

## RESEARCH COMMUNICATION

# Effect of regioisomers of hydroxystearic acids as peroxisomal proliferator-activated receptor agonists to boost the anti-ageing potential of retinoids

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## Abstract

**Introduction:** We report on the in vitro and ex vivo effects of chiral (*R*)-10-hydroxystearic acid (10-HSA) compared with other mono-hydroxystearic acid regioisomers and stearic acid (SA) together with its benefit when combined with retinol.

**Methods:** Following treatment with hydroxystearic acids peroxisomal proliferator-activated receptor alpha (PPAR $\alpha$ ) activity was determined in a luciferase reporter gene assay, collagen type I was assessed in primary human dermal fibroblasts by immunohistochemistry, modification of the intracellular fibroblast collagen proteome was studied by mass-spectrometry-based proteomics and collagen type III was assessed by immunohistochemistry on human ex vivo skin.

**Results:** 10-HSA was the most effective PPAR $\alpha$  agonist (15.7 $\times$  induction;  $p < 0.001$ ), followed by 9-HSA (10.1 $\times$  induction) and then 12-HSA (4.9 $\times$  induction) with 17-HSA (1.7 $\times$  induction) being similar to the effects of stearic acid (1.8 $\times$  induction). Collagen type I levels were increased in primary human fibroblasts by 2.12 $\times$  and 1.56 $\times$  for 10-HSA and 9-HSA, respectively, in vitro with the 10-HSA being significant ( $p < 0.05$ ), whereas 12-HSA and SA had no statistical effect over the untreated control. 10-HSA and 12-HSA modified the intracellular fibroblast collagen proteome slightly with significant increases in collagen alpha-1 (VI) and alpha-3 (VI) proteins but only 10-HSA increased levels of collagen alpha-2 (V), alpha-1 (III), alpha-1 (I) and alpha-2 (I) (all  $p < 0.05$ ) with the increases being significantly different between 10-HSA and 12-HSA for collagen alpha-1 (I), collagen-3 (VI) and collagen alpha-2 (I) ( $p < 0.01$ ). Collagen type III in ex vivo skin was increased +47% ( $p < 0.05$ ) by 0.05% (1.7 mM) retinol, +70% ( $p < 0.01$ ) by 0.01% (0.33 mM) 10-HSA and the combination increased levels by +240% ( $p < 0.01$  for either ingredient).

**Conclusion:** Chiral (*R*)-10-HSA has been shown to be superior to 9, 12 and 17-HSA as a PPAR $\alpha$  agonist. Moreover, 10-HSA stimulated collagen synthesis in

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monolayer fibroblast culture as assessed by proteomics and immunohistochemically. Furthermore, we also show the synergistic effects of 10-HSA with retinol on collagen III synthesis in skin explants. These results further highlight the efficacy of 10-HSA as a cosmetically acceptable PPAR $\alpha$  agonist and anti-ageing ingredient.

#### KEYWORDS

collagen, hydroxystearic acid, PPAR, proteomics, skin physiology/structure

#### Abstrait

**Introduction:** Nous rapportons les effets *in vitro* et *ex vivo* de l'acide chiral (R)-10-hydroxystéarique (10-HSA) par rapport à d'autres régioisomères d'acide monohydroxystéarique et à l'acide stéarique (SA) ainsi que ses avantages lorsqu'il est associé au rétinol.

**Méthodes:** Après un traitement avec des acides hydroxystéariques, l'activité du récepteur alpha activé par les proliférateurs peroxysonaux (PPAR $\alpha$ ) a été déterminée dans un test du gène rapporteur de la luciférase, le collagène de type I a été évalué dans les fibroblastes dermiques humains primaires par immunohistochimie, la modification du protéome du collagène des fibroblastes intracellulaires a été étudiée par spectrométrie de masse. La protéomique et le collagène de type III ont été évalués par immunohistochimie sur la peau humaine *ex vivo*.

**Résultats:** la 10-HSA était l'agoniste PPAR $\alpha$  le plus efficace (induction 15,7X ;  $p < 0,001$ ), suivi de la 9-HSA (induction 10,1X) puis de la 12-HSA (induction 4,9X) avec la 17-HSA (induction 1,7X) étant similaire aux effets de l'acide stéarique (induction 1,8X). Les niveaux de collagène de type I ont été augmentés dans les fibroblastes humains primaires de 2,12X et 1,56X pour la 10-HSA et la 9-HSA respectivement *in vitro*, la 10-HSA étant significative ( $p < 0,05$ ) : alors que la 12-HSA et la SA n'ont eu aucun effet statistique sur le témoin non traité. La 10-HSA et la 12-HSA ont légèrement modifié le protéome du collagène des fibroblastes intracellulaires avec des augmentations significatives des protéines de collagène alpha-1 (VI) et alpha-3 (VI), mais seule la 10-HSA a augmenté les niveaux de collagène alpha-2 (V), alpha-1 (III), alpha-1 (I) et alpha-2 (I) (tous  $p < 0,05$ ) avec des augmentations significativement différentes entre 10-HSA et 12-HSA pour le collagène alpha-1 (I), le collagène-3 (VI) et Collagène alpha-2 (I) ( $p < 0,01$ ). Le collagène de type III dans la peau *ex vivo* a augmenté de +47 % ( $p < 0,05$ ) de 0,05 % (1,7 mM) de rétinol, de +70 % ( $p < 0,01$ ) de 0,01 % (0,33 mM) de 10-HSA et la combinaison a augmenté les niveaux de +240 % ( $p < 0,01$  pour chaque ingrédient).

**Conclusion:** La chiral (R)-10-HSA s'est avérée supérieure à 9, 12 et 17-HSA en tant qu'agoniste de PPAR $\alpha$ . De plus, la 10-HSA a stimulé la synthèse de collagène dans la culture de fibroblastes monocouche telle qu'évaluée par protéomique et immunohistochimique. De plus, nous montrons également les effets synergiques de la 10-HSA avec le rétinol sur la synthèse du collagène III dans les explants de peau. Ces résultats soulignent en outre l'efficacité de la 10-HSA en tant qu'agoniste de PPAR $\alpha$  et ingrédient anti-âge cosmétiquement acceptable.

## INTRODUCTION

We have previously published the effect of the peroxisomal proliferator-activated receptor (PPAR) agonist, 10-hydroxystearic acid (10-HSA), as a novel anti-ageing ingredient that particularly reduces the appearance of pores and hyperpigmented lesions [1]. As reduced type I and III collagens are reported in both chronologically aged and in photodamaged skin, the biochemical induction of fibroblast collagens type I and III with 10-HSA indicated its potential skin anti-ageing effects [1–4]. Obviously, improvements in the overall skin ageing process are not limited to the induction of collagen proteins only. For example, the effects of tretinoin on the elastin and fibrillin-rich oxytalan fibres are well established [4]. However, 10-HSA is also known to induce and secrete other important extracellular matrix proteins in fibroblasts *in vitro* [1].

Effects of other hydroxystearic acid variants are reported in the literature [5, 6], and one, 12-HSA, has also been identified as a PPAR $\alpha$  agonist and has been used topically [7, 8]. However, the relative efficacies of these two hydroxystearic acid isomers as PPAR $\alpha$  agonists remain unreported as well as the phenotypic response they induce on fibroblast collagen synthesis. We also wanted to evaluate the structure–activity relationship of mono-hydroxy stearic acids that are naturally occurring or easy to produce such as from saturated fatty acids, either by enzymatic or by chemical synthesis. As a result, we report the efficacy of 9-HSA, 10-HSA, 12-HSA and 17-HSA together with stearic acid.

Moreover, retinoids are also used as anti-ageing ingredients that also induce collagen synthesis with type III collagen induction being more promoted by retinoic acid (RA) compared with retinol (ROH) [9]. As a result, the use of type III collagen as an RA-responsive marker maybe useful to identify ROH boosting strategies. Co-treatment of retinoids (retinoic acid or retinyl retinoate) with PPAR $\alpha$  agonists has been reported to reduce retinoid dermatitis suggesting benefits from combining agonists to the retinoid and PPAR transcription factors [10]. This could be the result of activating retinoid and PPAR pathways independently or may be the result of PPAR $\alpha$  interacting with the retinoid X receptor (RXR) [11]. As a result, the additional focus of this preliminary work was to also demonstrate the efficacy of ROH combined with 10-HSA on collagen synthesis *ex vivo*.

## MATERIALS AND METHODS

### Hydroxystearic acid variants

10-HSA was synthesized as previously described [1]. 9-HSA and 17-HSA were synthesized using a proprietary method.

12-HSA and SA were purchased from Sigma-Aldrich, Buchs, Switzerland. All variants were at least 95% pure.

### Experimental strategy

To determine the effectiveness of HSA isomers as PPAR $\alpha$  agonists, analyse the effects of 10-HSA and 12-HSA on extracellular matrix marker production (collagen type I) in fibroblast monolayer cultures, compare the collagen proteome by mass spectrometry proteomics and evaluate the synergistic response of 10-HSA together with retinol (ROH) on collagen type III synthesis in skin explants. All methods are similar to that published previously with the exception that the intracellular fibroblast collagen proteome is reported [1].

### PPAR $\alpha$ transactivation assay

A one-hybrid system was applied that used a hybrid construct of GAL4 DNA-binding part coupled to the human PPAR $\alpha$  ligand-binding domain, a luciferase reporter plasmid and a renilla-expressing plasmid to normalize transfection efficiency. For transient transfections, white 96-well cell culture plates with clear bottom (Corning, Basel, Switzerland) were used.  $7.5 \times 10^4$  HEK293 cells per well were plated in minimum essential medium (Eagle) at 37°C in 5% CO<sub>2</sub> without phenol red supplemented with 10% charcoal-treated foetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA). The cells were transiently transfected at 70%–80% confluence by polyethylene-mine-based transfection for 5 h at 37°C, 5% CO<sub>2</sub>, which was followed by respective applications of the test compounds (1.7  $\mu$ M) dissolved in DMSO as a vehicle (0.45% final DMSO concentration was achieved in the wells). The GW7647 (1  $\mu$ M) compound was used as a reference for human PPAR $\alpha$ . Stimulations lasted 16 h according to established protocols. All plates of a campaign were treated the same, and the only difference was the stimulation by the different compounds. All compounds were tested as 4 replicates with the exception of the vehicle ( $n = 52$ ) and positive ( $n = 24$ ) controls. The measurement of firefly and renilla luciferase luminescence was conducted sequentially in the same cell extract using buffers according to established protocols (Promega AG, Dübendorf, Switzerland). Relative transactivation values were calculated as the ratio of the luminescence of the firefly to renilla results. IBM SPSS software version 22 (SPSS; Chicago, IL, USA) was used for all statistical analysis. For multiple comparison of the means, we applied one-way ANOVA that was followed by Bonferroni multiple comparisons post hoc test with a significance level of  $p < 0.05$ .

## MEASUREMENT OF TOTAL CELLULAR COLLAGEN TYPE I CONTENTS IN NORMAL HUMAN FIBROBLASTS

Human dermal fibroblasts obtained from adult skin were seeded into 96-well plates (4000 cells/well) and cultured in Dulbecco's Modified Eagle's Medium high glucose (DMEM; Gibco Invitrogen, Basel, Switzerland) containing 10% foetal calf serum (FCS; Amimed BioConcept, Allschwil, Switzerland) and 1% penicillin/streptomycin (P/S; Invitrogen, Switzerland) at 37°C with 5% CO<sub>2</sub> for 24 h. Subsequently, the cells were starved in DMEM low glucose containing 0.2% FCS and 1% P/S for 2.5 days. Then, the starvation medium was refreshed together with the addition of the solubilized test compounds and incubated for another 48 h. Thereafter, HSA (5 µM) was diluted in the culture medium from a 10 mM dimethylsulphoxide (DMSO) stock solution. Transforming growth factor-beta 1 (TGF-β1; PeproTech, Hamburg, Germany) was used as positive control (10 ng/ml). After compound incubation cells were fixed in Dulbecco's phosphate-buffered saline (DPBS; Gibco Invitrogen, Switzerland) containing 4% formaldehyde (Life Technologies, Zug, Switzerland) for 15 min and permeabilized with 0.1% Triton-X100 (Sigma-Aldrich, Buchs, Switzerland) in DPBS for 90 s. Collagen type I or III were detected using mouse anti-human collagen I antibody (Millipore, Switzerland) and rabbit anti-human type III collagen (BioTrend, Cologne, Germany), respectively, followed by a AlexaFluor 488-conjugated secondary antibody (goat anti-mouse or anti-rabbit IgG; Life Technologies, Zug, Switzerland). We counterstained the nuclei with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Buchs, Switzerland). Image acquisition and quantitative analysis were performed using an ArrayScan VTI HCS imaging system (Thermo Scientific, Waltham, MA, USA) with 49 pictures per well with 10× objective. Collagen was measured intracellularly, and the fluorescence intensity values were normalized to the cell count. Data are based on a minimum of three independent measurements and were represented as mean values ± standard deviations (SD); differences between groups were evaluated by one-way ANOVA test followed by pairwise post hoc comparisons Dunnett's permutation test to the media control sample with a significance level of  $p < 0.05$ .

### Fibroblast collagen proteomics following treatment with 10-HSA and 12-HSA

Human dermal fibroblasts (HDF) isolated from a biopsy of an adult female donor (Fb-D) were maintained

in Dulbecco's modified Eagle's Medium high glucose (DMEM) containing 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). Cells were cultivated at 37°C in a humidified 5% CO<sub>2</sub>-air atmosphere. HDF were seeded in 6-well plates (120'000 cells per well) and cultured for 24 h in DMEM 10% FCS 1% P/S before being starved in DMEM low glucose containing 0.2% FCS and 1% P/S for 2.5 days. Starvation medium was then replaced, and solubilized compounds (5 µM) were added and incubated for 48 h. The preparation of the intracellular proteins from the treated cells was performed using 20 mM ammonium hydroxide and stringent washing with water. Intracellular protein isolates obtained from the cells were dissolved in 600 µl of 50 mM ammonium bicarbonate buffer containing 0.5 µg of trypsin was added in each culture plate well. Plates were incubated 1.5 h at 37°C. Total protein content was determined using the BCA kit from Thermo Scientific (Illkirch, France). Lysates from the same sample were pooled and acidified to block trypsin activity. Lysates were evaporated to dryness (SpeedVac, Thermo Scientific, France). Proteins were then reduced (DTT) and alkylated (IAA) and digested a second time with trypsin to complete proteins digestion. For all samples, peptides were purified by SPE chromatography (C18), dried and solubilized in 100 µl of 0.1% formic acid aqueous solution. Peptides digests (500 ng per run) were analysed by Eksigent Ultra Plus nano-LC 2D HPLC coupled to a TripleTOF 5600 (AB Sciex, Framingham, USA) mass spectrometer interfaced to a nano-spray III source. The absolute signal of peptide or protein was calculated by summing the extracted area of all unique fragment ions. For  $p$ -value calculation, a bilateral  $t$  test for homoscedastic variance was applied.  $p < 0.05$  was considered to be statistically significant.

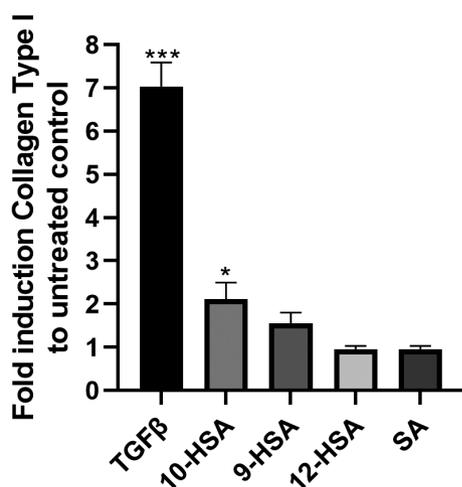
### Ex vivo skin sampling and type III collagen quantification

Human skin from abdominal plastic surgery was obtained from healthy Caucasian donors after their informed consent and was used for the analysis of collagen type III. Skin samples were cut to 8 × 3 mm (diameter × thickness) weighed and, if necessary, reduced in the dermal portion, to have approximately the same weight. They were maintained in an air-liquid interface in contact with culture medium (modified Williams' E medium, Thermo Fisher Scientific, Waltham, MA, USA) up to 6 days. Six skin samples for each treatment were cultured to perform the collagen assay and two samples for viability test. After 6 days of incubation and treatment, two samples per condition were processed with methylthiazolyldiphenyl-tetrazolium bromide (MTT) according to supplier's instructions (Roche Applied Science, Rotkreuz, Switzerland). Skin



one-hybrid test might be possible in HaCaTs or immortalized fibroblasts. However, we have never tested, nor established nor validated such assays. We believe that our established and validated PPAR $\alpha$  one-hybrid assay in HEK cells is sufficiently suited to demonstrate transactivation of PPAR $\alpha$  receptors and that the agonistic findings of the HSAs on PPAR $\alpha$  receptors are also transferable to keratinocytes and fibroblasts, as they express the PPAR $\alpha$  receptors.

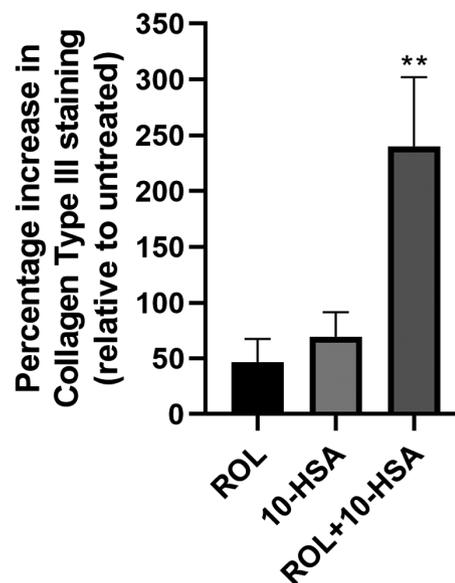
Transactivation of PPAR $\alpha$  in a reporter gene assay does not guarantee a cellular response in a PPAR $\alpha$  response gene as recruitment of coactivators or release of corepressors is not evaluated. As a result, demonstration of a marker protein is essential in the cell type of interest. We have previously demonstrated the type I and III collagen inducing activity of 10-HSA in fibroblast monolayer cultures and in skin explants [1]. Here, we compared the relative efficacy of 12-HSA, 9-HSA and stearic acid at inducing type I collagen in fibroblast



**FIGURE 1** Fold induction of collagen type I in fibroblast monolayer cultures by SA variants (5  $\mu$ M). Post hoc comparisons using Dunnett's test indicated TGF $\beta$  (10 ng/ml) was significantly different to untreated control ( $p < 0.001$ \*\*\*) as well as 10-HSA ( $p < 0.05$ \*)

monolayer cultures (all at 5  $\mu$ M). 10-HSA was shown to be numerically superior to the other acids tested and significantly increased collagen type I expression relative to the untreated control (Figure 1,  $p < 0.05$ ). 9-HSA responded similarly to that observed in the reporter gene assay compared with 10-HSA but not being significant to media control. Surprisingly, 12-HSA and stearic acid were not effective at all. This is very peculiar for 12-HSA, especially as it is reported to be a PPAR agonist [7, 8].

Proteomic analysis of fibroblast cellular proteins also demonstrated differences in collagen synthesis following 10-HSA and 12-HSA (5  $\mu$ M) treatment (Table 2). Induction of collagen alpha-1 (I) and collagen alpha-2 (I) following treatment with 10-HSA, but not 12-HSA was confirmed. 10-HSA also increased the levels of collagen alpha-2 (V) and collagen alpha-1 (III). Both acids increased the levels of collagen alpha-1 (VI) and collagen alpha-3 (VI), but



**FIGURE 2** Induction of collagen type III in skin explants by retinol (0.05%), 10-HSA (0.01%) and the combination. The combination was significantly different to the individual compounds ( $p < 0.01$ \*\*)

**TABLE 2** Fold change of collagen proteins induced by 10-HSA and 12-HSA (5  $\mu$ M) in primary fibroblast monolayer cultures determined by mass spectrometry proteomics

Protein	10-HSA Fold change	<i>p</i> -value 10-HSA to vehicle	12-HSA Fold change	<i>p</i> -value 12-HSA to vehicle	<i>p</i> -value 10-HSA to 12-HSA
Collagen alpha-2 (V)	1.35	$p < 0.05$	1.19	NS	NS
Collagen alpha-1 (III)	1.25	$p < 0.001$	1.03	NS	NS
Collagen alpha-1 (I)	1.16	$p < 0.01$	0.94	NS	$p < 0.001$
Collagen alpha-1 (VI)	1.14	$p < 0.01$	1.06	$p < 0.01$	NS
Collagen alpha-3 (VI)	1.12	$p < 0.001$	1.07	$p < 0.001$	$p < 0.01$
Collagen alpha-2 (I)	1.11	$p < 0.05$	0.97	NS	$p < 0.001$

10-HSA induced their levels more greatly. The increases in collagen peptides were significantly greater for 10-HSA compared with 12-HSA for collagen alpha-1 (I), collagen-3 (VI) and collagen alpha-2 (I) ( $p < 0.01$ ). Although these changes are small, these results are consistent with the immunohistochemistry of the fibroblast monolayer culture experiments.

Boosting the anti-ageing efficacy of ROH and/or its esters is of great interest topically. It is possible that this may be achieved with PPAR agonists. Moreover, synergy with PPAR agonists may offer routes to reduced skin irritation from retinoids. In this respect, a PPAR $\alpha$  agonist (WY14643) has been shown to reduce retinoid skin irritation [10]. Here, we were interested in the retinol boosting activity on collagen type III protein expression when combined with 10-HSA when applied topically to skin explants (Figure 2). Retinol (0.05%) and 10-HSA (0.01%) increased the protein expression of collagen type III by +47% and +70% respectively, when combined the expression increased by +240% at 0.05% ROH and 0.01% 10-HSA concentrations respectively. All tests were significantly different to the vehicle control ( $p < 0.05$ ), and the combinations were also significantly different to the individual compounds ( $p < 0.05$ ). Thus, 10-HSA cooperates with retinol in collagen biosynthesis probably by 10-HSA bound PPAR $\alpha$  partnering with RXR like that reported in astrocytes [12], although we cannot exclude an RAR-RXR response or both. Although fatty acids are also reported to bind to the retinoid X receptors, the transcriptional heterodimer partners involved in a PPAR response, hydroxy monounsaturated fatty acids do not [5, 11, 13]. Clearly, for the 10-HSA and retinol synergistic response, retinol must be converted to other retinoids for effects on RAR and/or RXR and for their effects in skin [14–16]. It is possible that the enzyme machinery for retinoid synthesis has been upregulated by 10-HSA bound PPAR $\alpha$  as has been shown for PPAR $\gamma$  in human dendritic cells [17]. We are in the process of determining the precise biochemical mechanisms for the effect between ROH and 10-HSA.

## CONCLUSIONS

In conclusion, chiral (*R*)-10-HSA has been shown to be superior to 9-HSA, 12-HSA and 17-HSA as a PPAR $\alpha$  agonist. Moreover, 10-HSA stimulated collagen synthesis in monolayer fibroblast culture as assessed by proteomics and immunohistochemically. Furthermore, we also show the synergistic effects of 10-HSA with retinol on collagen III synthesis in skin explants. These results further highlight the efficacy of 10-HSA as a cosmetically acceptable PPAR $\alpha$  agonist and anti-ageing ingredient.

## ACKNOWLEDGEMENTS

This work was fully funded by DSM Nutritional Products. AVR is a consultant to DSM. We thank Dr. Marco Massironi (Cutech, Italy) for his significant scientific support in performing the ex vivo studies.

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**How to cite this article:** A.V. Rawlings, E. Wandeler, I. Bendik, P. Fuchs, J.-M. Monneuse, D. Imfeld, R. Schütz, Effect of regioisomers of hydroxystearic acids as peroxisomal proliferator-activated receptor agonists to boost the anti-ageing potential of retinoids. *Int. J. Cosmet. Sci.* 43, 619–626 (2021). <https://doi.org/10.1111/ics.12730>