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# Damaging and protective properties of inorganic components of sunscreens applied to cultured human skin cells

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**Abstract**

Titanium dioxide particles are used in sunscreens to reflect UV radiation and are chemically modified to inhibit their natural photocatalytic ability. Titanium dioxide extracted from eight randomly selected commercial sunscreens and three titanium dioxide powders obtained from their manufacturers were investigated and crystal form and modification type of each identified. Under 3.5 mW/cm$^2$ UVA illumination, the type of particle modification and crystal form determined whether the particles were inert or rapidly photodegraded an aqueous dispersion of methylene blue dye (MB), or whether the particles killed or protected cultured human skin cells. Mixed anatase and rutile crystal forms of titanium dioxide coated with organosilane photodegraded MB at a similar rate (11.5 min MB half-life) to Degussa P25, a standard uncoated titanium dioxide powder (11.0 min MB half-life) and generated a 2–4.9-fold increase in cell death. In contrast, pure rutile particles with an alumina coat or manganese doped protected cells from UVA irradiation. This research concludes that mixed anatase and rutile crystal forms of titanium dioxide coated with organosilane or dimethicone may not be appropriate to use in sunscreen lotions.


*Keywords:* Titanium dioxide; Apoptosis; Photocatalysis

## 1. Introduction

Exposure to ultraviolet radiation in sunlight is the major cause of skin cancer [1–3]. To limit sun exposure, the government advises us to cover up with loose fitting, tightly woven clothing, stay in the shade between 11 a.m. and 3 p.m., and use a sunscreen with a sun protection factor (SPF) of 15 or higher [4]. Paradoxically, both skin cancer incidence and usage of sunscreens are rising together [5,6]. Many possible explanations for the paradox have been discussed [7–9], including insufficient application of sunscreens, but the actual reasons remain unknown. Here, evidence is provided for the potential harmful biological consequences of UV light induced chemical reactivity in the materials used in sunscreens, and alternative protective materials are investigated. Sunscreens contain a variety of organic and inorganic UV attenuating chemicals. Several UV absorbing, organic compounds used in sunscreens have been found to have side effects [10–14] including formation of cancer promoting components. Surprisingly, specific forms of titanium dioxide, the most widely used inorganic chemical component in sunscreens, have not been systematically tested for harmful side effects in the published literature [15]. Here, this work shows that some forms of titanium dioxide present in sunscreens have the capacity to cause substantial damage to cultured human skin cells whilst others are protective. The work does not test for a link between skin cancer and sunscreen use. Experiments in cultured epithelial cells, such as here, are necessary to identify potential risks before considering future tests with whole animals.

In the absence of ultraviolet light, titanium dioxide powder is inert and is used in a variety of applications, including toothpaste and foodstuffs. In the presence of UV light $\leq$400 nm, however, titanium dioxide is activated to produce reactive oxygen species such as hydroxyl radicals, superoxide anion and singlet oxygen [16–18]. Titanium dioxide is the most widely used photocatalyst and is capable of destroying virtually any organic matter. This property has many applications, as in self-cleaning glass, water

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and air purification, and antibacterial coatings [19–21]. The UV radiation present in sunlight is able to activate titanium dioxide, so it is surprising that it has been used in cosmetics and sunscreens. The justification is that titanium dioxide is a superior UV reflector due to its high refractive index and that the majority of the material is thought to lie on the uppermost part of the skin (in contrast to organic sunscreen chemicals that are absorbed into the lower layers of skin). We note, however that titanium dioxide can reach living skin cell layers [22]. There are two commonly found crystal forms of titanium dioxide: anatase and rutile. Rutile has a refractive index of 2.90 and anatase 2.49 [23]. Recent work has shown that, for titanium dioxide powders, the percent reflected versus absorbed light varies from 80:20 at 390 nm to 20:80 at 290 nm [24], which means that a significant amount of the UV in sunlight is absorbed by the powders rather than reflected; the absorbed UV can promote free radical formation.

Titanium dioxide particles used in some sunscreens are modified with inert coatings or are doped with specific ions, presumably in an effort to prevent free radical damage to skin cells. These modifications include coatings based on silicon oxides (silica), silicones and organosilanes, coatings based on aluminium oxides (alumina), and doping with manganese ions. The labels on sunscreens generally do not indicate which crystal forms of titanium dioxide are present or what types of coating are used.

Two sources of titanium dioxide particles were tested—commercially available particles of known crystal form and coating type and particles isolated directly from sunscreen lotions. A human skin cell line and other animal epithelial cell lines that are recognized as suitable for epithelial cell studies were used to determine the effects of these UVA irradiated titanium dioxide particles. In the presence of UVA irradiation ($\lambda = 315$–$400$ nm), this research found that some titanium dioxide particle types from both sunscreen extracted and manufactured sources are active photocatalysts that can damage cultured human skin and other animal epithelium cells, whilst other particle types from both sources are protective.

## 2. Materials and methods

The directly obtained, commercially available micronised powders sample-3, -9, -10, -d and -e were obtained from five different manufacturers. Most of these inorganic powders are coated or doped and are used in the manufacture of some sunscreens. Degussa P25 (sample-1, uncoated titanium dioxide) was supplied by Degussa Ltd. Titanium dioxide powders were serially solvent extracted or washed (acetone, chloroform, isopropanol, hexane) from 6 different sunscreens (sample-2, -4, -5, -6, -7 and -8) purchased from high street stores. Three mixed zinc oxide/titanium dioxide powders (sample-a, -b and -c) and one zinc oxide-only powder (sample-f) were also washed from different sunscreens.

### 2.1. Physical characterisation of the titanium dioxide particles

The crystal phase composition of the samples was determined using a Siemens D5000 X-ray diffractometer with a monochromated Cu K$\alpha$1 radiation source ($\lambda = 1.5406$) in the reflection mode over the range $20^\circ < 2\theta < 70^\circ$. Samples were placed on amorphous carbon coated copper TEM grids for imaging with a JEOL 100 CX microscope. Elemental composition of the powders was recorded with Electron Dispersive X-ray Analysis (Phillips XL30 ESEM). X-ray photoelectron spectroscopy using a VG ESCALAB 220i XL imaging spectrometer equipped with a monochromatic Al K$\alpha$ X-ray source was used to determine the amount and composition of the coating substance on the particles. High-resolution solid-state $^{13}C$, $^{29}Si$ and $^{27}Al$ NMR were recorded with a MSL300 (Bruker) with 7.05 T wide-bore magnet at 75.5, 59.6 and 78.2 MHz, respectively.

Two different approaches were taken in order to examine these coated powders. Firstly the chemical photocatalytic testing involved the degradation of methylene blue dye in the presence of the powders and UVA light (Section 2.2). This technique has previously been used to test the photocatalytic strength of titanium dioxide powders [25,26]. Secondly, biological testing of cultured epithelial cells (MDCK-1, PtK2 and HaCaT) to determine extent of apoptosis under similar treatment (Section 2.3).

### 2.2. Photocatalysis procedure

Titanium dioxide loading of 1 g/dm$^3$ with $1 \times 10^{-4}$ M methylene blue in water was used in these experiments. The suspension was stirred overnight in order to ensure adsorption equilibrium between the solid and the liquid. A 10 cm$^3$ aliquot was transferred to a 6 cm diameter petri-dish to be irradiated under air-equilibrated conditions whilst stirring vigorously in order to maintain the powder dispersion. The controls were methylene blue without titanium dioxide under UV irradiation and methylene blue with titanium dioxide without UV. The irradiation was carried out using an 8 W BLB light (maximum emission at $\lambda_m = 365$ nm) with an irradiance of 3.5 mW/cm$^2$ at a distance of 5.5 cm from the surface of the reaction mixture. Three milliliters aliquots were taken after 15-min time intervals, centrifuged at 3400 rpm for 5 min to remove the solid, and the solution analysed using a Helios single beam UV–vis spectrophotometer. This method was used to measure the variation in methylene blue concentration in each degraded sample at 660 nm. The decrease in absorption over time was then related to the photocatalytic ability of that particular sample.

### 2.3. Cell culture

All cell lines originated from the American Type Culture Collection, which is a standard repository for studying cell lines. The epithelial cell line MDCK-1 (dog kidney epithelium) used throughout this study was maintained at 37 °C/10% $CO_2$ in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 1% (v/v) penicillin/streptomycin and 10% (v/v) fetal bovine serum. The cells grew as confluent monolayers on 13 mm coverslips and were seeded at a density of $5 \times 10^4$ cells/ml for 36 h prior to the experiments. The PtK2 (potoroo kidney epithelium) and HaCaT (human skin epithelium) cell lines were maintained at 37 °C/5% $CO_2$ in DMEM.

### 2.4. Apoptosis assay

The biological effect of the titanium dioxide powders was investigated by quantifying the amount of apoptosis induced in cells. A monolayer of MDCK, PtK2 and HaCaT cells were used as in vitro models of simple epithelia. A 1 (or 3) mg of the titanium dioxide powder was suspended in 750 μl chamber media and added as a monolayer to the coverslip of cells on an area of 7.07 $cm^2$. To approximate (derived from first principles, explained in Sections 3 and 3.1) the effects of being in the sunlight, the cells were irradiated with 365 nm (UVA) light for 75 min, adding 150 μl of media every 30 min to account for evaporation losses which we tested empirically. After irradiation, the media was removed and replaced with 1.5 ml DMEM and incubated for 3 h at 37 °C/10% $CO_2$ (MDCK) and 5% $CO_2$ (PtK2 and HaCaT).

To stain for F-actin and DNA, the cells were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, rinsed with 0.1% Triton X-100 in PBS (PBSTx), permeabilised for 10 min with 0.5% Tx, rinsed with PBSTx, blocked with Antibody Diluting Solution (Abdil) for 20 min, stained with 0.1 μg/ml phalloidin 594 for 20 min, washed with PBSTx and finally stained for DNA with 1 μg/ml Hoescht 33342 in Abdil for 2 min. The coverslips were mounted onto glass slides and florescence microscopy used to view the stained cells. Florescence micrographs were obtained using a Nikon eclipse e800 microscope and captured using a SynSys cooled charge-coupled device (CCD) camera (Roper Scientific).

### 2.5. Caspase inhibitor assay

MDCK cells were incubated with 8 μM Zvad-FMK for 1 h then tested with Degussa P25 as above and incubated or 3 h in the presence of the inhibitor.

### 2.6. DNA damage assay

MDCK cells with sample-3 and -9 were irradiated as above. Immediately after irradiation, cells were fixed in methanol at −20 °C for 5 min, rinsed with PBS, washed with 0.1% Triton X-100 in PBSTx, permeabilised for 10 min with PBS-0.5% Tx, rinsed with PBS-0.1% Tx and blocked with Abdil for 20 min. The primary antibody, *ms antiphosphohistone 2AX* was added at a concentration of 9.3 μg/ml for 45 min. The cells were washed, 30 μg/ml of the secondary antibody, *FITC-goat-anti-mouse* administered for 30 min then finally stained for DNA.

## 3. Results

X-ray diffraction was used to identify crystal form, and X-ray line broadening to ascertain the crystallite size of the isolated titanium dioxide particles. To identify the coat-type, X-ray photoelectron spectroscopy, solid-state nuclear magnetic resonance ($^{13}C$, $^{29}Si$ and $^{27}Al$) and combustion microanalysis (see Table 1) were used. Coat-type was accurately identified as all methods gave comparable results. Importantly, several tests showed that the coated particles isolated from sunscreens retained an undamaged coat after organic-solvent-washing (see Section 2 for details): such washing did not alter subsequent identification of the respective coat-types or introduce holes, discontinuities or shelling of the coating detectable by electron microscopy. Moreover, X-ray photoelectron spectroscopy showed the presence of aluminium or silicon atoms on the surface of the washed particles. It was also found that organosilane coated particles supplied directly from the manufacturer and those subjected to the washing procedure showed the same activity in both chemical and biological tests. As expected, in the presence of UVA those titanium dioxide particles that we subsequently identified as damaging, catalyzed the production of free radicals including the superoxide anion and hydroperoxy radical as detected using powder EPR (A. Rampaul, I.P. Parkin and D.M. Murphy, unpublished work).

Before systematically testing any potential protective or damaging properties of the titania particles, the purity of the coated particles was determined, and appropriate conditions for laboratory tests devised. The isolated powders from sunscreens comprised 61–92% coated-titanium dioxide particles (Table 1), as revealed by elemental analysis and energy dispersive X-ray analysis; the remainder comprised known inert inorganic compounds such as magnesium oxide. In order to compare individual powders, the concentration of total powder tested was normalised such that the mass of titanium dioxide particles within the different powders was equal. To devise appropriate laboratory tests, the amount of titanium dioxide was considered within sunscreen lotions, and assumed a moderate exposure of human skin to sunlight (as described further below). The recommended sunscreen application that is used to determine its SPF value is 2 mg/$cm^2$ of skin. In Europe, up to 0.5 mg/$cm^2$ of titanium dioxide is allowed in sunscreens [27]. Consequently, various particles were tested at less than the recommended amount—0.1 and 0.4 mg/$cm^2$.

### 3.1. Photocatalytic activity

The UVA lamp (365 nm monochromatic) had a fluence rate of 3.5 mW/$cm^2$, and is an appropriate substitute for UVA occurring in natural sunlight; it most closely approximates the natural UVA irradiance during early to mid summer/autumn (average 3.7 mW/$cm^2$) in the UK [28]. In a direct test for the appropriateness of the experimental conditions, an anatase/rutile crystal mix, organosilane-coated particles isolated from sunscreen-2 (Table 1) were selected. In an experiment conducted outdoors, it was found that in an aqueous dispersion of these particles and 75 min exposure to natural sunlight during a sunny day in September, 52°N (London, UK), methylene blue was degraded (photobleached), with a near identical pseudo first order rate of reaction (Fig. 1A, ● -solid line) and similar rate constant of 0.0298 $min^{-1}$ to the same test performed in the laboratory with the UVA-lamp (Fig. 1A, ■ -solid line) with a rate constant of 0.0265 $min^{-1}$.

By 75 min, sunscreen-2-derived coated-titania particles in the presence of natural sunlight outdoors had degraded 88% of methylene blue whereas these particles in the presence of the UVA lamp had degraded 86%. As expected, both UV light and

Table 1
Properties of modified titanium dioxide particles

| Sample | Crystal form | Coating | Crystallite size (nm) | MB (half-life) (min ± 1.1 min) | % $TiO_2$ | Surface area ($m^2$/g) |
|---|---|---|---|---|---|---|
| 1 | 85% A<br>15% R | Uncoated | 30 | 11.7 | 100 | 45 |
| 2 | 90% A<br>10% R | Tri-<br>Methoxycaprylylsilane | 17 | 12.5 | 83 | UK |
| 3 | 85% A<br>15% R | Tri-<br>Methoxycaprylylsilane | 21 | 13.9 | 92 | 50 |
| 4 | 85% A<br>15% R | Dimethicone | 11 | 35.4 | 87 | UK |
| 5 | 75% A<br>25% R | Tri-<br>Methoxycaprylylsilane | 23 | 40.5 | 80 | 13 |
| 6 | 100% R | Aluminium oxide and Silica | 16 | 75.2 | 66 | 78 |
| 7 | 100% R | Aluminium stearate and dimethicone | 17 | >120 | 73 | UK |
| 8 | 100% R | Aluminium oxide | 16 | >120 | 61 | 14 |
| 9 | 100% R | Aluminium oxide | 10 | >120 | 91 | 40 |
| 10 | 100% R | Manganese doped | 14 | >120 | 87 | 20 |
| 11 Silica | – | – | 30 | >120 | 0 | 40 |
| 12 Control | – | – | – | >120 | – | – |
| a | 90% Zn<br>10% R | Uncoated | 20<br>16 | 11.3 | 67[a] | UK |
| b | 30% Zn<br>70% R | Aluminium oxide | 25<br>17 | 14.2 | 67[a] | UK |
| c | 80% Zn<br>20% R | Aluminium oxide | 28<br>16 | 23.2 | 70[a] | UK |
| d | 100% Zn | Uncoated | 14 | 26.1 | 100 | UK |
| e | 100% Zn | Dimethicone | 27 | 75.3 | 100 | UK |
| f | 100% Zn | Dimethicone | 27 | 76.2 | 85 | UK |

A: anatase; R: rutile. Samples 1, 3, 9, 10, d, e are directly from the manufacturers. Samples 2, 4, 5, 6, 7, 8, f are isolated from sunscreens. UK: Surface area measurement unknown; Zn: zincite (zinc oxide).
[a] Total percentage of Zn + Ti.

titanium dioxide are needed for the photodegradation to occur (Fig. 1): in either the absence of titanium dioxide under UV irradiation (Fig. 1A, ■ -dashed line) or the presence of titanium dioxide without irradiation (Fig. 1A, ● -dashed line), there was no measurable degradation of methylene blue.

As a baseline, the widely used, uncoated, Degussa P25 was found to be the most potent titania particle-type tested, rapidly degrading methylene blue (Fig. 1B, ▼ -solid line, rapid decrease in $C/C_0$) with a half-life of 12 min (Table 1, sample-1). Strikingly however, two anatase/rutile crystal mixtures, rich in anatase, organosilane-coated types of titanium dioxide, one directly obtained from manufacturers (sample-3) and one isolated from a sunscreen (sample-2) were similarly potent, degrading methylene blue with analogous half-lives (13–14 min, Table 1, Fig. 1B, + and ■ -solid lines). It is worth noting that the powders obtained directly from the manufacturers (sample-3, -9 and -10) were used in their original form, so it is clear that the organosilane coated titania (sample-3) is destructive. Other anatase/rutile crystal mix, dimethicone and organosilane coated titanium dioxide isolated from sunscreens (sample-4 and -5) were moderately potent, degrading methylene blue with half-lives of 40 and 35 min, respectively (Table 1; Fig. 1B, × and ▲ -solid lines). In contrast, the rutile crystal form, alumina-coated, or manganese-doped types of titanium dioxide, both those obtained directly from manufacturers as well as those isolated from sunscreen-7 and -8, were hardly active at all (half-lives of over 120 min, Table 1, Fig. 1B, × and △ -dashed lines). It is evident that both crystal form and coating type contribute to photodegradation, as a sunscreen-isolated, titanium dioxide particle of rutile crystal, but silica coat (sample-6), was intermediate (half-life of 75 min, Table 1, Fig. 1B, ● -solid line) between the most destructive and the most protective forms of titanium dioxide.

### 3.2. Surface area

Surface area can be an important criterion in photocatalytic function as catalysis proceeds on the surfaces of materials. The titanium dioxide particles used in this study varied from 20 to 50 nm in size with either a spherical or acicular morphology as determined by TEM. The BET surface area measurements of the most active powders sample-1, -3 and -5 are 45, 50 and 13 $m^2$/g whereas the least active powders, sample-8 and -9 have surface areas of 14 and 40 $m^2$/g. In contrast, one of the moderately destructive powders, sample-6 has a value of 78 $m^2$/g indicating that in this work, the photoactivity does not directly relate with surface area.

### 3.3. Zinc oxide

For similar reasons to titanium dioxide, zinc oxide is also used in sunscreens, particularly in children and babies' formulations. The sunscreen-isolated- zinc oxide alone, with a dimethicone (silicone polymer) coating, as well as titanium dioxide/zinc oxide mixtures of rutile/zincite crystal form with

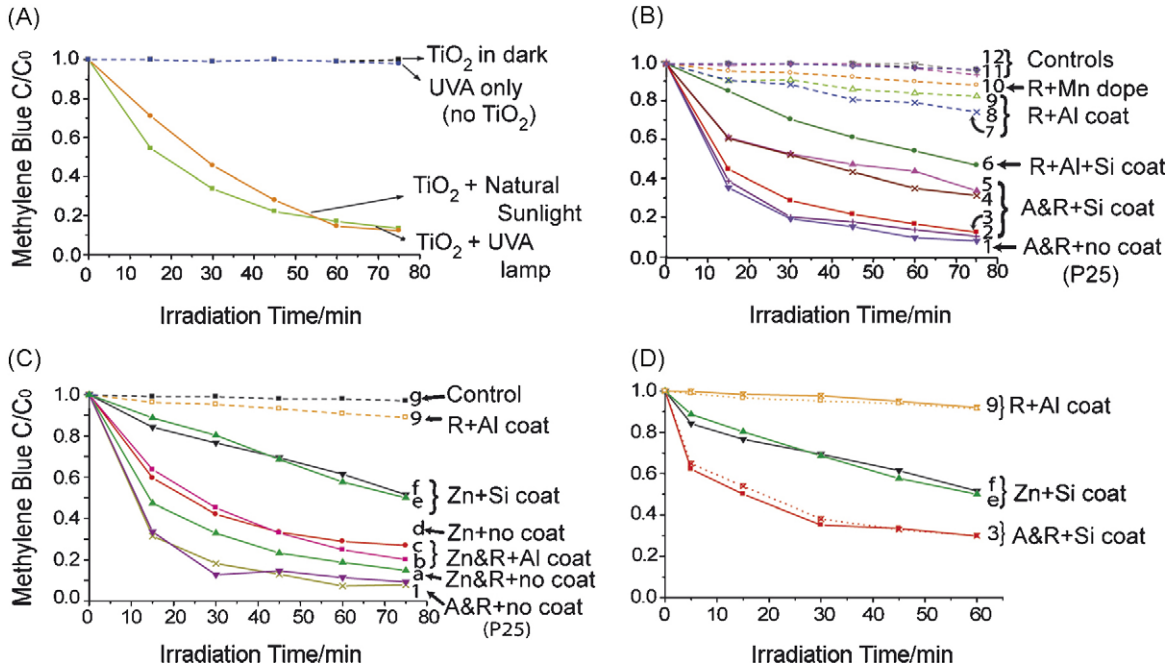


Fig. 1. Photocatalytic properties of coated or doped physical sunscreens vs. the industry standard photocatalyst, Degussa P25. Photodegradation of methylene blue (0.1 mM; $TiO_2$ loading 1 g/dm$^3$) measured by the reduction in concentration of the dye in aqueous $TiO_2$ dispersions by the decrease in the maximum UV absorption at 660 nm. A: anatase; R: rutile. (A) Similar rates of photocatalysis produced by UVA lamp (3.5 mW/cm$^2$; 75 min) and natural sunlight. Solid lines highlight more active samples. (B) All $TiO_2$ powders from both sunscreens and obtained from manufacturers-Anatase & Rutile (A&R) with organosilane coat is the most photocatalytically active. (C) All ZnO and mixed $TiO_2$/ZnO powders showing high methylene blue. (D) Control for extraction process-washed (dashed line) and unwashed powders (solid line) of sample-9 and -3 showing similar rates of methylene blue destruction; $n = 3$ repeat experiments.

an alumina coating, were extremely photoactive: they photobleached methylene blue as rapidly as uncoated-titanium dioxide (Degussa P25) (Fig. 1C, × and ▼ -solid lines). This was in contrast to the rutile form of titanium dioxide with an alumina coating, which was a poor photocatalyst (Table 1, sample-9; Fig. 1C, □ -dashed line). The presence of zinc oxide under certain conditions is known to enhance the photoactivity of titanium dioxide [29] and can also produce free radicals itself [30]. This makes a direct comparison of titanium dioxide particle types alone with those mixed with zinc oxide difficult. However, it remains that the mixed oxides tested in this study were damaging. Zinc oxide was not used in the cellular studies, as in the absence of UV irradiation; the powder changed the morphology of the nuclei under study.

### 3.4. Controls

In addition to earlier tests (above), further evidence was provided to show that the washing process used to isolate titanium dioxide and zinc oxide from sunscreens does not compromise the integrity of the coating. Firstly, two titania samples obtained directly from their manufacturers (sample-3 and -9) were tested with methylene blue. Portions of these samples were also put through the solvent wash procedure and photocatalytically tested. All washed samples showed near identical degradation rates to unwashed samples (Fig. 1D, sample-3, ■ -solid line and □ -dashed line; sample-9, □ -dashed line and ■ -solid line). If the coatings had been damaged, more potent photodegradation would be expected for the washed samples. Secondly, dimethicone-coated zinc oxide, sample-e (Fig. 1D, ▲ -solid line) obtained directly from its manufacturer is known to be the sole active ingredient in sunscreen-f (Fig. 1D, ▼ -solid line). Both showed similar rates of degradation of the methylene blue dye. Taken together these data show that the coating is not compromised by the solvent washing procedure.

### 3.5. Apoptosis assay

To test any damaging effect on cells induced by UVA-activated titanium dioxide particles two separate approaches were used: cellular damage assayed by apoptosis and activation of the DNA-damage-response pathway. Apoptosis is the main form of programmed cell death. A few minutes irradiation with the highly energetic UVC (λ: 100–280 nm) light alone is a standard experimental method to induce apoptosis in cultured cells within 2–4 h post-irradiation, with well-characterised alterations in cell morphology, including nuclear condensation and fragmentation. However, the timing and morphology of apoptotic nuclei induced by the less energetic, more penetrative UVA is less well characterised. With UVA irradiation, the earliest signs of apoptosis were detected in cultured cells around 3 h after 75 min of irradiation with monochromatic 365 nm light, and hence this timing was used in the experimental protocol. Like UVC, UVA induced condensed and fragmented nuclei (Fig. 2A–D). However, both UVA-only and UVA in the presence of titanium dioxide induced highly condensed nuclei that remained non-fragmented (Fig. 2E).

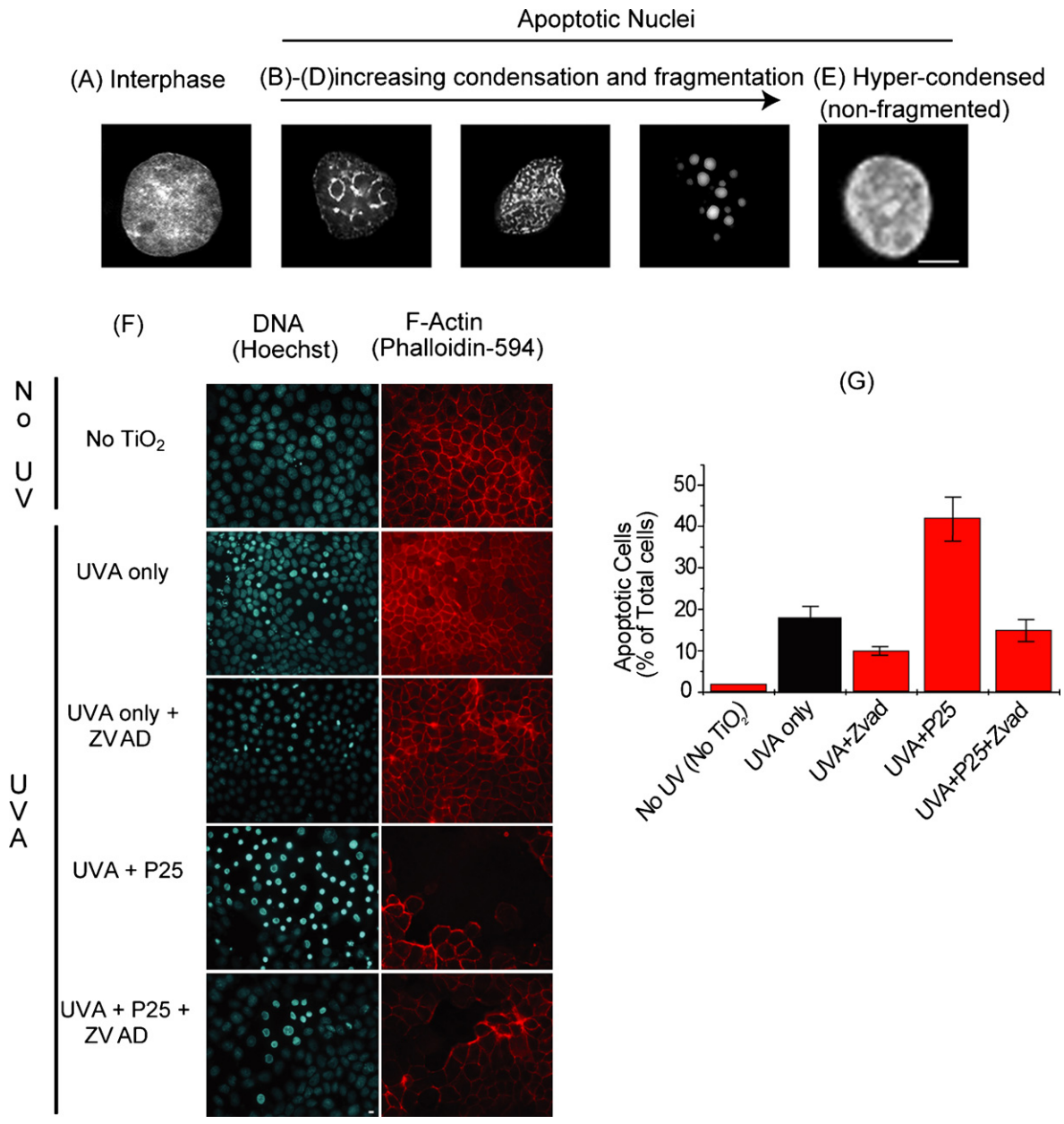


Fig. 2. Different morphology of cell nuclei undergoing apoptosis (A) normal non-apoptotic interphase, (B) early apoptosis, (C) mid-apoptosis, (D) late-apoptosis and (E) hypercondensed. Scale bar (A–D) 5 μm and (E) 3 μm. Hypercondensed nuclei are ~60% smaller than interphase nuclei. (F) Live MDCK cells pretreated with UVA (3.5 mW/cm$^2$; 75 min), Zvad-FMK and Degussa P25 then fixed and stained for DNA and F-actin; $n = 3$ repeat experiments, scale bar = 10 μm. Blocking apoptosis stops formation of hypercondensed nuclei. (G) Quantification of F (error bars are standard error of the mean).

Zvad-FMK, a caspase inhibitor that greatly slows apoptosis, blocked the induction of condensed non-fragmented nuclei, indicating that this nuclear change was caspase dependent (Fig. 2F and G). UVA also induced four other types of physiological changes that occur during apoptosis including cell shrinkage, membrane blebbing and phosphatidyl serine externalisation, a marker of late apoptotic nuclei (data not shown) and reduced F-actin staining in the monolayer (compare, Fig. 2F-UVA only and UVA + P25). In this study, nuclear morphology was chosen as a readily identifiable marker of apoptosis.

UVA-alone induced 10–30% apoptotic cells in the total cell population in individual repeat experiments. This increased up to 90% apoptotic cells with the most destructive titanium dioxide particles, dropping down to 1% with the most protective particles, which was similar to natural apoptosis in the absence of UVA. The 10–30% variation in UVA-only induced apoptosis between experiments is likely due to precise final cell density of cells between individual experiments and also due to natural variations in different batches of sera used to maintain the cells. Importantly however, when comparing the effect on cells of UVA-treated titanium dioxide particles to that of UVA-only in any individual repeat experiment, the increase or decrease in apoptosis was constant, reflected in small standard errors of the mean (see error bars, Fig. 3B for example). In order to compare

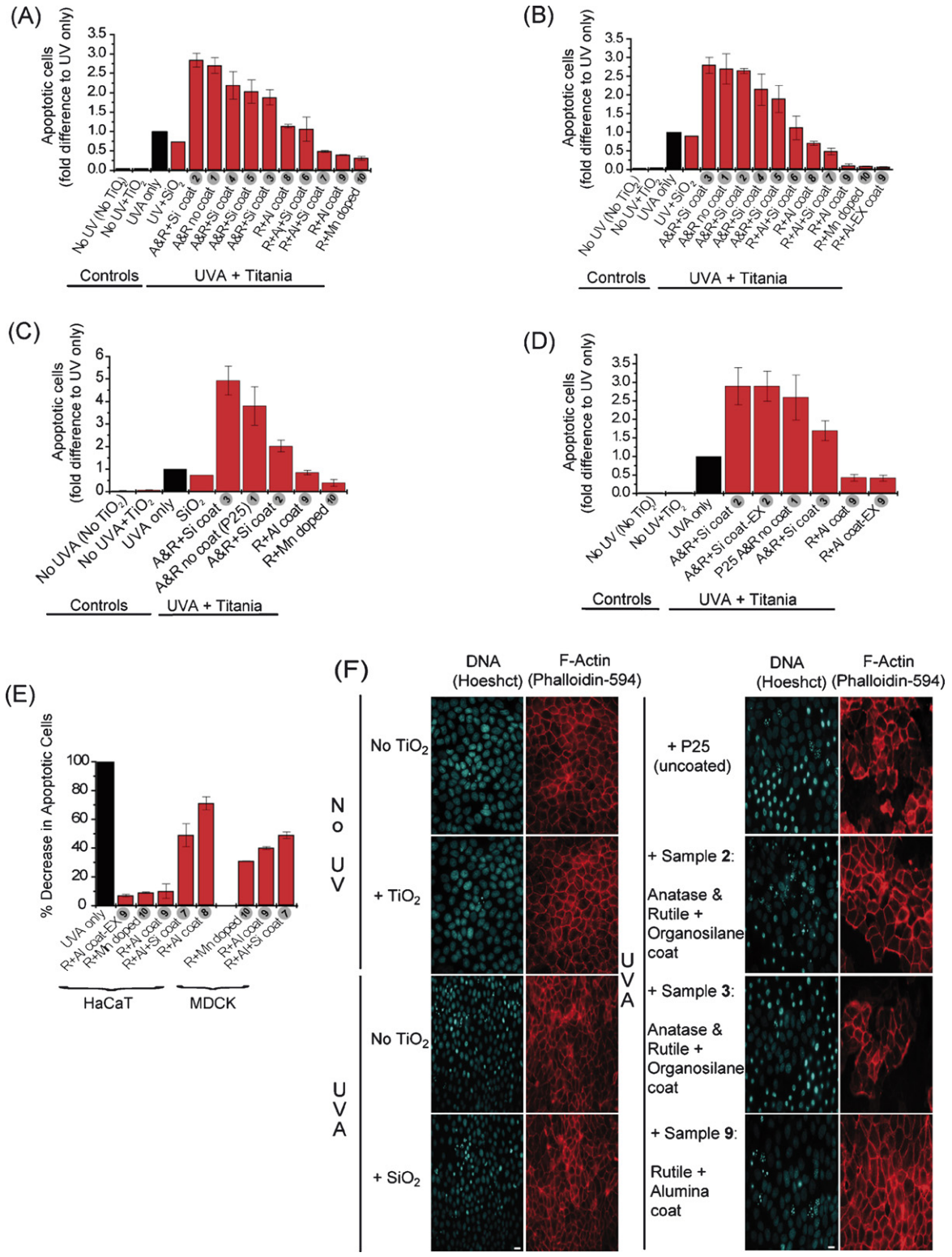


Fig. 3. Distinct UVA-treated titanium dioxide powders induce cell death or provide protection against cell death. (A) HaCaT (human skin cells) pretreated live with UVA and 0.4 mg/cm$^2$ $TiO_2$ then fixed and stained for DNA and F-Actin. (B) MDCK (dog epithelium cells) pretreated live with UVA and 0.4 mg/cm$^2$ $TiO_2$. (C) MDCK cells pretreated live with UVA and 0.1 mg/cm$^2$ of various $TiO_2$. (D) PtK2 (desert rat epithelial cells) pretreated live with UVA and 0.1 mg/cm$^2$ $TiO_2$. (E) Protection effect of those $TiO_2$ powders under UVA illumination that did not induce cell death in (A–D) for both human and animal cells. (F) Representative images of cells pretreated live with UVA and 0.1 mg/cm$^2$ of various $TiO_2$, illustrating controls (left hand panel) and the destructive (increased cell death) (P25, sample-2, sample-3) and protective (reduced cell death) (sample-9) effects of the powders (right hand panel) as compared with UVA-only (left hand panel). MDCK cells are shown (same results for the other cell types). $n = 3$ repeat experiments, 800–1000 total cells count per coverslip. Error bars are standard error of the mean. In each case, the amount of the powder added to cells was normalised to mass of titanium dioxide particle present in the powders, so that in each test the same final amount (0.1 or 0.4 mg/cm$^2$) of $TiO_2$ was added to cells. Scale bar, 10 μm.

the cell lines and individual repeat experiments, the data were quantified to the fold difference in apoptotic cells with respect to the UVA-only control.

In cultured, human skin cells (HaCaT) (Fig. 3A and E) and several other animal, epithelial cell lines (Fig. 3B–F) distinct UVA-treated titanium dioxide particles were either damaging, with a 2–4.9-fold increase in apoptosis above UVA-only (Fig. 3A–D sunscreen-2 and -3), or were protective, with a 2–10-fold decrease in apoptosis below UVA-only (Fig. 3E) or were incapable of inducing further apoptosis above the UVA-baseline (Fig. 3A sample-6 and -8 and Fig. 3B sample-6). The effects are similar across different animal species, for example compare human skin (HaCaT) (Fig. 3A) and dog epithelium (MDCK) (Fig. 3B) cell lines, both tested at 0.4 mg/cm$^2$. One cell line (MDCK) was then chosen to show that even when tested at lower concentration of 0.1 mg/cm$^2$ titanium dioxide was still as potent in destroying cells (Fig. 3C and F), and similarly this was also the case for a third epithelial cell line (PtK2) (Fig. 3D). Increase in apoptosis for the most destructive particles, P25 and sample-3 in the presence of UVA is likely underestimated as dying cells were detected lifting off the coverslip (also evidenced by holes remaining in the monolayer in fixed cells) or being extruded from the monolayer [31]. On the other hand, any protective effect of the titanium dioxide powders (decrease in apoptosis to UVA-only) is accurately represented, as on the day of the experiment there was no change in cell number before and after irradiation.

As observed in the methylene blue tests, uncoated (Degussa P25) and rutile/anatase crystal mix, organosilane and

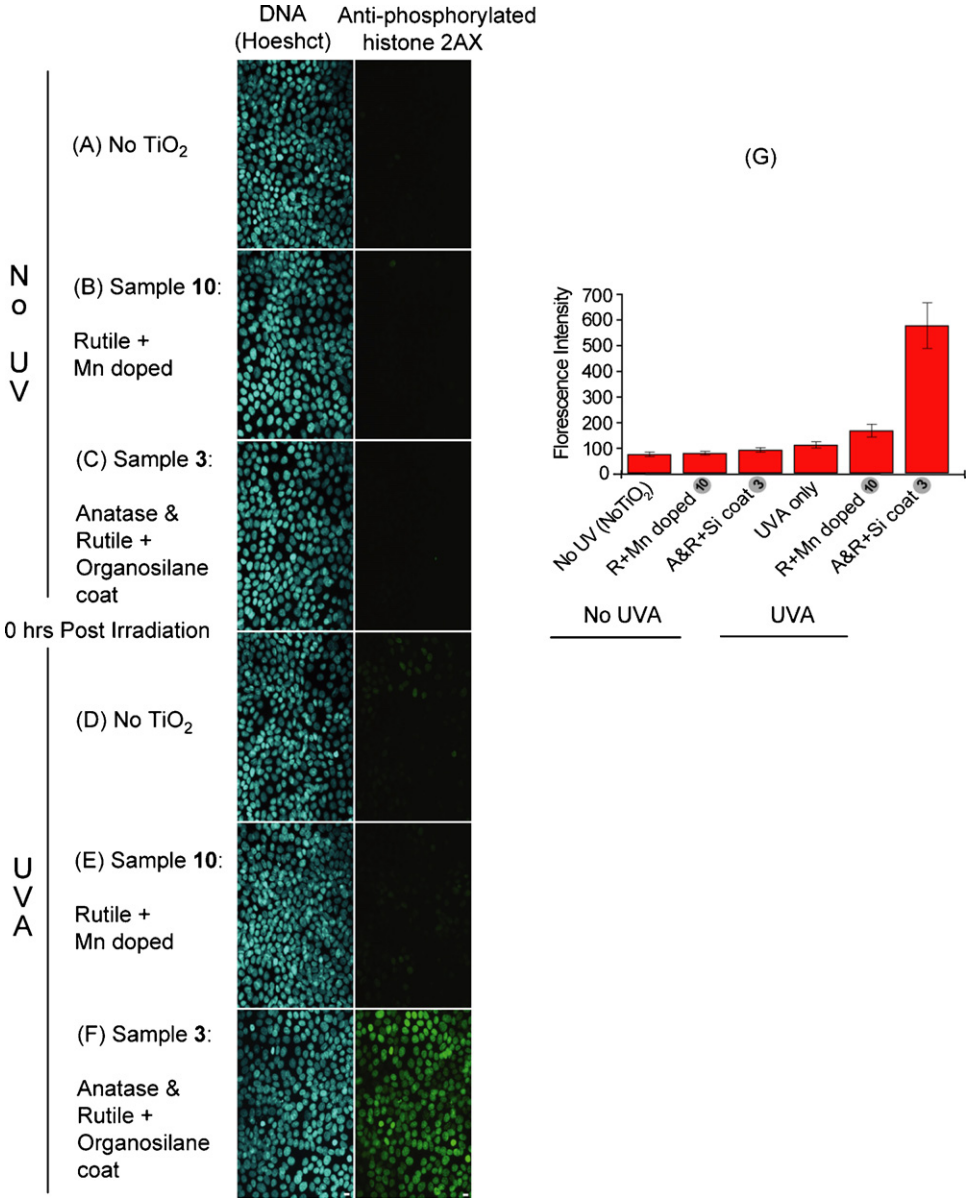


Fig. 4. (A–F) UVA induced DNA double stranded breaks in the presence of titanium dioxide. HaCaT cells were pretreated live with UVA (3.5 mW/cm; 75 min) and 0.1 mg/cm$^2$ sample-10 and -3 then fixed and stained for phoshorylated histone H2AX $n = 2$ repeat experiments. (G) Quantification for (A–F) (error bars are standard error of the mean). Scale bar, 10 μm.

dimethicone-coated titanium dioxide particles (sample-2, -3, -4 and -5) were destructive, whereas the rutile crystal form, either alumina-coated (sample-7 and -9) or manganese-doped (sample-10), were protective (Fig. 3A–D). Also, as with the methylene blue assays, UVA-activated-titania particles themselves were responsible for cell damage, as silica-alone in the presence of UVA, or titania particles-alone in the absence of irradiation had no effect compared with the relevant controls (UVA-only and no irradiation, respectively) (see Fig. 3A and B). As tested in the methylene blue assay, the coating material remained intact after treatment with the solvent-washing procedure; the most protective and destructive titanium dioxide particles obtained directly from the manufacturers, sample-9 and -3, respectively, showed near exact degradation rates as their washed counterparts (Fig. 1D). The solvent extracted powders (labelled R + Al coat-9-EX and A&R + Si coat-3-EX, Fig. 3B and D) had similar effects on cells as the unwashed R + Al coat-9 and A&R + Si coat-3.

### 3.6. DNA damage assay

In a separate approach, distinct titania particles were tested to determine whether they could activate the DNA damage response pathway. A key event in this pathway is phosphorylation of histone H2AX. As this response was rapid (within minutes of damage to DNA occurring) [33] and occurred prior to visible signs of apoptosis, and as the phosphorylation has a short half-life [34], cells were examined for elevated phospho-H2AX immediately after irradiation with UVA prior to the time (75 min post-irradiation), when apoptosis typically occurs in these cells. In the absence of UVA, both titanium dioxide samples were inert and did not activate this pathway in treated cells as measured by the lack of phosphorylation of H2AX (Fig. 4B and C) giving similar background phospho-H2AX to untreated cells (Fig. 4A and G). In the presence of UVA and rutile doped with manganese (Fig. 4E), the DNA damage response pathway was only marginally elevated, comparable to UVA-only (compare Fig. 4D and E; G). Strikingly however, UVA in the presence of anatase/rutile coated with organosilane activates this pathway about five-fold (Fig. 4G). Thus, consistent with the apoptosis findings above, this type of coated titanium dioxide particle was damaging to cells. Finally, Fig. 5 shows that there is a positive correlation between the chemical and biological experiments.

## 4. Discussion

Initial detailed characterisation of titanium dioxide particles and a number of careful controls, for example: determining that methylene blue was totally adsorbed onto the particle surface; that the coat material on the particles was unaltered by the solvent washing procedure; and that the powders did not provide protection by simply physically blocking out UV from cells; means that no technical reason can account for our observations that some titanium dioxide particles are protective and others destructive. Further careful controls showed that the observed effects were specific to UV-activation of particles.

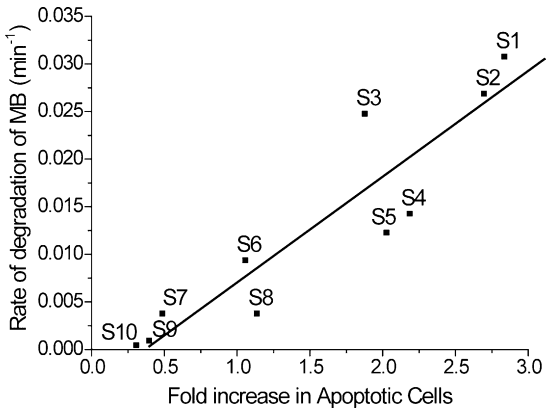


Fig. 5. Direct correlation between the chemical and biological experimentation. Initial rate of degradation of methylene blue dye vs. Fold increase in apoptotic nuclei for human skin cells. $n = 3$, $R^2 = 93\%$; $p < 0.0001$.

From our data then we conclude that there are two major factors in determining the photocatalytic activity of titanium dioxide: crystal phase and chemical modification (either coat-type or dopant). In the presence of UVA in aqueous solution, a mixed anatase and rutile crystal form of titanium dioxide with a dimethicone or organosilane coating is destructive (higher photocatalytic activity). In converse, a pure rutile crystal form with an alumina coat or manganese doped is protective (lower photocatalytic activity). We do not yet know whether crystal from or coat/dopant type is the more important in preventing natural photocatalytic activity of titanium dioxide. To test this requires pure anatase and pure rutile crystal forms, respectively, each with a range of coat-types/dopants. We could not locate an accessible source of such a set of titanium dioxide particles when searching globally for them and also did not identify pure anatase titanium dioxide in the sunscreens we tested. In the absence of any coat/dopant, although rutile titanium dioxide generally has a lower photocatalytic activity than anatase [21], in some cases, rutile has been found to be more photocatalytically active than anatase [35,36]. This difference in photoactivity could be due to the manufacturing process. Overall we would argue that each differently manufactured type of titanium dioxide particle needs to be tested for destructive/protective properties in the context of its use in sunscreens and this information made available to the general public.

Another consideration is surface area, as generally, a photocatalyst with a larger surface area tends to possess a greater activity potential. However when testing this (Table 1) for a selected range of particles, both pure and extracted (highest, lowest and moderate identified activities) showed no trend between surface area and particle photocatalytic activity. For example, sample-6 has one of the highest surface areas at 78 $m^2/g$ but is only moderately active (Fig. 1B; Fig. 3A and B). Apparently then coat-type/crystal form of titanium dioxide is more important than surface area in the context of photocatalytic activity for this particle.

When comparing the methylene blue tests and cellular studies (Fig. 5), remarkably the trends are very similar, arguing that the conclusions made (above) – that anatase/rutile mix crystal form with an organosilane or dimethicone-coat are destructive

and a rutile crystal form with alumina-coat or manganese doped are protective – are generally and widely applicable in both a chemical and biological context. One minor difference between the cellular studies and the methylene blue tests was the presence of inert powders, which had no major effect on the cells, for example, sample-6 (Fig. 3A and B). In the methylene blue studies, this rutile powder with an alumina and silica coat had some photobleaching effect (Fig. 1B, sample-6). It is possible that cells are able to overcome a low level of reactive oxygen species generation through cellular quenching; none the less it is clear that a mixed anatase and rutile crystal form of titanium dioxide with a dimethicone or organosilane coating is destructive to cells.

When comparing cell lines, as when comparing cellular studies with the methylene blue data, again the same trends occur, arguing that the effects are common to a range of animal species. When looking in more detail, comparing specific samples, the precise order of the specific samples is not always exactly the same in all of the cell lines tested. We do not yet know why this is the case, it may for example reflect slight differences in susceptibility to cell death. In contrast to our work where titanium dioxide particles were inactive in the absence of UVA, in one other study [32] titania particles themselves in the absence of UV apparently can cause oxidative DNA damage. However we note that it is not possible to assess if permanent cell damage would result from this oxidative damage as experimental measurements were made prior to when repair pathways in cells could have occurred.

The mixed ZnO/$TiO_2$ powders (Table 1) do not seem to follow the same trends as the $TiO_2$ powders regarding linking crystal form and coat/dopant-type with photocatalytic activity. Nevertheless, the photocatalytic tests show that some mixed ZnO/$TiO_2$ powders can be just as or more destructive than $TiO_2$ by itself. As zinc oxide is a photocatalyst [30] and can enhance $TiO_2$ photocatalytic activity [29], it is not possible in these experiments to determine what contribution the zinc component would have to the overall property (destructive or protective) of the mixed particles. It may turn out for example that the presence of zinc would thwart any protective effect of the alumina-coated rutile counter-part in the mixed particles. More research needs to be done in this area as many sunscreens contain both zinc oxide and titanium dioxide.

It must be emphasized that this work did not set out to address (nor does it prove) whether any particular type of titanium dioxide present in sunscreens can cause skin cancer. However, it clearly provides evidence that the titanium dioxide present in sunscreens can cause significant cellular damage in cultured cell lines, and this has several implications. Firstly, damage to skin tissue may compromise its primary function to act as a barrier to bacterial and viral pathogens and harmful chemicals. In addition, significant cellular damage can in itself activate cancer pathways. Thirdly, as an elevated DNA-damage response was detected in all cells treated with anatase/rutile coated with organosilane, there remains the possibility that a subset of cells escapes apoptosis and pass on mutations to their daughter cells. Our data together with other work [22] showing penetration of titanium dioxide – of particle size similar to that identified in our study – into living skin tissue layers means that these particles have the capacity to cause cell damage in whole animals, though this needs to be directly tested.

As cellular and DNA damage occurred in human skin cells and several other types of animal epithelial cell lines, this work questions whether the anatase/rutile crystal form of titanium dioxide with an organosilane or dimethicone coat, a common titania type identified in sunscreens, is appropriate to use in sunscreen lotions. It also argues that with further study, other types of titanium dioxide, such as manganese doped, or alumina-coated rutile, could potentially be safer alternatives.

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## References

[1] H.J.C.M. Stevenborg, J.C. Van der Leun, Photochem. Photobiol. 51 (1990) 325.
[2] R.B. Setlow, E. Grist, K. Thompson, A.D. Woodhead, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 6666.
[3] E.C. De Fabo, F.P. Noonan, T. Fears, G. Merlino, Cancer Res. 64 (2004) 6372.
[4] Health Protection Agency. http://www.hpa.org.uk/radiation.html.
[5] Cancer Research UK. Cancer Stats: Malignant Melanoma, March 2005.
[6] M. Quinn, P. Babb, A. Brock, E.A. Kirby, J. Jones, Cancer Trends in England and Wales 1950–1999, Studies on Medical and Population Subjects, vol. 66, The Stationary Office, London, 2001.
[7] P. Autier, et al., Int. J. Cancer 61 (1995) 749.
[8] R. Stokes, B. Diffey, Photodermatol. Photoimmunol. Photomed. 13 (1997) 186.
[9] R.P. Gallagher, et al., JAMA 283 (2000) 2955.
[10] H.G. Gonzalez, A. Farbrot, O. Larko, Exp. Dermatol. 27 (2002) 691.
[11] S.T. Butt, T. Christensen, Radiat. Prot. Dosim. 91 (2000) 283.
[12] A. Deflandre, G. Lang, Int. J. Cosmetic Sci. 10 (1988) 53.
[13] J. Knowland, E. Mckenzie, P.J. MeHugh, N.A. Cridland, FEBS Lett. 324 (1993) 309.
[14] J.J. lnbaraj, P. Bilski, C. Chignell, Photochem. Photobiol. 75 (2002) 102.
[15] R. Dunford, A. Salinaro, L. Cai, N. Serpone, FEBS Lett. 418 (1997) 87.
[16] J. Schwitzgebel, J.G. Ekerdt, H. Gerischer, A. Heller, J. Phys. Chem. 99 (1995) 5633.
[17] C.D. Jaeger, A.J. Bard, J. Phys. Chem. 83 (1979) 3146.
[18] R. Konaka, E. Kasahara, W.C. Dunlap, Y. Yamamoto, Free Radical Biol. Med. 27 (1999) 294.
[19] A. Mills, N. Elliott, I.P. Parkin, S.A. O'Neill, R.J. Clark, J. Photochem. Photobiol. A 151 (2002) 171.
[20] M.R. Hoffman, S.T. Martin, W. Choi, D.W. Bahnemann, Chem. Rev. 95 (1995) 69.
[21] N. Serpone, in: E. Pelizzetti (Ed.), Photocatalysis, Fundamentals and Applications, Wiley, New York, 1989.
[22] F. Menzel, T. Reinert, J. Vogt, T. Butz, Nucl. Instrum. Methods Phys. Res. Sect. A 219/220 (2004) 82.
[23] Titanium dioxide. http://www.azom.com.
[24] N. Serpone, A. Salinaro, A. Emeline, Paper presented at the Nanoparticles and Nanostructured Surfaces: Novel Reporters with Biological Applications, 2001.

[25] T. Zhang, et al., J. Photochem. Photobiol. A 140 (2001) 163.
[26] S. Lakshmi, R. Renganathan, S. Fujita, J. Photochem. Photobiol. A 88 (1995) 163.
[27] N.J. Lowe, N.A. Shaath, M.A. Pathak, Sunscreens, Development, Evaluation and Regulatory Aspects, Marcel Dekker, New York, 1997.
[28] A.J. Pearson, et al. National Radiation Protection Board, 2003. http://www.hpa.org.uk/radiation/publications/w_series_reports/2004/nrpb_w61.pdf.
[29] N. Serpone, P. Maruthamuthu, P. Pichat, E. Pelizzetti, H. Hidaka, J. Photochem. Photobiol. A 85 (1995) 247.
[30] L. Jing, Z. Xu, X. Sun, J. Shang, W. Cai, Appl. Surf. Sci. 180 (2001) 308.
[31] J. Rosenblatt, M.C. Raff, L.P. Cramer, Curr. Biol. 11 (2001) 1847.
[32] J.-R. Gurr, A.S.S. Wang, C.-H. Chen, K.-Y. Jan, Toxicology 213 (2005) 66.
[33] E.P. Rogakou, D.R. Pilch, A.H. Orr, V.S. Ivanova, W.M. Bonner, J. Biol. Chem. 10 (1998) 5858.
[34] C. Redon, et al., Curr. Opin. Genet. Dev. 12 (2002) 162.
[35] S.J. Kim, J. Sol–Gel Sci. Technol. 22 (2001) 63.
[36] T. Ohno, D. Haga, K. Fujihara, K. Kaizaki, M. Matsumura, J. Phys. Chem. B 101 (1997) 6415.