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Use of in vitro human keratinocyte models to study the effect of cooling on chemotherapy drug-induced cytotoxicity

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Abstract

A highly distressing side-effect of cancer chemotherapy is chemotherapy-induced alopecia (CIA). Scalp cooling remains the only treatment for CIA, yet there is no experimental evidence to support the cytoprotective capacity of cooling. We have established a series of in vitro models for the culture of human keratinocytes under conditions where they adopt a basal, highly-proliferative phenotype thus resembling the rapidly-dividing sub-population of native hair-matrix keratinocytes. Using a panel of chemotherapy drugs routinely used clinically (docetaxel, doxorubicin and the active metabolite of cyclophosphamide 4-OH-CP), we demonstrate that although these drugs are highly-cytotoxic, cooling can markedly reduce or completely inhibit drug cytotoxicity, in agreement with clinical observations. By contrast, we show that cytotoxicity caused by specific combinatorial drug treatments cannot be adequately attenuated by cooling, supporting data showing that such treatments do not always respond well to cooling clinically. Importantly, we provide evidence that the choice of temperature may be critical in determining the efficacy of cooling in rescuing cells from drug-mediated toxicity. Therefore, despite their reductive nature, these in vitro models have provided experimental evidence for the clinically-reported cytoprotective role of cooling and represent useful tools for future studies on the molecular mechanisms of cooling-mediated cytoprotection.

Abbreviations: BPE, bovine pituitary extract; CIA, chemotherapy-induced alopecia; EGF, epidermal growth factor; FBS, fetal bovine serum; HaCaTa, adapted HaCaT; HHFK, human hair follicular keratinocytes; 4-OH-CP, 4-hydroxycyclophosphamide; KSFM, keratinocyte serum-free medium; NHEK, normal human epidermal keratinocytes; ROS, Reactive Oxygen Species; TAC, taxotere-adriamycin-cyclophosphamide

Keywords: Chemotherapy; Cytotoxicity; Cooling; Cytoprotection; Keratinocytes

1 Introduction

Chemotherapy-induced alopecia (CIA) is the most common and distressing side effect of anticancer chemotherapy (Wang et al., 2006) and the anxiety caused by the prospect of CIA can cause patients to even refuse treatment in certain cases (Munstedt et al., 1997). Thus development of an effective CIA preventative regime represents an important challenge in oncology (Paus et al., 2013). CIA occurs due to damage to the hair follicles, which comprise various cell types including hair matrix keratinocytes, which represent the most rapidly dividing cell subset and contribute to follicular structure and function (Roh et al., 2005). As chemotherapeutic drugs such as taxanes (e.g. docetaxel), alkylating agents (e.g. cyclophosphamide) and anthracyclines/DNA intercalating agents (e.g. doxorubicin) target cancer cells due to their rapid division rate, these drugs also target the matrix keratinocytes which results in hair loss (Paus et al., 2013).

Currently the only available preventative treatment for CIA is head (scalp) cooling. Scalp cooling or hypothermia during the administration of chemotherapy drugs can substantially reduce hair loss (Protiere et al., 2002) and has been used since the 1970s (Dean et al., 1979). Clinically it has been shown that scalp cooling can substantially reduce the incidence of hair loss in response to individual drugs, including cyclophosphamide, doxorubicin and cisplatin (Breed et al., 2011; van den Hurk et al., 2012). However, for combined treatment regimens, such as sequential treatment with docetaxel (taxotere), doxorubicin (adriamycin) and cyclophosphamide (clinically also known as TAC), scalp cooling has limited reported efficacy (Grevelman and Breed, 2005). Despite the fact that scalp cooling can be effective, its overall mechanism of action is not fully understood.

In order to improve the efficacy of scalp cooling, particularly in the case of combinatorial drug treatments that do not respond well to cooling (such as TAC), it is necessary to achieve a better understanding of the cellular mechanisms that underlie drug-induced cytotoxicity and study the effects of cooling in this context. Several experimental in vivo models have been used to help understand CIA; however, rodent-based models demonstrate inherent physiological and practical limitations (Paus et al., 2013). *Ex vivo* models, such as those by Paus and colleagues that are based on isolation and culture of human hair follicles, represent an elegant model for studying cyclophosphamide-induced CIA (Bodo et al., 2007). A more reductive culture model to study chemotherapy drug-induced cytotoxicity involves the use of human neonatal epidermal keratinocytes and a previous report has provided some, though limited, evidence for an effect of culture temperature on the cytotoxicity of doxorubicin on such cells (Janssen et al., 2008). The principle behind the use of human epidermal keratinocytes is that they are maintained under culture conditions that render them highly-proliferative, thus resembling the rapidly-dividing population of native matrix keratinocytes. However, because of the finite nature of such primary cultures, the well-characterised cell line HaCaT, that shows similar characteristics and cell behaviour to normal keratinocytes ((Deyrieux and Wilson, 2007); and references therein), has also been used to study drug-induced cytotoxicity (Luanpitpong et al., 2011).

In this study we have used normal human epidermal keratinocytes (NHEK), human hair follicular keratinocytes (HHFK), the keratinocyte cell line HaCaT and HaCaT cells that were adapted to culture conditions identical to those for NHEK cells (serum-free and low-calcium medium) to examine the cytotoxicity of a panel of commonly used chemotherapeutic modalities and we investigated the effect of temperature on cytotoxicity. We show here for the first time that cooling dramatically reduces or completely prevents the cytotoxic capacity of docetaxel, doxorubicin and particularly cyclophosphamide, whilst combinatorial treatment (TAC) showed relatively poor response to cooling, findings that are in agreement with clinical observations. Moreover, we provide evidence that the minimum temperature achieved may be critical in improving the efficacy of cooling. The concordance of our observation of cytoprotection against chemotherapy drugs with the findings obtained in patients undergoing scalp cooling during chemotherapy indicates that, despite their reductive nature, these robust and reproducible in vitro models may improve our understanding of CIA and more importantly permit detailed investigations into the mechanisms that underpin cell cooling-mediated cytoprotection to chemotherapeutic drugs.

2 Materials and methods

2.1 Cell culture

Neonatal human epidermal keratinocytes (HEKn), referred to in this study as normal human epidermal keratinocytes (NHEK), were obtained from Life Technologies (supplied by Fisher Scientific) and were cultured in keratinocyte serum free medium (KSFM) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) as recommended by the manufacturer. Human hair follicular keratinocytes (HHFK) were purchased from ScienCell Research Laboratories (supplied by Caltag MedSystems) and were cultured in Keratinocyte Medium according to the manufacturer's recommendations. For all experiments, NHEK and HHFK cells were used at passages 1–3 to ensure maximal proliferative capacity. The keratinocyte cell line HaCaT was purchased from Cell Line Services (CLS) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 2 mM l-glutamine (all from Sigma Aldrich). Adapted HaCaT (HaCaTa) cells (see below) were cultured in the same medium as NHEK cells. All cells were routinely cultured at 37 °C in a humidified atmosphere of 5% CO_2, whereas for cooling experiments the temperature was altered as indicated. Cells were passaged at ~80–90% confluence by removing media, washing 0.1% (w/v) EDTA in PBS to aid disaggregation, and lifted using trypsin–EDTA solution (Sigma). In the case of NHEK, HHFK and HaCaTa cells the trypsin was inactivated using trypsin inhibitor (Sigma Aldrich). For routine maintenance and experiments, NHEK and HHFK cells were cultured in Primaria™ (Scientific Laboratory Supplies) or Cell Plus (Sarstedt) plasticware, whereas original and adapted HaCaT cells were maintained in standard plasticware (Sarstedt).

2.2 HaCaT adaptation to serum-free medium

A sequential adaptation methodology was followed to switch the culture conditions of HaCaT cells from a serum-supplemented to a serum-free, low calcium medium (KSFM). This involved culture and passaging whilst gradually reducing the proportion of standard culture medium DMEM/10% FBS (DMEM complete, DMEMc) and replacing it with KSFM/EGF/BPE (KSFM complete, KSFMc) and lasted a period of six passages. Briefly, this involved: medium-change of cells (p1) from DMEMc to 3:1 (v/v) DMEMc:KSFMc medium and passage (p2); medium-change to 1:1 (v/v) DMEMc:KSFMc followed by passage (p3); medium-change to 1:3 (v/v) DMEMc:KSFMc and passage in this medium (p4), medium-change to 1:9 (v/v) DMEMc:KSFMc (p4) and passage (p5); final medium-change to KSFMc and subsequent passage (p6). After this, HaCaT cells had fully adapted to the new culture medium and were named HaCaTa.

2.3 Assessment of the role of temperature conditions on chemotherapy drug-induced keratinocyte cytotoxicity

Keratinocytes were seeded into 96-well tissue culture plates at a density of 5 × 10^3 (HaCaT and HaCaTa) or 7.5 × 10^3 (NHEK and HHFK) cells per well – optimal density for each cell type was determined by pre-titration experiments – and incubated for 24-h at 37 °C.

For individual drugs treatment experiments, cells were subjected to a range of concentrations of docetaxel, doxorubicin (Sigma) and 4-hydroxycyclophosphamide (4-OH-CP), the active metabolite of cyclophosphamide (supplied by Niomech), for a period of 2-h at 37 °C (control conditions) or under cooling conditions (22, 18 or 14 °C) in the appropriate culture medium and as detailed in the Results. Solvent (DMSO) controls (representing the maximal amount of DMSO that corresponded to the highest drug concentration) were included in all experiments. Following treatment, drugs were removed, cells washed twice using PBS and fresh culture medium added. Cultures were then incubated at 37 °C for 72-h before cell growth was assessed (below).

For combinatorial TAC therapy experiments, sequential treatment with docetaxel (T), doxorubicin (A) and 4-OH-CP the active metabolite of cyclophosphamide (C) was carried out, with the exact concentration of each drug being dependent on the cell type (as explained in the main text). In particular:

(A) NHEK cells were challenged with the following three TAC regimes:

- **(a)** docetaxel 0.05 µg/ml (2-h), doxorubicin 3 µg/ml (1-h), and 4-OH-CP 25 µg/ml (1-h), termed ‘TAC’;
- **(b)** docetaxel 0.05 µg/ml (2-h), doxorubicin 3 µg/ml plus docetaxel 0.005 µg/ml (1-h), 4-OH-CP 25 µg/ml plus doxorubicin 0.3 µg/ml (1-h), termed ‘TAC (+10%)’; and
- **(c)** docetaxel 0.05 µg/ml (2-h), doxorubicin 3 µg/ml plus docetaxel 0.05 µg/ml (1-h), 4-OH-CP 25 µg/ml plus doxorubicin 3 µg/ml (1-h), termed ‘TAC (+100%)’.

(B) HaCaTa cells were challenged with the following three TAC regimes:

- **(a)** docetaxel 0.01 µg/ml (2-h), doxorubicin 0.3 µg/ml (1-h), and 4-OH-CP 5 µg/ml (1-h), termed ‘TAC’;
- **(b)** docetaxel 0.01 µg/ml (2-h), doxorubicin 0.3 µg/ml plus docetaxel 0.001 µg/ml (1-h), 4-OH-CP 5 µg/ml plus doxorubicin 0.03 µg/ml (1-h), termed ‘TAC (+10%)’; and
- **(c)** docetaxel 0.01 µg/ml (2-h), doxorubicin 0.3 µg/ml plus docetaxel 0.01 µg/ml (1-h), 4-OH-CP 5 µg/ml plus doxorubicin 0.3 µg/ml (1-h), termed ‘TAC (+100%)’.

Cells were then washed and medium was replaced as described above, before cell growth was assessed 72-h later.

2.4 Assessment of cell biomass

The effect of chemotherapy drugs on keratinocyte cell growth, and thus viability, was determined by measuring cell biomass using the CellTiter 96® AQueous One cell proliferation assay (Promega) and following the manufacturer’s instructions. Briefly, following treatment of cells as described above, 20 µl of reagent CellTiter reagent was added to each well and plates were incubated at 37 °C in 5% CO_2 conditions for 4-h. Total levels of formazan formation, which corresponds to relative cell biomass, were assessed using a FLUOstar OPTIMA (BMG Labtech) plate reader at a wavelength of 492 nm following background subtraction. Percentage (%) cell biomass was calculated using the formula: (Abs T/Abs C) × 100, where ‘Abs T’ is absorbance value for drug-treated cells and ‘Abs C’ corresponds to absorbance value for controls cultures.

In addition to CellTiter assays, cell viability was also monitored by phase contrast microscopy. In particular, HaCaT were seeded at 5 × 10^3 cells per well in 96-well tissue culture plates, whilst HaCaTa cells were seeded in 10 cm tissue culture dishes at 9 × 10^5 cells per dish. Test cultures were treated at 37 °C or 22 °C with the indicated drug concentrations alongside negative controls (as described above) and cells were incubated at 37 °C for the indicated time period. Phase contrast images were obtained using an EVOS XL core inverted microscope (Life Technologies) at 100× and 200× magnification.

2.5 Statistical analysis

Statistics were performed using SPSS software (v14, SPSS Inc). Parametric statistics (mean and SD) were used for descriptive purposes and tests of significance were by means of a two-tailed Student’s *t*-test; significance was assumed when $p \leqslant 0.05$.

3 Results

3.1 The effect of chemotherapy drugs on normal human epidermal keratinocyte growth and the role of cooling on drug-mediated effects

Only a limited number of studies have previously addressed the effect of chemotherapy drugs on normal human epidermal keratinocytes (NHEKs) and a single study assessed the effect of cooling on doxorubicin-mediated cytotoxicity on NHEKs (Janssen et al., 2008). Using NHEKs cultured in low calcium (0.09 mM) serum-free medium, conditions under which they adopt a highly-proliferative basal cell phenotype, we assessed the cytotoxic effect of a panel of three routinely used chemotherapy drugs (Grevelman and Breed, 2005), in particular the taxane docetaxel, the anthracycline doxorubicin and the alkylating agent cyclophosphamide, all of which are associated with CIA. Because in vivo cyclophosphamide requires enzymatic transformation to its active form 4-hydroxy-cyclophosphamide (4-OH-CP), we used this compound in our studies and as previously described elsewhere (Bodo et al., 2007).

We examined the effect of each of these drugs over a wide range of concentrations at standard culture temperature 37 °C and, to mimic cooling conditions, at 22 °C. This was primarily because it has been suggested by various studies that a scalp temperature of 22 °C or less is required for hair preservation (Komen et al., 2013) and also it was the temperature previously tested in vitro by Janssen et al. (2008). As shown in Fig. 1, all three drugs caused increasing cytotoxicity in NHEKs in a dose-dependent fashion, although in the case of docetaxel the maximum relative loss of cell biomass was ~75%. We also confirmed that the decrease in biomass was the result of active induction of cell death (apoptosis) using apoptosis detection assays (not shown). Interestingly, and despite the fact that these drugs act via different molecular mechanisms, cooling dramatically rescued cells from cytotoxicity for all three compounds (Fig. 1, upper panels), with the observation for doxorubicin being in agreement with previous studies (Janssen et al., 2008). The ability of cooling to protect from cytotoxicity was striking for several, mid-range drug concentrations, particularly for doxorubicin and 4-OH-CP; in fact for some drug doses cell biomass returned from below 20% to nearly 100% of the control value (Fig. 1, lower panels).

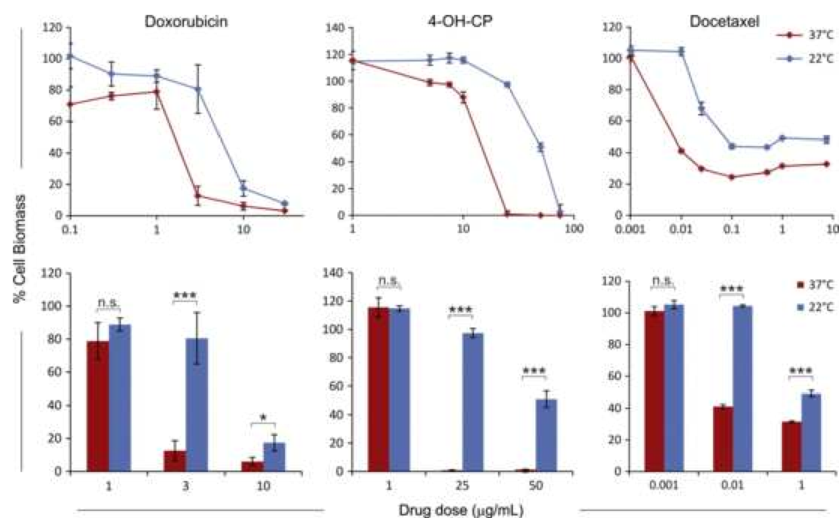

Fig. 1 Cytoprotective role of cooling against chemotherapy drug-mediated toxicity in NHEK cells. NHEK cells were treated with a range of concentrations of doxorubicin, 4-OH-CP and docetaxel at 37 °C and 22 °C (representing normal and cooling conditions, respectively) and % cell biomass was assessed 72-h post-treatment and calculated as described in Section 2. Representative results from the dose response curves (upper panels) are also shown in bar graph form (lower panels) for clarity and presentation of statistical significance. Data points correspond to mean % cell biomass (±S.E.M.) for three independent biological experiments, each consisting of 6–8 technical replicates. n.s., non-significant; *, $p < 0.05$; ***, $p < 0.001$.

3.2 The cytotoxicity of chemotherapy drugs on human hair follicular keratinocytes and the role of cooling on drug-mediated effects

A potential weakness of our approach of using keratinocytes of epidermal origin is that such cells, despite their ability to grow as a basal, highly-proliferative cell population (thus mimicking the rapidly-dividing population of native matrix keratinocytes), are not specifically isolated from the matrix keratinocyte niche. To circumvent this limitation, we used the only Human Hair Follicular Keratinocyte (HHFK) cultures currently commercially available (see Section 2). Upon routine culture, HHFK showed virtually identical morphological and growth properties to NHEK cells (not shown). When we tested the effects of doxorubicin and 4-OH-CP, as with keratinocytes of epidermal origin, we found that both drugs caused a dose-dependent cytotoxicity in HHFKs and cooling dramatically rescued HHFK cells from this (Fig. 2). Significantly, the response was strikingly similar to that observed for NHEK cells for these drugs (Fig. 1). Therefore, these experiments confirmed the validity of our approach of using NHEK cells as a representative keratinocyte in vitro model.

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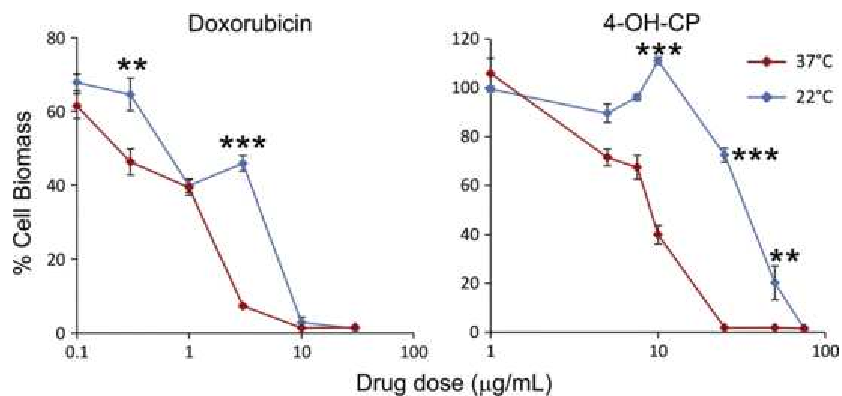


Fig. 2 Cytoprotective role of cooling against chemotherapy drug-mediated toxicity in HHFK cells. HHFK cells were treated with a range of concentrations of doxorubicin and 4-OH-CP at 37 °C and 22 °C (representing normal and cooling conditions, respectively) and % cell biomass was assessed 72-h post-treatment and calculated as described in Section 2. Data points correspond to mean % cell biomass (±S.E.M.) for three independent biological experiments, each consisting of 6–8 technical replicates. **, $p < 0.01$; ***, $p < 0.001$ (significance for only selected data points is shown for the purposes of clarity).

3.3 The effect of the combinatorial chemotherapy treatment TAC on normal human keratinocyte growth and the role of cooling

Having shown that cooling can protect keratinocytes from cytotoxicity caused by docetaxel, and particularly doxorubicin and cyclophosphamide, we investigated the combinatorial drug therapy TAC in this context. Clinical data demonstrate that, although scalp cooling provides good protection from CIA caused by these drugs when applied as monotherapies in the clinic (an observation mirrored by our in vitro findings), scalp cooling is less effective for patients undergoing combinatorial use of these drugs, i.e. TAC therapy (Grevelman and Breed, 2005; van den Hurk et al., 2012).

To test for the first time in vitro the potential cytotoxic effect of the TAC regimen and assess the influence of cooling, cells were treated with docetaxel (T), doxorubicin (A) and 4-OH-CP (C) as detailed in Section 2. One approach (‘TAC’) involved sequential treatment with the individual drugs at doses for which significant or maximal in vitro rescue was observed when each drug was tested separately (Fig. 1). In an attempt to simulate drug exposure in vivo (as during TAC therapy infusion not all the previous drug would be expected to be cleared systemically by the time the subsequent drug is infused), two modifications of this protocol were also employed (as outlined in detail in Section 2). Specifically, following initial treatment (T), cells were exposed to each subsequent drug (A and C) whilst being also supplemented with 10% (denoted ‘TAC (+10%)’) or 100% (denoted ‘TAC (+100%)’) of the previous drug. As shown in Fig. 3, combinatorial treatment at 37 °C resulted in nearly complete loss of NHEK biomass for all three TAC protocols. Notably, upon cooling (22 °C), although substantial (in some cases 100%) rescue was previously observed for individual drug treatments (Fig. 1), a modest degree of cytoprotection was observed following the ‘TAC’ and ‘TAC (+10%)’ treatments. By contrast, cooling provided little detectable rescue from the extensive cytotoxicity observed following ‘TAC (+100%)’ treatment (Fig. 3).

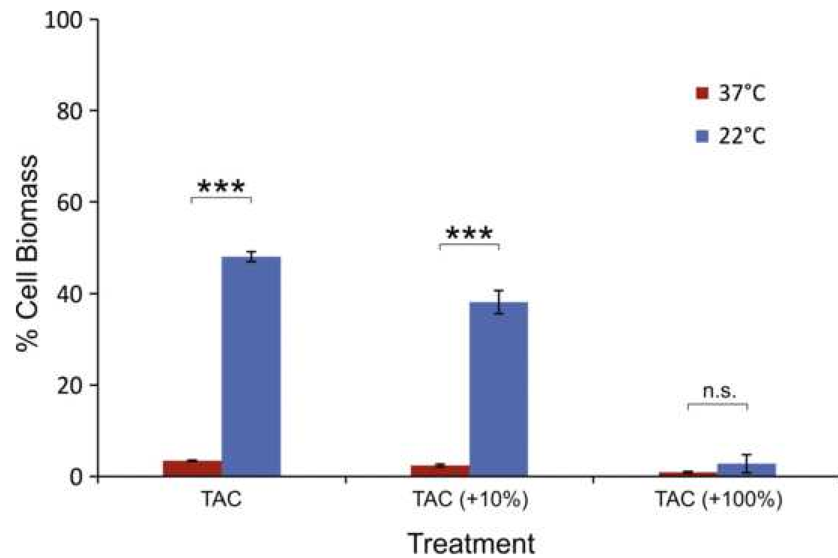


Fig. 3 Effect of cooling on TAC-mediated cytotoxicity in NHEK cells. NHEK cells were treated with the combinatorial drug protocols 'TAC', 'TAC (+10%)' or 'TAC (+100%)' at 37 °C and 22 °C as described in the text and detailed in Section 2, before % cell biomass was assessed and calculated as described in Fig. 1. Bars correspond to mean % cell biomass (±S.E.M.) for three independent biological experiments, each consisting of 6–8 technical replicates. n.s., non-significant; ***, $p < 0.001$.

3.4 Chemotherapy drug-mediated effects on HaCaT keratinocyte growth and the role of cell cooling

Our observation that by using cooling conditions highly-proliferative, cultured normal keratinocytes (a) could be efficiently rescued from chemotherapy drug-induced cytotoxicity, yet (b) could only be protected to a limited degree from combinatorial TAC treatment, are concordant with the data reported in the clinic (Grevelman and Breed, 2005). However, the finite nature of these cells (NHEK and HHFK cells display a decrease in proliferative potential by passages 5–6 and senesce soon after) poses a significant experimental obstacle. We therefore exploited the well-characterised, immortalised, non-malignant human keratinocyte line HaCaT, as it shows characteristics and behaviour similar to normal keratinocytes (Boukamp et al., 1988) and has been used to study drug-induced cytotoxicity (Luanpitpong et al., 2011).

Similarly to our studies using NHEK cells, we assessed the effect of the same panel of chemotherapy drugs on HaCaT cell growth and examined the potential protective role of cooling by comparing treatments at 37 °C and, to mimic cooling conditions, at 22 °C. Upon morphological examination, there was a clear correlation between drug concentration and number of phase-bright, non-adherent, apoptotic/dead cells; however, under cooling conditions there was a noticeable reduction in dead cell numbers for all three drugs, and particularly in the case of doxorubicin and 4-OH-CP. These observations are illustrated in Supplementary Fig. 1 which shows representative phase contrast photomicrographs of HaCaT cultures 72-h after treatment with doxorubicin, 4-OH-CP and docetaxel at both temperature conditions. When we assessed the role of cooling over a wide range of drug concentrations using cell biomass assays, we found that, in agreement with our NHEK experiments, cooling conditions provided significant and consistent protection against cytotoxicity (Fig. 4). However, the observed cooling-mediated rescue from drug cytotoxicity, although occurring over a wide range of drug doses, was noticeably less dramatic in HaCaT (Fig. 4) than in NHEK cells (Fig. 1).

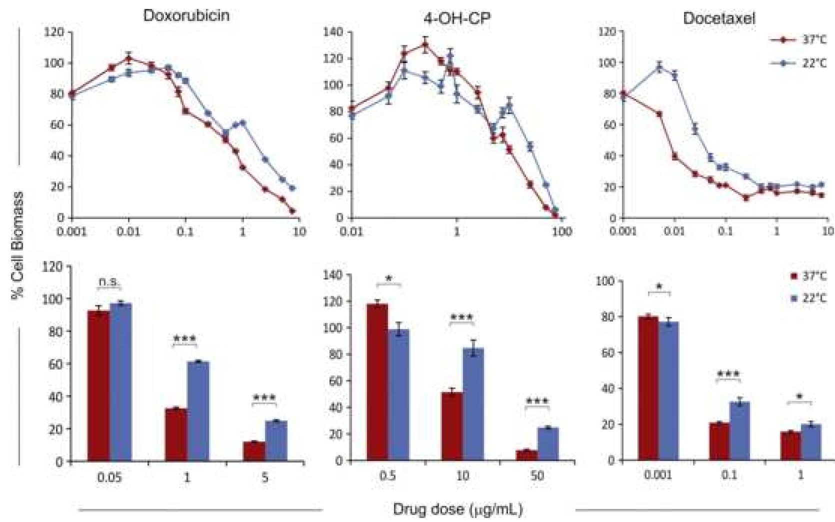
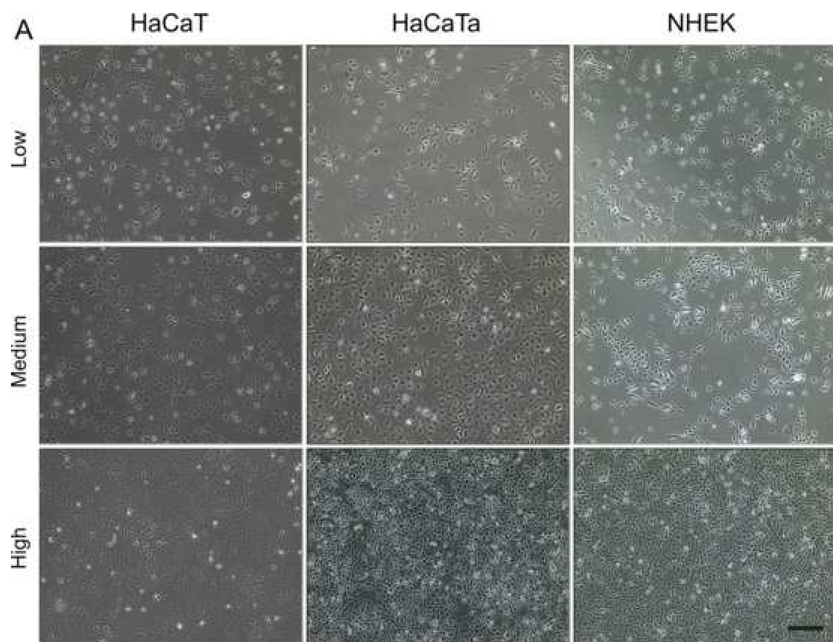


Fig. 4 Cytoprotective role of cooling against chemotherapy drug-mediated toxicity in HaCaT cells. HaCaT cells were treated with a range of concentrations of doxorubicin, 4-OH-CP and docetaxel at 37 °C and 22 °C and % cell biomass was assessed 72-h later and calculated as described in Section 2. Representative results from the dose response curves (upper panels) are also shown in bar graph form (lower panels) for clarity and presentation of statistical significance. Data points correspond to mean % cell biomass (±S.E.M.) for three independent biological experiments, each consisting of 6–8 technical replicates. n.s., non-significant; *, $p < 0.05$; ***, $p < 0.001$.

3.5 Adaptation of HaCaT keratinocytes to low-calcium, serum-free culture conditions and investigation of the role of cooling in chemotherapy drug-induced effects in the adapted cells

The differences in the ability of cooling to protect primary NHEK and HHFK cells versus the HaCaT cell line from drug-induced cytotoxicity, with NHEK showing a far more pronounced ‘response’ to cooling, prompted us to investigate the possibility of adapting the HaCaT cell line to culture conditions identical to those of NHEK. It has previously been reported that reduction of calcium levels in serum-containing culture medium allows HaCaT cells to adopt a more basal and less differentiated phenotype (Deyrieux and Wilson, 2007), consistent with the important role of calcium in mediating keratinocyte differentiation. In order to render the HaCaT cell model more closely representative to primary cells, we adapted HaCaT cells to grow in both low calcium (0.09 mM) and serum-free conditions (KSFM medium) by sequential medium adaptation and passage, as described in detail in Section 2, and named this new cell line HaCaTa.

Fig. 5A shows representative phase contrast photomicrographs of fully adapted HaCaTa cells during routine maintenance in comparison to the original HaCaT cell line as well as primary NHEK. By observing all three cell types at different culture densities (low, medium and high), it was apparent that HaCaTa cells exhibited a phenotype that was more representative of that of NHEKs. At low/medium densities (Fig. 5, upper and middle panels), HaCaT cells were less phase-bright, formed strong cell contacts and showed clear tendency to form colonies; by contrast, HaCaTa cells were very phase-bright and showed reduced tendency to form cell contacts. The similarity of HaCaTa cells to NHEK was even more pronounced when cultures approached full confluence (Fig. 5, lower panels); at such confluence HaCaTa cells remained phase-bright and were and nearly indistinguishable from NHEK cultures, in contrast to HaCaT cultures. More importantly, when we compared the proliferation rate of the HaCaTa derivative to the HaCaT line, cell biomass assays demonstrated that adapted cells grew much more rapidly than the parental cell line, with HaCaTa showing a nearly 50% higher proliferation rate (Fig. 5B), which was comparable to that of early-passage NHEK cells (not shown).



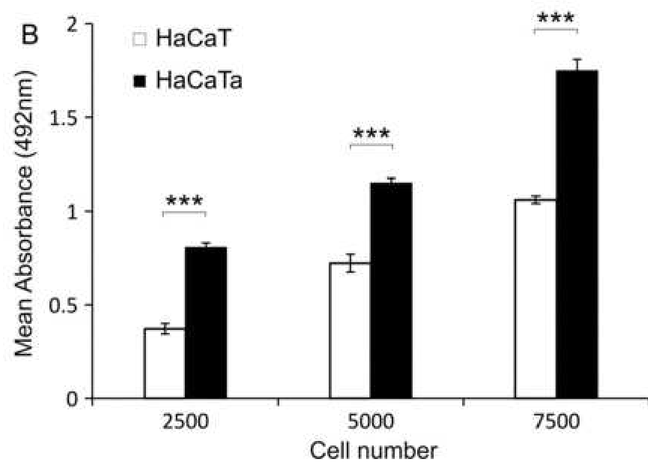


Fig. 5 Establishment of the HaCaTa cell line and assessment of its phenotypic and growth characteristics. (A) The HaCaTa cell line was derived as described in the text and detailed in Section 2. HaCaTa derivatives, the original cell line HaCaT and primary NHEK cells at increasing cell densities ('Low', 'Medium' and 'High') under routine culture conditions were examined by phase contrast microscopy and representative photomicrographs are provided. Scale bar: 100 µm. (B) Adapted HaCaTa and the original HaCaT cell line were seeded at the indicated cell densities ('Cell number' represents number of cells seeded per well) before cell growth was assessed 72-h later on the basis of total cell biomass as described in Section 2. Bars correspond to mean Absorbance at 492 nm (±SD) for three independent biological experiments, each consisting of 6–8 technical replicates. ***, $p < 0.001$.

We then investigated the response of the HaCaTa line to the same panel of chemotherapy drugs and using the cooling regime (22 °C) previously tested for HaCaT (and NHEK) cells. As shown in Fig. 6A, cooling during drug treatment provided consistent and significant protection from drug-mediated cytotoxicity for nearly all concentrations tested for doxorubicin, 4-OH-CP and, though to a lesser extent, docetaxel. Importantly, however, unlike our findings with HaCaT cells (Fig. 4), the cytoprotection observed for HaCaTa cells resembled more closely that seen with NHEK, with the results for 4-OH-CP (Fig. 6A) being particularly similar to those observed with NHEK (Fig. 1). In support of our quantitative assays, phase-contrast microscopic observation of HaCaTa cells treated with 4-OH-CP at 37 °C versus 22 °C showed clear, cooling-mediated protection against drug-mediated cytotoxicity (Fig. 6B).

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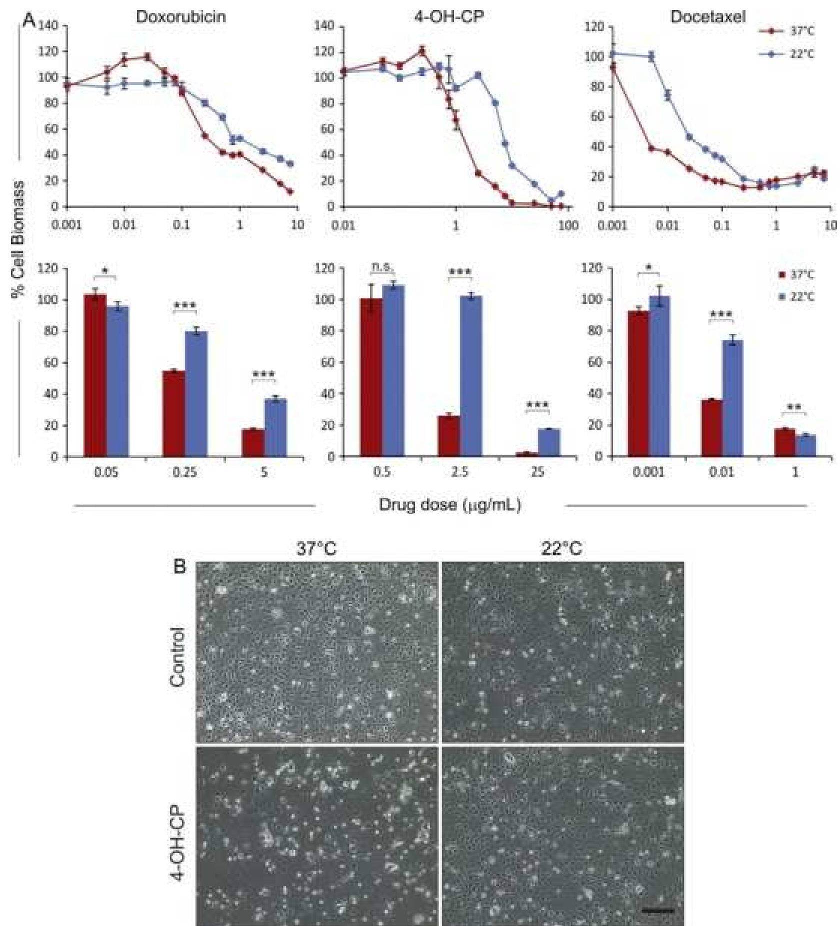

Fig. 6 Cytoprotective role of cooling against chemotherapy drug-mediated toxicity in HaCaTa cells. (A) HaCaTa cells were treated with a range of concentrations of doxorubicin, 4-OH-CP and docetaxel at 37 °C and 22 °C and % cell biomass was assessed 72-h later and calculated as described in Section 2. Representative results from the dose response curves (upper panels) are also shown in bar graph form (lower panels) for clarity and presentation of statistical significance. Data points correspond to mean % cell biomass (±S.E.M.) for four independent biological experiments, each consisting of 6–8 technical replicates. n.s., non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) HaCaTa cells were treated with 7.5 μg/mL of 4-OH-CP at 37 °C and 22 °C as described in Section 2. Solvent alone-treated (Control) HaCaTa cultures served as negative controls. Phase contrast light microscopy was used to assess the viability of control and drug-treated cultures 36-h post-treatment and representative photomicrographs from two independent experiments are provided. Scale bar: 100 μm.

3.6 The effect of the combinatorial TAC chemotherapy treatment on adapted HaCaT cell growth and the role of cooling

Because our observations above indicated that, unlike the parental cell line, HaCaTa cells closely resembled NHEK in their responses to chemotherapy drugs and in the extent of their protection by cooling, we tested the effect of combinatorial treatment TAC on HaCaTa. To do so, we followed the previously tested three treatment regimes, i.e. TAC, TAC (+10%) and TAC (+100%) and the drug concentrations chosen were doses that corresponded to the maximal observed cytoprotection upon cooling in HaCaTa cells (see Fig. 6). As shown in Fig. 7, treatment with all TAC protocols caused substantial loss of cell growth which was particularly evident following treatment using the ‘TAC (+100%)’ protocol. In concordance with our findings with NHEKs (Fig. 2), some, though modest, cytoprotection by cooling was observed for the ‘TAC’ and ‘TAC (+10%)’ treatments (~30–35% relative biomass). Moreover, cooling provided even less rescue from the extensive cytotoxicity observed following ‘TAC (+100%)’ treatment (Fig. 7).

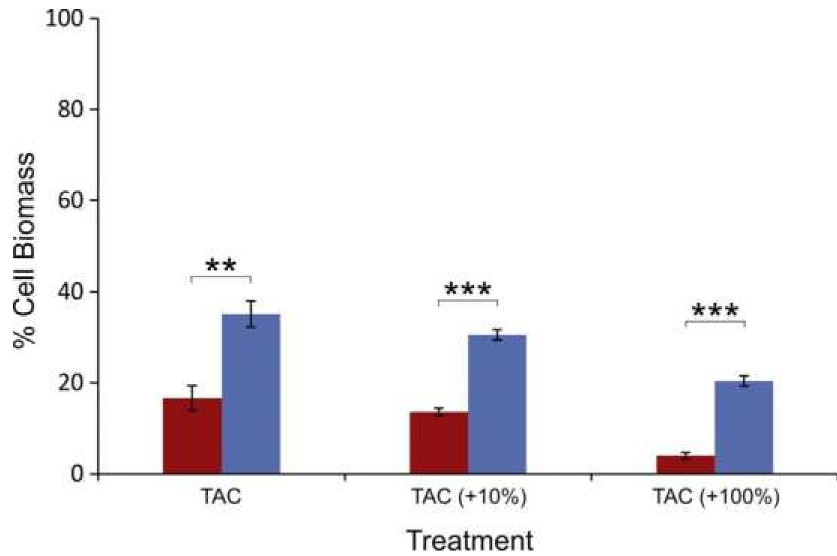

Fig. 7 Effect of cooling on TAC-mediated cytotoxicity in HaCaTa cells. HaCaTa cells were treated with the combinatorial drug protocols 'TAC', 'TAC (+10%)' or 'TAC (+100%)' at 37 °C and 22 °C as detailed in Section 2 (and explained in the text), before % cell biomass was assessed and calculated as described in Fig. 3. Bars correspond to mean % cell biomass (±S.E.M.) for three independent biological experiments, each consisting of 6–8 technical replicates. **, $p < 0.01$; ***, $p < 0.001$.

3.7 The importance of temperature in determining the efficacy of cooling in protecting from chemotherapy drug-mediated cytotoxicity

It has previously been reported that cooling below 22 °C did not provide any further protection against doxorubicin-mediated keratinocyte cytotoxicity, even when the culture temperature was reduced to 10 °C during drug treatment (Janssen et al., 2008). Using HaCaTa cells, we tested whether temperature values below 22 °C could provide further protection against doxorubicin, 4-OH-CP and docetaxel. As evident by representative results shown in Fig. 8, lowering the temperature from 22 °C to 18 °C and even further to 14 °C, resulted incrementally in a better degree of rescue from drug cytotoxicity. Strikingly, cytoprotection was detectable even for the maximal drug doses tested, which had previously resulted in complete loss in cell biomass that was non-recoverable by cooling at 22 °C. Decreasing the temperature to 10 °C during cooling did not provide any significantly better protection than did cooling at 14 °C (not shown). Interestingly, cooling even at 14 °C did not substantially improve the relatively modest protection observed for the 'TAC' and 'TAC (+10%)' protocols or the minimal protection from the 'TAC (+100%)' treatment when experiments were performed in either NHEK or HaCaTa cells (data not shown).

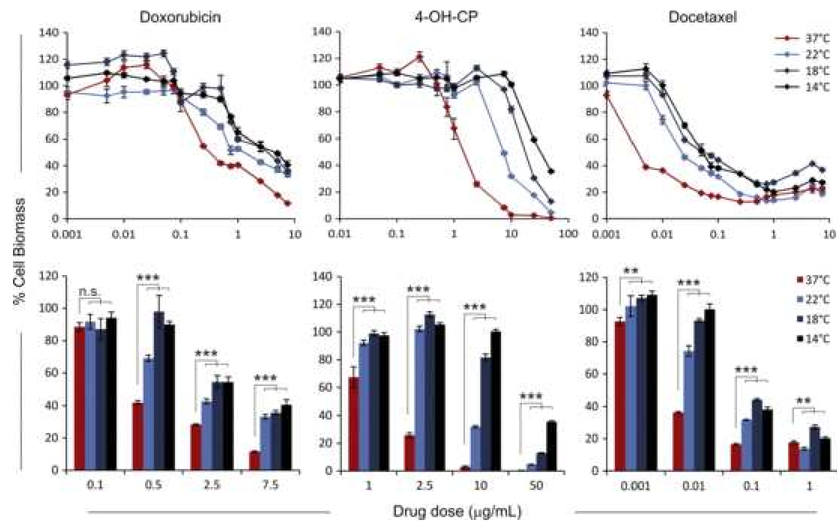

Fig. 8 The role of temperature on the efficacy of cooling in protecting from chemotherapy drug-mediated cytotoxicity. HaCaTa cells were treated with a range of concentrations of doxorubicin, 4-OH-CP and docetaxel at 37 °C as well as 22 °C, 18 °C and 14 °C. % cell biomass was assessed 72-h

post-treatment and calculated as described in Section 2. Representative results from the dose response curves (upper panels) are also shown in bar graph form (lower panels) for clarity and presentation of statistical significance. Data points correspond to mean % cell biomass (±S.E.M.) for three independent biological experiments, each consisting of 6 technical replicates. n.s., non-significant; **, $p < 0.01$; ***, $p < 0.001$.

4 Discussion

The only currently available preventative treatment for CIA is head (scalp) cooling. Scalp cooling or scalp hypothermia during the administration of chemotherapy drugs can substantially reduce hair loss (Protiere et al., 2002). Clinically it has been shown that scalp cooling can substantially reduce the incidence of hair loss in response to individual drugs, including cyclophosphamide, doxorubicin and cisplatin (Grevelman and Breed, 2005). However, for combined treatment regimens, and in particular treatment with docetaxel, doxorubicin and cyclophosphamide (clinically known as Taxotere, Adriamycin and Endoxan, or TAC), scalp cooling shows little reported efficacy (Breed et al., 2011; van den Hurk et al., 2012).

Despite the fact that scalp cooling can be effective, its principal mechanism of action is not understood and several hypotheses have been raised to explain its role. Firstly, as cooling causes blood vessel vasoconstriction, which dramatically reduces blood flow to the scalp (Janssen et al., 2007), it has been suggested that less chemotherapeutic drug is 'delivered' to the hair follicles (Bulow et al., 1985). Another possibility is that the rate of drug diffusion across a plasma membrane may be reduced and thus lower 'effective' drug doses may enter the cells (Lane et al., 1987). Finally, as cell division is metabolism-driven, it is possible that this process could be decelerated by cooling as temperature can particularly affect phases G1 and S (Watanabe and Okada, 1967), which could be particularly important for drugs that target specific phases of the cell cycle, such as microtubule-destructive drugs targeting mitosis. It is possible that cooling involves a combination of these three mechanisms to exert its protective effect.

Several experimental in vivo models have been used to help understand CIA, however, and despite their clear physiological relevance, rodent-based models demonstrate a number of inherent limitations (Paus et al., 2013). For this reason *ex vivo* models have been developed, such as those by Paus and colleagues that are based on the isolation and culture of human hair follicles, which represents a physiologically-relevant and elegant approach to mimic and study cyclophosphamide-induced CIA (Bodo et al., 2007). A more reductive in vitro culture approach involves studying chemotherapy drug-induced cytotoxicity using primary normal human epidermal keratinocytes (NHEK). The principle behind the use of such keratinocytes is that they are maintained under culture conditions that render them highly-proliferative, thus resembling the rapidly-dividing population of native matrix keratinocytes. Using such primary NHEKs, a previous report has provided evidence that doxorubicin induces NHEK cytotoxicity over a range of concentrations (Janssen et al., 2008). Another in vitro approach uses the well-characterised cell line HaCaT, that shows similar characteristics and cell behaviour to normal keratinocytes (Deyrieux and Wilson, 2007). These cells have been used to study chemotherapy drug-induced keratinocyte cytotoxicity and such studies provided evidence on cisplatin-induced cytotoxicity by demonstrating Reactive Oxygen Species (ROS)-mediated mechanisms that induce Bcl-2 down-regulation, Bax up-regulation and subsequent induction of apoptosis (Luanpitpong et al., 2011).

A previous report has provided some evidence that cooling NHEKs during treatment with doxorubicin can reduce drug-mediated cytotoxicity (Janssen et al., 2008). In our study we have for the first time examined the cytotoxicity of a panel of chemotherapeutic modalities, including doxorubicin, docetaxel and the active metabolite of cyclophosphamide (4-OH-CP), on NHEK cultures and investigated the possible influence of culture temperature on drug-mediated cytotoxicity. Moreover, our study has been the first to test these effects on human hair follicular keratinocytes (HHFKs). Our choice of 22 °C to mimic cooling was, because (a) this is the temperature the skin reaches on average in patients during scalp cooling (Komen, unpublished observations), (b) various studies have shown that a scalp temperature of 22 °C or less is required for hair preservation (reviewed in (Komen et al., 2013)) (c) 22 °C was the temperature previously tested in vitro (Janssen et al., 2008).

The results of our study demonstrate that cooling can rescue NHEKs (and HHFKs) from drug-mediated cytotoxicity. The cytoprotective effect of cooling was striking for a series of drug concentrations, particularly for doxorubicin and 4-OH-CP where for some doses cell biomass returned from below 20% to nearly 100% of the control value. For doxorubicin this effect was particularly marked for 3 µg/mL. Interestingly, the maximal plasma concentration (C_{max}) following routine infusion with doxorubicin in patients undergoing chemotherapy is reported to be ~2–4 µg/mL (Itoh et al., 2000; Nabholtz et al., 2001; Brunsvig et al., 2007). Although measured drug concentrations in the overall circulation may not necessarily correspond to those at the hair follicles, our finding that even at this maximal concentration cooling can fully protect from cytotoxicity at 22 °C, explains the success of head cooling in preventing CIA clinically. We also show for the first time that both the active metabolite of cyclophosphamide (4-hydroxy-cyclophosphamide, 4-OH-CP) and docetaxel induce dose-dependent cytotoxicity in NHEKs, an effect which is consistent with the association of these drugs with CIA clinically. The dose-dependence of this toxicity is also in agreement with a number of clinical reports that slower infusion speeds which will lead to a reduced C_{max} cause less severe CIA (Breed et al., 2011). More importantly, our findings that cooling can rescue from cytotoxicity are in concordance with clinical observations that scalp cooling significantly reduces the incidence of CIA following treatment with these drugs (van den Hurk et al., 2012).

Having demonstrated that exposure to individual chemotherapeutic drugs caused dose-dependent toxicity in human keratinocytes of both epidermal and follicular origin and that this could be significantly attenuated by cooling, we examined the response to the combinatorial TAC therapy (T = taxotere/docetaxel, A = adriamycin/doxorubicin, C = cyclophosphamide/endoxan). This regimen is being increasingly used in early stage breast cancer but almost always results in CIA and it has been reported that head cooling has very limited success in preventing TAC-mediated CIA, with approximately 90% of patients exhibiting CIA despite using scalp cooling (Breed et al., 2011). Treatment of cells in vitro to mimic clinical administration of TAC posed challenges. One challenge was the choice of concentration of each chemotherapy drug. Equally, as the drugs are administered sequentially in the clinic, it would be expected (based on published

pharmacokinetic data) that following administration of the first drug, infusion of the next drug would take place whilst the previous drug may still be present at high concentrations in the blood. Also, infusion of these three drugs takes place for different time periods during TAC treatment; often this involves docetaxel for 60 min followed by doxorubicin for 15 min and cyclophosphamide for 15 min (van den Hurk et al., 2012). However, precise timings and the order of administration can vary between different clinics.

In an attempt to incorporate these parameters and make the in vitro TAC treatment as relevant as possible, we adopted three approaches. In all cases cells were treated with chemotherapy drug doses for which significant or maximal in vitro rescue was observed when each drug was tested individually. The first approach ('TAC') involved sequential treatment with the individual drugs. The other two approaches involved initial treatment with the first drug (T), followed by treatment with each subsequent drug (A and C) whilst being also supplemented with either 10% (denoted 'TAC (+10%)') or 100% (denoted 'TAC (+100%)') of the previous drug. The use of 'TAC (+100%)' aimed at addressing the scenario where infusion with the second or third drug would take place whilst the previous drug was still present at maximal concentration. The rationale behind the 'TAC (+10%)' protocol was based on the reported observation that the plasma levels of doxorubicin and docetaxel fall on average by approximately 10-fold within 1–2 h post-infusion in comparison to the C_{max} (Itoh et al., 2000; Nabholtz et al., 2001; Brunsvig et al., 2007). Interestingly, all three TAC protocols tested caused significant toxicity to NHEKs at 37 °C consistent with the high levels of CIA reported for individuals undergoing TAC therapy in the clinic (van den Hurk et al., 2012). Upon cooling to 22 °C, despite some degree of cytoprotection, and although the drug concentrations used had previously 'responded' to cooling in individual treatment experiments, following combinatorial treatment the protection observed was modest at best.

Of note, despite observing a cell biomass between 40% and 50% for the TAC only and TAC (+10%) protocols, we found little detectable rescue following treatment with the TAC (+100%) protocol, suggesting that this may represent the protocol that more appropriately reflects cytotoxicity in vivo. It is most striking that, despite the assumptions made when constructing the TAC protocols for cell treatment in vitro, our observations reflect the clinically reported inability of cooling to attenuate TAC-mediated toxicity that results in CIA. Also in support of these findings, is the observation that, in addition to the TAC protocols reported in the Results section, when we tested further possible alternatives as TAC treatments (for instance by altering drug treatment duration and/or drug dose) our results remained unchanged (data not shown).

Due to the finite nature of NHEK and HHFK cells, we explored the possibility of using the well-characterised, immortalised, non-malignant human keratinocyte line HaCaT for our studies, as HaCaT cells show characteristics and behaviour similar to normal keratinocytes (Boukamp et al., 1988; Itami et al., 1995; Inui et al., 2000; Deyrieux and Wilson, 2007), they have previously been extensively used in both 2D and organotypic cultures for molecular studies on skin (Margulis et al., 2005). They adopt a phenotype similar to cell sub-populations of the human hair follicle (Inui et al., 2000) and have been used to study drug-induced (Luanpitpong et al., 2011) or ROS-mediated (Liu et al., 2012) cytotoxicity. At 37 °C the response of HaCaT cells to docetaxel was similar to that in NHEK and HHFK. However, for both doxorubicin and 4-OH-CP, although the general pattern of toxicity was similar, qualitatively there were some differences and HaCaT cells were somewhat more drug-sensitive than primary keratinocytes. Interestingly, we observed that low concentrations of doxorubicin and 4-OH-CP stimulated an overall increase in biomass (Fig. 4) compared to controls in HaCaT (a response that was also observed for 4-OH-CP in NHEK and HHFK – see Figs. 1 and 2). This increase in cell growth at sub-lethal concentrations of otherwise cytotoxic drugs has been reported previously in different cell types (Kayamba et al., 2013; Paus et al., 2013). Cooling HaCaT cells to 22 °C during drug exposure produced a general reduction in the toxicity of the drugs, however this was less dramatic than NHEK cells and at no concentration was the nearly 100% cell rescue that we had observed with NHEK for singularly administered drugs replicated. These findings indicated that HaCaT cells do not represent an appropriate in vitro model for investigations on the role of cooling in modulating chemotherapy drug-induced cytotoxicity.

The reduced efficacy of cooling in HaCaT compared to NHEK and HHFK cells, led us to hypothesise that the differences in responses may be due to the lower proliferative capacity and more differentiated phenotype of HaCaT compared to NHEK. HaCaT cells are maintained in standard, serum-containing medium whereas NHEK are cultured in Keratinocyte Serum Free Medium (KSFM) which contains low calcium concentration (~0.09 mM) – in comparison to physiological (~2 mM) calcium (Georgopoulos et al., 2010). The concentration of extracellular calcium has a profound role as a switch between epithelial growth and differentiation of keratinocytes, as calcium induces terminal differentiation, including specific structural changes, cell cycle withdrawal and induction of terminal differentiation-related genes (Boelsma et al., 1999). It has previously been reported that reducing the level of calcium in the culture media of HaCaT cells resulted in a more proliferative/basal and less differentiated phenotype evident by the reduction of molecular differentiation markers, such as K1 and involucrin (Deyrieux and Wilson, 2007).

When we adapted HaCaT cells to grow in low calcium (0.09 mM), serum-free conditions, the new cell line (HaCaTa) was phenotypically very similar to NHEKs and exhibited higher proliferation rates than HaCaT cells that were comparable to those of early-passage NHEKs. More importantly, when we tested the effects of the panel of chemotherapy drugs and the capacity of cooling to protect from cytotoxicity, the results with the HaCaTa cell line closely resembled our observations with NHEKs, as exemplified by the results using 4-OH-CP. When we tested the different TAC protocols on these cells and examined the ability of cooling to protect, again the findings with the HaCaTa cells were in concordance with those for NHEKs, as only modest cytoprotection by cooling was observed for the 'TAC' and 'TAC (+10%)' treatments and cooling provided even less rescue from the extensive cytotoxicity observed following the 'TAC (+100%)' protocol. Therefore, this novel, adapted HaCaTa cell line represents an in vitro model that more closely resembles NHEKs but has the advantage of maintaining a proliferative phenotype over an extended number of passages and could be used for investigations on the influence of cooling on chemotherapy drug-induced cytotoxicity.

Previous in vitro studies by Janssen et al. have reported that reducing culture temperatures below 22 °C did not provide any further protection against doxorubicin-induced cytotoxicity in NHEKs (Janssen et al., 2008). Using the HaCaTa line as a model, our study has demonstrated that further reduction of culture temperature to 18 °C and 14 °C provided significantly more cytoprotection (than that at 22 °C) from all three drugs tested, and resulted in particularly more rescue from

4-OH-CP and doxorubicin-induced cytotoxicity. One possibility for the discrepancy between our findings and those of Janssen et al. could be that those studies used NHEKs whereas in our experiments we used the HaCaTa line. However, we have excluded this possibility by demonstrating that lowering the culture temperature below 22 °C in experiments where NHEK cells were treated with an individual exposure to all three drugs employed in this study also resulted in improved cytoprotection (data not shown). A more likely explanation for the difference in observations is that in the studies by Janssen et al., the cell density used in all cell biomass measurement experiments was substantially lower than those we used in our studies; it is possible that this could result in much higher cellular stress during drug treatment that could 'mask' the real potential of cooling in rescuing from cytotoxicity.

Our finding that lowering the temperature further results in improved cytoprotection implies that the scalp temperature that is achieved clinically may be important in dictating the success of head cooling in CIA prevention. Despite the lack of appropriate clinical data to formally demonstrate the precise temperature conditions that determine the efficacy of cooling, it has previously been suggested by various studies that a scalp temperature of 22 °C or less is required for hair preservation during chemotherapy (Gregory et al., 1982) (and reviewed in (Komen et al., 2013)), whilst we have evidence that a scalp temperature <16 °C can be achieved during cooling with commercially available scalp cooling devices, yet not all subjects undergoing scalp cooling can achieve this potentially critical temperature threshold (Hussain et al., unpublished observations). Thus, the ability to reach a temperature low enough to achieve cytoprotection may hold the key in determining the success of scalp cooling in preventing CIA in the clinic. Nevertheless, the lack of improvement in protecting from TAC-mediated cytotoxicity despite cooling at even lower temperatures indicates that for treatment types that do not respond well, further cooling alone might not provide an effective solution. Understanding the mechanisms of cooling-mediated cytoprotection may therefore provide novel avenues for combinatorial intervention in the future.

In conclusion, using human keratinocytes as in vitro models, we have shown that cooling dramatically reduces or prevents the cytotoxic capacity of chemotherapy drugs routinely used for anticancer therapy, specifically docetaxel, doxorubicin and particularly cyclophosphamide, thus providing for the first time direct experimental evidence that supports the clinical findings demonstrating that cooling can protect from the cytotoxic effect of such drugs in the scalp. We also demonstrate that combinatorial drug treatment showed relatively poor response to cooling, findings that are also in agreement with clinical observations. Moreover, we have provided evidence that temperature may be critical in determining the capacity of cooling to rescue from cytotoxicity. The concordance of our observation of cytoprotection with the findings from patients undergoing scalp cooling during chemotherapy indicates that, despite their reductive nature, these robust and reproducible in vitro models may improve our understanding of CIA. In the long term, such investigations may not only permit the improvement of the efficacy of current cooling-based methodologies, but also help provide a basis for the design of novel CIA-prevention methods.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2014.07.011.

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E-Extra

Appendix A. Supplementary material

Supplementary Fig. 1A–C Microscopic observation of cooling-mediated cytoprotection of HaCaT cells from chemotherapy drug-mediated toxicity. HaCaT cells were treated with the indicated doses of doxorubicin (A), 4-OH-CP (B) and docetaxel (C) at 37 °C and 22 °C. Solvent alone-treated (Control) HaCaT cultures served as negative controls. Phase contrast light microscopy was used to assess the viability of control and drug-treated cultures and representative photomicrographs are provided from two independent series of experiments. The presence of non-adherent, phase-bright cells following drug treatment was indicative of drug-mediated cytotoxicity. Scale bar: 100 µm.

E-component

Multimedia Component 1

Transparency document

Highlights

- Cooling protects human keratinocytes from chemotherapy drug-mediated toxicity.
- Used epidermal keratinocytes, follicular keratinocytes and established cell lines.
- Cooling can fully inhibit toxicity triggered by certain chemotherapeutic modalities.
- In vitro findings are in striking concordance to clinical observations.

Queries and Answers

Query: Please confirm that given name(s) and surname(s) have been identified correctly.

Answer: Yes, all correct

Query: Please check the hierarchy of the section headings.

Answer: All fine.