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Letter to the Editor

Inhibition of ATP binding cassette transporter B1 sensitizes human hair follicles to chemotherapy-induced damage

Hair follicles (HFs) are sensitive to xenobiotic damage, which can ultimately lead to hair loss such as in chemotherapy-induced alopecia (CIA) [1]. Members of the ATP-binding cassette (ABC) transporter family fulfil a crucial role in xenobiotic handling, where they actively extrude drugs thereby reducing intracellular drug accumulation and limiting cellular toxicity [2]. Recently, we demonstrated that human scalp hair follicles (HFs) express an array of ABC transporter proteins, many of which localise to the anagen hair matrix (HM) and the bulge [3]. One predicted, but as yet unproven role for these ABC transporters may be to protect defined HF cell populations against chemotherapy-induced damage and thus provide a novel solution to this unmet clinical problem [3,4]. Although the physiological role for these transporters in human HF biology remains largely elusive, dye exclusion assays suggest that ABCB1, ABCG2 and ABCC1 can reduce substrate accumulation in key regions of the human HF epithelium [3]. The current pilot study examines whether ABC transporter activity in the human HF impacts on the cytotoxicity of anticancer drugs known to cause extensive hair loss [1].

Well-established models for examining chemotherapy-induced damage in organ-cultured human HFs were utilised, namely exposure to the cyclophosphamide metabolite 4-hydroxycyclophosphamide [5] (4HC) and the anthracycline doxorubicin [6] (DOX). HFs were obtained from patients undergoing hair transplant surgery following written informed consent, under an institutional ethics licence and cultured in serum-free supplemented medium with inhibitors of ABCB1 (100 μM verapamil [3]), ABCG2 (1 μM Ko143 [3]) or ABCC1 (10 μM reversan [7]) for 30 min prior to exposure to either 30 μM 4HC for 48 h or 1 μM DOX for 1 h followed by a 48 h washout, using doses and timepoints as previously described [5,6].

Quantitative immunohistomorphometry was utilised to examine proliferation and apoptosis in the HF bulb [8] (Fig. 1a). There was an increase in the proportion of HFs entering catagen following 4HC exposure, but this was unchanged by ABC transporter inhibition (Fig. 1b). Interestingly, the percentage of apoptotic (TUNEL+) cells increased significantly in both the hair bulb (Fig. 1d; $p < 0.01$) and the dermal papilla (DP; Fig. 1e; $p < 0.01$) when ABCB1 was inhibited during 4HC exposure, whereas verapamil alone did not alter TUNEL levels in these regions (Fig. 1c, d). Although Ki-67 immunofluorescence demonstrated that inhibition of ABCB1 with verapamil significantly decreased proliferation in the HF following 4HC exposure (Fig. 1c, $p < 0.001$). This is likely the result of calcium channel blockade, as verapamil alone also significantly reduced HF bulb proliferation (Fig. 1c, $p < 0.05$). We therefore used the alternate ABCB1 inhibitor tariquidar, showing no impact on proliferation when administered alone or in combination with 4HC (Supplementary Fig1a). It did however show a strikingly similar effect on HF apoptosis to verapamil (Supplementary Fig. 1b), indicating that the apoptotic response is greater in human HFs following inhibition of ABCB1. This response was further examined by cleaved caspase-3 (CC3) immunofluorescence. Whilst 4HC strongly up-regulated CC3 expression in the HM ($p < 0.001$) (Fig. 2a), verapamil did not further enhance this response, most likely because this apoptotic pathway was already maximally induced by the dose of 4HC employed at the timepoint assessed [5]. Notwithstanding the possibility of temporal differences in CC3 activation and upregulation of TUNEL labelling, these markers represent discrete processes within the apoptotic pathway, which could explain differences in the impact of verapamil.

Since pigmentary abnormalities are a sensitive indicator of chemotherapy-induced HF damage [1,5], Masson-Fontana histochemistry (MF) was performed. Ectopic melanin granules were observed, extending beyond the pigmentary unit and into the proximal HM following 4HC exposure (Fig. 2b). Furthermore, a loss of melanocyte dendrites was apparent, coupled to an increase in smaller, dense melanin clumps. Co-treatment with verapamil and 4HC enhanced the appearance of these widespread dense melanin clumps and increased the level of ectopic melanin observed in the HM beyond that of 4HC alone (Fig. 2b).

Results obtained when exposing HFs to the anthracycline DOX in the presence/absence of verapamil followed a strikingly similar pattern, although there was a high degree of interindividual variability in donor response to DOX-induced HF damage. Inhibition of ABCB1 with verapamil prior to DOX exposure showed an increase in the percentage of HFs in catagen (Fig. 1b) and a consistent increased in the percentage of TUNEL + cells in both the HF Bulb and DP (Fig. 1d,e) above those of DOX alone, with no change in proliferation (Fig. 1b). CC3 analysis revealed a significant increase in apoptosis in DOX-treated HFs and a (non-significant) trend towards a further increase with verapamil co-administration (Fig. 2a). DOX also induced the pigmentary abnormalities observed following 4HC exposure, namely melanin clumping and ectopic melanin in the proximal HM, which was further enhanced by verapamil exposure alongside DOX (Fig. 2b).

Since there was also a (non-significant) trend towards increased apoptosis in the DP following reversan treatment with either 4HC or DOX (Fig. 1e), one cannot exclude that ABCC1 may also play some role in HF protection, although perhaps less prominently than ABCB1. There was no difference in any of the parameters examined when ABCG2 was inhibited with Ko143, in comparison to 4HC or DOX treatment (Fig. 1b,c,d), suggesting that this transporter does not modulate human HF responses to the tested chemotherapy agent, even though ABCG2 is highly expressed in anagen HM [3], the key HF region targeted during CIA [1].

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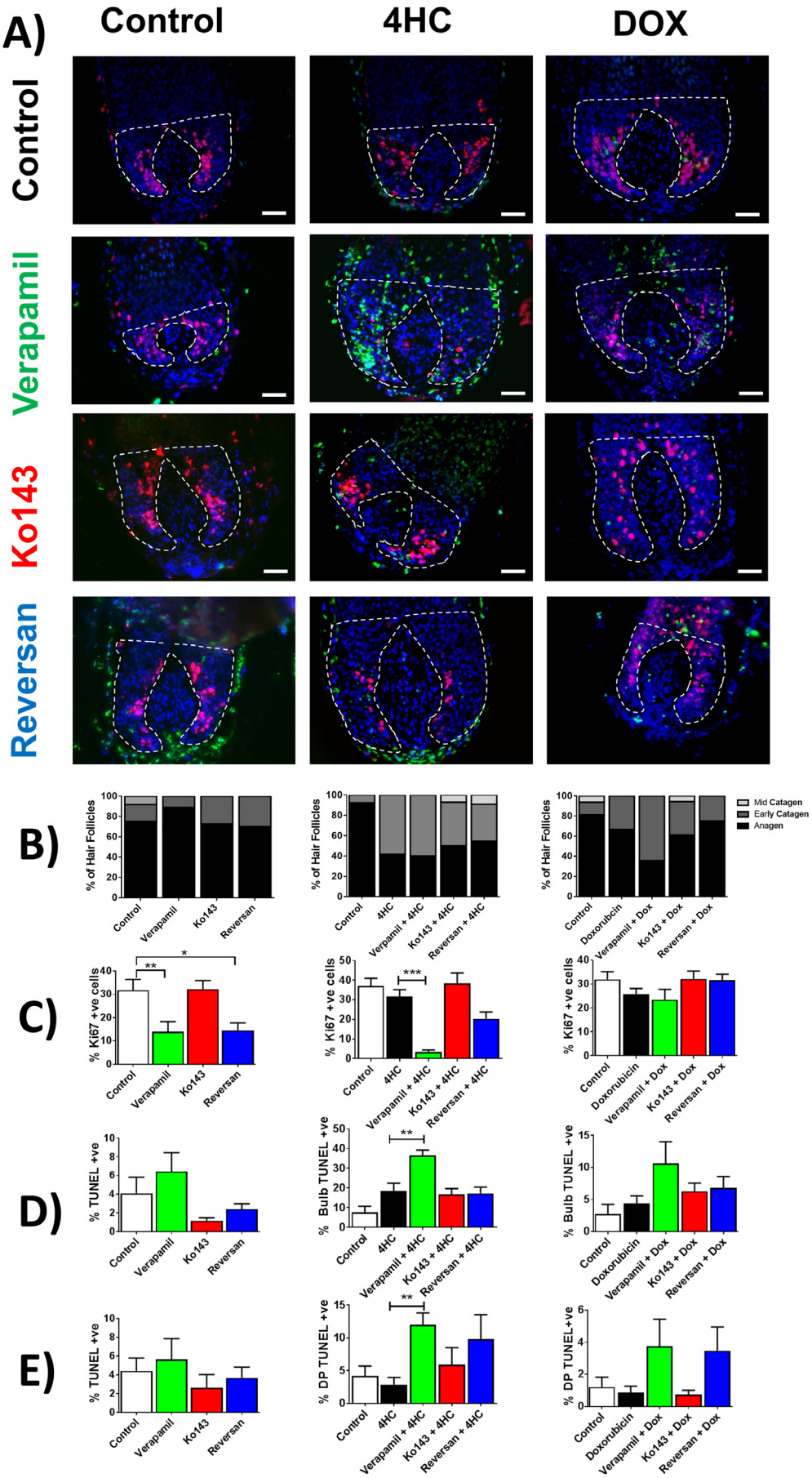
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Reversan
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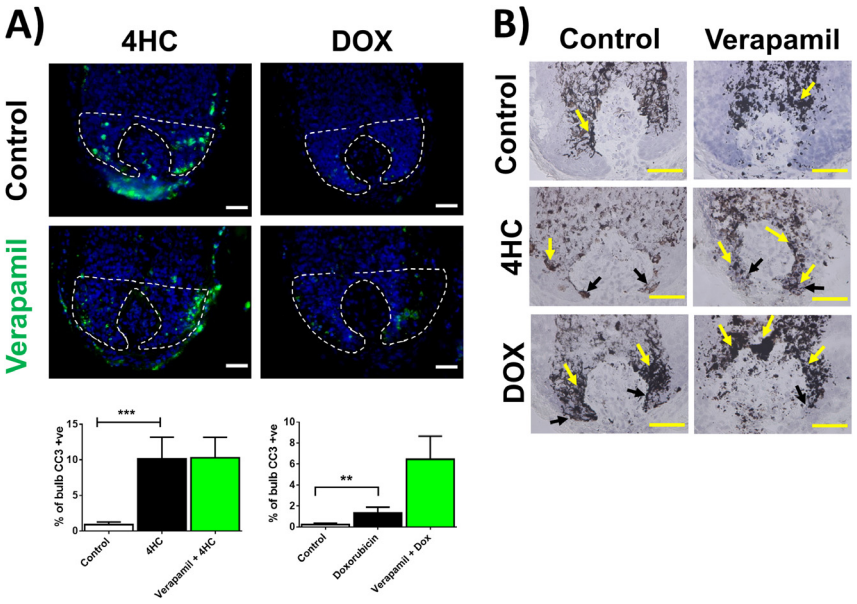

Fig. 2. Impact of verapamil on intrafollicular apoptosis and HF pigmentary abnormalities following chemotherapy. Human hair follicles (HFs) were cultured for 30 min with ABC transporter inhibitors verapamil (100μM, ABCB1) followed by the addition of 30 μM 4HC for 48 h or 1 μM Doxorubicin for 1 h followed by a 48 h washout. (A) HFs were stained with CC3 (green) and counterstained with DAPI (blue) and the proportion of the HF bulb positive for CC3 quantified. A Kruskal–Wallis test was performed, reached significance ($p < 0.001$) and was followed by Dunn's multiple comparison tests (n = 13–17 HFs; pooled from 3 patients). White dashed line indicates HF bulb, which was the area of analysis. Scale Bars = 50μm. (B) HFs were stained with Masson Fontana (n = 10–16 HFs; pooled from 3 patients). Black arrows indicate ectopic melanin in the proximal HM and yellow arrows indicate melanin clumps. Scale Bars = 50μm.

Collectively, the current study provides the first functional evidence that the inhibition of ABCB1 sensitizes human scalp HFs to chemotherapy-induced damage *ex vivo* and suggests that selected ABC transporters are functionally important HF protectants, that may be manipulated to alter HF toxicity against xenobiotics [4]. Cyclophosphamide and its metabolites are not classical ABC transporter substrates, however the current data is in line with a growing body of evidence that ABC transporters can mediate resistance to cyclophosphamide [9,10]. In addition, our pilot data further support the previously proposed strategy to therapeutically increase the expression and activity of selected ABC transporters such as ABCB1 to protect against chemotherapy-induced alopecia, and could be developed into a targeted preventative strategy to alleviate this major burden of cancer treatment.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.06.003.

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Fig. 1. ABC transporter inhibition augments the hair growth-inhibitory effects of 4HC and DOX. Human hair follicles (HFs) were cultured for 30 min with ABC transporter inhibitors verapamil (100μM, ABCB1), ko143 (1μM, ABCG2) or reversan (10μM ABCC1) followed by the addition of 30μM 4HC for 48 h or 1 μM Doxorubicin for 1 h followed by a 48 h washout. HF sections were stained with Ki-67 (red) and TUNEL (green) and counterstained with DAPI (blue). Scale Bars = 50μm (n = 10–18 HFs pooled from 3 patients) (A). The percentage of HFs in each hair cycle stage was determined (B) and the percentage of Ki-67+ cells was examined in the HF bulb (C) along with the proportion of the total bulb epithelium (D) and DP (E) that was TUNEL+ DP=dermal papilla. White dashed line indicated the hair bulb reference area used for morphometric analysis. Data is expressed as mean ± SEM. For data that was not normally distributed (All TUNEL and Ki-67 DOX) a Kruskal–Wallis test was performed and those that reached significance were followed by Dunn's multiple comparison tests between 4HC and each other group. For data that was normally distributed a One-way ANOVA was performed followed by Sidak's multiple comparison test.
*p<0.05, ** p<0.01, *** p<0.001.

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