

Title:

Determination of the action spectrum of UVR-induced mitochondrial DNA damage in human skin Cells

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Short Title:

UVR-induced mtDNA damage action spectrum

Abbreviations:

(C(t)), cycle threshold; HDfn, human dermal fibroblast neonatal; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; PK, primary keratinocyte; UVR, ultraviolet radiation.

Abstract: (199 words)

Biological responses of human skin to ultraviolet radiation (UVR) including cancer and ageing are largely wavelength-dependent as shown by the action spectra of UVR-induced erythema and nuclear DNA (nDNA) damage. A molecular dosimeter of UVR exposure is therefore required. While mitochondrial DNA (mtDNA) damage has been shown to be a reliable and sensitive biomarker of UVR exposure in human skin, its wavelength dependency is unknown. The current study solves this problem by determining, for the first time, the action spectrum of UVR-induced mtDNA damage in human skin. Human neonatal dermal fibroblasts and primary human adult keratinocyte cells were irradiated with increasing doses of UVR. Dose-response curves of mtDNA damage were produced for each of the UVR sources and cell types and an action spectrum for each cell type was determined by mathematical induction. Similarities between these mtDNA damage action spectra and previously determined nDNA damage were observed, with the most detrimental effects occurring over the shorter UVR wavelengths. Notably, a statistically significant ($p < 0.0001$) greater sensitivity to mtDNA damage was observed in dermal fibroblasts compared to keratinocytes at wavelengths $> 300\text{nm}$, possibly indicating a wider picture of depth-dependence in sensitivity. This finding has implications for disease / photo-damage mechanisms and interventions.

Introduction

Most biological endpoints induced by exposure to ultraviolet radiation (UVR) show a highly wavelength-specific response, often encompassing 3-4 orders of sensitivity over the spectral waveband of solar UVR (290-400nm). These include erythema and tanning in human skin as well as nuclear DNA (nDNA) damage and carcinogenesis (Diffey, 1991).

In addition to the nucleus, mitochondria also contain their own DNA (mtDNA), linked indirectly with longevity. Both mtDNA mutations and deletions have been implicated in a number of human pathologies including cancer, and a cause-and-effect relationship between mutated / deleted mtDNA and ageing has been reported in various cell types (Birch-Machin *et al.*, 1998; Schroeder *et al.*, 2008).

Skin is our main environmental interface with a significant increased risk of insult versus most other tissues. Age-associated features including wrinkling, roughness, laxity, pigmented spots and diffuse hyper-pigmentation are most prominent in areas of the body which are most exposed to solar UVR, such as the face and hands. This suggests that the expression of these characteristics is driven primarily by exposure to solar radiation, a phenomenon known as photo-ageing (Berneburg *et al.*, 2004; Schroeder *et al.*, 2008). Because of the permanency of mtDNA damage, it is conceivable that exposure to UVR results in an accumulation of associated damage endpoints leading to an acceleration in ageing, a notion which has led to the development of mtDNA as a biomarker of UVR-induced damage (using techniques developed by our group and adopted by others (Berneburg *et al.*, 2004; Birch-Machin, 2000; Sligh *et al.*, 2002)). In relation to this, several studies have found an increase in incidence of specific

deletions of mtDNA in skin cancer as well as in sun-exposed skin when compared to sun-protected skin (Birch-Machin *et al.*, 2013; Birch-Machin *et al.*, 1998; Koch *et al.*, 2001; Krishnan *et al.*, 2004). Furthermore, damage to mtDNA can be induced *in vitro* by UVR-irradiation of cultured human skin cells and skin equivalents (Birch-Machin *et al.*, 1998; Birket and Birch-Machin, 2007).

Although the action spectra of UVR-induced erythema (McKinlay and Diffey, 1987) and UVR-induced nuclear DNA (nDNA) damage (Setlow, 1974) have been investigated previously, UVR-induced mtDNA damage has yet to be studied. This is a significant omission as, apart from mitochondria being the source of cellular energy and major determinant of cellular oxidative stress, mtDNA has been established as a marker of UVR exposure in skin and has a strong association with ageing as well as with skin cancer. Studying the action spectrum of UVR-induced mtDNA damage will provide important mechanistic insight into this phenomenon and may indicate new biomarkers of carcinogenesis, as well as helping guide the development of new therapeutic and behavioural approaches to modulating sun damage.

Results

UVR-Induced mtDNA Damage

The initial damage created in mtDNA was determined by the occurrence of strand breaks / lesions within an 11 kb section of the 16.5 kb mitochondrial genome. This was quantified by the reduction in efficiency of the amplification of this product by quantitative real-time PCR (QPCR)

in the damaged DNA. Damage was induced by irradiating cells with an increasing dose of UVR from UVR sources with a range of spectral output, in order to construct a dose-response damage curve.

The complete set of dose-response damage curves created by the various UVR sources in primary human adult keratinocytes (PK) and human dermal neonatal fibroblasts (HDFn) cells is recorded in Fig. 1. A and B, respectively. The dose ranges used were as follows, 0.01-3 J/cm² (TL12), 0.1-4 J/cm² (UV6), 0.1-14 J/cm² (TL01), 0.3-30 J/cm² (Helarium), 1-73 J/cm² (Arimed B), 4-74 J/cm² (Cleo - filter) and 3-37 J/cm² (Cleo + filter); this equates to maximum Standard Erythemal Dose (SED) values of 80, 50, 80, 60, 40, 10 and 2 respectively. The SED (CIE Standard, 1998; Diffey *et al.*, 1997) is a unit which is being introduced progressively as an erythemally-weighted measure of radiant exposure equivalent to 100Jm⁻² (in contrast with the Minimum Erythema Dose which depends upon several factors such as susceptibility to sunburn, anatomical site, time of observation, etc.). Each dose-response curve represents the mtDNA damage induced by an independent UVR source and each data point on the curve denotes a minimum of three biologically-independent experiments, each of which was analysed three times. Each dose-response curve, therefore, comprises 72 individual data points.

An index of mtDNA damage was derived by calculating the percentage change in cycle threshold compared to UVR-protected control cells. Characteristic dose effect curves could be plotted for all UVR sources in all cell types. A consistent pattern in results emerged where curves shifted towards higher log dose values for UVR sources emitting longer wavelengths.

Action Spectrum Modelling

We tested the null hypothesis that the mechanism of the responses observed in Fig 1 was the same for all UVR sources (in other words, that the maximum response and slope of the dose response curves were independent of the lamp used for irradiation). Maximum response A and slope parameter c are shown in Fig 2.

An analysis of variance test (Kruskall-Wallis) was applied to all lamp data, excluding the effect of the Cleo lamp because of differences due to issues of cell viability and also omitting a single value of c (0.59) from TL12 (HDFn), that appeared to be an outlier. It was concluded that there was no significant difference ($p > 0.05$) in A and c between lamps, with mean \pm SD c values of 0.22 ± 0.078 and 0.25 ± 0.061 for HDFn and PK respectively. Finally, the spectral sensitivity factor b was examined and a significant difference ($p = 0.0015$) between lamps was found for both HDFn and PK cells.

Consequently, it was concluded that the mechanism of response was independent of UVR lamp and could, therefore, be expressed as the dose response curve from the i^{th} UVR source as: $Y_i = 126 / \{1 + \exp[-(x-b_i)/0.22]\}$ and $Y_i = 157 / \{1 + \exp[-(x-b_i)/0.25]\}$ for HDFn and PK cells respectively. These families of curves are represented in Fig 3.

The action spectrum of UVR-induced mtDNA damage in HDFn cells (Figure 4) was derived by a process of mathematical induction. The basis of the induction approach is to estimate an action spectrum that is described by fewer parameters than the number of sources used, calculate the logarithm of the dose expected for a given change in cycle threshold ($C(t)$), and compare these calculated doses with those actually observed (Flockhart *et al.*, 2008). The optimisation process,

using the SOLVER facility in Excel, involves repeated adjustment of the various parameters until the closest agreement between the modelled and the observed values is achieved.

The logarithm of the observed dose (x) for which a change in cycle threshold is $A/2$ (that is, the point of maximum slope) is given by $x = b$ with a standard deviation Δx equal to that of the standard deviation on b .

The action spectrum for DNA damage derived by Setlow (Setlow, 1974) was used as the initial candidate. This can be expressed by the equation:

$$A(\lambda) = 1.17 * \exp(K * (1 / (1 + \exp((\lambda - \lambda_c) / \sigma)) - 1))$$

where $K=13.82$, $\lambda_c=310\text{nm}$ and $\sigma=9$

The resulting action spectrum is normalised to unity at 270 nm.

The optimisation process, using the SOLVER facility in Excel, involved repeated adjustment of the parameters of this spectrum until a minimum was found in the chi-squared value, defined as:

$$\chi^2 = \sum_{i=1}^N (O_i - E_i)^2 / E_i$$

where O_i and E_i are the observed and expected log dose for the i^{th} lamp, respectively.

E_i is given by the expression:

$$E_i = \log \left\{ \frac{\int_{270}^{400} S(\lambda)_i}{\int_{270}^{400} S(\lambda)_i \varepsilon(\lambda)} \right\}$$

$S(\lambda)_i$ is the relative spectral irradiance of the i^{th} lamp, and $\epsilon(\lambda)$ is the action spectrum for mtDNA damage expressed as the reciprocal of the UV dose (in $\text{cm}^2\text{mJ}^{-1}$) that results in a change in cycle threshold that is 50% of the plateau value (A).

Once a minimum in χ^2 was found, the probability (P_i) that the difference between E_i and O_i was statistically significant was determined as $2 \times \text{NORMSDIST}[-\text{ABS}(O_i - E_i) / \Delta x]$. The combined significance level (P) over all 7 lamps is expressed as:

$$P = K \sum_{i=0}^6 (-\ln(K))^i / i!$$

where $K = P_1 \times P_2 \times P_3 \times P_4 \times P_5 \times P_6 \times P_7$

This analysis resulted in the following expression for the action spectrum for UVR-induced mtDNA damage in HDFn cells:

$$\epsilon(\lambda) = \exp(8.64 * (1 / (1 + \exp((\lambda - 295.5) / 10.7)) - 1)) \quad \text{cm}^2\text{mJ}^{-1}$$

The combined significance level (P) was calculated as 0.46 (i.e., no statistically significant difference between the cohort of observed and expected doses).

Repeating the analysis for data obtained with the PK cells resulted in an action spectrum (Figure 4) defined by:

$$\epsilon(\lambda) = \exp(12.2 * (1 / (1 + \exp((\lambda - 301.9) / 9.68)) - 1)) \quad \text{cm}^2\text{mJ}^{-1}$$

The combined significance level (P) was calculated as 0.47 (i.e., no statistically significant difference between the cohort of observed and expected doses).

Overall, it is apparent that short-wavelength UVR has the greatest relative effectiveness in inducing mtDNA damage action spectra in both PK and HDFn cell types. The same is true for the action spectra reported previously for nDNA (Setlow, 1974). mtDNA was found to be more sensitive than nDNA to damage from UVR >320 nm, in both cell types.

Importantly, HDFn cells had a statistically significantly greater sensitivity to UVR >300 nm than PK cells.

Discussion

Solar UVR is an environmental insult leading to photo-ageing and carcinogenesis in human skin. UVR covers a wide spectrum of wavelengths which have differential effects on human skin, demonstrated by the determination of a variety of action spectra, including UVR-induced erythema and nDNA damage. Over the last 15 years, mtDNA damage has been shown increasingly to be a reliable and sensitive biomarker of UVR exposure in human skin. Although mitochondria are thought to be involved in the ageing process and a link between skin carcinogenesis and mitochondrial damage has been reported, the wavelength dependence of this effect has not been reported. Therefore, the aim of this novel study was to determine, for the first time, the action spectrum for UVR-induced mtDNA damage in human skin cells.

To rule out any confounding factors the following steps were performed. First, differences in mtDNA copy number due to UVR exposure or different skin cell type were determined using the 83 bp assay, amplifying a short amplicon of mtDNA using QPCR. $C(t)$ values from the QPCR were

then plotted and analysed by linear regression (Figure 5). No change in mtDNA copy number was found in either PK or HDFn cells following irradiation by any of the UVR sources compared with the control and, therefore, no apparent confounding effect of increasing doses of UVR were observed.

Further investigation ruled out differences in mtDNA damage due to media type or to thermal effects of the UVR sources. For example, $C(t)$ values did not change in cells exposed to 48°C for prolonged exposures (even up to 2 hours). We also found there to be no significant difference in broadband UVR induced threshold damage between the primary keratinocyte donors.

Clearly, in this present study, native cell types were harvested from their natural environment and compared under the same conditions as single monolayer cultures – whereas, *in situ* in living skin, these cell types have different anatomical locations within the skin compartment, i.e., basal keratinocytes at an approximate depth of 100µm and dermal fibroblasts located within a depth range of 100µm to 2mm. *In vivo*, therefore, dermal fibroblasts are exposed to only relatively small doses of shorter UVB wavelengths (<10% of incident radiation), due to Rayleigh scattering and absorption (Bruls *et al.*, 1984). It might be hypothesised, therefore, that the mtDNA of dermal fibroblasts cultured in monolayer might be more sensitive to short-wave UVR than keratinocytes, given that they are normally exposed to much lower fluxes of these wavelengths and perhaps, therefore, not developed appropriate defence / repair mechanisms. In this study, little difference in sensitivity was seen between the two cell types at wavelengths <300nm. Importantly, however, differences in sensitivity to UVR-induced mtDNA damage emerged at UVR wavelengths >300 nm where a greater sensitivity was seen in fibroblasts

compared to keratinocytes. Cell type-dependent response to UVR has been reported by others. Derrico *et al.*, 2007 reported that keratinocytes were more resistant than fibroblasts to the lethal effects of UVR and more efficient in the removal of cyclobutane pyrimidine dimers (D'Errico *et al.*, 2007). Marionnet *et al.*, 2010 found that there was a greater oxidative response in dermal fibroblasts in comparison to keratinocytes (demonstrated by a more rapid induction of related gene expression in these cells when irradiated with a solar simulator producing UVR approximating daily, non-zenithal sunlight, proportionally richer in UVA wavelengths (Marionnet *et al.*, 2010). Bernerd and Asselineau, 1998 have also suggested differential cell type sensitivity to UVA supporting the opinion that dermal fibroblasts are less resistant to UVA in experiments that exposed skin equivalents to 30 J/cm² UVA1 (Bernerd and Asselineau, 1998). One hypothesis that might explain this phenomenon is that keratinocytes contain higher levels of ferritin, involved in oxidative stress response. Ferritin provides a protective effect by chelating iron which might otherwise catalyse the formation of damaging hydroxyl radicals induced by UVA (Qian and Van Houten, 2010). Bernerd and Asselineau 1998 hypothesised that keratinocytes could be “programmed” for stress-resistance, due to their superficial location in the epidermis and resulting chronic exposure to higher doses of UVR. An example of keratinocytes being physiologically fit-for-purpose can be seen in the high concentrations of keratin protein found in these cells, providing a degree of protection against short-wave UVR due to absorption by constitutive amino acids. This notion is supported by Otto *et al.*, 1999 who reported better survival rates in keratinocytes vs dermal fibroblasts after exposure to UVR (Otto *et al.*, 1999).

The action spectra for UVR-induced mtDNA damage in both cell types indicated greater sensitivity to UVR wavelengths >320 nm vs nDNA (Setlow, 1974). Direct comparisons with the action spectrum for UVR-induced nDNA damage should be made with some caution, however, since that work was performed in a prokaryotic model (notably, the bacterium *Escherichia coli*). Nonetheless, aside from anatomical differences such as the presence / absence of a nuclear membrane, the inferred relative greater sensitivity of mtDNA vs nDNA to UVR damage may be due to the close proximity of mtDNA to the intra-mitochondrial electron transport chain (ETC). Reactive oxygen species (ROS) generated by longer UVR wavelengths would be incremental to the significant source of ROS already generated by the ETC.

Furthermore, it has been reported previously that there is an increase in nucleotide excision repair (NER), a major DNA repair pathway, in keratinocytes exposed to low dose UVB (Maeda *et al.*, 2001). When compared with nDNA, the repair of mtDNA is significantly more limited because of a lack of NER (Birch-Machin *et al.*, 2013) and, therefore, the apparent relative difference in mtDNA and nDNA damage may also be due, in part, to differing repair mechanisms / efficiency. In addition to limited repair mechanisms mtDNA is also more vulnerable to damage as it lacks histones that are associated with nDNA protection (Birch-Machin *et al.*, 2013).

There is an established link between ROS production, mtDNA damage and skin ageing (Anderson *et al.*, 2014). These new data clearly support the continued need for systems to manage sun exposure (including appropriate clothing, behaviour and sunscreens). Furthermore, it is not unreasonable to hypothesise that topical supplementation with antioxidant species

designed specifically to reduce mtDNA damage from oxidative stress may yet prove to play a useful role in the prevention and management of skin ageing. This is especially important given that there is increasing evidence of a link between mtDNA dysfunction and a spectrum of deleterious skin manifestations (Boulton *et al.*, 2014).

This study has confirmed, as seen with the action spectra of UVR-induced erythema and nDNA damage, that the shorter UVR wavelengths are the most detrimental to human skin. Importantly, a novel observation found that there was a statistically significant ($p < 0.0001$) greater sensitivity to UVR-induced mtDNA damage observed in human dermal fibroblasts compared to human keratinocyte cells. This finding has important implications for disease and photo-damage mechanisms and interventions as it may indicate a depth-dependence in sensitivity to UVR-induced mtDNA damage in human skin.

Materials and Methods

Cell culture

Human neonatal dermal fibroblast cells, HDFn (Invitrogen, UK), were maintained in Dulbecco's Modified Eagle Medium (DMEM; Lonza, UK) containing 10 % fetal calf serum (FCS; Lonza, UK), 5 IU/ml Penicillin and 5 g/ml streptomycin (Lonza; UK), at 37 °C with 5 % CO₂. Human keratinocyte cells were obtained from 15 adult male patient samples (aged 24 – 74 years) from the RVI, Newcastle. Cells were maintained in EpiLife medium (Gibco, UK) supplemented with 0.2 % Human Keratinocyte Growth Supplement (HKGS; Gibco, UK) 5 IU/ml.

Penicillin and 5 g/ml streptomycin (Lonza, UK) at 37 °C with 5 % CO₂.

Irradiation Method

Cells were irradiated in 60 mm dishes when they had been grown to confluent monolayers. Prior to irradiation, medium was removed and cells were washed with phosphate buffered saline (PBS). Either phenol red free DMEM minus FCS (HDFns) or PBS (PK) was then added and the lids of the dishes were removed. For the mock control, cells were covered in aluminium foil and placed under the lamps for the same duration as the irradiated cells. For PK experiments a minimum of three different donors were used per irradiation source. The cells were irradiated with various UVR sources, as shown in Fig 6 and as follows: TL01 (Philips TL 20W/01 RS; 22 % UVA), TL12 (Philips TL 20W/12 RS; 48 % UVA), UV6 (Waldmann F85/100W-UV6; 63 % UVA), Helarium (Helarium R1.01; 88 % UVA), Arimed B (Wolff System Helarium B1-12-40W/BPIN; 96 % UVA), Cleo (Cleo performance 100W-R; 99.3 %; 100 % UVA when used with a glass filter). To induce the required threshold damage irradiation times varied from 0.5 min to 30 min. Immediately following UVR treatment, medium was removed and the cells were washed with PBS before further analysis.

Quantification of mtDNA damage

For damage assessment, DNA was extracted using a QIAamp DNA mini kit as per manufacturer's instructions (Qiagen, UK). The content of mtDNA was determined by amplification of a 83bp fragment of the 16.5kb mitochondrial genome by quantitative real-time PCR (QPCR) as shown previously (Koch *et al.*, 2001; Oyewole *et al.*, 2014) and no difference between samples was found. Damage within mtDNA was established by amplification of a 11kb

segment of the 16.5kb mitochondrial genome by QPCR as previously described (Oyewole *et al.*, 2014; Passos *et al.*, 2007).

Analysis of mtDNA Damage

The number of cycles of PCR required to amplify a consistent amount of the 11kb product was measured by fluorescence and is known as the $C(t)$. The change in the $C(t)$ value in samples exposed to a range of doses of the various UVR sources with differing spectral emissions was expressed relative to un-irradiated controls. These changes in $C(t)$ were assumed to be related to UVR dose by a sigmoid curve of the form:

$$Y = A / \{1 + \exp[-(x-b)/c]\}$$

where x is the logarithm to the base 10 of the UV dose at which the change in cycle threshold is Y , A is the maximum response corresponding to the maximum damage created by each UVR source, b is a parameter relating to the spectral sensitivity of a particular UV source in initiating the effect (i.e. aligned to the action spectrum of UVR induced mtDNA damage) and c is related to the maximum slope of the dose response curve by the expression $c = A / (4 \times \text{max slope})$.

Conflict of interest.

The authors state no conflict of interest.

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Figure Legends.

Fig 1. UVR Induced Dose Curves of Damage. Primary Keratinocyte (A) and HDFn (B) cells were irradiated with increasing doses of (from left to right) the TL12 (-), UV6 (-), TL01 (-), Helarium (-), Arimed B (-), Cleo – filter (-) and Cleo + filter (-) UVR sources. Damage was assessed by a reduction in amplification of an 11 kb product by QPCR and is expressed as a percentage increase when compared to control (UVR protected) cells ($n \geq 3 \pm \text{SEM}$).

Fig 2. Differences in maximum response, spectral sensitivity and slope of the UVR Induced Dose Curves of Damage. Primary Keratinocyte (A) and HDFn (B) Values taken from those shown in Fig 1.

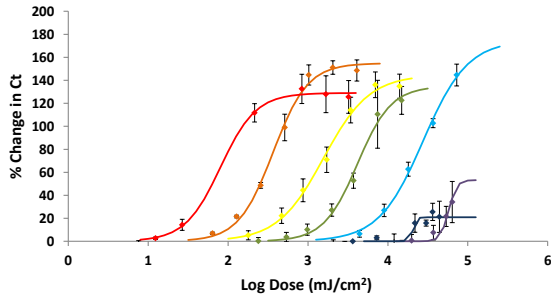
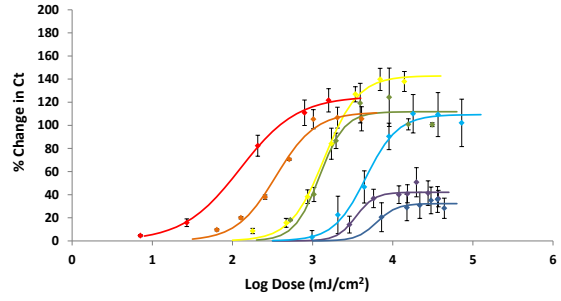
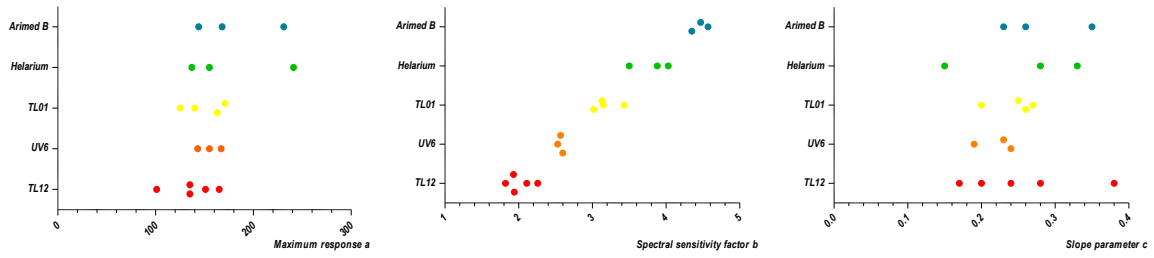
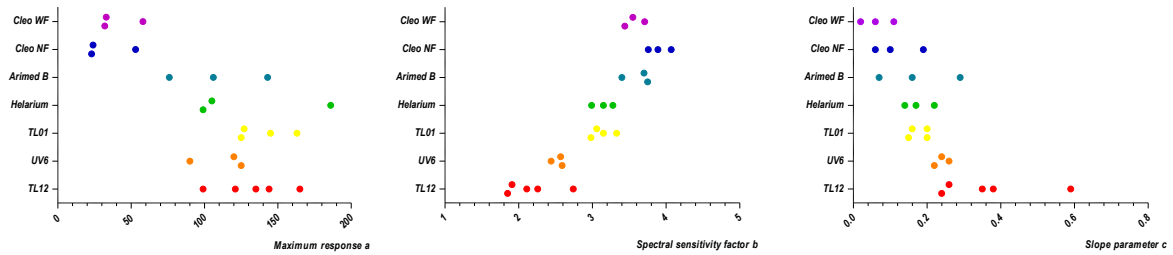
Fig 3. Normalised UVR Induced dose curves of damage. Primary Keratinocyte (A) and HDFn (B) cells were irradiated with increasing doses of various UVR sources. Curves were normalised from those shown in Fig 1 by incorporating a common maximum response and slope.

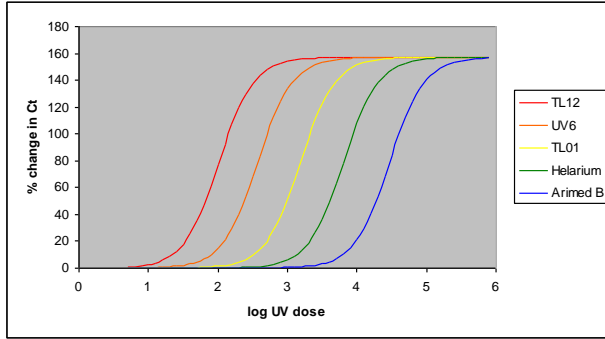
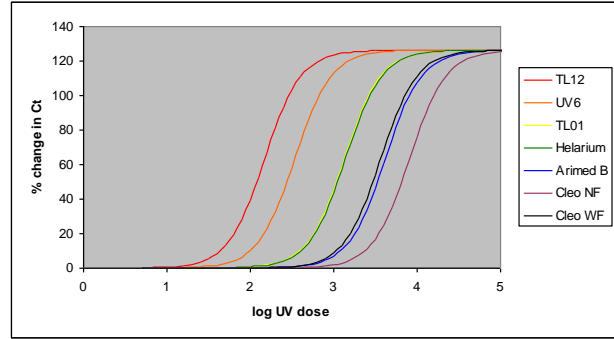
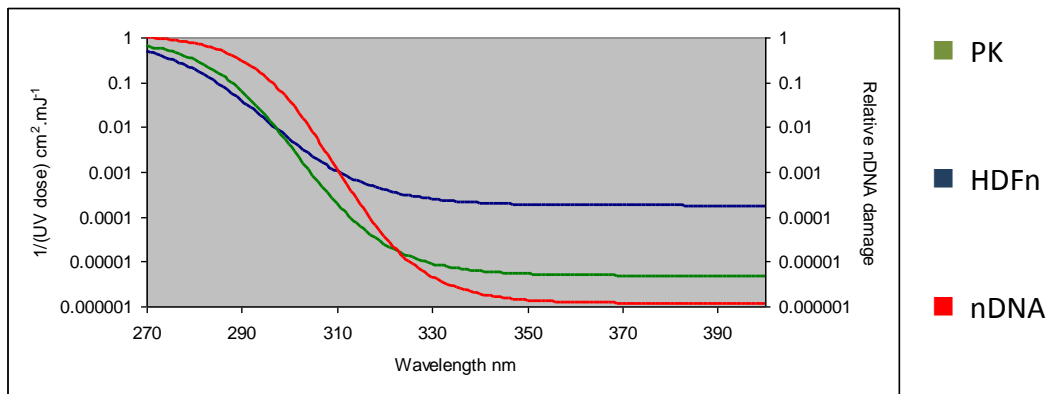
Fig 4. Action Spectra of UVR-Induced mtDNA Damage. The action spectrum for mtDNA damage in dermal fibroblasts and keratinocytes expressed as the reciprocal of the UV dose (in $\text{cm}^2\text{mJ}^{-1}$) that results in a change in cycle threshold ($C(t)$) that is 50% of the plateau value (A). The action spectrum for DNA damage (Setlow, 1974) is shown in red for comparison.

Fig 5. Determination of mtDNA Content Following UVR Exposure. Primary Keratinocyte (A) and HDFn (B) cells were exposed to increasing doses of the broadband UVR sources Arimed B and TL12 respectively. mtDNA content was assessed using a QPCR method to amplify an 83 bp section of mtDNA. Data expressed as the mean $n=3 \pm \text{SEM}$. Linear regression analysis

of this data showed no statistically significant deviation from control ($P=0.054$ and $P=0.919$, respectively).

Fig 6. Spectral Chart. This figure shows the spectral output of the different UVR lamps used in the study.

A**B****Fig 1.****A****B****Fig 2.**

A**B****Fig 3.****Fig 4.**

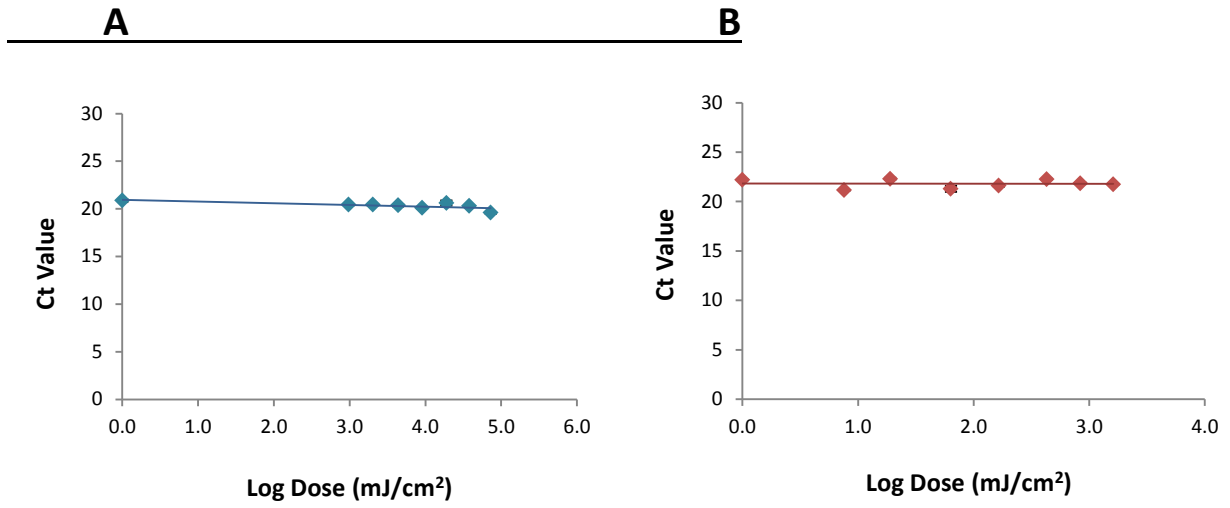


Fig 5.

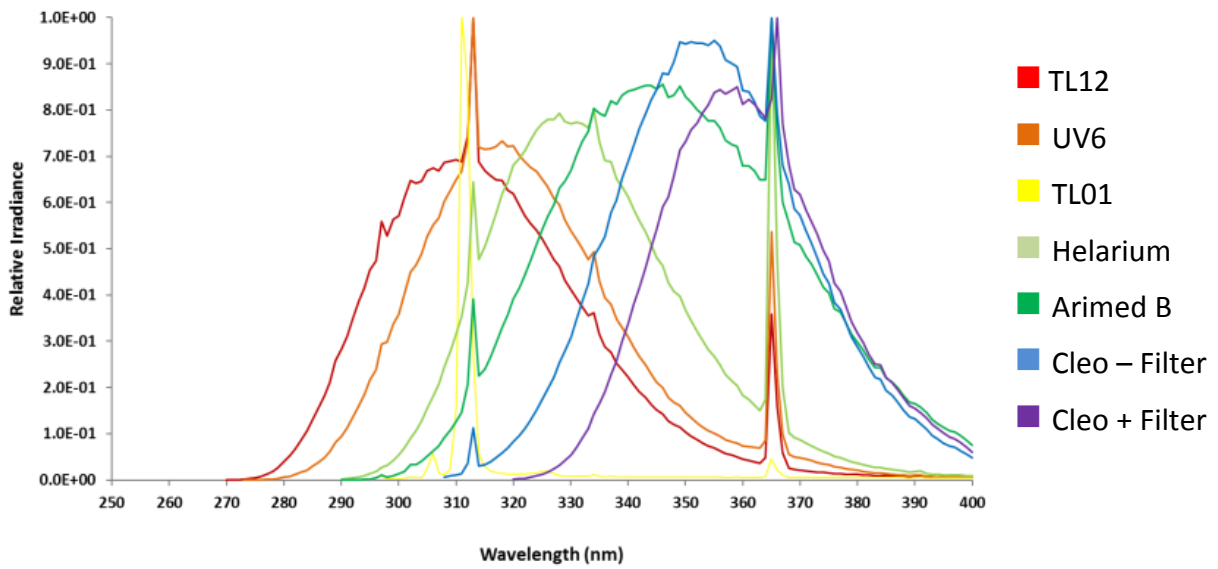


Fig 6.