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**Advanced inhibition of undesired human hair growth by
PPAR γ modulation?**

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Short title: GMG-43AC induces catagen

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Abbreviations: HF, hair follicle; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; LPP, lichem planopilaris; NHK, normal human keratinocyte; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor

TO THE EDITOR

The inhibition of unwanted hair growth (hirsutism, hypertrichosis) by safe, but effective agents that do not destroy the hair follicle (HF) remains an important challenge in clinical dermatology. Being interested in identifying alternative therapies to depilation, laser therapy, anti-androgens and eflornithine (Blume-Peytavi, 2013), we explored whether modulating peroxisome proliferator-activated receptors (PPAR γ , α , and β/δ), namely PPAR γ , may provide a promising novel strategy for the inhibition of excessive human hair growth.

As part of the nuclear receptor super-gene family, PPARs control the expression of genes which regulate inflammation and lipid metabolism (*s8, s15*). Activation of all three PPARs alters the expression of inflammatory mediators including cytokines, receptors and adhesion molecules, thereby exhibiting an overall strong anti-inflammatory effect (Choi and Bothwell, 2012). Moreover, PPAR γ activation stimulates keratinocyte and sebocyte differentiation as well as epidermal lipid synthesis and regulates epidermal barrier homeostasis (*s4, s12*). From a hair growth perspective, PPAR γ agonists are particularly interesting since PPAR γ -signaling is required for the maintenance of HF epithelial stem cells in mice, while insufficient PPAR γ -signaling may predispose patients to develop the inflammatory cicatricial alopecia, lichen planopilaris (LPP) (Karnik *et al.*, 2009, *s2*), even though the occurrence of individual LPP lesions may not be PPAR γ -driven (Harries *et al.*, 2013).

Hence, PPAR γ agonists are interesting from a hair and stem cell biology perspective as well as in scarring alopecia. Additionally, given that there is growing interest in producing selective PPAR γ modulators that retain the desirable therapeutic activities without the unwanted effects (Higgins and Depaoli, 2010), we tested the hair growth-modulatory effects of a newly designed anti-inflammatory PPAR γ modulator, GMG-43AC, a new chemical entity with structural similarity to propionic acid, with a favorable toxicological profile (Pirat *et al.*, 2012) and documented selective action as a PPAR γ -modulator (see Supplementary Text and Supplementary Figure S1a-c).

This was studied in microdissected, organ-cultured female human scalp HFs (Kloepper *et al.*, 2010), while potential anti-inflammatory effects of GMG-43AC were assessed in normal human keratinocytes (NHKs) exposed to defined inflammatory stimuli.

Measurement of lactate dehydrogenase (LDH) levels in the HF organ culture supernatants showed only a slight increase in LDH activity by day 6 (Supplementary Figure S2a), suggesting only low-level GMG-43AC-associated general tissue toxicity. While administration of GMG-43AC for 6 days did not alter hair shaft elongation (Supplementary Figure S2b), HF cycle analyses revealed that all tested GMG-43AC doses strongly decreased the percentage of HFs in anagen, and induced premature HF regression (catagen development) (Figure 1a): while only 55% of the HFs treated with vehicle transformed into the catagen phase after 6 days, almost all (85%) of the

0.01mM GMG-43AC-treated HFs were in this phase. In the two higher doses of GMG-43AC, 70% of HFs were in the catagen phase. These results were independently confirmed by measuring the hair cycle score (s_3), which reached significance in the 0.01 mM dose (Figure 1b), supporting catagen promotion. While GMG-43AC did not significantly alter the percentage of proliferating hair matrix keratinocytes in anagen VI HFs, GMG-43AC tended to stimulate keratinocyte apoptosis in the hair matrix (significance reached at 0.1 mM) (Supplementary Figure S2c).

Since PPAR γ -mediated signaling is important for the maintenance of murine HF epithelial stem cells and is defective in human LPP patients (Karnik *et al.*, 2009) we also explored whether GMG-43AC modulates *in situ* the expression of two signature keratins that are up-regulated by human HF progenitor cells, i.e. keratins K15 and K19 (Kloepper *et al.*, 2008; Lyle *et al.*, 1998; Michel *et al.*, 1996). Interestingly, GMG-43AC significantly upregulated K15-immunoreactivity in all tested doses (Figure 1c), but did not significantly alter the number of K15-positive cells (Figure 1c). A positive impact on human HF epithelial progenitor cells was independently corroborated by demonstrating that K19-immunoreactivity was also significantly upregulated by GMG-43AC (Figure 1d). This PPAR γ -modulator also elevated the relative number of K19-positive cells in the lower outer root sheath (Figure 1d). These observations suggest that GMG-43AC may effectively inhibit unwanted human hair growth by inducing catagen while simultaneously preserving (or possibly even promoting) the HF's epithelial progenitor cell pools.

As a first attempt towards identifying early-response candidate target genes of GMG-43AC-mediated stimulation, two independent sets of organ-cultured female human scalp HFs were compared by genome-wide DNA microarray analysis (see Supplementary Text). This identified 39 genes as being substantially *upregulated* (i.e. >3 fold, $p < 0.05$) in the HFs of *both* female patients investigated by stimulation with 0.01 mM GMG-43AC for 6 hours (Supplementary Table S1). Among these upregulated genes, none was a gene recognized to be specific for epithelial HF stem cells. However, *COL6A1*, which encodes the alpha 1 chain of collagen VI, is known to be expressed in very close proximity to the HF (Watson *et al.*, 2001) and may be involved in HF development during embryogenesis (Smith, 1994). Therefore, we validated this maximally regulated gene by quantitative RT-PCR, which independently confirmed that 0.01 mM GMG-43AC significantly increased *COL6A1* mRNA expression (Supplementary Figure S2d). While the mechanisms that underlie the observed HF and HF stem cell effects of GMG-43AC remain to be systematically dissected, the current microarray analysis has at least identified some candidate early-response target genes (for extended discussion of microarray results, see legend of Supplementary Table S1).

Since PPAR γ activation is known to elicit an anti-inflammatory response (Choi and Bothwell, 2012), we finally investigated the effect of GMG-43AC on mRNA expression and protein secretion of interleukin (IL)-6 in NHKs treated with interferon

(IFN)- γ or tumor necrosis factor (TNF)- α . As expected, treatment of NHKs with IFN- γ (30 ng/ml) or TNF- α (10 ng/ml) for 6 h markedly increased the mRNA level as well as the released amount of IL-6, while co-administration of GMG-43AC prevented the above effects both at the mRNA (Figure 2a,b) and protein levels (Figure 2c,d). Notably, no significant change in the mRNA expression or the secreted amount of IL-6 was seen following treatment with GMG-43AC (Figure 2). The mechanism of this anti-inflammatory action of GMG-43AC involves a trans-repression of NF- κ B signaling pathway (data not shown).

The current pilot study shows that PPAR γ activation via GMG-43AC (Figure S1) represents an interesting novel hair growth-inhibitory strategy that constitutes a substantial advance over currently practiced therapies for managing undesired hair growth: Our results with this novel PPAR γ -modulator suggest that stimulating PPAR γ -signaling can effectively abrogate hair growth by premature catagen induction. While other hair growth-inhibitory compounds also promote catagen [e.g. tretinoin (Foitzik *et al.*, 2005), eflornithine (Kloepper *et al.*, 2010) or dopamine (Langan *et al.*, 2013)], GMG-43AC appears to actually protect human HF epithelial stem cells, as indicated by its stimulatory effects on K15 and K19 protein expression and the number of K19+ cells.

Along with the well-appreciated anti-inflammatory effects of PPAR γ agonists (here exemplarily demonstrated for GMG-43AC in cultured NHKs), this promises that PPAR γ -modulators like GMG-43AC may allow to optimize human hair growth inhibition by effectively suppressing unwanted hair growth by pushing anagen HFs into catagen while simultaneously preserving the HF's epithelial progenitor cells pool. The latter appears important to maintain the HF and its stem cells as a regeneration resource for optimal skin wound healing (Plikus *et al.*, 2012; Xiong *et al.*, 2013). If applied together with standard depilatory therapies, the anti-inflammatory effects of PPAR γ -modulators like GMG-43AC may also reduce the pro-inflammatory sequelae of depilation. Together with its favorable toxicity profile (Pirat *et al.*, 2012), this renders topically applied GMG-43AC an interesting candidate agent for clinical testing in the future management of hirsutism. Moreover, the anti-inflammatory and candidate "stem cell-protective" properties of this PPAR γ modulator also invite the future testing of GMG43-AC in cicatricial alopecia such as LPP [e.g., it is conceivable that GMG-43AC might inhibit LPP progression based on irreversible HF stem cell damage (Harries *et al.*, 2013; Karnik *et al.*, 2009)].

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Figure legends

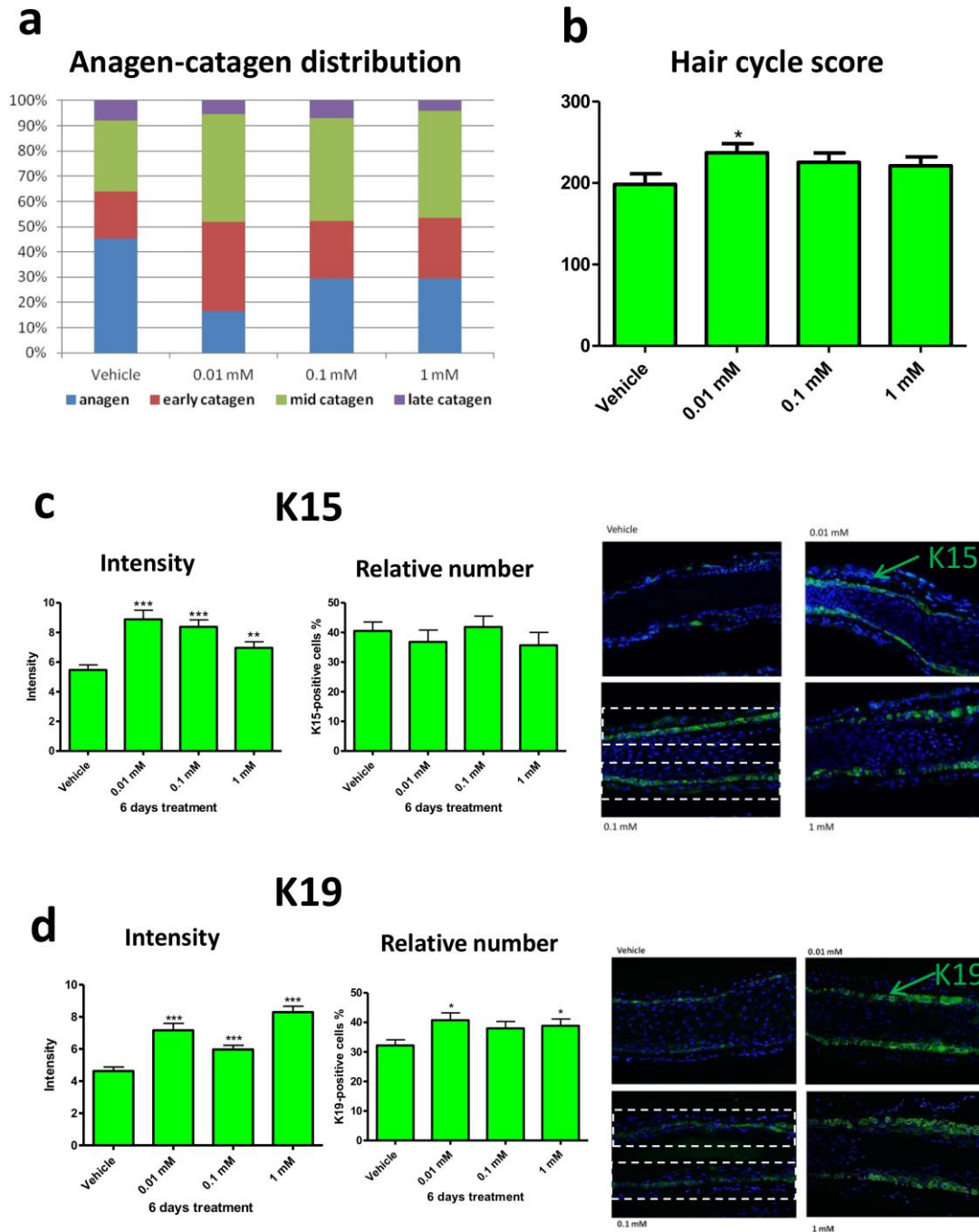


Figure 1. GMG-43AC enhances premature catagen, but stimulates keratins K15 and K19. (a) Percentage of HF in anagen, early, mid or late catagen. GMG43-AC administration induced catagen. (b) Hair cycle score, determined as previously described (s4). Higher score means catagen induction. Columns represent means±SEM; data were pooled from 5 independent experiments, n=54-71 HF for each group. (c-d) GMG43-AC stimulated immunoreactivity of K15 (c) and K19 (d) in organ-cultured human scalp HF. Staining intensities were measured in defined reference areas (white rectangles) using ImageJ software. Additionally, the number of

K15- (c) and K19-positive (d) cells was counted relative to the total number of DAPI-positive cells. Columns represent means \pm SEM; n=22-44 HF/group. * P <0.05, ** P <0.01, *** P <0.001; unpaired two-tailed Student's t-test.

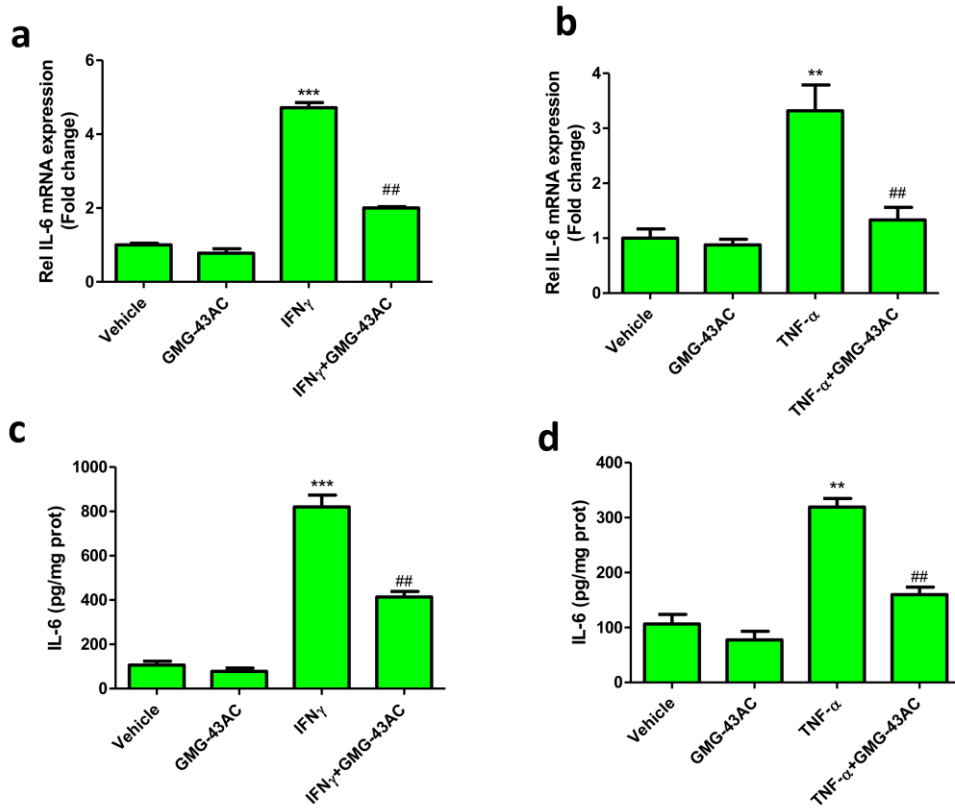


Figure 2. Suppression of IL-6 in response to GMG-43AC. (a-b) mRNA transcript level of *IL-6* evaluated by real time PCR in normal human keartinocytes (NHKs) stimulated with IFN γ (30ng/ml) and TNF- α (10ng/ml) in the presence of GMG-43AC (0.5 mM) for 6 h. Values are normalized to *GAPDH* and expressed relative to untreated cells. (c-d) IL-6 protein level measured by ELISA assay in NHKs supernatants treated with IFN γ (30ng/ml) and TNF- α (10ng/ml) in the presence of GMG-43AC (0.5 mM) for 24 h. Cytokine levels were measured in duplicates for each condition and expressed as pg/mg protein. Statistical significance between groups was assessed using paired Student's *t*-test ** $P < 0.01$; *** $P < 0.001$ vs Ctr ## $P < 0.01$ vs stimulus.