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Efficacy of an agonist of 03B1-MSH, the palmitoyl tetrapeptide-20, in hair pigmentation

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ABSTRACT

OBJECTIVE: Hair greying (i.e., canities) is a component of chronological aging and occurs regardless of gender or ethnicity. Canities is directly linked to the loss of melanin and increase in oxidative stress in the hair follicle and shaft. To promote hair pigmentation and reduce the hair greying process, an agonist of αmelanocyte-stimulating hormone (αMSH), a biomimetic peptide (palmitoyl tetrapeptide-20; PTP20) was developed. The aim of this study was to describe the effects of the designed peptide on hair greying.

METHODS: Effect of the PTP20 on the enzymatic activity of catalase and the production of H_2O_2 by Human Follicle Dermal Papilla Cells (HFDPC) was evaluated. Influence of PTP20 on the expression of melanocortin receptor-1 (MC1-R) and the production of melanin were investigated. Enzymatic activity of sirtuin 1 (SIRT1) after treatment with PTP20 was also determined. *Ex vivo* studies using human micro-dissected hairs allowed to visualise the effect of PTP20 on the expression in hair follicle of catalase, TRP-1, TRP-2, Melan-A, ASIP and MC1-R. These investigations were completed by a clinical study on 15 human male volunteers suffering from premature canities.

RESULTS: The *in vitro* and *ex vivo* studies revealed the capacity of the examined PTP20 peptide to enhance the expression of catalase and to decrease (30%) the intracellular level of H_2O_2. Moreover, PTP20 was shown to activate *in vitro* and *ex vivo* the melanogenesis process. In fact, an increase in the production of melanin was shown to be correlated with elevated expression of MC1-R, TRP-1 and Melan-A, and with the reduction in ASIP expression. A modulation on TRP-2 was also observed. The pivotal role of MC1-R was confirmed on protein expression analyzed on volunteer's plucked hairs after 3 months of the daily application of lotion containing 10 ppm of PTP20 peptide.

CONCLUSION: The current findings demonstrate the ability of the biomimetic PTP20 peptide to preserve the function of follicular melanocytes. The present results suggest potential cosmetic application of this newly designed agonist of αMSH to promote hair pigmentation and thus, reduce the hair greying process.

INTRODUCTION

The visual appearance of humans derives predominantly from their hair and skin colour. As hair colour is considered socially important, numerous strategies have been developed to hide, prevent or reverse hair greying. Hair greying (canities) is a natural physiological process that is closely related with aging. Recent epidemiologic studies of men and women of various ethnicities revealed that between 45 and 65 years of age, 74% of people were affected by grey hair with a mean intensity of 27% [1].

Hair colour is due to the presence of a pigment, melanin, produced by melanocytes and transferred to keratinocytes. In fact, the hair follicle pigmentation results from precise sequential interactions between follicular melanocytes, matrix keratinocytes, and dermal papilla fibroblasts. It involves successively the melanogenic activity of follicular melanocytes, the transfer of their product,

melanin granules, into cortical and medullary keratinocytes, and the formation of pigmented hair shafts [2].

Numerous mechanisms, controlled by a great number of various protein factors, acting at different stages of follicular melanogenesis, contribute to hair greying, including the loss of melanocyte stem cells or their failure to differentiate, melanocyte migration defects, melanocyte apoptosis, and pigmentary machinery malfunction [3]. Indeed, as reported previously, the appearance of white hair is a phenomenon directly associated with a decrease in follicular melanocyte population and a decrease in melanin content [4]. A buildup of reactive oxygen species with a decreased ability to handle oxidative damage is also implicated in the process of hair greying [5].

The recently proven concept of H_2O_2-induced oxidative damage in the entire hair follicle, including the hair shaft, is considered as one of key elements in senile hair greying. As reported by Wood *et al*., [6] human grey/white scalp hair shafts accumulate hydrogen peroxide (H_2O_2) in millimolar concentrations. In fact, FT-Raman spectra showed *in vivo* the presence of 10^{-3}mol/L H_2O_2 concentrations in grey and completely white hair.

It is well known that catalase, one major degrading enzyme for H_2O_2, constitutes the body's primary defense against hydrogen peroxide damage. However, recent investigations revealed that catalase protein expression and hydroxyl radical scavenging activities are strongly repressed in unpigmented hair follicles leading consequently to accumulation of hydrogen peroxide, which is a chemical compound with strong oxidizing properties [8]. In addition to its direct bleaching effect (oxidation of endogenous melanin), H_2O_2 also inhibits the synthesis of melanin. Indeed, the structural modification of tyrosinase due to the oxidation of its active site Met 374 limits the functionality of this key enzyme in melanogenesis, which leads to gradual loss of hair colour [6]. In this context, it is noteworthy that oxidation and deactivation of catalase by its own substrate, hydrogen peroxide, also occurs [7]. H_2O_2-mediated oxidation resulting in the loss of biological activity has been also documented for many other important regulators of pigmentation, including the pro-eumelanogenic peptide αmelanocyte-stimulating hormone (αMSH) [8].

It is well documented that αMSH and its receptor MC1-R (melanocortin 1 receptor) are the key regulators of melanin pigment generation in both skin and hair [9]. Thus, each structural and functional perturbation of this hormone or/and its receptor will result in the decline in hair pigmentation.

The effects of αMSH are mediated by binding to MC1-R, expressed preferentially on normal human melanocytes and recognized as a key-signaling molecule of cutaneous melanogenesis. In fact, it plays a pivotal role in the activation of downstream factors, followed by sequential activation of MITF (microphthalmia-associated transcription factor), which regulates the expression of tyrosinase, TRP-1 (tyrosinase related protein 1) and TRP-2 (tyrosinase related protein 2) [10].

It has been shown that MC1-R signaling is negatively regulated by an endogenous ligand ASIP (agouti signaling protein). ASIP blocks the stimulatory effect of αMSH on tyrosinase and decreases TRP-1 and TRP-2 gene expression and total melanin production [11]. Although ASIP's primary sequence has no similarities to αMSH, it binds to MC1-R with almost equal affinity [12]. Thus, the secreted ASIP protein could affect the quality of hair pigmentation. Indeed, polymorphisms in ASIP are associated with skin, hair, and eye pigmentation phenotypic changes [13].

Emerging evidence suggests also a role of sirtuin 1 (SIRT1) in maintaining capillary homeostasis. This nuclear protein belonging to the family of sirtuins plays a key role in the control of

the aging process reducing the incidence of age-related disorders [14]. It was reported that animals characterized by reduced levels of SIRT1 had a phenotype of accelerated aging and exhibited several features of premature skin and hair aging [15]. Thus, the enhanced expression of SIRT1 could antagonize hair pigmentation age-related decline.

The constantly growing knowledge concerning hair follicle biology and the causes of hair depigmentation open new strategies for intervention and reversal of the hair greying process. To fight against canities we developed an agonist of αMSH, a biomimetic peptide (palmitoyl tetrapeptide-20; PTP20). In the current paper, we describe the effects of this peptide on different molecular targets related to hair greying and we provide the results of clinical studies on the anti-greying efficacy of a topical formulation containing this bioactive peptide.

MATERIAL AND METHODS

Peptide design

The palmitoyl tetrapeptide-20 (PTP20) is a biomimetic peptide derived from the α melanocyte-stimulating hormone (αMSH). The sequence was chosen to interact with its receptor, the MC1-R.

Palmitoyl tetrapeptide-20 synthesis

Briefly, the peptide was synthesized following the fluorenyl-methoxycarbonyl strategy, by solid phase assembling, using diisopropylcarbodiimide/ ethyl 2-cyano-2-(hydroxyimino) acetate (DIC/Oxyma) as coupling reagents, on DEG-aminomethyl (DEG-AM) resin, with Ramage linker. Cleavage from the resin was performed with trifluoroacetic acid (TFA)/H_2O/dithiothreitol (DTT). Then, the peptide was decarboxylated and eluted from resin. After that, ion exchange was achieved in order to converse trifluoroacetate salt into acetate salt. It also acts as purification. Peptide purity was assessed by reverse-phase high-pressure liquid chromatography using a Symmetry column (250 x 4.6 mm), applying a linear gradient (A: 0.1% TFA/water / B: 0.1% TFA/acetonitrile; B from 40 to 100% over 30 minutes). Its purity exceeded 90% and it was further characterized by mass spectrometry.

Cell culture

Primary human melanocytes (HEMas) isolated from adult donor material were purchased from CellnTec (Bern, Switzerland) and cultured according to the supplier's instruction in Cnt-40 medium benefiting from the presence of PCT factors and other cofactors that provided strong proliferation and extended longevity. Human Embryonic Kidney (HEK293) cells were grown in DMEM with 10% fetal bovine serum with sodium pyruvate according to the supplier's instruction. Human Follicle Dermal Papilla Cells (HFDPC) isolated from human dermis originating from lateral scalp were purchased from Tebu-Bio (Le Perray en Yvelines, France) and grown in Follicle Dermal Papilla Cells Medium containing 4% FCS, 0.4% bovine pituitary extract, 1 ng/mL bFGF and 5 µg/mL insulin (Tebu-Bio). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Evaluation of the MC1-R receptor transactivation

Human Embryonic Kidney (HEK293) cells were co-transfected using calcium phosphate and selected in 200 µg/ml hygromycin, with two vectors, one containing the gene that codes for MC1-R (hMC1-R) and the other containing the gene that codes for luciferase (CRE-Luc). The cDNA for human MC1-R (hMC1-R) receptor is derived from the mRNA of Normal Human Epidermal Melanocytes amplified by PCR.

When the plasmid construct was activated by PTP20, there was a luminescent signal measured and quantified by a luminometer. The AC_{50} was determined. It corresponds to the concentration which activates 50% of the luminescence compared to control.

Evaluation of melanin synthesis

HEMa cells have been seeded in 6-well plates at the concentration of 3.10^5 cells/ well. After 24 hours, the medium was removed and the cells were treated with PTP20 (10^{-6} and

10^{-7} M) or αMSH (10^{-6}M). After 72 hours of treatment, cells were detached and incubated 10 minutes at 100°C, with NaOH at 1M, for melanin extraction. Activity of tested products relatively to αMSH activation was evaluated by spectrophotometry at λ = 405nm. Melanin content is expressed as the percentage of melanin production, compared to cell number. Experiments were performed in triplicate.

Determination of catalase enzymatic activity

The direct effect of PTP20 on the enzymatic activity of catalase was evaluated using Amplex Red Catalase Assay Kit purchased from Molecular Probes (Invitrogen, Cergy-Pontoise, France) according to the instructions of the manufacturer. In brief, 10^{-5}M PTP20 solution was incubated with 6.25 mU of catalase in a 96-well plate for 10 minutes at room temperature. Then an extemporaneously prepared solution of H_2O_2 (20µM final concentration) was added and the mixture was incubated 30 minutes at room temperature in the dark. A mixture of HRP (0.2U/mL final concentration) and Amplex Red fluorogenic probe (50µM final concentration) was then added and plates were incubated in the dark at 37°C for 60 minutes. The generated fluorescence of formed resorufin was immediately recorded (λ_{ex} = 544 nm, λ_{em} = 590 nm) using a Polar Star Omega (BMG, Ortenberg, Germany) reader.

The effect of PTP20 on the enzymatic activity of catalase was also investigated by evaluation of intracellular levels of H_2O_2 using a fluorescent dichlorofluorescein assay adapted to flow cytometry which allows detection of picomole levels of hydroperoxides [16].

Using the fluorogenic probe, the 2',7'-dichlorofluorescin diacetate (DCFH-DA) from Sigma (Saint-Quentin Fallavier, France), we assessed the levels of intracellular hydrogen peroxide within the control and treated cells. Briefly, HFDPC cells were seeded at 50 000 cells/well in 12-well plates with 500 µl of complete medium and incubated for 24 hours at 37°C. PTP20 (10^{-5}M final concentration) was then added and incubated for 18 hours at 37°C. At the end of incubation, DCFH-DA probe (10 µM final concentration) was added for additive 15 minutes. Cells were then trypsinized, washed and immediately scanned on FACS (FC500 flow

cytometer from Beckman-Coulter, Villepinte, France) with excitation and emission settings of 485 and 529 nm, respectively. The emitted fluorescence is assumed to be proportional to the concentration of hydrogen peroxide in the cells [17]. The results are expressed as percentage of decrease in the H_2O_2 production by HFDPC, in comparison with the untreated cells.

SIRT1 activity evaluation

The effect of PTP20 on the enzymatic activity of SIRT1 was studied using HTRF® SIRT1 assay (Cisbio, Bedford, USA) according to the manufacturer's instructions. This assay is a homogeneous method to directly measure SIRT1 deacetylation activity. Briefly, the purified enzyme was incubated at room temperature in presence of its acetylated substrate, NAD^+ (cofactor) and PTP20 compound at concentrations varying from 10^{-5}M to 10^{-11}M. The controls were realized in the absence of PTP20. SIRT1 Activator 3 (Santa Cruz Biotechnology, USA) was used as a positive control.

After 30 minutes of incubation, the enzymatic reaction was stopped and the amount of remaining acetylated substrate was assessed using a specific antibody coupled to a fluorophore. The quantification of emitted fluorescence allows to measure the activity of SIRT1. The results are expressed as percentage of increase in SIRT1 activity in comparison to the control values obtained without addition of the tested peptide.

Microdissected human hairs *ex vivo*

The human grey-hair scalp plasty was obtained from a healthy 71-year-old female donor undergoing a face-lift. The hairs were isolated by microdissection from the bulb to the hair tip. All hairs were individually transferred into 48-well plates using one hair with 300 µl of medium per well. The hairs were cultured in William's medium (Sigma-Aldrich, ref. W1878), supplemented with 2 mM L-glutamine (Gibco, ref. 25030), insulin-transferrin-selenium G (Gibco, ref. 41400), 20 ng/ml hydrocortisone (Sigma, H-0888), penicillin/ streptomycin (Gibco, ref. 15140) and 1% fetal calf serum (Hyclone, ref. SH3010903) for 7 days at 37 °C in a humidified atmosphere containing 5% CO_2.

11 hairs were treated with the product PTP20 added to the culture medium at 10^{-9}M on day 0, day 1, day 4 and day 6 while the culture media of the 11 untreated control hairs were completely renewed on the same days.

On day 0 and day 7, the hairs were harvested for histological analysis. For each condition, half of the hairs were fixed in standard formalin-buffered solution for 24 hours, dehydrated and embedded in paraffin; the other half were frozen at -80°C for immunostainings.

***In vivo* clinical study**

Inclusion

Fifteen healthy Caucasian male volunteers were included in a clinical study after informed consent. They were between 18 and 38 year-old (average age 33 ± 5 years) and exhibited a premature hair greying, with more than 20% of white hairs.

Formulation

The active peptide PTP20 was added at 10 ppm in the following colorless lotion, using the described formulation process:

PHASE	INGREDIENT	SUPPLIER	%
A	DEIONIZED WATER		78.51
B1	ALCOHOL	CHARBONNEAUX - BRABANT	14.73
B1	PROPYLENE GLYCOL	INTERCHIMIE	2
B1	LECITHIN	LUCAS MEYER	1
B2	500ppm PTP20 SOLUTION	LUCAS MEYER	2
C	PEG-40 HYDROGENATED CASTOR OIL	SAFIC-ALCAN	1.5
C	TOCOPHEROL	BASF	0,01
D1	PVP	ASHLAND	0.15
D3	PANTHENOL	BASF	0.1

1. In a container solubilize lecithin in propylene glycol and in alcohol at room temperature with gentle agitation. The solution must be clear and homogeneous.

2. Add the peptide solution at room temperature with gentle stirring. The solution must be homogeneous.

3. Add phase B to phase A at room temperature with medium agitation.

4. Pre-mix phase C in an auxiliary container, with gentle stirring, until complete solubilization. The mixture should be clear and homogeneous.

5. Then add phase C to the manufacturing vessel at 30 ° C with medium agitation. Thoroughly homogenize the solution.

6. Then add the ingredients of phase D, one by one, to the main container, with medium agitation. Check the homogeneity of the solution between each addition.

Thoroughly homogenize the solution.

Treatment

The described above lotion was applied topically on dry hair scalp, at the dose of 3 ml dispensed into 5 pipettes with a massage to facilitate the hair scalp distribution, without rinse. The application was repeated every day for 3 months (from Mai to July), except the last day of sampling.

Plucked hairs

On day 0, and after 3 months treatment, several hairs were plucked from each volunteer hair scalp. For each volunteer, half of the plucked hairs were fixed in standard formalin-buffered solution for 24 hours, dehydrated and embedded in paraffin, and half were frozen at -80°C for immunostainings.

Stainings & fluorescent immunostainings & immunohistochemistry

7 µm sections of frozen hairs were fixed with acetone at -20°C for 10 minutes, while 5 µm sections of paraffin-embedded hairs were deparaffinized and incubated with pH6-antigen retrieval solution for 20 minutes at 98°C (Dako, ref. K8005) for immunostainings. The hair sections were incubated for 30 minutes with 3% normal rabbit, goat or horse serum (Vector, Vectastain® Universal ABC kit, ref. PK7200) in PBS to block nonspecific binding sites and stained with specific primary antibodies diluted in PBS with 3% BSA.

For fluorescent immunostainings, the primary antibodies used were anti-PARD3/ASIP rabbit antibody (Abcam, ref. ab64646) diluted at 1/200 for 1h at RT and anti-catalase goat antibody (Santa-Cruz biotechnologies, ref. sc-34280) diluted at 1/100 overnight at 4°C. The sections were then incubated for 30 minutes at room temperature with Alexa Fluor 488-labeled rabbit anti-goat antibody (Lifetechnologies, ref. A11078) or AF488-labeled goat anti-rabbit antibody (Lifetechnologies, ref. A11008) diluted at 1/1000 in PBS with 3% BSA. The nuclei were counterstained using propidium iodide at 0.2µg/ml for 2 minutes. The sections were finally mounted using Vectashield® mounting medium (Vector, ref. H-1400) or Fluoromount-G® mounting medium (Southern Biotech, cat n°0100-01).

For Immunohistochemistry, the hair sections were preincubated with hydrogen peroxide (VWR, ref. 23619.264) at 0.3% in PBS for 10 minutes to inactivate endogenous peroxidase activity. The primary antibodies used were anti-TRP-1 mouse antibody (Eurogentec, ref. SIG-38150-1000) diluted at 1/200 for 1h at RT, anti-MC1-R rabbit antibody (Santa Cruz biotechnologies, ref. sc-28990) diluted at 1/200 for 1h at RT, anti-Melan-A mouse antibody (Santa Cruz biotechnologies, ref. sc-

20032) diluted at 1/100 for 1h at RT and anti-TRP-2 mouse antibody (Santa Cruz biotechnologies, ref. sc-74439) diluted at 1/500 for 1h at RT. The sections were incubated for 30 minutes at room temperature with a horse anti-mouse/rabbit biotinylated secondary antibody, then incubated for 30 minutes at room temperature with a streptavidin- labeled peroxidase (Vector, Vectastain® Universal ABC kit, ref. PK7200). The staining was revealed by a violet substrate of peroxidase, VIP (Vector, ref. SK-4600) from 2 to 4 minutes. The nuclei could be counterstained using Mayer hemalun (RAL diagnostics, ref. 320550). The sections were finally dehydrated and mounted using Eukitt mounting medium (VWR, ref. KIND01250).

The microscopical observations were realized using a Leica DMLB or Olympus BX43 microscope. Pictures were digitized with a numeric DP72 Olympus camera with CellD storing software.

Statistical analysis

The obtained data and percentage variations were submitted to Student t-test. The statistical significance value was considered at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

RESULTS

Effect of PTP20 on MC1-R receptor transactivation

The ability of the peptide to modulate the transactivation of MC1-R was evaluated after co-transfection of HEK293 cells. The dose-response of MC1-R receptor activation by the PTP20 is presented in Fig. 1. The maximum activation (137%) was obtained by 10 µM of peptide with an AC_{50} = 0.16 nM. The lower is the AC_{50}, the better is the affinity of the molecule for its receptor.

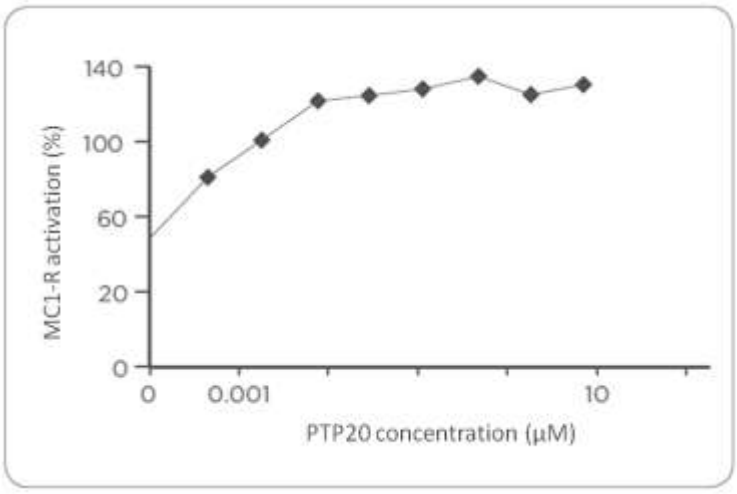

Figure 1. MC1-R activation by PTP20. The dose-response of PTP20 was tested on the transactivation studies in HEK293-hMC1-R.

Effect of PTP20 on melanin production

To investigate the peptide activity mediated by MC1-R, the effect of PTP20 on melanin production was analysed in human melanocyte culture. After 72h of treatment, the peptide increased significantly melanin synthesis by 19% and 39% at the concentration of

10^{-7} M and 10^{-6} M respectively (Fig. 2).

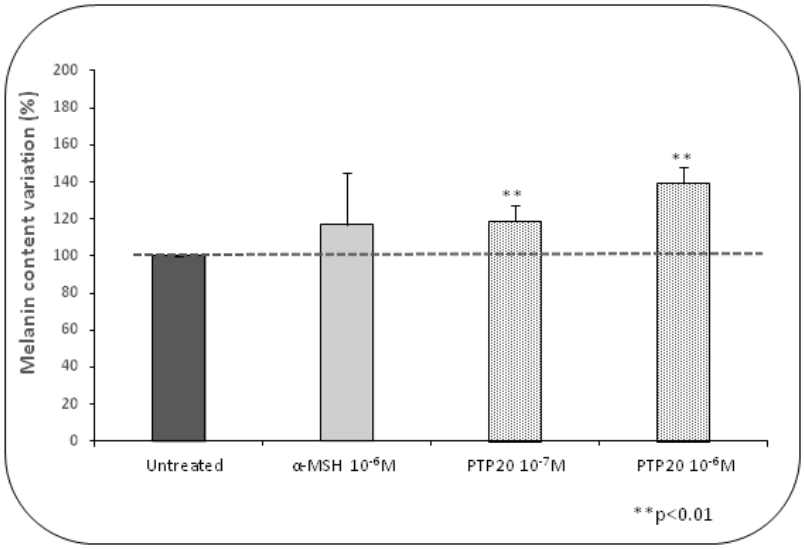

Figure 2. PTP20 peptide increases melanin synthesis in comparison to the untreated control (**p<0.01).

Effect of PTP20 on the enzymatic activity of catalase

Two different experimental approaches were used to evaluate the catalase activity. A preliminary assessment was done with the commercial kit « Amplex Red Catalase Assay » and allowed to demonstrate an increase of 10% in the enzymatic activity of catalase incubated with a 10^{-5}M PTP20 solution.

The effect of PTP20 peptide on the enzymatic activity of catalase investigated by evaluation of intracellular levels of H_2O_2, using a fluorescent assay adapted to flow cytometry, stays in line with earlier finding. Indeed, the incubation of hair follicle dermal papilla cells for 18 hours with PTP20 peptide resulted in a significant decrease of 30% in the intracellular level of H_2O_2 (Fig. 3).

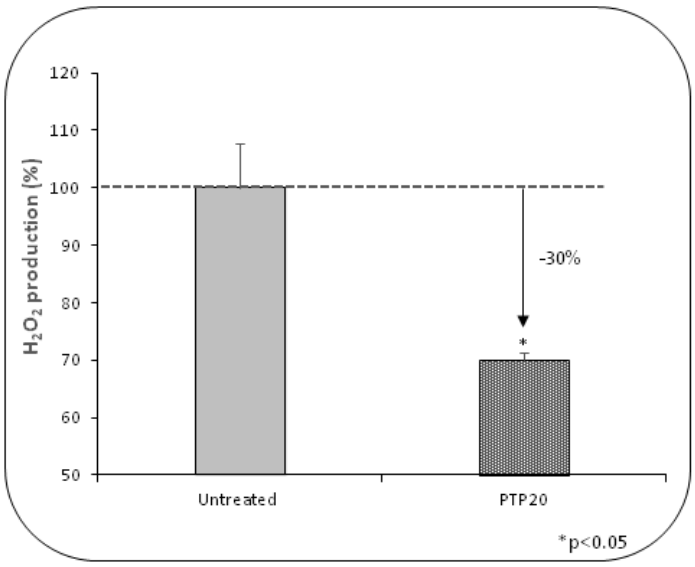

Figure 3. PTP20 peptide decreases the intracellular H_2O_2 production.

Effect of PTP20 peptide on the enzymatic activity of SIRT1

A high-throughput HTRF SIRT1 assay was used to study the impact of PTP20 on the activity of SIRT1. The obtained results (Fig. 4) showed that the treatment of purified SIRT1 with PTP20 stimulated SIRT1 activity. Indeed, a significant increase from 12.5% to 16% in the measured activity was noted for PTP20 tested from 10^{-9}M to 10^{-5}M concentration, respectively.

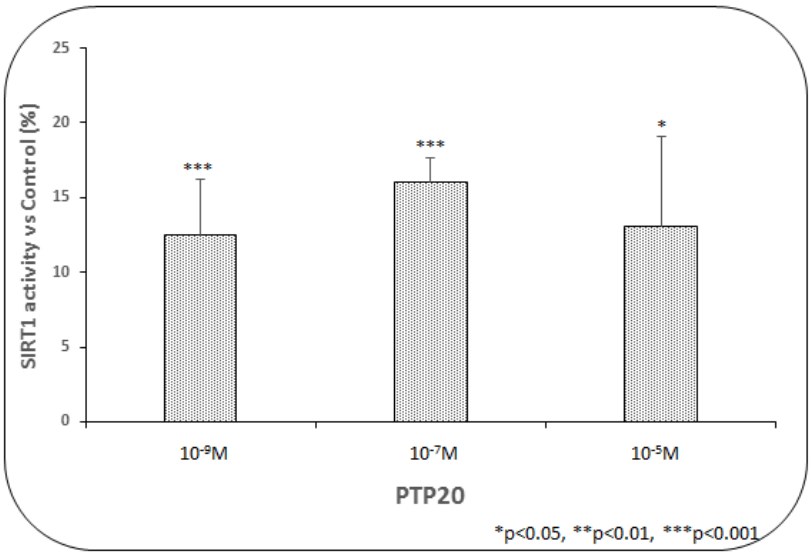

Figure 4. Effect of PTP20 on the enzymatic activity of SIRT1.

Effect of PTP20 peptide on human hairs *ex vivo*

After 7 days of treatments with 10^{-9}M of PTP20, a stimulation of hair melanosome biogenesis, eumelanin synthesis and anti-oxidant activity was observed by immunostainings on human microdissected hairs. The expression of the melanosomal protein, Melan-A was slightly increased especially in the bulge area, a location of melanocyte stem cells. The expression of αMSH receptor, MC1-R, was also slightly increased whereas the expression of the Agouti signalling protein, ASIP/PARD3, was slightly decreased in the lower part of the hair follicle (lower root sheaths and bulb). Two other melanosomal proteins, tyrosinase-related proteins TRP-1 and TRP-2, were slightly induced in the upper root sheaths and the bulge, respectively. The anti-oxidant enzyme catalase, known to block the deleterious whitening effect of hydrogen peroxide in hair, was moderately increased in the lower root sheaths (**Table I** & **Fig. 5**).

Table I. Immunostaining variation (intensity and frequency) after PTP20 *ex vivo* treatment of human hair follicles.

Markers with variation of staining vs untreated hairs	**10^{-9} M PTP20 after 7 days**
Melan-A	+ bulge
TRP-1	+ upper root sheaths
TRP-2	+ bulge
MC1-R	+ lower root sheaths + bulb
ASIP (PARD3) **(weak detection)**	- lower root sheaths - bulb
Catalase	+ lower root sheaths
- decrease; + increase	

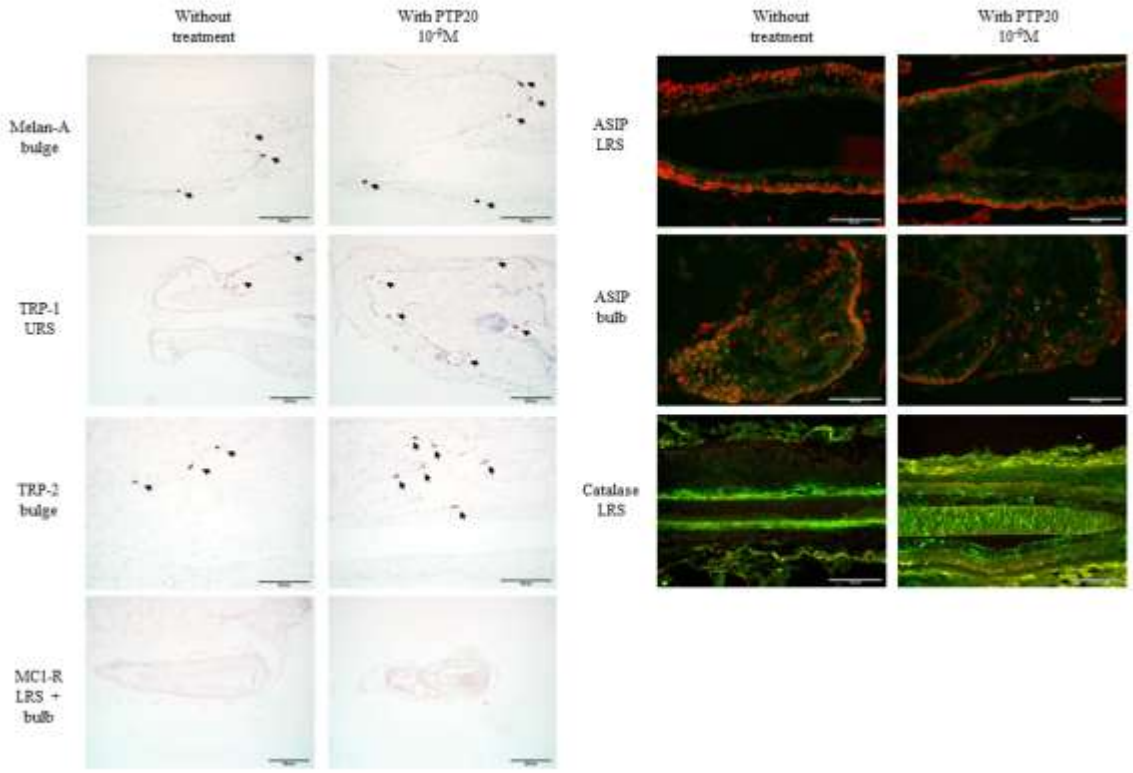

Figure 5. Immunostaining of Melan-A, TRP-1, TRP-2 (see arrows) and MC1-R (in violet) and immunostaining of ASIP/PARD3 and catalase (in green with nuclei in red) on *ex vivo* control or treated for 7 days with PTP20 human hair follicles. *URS: upper root sheaths; LRS: lower root sheaths. Scale bar 100 µm (Melan-A, TRP-2, ASIP/PARD3 and catalase); scale bar 200 µm (TRP-1 and MC1-R).*

Effect of PTP20 lotion observed during *in vivo* clinical study

After 3 months treatment with the lotion containing PTP20 active peptide, we observed a stimulation of melanosome biogenesis and eumelanin synthesis in plucked hairs by immunostainings. Indeed, MC1-R expression was slightly increased in the bulb and moderately in the lower root sheaths, whereas ASIP/PARD3 was moderately decreased in the lower root sheaths and bulb, in more than 50% of the volunteers (**Table II** & **Fig. 6**).

Table II: Immunostaining variation (intensity and frequency) of MC1-R and ASIP/PARD3 on plucked hairs after 3 months *in vivo* treatment with PTP20 lotion (n=15), compared to T0.

Markers with variation of staining vs T0	**PTP20 active lotion after 3 months**
MC1-R	+ lower root sheaths (67% of volunteers) + bulb (53% of volunteers)
ASIP (PARD3)	- lower root sheaths (62% of volunteers) - bulb (64% of volunteers)
- decrease; + increase	

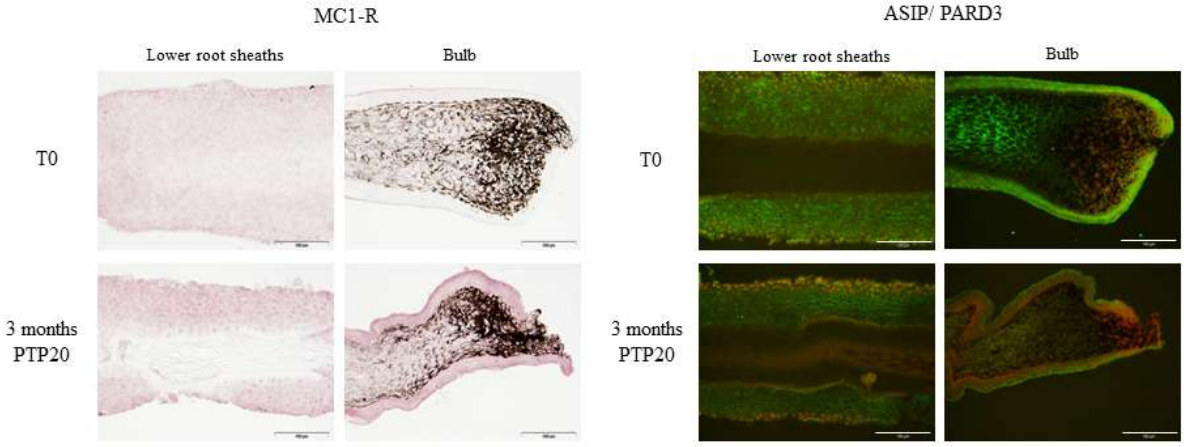

Figure 6: Immunostaining of MC1-R (in violet) and immunostaining of ASIP/PARD3 (in green with nuclei in red) on human plucked hairs before (T0) and after 3 months *in vivo* treatment with PTP20 lotion (volunteer 13). *Scale bar 100 µm.*

DISCUSSION

During the past decade, melanins and melanogenesis have attracted growing interest for a broad range of biomedical and technological applications designed in great part for skin and hair care. Hair colour plays an important role in people's physical appearance and self-perception. With today's increasing life expectation, the desire to look youthful plays a bigger role than ever. The hair care industry has become aware of this and is also more and more capable of delivering active products that are directed toward meeting this consumers demand. Hair aging comprises weathering of the hair shaft and aging of the hair follicle. The latter manifests as decrease of melanocyte function or greying. The pigmentation of hair fibers is affected by numerous intrinsic factors including hair-cycle dependent changes, ethnic and gender differences, variable hormone-responsiveness, genetic defects and finally by age-associated changes. The progressive loss of capillary pigmentation is a normal sign of aging of the follicular units. However, in some ethnic groups and in certain families, canities can

develop early before the age of 20, causing a suspicion of a genetic origin. Knowing more about the pigmentation process and all factors involved should help finding a compound to down-regulate canities.

Thus, taking into account the major role of αmelanocyte-stimulating hormone (αMSH) in the hormonal regulation of melanogenesis, we developed an agonist of αMSH, a biomimetic peptide (palmitoyl tetrapeptide-20, PTP20). Our *in vitro*, *ex-vivo* and *in vivo* investigations revealed a great number of biological activities of the studied peptide, which contribute to the process of hair pigmentation. Indeed, the results of *in vitro* studies confirmed the MC1-R agonist property of PTP20 peptide and revealed its capacity to increase significantly melanin synthesis in cultured human melanocytes. Moreover, MC1-R expression was up-regulated by PTP20 in *ex vivo* experiments as well as in clinical studies.

It is well known that besides αMSH, its receptor MC1-R and tyrosinase are the major targets in the medicinal/cosmetic hair greying treatment. It has been previously shown that mice with knockout of the MC1-R had premature greying of their fur [18]. Furthermore, recent reports on the potency of natural root extract to induce melanin synthesis point out the activation of MC1-R/MITF/tyrosinase- signaling pathway and an increase in MC1-R expression as key elements of observed promotion of hair pigmentation. Moreover, it was found that early hair greying phenomenon may be related to downregulation of this pathway [19]. The activation of MC1-R pathway can be inhibited by agouti signaling protein (ASIP). Indeed, ASIP is an important negative regulator of hair pigmentation as it competitively prevents the binding of αMSH to MC1-R and inhibits melanogenesis [11]. Considering the pivotal role of MC1-R in inducing expression of melanogenic enzymes [10], the melanin level increase reported here, together with the stimulation of MC1-R, TRP-1 and TRP-2 expression, is consistent with the observed down-regulation of ASIP expression in the human follicle following PTP20 treatment *ex vivo* and *in vivo*.

Moreover, the agouti gene encodes a paracrine signalling molecule that causes hair follicle melanocytes to synthesize the yellow pigment pheomelanin instead of the black or brown pigment eumelanin. In addition, TRP-2 converts the melanogenic intermediate DOPAchrome to DHICA (5,6-dihydroxyindole-2-carboxylic acid), therefore affecting eumelanin, but not pheomelanin synthesis. TRP-2 is downregulated during pheomelanin synthesis [20]. *Ex vivo* and clinical data showing the decreasing effect of PTP20 on ASIP expression and its increasing effect on TRP-2 expression, suggested the potential efficacy of PTP20 to repigment the grey hair with brown pigments.

The physiological hair greying that occurs with aging is also known to result, in part, from accumulation of oxidative damage generated during normal metabolism [21]. The growing number of data provided the clear evidence of primordial role of catalase deficiency and consequent hydrogen peroxide accumulation in the phenomenon of hair greying [6, 19].

In vivo identification of massive H_2O_2 concentrations in the grey hair shaft introduced a new step in the understanding of human hair greying at the biochemical and molecular level [6]. Depigmentation of the hair is in fact related to failure of the action of tyrosinase and αMSH, structurally damaged and functionally altered by H_2O_2-mediated oxidation as well as to direct oxidation of melanin leading to the pigment degradation (bleaching effect). This advanced insight opens new strategies for intervention and reversal of the hair greying process. It might be expected that enhanced expression/activity of catalase could result in the reduction of canities. One should add that a particular target of research on canities is the nature of the melanocyte stem compartment and a

failure of melanocyte stem cell renewal due to oxidative stress. Taking into consideration the reported here abilities of PTP20 peptide to enhance catalase expression and reduce the intracellular level of H_2O_2, it is tempting to speculate on the ability of PTP20 to also protect melanocytes stem cell population against oxidative damage.

The data from *ex vivo* studies indicating that PTP20 had enhanced TRP-2 expression, consolidate the PTP20 potential to reduce the incidence and the severity of hair greying. TRP-2 expression was shown to be uncoupled to that of tyrosinase and TRP-1 in hair bulb melanocytes, whatever the hair colour and ethnic origin [22], suggesting a specific role of this enzyme apart from pigmentation through melanin synthesis. Furthermore, TRP2 expression dramatically declined in senescent melanocytes, whereas tyrosinase and TRP-1 levels remained unaltered [23]. These observations sustain a role for TRP-2 in age-associated pigmentation disorders. Indeed, unlike tyrosinase and TRP-1, TRP2 is expressed outside of the melanosome, in the melanocyte trans-Golgi network, suggesting a potential role in a recycling pathway [24]. Therefore, Michard *et al*. [25] investigated on TRP-2 effects on oxidative stress-mediated toxicity. They demonstrated that TRP-2 overexpression reduced H_2O_2-induced DNA damage, with a significant improvement of respiration and survival rate without any impact on cell proliferation. Moreover, they demonstrated that TRP-2 silencing partially restored sensitivity to oxidative stress. They explain these results by an increase of intracellular glutathione in TRP-2-overexpressing cells, which would imbalance the basal intracellular redox homeostasis and result in an enhanced cell response to oxidative stress. The importance of TRP-2 as a protector for hair follicle and hair colour preservation is further confirmed by the fact that TRP-2 expression is preserved in eyelash follicle melanocytes [26]: it would explain eyelash protection to hair greying. Altogether, we can seriously presume that TRP-2 contributes to hair follicle melanocytes protection from oxidation, its specific lack leading to hair follicle susceptibility to oxidation and contributing mainly to hair greying.

It is also worth emphasizing the role of growth factors as key regulators of hair follicle homeostasis and melanogenesis [27-32]. Indeed, it has been shown that Stem Cell Factor (SCF) expression positively correlates with the rise of tyrosinase activity. Moreover, SCF is crucial for melanocyte survival during development, and its gene mutation results in unpigmented hairs [27]. Hepatocyte Growth Factor (HGF) is known to promote *in vivo* survival, proliferation and differentiation of melanocyte precursors [28] and its expression prevents hair greying [29]. It was also reported that Platelet Derived Growth Factor (PDGF) and Vascular Endothelial Growth Factor (VEGF) both activate melanocyte stem cells, and are efficient for reversal of hair greying [30, 31]. Finally, Nerve Growth Factor (NGF) was found to rescue melanocytes from apoptosis [32]. The results of preliminary one shoot experiment carried out *in vitro* with HFDPC cells revealed that the cell treatment with 10^{-9}M to 10^{-5}M PTP20 enhanced to a different degree (17-90%) the production of all these growth factors. These findings corroborate the anti-greying effect of PTP20. However, complementary studies are needed to precise the individual effect of PTP20 on the expression and production of each of these factors.

An age-related hair pigment loss becomes the inescapable signal of our disappearing youth. Over the past decade, a large number of regulators and signaling pathways have been identified for the control of aging and longevity. They include among others SIRT1, a nuclear enzyme belonging to the family of sirtuins and recognized as a longevity protein and key regulator of cell survival in response to stress [14]. The bibliographic data clearly indicate that the SIRT1 protein prolongs the life of fibroblasts and protects keratinocytes against UVB and H_2O_2-induced cell death [33, 34]. The data from *in vitro* experiments support the idea

that SIRT1 activity is critically required for melanocyte cell proliferation. Indeed, SIRT1 suppression induced a senescence-like phenotype and its associated cell proliferation arrest of human melanoma cells [35]. Thus, the activation of SIRT1 by PTP20 could have an anti-aging effect favoring the survival of both melanocytes and cells forming the hair follicle and exhibiting a potential protective effect against hair greying.

CONCLUSIONS

Hair greying is one of the most dramatic phenotypic changes during aging and therefore much cosmetic research is focused on preventing this process. Apart from various hair dyes of varying efficacy and duration, fully satisfactory solutions for the greying problem remain to be brought to market. Therefore, searching for effective and safe hair greying prevention and efficient compounds has huge social and economic benefits.

The purpose of this study was to evaluate the effects of a new agonist of αMSH, the tetrapeptide PTP20, on hair greying. The findings presented in this paper provide several concordant lines of evidence, *in vitro*, *ex vivo* and *in vivo* in support of biological efficacy of this peptide to stimulate the process of hair pigmentation. In fact, the data we are reporting might yield clues to possible therapies for the prevention and/or the reduction of hair greying process and strongly suggest potential cosmetic applications of this new mime of αMSH.

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