5 µM.

2.2 Serum levels of Leoligin

Serum samples from 51 mice (baseline/no treatment with Leoligin) and 56 mice (treated with the drug) were analysed. Leoligin could not be detected in any of these samples. Consequently, the Leoligin serum concentrations were below the limit of detection (0.5 µM).

2.3 Leoligin concentrations in mouse liver

Liver tissue samples from 34 mice were analysed. Leoligin could not be detected in any of these samples. Consequently, the Leoligin concentrations were below the limit of detection of 1 µM.

2.4 Atherosclerotic plaque histology

Atherosclerotic plaque morphology (plaque structure, cap thickness) was analysed to identify possible effects of leoligin on plaque stability. To visualise smooth muscle cells, endothelial cells, and cell nuclei, aortic arch tissue sections with atherosclerotic plaques were stained for alpha-smooth muscle actin, von-Willebrand Factor, and TO-PRO3, respectively. Quantification of alpha-smooth muscle actin positive area (in percent) in relation to whole plaque area revealed no difference between the vehicle control group and leoligin treated groups in regard to cap thickness (Supplementary Fig. S1A and S1B).
Figure S1
Supplementary Figure S1: Leoligin does not influence atherosclerotic plaque stability and plaque-cap thickness

(A) Atherosclerotic plaque-cap thickness is given as alpha-SMA positive area in percentage of whole plaque area after 16 weeks of treatment with leoligin and vehicle control (control: n=3, Leoligin 1 µM: n=6, Leoligin 10 µM: n=8, Leoligin 50 µM: n=4). (B) Cryosections of plaques from aortic arches were stained with anti-vWF antibody (red signal), anti-alpha-SMA antibody (green signal) and appropriate secondary antibodies. Cell nuclei were stained with TO-PRO-3 (blue signal). Representative images are shown.

2.5 HDL-uptake and cholesterol efflux measurements

In order to determine alterations in hepatic HDL-uptake, the effect of leoligin on HEK293 cells was tested. Leoligin, in this *in vitro* setting, did not affect HDL-cholesterol uptake (Supplementary Fig. S2A). Further, additional *in vitro* HDL-mediated cholesterol efflux measurements in J774 mouse macrophages were performed. No effect on cholesterol efflux by leoligin was observed (Supplementary Fig. S2B).
Supplementary Figure S2: Leoligin does not influence HDL-uptake and cholesterol efflux in vitro

Results are given in % of the positive control. (A) Leoligin in varying concentrations does neither affect HDL-cholesterol uptake in HEK 293 cells (B) nor does it affect HDL-mediated cholesterol efflux in J774 macrophages.
2.6 Effect of leoligin on hepatic ABCA-1, SR-B1 and LDL-R

To test for potential alterations in liver cholesterol uptake and metabolism, Western blot analyses of liver tissue homogenate were performed. Analysis of liver tissue showed at least a trend towards an increased LDL-R expression in mice treated with 1 and 10 µM leoligin (Supplementary Fig. S3C). No significantly difference in expression of the proteins ABCA-1 and SR-B1 was observed between the leoligin treated groups and the vehicle control group (Supplementary Fig. S3A and S3B).
Figure S3
Supplementary Figure S3: Leoligin does not influence expression of hepatic ABCA-1.

SR-B1 and LDL-receptor

ApoE -/- mouse liver lysates were separated on an SDS-PAGE, blotted onto a nitrocellulose membrane and then stained with different antibodies. Protein expression is given as specific band intensity. (A) Quantification of ABCA-1 expression in liver lysates. (B) Quantification of SR-B1 expression. (C) Quantification of SR-B1 expression.

2.7 Feed consumption

To exclude an influence of differential energy intake on the effects of weight gain, nutrition intake was measured over the time period of 16 weeks. No significant difference in feed consumption between the leoligin treated mice and the vehicle control group could be observed (see Supplementary Fig. S4A).

Supplementary Figure S4: Mean total feed consumption of mice over 16 weeks

(A) Feed consumption is given as mean in gram per mouse over 16 weeks.
REFERENCES

