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# THE THYROID HORMONE ANALOGUE KB2115 (EPROTIROME) PROLONGS HUMAN HAIR GROWTH (ANAGEN) *EX VIVO*

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*Running title:* Eprotirome prolongs anagen

*Key words:* hair follicle, hair cycle, anagen phase, thyroid hormone analogue

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*Abbreviations:* HF: hair follicle; TH: thyroid hormone; TR: thyroid hormone receptor; T4: thyroxine

**TO THE EDITOR**

Premature termination of the active growth phase of the human hair follicle (HF) cycle (anagen) lies at the base of most clinically important hair loss disorders. Therefore, the development of effective, topically applied and safe anagen-prolonging agents constitutes one of the principle challenges in translational human hair research (Paus, 2006; s1-s2).

Previously, we had shown that thyroid hormones (THs) significantly prolong anagen phase, and retard the onset of apoptosis-driven HF involution (catagen) (van Beek et al., 2008; Gáspár et al., 2010), strongly supporting the concept that TH receptors (TRs) are promising targets for treating HF cycle-based hair loss disorders (Billoni et al., 2000; s3). However, due to their potential systemic adverse effects, such as cardiovascular ones (Jameson, 2013; s4) and overstimulation of mitochondrial activity (Cioffi et al., 2013), administration of "classical" TR agonists, especially those that fully target the myocardially expressed TRα (Mullur et al., 2014), are difficult to justify for non-life-threatening hair loss disorders.

Therefore, in the current study we asked whether a synthetic TR-modulator, KB2115 (eprotirome), which shows higher affinity for TRβ than for TRα (Shiohara et al., 2012) and thus is less likely to exert cardiovascular adverse effects than thyroxine (T4) (Ladenson et al., 2010), may also prolong anagen. This was tested in microdissected, organ-cultured human scalp HFs in serum-free medium and without addition of natural THs (Kloepper et al., 2010; Langan et al., 2015). The study was approved by the Ethics

Committee of the University of Lübeck and the University of Münster (*reference No. 06-109, 2014-041-b-N*, *2015-602-f-S*) and written informed consent was obtained from the patients (for details, see **Supplementary Materials and methods** section and **Supplementary Figure S1a-f**).

First, we probed whether KB2115 stimulated mitochondrial function in the HF epithelium by assessing how low concentrations (1 and 100 pM) of KB2115 influence the expression of mitochondrially encoded cytochrome C oxidase I (MTCO1; a key enzyme of respiratory complex IV). We had previously documented that the immunoreactivity intensity of MTCO1 correlates well with respiratory complex IV activity and even mitochondrial biogenesis (Knuever et al., 2012; Poeggeler et al., 2010; Vidali et al., 2014); moreover classical THs up-regulate complex IV activity as well as MTCO1 immunoreactivity in human HF epithelium (van Beek et al., 2008). This showed that KB2115 did not significantly alter MTCO1 immunoreactivity (**Supplementary Figure S2a-b**) suggesting that the tested picomolar concentrations may not exert major effects on energy metabolism.

Preliminary results from microarray analysis (data not shown), followed-up by qRT-PCR for four selected TH-responsive, mitochondrial biology-related genes (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 [PGC1]-α and -β as well as sarco/endoplasmic reticulum $Ca^{2+}$-ATPase 2 [SERCA2]) (s5-s7) and mitochondrial creatine kinase 1A (CMKT1A) showed that KB2115 induced significantly smaller (if any) up-regulation of the above genes in human HFs, compared to T4 (**Supplementary Figure S3**). This supports the concept that KB2115 is less likely to exert unwanted

systemic, mitochondrial activity-related adverse effects than T4 (see **Supplementary Discussion 1** for details).

Therefore, we studied the effects of these (presumably "mitochondrially safe") concentrations of KB2115 on the spontaneous anagen-catagen transition of human scalp HFs *ex vivo*. Quantitative hair cycle histomorphometry (Kloepper et al., 2010) showed that KB2115 treatment significantly increased the percentage of anagen HFs compared to vehicle controls (**Figure 1a**). Given that KB2115 thus effectively countered the tendency of organ-cultured HFs to spontaneously enter into catagen (Kloepper et al., 2010; Langan et al., 2015), our findings support the concept that KB2115 may prevent or reduce premature catagen entry leading to telogen effluvium *in vivo*.

The anagen-prolonging effect of KB2115 was supported by the finding that the percentage of Ki67+ (i.e. proliferating) cells in the hair matrix was also significantly increased; whereas, the percentage of apoptotic (TUNEL+) hair matrix cells remained unchanged (**Figure 1b-c**).

KB2115 also slightly, but non-significantly increased hair bulb melanin content compared to vehicle control HFs, as assessed by quantitative Masson-Fontana histochemistry (**Figure 2a-b**). Moreover, KB2115 significantly increased the intrafollicular activity of tyrosinase *in situ* (s8; **Figure 2c-d**), the rate-limiting enzyme of melanogenesis (s9). Since none of the tested KB2115 concentrations significantly altered the histochemically detected hair bulb melanin content or tyrosinase activity *in situ* to controls, when exclusively anagen VI HFs were compared with each other (data not shown), this

pigmentary effect of KB2115 most likely reflects prolonged anagen-associated intrafollicular melanogenesis (Kloepper et al., 2010; s8). However, it deserves further scrutiny whether long-term administration of KB2115 may also stimulate HF pigmentation in a hair cycle-independent manner and thus may also exert “anti-graying” actions.

Finally, we asked, whether and how KB2115 impacts on the two best-studied human hair cycle-regulatory growth factors, i.e. transforming growth factor-β2 (TGF-β2), which promotes catagen, and insulin-like growth factor-1 (IGF-1), which maintains anagen (Fischer et al., 2014; Gáspár et al., 2010; s10-s11). This showed that KB2115 significantly decreased TGF-β2 protein expression in the outer root sheath (**Fig. 2e-f**). Surprisingly, intrafollicular IGF-1 protein expression was slightly decreased (**Supplementary Fig. 4a-b**), suggesting that KB2115 may prolong anagen in an IGF-1-independent manner.

That the synthetic TR-modulator, KB2115, which is expected to show reduced adverse effects compared to T4, promotes human HF growth *ex vivo* corroborates the recognized anagen-prolonging effect of endogenous THs (van Beek et al., 2008; Billoni et al., 2000). The apparently much lower impact of KB2115 than of T4 on mitochondrial activity parameters in human HF keratinocytes *in situ* suggests a favorable profile of systemic adverse effects, which may be further reduced by employing a HF-targeting topical application vehicle (s12-s13). Also unlike T4 (van Beek et al., 2008), KB2115 did not increase HF pigmentation under the current assay conditions.

Taken together, our *ex vivo* pilot data suggest that KB2115 is a promising candidate for the future treatment of hair loss disorders characterized by premature catagen entry, such as various forms of telogen effluvium and androgenetic alopecia (Paus, 2006; s1) ideally after topical application, and that KB2115 may serve as a lead compound for the development of other hair growth-promoting agents with reduced toxicity compared to T4. However, given that systemic KB2115 treatment may lead to cartilage damage in dogs (s14), further rigorous toxicological and pharmacokinetic screening is required before KB2115 is applied topically to human skin (see **Supplementary Discussion section 2** for details).

## CONFLICT OF INTEREST

This study was supported by an industry research grant (see Acknowledgement), and two of the authors (MS and CA) are employees of the sponsor. AO was employed by the sponsor between 02/01/2014 and 01/31/2015, and RP has previously served in a consultancy function for the sponsor. MS and CA are named as inventors on a patent claiming the use of eprotirome in the prevention and/or treatment of hair disorders.

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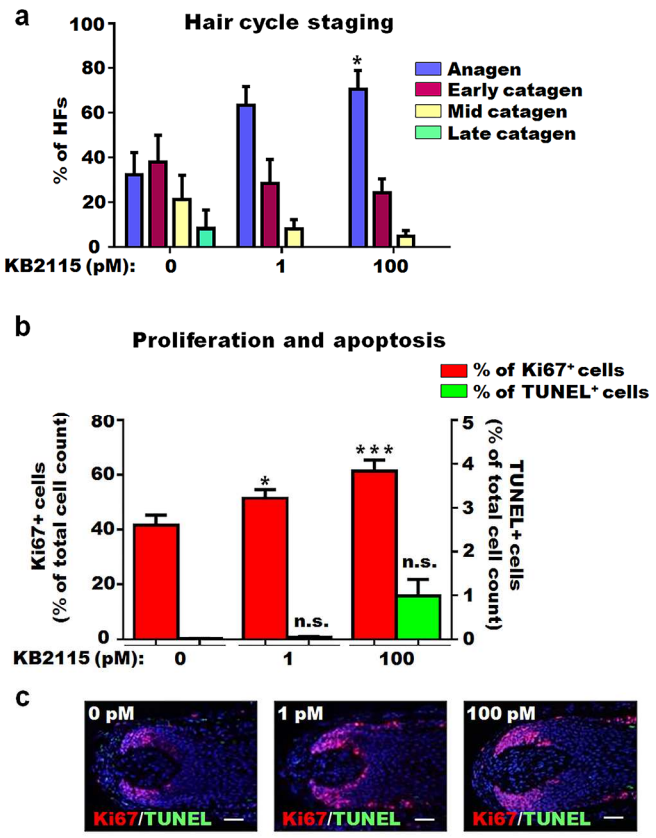
## FIGURE LEGENDS

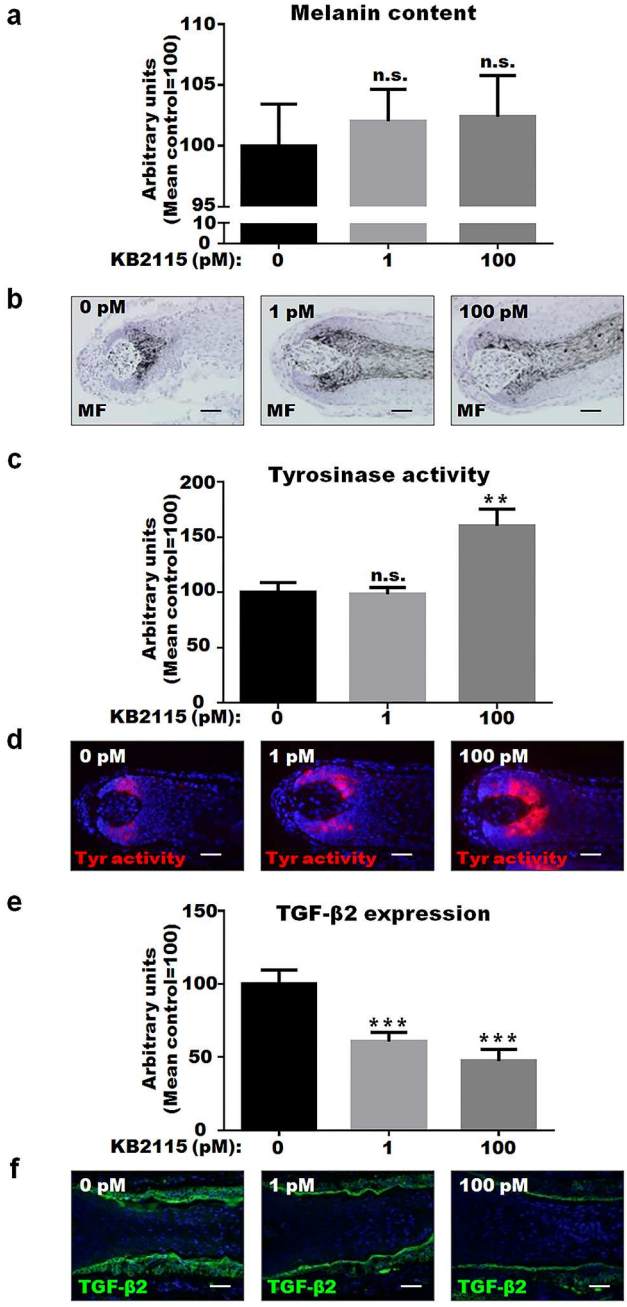
**Figure 1** – *KB2115 promotes the anagen phase, and increases proliferation of matrix keratinocytes in human HFs*

(**a**) Results of microscopic hair cycle staging of three independent donors. Ratio of anagen, early, mid and late catagen HFs was calculated in each case. Data are shown as mean±SEM of the three donors. (**b**) Percentages of proliferating (Ki67 positive) and apoptotic (TUNEL positive) matrix keratinocytes were determined. Data are expressed as mean±SEM of N=36-37 HFs from three donors. *$p$<0.05 and ***$p$<0.001 vs. control group, respectively. (**c**) Representative pictures of Ki67 (red) and TUNEL (green) double staining following the indicated treatments. Scale bar: 50 μm. Nuclei were counterstained by 4'-6-diamidino-2-phenylindole (DAPI; blue).

**Figure 2** – *KB2115 tends to increase melanin synthesis, significantly increases tyrosinase activity, and decreases TGF-β2 immunoreactivity*

(**a**, **c**, **e**) Signal intensities (determined as arbitrary units) of Masson-Fontana (MF) histochemistry (**a**), tyrosinase activity assay (**c**) and TGF-β2 immunofluorescence (**e**) were normalized to the control. Data are expressed as mean±SEM of N=37-42 (**a**), N=28-39 (**c**) or N=29-36 (**e**) HFs of three donors. **$p$<0.01, ***$p$<0.001 vs. control group. (**b**, **d**, **f**) Representative images of Masson-Fontana (black) histochemistry (**b**), tyrosinase activity (red) assay (**d**) and TGF-β2 (green) immunofluorescence (**f**). Nuclei were counterstained by hematoxylin (blue) (**b**) or by DAPI (blue) (**d**, **f**). Scale bar: 50 μm.




a
Melanin content
Arbitrary units (Mean control=100)
110
105
100
95
10
0
n.s.
n.s.
KB2115 (pM): 0 1 100
b
0 pM
1 pM
100 pM
MF
c
Tyrosinase activity
Arbitrary units (Mean control=100)
200
150
100
50
0
n.s.
**
KB2115 (pM): 0 1 100
d
0 pM
1 pM
100 pM
Tyr activity
e
TGF-β2 expression
Arbitrary units (Mean control=100)
150
100
50
0
***
***
KB2115 (pM): 0 1 100
f
0 pM
1 pM
100 pM
TGF-β2