[For submission as Letter, J Invest Dermatol]

Limits: 1000 words (1258), 2 figures or tables, 15 references (16)

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Advanced inhibition of undesired human hair growth by PPARγ 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 (PPARγ, α, and β/δ), namely PPARγ, 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, PPARγ activation stimulates keratinocyte and sebocyte differentiation as well as epidermal lipid synthesis and regulates epidermal barrier homeostasis (*s4*, *s12*). From a hair growth perspective, PPARγ agonists are particularly interesting since PPARγ-signaling is required for the maintenance of HF epithelial stem cells in mice, while insufficient PPARγ-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 PPARγ-driven (Harries et al., 2013).

Hence, PPARγ 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 PPARγ 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 PPARγ 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 PPARγ-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 (*s3*), 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 PPARγ-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 PPARγ-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 PPARγ 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-kB signaling pathway (data not shown).

The current pilot study shows that PPARγ 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 PPARγ-modulator suggest that stimulating PPARγ-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 PPARγ agonists (here exemplarily demonstrated for GMG-43AC in cultured NHKs), this promises that PPARγ-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 PPARγ-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 PPARγ 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)].

Funding resources: This study was supported in part by a research grant from Giuliani S.p.A, Milano, Italy, to R.P. and M.P.

Conflict of interest: R.P. and M.P. have received basic research grants from Giuliani S.p.A., Milano, Italy, and have served in a consultancy function for this company. Y.R. has received travel support from Giuliani S.p.A.

Acknowledgements

The excellent technical assistance of Astrid Becker and Heike Krauth is gratefully acknowledged. This study was supported by a basic research grant from Giuliani Srl, Milano, to RP.

References

Blume-Peytavi U (2013) How to diagnose and treat medically women with excessive hair. *Dermatologic clinics* 31:57-65.

Choi JM, Bothwell AL (2012) The nuclear receptor PPARs as important regulators of T-cell functions and autoimmune diseases. *Mol Cells* 33:217-22.

Foitzik K, Spexard T, Nakamura M, *et al.* (2005) Towards dissecting the pathogenesis of retinoid-induced hair loss: all-trans retinoic acid induces premature hair follicle regression (catagen) by upregulation of transforming growth factor-beta2 in the dermal papilla. *J Invest Dermatol* 124:1119-26.

Harries MJ, Meyer K, Chaudhry I, *et al.* (2013) Lichen planopilaris is characterized by immune privilege collapse of the hair follicle's epithelial stem cell niche. *The Journal of pathology*.

Higgins LS, Depaoli AM (2010) Selective peroxisome proliferator-activated receptor gamma (PPARgamma) modulation as a strategy for safer therapeutic PPARgamma activation. *Am J Clin Nutr* 91:267S-72S.

Karnik P, Tekeste Z, McCormick TS, *et al.* (2009) Hair follicle stem cell-specific PPARgamma deletion causes scarring alopecia. *J Invest Dermatol* 129:1243-57.

Kloepper JE, Sugawara K, Al-Nuaimi Y, *et al.* (2010) Methods in hair research: how to objectively distinguish between anagen and catagen in human hair follicle organ culture. *Exp Dermatol* 19:305-12.

Kloepper JE, Tiede S, Brinckmann J, *et al.* (2008) Immunophenotyping of the human bulge region: the quest to define useful in situ markers for human epithelial hair follicle stem cells and their niche. *Exp Dermatol* 17:592-609.

Langan EA, Lisztes E, Biro T, *et al.* (2013) Dopamine is a novel, direct inducer of catagen in human scalp hair follicles in vitro. *Br J Dermatol* 168:520-5.

Lyle S, Christofidou-Solomidou M, Liu Y, *et al.* (1998) The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. *Journal of cell science* 111 (Pt 21):3179-88.

Michel M, Torok N, Godbout MJ, *et al.* (1996) Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. *Journal of cell science* 109 (Pt 5):1017-28.

Pirat C, Farce A, Lebegue N, *et al.* (2012) Targeting peroxisome proliferator-activated receptors (PPARs): development of modulators. *J Med Chem* 55:4027-61.

Plikus MV, Gay DL, Treffeisen E, *et al.* (2012) Epithelial stem cells and implications for wound repair. *Seminars in cell & developmental biology* 23:946-53.

Smith LT (1994) Patterns of type VI collagen compared to types I, III and V collagen in human embryonic and fetal skin and in fetal skin-derived cell cultures. *Matrix Biol* 14:159-70.

Watson RE, Ball SG, Craven NM, *et al.* (2001) Distribution and expression of type VI collagen in photoaged skin. *Br J Dermatol* 144:751-9.

Xiong Y, Li W, Shang C, *et al.* (2013) Brg1 governs a positive feedback circuit in the hair follicle for tissue regeneration and repair. *Developmental cell* 25:169-81.

Figure legends

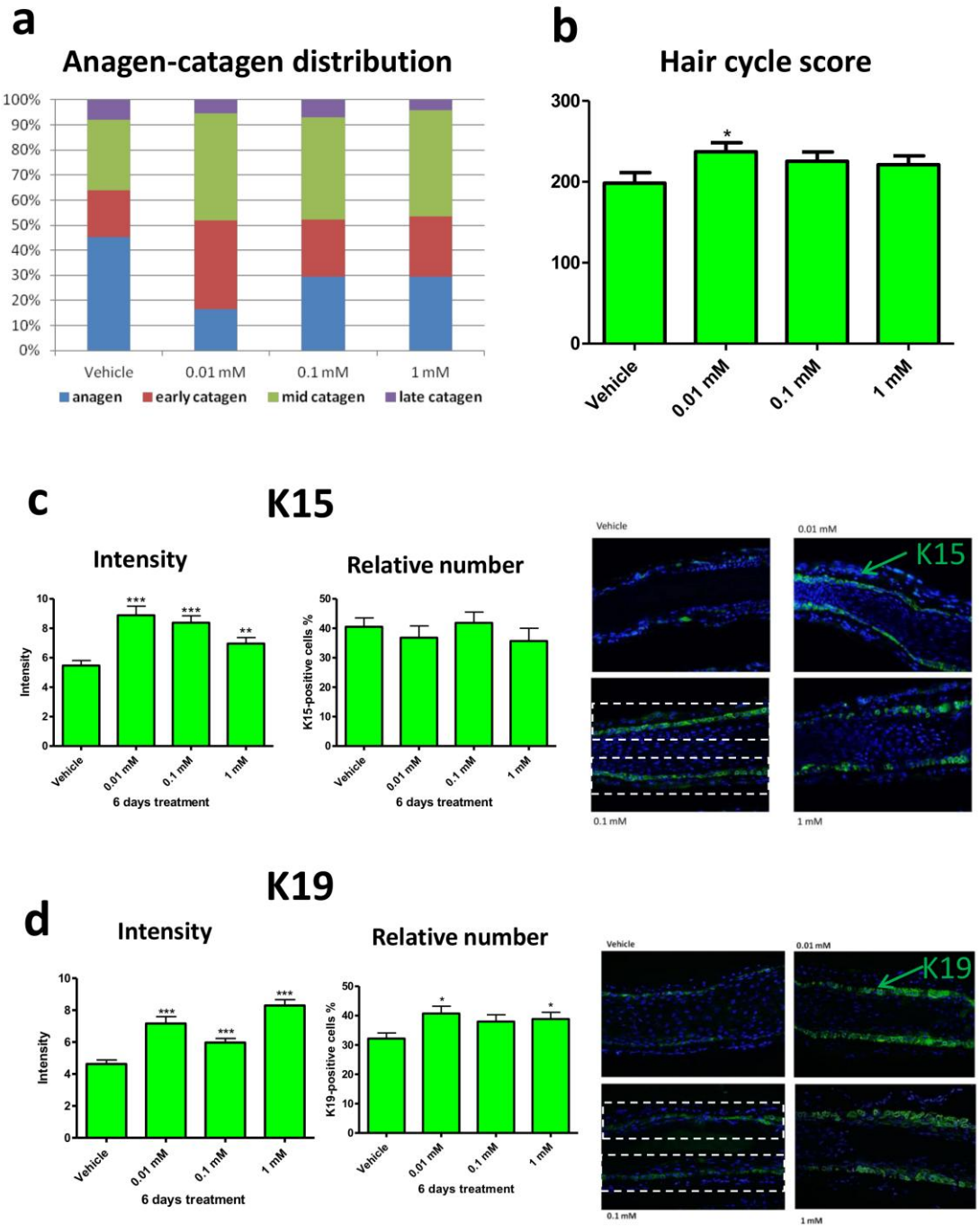

Figure 1. GMG-43AC enhances premature catagen, but stimulates keratins K15 and K19. (a) Percentage of HFs in anagen, early, mid or late catagen. GMG43-AC administration induced catagen. **(b)** Hair cycle score, determined as previously described (*s4*). Higher score means catagen induction. Columns represent means±SEM; data were pooled from 5 independent experiments, n=54-71 HFs for each group. **(c-d)** GMG43-AC stimulated immunoreactivity of K15 (c) and K19 (d) in organ-cultured human scalp HFs. 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±SEM; n=22-44 HFs/group. *P<0.05, **P<0.01, ***P<0.001; unpaired two-tailed Student's t-test.

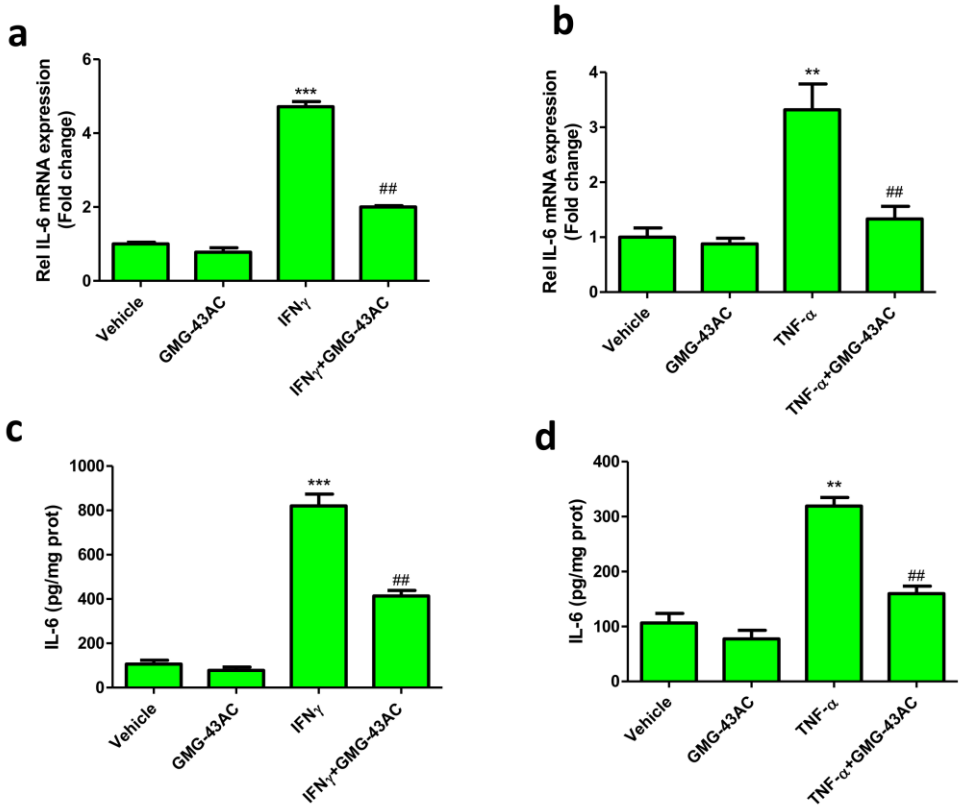

Figure 2. Suppression of IL-6 in response to GMG-43AC. (a-b) mRNA transcript level of *IL-6* evaluated by real time PCR in normal human keartinocytes (NHKs) stimulated with IFNγ (30ng/ml) and TNF-α (10ng/ml) in the presence of GMG-43AC (0.5 mM) for 6 h. Values are normalized to *GAPDH* and expressed relative to untreated cells. **(c-d)** IL-6 protein level measured by ELISA assay in NHKs supernatants treated with IFNγ (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.