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Implications of using the fluorescent probes, dihydrorhodamine 123 and 2′,7′-dichlorodihydrofluorescein diacetate, for the detection of UVA-induced reactive oxygen species

SARAHJAYNE BOULTON[1*], ALASDAIR ANDERSON[2*], HELEN SWALWELL[2], JAMES R. HENDERSON[1], PHILIP MANNING[1] & MARK A BIRCH-MACHIN[2]

[1]*Diagnostic and Therapeutic Technologies, and* [2]*Dermatological Sciences, Institute of Cellular Medicine, Newcastle University, Newcastle NE2 4HH, UK*

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Abstract

During investigation of UVA-induced oxidative stress in HaCaT keratinocytes with dihydrorhodamine 123 (DHR123) and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), exaggerated baseline values were observed within control samples, suggesting a mechanism of probe oxidation and subsequent change in fluorescence intensity (FI) independent of cellular ROS generation. The effects of diluent, UVA pre-treatment and loading protocols upon the FI of the probes have therefore been investigated. The study confirmed the capacity of Dulbecco's Modified Eagle's Medium (DMEM) to confer fluorescence intensity changes in both probes, most notably DCF-DA. In addition, UVA pre-treatment compromises the effectiveness of DHR123 and DCF-DA to detect ROS generated in a cell-free system. *In vitro* data shows a greater UVA-induced FI increase in HaCaT cells loaded with probe before rather than after UVA treatment. This study has important implications for future research, the understanding of previous studies and associated confounding effects using DHR123 and DCF-DA as ROS sensitive probes.

Keywords: *Reactive oxygen species, DHR123, DCF-DA, UVA, Fluorescence, FACS*

Abbreviations: *DHR123, dihydrorhodamine 123; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; FACS, fluorescence assisted cell sorting; FI, fluorescence intensity; ROS, reactive oxygen species; R-123, rhodamine 123; RNS, reactive nitrogen species; XOD, xanthine oxidase; DMEM, Dulbecco's Modified Eagle's Medium; FCS, foetal calf serum; PBS, phosphate buffered saline.*

Introduction

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are known to be key signalling species of cellular stress, damage and death [1–5]. In addition to their involvement in communication and oxidative damage, it is now thought that these short-lived and potentially highly reactive molecules may harbour a more intrinsic and sophisticated role in ubiquitous cellular processes [6–9].

The development of sensors capable of reporting and quantifying changes in ROS has been the focus of a considerable amount of research [7,10–16], although the realization of a real-time, direct and dynamic sensor method remains somewhat elusive. The fluorescent probes dihydrorhodamine 123 (DHR123) and 2′,7′-dichlorodihydrofluorescein diacetate are commonly used to detect production of cellular ROS and RNS in a variety of cell types. In this process DHR123 is oxidized from non-fluorescent DHR123 to fluorescent rhodamine 123 (R-123) and DCF-DA is similarly oxidized to its fluorescent product DCF.

Whilst studying ultraviolet A (UVA) light-induced oxidative stress with DHR123 and DCFDA, the current study observed an unexpected and abnormally

*Boulton and Anderson are joint first authors.

Correspondence: Professor Mark A. Birch-Machin, Dermatological Sciences, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne NE2 4HH, UK. Tel: +44 (0)1912225841. Email: m.a.birch-machin@newcastle.ac.uk

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large difference in the trend and magnitude of response between human skin keratinocytes (i.e., HaCaTs) loaded with ROS-sensitive probe either immediately before or after UVA exposure of the cultured cells. This suggested that UVA light may itself exhibit an effect on DHR123 and DCF-DA that is independent of cellular ROS and RNS production. A possible explanation for this observation may in part be due to a non-specific interaction with the cell culture medium as a focused review of previous studies identified similar anomalies which may be attributed to the nature or content of the cell culture medium [17–19]. Therefore, this current study addresses three important questions regarding the optimization and good laboratory practice surrounding the future use of DHR123 and DCF-DA in detecting ROS production in cell biology investigations, building upon a foundation of peer reviewed investigatory work surrounding potential pitfalls of fluorescent ROS and RNS measurement [20–23]. First, does the experimental diluent (e.g. culture medium/buffer) interact with the probes, thereby modifying its response to ROS? Second, does UVA irradiation affect the fluorescence of DHR123 and DCF-DA and, if so, is this effect independent of ROS/RNS interaction? Third, does the effect on the probes alter their capacity or sensitivity for ROS/RNS detection?

Materials and methods

Reagents

Propidium iodide (PI), DHR123, DCF-DA, PBS tablets, xanthine and xanthine oxidase (XOD) were all purchased from Sigma-Aldrich (Poole, UK).

Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT, a gift from Dr N. E. Fusenig (German Cancer Research Centre, Heidelberg, Germany) was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS) in a humidified atmosphere with 5% (v/v) CO_2 at 37°C. The cells were grown to 70–80% confluence in 75cm^2 flasks through no more than six passages during experimentation.

Cell-free fluorimeter measurements

Unless otherwise stated, the following settings were used for all fluorescence intensity measurements: DCF-DA: excitation = 488 nm, emission = 535 nm; DHR123: excitation = 488 nm, emission = 520 nm. All DMEM used in fluorescence-based measurements was phenol red free and without any modification; 'complete DMEM' refers to the modification of DMEM with 10% FCS (therefore 'plain DMEM' is DMEM without the FCS addition). Working concentrations for all methods were 10 μM and 25 μM for DCF-DA and DHR123, respectively.

UVA exposure dose response

Previous work by Gniadecki et al. [24] was used as a basis for the current UVA dose regime together with previous experience obtained from our studies using sub-lethal doses of ultraviolet radiation in cultured skin cells [10,25]. A dose of 10 J/cm^2 was found to be sublethal, but sufficient to induce increased cellular ROS generation. The source of the UVA irradiation was a glass filtered TL09 (Phillips TL100/09) providing a peak output of 350 nm with a range of 315–410 nm. DHR123 and DCF-DA were made to 25 μM and 10 μM, respectively, in plain DMEM, complete DMEM and PBS. Probe solutions were aliquoted into a white 96-well plate (Corning, VWR, Lutterworth, Leicestershire, UK), with the UVA negative control aliquots subsequently heavily masked from any UVA exposure. The plates were exposed to increasing doses of UVA from 0 to 10 J/cm^2. Once all wells had received their allocated exposure, the UVA untreated wells were unmasked and the plate transferred to a Tecan Infinite M200 fluorimeter (Tecan, Grodig, Austria) for FI measurement.

Xanthine/XOD ROS generation and sensation

Immediately prior to use, each probe was irradiated with 10 J/cm^2 UVA, diluted in PBS to the working concentration and then transferred (50 μl per well) to a white 96-well plate (Corning, UK). Each well in the 'baseline' column (i.e. nine replicate wells) of the plate received a further 50 μl PBS; thereby diluting the probe to a working concentration to account for baseline FI. Four columns then received 40 μl 10 mM xanthine in PBS, one column received a further 10 μl PBS and served as a control for any FI changes elicited by xanthine. The remaining three columns were treated with 10 μl of differing XOD concentrations making the final concentration range of enzyme across the plate 0.1, 0.5 and 2.5 U/ml. Once all these additions were completed, the plate was incubated at room temperature for 5 min prior to being transferred to the fluorimeter for FI measurement. As XOD exhibits emission reduction capabilities at 520 nm (data not shown), DHR123 fluorescent emission was determined at a wavelength of 535 nm as opposed to the more conventional 520 nm in order to avoid any interference whilst maintaining sufficient sensitivity of the FI readings.

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FACS analysis of DCF-DA and DHR123 FI in HaCaT cells

HaCaT cells were treated with trypsin, washed and re-suspended in PBS. Half of the cells were incubated with either DCF-DA for 30 min or DHR123 for 20 min in complete darkness and designated as 'pre-UV loaded probe'. All the cells were then irradiated with 10 J/cm^2 UVA during which the cells were gently agitated every 5 min to prevent adherence (control samples were treated identically but heavily masked to prevent exposure to UVA). The remaining unstained cells (i.e. the other half of the cells) were incubated with either DCF-DA or DHR123 as described above and designated as 'post-UV loaded probe'. All cells were then washed and re-suspended in PBS with 10 μM propidium iodide and incubated at room temperature for 5 min prior to FACS analysis using the FL1 channel of a BD FACScan (Becton Dickinson, Oxfordshire, UK). Analysis was performed on PI negative cells using Venturi 1 software (Applied Cytometry Inc., Plano, TX).

Fluorimetric analysis of DCF-DA and DHR123 FI in HaCaT cells

HaCaT cells were plated into a white 96-well plate (Corning, VWR, Lutterworth, Leicestershire, UK) at a density of 5000 cells per well and were incubated overnight to allow adherence to the culture flask. Cells investigated as 'pre-loaded' with probes were incubated as for FACS analysis prior to UVA irradiation. A sub-sample of 'pre-loaded' cells was heavily masked to serve as UVA negative controls. All cells were washed and covered with PBS for the duration of the 10 J/cm^2 UVA irradiation. The remaining unstained sample cells (i.e. the 'post-UVA loaded' cells) were incubated with the probes as previously described. The plate was then loaded into the fluorimeter for FI measurement.

Results

Effect of diluent upon fluorescence of DHR123 and DCF-DA

The data in Figure 1 investigates the question of whether the experimental diluent (e.g. culture medium/buffer) interacts with the fluorescent probes and consequently modifies their response to ROS. At time zero, increased fluorescence was observed in solutions of DHR123 and DCF-DA diluted in plain and complete DMEM when compared with probe diluted in PBS. When exposed to UVA, DCF-DA in plain DMEM exhibited a striking increase in FI within 1 J/cm^2 of UVA exposure. The increase in fluorescence continued for the duration of the UVA exposure, resulting in a 10-fold increase in fluorescence at a 10 J/cm^2 dose of UVA. In contrast, other combinations of probes and diluents did not provide such an amplified response. For example, DCF-DA in complete DMEM exhibited an ~2-fold increase in FI following 10 J/cm^2 UVA. Interestingly, a very similar profile of dose curves was observed for the UVA irradiation of DHR123 in both plain and complete DMEM where again an ~2-fold increase in FI was recorded following 10 J/cm^2 UVA. In complete contrast to the behaviour of the probes in plain and complete DMEM, the probes diluted in PBS exhibited only a slight increase in FI when exposed to UVA.

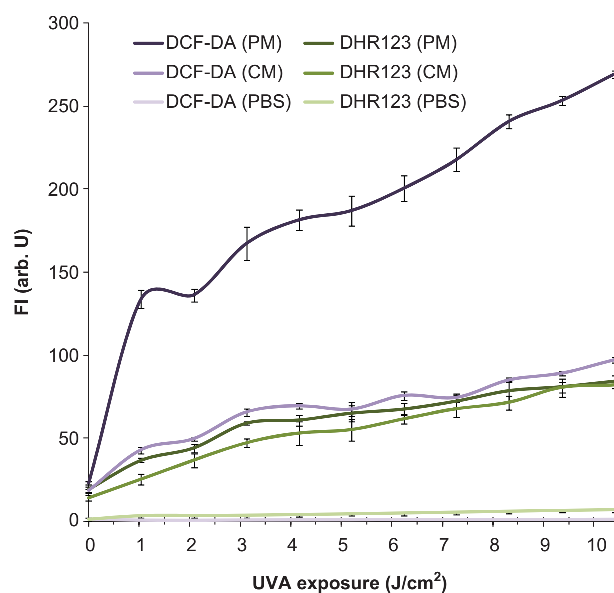

Figure 1. Differential fluorescence intensity (FI) responses of DCF-DA and DHR123 diluted in PBS, Plain (Phenol Red Free) DMEM (PM) or Complete (Phenol Red Free) DMEM (CM) resulting from increasing UVA exposure. Fluorescence intensity (FI) was measured using a fluorimeter as described in Materials and methods. Data presented ± standard error of the mean, $n = 6$.

Effect of UVA pre-treatment upon the response of DHR123/DCF-DA as a ROS sensitive probe

In order to address the other two questions of the study (namely does UVA irradiation affect the fluorescence of DHR123 and DCF-DA and, if so, is this effect independent of ROS interaction and does it alter probe sensitivity for ROS?), a previously established system for the generation of H_2O_2 and O_2^- using xanthine and XOD [15] was used to determine the ROS responses of DHR123 and DCF-DA both in the absence and presence of UVA pre-treatment. The study took into consideration any effects of xanthine or XOD upon the probes (see methods).

The data in Figure 2 clearly show that DCF-DA exhibits a decreased response to xanthine/XOD generated ROS following UVA irradiation when compared with the absence of any UVA pre-treatment. There was a significant difference in the response ($p < 0.005$) between the irradiated and non-irradiated samples at all enzyme concentrations (taking into account the

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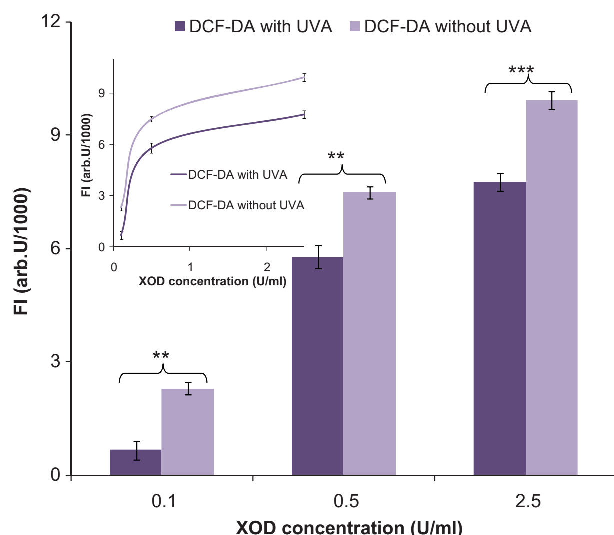

Figure 2. XOD dose-dependent fluorescence of DCF-DA with or without UVA pre-treatment (10 J/cm^2) in a cell-free system. Fluorescence intensity (FI) was determined as described in Materials and methods. Significant difference between data sets denoted by ***($p < 0.001$) and **($p < 0.01$) as determined by one way ANOVA. Data presented ± standard error of the mean, $n = 8$.

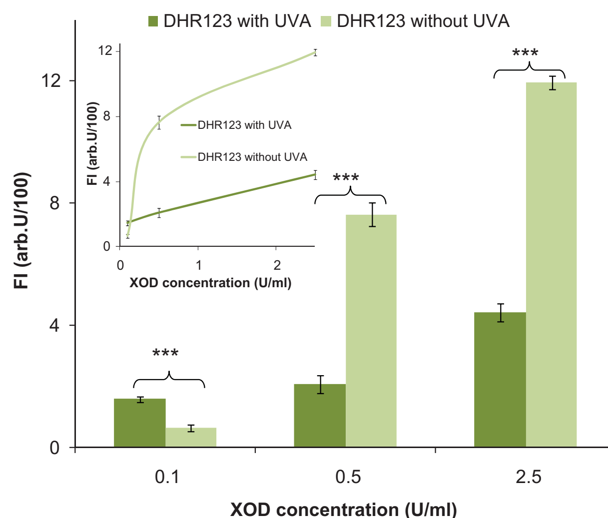

Figure 3. XOD (i.e. cell-free) dose-dependent fluorescence of DHR123 with or without UVA pretreatment (10 J/cm^2) in a cell-free system. Fluorescence intensity (FI) was determined as described in Materials and methods. Significant difference between data sets denoted by ***($p < 0.001$) as determined by one way ANOVA. Data presented ± standard error of the mean, $n = 8$.

absolute FI for the XOD free controls in which the irradiated DCF-DA samples exhibited a higher absolute FI values than the non-irradiated counterparts). The inset in Figure 2 emphasizes the fact that both UVA irradiated and non-irradiated sample sets show a similar trend of DCF-DA response to the generated ROS (although the amplitude is more pronounced in the non-irradiated dataset, implying an increased response to ROS in the absence of any UVA pre-treatment).

The data in Figure 3 represents the same experimental protocol used in Figure 2 with the exception that the probe used was DHR123 rather than DCF-DA. In a similar fashion to the observations reported in Figure 2, DHR123 exhibited a decreased response to xanthine/XOD generated ROS following UVA irradiation when compared with the absence of any UVA pre-treatment. Again, taking into account the absolute FI for the XOD free controls (in which the irradiated DHR123 samples exhibited higher absolute FI values than the non-irradiated counterparts), there was a significant difference between irradiated and non-irradiated probe, most notably at 0.5 and 2.5 U/ml XOD ($p < 0.0001$) (the trend was not observed at the lowest XOD concentration for which similarly low responses were exhibited). The inset in Figure 3 emphasizes the decreased response of the probe following UVA pretreatment, although the actual profile of the DHR123 response was different to that observed for DCF-DA (i.e. UVA appeared to decrease the response of DHR123 more than DCF-DA). Despite this issue, all the data displayed in Figures 2 and 3 clearly suggested that UVA pre-treatment had a compromising effect on the ability of the probes to respond reliably to ROS generated in a cell-free system.

In vitro methods using DHR123/DCF-DA as probes for UVA-induced oxidative stress

The next question to address was whether UVA treatment had a compromising effect on the ability of the probes to respond reliably to ROS generated *in vitro* from cultured human skin cells when compared with the cell-free environment described in Figures 2 and 3. Additionally, in order to study the dynamics of the UVA effect on the probes, HaCaT cells were loaded with DHR123 or DCF-DA either before or after exposure to 10 J/cm^2 UVA and the resulting changes in FI were detected using fluorimetry. A significantly greater UVA induced FI was observed in those HaCaT cells loaded with DHR123 or DCF-DA prior to UVA treatment (i.e. pre-UVA loaded probe, Figure 4) compared with loading the cells with the probe post-UVA treatment (i.e. post-UVA loaded probe) ($p < 0.005$ (DHR123), $p < 0.0005$ (DCF-DA)). Control fluorescence data from non-irradiated HaCaT cells were used to normalize the FI in both sample sets.

Even though the UVA treatment was sub-lethal to the HaCaT cells, a further experiment was performed in order to account for any confounding effects due to cell death or lack of cell viability. This involved flow cytometric analysis of cells double labelled with the ROS probe and propidium iodide to indicate any dead cells which might have taken up the probe and would therefore contribute to the overall FI reading. Table I shows the results of the flow cytometric measurement of HaCaT cells loaded with both probes either before or after exposure to 10 J/cm^2 UVA. The results of this experiment confirm the data displayed in Figure 4 by demonstrating a significantly greater UVA-induced

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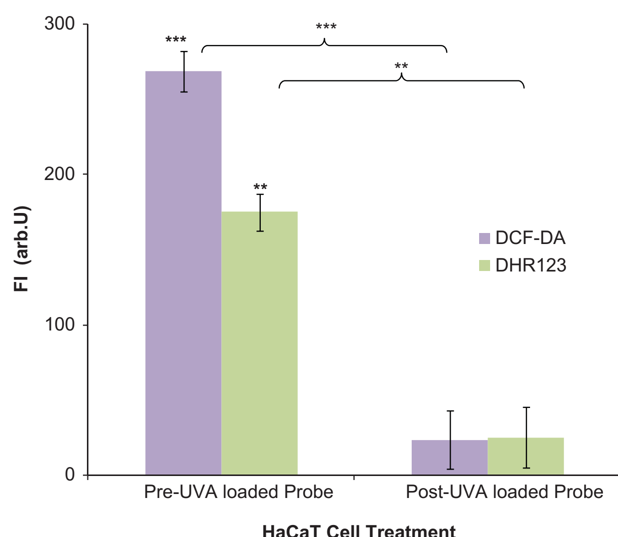

Figure 4. Fluorimetric analysis of the loading-dependent responses of DCF-DA and DHR123 to UVA-induced ROS production *in vitro*. HaCaT cells were loaded with DHR123 or DCF-DA either before or after exposure to 10 J/cm^2 UVA with the resulting changes in FI detected using a fluorimeter as described in Materials and methods. Significant difference between data sets denoted by $^{***}(p < 0.001)$ and $^{**}(p < 0.01)$ as determined by one way ANOVA. Data presented ± standard error of the mean, $n = 8$.

FI in those HaCaT cells loaded with DHR123 or DCF-DA prior to UVA treatment (i.e. pre-UVA loaded) compared with loading the cells with the probe post UVA treatment (i.e. post-UVA loaded) ($p < 0.005$ (DHR123), $p < 0.05$ (DCF-DA)). As in Figure 4, control fluorescence data from non-irradiated HaCaT cells were used to normalize the FI in both sample sets.

Discussion

The experimental diluent interacts with the DHR123 and DCF-DA probes, thereby modifying their response to ROS

This study set out to address three questions, the first was whether the experimental diluent (e.g. culture medium/buffer) interacted with the probes, thereby modifying their response to ROS? By exposing the naked probe to increasing UVA exposure in a range of media, Figure 1 shows that the diluent used during irradiation will impact upon the magnitude of fluorescence determined by both DHR123 and DCF-DA. Long and Halliwell [19] have shown that components of DMEM can catalyse the production of H_2O_2 on the addition of phenolic compounds such as epigallocatechin gallate. This may provide some indication as to why probes such as DHR123 and DCF-DA, themselves phenolic compounds, may interact with components of DMEM and were observed to exhibit increased fluorescence intensity. Spontaneous oxidation of the probes has been demonstrated to be in the order of ~0.02%/min [26] in a ROS-free system, thus the observed changes in fluorescence intensity are likely to originate from the generation of an oxidative species. Riboflavin is a key component of DMEM and not only has it been shown to produce ROS it has also been identified as an intracellular source of O_2^- upon exposure to sunlight [27,28]. The inherent redox activity of riboflavin which is known to interfere with cytotoxicity assays [17] may also play a part in the interaction between the media content and the ROS sensing probes.

A number of studies have demonstrated that DMEM, when exposed to ambient light such as that from a non-UV safety cabinet bulb, can produce ROS catalysed by trace metal ions such as iron, magnesium, sodium, potassium and calcium [27–29]. These metal ions also accelerate ROS production through the auto-oxidation of glucose. Mammalian serum albumins such as those present in FCS have been shown to exhibit non-specific binding properties with a wide range of different ions including Cu^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} due to the N-terminal 3 residues that form a strong square planar binding site [30,31]. Other studies have suggested that there is more than one metal-specific binding site on serum albumin [29,32]. This is in keeping with the difference in probe fluorescence observed in plain DMEM compared with complete DMEM reported in this study. This difference in FI levels may be attributed to the presence of FCS in the media. For example, in complete DMEM nonspecific interactions between serum protein and the metal ions may attenuate the catalysis of peroxide generation (and decrease the production of hydroxyl radicals from hydrogen peroxide catalysed by transition metals such as iron and copper via the Fenton reaction [33]), thereby leading to reduced FI as shown in Figure 1.

Table I. FACS-defined, loading-dependent responses of DCF-DA and DHR123 to UVA-induced ROS production *in vitro*. HaCaT cells were loaded with DHR123 or DCF-DA either before or after exposure to 10 J/cm^2 UVA with the resulting changes in FI quantified in living cells (i.e. propidium iodide negative cells) using flow cytometry as described in Materials and methods.

	DHR123		DCF-DA		
	χ^2	σ	χ^2	σ	*p*-value
Pre-UVA loaded	386.79	2.870 79	1115.313	147.813 3	0.001 537 1
Post-UVA loaded	78.543 34	1.581 529	47.369 92	50.973 85	0.136 548 1
p-value	$2.205\ 08 \times 10^{-5}$		$2.117\ 05 \times 10^{-3}$		

Data presented ± standard error of the mean, $n = 3$.

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However, this is clearly not the entire explanation as there was a greater amplitude of response with DCF-DA compared with DHR123 which may in part be related to additional structural or stochastic interactions. In complete contrast to the behaviour of the probes in plain and complete DMEM, the probes diluted in PBS exhibited only a slight increase in FI when exposed to UVA. The simple composition and lack of any metal ions in PBS maintains a 'neutral' environment for the irradiation of probes, thereby removing the opportunity for any exacerbation of fluorescence by the presence of such components in DMEM.

UVA pre-treatment compromises the effectiveness of DHR123 and DCF-DA to detect ROS generated in a cell-free system

In order to address the other two questions of the study (namely does UVA irradiation affect the fluorescence of DHR123 and DCF-DA and, if so, is this effect independent of ROS interaction and does it alter probe sensitivity for ROS?), a previously established cell free system for the generation of H_2O_2 and O_2^- using xanthine and XOD [15] was used to determine the responses of DHR123 and DCF-DA in the absence and presence of UVA pretreatment. The generation of O_2^- using xanthine and XOD [15] was used to determine the responses of DHR123 and DCF-DA in the absence and presence of UVA pre-treatment. The generation of O_2^- and H_2O_2 (and uric acid) via the decomposition of xanthine by XOD has been well characterized and has been used extensively as a calibration technique for amperometric superoxide sensors [12–15] and as a testing platform for ROS-sensitive nanosensors [34]. By introducing the probes into an environment in which ROS are being enzymatically generated, a response can be measured dependent on the turnover of the reaction. As the concentration of XOD was increased, it was expected that an increase in FI would also be observed for both DHR123 and DCF-DA. This was indeed the case, although the profile of the response appeared to be dependent upon the type of probe as well as UVA treatment.

The data in Figures 2 and 3 clearly showed that while both probes detected increasing ROS generated by greater concentrations of XOD, they also exhibited a decreased response to xanthine/XOD generated ROS following UVA irradiation when compared with the absence of any UVA pre-treatment. It appears that the UVA pre-treatment had a compromising effect on the ability of the probes to respond reliably to additional ROS generated in a cell-free system. This effect was more pronounced with DHR123 than DCF-DA. It appears therefore that the probe may be excited and sensitized directly by the UVA light, resulting in a decreased proportion of the non-excited state remnant of the probe being available for interaction with the ROS generated by the xanthine/XOD system. This reduced capacity to interact with any XOD generated ROS would explain the decreased ability of the probes to sense ROS following UVA treatment as there was a smaller residue of non-reduced probe able to respond to the XOD generated ROS. Hence, as XOD was introduced to the probe in increasing concentrations, only a relatively small change in FI was observed in the irradiated probe compared with the non-irradiated probe. Against the background of this 'desensitization-like effect' exhibited by the two probes, it is interesting to note that there was a different profile of response between DCF-DA and DHR123 and again this may depend in part on additional structural or stochastic interactions. This 'desensitization-like effect' is important as it suggests that the increase in fluorescence induced by UVA treatment of DHR123, and to a lesser extent DCF-DA, could in fact be masking the more subtle but nevertheless key responses in ROS mediated pathways and events.

In vitro *data shows a greater UVA-induced FI in HaCaT cells loaded with probe before rather than after UVA treatment*

Previous publications have indicated that there was little or no difference when loading DCFDA and DHR123 into cells before rather than after UVA exposure for longer irradiations and at the concentrations used for this investigation [16,35]. However, the data reported in this study demonstrates a significantly increased FI for both probes when loaded into HaCaT keratinocytes before UVA exposure (Figure 4 and Table I). The loading of DHR123 and DCF-DA before UVA exposure is preferable to loading afterwards as the probe is able to report ROS/RNS production in the cell immediately, with no lag time, which would not be the case with cells loaded with probe after UVA irradiation. This is because loading of the probe involves standard incubation times of 20 min, during which time UVA-induced ROS/RNS production may have exhibited an initial burst which therefore cannot be reported.

Implications for future research and the understanding of previous studies using DHR123 and DCF-DA

Whilst there is little detailed published literature on the photo-stability of DHR123 and DCFDA, particularly in relation to UVA, this study provides evidence that UVA irradiation increases conversion of DHR123 to R123 and DCF-DA to DCF in a cellular ROS/RNS independent manner. This has important implications for those studies requiring the use of a fluorescent probe such as DCF-DA and DHR123 when UVA is the inducer for oxidative stress in the cells. Small differences in cellular ROS production (which may in

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fact represent significant events in cellular signalling responses) are likely to be masked by an exaggerated response of dyes to UVA, especially when diluted in DMEM. This may be particularly important if such probes are to be loaded into cells prior to UV exposure. The data presented in this study suggests that such probes should be loaded preferably in PBS and post-UV irradiation. However, loading dyes after UV exposure may cause a trade-off in measuring the time course of a response when ROS production is measured after the initial UV-induced burst of ROS. If loaded pre-UV irradiation, then adequate control conditions must be included to account for the potential confounding effects reported in this work.

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Declaration of interest

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