











Peroxisome proliferator-activated receptor- γ signalling protects hair follicle stem cells from chemotherapy-induced apoptosis and epithelial–mesenchymal transition*

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Summary

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Funding sources

Monasterium Laboratory has received preclinical research grants to study the effects of N-acetyl-GED-0507-34-Levo (NAGED) on human hair follicles from Nogra Pharma Ltd, Ireland, which has filed a patent on the use of NAGED as a treatment for scarring alopecia.

Conflicts of interest

R.P. is the Founder and CEO of Monasterium Laboratory GmbH, Münster, a dermatology contract research organization. I.P., L.B., J.C., S.G., M.A. and M.B. are or have been employees of Monasterium Laboratory, for which Y.R., F.J. and J.H. serve or served as consultants.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

M.A.'s current affiliations are Department of Dermatology and Venereology, Hamad Medical Corporation, Doha, Qatar; and Translational Research Institute, Academic Health System, Doha, Qatar.

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Background Permanent chemotherapy-induced alopecia (pCIA), for which preventive interventions remain limited, can manifest with scarring. While the underlying pathomechanisms of pCIA are unclear, depletion of epithelial hair follicle (HF) stem cells (eHFSCs) is likely to play a role.

Objectives To explore the hypothesis that, besides apoptosis, eHFSCs undergo pathological epithelial–mesenchymal transition (EMT) in pCIA, thus explaining the scarring phenotype. Furthermore, we tested whether a peroxisome proliferator-activated receptor (PPAR)- γ modulator could prevent pCIA-associated pathomechanisms.

Methods Organ-cultured human scalp HFs were treated with the cyclophosphamide metabolite 4-hydroperoxycyclophosphamide (4-HC). Additionally, HFs were pretreated with the agonistic PPAR- γ modulator N-acetyl-GED-0507-34-Levo (NAGED), which has previously been shown to promote K15 expression and antagonize EMT in eHFSCs.

Results In accordance with anticipated hair bulb cytotoxicity, dystrophy and catagen induction, 4-HC promoted apoptosis along with increased p53 expression, DNA damage and pathological EMT in keratin 15⁺ (K15) eHFSCs, as evidenced by decreased E-cadherin expression and the appearance of fibronectin⁺ and vimentin⁺ cells in the hair bulge. Pretreatment with NAGED protected against 4-HC-induced hair bulb cytotoxicity/dystrophy, and apoptosis, p53 upregulation and EMT in the bulge, thereby significantly preventing depletion of K15⁺ human eHFSCs *ex vivo*.

Conclusions Since a key cyclophosphamide metabolite alone suffices to damage and deplete human scalp eHFSCs by promoting apoptosis, DNA damage and EMT *ex vivo*, strategies to prevent pCIA need to target these pathomechanisms. Given the ability of NAGED to prevent chemotherapy-induced eHFSCs damage *ex vivo*, our study introduces the stimulation of PPAR- γ signalling as a novel intervention strategy for the prevention of pCIA.

What is already known about this topic?

- Chemotherapy can lead to permanent hair loss, which can manifest as scarring alopecia. Unfortunately, the pathomechanism of this phenomenon is unknown, and efficient preventive interventions are lacking.

R.P. and M.B. contributed equally.

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What does this study add?

- Repeated treatment of organ-cultured human hair follicles (HFs) with 4-hydroperoxycyclophosphamide (4-HC) led not only to expected dystrophic changes in the bulb, but also resulted in DNA damage, apoptosis and pathological epithelial–mesenchymal transition (EMT) of epithelial HF stem cells (eHFSCs).
- Pretreatment with a peroxisome proliferator-activated receptor (PPAR)- γ modulator hindered 4-HC-induced hair bulb cytotoxicity/dystrophy, and protected K15⁺ human eHFSCs from apoptosis and EMT, thereby significantly preventing their depletion *ex vivo*.

What is the translational message?

- Stimulation of PPAR- γ signalling is a promising novel intervention strategy for the prevention of permanent chemotherapy-induced alopecia.

Chemotherapy-induced alopecia (CIA) is regarded by patients as one of the most severe adverse effects of oncological therapy, which may even lead to the rejection of treatment, especially by women.^{1–3} In most cases, CIA is transient, with hair regrowth seen within 3 months.² However, chemotherapy can also lead to permanent hair loss, and permanent CIA (pCIA) is diagnosed when there is no or incomplete hair regrowth 6 months after chemotherapy is stopped.⁴ The incidence of pCIA can reach 42% in adult breast cancer survivors,⁵ and can be extremely distressing.⁶ Although scalp hypothermia may reduce the occurrence of pCIA,^{7–10} reliably effective pCIA prevention strategies remain to be developed, and there are no generally accepted guidelines for the prevention and management of pCIA.⁷

The pathomechanisms of pCIA are unclear and are likely to vary between chemotherapeutic agents.¹¹ Other forms of permanent hair loss, namely scarring alopecias, are associated with damage [i.e. apoptosis and epithelial–mesenchymal transition (EMT)] to the epithelial hair follicle (HF) stem cell (eHFSC) and the replacement of HFs by fibrotic tissue.^{12,13} Therefore, it is plausible that pCIA results from irreversible drug-induced damage to eHFSCs located in the hair bulge,^{11,14} especially when a fibrotic (scarring) phenotype is seen in the hair loss lesions of some patients with pCIA.^{15,16}

The alkylating agent cyclophosphamide is a commonly used chemotherapeutic agent. While cyclophosphamide promotes reversible CIA in the majority of treated patients, it has been implicated in cases of pCIA.^{17–21} Moreover, cyclophosphamide and its toxicologically active metabolite 4-hydroperoxycyclophosphamide (4-HC)²² have proven to be excellent reference agents for investigating the pathobiology of CIA, both in mice *in vivo* and in human scalp HFs *ex vivo*.^{22–24} These studies have helped elucidate how HFs respond to chemotherapy-induced damage.^{11,23,25} A humanized mouse model has recently joined these two model systems by showing that the administration of a single dose of cyclophosphamide to immunodeficient mice transplanted with human HFs induced typical HF changes seen in patients with CIA.²⁶

Moreover, repeated treatment with busulfan and cyclophosphamide, but not the single administration of cyclophosphamide alone, reportedly results in profound apoptosis of keratin 15 (K15)⁺ eHFSCs,²⁷ which are crucial for HF cycling and regeneration.^{13,27–29} Similar results were seen with busulfan and 4-HC co-administration to organ-cultured human scalp HFs.²⁷ Moreover, we have shown that paclitaxel and docetaxel also induce pathological mitosis, apoptosis, and DNA damage of human eHFSCs *ex vivo*,³⁰ which may explain the pCIA frequently seen with taxane therapy.³¹

Therefore, all currently available evidence clearly points to irreversible eHFSC damage as a chief cause of pCIA. However, whether EMT also occurs in eHFSCs after chemotherapy treatment, explaining scarring hair loss lesions in certain patients with pCIA, remains to be investigated.^{15–21}

A key signalling pathway that suppresses EMT is the peroxisome proliferator-activated receptor (PPAR)- γ pathway.^{32–34} PPAR- γ is abundantly expressed in human HFs, including in the hair bulge eHFSC niche.³³ PPAR- γ plays a major role in HF homeostasis, as its stimulation results in enhanced mitochondrial energy metabolism, premature catagen development and the inhibition of intra- and perifollicular inflammation towards the hair bulge in HFs affected by lichen planopilaris.^{33,35–37} Notably, stimulation of PPAR- γ -mediated signalling by *N*-acetyl-GED-0507-34-Levo (NAGED)³⁸, a novel, topically applicable and selective PPAR- γ modulator with agonistic activity, resulted in higher K15 expression and/or an increased number of K15⁺ cells both in normal human scalp HFs and in lesional HFs from patients with scarring alopecia *ex vivo*.^{32,34} In addition, NAGED protected against and partially reversed experimentally induced EMT in eHFSCs in healthy human scalp HFs and reduced the EMT phenotype in the bulge of lesional HFs from scarring alopecia skin biopsies *ex vivo*.^{32,34}

This prompted us to examine whether NAGED exerts protective properties against pCIA-associated pathomechanisms directly within the main human target organ (i.e. terminal anagen VI scalp HFs).²⁸ To test this hypothesis, we

experimentally induced HF dystrophy and bulge eHFSC apoptosis and EMT by repetitive 4-HC treatment of healthy, organ-cultured full-length human scalp HF, ^{22,27,30,32,34} and then tested the protective effects of NAGED under these experimental conditions.

Materials and methods

Human samples

Temporal and occipital human scalp skin or follicular units were obtained from healthy donors (both men and women, aged 21–56 years) undergoing cosmetic surgery after informed consent and ethical approval was obtained (University of Muenster, no. 2015-602-f-S).

Hair follicle organ culture

Full-length anagen VI HF were microdissected and individually cultured for 5 or 6 days *ex vivo* at 37°C with 5% CO₂ in William's E medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 2 mmol L⁻¹ L-glutamine (Gibco), 10 ng mL⁻¹ hydrocortisone (Sigma-Aldrich, St Louis, MO, USA), 10 μ g mL⁻¹ insulin (Sigma-Aldrich) and 1% penicillin/streptomycin mix (Gibco).

After either 1 or 2 days of rest, 3 μ mol L⁻¹ or 30 μ mol L⁻¹ 4-HC was added to the culture medium, and then administered every other day (4 days of treatment) (Figure S1a; see Supporting Information). The concentrations for 4-HC were selected based on previously published results.²² For protection experiments, NAGED was added at three different concentrations (0.01, 0.1 and 1 mmol L⁻¹) to the medium after a day of rest of the *ex vivo* culture, and the medium was replaced on days 2 and 4 with NAGED and 30 μ mol L⁻¹ 4-HC (5-day

treatment with NAGED, including 4 days of co-treatment with 4-HC) (Figure S1b). The concentrations for NAGED were selected based on previously published results.^{32,34,35,37} Vehicle-treated HF were exposed to 0.1–0.3% dimethyl sulfoxide. The culture was terminated by embedding the HF in optimal cutting temperature compound (OCT) and freezing them in liquid nitrogen. Cryosections of 6 μ m were obtained with a Leica cryostat (Leica Biosystems, Wetzlar, Germany) and stored at -80°C.

Histology

For histochemical visualization of melanin, Masson Fontana staining was performed. Briefly, cryosections were fixed in ethanol + acetic acid (glacial) for 10 min at -20°C, incubated for 40 min in 5% silver nitrate solution at 56°C in the dark, followed by 1 min in 5% sodium thiosulfate and then counterstained for 5 min with haematoxylin solution.^{22,39}

Immunofluorescence

Tissue cryosections were fixed, blocked and incubated with the corresponding primary antibodies at 4°C overnight (Table 1). Secondary antibody incubation was performed at RT for 45 min.^{30,32,34,35} To stain terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)⁺ K15⁺ cells, we used the ApopTag[®] kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's protocol, and K15 primary antibody was incubated overnight after the terminal deoxynucleotidyl transferase step. The secondary antibody was incubated for 45 min at RT after the fluorescent-labelled anti-digoxigenin step of the ApopTag kit.^{22,39} Counterstaining with 4',6-diamidino-2-phenylindole (1 μ g mL⁻¹) was performed to visualize nuclei.

Table 1 List of primary antibodies used in the study

Fixation	First primary			Second primary			Blocking
	antibody	Company	Dilution	antibody	Company	Dilution	
Acetone	Mouse K15	Millipore (CBL272)	1:200	Rabbit Ki67	Abcam (ab15580) ^a	1:200	2% BSA
4% PFA	Mouse K15	Millipore (CBL272)	1:200	Rabbit cCasp3	Cell Signaling Technology (9661S) ^b	1:400	10% GNS
4% PFA	Mouse K15	Millipore (CBL272)	1:200	γ H2A.X	Cell Signaling Technology (2577S)	1:1500	10% GNS
Acetone	Mouse E-cadherin	Abcam (ab1416)	1:100	Rat ITGA6	Millipore (MAB1378) ^c	1:200	2% BSA
Acetone	Mouse vimentin	Millipore (MAB3400)	1:200	Rat ITGA6	Millipore (MAB1378)	1:200	2% BSA
Acetone	Rabbit fibronectin	Abcam (ab23750)	1:200	Rat ITGA6	Millipore (MAB1378)	1:200	2% BSA
4% PFA	Mouse p53	Biologend (645701)	1:200	Mouse K15	Millipore (CBL272)	1:200	–

BSA, bovine serum albumin; GNS, goat normal serum; PFA, paraformaldehyde. ^aAbcam (Cambridge, UK); ^bCell Signaling Technology (Danvers, MA, USA); ^cMerck Millipore (Darmstadt, Germany).

Quantitative (immuno-)histomorphometry

Images were taken using a Keyence fluorescence microscope BZ9100 (Osaka, Japan) maintaining a constant set exposure time throughout imaging for further analysis. Staining immunoreactivity or the number of positive cells were counted in the corresponding reference areas depicted in the different figures. Analyses were carried out with IMAGEJ (National Institutes of Health, Bethesda, MD, USA).^{22,30,32,34,35} Hair cycle staging and scoring were performed as previously described.^{39,40} For hair cycle scoring, each hair cycle phase receives an arbitrary score: anagen VI = 100; early catagen = 200; mid-catagen = 300; and late catagen = 400.⁴⁰

Statistics

Statistical analyses were performed with GraphPad Prism 6 (GraphPad, La Jolla, CA, USA). The D'Agostino and Pearson omnibus normality test was used to determine whether the data followed a Gaussian distribution. An unpaired parametric Student's *t*-test or nonparametric Mann–Whitney *U*-test was used to compare the results between two experimental groups, while a parametric one-way ANOVA and Tukey's multiple comparison test or nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison test were used to compare the results deriving from more than two experimental groups. Data were expressed as mean \pm SEM; *P*-values < 0.05 were considered to be statistically significant. Only relevant comparisons were reported (i.e. 3 $\mu\text{mol L}^{-1}$ or 30 $\mu\text{mol L}^{-1}$ 4-HC vs. vehicle; 3 $\mu\text{mol L}^{-1}$ 4-HC vs. 30 $\mu\text{mol L}^{-1}$ 4-HC; and 4-HC + NAGED vs. 4-HC alone).

Results

Repeated treatment with 4-hydroperoxycyclophosphamide induces hair follicle cytotoxicity/dystrophy and premature catagen development

We pursued to confirm that repeated treatment with 4-HC induces changes in the hair bulb of full-length human scalp

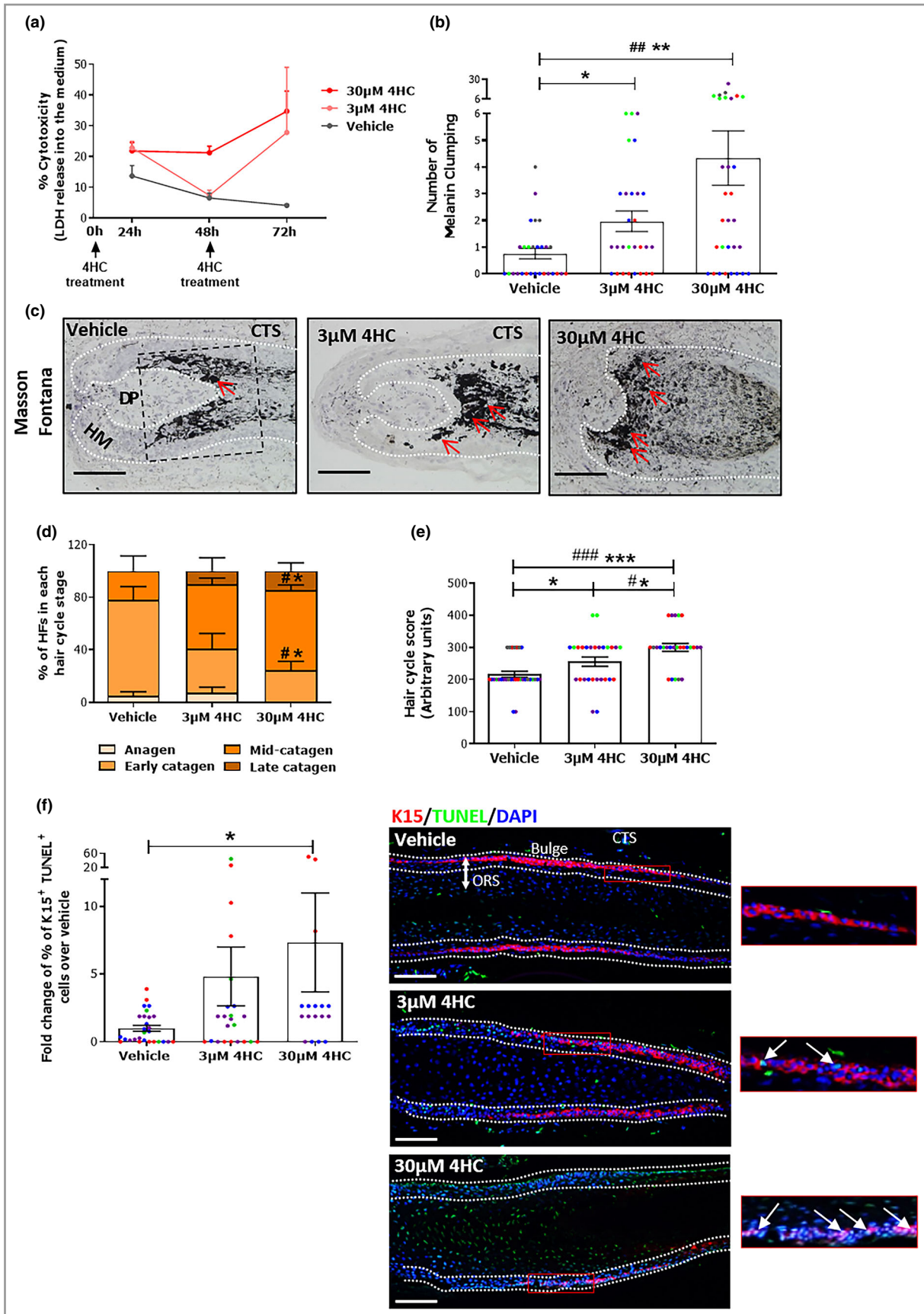
HF, recapitulating the clinical presentation of CIA in patients, as previously shown with a similar model using microdissected HFs amputated below the hair bulge.²² HFs were treated with repetitive exposure to two concentrations of 4-HC (3 $\mu\text{mol L}^{-1}$ and 30 $\mu\text{mol L}^{-1}$) for 4 days, and the changes were compared to vehicle-treated HFs. As a sensitive indicator of general HF cytotoxicity, we measured lactate dehydrogenase (LDH) activity in the supernatant throughout the study period.^{40,41} Treatment with both concentrations of 4-HC induced a gradual increase in LDH activity, demonstrating the expected cumulative HF cytotoxicity of repeated 4-HC treatment over time (Figure 1a).

As a sensitive marker for HF dystrophy, we next evaluated the number of melanin clumps (i.e. the pathological extracellular deposition of melanin and melanin granule fusion in the hair bulb).^{22,25,39} 4-HC significantly and dose dependently increased the number of melanin clumps (Figure 1b, c), thus attesting to the induction of major HF dystrophy. Finally, we tested the effect of 4-HC on catagen induction, the regression phase of the HF cycle, which can be accurately assessed *ex vivo*, as previously described.^{22,40,42} 4-HC induced premature catagen in a dose-dependent manner, and the hair cycle score was significantly higher with increasing concentrations of 4-HC (Figure 1d, e). These results are in line with previously published findings that repeated treatment of HFs *ex vivo* with 4-HC leads to HF cytotoxicity and dystrophy,²² thus attesting to the suitability of full-length HF organ culture as a reliable pre-clinical model for CIA research in the human system.

Repeated treatment with 4-hydroperoxycyclophosphamide induces DNA damage and apoptosis in bulge keratin 15⁺ cells

Next, we studied the suitability of our assay to interrogate eHFSC damage in the bulge.³⁰ K15/TUNEL and K15/cleaved caspase-3 double immunostaining and quantitative (immuno-) histomorphometry showed a tendency to (3 $\mu\text{mol L}^{-1}$) or significant (30 $\mu\text{mol L}^{-1}$) increase in the percentage of apoptotic TUNEL⁺ and cleaved caspase-3⁺ K15⁺ cells in the bulge

Figure 1 Repeated treatment with 4-hydroperoxycyclophosphamide (4-HC) induces hair follicle (HF) cytotoxicity/dystrophy and premature catagen development, accompanied by apoptosis in keratin 15 (K15)⁺ hair bulge cells. (a) Percentage cytotoxicity (i.e. lactate dehydrogenase levels released into medium). Data are shown as mean \pm SEM; *n* = 10–12 HFs per group from three independent experiments (three donors). Kruskal–Wallis test (*P* < 0.001) followed by Dunn's multiple comparison test (not significant) or Mann–Whitney *U*-test (not significant). (b) Quantification of melanin clumping. Data are shown as mean \pm SEM; *n* = 28–31 HFs/group from 4–5 independent experiments (4–5 donors). Kruskal–Wallis test (*P* = 0.029) followed by Dunn's multiple comparison test (^{##}*P* < 0.01) or Mann–Whitney *U*-test (^{*}*P* < 0.05; ^{**}*P* < 0.01). (c) Representative Masson Fontana histochemistry images. Red arrows indicate melanin clumps. (d, e) Hair cycle staging: mean \pm SEM (SEM), *n* = 4–5 different experiments (4–5 donors). Kruskal–Wallis test for comparison for anagen HFs (not significant); comparison for early catagen HFs (*P* = 0.016); comparison for mid-catagen HFs (*P* = 0.021); comparison for late catagen HFs (not significant) vs. vehicle followed by Dunn's multiple comparison test ([#]*P* < 0.05) or Mann–Whitney *U*-test (^{*}*P* < 0.05). Hair cycle score: mean \pm SEM, *n* = 26–31 HFs per group from 4–5 different donors (4–5 experiments). One-way ANOVA (*P* < 0.001) followed by Tukey's multiple comparison test ([#]*P* < 0.05, ^{###}*P* < 0.001) or unpaired *t*-test (^{*}*P* < 0.05 and ^{***}*P* < 0.001). (f) Fold change in percentage of apoptotic epithelial hair follicle stem cells (eHFSCs) [terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)⁺/K15⁺] over vehicle in the hair bulge. Mean \pm SEM, *n* = 17–28 HFs/group from 3–4 independent experiments (3–4 donors). Kruskal–Wallis test (not significant) or Mann–Whitney test, ^{*}*P* < 0.05. Representative images of K15 (red)/TUNEL (green) double staining with DAPI (blue) counterstaining. White arrows indicate K15⁺TUNEL⁺ cells in the bulge. Dashed lines indicate reference areas. AU, arbitrary units; CTS, connective tissue sheath; DAPI, 4',6'-diamidino-2-phenylindole; DP, dermal papilla; HM, hair matrix; ORS, outer root sheath. Scale bar = 100 μm .



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[Figure 1f; Figures S2a and S3 (see Supporting Information)]. Double staining for K15 and the well-established DNA damage marker γ -H2A.X showed that 30 $\mu\text{mol L}^{-1}$ 4-HC led to a significant increase in the percentage of γ -H2A.X⁺ K15 cells (Figure S2b),^{30,43} thus demonstrating direct DNA damage to eHFSCs by 4-HC. Therefore, the highest tested dose of this toxic cyclophosphamide metabolite induces both apoptosis and major DNA damage to eHFSCs in the human bulge *ex vivo*.

Repeated treatment with 4-hydroperoxycyclophosphamide stimulates epithelial–mesenchymal transition in bulge keratin 15⁺ cells

To test whether the administration of 4-HC and the resulting scarring phenotype observed in patients with pCIA⁷ is due, at least in part, to the induction of EMT in eHFSCs, we also evaluated established markers for intrafollicular EMT, namely E-cadherin downregulation and abnormal vimentin and/or fibronectin expression in the bulge epithelium.^{13,32,34} The evaluation of these markers in the bulge area was consistent with the induction of EMT. Namely, 30 $\mu\text{mol L}^{-1}$ 4-HC led to a statistically significant decrease in the expression of the epithelial marker E-cadherin (Figure 2a) – a key event during EMT⁴⁴ – and also increased the number of intrabulge cells that abnormally expressed the mesenchymal markers vimentin or fibronectin (Figure 2b, c). Importantly, some intrabulge vimentin⁺ cells were double-positive for K15⁺ (Figure 2d; Figure S2c), which demonstrated that eHFSCs undergo EMT in response to 4-HC repeated treatment, as observed in the primary scarring alopecia, lichen planopilaris.^{13,34} To the best of our knowledge, this represents the first demonstration that chemotherapy can induce EMT in the bulge of human HF.

Repeated treatment with 4-hydroperoxycyclophosphamide depletes human epithelial hair follicle stem cells *ex vivo*

Given that K15⁺ cells undergo DNA damage, apoptosis and EMT after repeated treatment with 4-HC, we subsequently investigated whether these phenomena lead to the depletion of the human eHFSC pool in the bulge by evaluating the

number of K15⁺ cells and the expression of K15 in the bulge.^{30,32} Contrary to previous results based on a single treatment with 4-HC,²⁷ quantitative (immuno)histomorphometry showed that repeated treatment of full-length human HF resulted in a significant decrease in both the overall expression level of K15 protein and in the number of K15⁺ cells in the hair bulge (Figure 2e), thus demonstrating that eHFSC damage by 4-HC is enough to deplete the human bulge stem cell niche *ex vivo*. This renders it likely that such a depletion phenomenon also occurs *in vivo*, at least under conditions of high-dose cyclophosphamide therapy, which is likely to be aggravated when combined with other eHFSC-damaging therapies such as busulfane and taxanes,^{27,30} or with radiotherapy.⁴⁵

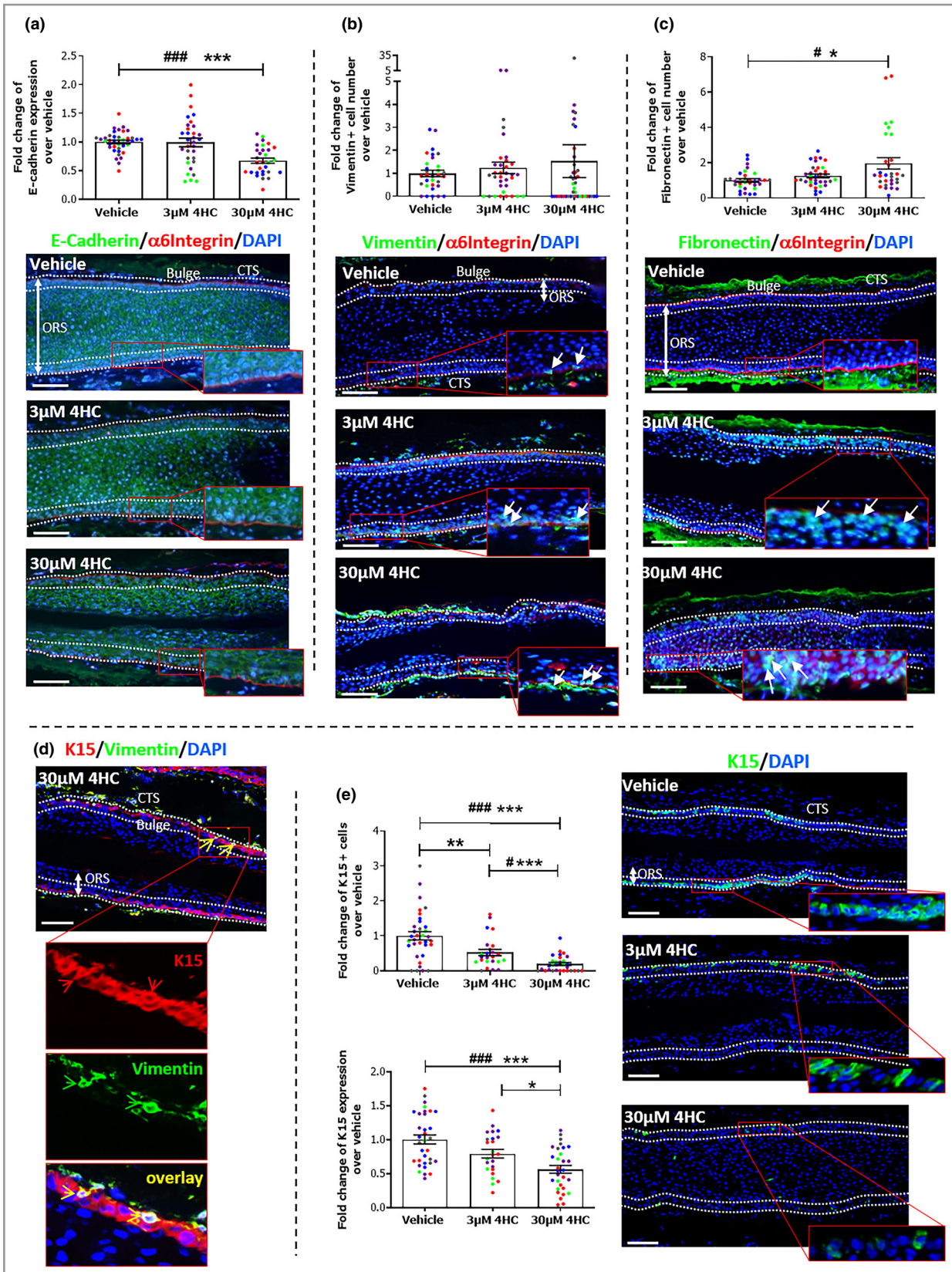
N-acetyl-GED-0507-34-Levo treatment reduces 4-hydroperoxycyclophosphamide-induced hair follicle cytotoxicity and dystrophy

Next, we tested the ability of the novel, selective and topically applicable PPAR- γ modulator NAGED to prevent HF cytotoxicity/dystrophy.^{22,32,34,38} We pretreated anagen VI scalp HF with NAGED (0.01, 0.1 and 1 mmol L⁻¹) for 24 h, and then co-administered NAGED with 4-HC for the remaining 4-day treatment period. This showed that NAGED prevented the formation of melanin clumps induced by 30 $\mu\text{mol L}^{-1}$ 4-HC (Figure 3a) and inhibited LDH release into the medium, when this was increased by 4-HC treatment, at day 6 of organ culture (Figure 3b). However, as expected from the known catagen-promoting effects of NAGED on normal HF *ex vivo*,^{33,37} the addition of NAGED did not change the ratio of 4-HC-treated HF in catagen vs. anagen, or the hair cycle score (Figure 3c, d). These data show that, while the stimulation of PPAR- γ signalling does not prevent chemotherapy-induced catagen induction, it reduces the level of 4-HC-induced hair bulb dystrophy *ex vivo*.

N-acetyl-GED-0507-34-Levo pretreatment prevents 4-hydroperoxycyclophosphamide-induced keratin 15⁺ cell apoptosis and p53 induction in the bulge

We next tested whether the administration of NAGED can reduce chemotherapy-induced eHFSC apoptosis in the bulge.

Figure 2 Repeated treatment with 4-hydroperoxycyclophosphamide (4-HC) stimulates epithelial–mesenchymal transition (EMT) and induces keratin 15 (K15)⁺ cell depletion in the hair bulge. (a) Fold change in E-cadherin expression in the hair bulge region. Mean \pm SEM, n = 30–39 hair follicles (HF) per group from five independent experiments (five donors). One-way ANOVA ($P < 0.001$) followed by Tukey's multiple comparisons test (#### $P < 0.001$) or unpaired Student's t-test (*** $P < 0.001$). (b) Fold change in number of vimentin⁺ cells in the hair bulge region. Mean \pm SEM, n = 31–41 HF per group from five independent experiments (five donors). Kruskal–Wallis test (not significant) or Mann–Whitney U-test (not significant). White arrows indicate vimentin⁺ cells in the bulge. (c) Fold change in fibronectin⁺ cell number in the hair bulge. Mean \pm SEM, n = 30–35 HF per group from five independent experiments (five donors). Kruskal–Wallis test ($P = 0.057$) followed by Dunn's multiple comparisons test ([#] $P < 0.05$) or Mann–Whitney U-test (* $P < 0.05$). White arrows indicate fibronectin⁺ cells in the bulge. (d) Representative images of HF treated with 30 $\mu\text{mol L}^{-1}$ 4-HC. Red arrows indicate K15⁺ cells, green arrows indicate vimentin⁺ cells and yellow arrows indicate K15⁺ vimentin⁺ cells. (e) Fold change in number of K15⁺ cells and K15 expression within the hair bulge. Mean \pm SEM, n = 24–34 HF per group from five independent experiments (five donors). Kruskal–Wallis test ($P < 0.001$) followed by Dunn's multiple comparisons test ([#] $P < 0.05$, #### $P < 0.001$) or Mann–Whitney U-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Dashed lines indicate reference areas. CTS, connective tissue sheath; DAPI, 4',6-diamidino-2-phenylindole; ORS, outer root sheath. Scale bar = 100 μm .



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Our data showed that all concentrations, particularly the highest (1 mmol L⁻¹), of NAGED tended to reduce 4-HC-induced apoptosis in K15⁺ hair bulge eHFSCs, as

demonstrated by a trend towards a decrease in K15/TUNEL double-positive cell number [Figure 4a; Figure S3 (see Supporting Information)]. Previous studies have demonstrated

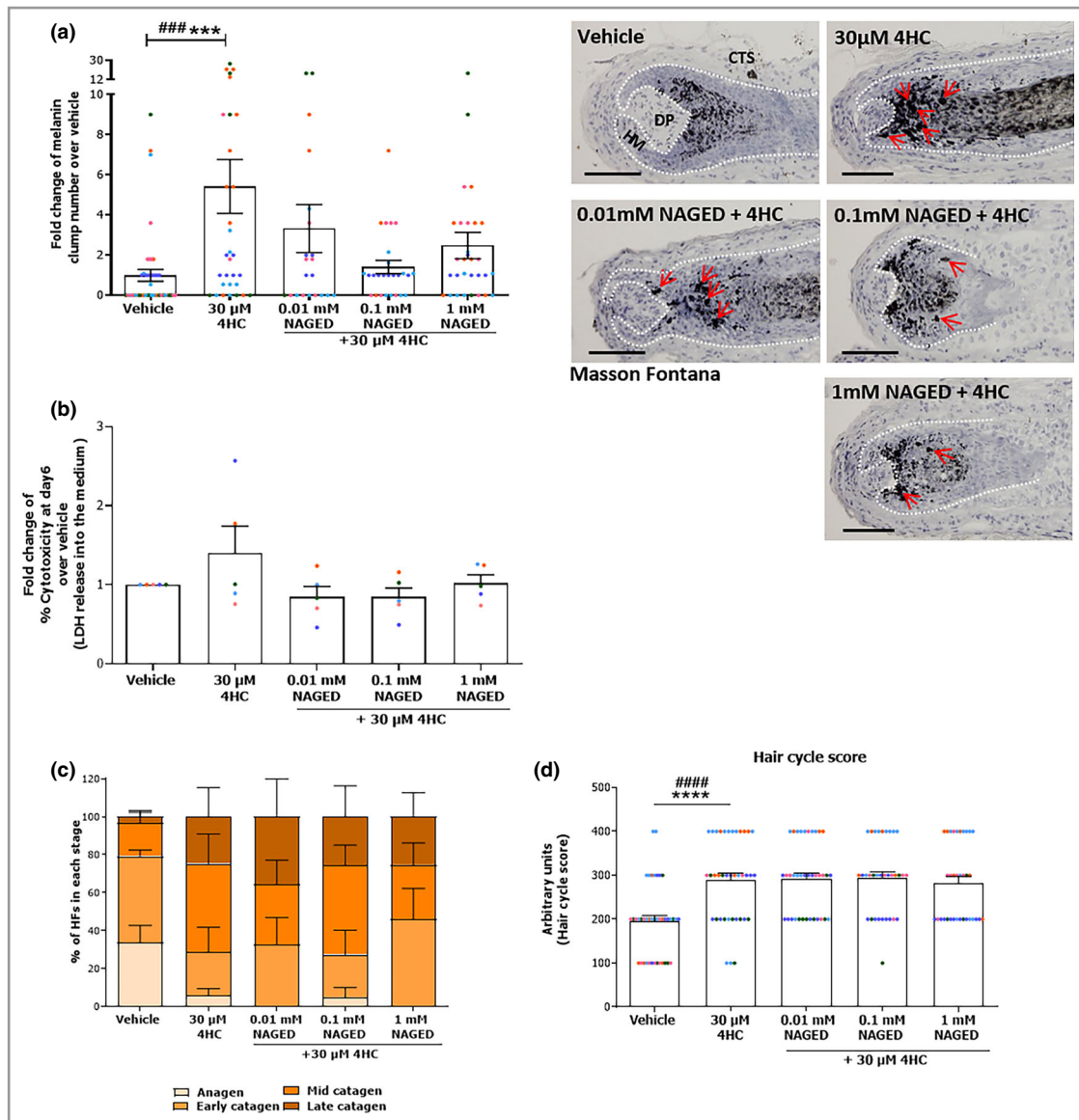


Figure 3 N-acetyl-GED-0507-34-Levo (NAGED) treatment prevents 4-hydroperoxycyclophosphamide (4-HC)-induced hair follicle (HF) cytotoxicity and dystrophy but not premature catagen development. (a) Fold change in melanin clumps. Mean \pm SEM, $n = 21$ –45 HF per group from 4–5 independent experiments (4–5 different donors). Kruskal–Wallis test ($P = 0.002$) followed by Dunn’s multiple comparisons test ($^{##}P < 0.01$) or Mann–Whitney U-test ($^{***}P < 0.001$). Representative images of Masson Fontana staining. Red arrows point to melanin clumping. (b) Fold change in the percentage of cytotoxicity (lactate dehydrogenase released into the medium) of cultured HF. Mean \pm SEM, $n = 5$ independent experiments (five donors). Kruskal–Wallis test (not significant) or Mann–Whitney U-test (not significant). (c) Hair cycle staging. Mean \pm SEM, $n = 5$ independent experiments (five donors) deriving from 33–41 HF per group. Kruskal–Wallis test (not significant) or Mann–Whitney U-test (nonsignificant). (d) Calculated hair cycle score. Mean \pm SEM, $n = 33$ –41 HF per group from five independent experiments (five donors). Kruskal–Wallis test ($P < 0.001$) followed by Dunn’s multiple comparisons test ($^{####}P < 0.0001$) or Mann–Whitney U-test ($^{****}P < 0.0001$). AU, arbitrary units; CTS, connective tissue sheath; DP, dermal papilla; HM, hair matrix. Scale bar = 100 μ m.

that cyclophosphamide-induced apoptosis is accompanied by the induction of p53 expression,^{22,27,46–50} and that p53 is an essential part of CIA pathogenesis.⁴⁶ Therefore, we tested whether pretreatment with NAGED can protect against the induction of p53 by 4-HC in K15⁺ bulge cells. 4-HC (30 μ mol L⁻¹) significantly increased the expression of p53

in K15⁺ cells in the bulge, as expected (Figure 4b). Treatment with NAGED at 0.1 mmol L⁻¹ and 1 mmol L⁻¹ concentrations hindered the upregulation of p53 expression in K15⁺ bulge eHFSCs (Figure 4b). Thus, NAGED inhibits the induction of p53-dependent apoptosis in K15⁺ bulge eHFSCs mediated by 4-HC administration.

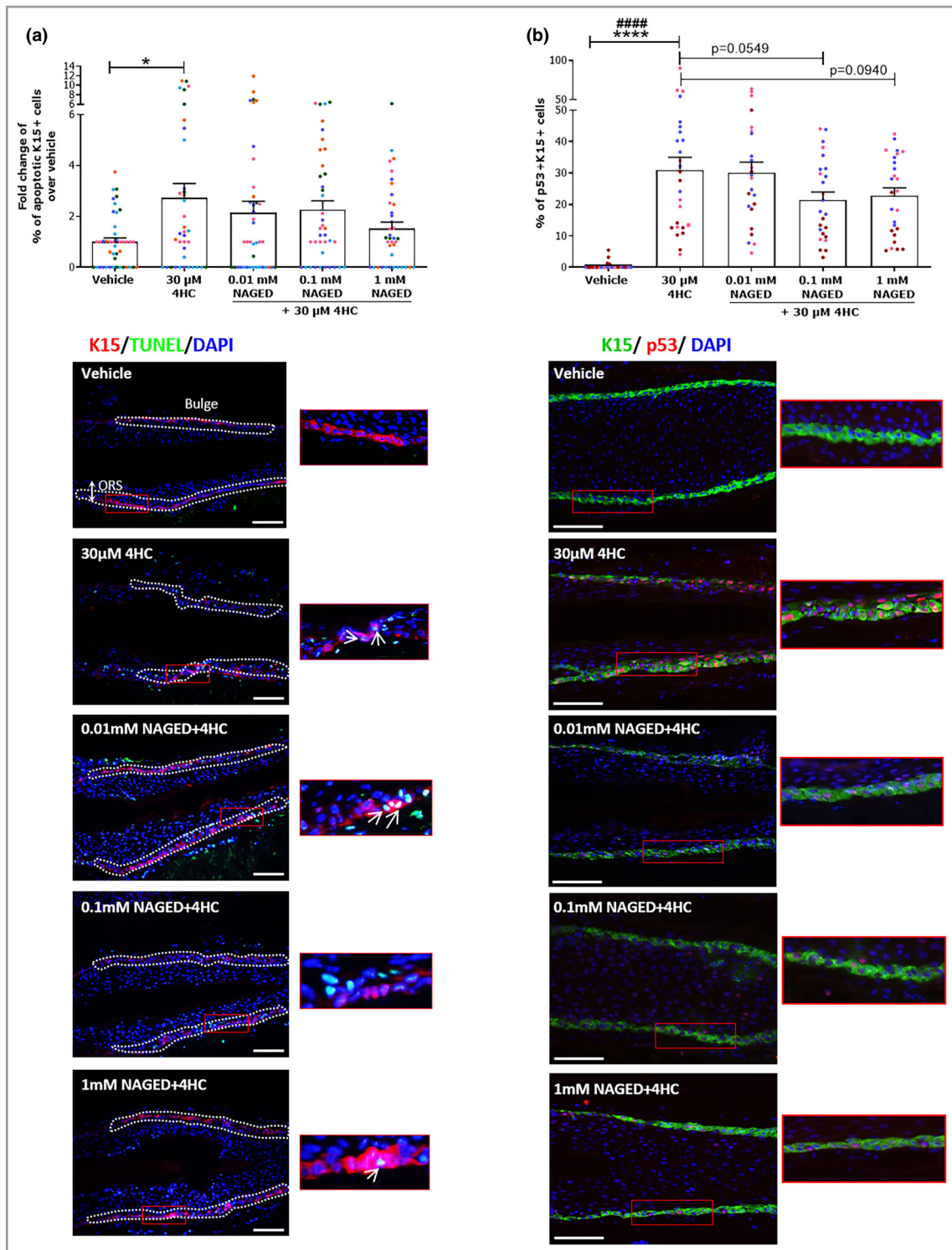


Figure 4 Pretreatment with N-acetyl-GED-0507-34-Levo (NAGED) protects from 4-hydroperoxycyclophosphamide (4-HC)-induced keratin 15 (K15)⁺ cell apoptosis and induction of p53 in the hair bulge. (a) Fold change in the percentage of apoptotic epithelial hair follicle stem cells (eHFSCs) [terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)⁺/K15⁺] in the hair bulge region. Mean \pm SEM, n = 37–43 hair follicles (HF) per group from five independent experiments (five donors). Kruskal–Wallis test (not significant) or Mann–Whitney U-test (*P < 0.05). White arrows indicate TUNEL⁺/K15⁺ cells in the hair bulge. (b) p53 expression in K15⁺ cells after 4-HC treatment and NAGED pretreatment. Mean \pm SEM, n = 24–26 HF per group from three independent experiments (three donors). Kruskal–Wallis test (P < 0.001) followed by Dunn’s multiple comparisons test (####P < 0.0001) or Mann–Whitney U-test (****P < 0.0001). Dashed lines indicate the reference areas. DAPI, 4’,6-diamidino-2-phenylindole; ORS, outer root sheath.

Pretreatment with *N*-acetyl-GED-0507-34-Levo prevents 4-hydroperoxycyclophosphamide-induced bulge epithelial-mesenchymal transition

Given the recognized protective effects of NAGED in inhibiting pathological EMT in the bulge *ex vivo*,^{32,34} we evaluated whether this PPAR- γ modulator also prevents 4-HC-induced EMT. NAGED significantly inhibited the decrease in E-cadherin expression and the increase in vimentin⁺ cells in the bulge epithelium stimulated by 4-HC treatment [Figure 5a, b; Figure S4a, b (see Supporting Information)]. Interestingly, these EMT-protective effects were mainly seen after treatment with low concentrations of NAGED (0.01 and 0.1 mmol L⁻¹) (Figure 5a, b; Figure S4a, b). Therefore, the evaluation of EMT-associated markers indicated that NAGED significantly prevented 4-HC-induced EMT.

Pretreatment with *N*-acetyl-GED-0507-34-Levo inhibits 4-hydroperoxycyclophosphamide-induced depletion of bulge epithelial hair follicle stem cells

Lastly, we investigated whether NAGED could prevent 4-HC-induced eHFSC depletion, seen as a result of K15⁺ cells apoptosis and/or EMT. Pretreatment with NAGED significantly prevented the decrease of K15 expression in the bulge (Figure 5c; Figure S3) and an accompanying decline in the percentage of K15⁺ cells in the bulge induced after the administration of 4-HC (Figure 5d; Figure S3). Thus, the selective stimulation of PPAR- γ signalling significantly prevented chemotherapy-induced eHFSC depletion by providing relative protection from apoptosis and the induction of EMT.

Discussion

Our study reveals that repeated administration of a cyclophosphamide metabolite alone is enough to damage human scalp eHFSCs severely by promoting pathological bulge K15⁺ cells apoptosis, DNA damage, and EMT *ex vivo*. This strongly supports the concept that the clinical presentation of pCIA reflects persistent or even irreversible eHFSC damage and eventual depletion,^{13,32,34,51} and highlights that future pCIA preventive strategies need to be tailored to target and reduce not only chemotherapy-induced eHFSC apoptosis and DNA damage, but also pathological EMT. Even if eHFSC are not completely depleted, this damage likely suffices to reduce their capacity to generate transit amplifying cells, which could contribute HF miniaturization in pCIA.

It is therefore encouraging that our study suggests that stimulation of PPAR- γ signalling in eHFSCs is a promising novel intervention strategy for the prevention of pCIA. Specifically, we show here that a PPAR- γ -stimulating agent (NAGED) reduces key aspects of chemotherapy-induced bulge damage (i.e. eHFSC apoptosis and EMT). Thus, it is conceivable that NAGED can also positively impact on the survival or repair of bulge eHFSCs under clinical conditions of CIA and pCIA.

Our work also raises the question of whether subtle indications of fibrotic changes in the bulge, consistent with EMT, may have been overlooked in previous histopathological studies of pCIA skin biopsies,^{19,21,31,52} especially as standard immunohistological and ultrastructural EMT markers were not investigated.³⁴

NAGED demonstrated an apoptosis-protective effect in human eHFSCs *ex vivo*, which is in agreement with previous work that showed that NAGED increases K15⁺ cell number in human scalp HF *ex vivo*, both under physiological and pathological conditions.^{32,33,37} The significant prevention of p53 upregulation in the bulge in NAGED-treated HF further corroborates these data and supports the hypothesis that the anti-apoptotic effect might be mediated, in part, by inhibiting p53 induction in our model, confirming previous data.^{22,27,46–50} Therefore, our data highlight that stimulation of PPAR- γ signalling in resting K15⁺ human eHFSCs can be robustly stem cell protective.

That NAGED could not prevent CIA-induced premature catagen induction was expected as we had shown previously that it promotes catagen development in healthy human HF *ex vivo*.³⁷ However, our results suggest that pretreatment with NAGED promotes the induction of physiological catagen (absence of melanin clumping) and prevents the 'dystrophic catagen' (presence of melanin clumping) pathway triggered by 4-HC.^{11,25} Therefore, NAGED may induce faster recovery after chemotherapy, thus shortening the time until normal, fully pigmented hair shafts regrow, despite initial hair loss. However, the latter could be minimized by the application of a topical formulation limiting NAGED penetration into the skin, targeting mostly the hair bulge and sparing the proximal HF epithelium.

To the best of our knowledge, PPAR- γ agonists have not previously been tested for their capacity to prevent or reverse human CIA. Physicians have been hesitant to use classical systemic PPAR- γ agonists for dermatological indications owing to the associated adverse effects.⁵³ However, NAGED can be used topically, thus greatly reducing the risk of systemic drug toxicity and overcoming drug absorption limitations caused by chemotherapy-induced side-effects; it has a favourable toxicology profile and is currently being tested in clinical trials as a topical anti-inflammatory agent for the treatment of psoriasis and acne (EudraCT 2014-005244-17, EudraCT 2016-000540-33, EudraCT 2017-003796-58 and EudraCT 2014-002913-43).

Although our study provides only pilot evidence from *ex vivo* assays, which imitate systemic drug administration, the current results strongly encourage further investigation of the novel PPAR- γ -targeting, pCIA-preventive pharmacological strategy reported here at the clinical trial level. Moreover, our study calls for a systematic search for other suitable agents that effectively counteract chemotherapy-induced eHFSC apoptosis, DNA damage and EMT. These may be co-administered with scalp-cooling devices,^{8,54,55} and should be topically applicable so as to minimize the risk that clinically unapparent scalp micrometastases may profit from such therapeutics.¹¹

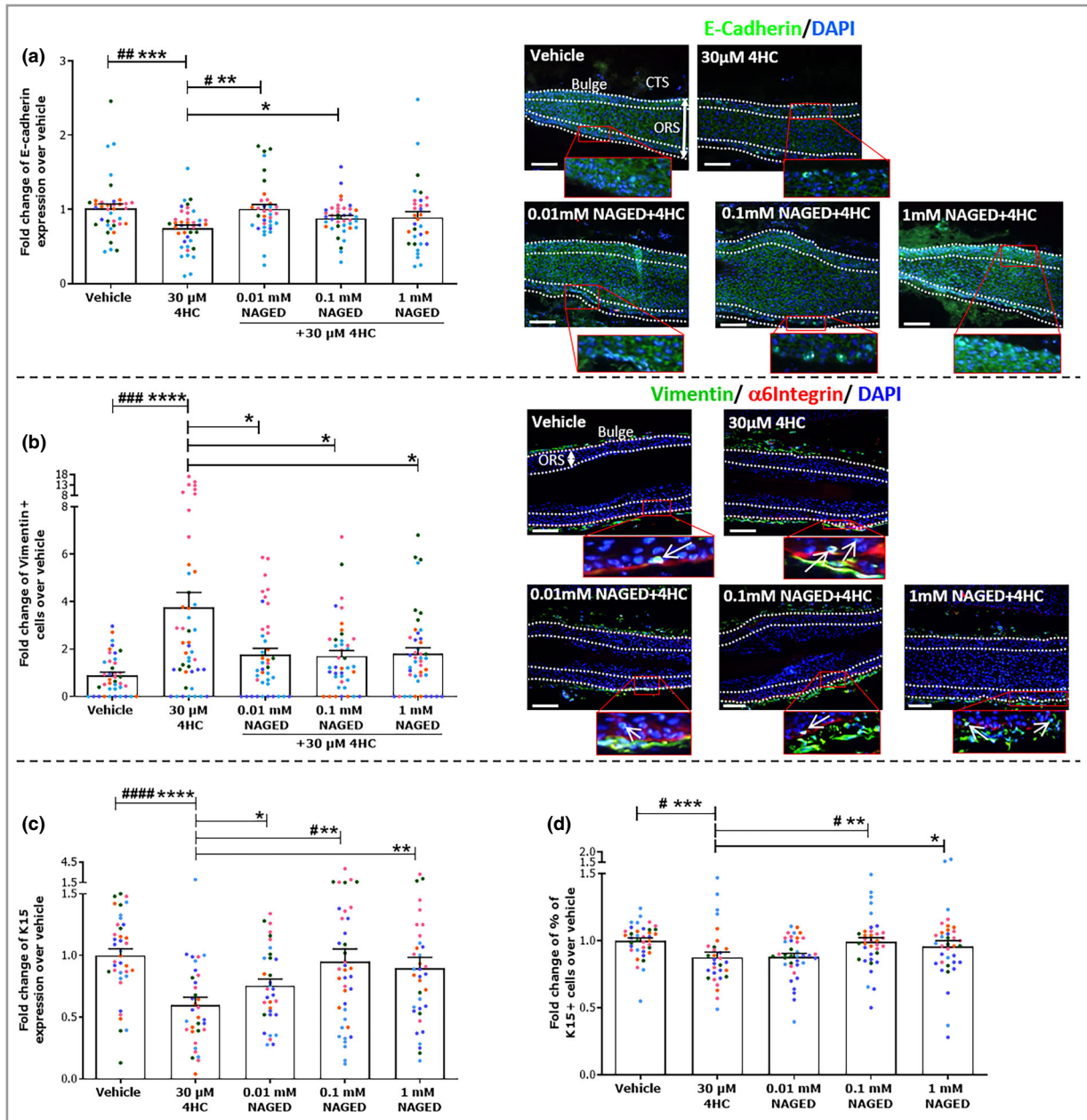


Figure 5 Treatment with N-acetyl-GED-0507-34-Levo (NAGED) prevents 4-hydroperoxycyclophosphamide (4-HC)-induced epithelial–mesenchymal transition (EMT) and keratin 15 (K15)⁺ cell depletion in the hair bulge. (a) Fold change in E-cadherin expression within the bulge region. Mean±SEM, n = 32–40 hair follicles (HF) per group from five independent experiments (five donors). Kruskal–Wallis test (P = 0.005), followed by Dunn’s multiple comparisons test ([#]P < 0.05, ^{##}P < 0.01) or Mann–Whitney U-test (*P < 0.05, **P < 0.01, ***P < 0.001). (b) Fold change in vimentin⁺ cells within the hair bulge region. Mean±SEM, n = 40–46 HF per group from five independent experiments (five donors). Kruskal–Wallis test (P = 0.001) followed by Dunn’s multiple comparisons test (^{###}P < 0.001) or Mann–Whitney U-test (*P < 0.05, ****P < 0.0001). White arrows indicate vimentin⁺ cells in the bulge. (c) Fold change in K15 expression in the hair bulge region. Mean±SEM, n = 33–39 HF per group from five independent experiments (five donors). Kruskal–Wallis test (P < 0.001) followed by Dunn’s multiple comparisons test ([#]P < 0.05, ^{####}P < 0.0001) or Mann–Whitney U-test (*P < 0.05, **P < 0.01, ****P < 0.0001). (d) Fold change in percentage of K15⁺ cells in the hair bulge region. Mean±SEM, n = 31–38 HF per group from five independent experiments (five donors). Kruskal–Wallis test (P < 0.001) followed by Dunn’s multiple comparisons test ([#]P < 0.05) or Mann–Whitney U-test (*P < 0.05, **P < 0.01, ***P < 0.001). Dashed lines indicate reference areas. CTS, connective tissue sheath; DAPI, 4’,6-diamidino-2-phenylindole; ORS, outer root sheath. Scale bar = 100 μm.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 Experimental design of chemotherapy-induced alopecia (CIA) and permanent CIA induction by (a) 4-hydroperoxycyclophosphamide, and the (b) N-acetyl-GED-0507-34-Levo protection experiments.

Figure S2 Repeated treatment with 4-hydroperoxycyclophosphamide induces DNA damage and apoptosis in keratin 15⁺ hair bulge cells.

Figure S3 Selected images of full-length hair follicles treated with 4-hydroperoxycyclophosphamide and N-acetyl-GED-0507-34-Levo, showing terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)- and keratin 15⁺ cells.

Figure S4 Selected images of full-length hair follicles treated with 4-hydroperoxycyclophosphamide and N-acetyl-GED-0507-34-Levo, showing cells positive for α 6Integrin, and (a) E-cadherin and (b) vimentin (B).

Powerpoint S1 Journal Club Slide Set.

Video S1 Author video.