Boosting of retinol activity using novel lecithin:retinol acyltransferase inhibitors

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Abstract.

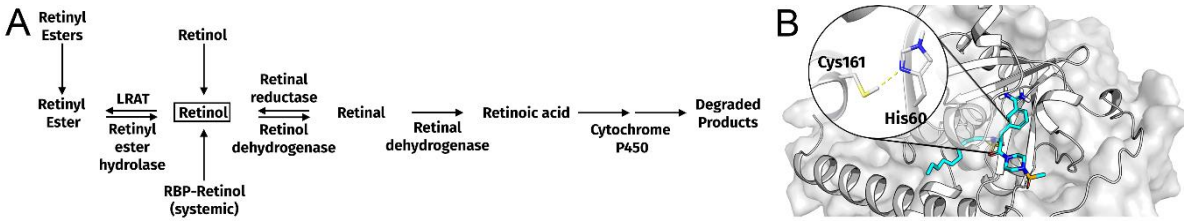
Lecithin:retinol acyltransferase (LRAT) is the main enzyme catalyzing the esterification of retinol to retinyl esters and, hence, is of central importance for retinol homeostasis. As retinol stimulates fibroblasts to synthesize collagen fibers and inhibits collagen-degrading enzymes, the inhibition of LRAT presents an intriguing strategy for anti-aging ingredients by increasing the available retinol in the skin. Here, we synthesized several derivatives mimicking natural lecithin substrates as potential LRAT inhibitors. By exploring various chemical modifications of the core scaffold consisting of a central amino acid and an N-terminal acylsulfone, we explored 10 different compounds in a biochemical assay, resulting in two compounds with IC_{50} values of 21.1 and 32.7 µM. Supported by computational methods, we investigated their structure-activity relationship, resulting in the identification several structural features associated with high inhibition of LRAT. Ultimately, we conducted an ex vivo study with human skin, demonstrating an increase of collagen III associated with a reduction of the skin aging process. In conclusion, the reported compounds offer a promising approach to boost retinol in human skin and might present a new generation of anti-aging ingredients for cosmetic application.

Introduction.

The human skin is largely composed of connective tissue rich in collagen fibers making up the extracellular matrix. As reduced thickness and strength of type I & III collagens are reported in both chronologically aged and in photodamaged skin, neocollagenesis is important for skin antiaging effects [1-4]. Retinoids stimulate the collagen synthesis of fibroblasts by increasing their number as well as their activity, promote skin elasticity by removing degenerated elastin fibers, and reduce collagen degradation by inhibiting matrix metalloproteinases. The effects of retinoids are primarily mediated by nuclear receptors including retinoic acid receptor and retinoid X receptor. As type III collagen is induced to a higher degree by retinoic acid (RA) as opposed to retinol (ROH) [5] the use of type III Collagen as an RA-responsive marker may be useful to identify retinoid boosting strategies. ROH and its esters are well accepted as efficacious skin anti-aging ingredients [6]. However, there is an ongoing desire in the cosmetic industry to boost the efficacy of either exogenous or endogenous ROH in order to be able to reduce the concentration in topical compositions mitigating its irritative effects and/or increase concentrations maximise its efficacy. In the skin, ROH is metabolized to

retinoic acid using a variety of metabolic steps (Figure 1A). Thus, activation or inhibition of these enzymes offers routes to improve the efficacy of retinol. Indeed, these routes have been shown to improve the antiaging efficacy of ROH and its esters [7-9]. However, only currently available cosmetic ingredients were investigated in this latter strategy.

Figure 1. *Role and functionality of LRAT. (A) Generalised metabolism of ROH and its esters to RA. (B) Structural overview of LRAT and catalytic diad.*



ROH is mostly converted to retinyl esters in skin cells to become storage reserves and thus its esterification mechanism is the rate-limiting step to manipulate its levels [10]. It has been shown that lecithin:retinol acyltransferase (LRAT) is a main enzyme responsible for ROH esterification [11]. The enzyme transfers the sn-1 fatty acyl group, largely linoleic acid, from phosphatidylcholine to retinol [12,13]. Thus, inhibition of LRAT would allow more ROH to be available for subsequent conversion to retinoic acid, making LRAT an attractive target for the development of very effective anti-ageing skin actives. Reactions by LRAT are facilitated by a catalytic diad consisting of His60 and Cys161 and take place in two distinct steps (Figure 1B). First, the ester group phosphatidylcholine substrate is attacked by the thiolate and covalently attached to the catalytic cysteine residue. In a next step, ROH attacks this newly formed thioester, resulting in the ROH ester product [12]. In contrast to other acyltransferases, LRAT does not require a coenzyme A intermediate. The focus of this preliminary work was to identify novel LRAT inhibitors and to demonstrate their retinoid boosting and resulting effects on collagen III synthesis. Further, we deduced structure-activity relationships of the inhibitors to explore observed activity differences with computational methods.

Methods.

***In vitro* assay.** Selected ingredients were assayed *in vitro* for their LRAT inhibitory potential in a phosphate buffer containing retinol, dilaurylphosphatidylcholine, dithioerythritol and bovine serum albumin (pH 7.0) for 60 min at 37˚C. The reaction was quenched with ethanol before extraction with pentane and the resulting extracted retinoids were determined by reverse phase high performance liquid chromatography.

***Ex vivo* assay.** As a proof-of-concept study to underline the promising potential based on LRAT inhibition we performed an *ex vivo* study on human skin via topical application. The inhibitors were applied 3 times (at day 0, 1, 4, 6) and the skin was harvested for histologic analysis at day 8. The collagen III level was assessed by immunohistologic staining and % stained surface of papillary

dermis was quantified by image analysis using CellSense software. For each condition nine samples were stained and analysed (n=9). Data was collected in Microsoft Excel and statistics was calculated using unpaired student t-test.

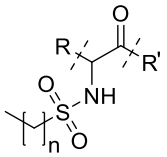
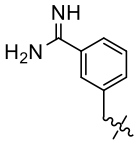
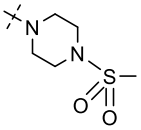
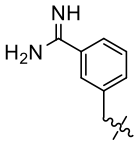
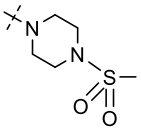
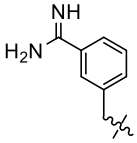
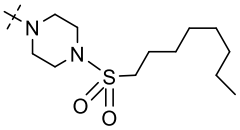
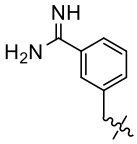
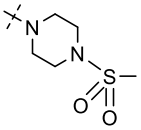
Molecular modeling. Homology models of LRAT were generated using the SWISS-MODEL2 webserver using the input sequence in FASTA format derived from the UniProt database (Accession code: O95237). Models were constructed using the top-3 ranked template structures (PDB IDs: 4Q95, 2KYT, and 4DPZ) according to the global quality estimation score [14]. Furthermore, an alternative structure was obtained from the AlphaFold Protein Structure Database [15]. The protein structures were preprocessed using the Protein Preparation Wizard [16] within the Maestro Small-Molecule Drug Discovery Suite. Ligands were preprocessed using the LigPrep routine on default parameters. Docking was conducted using the Glide standard precision (SP) [17] and AutoDock Vina [18] docking engines. The centroid for these docking procedures was defined based on the mass center of the cocrystallized ligand in the homologous HRASLS3/LRAT chimeric protein (PDB ID: 4Q95). We visually inspected the binding modes regarding the proximity of the reactive centers (sn-1 ester carbonyl carbon and cysteine thiolate) involved in catalysis. The combination of the structural model obtained from AlphaFold together with the AutoDock Vina docking engine produced the most consistent results. Using the validated model, the stereoisomers of inhibitors 1 and 2 were docked to the active site of LRAT. Based on the introduced characteristics of binding modes of different lecithin derivatives, we selected poses of the inhibitors in accordance with this rationale by visual inspection.

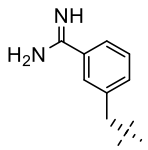
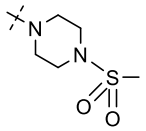
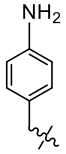
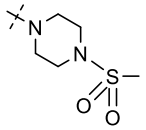
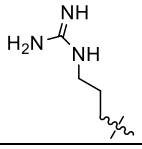
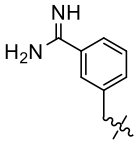
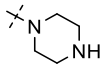
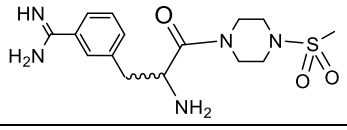
Results.

We developed an enzymatic screening method for LRAT *in vitro* and analyzed the inhibitory activity of 80 compounds derived from our chemical and peptide libraries. By setting the threshold at 80% inhibitory activity, we obtained a hit list of 10 novel LRAT inhibitors. A common lead structure of a modified phenylalanine was identified (Table 1). The inhibitors likely interfere with the formation of the thioester intermediate due to their similarity to the lecithin substrate. As no crystal structures are available for LRAT, a structural model had to be established and validated. The combination of the structural model obtained from AlphaFold together with the AutoDock Vina docking engine produced the most consistent results. Interestingly, the binding modes fulfilling the proximity of the reactive centers were highly similar for different phosphatidylcholine lipids, as they: (i.) placed the sn-1 lipid tail at least partly in the same hydrophobic pocket flanked by Leu17, Leu18, Leu58, and Leu156, (ii.) placed the negatively charged phosphate group in proximity to Arg55 forming an ionic interaction, and (iii.) placed the positively charged choline headgroup in proximity to Asp111 forming an additional ionic interaction (Figure S1). Docking of inhibitors to the established model revealed a high similarity of their binding modes to the ones of different lecithins. Remarkably, the fatty acid chain of the inhibitors fits in the same hydrophobic pocket as the sn-1 chain of the lecithins (Figure 2).

Furthermore, the positively charged amidino moiety resembled the choline head group of the lecithins and, similarly, underwent an ionic interaction with Asp111. Depending on the stereochemistry, a cation-pi interaction between the phenyl ring of the inhibitors and Arg55 was established. Lastly, the sulfomethyl moiety of the inhibitors formed hydrogen bonding interactions with either Tyr118 or Arg55. The resulting structure-activity relationship (SAR) analysis showed that a long-chain fatty acid residue as DODS (dodecansulfonyl) or DECS (decansulfonyl) was essential for activity whereas compound 10 with a free N-terminus exerted very low activity (Table 1). Further, the replacement of the amidino moiety with an amine resulted in a complete loss of inhibition. Regarding the acyl moiety of the compounds, a length of 10-12 carbon atoms produced good results, while the elongation to 16 atoms resulted in lower inhibition. Modifications of the piperazine moiety only had mild effects on LRAT inhibition. Based on initial single-concentration measurements, we selected the two promising inhibitors 1 and 2 for dose-response experiments to determine their IC_{50} for LRAT (Figure 2).

Table 1. *Structures and effect of LRAT inhibitors and controls in single-concentration measurements.*

Compound	**Structure**			**Inhibition at 100 µM (%)**
				
	n=	R=	R'=	
1	11			94.3
2	9			91.5
3	7			89.9
4	15			39.3

5	11			96.7
7	11			0
8	11			84.6
9	11			44.6
10				6.8

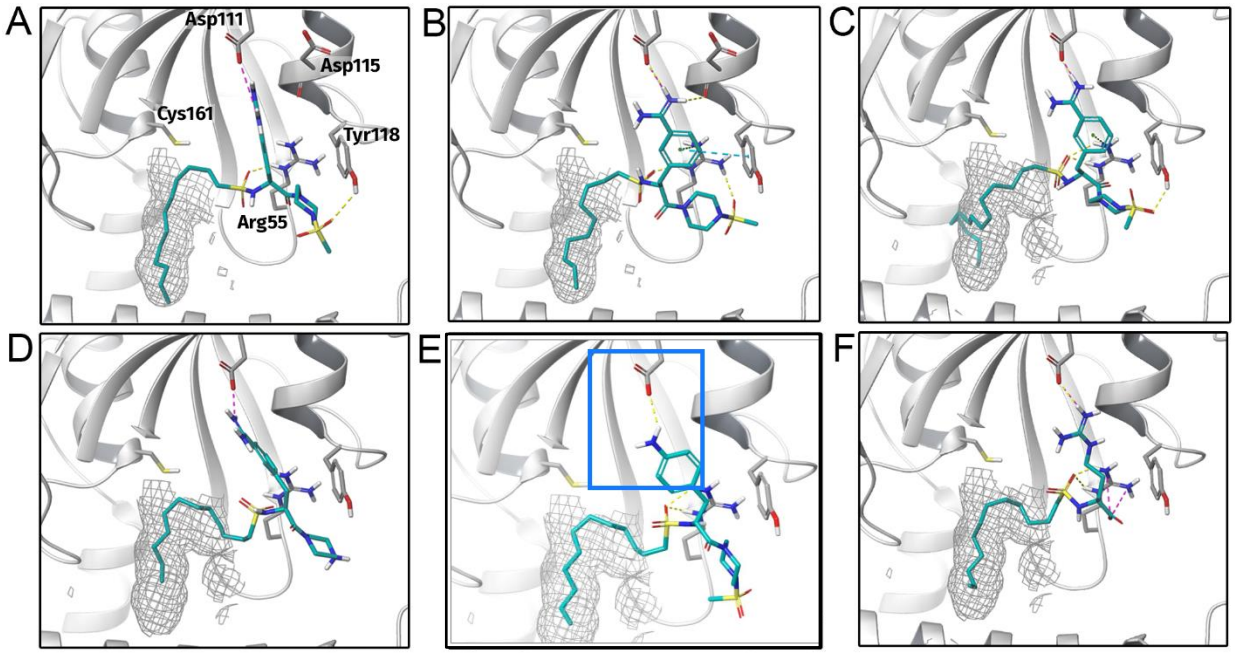


Figure 2. *Structural insights into the binding of LRAT inhibitors. Binding modes of (A) compound 1, (B) compound 2, (C) compound 4, (D) compound 9, (E) compound 7, (F) compound 8 determined by molecular docking. A hydrophobic cavity occupied by the aliphatic tails is shown in a surface representation.*

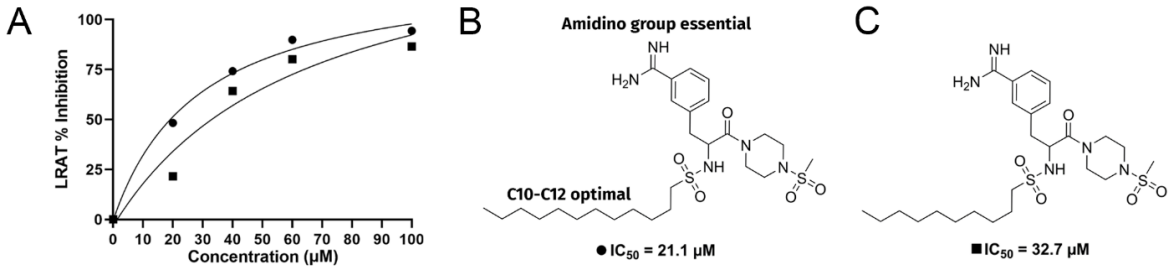


Figure 3. *Dose response effect of inhibitors 1 and 2. Inhibition curves of inhibitors 1 and 2 for IC50 determination. (B) Structure of inhibitor 1 with SAR information depicted. (C) Structure of inhibitor 2.*

In the *ex-vivo* studies, ROH alone at 0.05% exerted as expected a moderate positive effect on collagen III synthesis. The LRAT inhibitors were tested alone at 0.005% and 0.1% with a slight positive effect that was dose-dependent and the same inhibitors were tested in combination with 0.05% ROH. These combined tests revealed the strongest and also dose dependent increase on collagen III and the inhibitor 2 was even slightly better than inhibitor 1 consistent with their IC50 values (Figure 4).

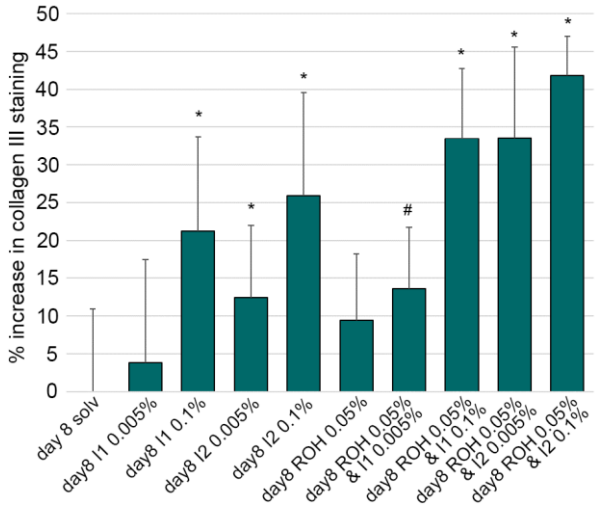


Figure 4*. ex vivo collagen III immunostaining after topical application of test compounds I1/I2 = inhibitor 1 or 2, ROH = retinol; statistics t-test # p<0.02, *p<0.01 versus vehicle control at day 8, control was normalized to level 0, error bars represent SD.*

Discussion.

ROH and its esters are well established antiaging ingredients [6]. However, their optimal efficacy is limited by the skin's capacity to storage them in an ester form, even when the retinyl esters are hydrolysed in the stratum corneum. Although several enzymes are involved in the conversion of ROH to RA which then interacts with the retinoid A and X receptors to trigger gene expression [19], we propose that the ROH esterification step is key in limiting the flux of ROH to RA. Others have demonstrated that the approach is valid, although only general cosmetic ingredients were used at that time. Hence, we set out to develop novel ingredients. [7]

As LRAT can be inhibited by phenylmethylsulphonyl fluoride, an inhibitor of serine and cysteine proteases as well as several esterases [20], we proposed that inhibitors of this class may be useful starting scaffolds for the development of LRAT inhibitors [20].

Interestingly, the acyl chain length of our LRAT inhibitors affected their inhibitory activity with the shorter variants being more active in accordance with the substrate specificity reported by Horchani *et al.* [21]. Using molecular modeling, the above-described SAR could be investigated in structural setting. The influence of the length of the acyl side chain, which presented best inhibition with 8-12 carbon atoms, could be explained by the size of the respective hydrophobic pocket where it resides (Figures 2A and 2B). As indicated by the binding mode of inhibitor 4 (Figure 2C), the C16 acyl moiety could not be completely accommodated in the hydrophobic pocket. In contrast, the binding mode of inhibitor 1 (Figure 2A) showed that the C12 fatty acid nearly completely occupied the hydrophobic pocket. Hence, longer acyl moieties result in reduced inhibition likely due to steric limitations. The loss of inhibition by compounds without the amidino group could be explained by the lack of a potent ionic interaction in the active site. The mildly reduced inhibition resulting from the removal of the sulfomethyl moiety attached to the piperazine ring might be caused by the lack of hydrogen bonding interactions with Arg55 or Tyr118. Inhibitor 1 composed of DODS linked to the N-terminus and a piperazine moiety with a methylsulfonamide modification at the C-terminus of modified phenylalanine moiety was found to be a particularly effective LRAT inhibitor i*n vitro* and *ex vivo*. In the *ex vivo* experiment this ultimately led to a synergistic stimulation of Type III collagen production in combination with ROH (Figure 4). For example, the application of inhibitor 2 and ROH alone increased type III collagen production by 25.9% and 9.4%, respectively, while their combination resulted in an increase of 41.8%.

Conclusion.

The inhibition of LRAT involved in the esterification of ROH is a promising strategy to increase the effects of retinoids on the production of collagens, which present a decreased thickness in aged and photodamaged skin. Hence, LRAT inhibitors might be relevant as cosmetic ingredients for anti-aging treatment. Upon screening of several structures, we found that amidino substituted amino acid

derivatives are highly effective LRAT inhibitors and are in particular suitable to enhance the beneficial skin-ageing effects of ROH, reflected by increased neocollagenesis levels, particularly Type III collagen. Using molecular modeling, we could identify the SAR of this compound series by investigating key ligand-protein contacts such as the size of the hydrophobic pocket the acyl moieties reside in and an ionic interaction of the amidino group. We confirmed that the LRAT inhibitors reduced the formation of retinyl esters from ROH and that the anticipated increased flux to retinoic acid in human skin resulted in increased retinoid responsive collagen III. Further work will be needed to establish if RA is effectively increased and in which cell types and/or whether a paracrine response has occurring following the ingredient application leading to neocollagenesis. Overall, LRAT inhibition using the reported compounds based on a common scaffold is a very promising approach to further boost the efficacy of ROH in future products, delivering superior anti-aging results.

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