Vitamin E inhibits the UVAI induction of “light” and “dark” cyclobutane pyrimidine dimers, and oxidatively generated DNA damage, in keratinocytes

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Solar ultraviolet radiation (UVR)-induced DNA damage has acute, and long-term adverse effects in the skin. This damage arises directly by absorption of UVR, and indirectly via photosensitization reactions. The aim of the present study was to assess the effects of vitamin E on UVAI-induced DNA damage in keratinocytes in vitro. Incubation with vitamin E before UVAI exposure decreased the formation of oxidized purines (with a decrease in intracellular oxidizing species), and cyclobutane pyrimidine dimers (CPD). A possible sunscreening effect was excluded when similar results were obtained following vitamin E addition after UVAI exposure. Our data showed that DNA damage by UVA-induced photosensitization reactions can be inhibited by the introduction of vitamin E either pre- or post-irradiation, for both oxidized purines and CPD (including so-called “dark” CPDs). These data validate the evidence that some CPD are induced by UVAI initially via photosensitization, and some via chemoexcitation, and support the evidence that vitamin E can intervene in this pathway to prevent CPD formation in keratinocytes. We propose the inclusion of similar agents into topical sunscreens and aftersun preparations which, for the latter in particular, represents a means to mitigate on-going DNA damage formation, even after sun exposure has ended.
A UVA dose-dependent increase in oxidizing species, determined by H$_2$DCFDA fluorescence, was observed (Fig. 1A). Pre-UV AI protective effect of vitamin E was also evident on DNA damage. Vitamin E treatment significantly decreased the viability was found to be unaffected by UV AI exposure and/or vitamin E treatment (Table 1). A UV A dose-dependent increase in both oxidized purines and CPD formation was demonstrated at all cases (p < 0.05), with the effect being more evident at higher UV A doses. The effect was highly significant at 40 J/cm$^2$ with a 35% decrease in oxidizing species compared to control (p < 0.001). Vitamin E did not alter the level of intracellular oxidizing species in unirradiated cells. Incubating HaCaTs with vitamin E for 24 h, prior to irradiation, significantly increased intracellular GSH levels by 2.3-fold (p = 0.002), and protected against UV AI-induced CPD (Fig. 1B). The post-UV A vitamin E treatment was found to also inhibit UV AI-induced CPD formation, at both UV A doses tested (60% and 23% decrease at 5 and 10 J/cm$^2$, with p < 0.001 and p < 0.01, respectively).

### Results

**Pre-UV AI treatment with vitamin E protects against oxidizing species and DNA damage.** Cell viability was found to be unaffected by UV AI exposure and/or vitamin E treatment (Table 1). A UVA dependence in oxidizing species, determined by H$_2$DCFDA fluorescence, was observed (Fig. 1A). Pre-UV AI treatment with vitamin E was found to offer significant protection at all UV A doses tested. This was demonstrated at all cases (p < 0.05), with the effect being more evident at higher UV A doses. The effect was highly significant at 40 J/cm$^2$ with a 35% decrease in oxidizing species compared to control (p < 0.001). Vitamin E did not alter the level of intracellular oxidizing species in unirradiated cells. Incubating HaCaTs with vitamin E for 24 h, prior to irradiation, significantly increased intracellular GSH levels by 2.3-fold (p = 0.002), and protected against UV AI-induced CPD (Fig. 1B). The post-UV A vitamin E treatment was found to also inhibit UV AI-induced CPD formation, at both UV A doses tested (60% and 23% decrease at 5 and 10 J/cm$^2$, with p < 0.001 and p < 0.01, respectively).

### Post-UV AI treatment with vitamin E protects against oxidizing species and DNA damage.

A time-course study showed that all DNA lesions increased after UV AI exposure (0 h) with a peak of formation at 1 h (Fig. 3). This was partly expected for oxidized purines and ALS/SP, due to their formation by reactive intermediates, but was surprising for CPD. This reveals the delayed induction of dark CPD in keratinocytes, even after removal from UV AI exposure (dotted line), and their repair, contrasting with the expected repair of CPD (dashed line; Fig. 3). The increase and decrease in CPD levels during the 0–2.5 h period was the basis for determining the time for the post-UV AI vitamin E incubations in subsequent studies (i.e. Figs 4 and 5).

Cells treated with vitamin E exhibited lower levels of oxidizing species compared to their untreated counterparts (Fig. 4). This clear protective effect of vitamin E was found to be greater following doses of 20 and 40 J/cm$^2$ (maximal inhibition of the production of oxidizing species was 38%, at 40 J/cm$^2$: p < 0.05). The post-UV AI protective effect of vitamin E was also evident on DNA damage. Vitamin E treatment significantly decreased the

| Treatment            | Viable cells (%) TB | Viable cells (%) MTT |
|----------------------|---------------------|----------------------|
| Control*             | 87.3 ± 3.2          | 88.7 ± 2.5           |
| 20 J/cm$^2$ UV AI*   | 81.8 ± 4.3          | 80.1 ± 3.6           |
| Vitamin E            | 88.6 ± 2.5          | 89.2 ± 2.0           |
| 20 J/cm$^2$ UV A + Vitamin E | 85.9 ± 1.6 | 86.5 ± 2.5 |

Table 1. HaCaT cell viability following UVA ± vit E. Cell viability was assessed by the trypan blue (TB) exclusion and the MTT assays. Data represent the mean of three independent experiments ± SEM. One-way ANOVA with Bonferroni correction showed significant differences between all groups (p < 0.05). *Cells incubated with EtOH for 24 h.
formation of oxidized purines, compared to non-treated cells (70% and 32% decreases at 5 and 10 J/cm², with \( p < 0.001 \) and \( p < 0.01 \), respectively) (Fig. 5). Post-UV A vitamin E treatment was also found to decrease CPD formation, an observation that cannot be attributed to a possible sunscreen property, since vitamin E was added after UV A exposure. CPD values were decreased at both UV A doses tested (52% and 44% decrease at 5 and 10 J/cm², with \( p < 0.05 \) and \( p < 0.01 \), respectively). Induced DNA damage was lower in the post-UV A incubation control experiments compared to incubation pre-UV A, especially at 5 J/cm². This suggests DNA repair occurs within 2.5 h of exposure.

**Vitamin E prevents UVC-induced formation of oxidized purines, but not CPD.** Monochromatic UVC (254 nm) was employed to assess a possible sunscreening role of vitamin E (see discussion section for fuller rationale). As expected; UVC induced high CPD levels, together with some oxidized purines, ALS and SB. Incubation with vitamin E pre-UVC led to a significant decrease (Fig. 6) in ALS and SB and oxidized purines (66%, \( p < 0.05 \)) but had no effect on CPD formation.

**Discussion**

UVAI is by far the major spectral component of solar UVR and penetrates deeper into the skin than UVB. A study of DNA damage depth profiles in human skin in vivo shows attenuation of CPD and pyrimidine (6–4) photoproduct formation with increasing with skin (epidermis and dermis) depth, but the reverse is true, for CPD at least, with UVAI²⁴. This results in greater sensitivity of the keratinocyte stem cell and melanocyte containing basal layer to UVAI exposure. UVAI-induced mutations are more prevalent in the basal layer than the supra-basal layers²⁵. It is therefore important to find new strategies to protect the skin, especially the basal layer, from UVAI-induced DNA damage. Vitamin E has been established as a UVR-induced ROS scavenger. To the best of our knowledge, we provide the first evidence that UVAI-induced CPD (including dark CPD), as well as oxidatively-generated DNA lesions, can be inhibited by vitamin E in HaCaT keratinocytes. Whilst the latter is in line with its classical role as an antioxidant, the former is an important finding, given earlier, similar findings, but
in melanocytes\(^{13}\). Our UVA doses (5 and 10 J/cm\(^2\)) were sub-erythemal and physiologically and environmentally relevant; a minimal erythema dose (MED) of UVA is about 50 J/cm\(^2\) in fair skin types\(^{26}\).

Carboxy-H\(_2\)DCFDA was selected to study the formation of oxidizing species because it can be oxidized by several UV A-induced reactive oxygen species and free radicals (including H\(_2\)O\(_2\), NO and peroxides)\(^{27}\). The data showed a UVA dose-dependent increase in oxidizing species formation in HaCaT cells. Cells treated with vitamin E, prior to UVA, produced less oxidizing species compared to controls at all tested doses. Under these same conditions, levels of intracellular GSH increased, and subsequently provided some protection against the UVA induction of oxidizing species suggesting that at least some of these oxidizing species are ROS.

Interestingly, a protective effect was also seen when vitamin E was administered post-UVA. It is noteworthy that more oxidizing species were generated post- versus pre-UVA incubation. This observation may be attributed to the formation of oxidizing species via secondary biochemical pathways\(^{28}\). These species can result from lipid peroxidation (which can be inhibited by vitamin E), which in turn might initiate the formation of further oxidatively damaged DNA.

The hOGG1-modified comet assay showed that UVA induced the formation of oxidized purines in a dose-dependent manner. Vitamin E offered a significant protective effect with pre- and post-exposure incubation. Since the UVA-induced oxidation of purines are formed indirectly, predominantly via the

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**Figure 2.** Effect of pre-UVAI incubation with vitamin E on the formation of oxidized purines and CPD. Mean percentage of tail DNA was determined following UVAI doses of 5 and 10 J/cm\(^2\). Results are the mean ± SEM of three independent experiments; ***p < 0.001, **p < 0.01 for selected comparisons.

**Figure 3.** Induction and repair of UVAI-induced oxidized purines and CPDs, determined by the T4endoV- and hOGG1-modified comet assay. HaCaT keratinocytes were irradiated with 5 J/cm\(^2\) UVA and were left to repair for different time periods. At 0 h, the dotted line mainly represents the formation of “light” CPD. The subsequent increase, with a peak at 1 h, represents the formation of “dark” CPD and their repair (1–2.5 h). The dashed line, which joins the dotted line, represents the proposed, differential repair of “light” CPD. The results are the mean ± SEM of three independent experiments.
photosensitizer-dependent induction of $^{1}\text{O}_2^{29,30}$, the antioxidant/scavenging properties of vitamin E were not surprising, although this protective effect has not previously been demonstrated for oxidized purines by the comet assay, especially with post-UV A incubation. This suggests that, after the initial ROS generation via photochemical processes (energy transfer to molecular oxygen, electron abstraction, etc), biochemical pathways are then responsible for the generation of secondary ROS, possibly through $^{1}\text{O}_2$ production$^{31,32}$. This may occur via UV A-induced enzyme activity, e.g. activation of NADPH oxidase$^{28}$. NADPH oxidase increases UV A-induced superoxide, in mouse, monkey and human cell lines$^{33}$, which can be converted to other ROS. Other studies show evidence of a protective effect of vitamin E against ovulation-induced 8-oxoGua in ovarian epithelial cells$^{34}$, as well as ozone-induced 8-oxoGua$^{35}$. The antioxidant role of vitamin E has been reported to protect against cis-urocanic acid-induced ROS$^{36}$. It is worth noting that $^{1}\text{O}_2$ does not generate strand breaks, although some alkali-labile sites (both of which may be evaluated by the comet assay) can be produced (as noted in Cooke et al.$^{37}$, but predominantly it is the nucleobase modification, 8-oxoGua, that is generated$^{38}$.

CPDs, assessed by the T4endoV-modified comet assay, were readily induced by UV A irradiation, and their formation was significantly inhibited by incubation with vitamin E before and after irradiation. The ability of
post-UVAI vitamin E incubation to inhibit CPD excludes a sunscreening effect, but also alludes to an indirect mechanism for UVAI-induced CPD. This is supported by the irradiation studies with UVC (254 nm), which is close to the action spectrum maximum for CPD induction in vitro. Action spectroscopy shows that production of CPD at 300–310 nm is three orders of magnitude lower than at 254 nm. Figure 6 shows that 254 nm induced high levels of CPD as well as oxidized purines. Although UVC-induced 8-oxoGua has been previously reported, there is little literature on UVC-induced cellular oxidative stress. One report has suggested that UVC-induced 8-oxoGua formation is via 1O2, although it is not clear how this would occur, and the involvement of guanine radical cations are perhaps a more likely mechanism. Pre-UVC incubation with vitamin E significantly protected against the formation of oxidized purines but not against CPD (Fig. 6). As can be seen in Fig. 7, vitamin E absorption at 254 and ~300 nm is similar, but higher than at longer wavelengths in the UVAI source. Thus, one might expect a comparable or better sunscreensing effect for CPD at 254 nm than with UVAI. The lack of such an effect for CPD suggests that protection against the formation of oxidized purines is via mechanisms

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**Figure 6.** Effect of vitamin E pre-incubation on UVC-induced formation of CPD and oxidized purines, determined by T4endoV- and hOGG1-modified comet assays, respectively. The results represent mean (±SEM) percentage tail DNA in HaCaT cells exposed to UVC for 10 s, and have been corrected for baseline levels of damage and SB/ALS. Results are the mean ± SEM of three independent experiments; *p < 0.05 for selected comparisons.

**Figure 7.** Emission spectrum of the "UVA spot". This was determined by a Bentham DM150 double monochromator spectroradiometer through the plastic lid of a petri dish in which cells were irradiated, at a distance of 39 cm. Also shown is the absorption spectrum of a vitamin E solution (0.1 mg/mL in ethanol).
other than sunscreening. Furthermore, the action spectrum for 8-oxoGua formation shows a peak in the UV AI ($\lambda_{\text{max}} = 365 \text{ nm}$) region 40, although UVB may also induce this lesion 7. We recognize that the very small UVB content (0.1%) of our UV AI source (Table 2) may have caused a disproportional large number of CPD 45 but the complete lack of effect of vitamin E on UVC-induced CPD supports a different mechanism for UV AI-induced CPD.

CPDs are formed mainly via direct photon absorption by DNA in the UVC and UVB regions. DNA shows some direct UV A absorption 46 which is well established to result in CPD formation, time-course studies with 5 J/cm² (Fig. 3) that showed a t½ of about 4 h for both lesions (achieving a maximum at 2.5 h) suggests some repair of oxidized purines and CPD during the 2.5 h post-UV A period. This was confirmed in dermatology research.

Table 2. Spectroradiometric distribution of the emission spectrum (see Fig. 1) of the UV spot (measured through the plastic lid of a 6-well plate used in the experiments), and their respective erythemally effective energies (EEE), obtained by multiplying with the CIE action spectrum for erythema 47. This shows that the majority of the EEE was in the UV AI region. *Official CIE definition of UVB, but a cut-off at 320 nm is used in dermatology research.

| Spectral Region | Wavelength (nm) | % of total irradiance | % EEE |
|-----------------|-----------------|----------------------|--------|
| UVA             | 321–400         | 99.8                 | 87.5   |
| UV AI           | 340–400         | 97.5                 | 78.4   |
| UV AII          | 321–340         | 2.3                  | 9.1    |
| UVB (CIE)*      | 281–315         | 0.1                  | 10.4   |
| UVB             | 281–320         | 0.2                  | 12.7   |
| UVC             | 200–280         | 0.0                  | 0      |
| Total UVR       | 200–400         | 100                  | 100    |

A comparison of the comet assay data from the pre- and post-UV A vitamin E incubation studies (Figs 2 and 5) suggests some repair of oxidized purines and CPD during the 2.5 h post-UV A period. This was confirmed in time-course studies with 5 J/cm² (Fig. 3) that showed a t½ of about 4 h for both lesions (achieving a maximum at 1 h). This is similar to the t½ of ~0.5 h reported for UV A-induced CPD in HaCaTs in a recent publication from our group 44. However, the t½ for oxidized purines in the current study is faster than that of about 10 h in our previous study, in which we also showed that DNA repair kinetics were dependent on UV spectrum. The times for t½ are of course longer when using 0 h as a reference point. It should also be noted that very different UV spectra were used in the two studies. Our present time-course study demonstrates the formation of “dark DNA photodamage” that have been recently reported for CPD in melanocytes 13 but not for oxidatively-induced DNA lesions.

It is possible that vitamin E incubation post-UV A exposure decreases CPD by enhancing their repair by inhibiting ROS 49 which, along with RNS, can damage/inhibit DNA repair enzymes and DNA polymerases (associated with both nucleotide excision repair (NER) and base excision repair (BER)) 16,67. This concept is supported by studies that show that post-UV A treatment with vitamin D₃ suppresses nitric oxide products resulting in enhanced DNA repair; with a consequent reduction of CPD, immunosuppression and photocarcinogenesis 48. However, while UV A induces oxidatively-generated crosslinking (through 1O₂ production) between the subunits of the replication and repair protein, PCNA 46. Furthermore, UV A-induced ROS are also known to activate several MAPKs 49, therefore, by affecting various downstream effectors, such as AP-1 and NFκB, they may alter DNA repair responses, cell cycle arrest or apoptosis 1. One study showed that vitamin E treatment post-UV A (broad spectrum) irradiation increased CPD repair in mouse skin in vivo and this result was correlated with decreased p53 protein levels 72.

In the present study, we showed that vitamin E can inhibit UV AI-induced oxidizing species production and induction of DNA damage, even when human keratinocytes are treated after irradiation. Whilst perhaps better established for ROS-induced DNA damage, the important implication of this is that the process of CPD formation continues after irradiation has ended, implying a mechanism similar to that seen in melanocytes 13,73. Several questions remain about the chemistry behind the protection offered by vitamin E, and it is important to establish whether similar protection levels can be demonstrated in human skin.
An ideal sunscreen protects against UVR-induced, direct and indirect DNA damage, together with oxidative stress, and erythema. Our data, and those from other groups, support the addition of antioxidants to sunscreens and after-sun preparations. Although relatively small, our demonstration of ‘dark’ CPD formation in keratinocytes, in the absence of melanin, indicates that such preparations should also contain agents which are both antioxidants and, for completeness, triplet state quenchers to decrease the formation of CPD in skin, which continues even after sunlight exposure has ended.

Figure 3 shows the induction and repair of CPD. The delayed, secondary increase, peaking at 0.5–1 h after the end of the UVR exposure, is very similar to that reported by Premi et al. for ‘dark’ CPD. Whilst it is not possible to distinguish between ‘dark’ CPD and the formation of those from direct UVR absorption (‘light’ CPD), we speculate that CPD burden at time 0 h primarily represents the latter and that the peak at 0.5–1 h represents the addition of ‘dark’ to ‘light’ CPD. We further speculate that there are two overlapping CPD repair kinetics in Fig. 3. The dashed line from 0 to 2.5 h, and continuing to the dotted line, mainly reflect the typical NER kinetics of ‘light’ CPD that is relatively slow with a half-life of 33.3 h in human epidermis in vivo, but the rapid decline from the peak to the 3 h timepoint suggests a different, faster repair process for the ‘dark’ CPD, more akin to the kinetics of the pyrimidine (6–4) pyrimidone photoproduct, with a half-life of 2.3 h in human epidermis in vivo. Although fast repair of CPD, under particular conditions, is not without precedent. The reasons for these differences remain to be elucidated. ‘Light’ and ‘dark’ CPD may have different preferential nucleobases locations or properties that differentially activate NER. For example, ‘dark’ CPD are reported to include a higher ratio of cytosine-containing (T < > C and C < > T), to thymine-thymine CPD and cytosine-containing CPD are more rapidly repaired. It is clear that ‘dark’ CPD remain an intriguing phenomenon, and about which there is no doubt much more for us to learn.

Materials and Methods
Cell culture and vitamin E treatment. The HaCaT cell line (spontaneously immortalized keratinocytes) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (Sigma-Aldrich, Poole, UK), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) and maintained at 37°C in 95% air/5% CO₂. Vitamin E (D-α-tocopherol; Sigma-Aldrich, Poole, UK) concentration was fixed at 1 μM and a number of caveats need to be considered. Specifically, oxidizing species other than ROS may also oxidize keratinocytes, in the absence of melanin, indicates that such preparations should also contain agents which are both antioxidants and, for completeness, triplet state quenchers to decrease the formation of CPD in skin, which continues even after sunlight exposure has ended.

Irradiation and dosimetry. The UVA source used was a UVASpot (400/T, Dr K Hönle UVTechnology, Munich, Germany), the spectrum of which is shown in Fig. 7, and described in Table 2. The erythemal effective energy (EEE) was calculated using the erythemal action spectrum of the International Commission on Illumination (CIE). Irradiance was determined with an International Light IL 442 A radiometer (Newbury Port, MA, USA) with a UVA detector calibrated against the measurements made with a double-monochromator spectroradiometer (Bentham Instruments, Reading, UK), which was calibrated against a UK national standard. Experiments to exclude any sunscreening effect of vitamin E were carried out with UVC (254 nm) using an XX-15s UV Bench Lamp (UVP, Cambridge, UK).

Spectroscopy. UVR absorbance of vitamin E (0.1 mg/mL in ethanol) was determined with a UV/Vis Spectrophotometer (ATI Unicam, UK) between wavelengths 250–300 nm, to assess for a possible sunscreening effect. An overlap between the emission spectrum of the UVAspot and the vitamin E absorption spectrum can be observed in the 300–320 nm UBV region (Fig. 7).

Cell viability. Twenty-four hours after UVA exposure or vitamin E treatment, cell viability was determined using both the trypan blue exclusion assay, and the MTT assay, to ensure the absence of any significant cytotoxicity. Trypan blue (Sigma-Aldrich, Poole, UK) (0.04% final concentration) was added to cell suspensions and cells were counted in a haemacytometer. For the MTT assay, aliquots (20 μL) of MTT solution (10 mg/mL PBS) were added to the cells, and incubated for 4 h at 37°C. After this, 100 μL of lysis solution (0.04 M HCl in absolute isopropanol) were added, and the cells shaken for approximately 10 min. The plates were subsequently read on a plate reader at 550 nm.

Measurement of oxidizing species. We aimed to evaluate total intracellular ROS generation was detected using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA; Invitrogen, Paisley, UK). The reliability of this approach to measure intracellular H₂O₂ and ROS has been called into question, and a number of caveats need to be considered. Specifically, oxidizing species other than ROS may also oxidize carboxy-H₂DCFDA to form a fluorescent product. We acknowledge this caveat and use the term “oxidizing species” accordingly.
After UVA exposure, 2 mL of 5 μM carboxy-
H 2DCFDA, diluted in PBS (containing 1 g/L glucose; Gibco) were added to cell suspensions (PBS-only was added in control samples). Cells were incubated for 20 min, in the dark, at 37 °C in a humidified incubator with 95% air/5% CO₂. Plates were subsequently washed twice with PBS to completely remove any dye not internalized by the cells.

For the pre-UVR vitamin E incubation experiments, cells were analyzed immediately following irradiation. Following trypsinisation, cells were centrifuged at 400 × g for 4 min at 4 °C and resuspended in 0.5 mL of PBS + 0.1% (w/v) bovine serum albumin (BSA). Samples were then transferred to FACS tubes and analyzed with a Becton Dickinson FACSria II instrument (BD Biosciences, San Jose, USA), using the FL1 channel (green fluorescence). The viable portion of the cell population was quantified by addition of 2.5 μg/mL propidium iodide (PI) immediately before the analysis. Cells were then subjected to analysis by flow cytometry.

**Total glutathione measurement.** Intracellular concentrations of reduced glutathione (GSH) were determined using the GSH/GSSG kit (Calbiochem, La Jolla, CA, USA). Cell pellets were homogenized in 50 μL of cold metaphosphoric acid (5% w/v) and resuspended in a total volume of 500 μL. The homogenate was centrifuged for 10 min (3,000 × g) at 4 °C, before 100 μL of supernatant was combined with the kit, and analyzed using a UV-vis spectrophotometer.

**Measurement of DNA damage by the comet assay.** DNA damage was assessed using the alkaline comet assay with specific protocol modifications, according to the type of damage investigated. Oxidatively-induced DNA damage was measured using the human 8-oxoguanine DNA glycosylase 1 (hOGG1)-modified comet assay. hOGG1 recognizes 8-oxoGua, together with 2,6-diamino-4-hydroxy-5-formamidopyrimidine, with minimal activity towards 4,6-diamino-5-formamidopyrimidine. On this basis, and as previously, we have used the term ‘oxidised purines’ to describe the damage recognized by the hOGG1-modified comet assay. CPDs were assessed using the T4 endonuclease V (T4endoV)-modified comet assay, as described elsewhere. As we have noted previously, there are no specific data concerning the preferential activity of T4endoV towards the potential combinations of pyrimidines in CPD. However, inferences can be made from the ability of the enzyme to incise at all combinations of CPD, in plasmids and small bacteriophage vectors, suggesting all are equal substrates.

Cells were counted before UVR exposure and distributed in the wells of a 9-well plate (~10⁴ cells per well). After irradiation, cells were collected and centrifuged at 400 × g for 4 min, at 4 °C. The supernatant was discarded and pellets were kept on ice. Cells were mixed with 200 μL of low-melting point agarose and 75 μL of the gel was quickly poured onto slides pre-coated with agarose. Coverslips were placed over the gels, and the slides were kept on a tray on ice for 10 min to allow the agarose to set. Coverslips were then removed and the slides were placed in a tank filled with lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM acid Tris, 1% sodium sarcosinate, pH 10, 1% Triton X-100, and 10% dimethyl sulphoxide) for 16 h at 4 °C, in the dark.

After removing the lysis buffer, slides were washed with ice-cold ddH₂O for 10 min (in the dark, to prevent adventitious DNA damage). Slides were immersed twice in enzyme reaction buffer (40 mM Heps, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8), for 5 min each, at room temperature. T4endoV (0.1 U/mL), or hOGG1 (3.2 U/mL), or enzyme reaction buffer alone, was added to each gel (both enzymes were purchased from New England Biolabs, Hitchin, UK). Coverslips were placed on top of the gels to ensure equal distribution of the enzymes, and slides were incubated at 37 °C in a humid atmosphere for 45 min. Slides were subsequently transferred to ice-cold electrophoresis buffer (NaOH 10 M, EDTA 200 mM, pH 13 in ddH₂O) and incubated for 20 min in the dark. Electrophoresis was then performed for 20 min at 25 V, 300 mA.

Finally, slides were rinsed with neutralization solution (0.4 M Trizma Base, pH 7.5; Sigma) for 20 min and then washed with ddH₂O for 10 min. Slides were allowed to dry at room temperature overnight. DNA was stained using 1 mL of propidium iodide solution at 2.5 μg/mL in PBS per slide for 20 min. Slides were then washed with ddH₂O for 20 min. After drying, slides were examined at a magnification of 40 ×, using a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Germany) equipped with a green excitation filter. Images of the whole of each slide were taken with a Nikon camera linked to the microscope. Fifty randomly selected cells per gel and three
gels per condition were analysed (n = 150) using Comet Score (TriTek Corp., Summerduck, VA, USA). The percentage tail DNA (%DNA) was measured for each nucleoid body scored.

Representative images of comets are shown in Fig. 8.

Statistical analysis. All experiments were conducted in triplicate and values are presented as mean ± standard error of the mean (SEM). Comet assay values were compared for statistical significance with the Mann–Whitney non-parametric test. For the measurement of oxidizing species, a Student’s t-test was used to determine the degree of statistical significance between values from different experimental groups; results were plotted. All analyses and graphs were performed using the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as p < 0.05.

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**Author Contributions**

G.D. performed the experiments, with advice from M.K. G.D., A.R.Y. and M.S.C. wrote the manuscript. All authors contributed to, and reviewed, the manuscript.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing interests.

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