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HIF-1α Stimulators Function Equally to Leading Hair Loss Agents in Enhancing Dermal Papilla Growth

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Keywords
Dermal papilla · Androgenic alopecia · Hair loss · HIF-1α · Hypoxia-inducible factor · Deferoxamine · Deferiprone · Minoxidil · Caffeine

Abstract
Introduction: Androgenic alopecia (AGA) occurs due to progressive miniaturization of the dermal papilla (DP). During this process the hair follicle loses nutrition over time and eventually dies, causing the hair to fall out. Recent evidence suggests that hypoxia-inducible factor-1a (HIF-1α) modulation may counteract hair loss. This study aims to evaluate the proliferation of dermal papilla cells (DPCs) under the influence of a selection of commercially available topical hair loss drugs, compared to HIF-1α-stimulating agents. ***Materials and Methods:*** Using the hanging drop method, DPCs self-organized into spheroid shape, mirroring the three-dimensional (3D) structure of the DP in vivo. DP analogs were treated with established substances against AGA (minoxidil and caffeine) compared to HIF-1α-stimulating agents (deferoxamine [DFO] and deferiprone [DFP]), at 10 mM doses. DP analogs were simultaneously stained with 5-bromo-2′-deoxyuridine (BrdU) to evaluate impact of drug compounds on DP daughter cell production. Concurrently, fluorescent microscopy visualization of migration of daughter cells after 48 h in culture was performed. ***Results:*** DPC proliferation within the spheroid structure was significantly enhanced by caffeine, minoxidil, and the HIF-1α-stimulating agent DFP when compared to control. Highest proliferation was seen in the DFP-treated DP analogs. Migration of peripheral DP daughter cells was highest in control and DFO groups. ***Conclusion:*** Here we demonstrate a significantly enhanced proliferative activity for both established substances against AGA (minoxidil and caffeine) and the HIF-1α-stimulating agent DFP in a 3D DPC spheroid culture model with equal results for DFP and minoxidil. These favorable characteristics make such compounds potential water-soluble alternatives to minoxidil.

Introduction

Hair is a defining feature of humans and has critical functions, including protection, production of sebum, apocrine sweat and pheromones, thermoregulation, as well as skin homeostasis, regeneration, and repair [1].

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The hair follicle (HF) is considered a "mini-organ" comprising dermal papilla cells (DPCs), epithelial cells, and stem cells. DPCs are the main components of the mesenchymal compartments in the hair bulb and are instrumental in generating signals to regulate the hair cycle [2]. The dermal papilla (DP) niche drives the hair growth and regeneration cycle. Hair growth undergoes repetitive cycling, alternating between phases of active growth (anagen), regression (catagen), and relative "quiescence" (telogen) [3]. Androgenic alopecia (AGA), also known as male or female pattern hair loss is a progressive, genetically programmed loss of hair that affects the vast majority of the male and almost half the female population and is a huge burden on those concerned [4]. AGA occurs due to demise of DP, leading to consequential hair loss. The cause for this is not yet fully understood; however, AGA is associated with progressive shortening of the anagen phase (hair production) and an elongation of the telogen phase (HF dormancy) [5]. Furthermore, in AGA the HF undergoes a process known as miniaturization during which the hair shaft becomes smaller. This reduction in size of the hair shaft is coupled with a reduction of the size of the DP, a spheroid-shaped structure, that can be found at the base of the HF which provides the HF with nutrition and oxygen. The size of the DP is proportional to the diameter of the hair shaft [6]. If untreated, the HF loses nutrition over time, triggering the process of miniaturization, and is thus unable to reenter anagen phase and eventually dies.

Hair development, repair, and regeneration are dependent on cell signaling pathways governing these processes. Recent studies identified the hypoxia-inducible factor (HIF) pathway as a novel paradigm of molecular signaling governing the DP. Hair growth bears resemblance to wound healing in that it requires a highly coordinated interplay between tissue formation, cell growth, and cell migration. Hypoxia-inducible factor-1a (HIF-1α) drives neovascularization and production of collagen and elastin during wound healing. AGA has been linked to a lack of blood vessels and nutrient supply of the hair bulb and stimulating the HIF pathway can enhance both neovascularization and tissue regeneration with DPCs being reactive to hypoxia [7–9].

There are numerous topical drugs/compounds on the market that claim to prevent, stop, and even partially reverse hair loss. Minoxidil is currently the mainstay in hair loss treatment [10]. Formerly taken orally as a medication to treat hypertension, the drug was seen to cause hypertrichosis. It was formulated into a hair loss treatment and is available in a 2 and 5% tonic or foam that has to be applied topically twice daily. The exact cause of its effect on hair growth has not been firmly established but has widely been attributed to its vasodilating properties. Interestingly, it has recently been shown that mechanism of action of minoxidil is the direct inhibition of PHD-2 (prolyl-hydroxylase 2) which hydroxylates HIF-1α and thus causes its degradation. Blocking PHD-2 leads to the induction of HIF-1α and the transcription of hypoxia-response element genes such as VEGF (Fig. 1) [11]. Accordingly, minoxidil acts to stimulate the HIF pathway and all angiogenic components thereof. The anagen phase of the hair cycle is characterized by increased vascularization [12]; hence, neovascularization due to minoxidil triggers dormant HFs to reenter the anagen phase. The effect of minoxidil could be abrogated by the addition of ascorbate implying that it is a competitive inhibitor of PHD at the binding site of ascorbate, a co-factor of PHD [7].

Caffeine, another compound commonly used as a topical treatment against hair loss, can often be found in shampoos or as a tonic. Herman and Herman [13] discuss the effect of caffeine on hair growth, specifically its inhibition of 5-α-reductase, the enzyme that converts testosterone into the more active dihydrotestosterone (DHT). This serves to prevent the accumulation of DHT in the scalp to which especially HFs are very sensitive. Furthermore, caffeine was seen to increase blood circulation in the area applied.

Deferoxamine (DFO) and deferiprone (DFP) are iron chelators that are administered intravenously to treat iron toxicity. Iron is an important co-factor of PHD, and by chelating iron, activation of PHD is inhibited. This leads to high levels of undegraded HIF-1α, which is then free to dimerize with HIF-1β, forming the transcription factor HIF-1. The transcription of all genes carrying hypoxic-response elements means a strong pro-angiogenic response. DFO is currently attracting a lot of attention in the field of wound healing. Applied topically, the small molecule is able to penetrate the skin [14]. Since the decrease of blood flow to the HF is a major contributor to hair loss, using angiogenic pharmaceutical agents is a logical path in preventing hair loss.

DPCs have been studied extensively in the conventional monolayer [15]. However, due to great deviation from the real in vivo three-dimensional (3D) environment, these two-dimensional grown cells tend to lose their hair-inducible capability during passaging [15]. These restrictions motivated the development of a novel culture technique, the "hanging drop method" [16]. This model allows for the testing of the aforementioned drugs/compounds and their effects on the DPCs in their native

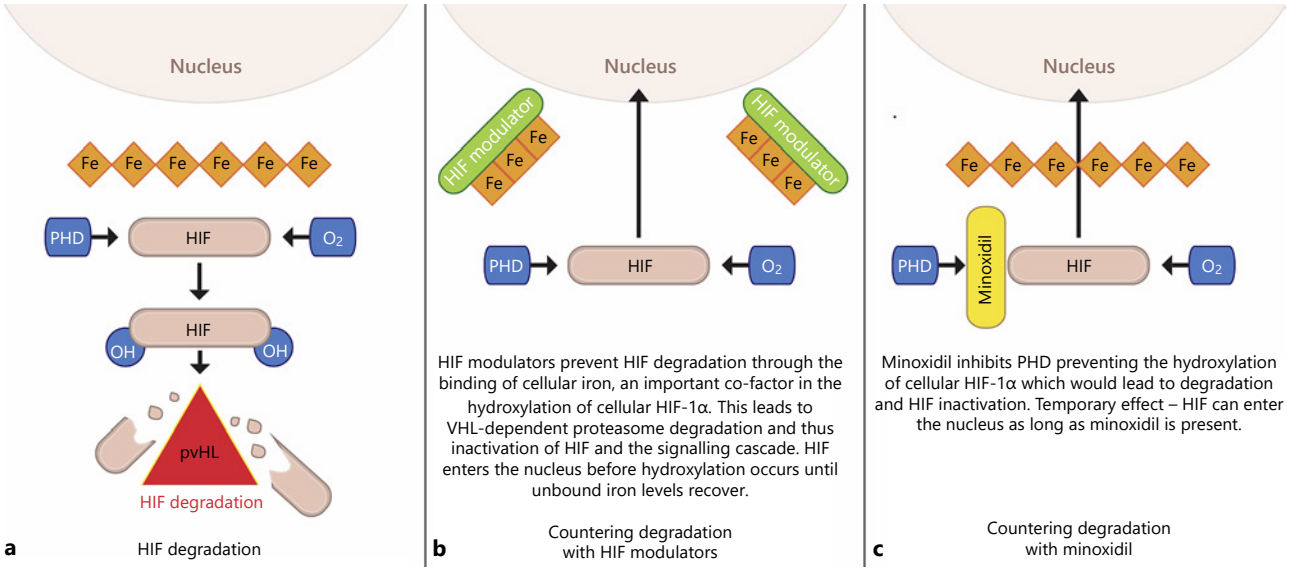

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Fig. 1. HIF pathway regulation. **a** HIF hydroxylation occurs by PHD, followed by ubiquitylation by VHL, which facilitates enzymatic degradation of HIF. **b** Truncated HIF breakdown pathway in the presence of HIF modulating iron chelators, allowing HIF to remain intact and free to dimerize for downstream HIF pathway activation. **c** PHD is temporarily inhibited by minoxidil [11]. HIF, hypoxia-inducible factor.

spheroidal structure. The purpose of this study is threefold: (1) to examine the formation of DPCs into spheroids, (2) the development of a novel method of simultaneous drug dosing, (3) comparing the impact of HIF-1α-stimulating agents with the established substances for boosting DP maintenance, caffeine, and minoxidil.

Materials and Methods

Cell Acquisition, Culture, and Staining

HF DPCs were purchased from Sigma-Aldrich and cultured in Follicle Dermal Papilla Cell Growth Medium (Promo Cell, Heidelberg, Germany), supplemented with Supplement Mix for Follicle Dermal Papilla Cell GM (Promo Cell, Heidelberg, Germany) and 1% antibiotic/antimycotic (penicillin G, streptomycin, and amphotericin C), (Capricorn Scientific). Media were changed every 2–3 days, and cells were passaged after reaching 80–90% confluence with 0.25/0.02% trypsin/EDTA (PAN Biotech). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were used for experimentation immediately after passaging and were not grown above P4.

Hanging Drop Method

Using the hanging drop method, DPCs can self-organize into spheroid shape, mirroring their natural structure in vivo (Fig. 2). This 3D culturing method allows the DPCs to maintain their transcriptional signature and associated inductivity [16]. To prepare the DPCs for spheroid formation, cells were counted and separated into a cell suspension of 300,000/mL DPCs. Droplets of 10 µL, each containing 3,000 DPCs, were placed onto the inverted lid of a 10-cm culture dish (Falcon). The bottom of the culture dish was filled with 7 mL of PBS to prevent droplet evaporation. The lid was carefully inverted on the dish and incubated. After 48 h, 6 µL of fresh medium was added to each droplet and cultured for a further 48 h. After the 96 h culture period, DPCS formed coherent 3D spheroids and were ready for experimentation as DP analogs.

Spheroid Transfer and Culture

When observing carefully the inside of the lid, successfully formed spheroids can be seen at the base of their droplets with the naked eye. A 100 µL pipette was used to transfer single spheroids into their own individual well of a flat-bottomed 96-well plate, containing 75 µL of medium. Next the well plate was incubated for 4 h, to allow successful adherence of the spheroid to the base of the well.

BrdU Staining and Drug Dosing

The wells, each containing 1 spheroid, were divided into 5 groups (control, minoxidil, caffeine, DFO, and DFP). Twenty millimolar solutions of each drug/compound were prepared. The spheroids were each treated with a 10 mM solution of the assigned drug/compound while simultaneously supplied with 5-bromo-2′-deoxyuridine (BrdU). The effect of the drugs was measured over 24 h. At the end of each time point, spheroids were aldehyde fixed as per the manufacturer's protocol and prepared for fluorescent detection of the incorporated uracil base in the proliferating daughter cells of the treated spheroids. Upon completion of BrdU

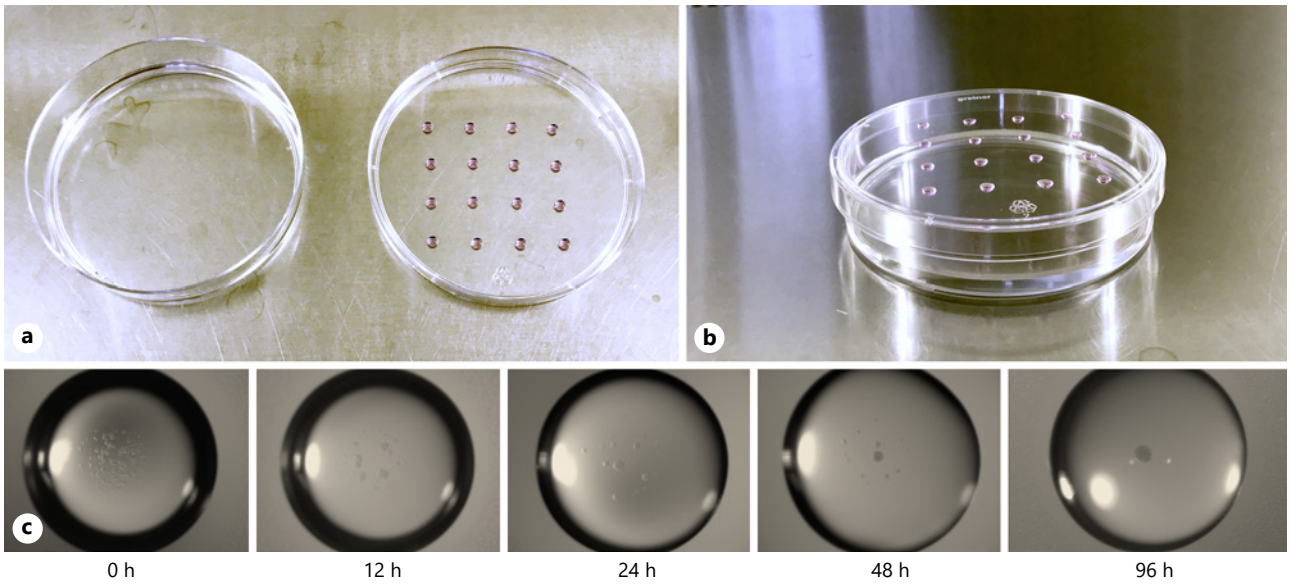

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Fig. 2. 3D hanging drop culture of DPCs. **a** 10 µL media droplets each containing 3,000 DPCs placed onto the inside of a 10-cm culture dish (lid placed back on top of the base of the culture dish). **b** The DPCs aggregate to stable spheroids over a period of 96 h. **c** Time lapse of spheroid formation showing collection of cells at the base of the droplet over 96 h. After spheroid formation is complete the samples are transferred into a 96-well plate for further cultivation and drug exposure. 3D, three dimensional; DPCs, dermal papilla cells.

staining, spheroids were counter stained with DAPI and imaged under brightfield and fluorescent microscopy (Zeiss, Oberkochen, Germany).

Image Analysis and Statistics

Quantitation of green fluorescence and area of migration by the cells of the spheroid were both analyzed by ImageJ (NIH). Each group has $n = 3$. Two-tailed Student's t tests were used to perform direct comparisons between 2 groups, and a p value of <0.05 was considered statistically significant. Data are presented as mean ± SD. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA, http://www.graphpad.com).

Results

Spheroid Formation Is Successful after Three Days of Culture

Using the hanging drop method (Fig. 2a, b), spheroids of DPCs consistently formed over 3 days. Images were taken using a dissection microscope to monitor the successful collection of cells. At 96 h, the spheroid is clearly visible at the apex of the droplet and ready for transfer to the respective well plates (Fig. 2c).

Daughter Cell Production of DFP and Minoxidil Is Not Significantly Different

In a test involving BrdU staining, it was observed that caffeine, minoxidil, and DFP triggered significantly higher production of daughter cells compared to the drug-free control, with DFP showing the highest proliferation. While an increase of proliferation was visible in the DFO-treated group, the difference was not statistically different from the untreated control ($^*p < 0.005$, $^{**}p < 0.01$) (Fig. 3a). It is important to note the high proliferative influence of DFP on the core of the spheroid illustrated by deep green coloration (Fig. 3b).

Migration of Peripheral DPCs Is Highest in DFO-Treated Spheroids

After 24 h of drug dosing, fluorescent microscopy revealed the extent of migration under the influence of each particular compound (Fig. 4a). DFO showed the highest migration, closest to control. Meanwhile, the migration pattern of DFP-, caffeine-, and minoxidil-dosed spheroids are not different from one another and approximately one-third of the migration of DFO/control (Fig. 4b).

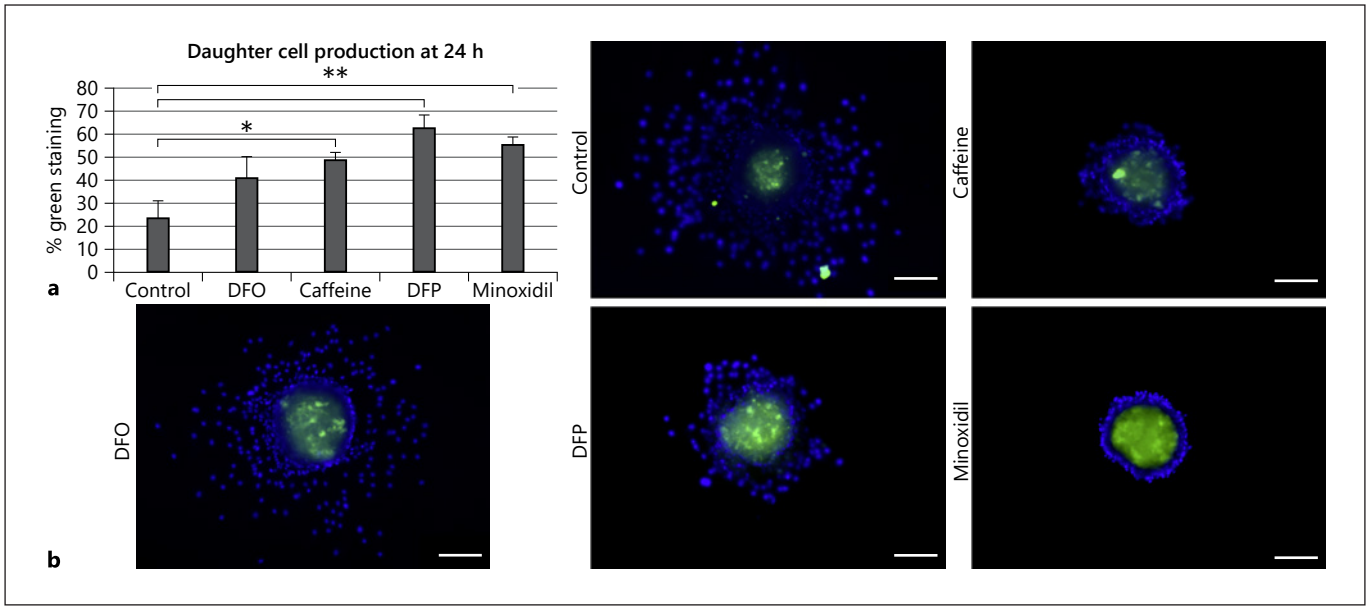

Fig. 3. a Both minoxidil and DFP equally enhance DPC proliferation in a 3D spheroid culture model. **b** Representative images of spheroids stained for newly synthesized cells were taken after drug exposure for 24 h respectively. Evaluating the synthesis of new daughter cells demonstrated an equally significantly enhanced proliferative activity for both minoxidil and DFP and lesser also of caffeine (*$p < 0.005$, **$p < 0.01$). Scale bar, 200 µm. DFP, deferiprone; DPC, dermal papilla cell; DFO, deferoxamine.

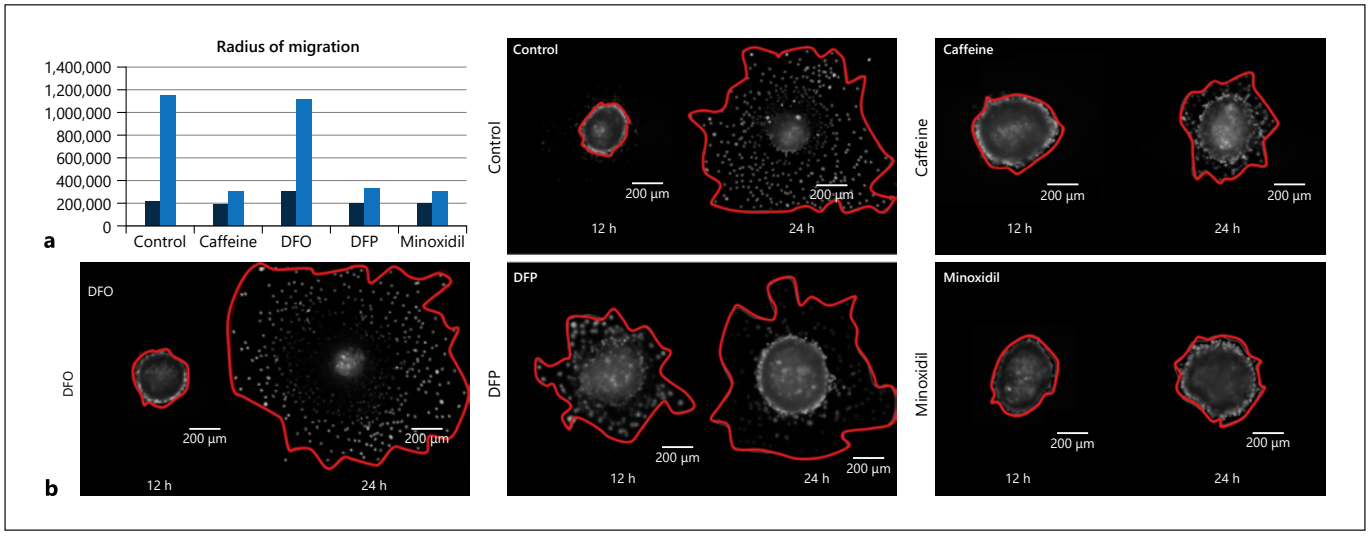

Fig. 4. Radius of peripheral cell migration is highest in DFO-treated spheroids. **a** Graph showing quantification of migration for each condition at time zero at 24 h after dosing. **b** Binary images show spheroid at time zero of drug dosage (left) and 24 h later (right). Scale bar, 200 µm. Red delineation shows total surface area covered by cells migrating from the spheroid. DFP, deferiprone; DFO, deferoxamine.

Discussion

This work aims to shed light on the differences of drug impact on the functional unit of the HF, the DP. Another goal of this work is to add knowledge to the field on 2 under-researched iron-chelating compounds, DFO and DFP, in the context of hair loss. Corroborating the results of other research groups [17], we were able to detect a significantly enhanced cell proliferation of minoxidil-treated DPCs after 24 h of drug exposure. However, this

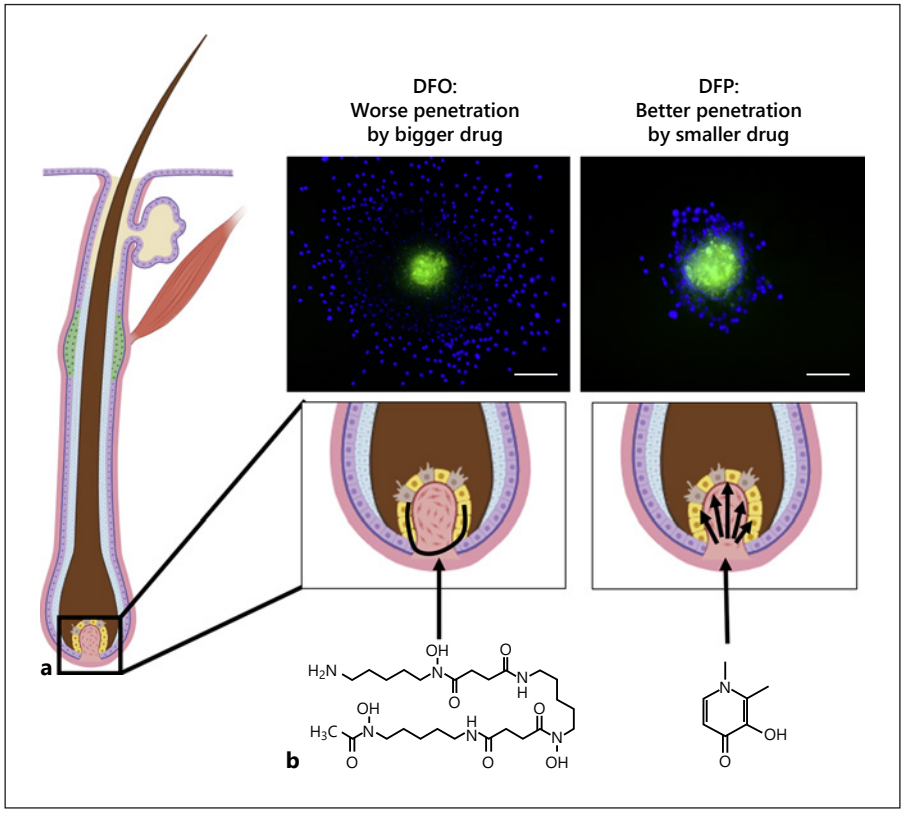

Fig. 5. Graphical hypothesis: drug compound size is a factor in DP penetration. **a** Schematic shows hair follicle with zoomed in aspects of the DP and drug compound structures. **b** Depicts representative fluorescent images. The drug compounds of both DFO and DFP are below, showing hypothesized physical interactions with the tightly formed DP. We explain our results of higher outer migration of cells treated with DFO, and higher internal daughter cell production of DP analogs with DFP, as DFO having most effect on the outer layer of cells, while DFP is small enough to penetrate through and have a profound effect on the proliferation of the DP from the inside. Figure made with BioRender.com. Scale bar, 200 μm. DP, dermal papilla; DFO, deferoxamine; DFP, deferiprone.

study demonstrated for the first time the effect of these compounds in a 3D spheroid model of DP fibroblasts. Interestingly, we observed an equal effect for the DFP-treated group, suggesting a comparable stimulating effect of both agents via the common denominator of minoxidil/DFP/DFO, the HIF pathway.

To test the hypothesis that manipulating the HIF-1-VEGF axis will lead to significant effects on a cellular level a previously described DP organoid model system was employed [16]. The DPCs' natural formation pattern is of a spheroid shape; however, when transferring the spheroid into a flat-bottom well the DPCs will naturally start to spread out over time. While most cell migration takes place in the control group, the DFO-treated group is extremely similar. This is likely due to the pro-migratory impact of HIF-1α [18] which is the target pathway of DFO.

It was seen that all 4 treatments led to more proliferation compared to the control group. One fact that has to be taken into consideration is that when applying caffeine onto the scalp as a shampoo when washing the hair, as is the recommended usage per shampoo manufacturer, the caffeine shampoo usually does not stay in contact with the skin for longer than 1–2 min. In our experiment, the spheroids remained in the solution for 24 h, respectively. Therefore, one could assume that the proliferation would have been less had the spheroids only been in contact with the caffeine for 2 min. Still, in a study, Otberg et al. [19] show that 2 min of skin contact are sufficient for caffeine to penetrate deeply into the HF and also remain there for up to 48 h, even after washing the hair again.

Pharmaceutically, minoxidil is associated with a specific transcriptional loss of lysyl hydroxylase activity; this loss is reversed by removing minoxidil [20, 21]. This enzyme is essential for collagen stabilization. Treatment of cells with minoxidil is also associated with inhibition of cell proliferation [22]. This effect of minoxidil is accompanied by inhibition of DNA synthesis in skin cells. Since collagen is the major product of fibroblast activity, and lysyl hydroxylase catalyzes a crucial reaction in collagen biosynthesis, minoxidil could be harmful to fibroblast functionality and consequentially skin homeostasis [23].

An important consideration, if the drug passes transdermal, is the impact of the drug/drug vehicle on the dermis. A major problematic aspect of minoxidil treatment is its solubility in alcohol, which damages the skin. For in vivo minoxidil treatment, patients must apply the alcohol vehicle to the scalp every 12 h to achieve the desired effect of hair growth [7]. Minoxidil is dissolved in alcohol, not

water, and sold as such. Applying it twice daily can hence lead to irritation of the skin since alcohol is deeply dehydrating [24, 25].

All HIF-pathway influencing compounds (minoxidil, DFO, and DFP) have a considerable effect on the DPCs in terms of proliferation, with DFP leading to the most daughter cell production, closely followed by minoxidil. Given that the target pathway for both minoxidil and the iron chelators (DFO/DFP) is the same HIF pathway, it would make sense to examine DFO/DFP as water-soluble alternatives to minoxidil.

Another medication that counteracts hair loss which is frequently used is finasteride. Finasteride is taken orally to treat benign prostatic hyperplasia and exhibits hypertrichosis as a side effect. It is a 5-α-reductase inhibitor and prevents the conversion of testosterone into DHT [26]. We initially considered it as another test group in our experiment; however, as it is completely insoluble in water, it was excluded from our study.

DFO and DFP are both iron chelators, sharing the same pharmacokinetics [27]; however, there are differences between them in promoting proliferation and migration in our study. A possible reason for this discrepancy could be the size of the molecule. DFO is structurally larger than DFP. One could assume that the inherent structure/polarity of DFO [28] creates difficulty in penetrating the spheroid structure and will thus work to chelate iron around periphery of the spheroid. On the other hand, a smaller/polar molecule like DFP could easily infiltrate the spheroid [29] and so have a higher impact in iron chelation and pathway activation, bringing about a high daughter cell production (Fig. 5).

Conclusion

One major factor contributing to hair loss is the slow, progressive decline of blood supply to the HF. Therefore, treating affected areas with drugs/compounds that promote blood flow is a reasonable approach to combat hair loss. This study shows that iron-chelating agents such as DFO and DFP are water-soluble alternative activators of the HIF pathway, which promotes proliferation of DP fibroblasts.

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Statement of Ethics

The paper is exempt from Ethics Committee approval as it does not require human testing or procurement of primary tissue.

Conflict of Interest Statement

D.T. and D.D. are co-founders of and have equity positions in Tomorrowlabs GmbH, a commercial-stage biotech company that produces products based on HSF (HIF Stimulating Factor) Technology. Tomorrowlabs GmbH provided research grant support for the study. The other authors have no conflicts of interest to declare. This study was further supported by the Translational Medicine Program of the TUM School of Medicine and by a grant from the TUM Commission of Clinical Research to Dominik Duscher. The authors listed expressly wrote the content of this article. No ghostwriters were used to write this article.

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Author Contributions

J.B.: lab work and data collection, data analysis, and manuscript writing. D.P.: study support and manuscript editing. D.T.: study support and manuscript writing. D.D.: study support and manuscript editing. E.B.: project design and oversight, manuscript writing and editing.

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