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Leoligin, the major lignan from Edelweiss, activates cholesteryl ester transfer protein

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ABSTRACT

Objective: Cholesteryl ester transfer protein (CETP) plays a central role in the metabolism of high-density lipoprotein particles. Therefore, we searched for new drugs that bind to CETP and modulate its activity.
Methods: A preliminary pharmacophore-based parallel screening approach indicated that leoligin, a major lignan of Edelweiss (*Leontopodium alpinum* Cass.), might bind to CETP. Therefore we incubated leoligin *ex vivo* at different concentrations with human ($n = 20$) and rabbit plasma ($n = 3$), and quantified the CETP activity by fluorimeter. Probucol served as positive control. Furthermore, we dosed CETP transgenic mice with leoligin and vehicle control by oral gavage for 7 days and measured subsequently the *in vivo* modulation of CETP activity ($n = 5$ for each treatment group).
Results: In vitro, leoligin significantly activated CETP in human plasma at 100 pM ($p = 0.023$) and 1 nM ($p = 0.042$), respectively, whereas leoligin concentrations of 1 mM inhibited CETP activity ($p = 0.012$). The observed CETP activation was not species specific, as it was similar in magnitude for rabbit CETP. *In vivo*, there was also a higher CETP activity after oral dosage of CETP transgenic mice with leoligin ($p = 0.015$). There was no short-term toxicity apparent in mice treated with leoligin.
Conclusion: CETP agonism by leoligin appears to be safe and effective, and may prove to be a useful modality to alter high-density lipoprotein metabolism.

1. Introduction

Cardiovascular diseases remain to be the leading cause of death in the developed world. The inverse relation between high-density lipoprotein cholesterol (HDL-C) and coronary artery disease (CAD) has brought attention to pharmacological interventions increasing plasma HDL-C [1,2]. Cholesteryl ester transfer protein (CETP) exchanges cholesteryl esters of high-density lipoproteins (HDL) for triglycerides of apolipoprotein B containing lipoproteins. In the presence of CETP, large HDL_2 particles lose their cholesteryl esters and shrink in size, whereas triglyceride-rich very low density lipoproteins (VLDL) transform into cholesteryl ester rich intermediate density lipoproteins [2–4]. CETP, thus, plays a central role in lipoprotein metabolism and was pursued for several years as a prime target for pharmacological intervention in order to treat dyslipidemia and to prevent atherosclerosis [5–8].

Animal studies suggested that rodents lacking plasma CETP activity or getting a CETP inhibitor held elevated HDL-C levels and showed resistance to diet-induced atherosclerosis. Furthermore, patients with CETP mutations tended to have increased HDL-C plasma concentrations and less CAD [4]. Thus, the pharmacological inhibition of CETP seemed to be a promising strategy to fight CAD. First pilot studies with CETP inhibitors showed indeed higher HDL-C and minor low-density lipoprotein cholesterol (LDL-C) levels without serious adverse events [5,6]. However, the first large multicenter randomized controlled clinical trial with the CETP inhibitor torcetrapib was disrupted prior to schedule because of an excess of cardiovascular and overall mortality in the active treatment group [7]. Moreover, recent work from Vasan et al. and us revealed that low endogenous CETP plasma levels *per se* were associated with

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increased cardiovascular and all-cause mortality in the Framingham, the LURIC, and the KAROLA population [9–11].

Having focussed on CETP inhibitors up to now, there is much less information on potential benefits of a pharmacological increase of CETP activity. Hitherto, the only known CETP-activator was probucol [12]. Probucol was initially developed as an anti-oxidant to be used in the manufacturing of tires, but was found to have cholesterol-lowering properties and thus marketed for a number of years as hypolipidemic agent. A remarkable property of probucol is its ability to lower cholesterol in patients with homozygous familial hypercholesterolemia [13]. In such patients, probucol causes a dramatic decrease in tendon and planar xanthomas, seemingly out of proportion to the degree of cholesterol lowering. Probucol increases both the amount and the activity of CETP, and enhances the reverse cholesterol transport [12,14]. Unfortunately, probucol has severe untoward effects including prolongation of the QT_c interval. Therefore, the substance was retracted from the market in the USA and Europe, but is still in use in Japan [15].

As the role of CETP in the pathogenesis of atherosclerosis is still open to debate [16], we looked for new biological compounds to alter CETP activity. Our data from *in silico* screenings indicated that leoligin, the major lignan of the alpine flower Edelweiss (*Leontopodium alpinum* Cass.), may bind to CETP. This natural lignan belongs to the class of lariciresinol derivatives and was shown to inhibit intimal hyperplasia of venous bypass grafts [17], as well as the *in vitro* leukotriene biosynthesis [18]. In the present study, we examined the effects on blood lipids of this unique compound providing clear evidence that leoligin enhances CETP activity *ex vivo* and *in vivo*.

2. Materials and methods

2.1. Plant material, isolation, and purification of leoligin

Leoligin (Fig. 1A) was isolated as described previously from 5.3 kg sub-aerial parts of Edelweiss (*L. alpinum* Cass.) which were obtained from Swiss horticultures [17]. The purity of leoligin according to LC-DAD/MS- and NMR examination was >98%. Furthermore, a voucher specimen (CH 5002) has been deposited at the herbarium of the Institute of Pharmacy/Pharmacognosy, University of Innsbruck. In addition, two further natural derivatives of leoligin 5-methoxyleoligin (=[(2*S*,3*R*,4*R*)-4-(3,4-dimethoxybenzyl)-2-(3,4,5-trimethoxyphenyl)tetrahydrofuran-3-yl]methyl-(2*Z*)-2-methylbut-2-en-oate and 5,5′-dimethoxyleoligin (=[(2*S*,3*R*,4*R*)-4-(3,4,5-trimethoxybenzyl)-2-(3,4,5-trimethoxyphenyl)tetrahydro-furan-3-yl]methyl-(2*Z*)-2-methylbut-2-en-oate were isolated as described in [18] and used for testing. The purity of both compounds was comparable to that of leoligin.

2.2. In silico screening

We performed the pharmacophore-based parallel screening as previously described [19]. In brief, each pharmacological target is represented by one or more pharmacophore models. A pharmacophore model is an abstract representation of the lock-and-key hypothesis of protein–ligand interactions. It consists of the steric and electronic features that are necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger or block its biological response. A pharmacophore is composed of chemical features that describe types of protein–ligand interactions such as hydrogen bonds, charged groups, aromatic structures and hydrophobic areas. The technology to perform simultaneous, parallel screening of one compound against a multitude of pharmacophore models is available as a Pipeline Pilot-based program protocol in Discovery Studio 2.01 (Accelrys Inc., San Diego, CA, USA). Together with this software, the Inte:Ligand (www.inteligand.com) pharmacophore model collection of 2208 in-house generated pharmacophore models was used for the virtual screening. The CETP model from the InteLigand pharmacophore model database (www.inteligand.com/pharmdb/) and validation studies were performed using the CATALYST software package (Accelrys Inc., San Diego, CA, USA). Anacetrapib, a CETP inhibitor (IC_{50} = 13 nM) that recently proceeded to phase III clinical trials, was selected as template for ligand-based pharmacophore elucidation [8]. Applying the feature mapping algorithm of CATALYST, several models were generated by placing pharmacophoric features on the chemical moieties of the energetically minimized 3D structure of anacetrapib. The ability of the generated models to enrich CETP ligands among biologically inactive compounds was validated by virtually screening them against a test set. The test set comprised 14 CETP ligands and 32 compounds that did not show biological activity for CETP below 100 μM (Suppl. Table 1). The latter were chosen as inactive ligands for model validation. The best model was kept for virtual activity profiling within the InteLigand pharmacophore model collection. The CETP model comprised a hydrogen bond acceptor fitted to the oxazolidin-2-one moiety of anacetrapib, as well as two aromatic and two hydrophobic interactions. The model retrieved 13 test set inhibitors (93%) and two inactive compounds (6%). The enrichment of actives compared to a random selection was measured using the enrichment factor (EF) calculated by the following equation [19]: $EF = (TP/n)/(A/N)$. *TP* is the number of actives retrieved by the model, *n* is the number of actives and inactive compounds retrieved by the model, *A* is the number of actives in the test set, and *N* is the number of all compounds in the test set. The *EF* determined for the CETP model was 2.85. The maximum *EF* for this dataset, where a model would find only active compounds, would have been 3.29. The high *EF* of this model indicated high quality and restrictivity.

2.3. Lipidiological analyses

The study protocol was approved by the university ethics review board (# AN4143 294/4.1) and complies with the Declaration of Helsinki. All participants gave written informed consent before the trial and filled standardized questionnaires for baseline information. Blood samples were collected from 20 healthy volunteers after an overnight fast. Total plasma cholesterol, triglycerides, and HDL-C were measured in whole plasma using Roche Diagnostics commercial kits (Roche Diagnostics, Mannheim, Germany). Due to the small volume size of available plasma samples from CETP transgenic mice, single measurements of HDL- and LDL-cholesterol for each animal were not possible. As a surrogate, we used FPLC lipid profiles from pooled murine plasma samples to differentiate between the high-density and low-density lipoprotein cholesterol fractions in the different treatment arms. The murine plasma samples were subjected to FPLC fractionation analysis with two tandem superose 6 columns (GE Healthcare, Vienna, Austria), as described previously [20]. CETP activity was measured using a commercial CETP activity assay kit (BioVision, Mountain View, CA, USA). Briefly, CETP activity was determined in microplates by a fluorescent method using a donor molecule containing a fluorescent self-quenched neutral lipid that is transferred to an acceptor molecule in the presence of CETP. Three μL of human plasma were used per well and the plate was incubated for 30 min at 37 °C. CETP-mediated transfer of the fluorescent neutral lipid to the acceptor molecule results in an increase in fluorescence which was measured with the fluorimeter Tecan infinite M200 and the appropriate software i-control 1.6 (Tecan Group, Maennedorf, Switzerland). CETP plasma levels were determined using an enzyme-linked immunosorbent assay (ELISA) employing a CETP-specific recombinant single-chain antibody