A novel non-drug SFRP1 antagonist inhibits catagen development in human hair follicles *ex vivo*

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Conflict of Interest: MB, JC, NH, LP are or were employees or consultants of Monasterium Laboratory, a CRO performing contracted research in the field of dermatology, for which RP serves as CEO. RP is also a consultant of Giuliani S.p.A., while DP is an employee, which has filed a patent (P020378WO-01) for the use of iminooxothiazolidine derivatives in the cosmetic and trichological field.

Wnt signalling is one of the most fundamental molecular pathways for modulating the human hair cycle and is essential for maintaining hair growth[1]. We recently identified that the Wnt

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antagonist, secreted frizzled related protein 1 (SFRP1), prematurely induces catagen in microdissected human scalp hair follicles (HFs) *ex vivo*[1]. SFRP1 is inhibited by the potent hypertrichosis inducing immunosuppressant, cyclosporine A (CsA)[2], and the specific SFRP1 antagonist, WAY-316606, both of which prevent spontaneous catagen development[1]. Collectively, this establishes SFRP1 antagonists as attractive candidate therapeutics for management of hair loss disorders.

However, the immunosuppressive potency and overall toxicity of CsA[2] prohibits its long-term application for hair loss indications, while the hair growth-promoting candidate WAY-316606[1], awaits licensing for clinical use. Therefore, it would be optimal to develop topically applicable, non-drug SFRP1 antagonists, whose long-term application in cosmetic formulations is expected to limit agent toxicity. Therefore, we have explored a group of potentially cosmetically applicable iminooxothiazolidine derivates that are known to inhibit SFRP1 activity *in vitro*[3] as candidate human hair growth promoters.

First, we evaluated the toxicological profile of selected iminooxothiazolidine derivates[3] using VEGA, a quantitative *in silico* platform that predicts a range of compound toxicity parameters. This revealed compound C1 (methyl 2-[[2-(2-imino-4-oxo-thiazolidin-5-yl)acetyl]amino]-4,5-dimethyl-thiophene-3-carboxylate)[3] to be the most favourable derivate due to the lack of toxicological alerts. C1 was next assessed for its safety in dermatological applications. Specifically, *in vitro* assays determined that C1 is not a skin irritant (OECD Test method 439), ocular irritant (OECD 492) or a sensitizer (RHE IL-18)[4]. Next, genotoxic evaluation *in silico* (VegaNic version 1.0.8) and in three different *in vitro* tests (Ames test, micronuclei assay and comet assay)[4] demonstrated that C1 is not predicted to be genotoxic. Frog Embryo Teratogenesis Assay in Xenopus (FETAX)[4] was also performed to exclude effect on

embryogenesis, which resulted in no observable adverse effect level (NOAEL) of 50 µM. Finally, we assessed whether C1 is immunosuppressive, namely inhibits T-cell proliferation and IL-2 production, i.e. two well-established key downstream indicators of CsA-associated immunosuppression. This showed that C1 had NOAEL at 45 µM. Furthermore, with a molecular weight of 341.4 Da and logP=0.6, C1 is expected to be minimally absorbed systematically and not accumulating[5]. Collectively, these data suggest that C1 is suitable for cosmetic applications. (All toxicology and dermatological safety data are available upon request).

Next, we determined if C1 (15 µM[3]) prolongs anagen in microdissected, organ-cultured human occipital/temporal scalp HFs cultured over 5-6 days[1,2] compared to the vehicle control (0.1% DMSO), using WAY-316606 (2 µM) as positive control[1]. Standardised quantitative (immuno-)histomorphometry[1] revealed that a higher percentage of HFs remained in anagen VI after treatment with either C1 or WAY-316606, compared to the vehicle control (**Figure 1**). Despite the substantial inhibition of catagen development exerted by C1, only treatment with WAY-316606 significantly reduced the hair cycle score (**Figure 1**).

To validate this effect, we quantified the proliferation and apoptosis of hair matrix keratinocytes (HMKs) using quantitative Ki-67/TUNEL immunohistomorphometry[1,2]. The percentage of Ki-67$^+$ germinative HMKs tendentially increased after C1 treatment as compared to vehicle control, to a similar extent to that of WAY-316606 (**Figure 1**). Conversely, there was no significant changes in the percentage of TUNEL$^+$ cells within the HM of treated HFs (**Figure 1**), validating our previous report that SFRP1 inhibition does not affect apoptosis in human HFs[1]. This observation also corroborates our C1 toxicology data, as we did not detect indicators of HF cytotoxicity, such as enhanced apoptosis and reduced proliferation of

HMKs (**Figure 1)**, or a disruption of the HF pigmentary unit (data not shown), which are typically seen with cytotoxic compounds[6].

Since SFRP1 is a prominent inhibitor of Wnt activity in the human HM[1], we finally assessed whether C1 increases Wnt activity. 6hr' treatment with C1 significantly increased the transcription of the Wnt target gene, *LEF1*[1]*, ex vivo,* and this even to a greater extent than WAY-316606 (**Figure 1**), indicating that C1 indeed stimulates Wnt activity in human HF.

The fact that C1 is not as beneficial as WAY-316606 in positively regulating human hair cycle *ex vivo* despite the higher stimulation of Wnt signalling may be related to the short ex vivo treatment, suboptimal concentration, or the importance of fine-tuning Wnt signalling during regeneration processes[7].

Collectively, we unveil a new cosmeceutically applicable SFRP1 antagonist, the Wnt-activating iminooxothiazolidine derivate, C1[3], which prolongs anagen and stimulates HMK proliferation *ex vivo*. Given the favourable toxicological profile of C1, its topical application therefore deserves to be clinically explored as an adjuvant cosmetic therapy for hair loss disorders associated with premature catagen development leading to telogen effluvium, such androgenetic alopecia[8].

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Figure Legend

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Figure legend

Figure 1. Compound C1 stimulates Wnt signaling and prolongs anagen in the human hair follicle. Microscopic quantification of hair cycle staging and hair cycle score (arbitrary assignment of a score to HFs in anagen=100, early catagen=200, and mid catagen=300) using Masson-Fontana (images not shown), and Ki-67/TUNEL. Mean ± SEM, *N*=27HFs/experimental group from 3 independent experiments (donors). Quantification of Ki-67+ and TUNEL+ cells in the hair matrix. Mean ± SEM, *N*=16-24HFs/experimental group from 3 independent experiments (donors). Representative images of Ki-67/TUNEL immunofluorescence showing reference areas for evaluation of Ki-67 (yellow line) and TUNEL (yellow and green lines) and Auber's Line (white line).

QRT-PCR analysis of *LEF1* mRNA. Mean ± SEM, *N*=3 technical replicates/donor from 2 independent experiments (donors). RNA isolated from n=8 HFs/experimental group/donor. Kruskal-Wallis test and Dunn's multiple comparison test or One-WAY ANOVA and Tukey's multiple comparison test, ###p<0.001, and Mann Whitney test or Student's *t*-test, *p<0.05, **p<0.01. DP= dermal papilla, HM= hair matrix, WAY= WAY-316606. Scale bar: 50µm.