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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.



## 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

*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.

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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 $5 \times 10^3$ (HaCaT and HaCaTa) or $7.5 \times 10^3$ (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: