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Photogenotoxicity of hypericin in HaCaT keratinocytes: Implications for St. John's Wort supplements and high dose UVA-1 therapy

N.J. Traynor, P.E. Beattie, S.H. Ibbotson, H. Moseley, J. Ferguson, J.A. Woods *

Photobiology Unit, Department of Dermatology, Ninewells Hospital and Medical School, University of Dundee, Scotland, Dundee DD1 9SY, UK

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Abstract

Extract of St. John's Wort (*Hypericum perforatum*) is commonly used as natural remedy for treatment of mild to moderate depression. However, it contains a powerful photoactive component, hypericin, which can cause a severe photodermatitis when eaten by grazing animals (hypericism). In humans, there is evidence that supplementation with St. John's Wort can reduce the minimal erythemal dose (MED) in patients undergoing high dose UVA-1 phototherapy. This is a recent development in phototherapy where the most erythemogenic parts of the UVA spectrum are filtered out, allowing delivery of higher doses of the longer wavelengths of UVA. Although current published evidence suggests that the plasma levels of hypericin are unlikely to cause clinical phototoxicity, it has been established that photoactive compounds can cause DNA damage at sub-toxic and sub-erythemal doses, the effects of which might not be apparent for many years after the event. The present study used HaCaT keratinocytes to investigate the photoclastogenic ability of hypericin on irradiation with UVA. The results show that although the combination of hypericin and UVA light increased the genotoxic burden, when all factors are taken into account, the risk of significant photogenotoxic damage incurred by the combination of *Hypericum* extracts and UVA phototherapy may be low in the majority of individuals.

Keywords: Hypericin; Comet assay; Photogenotoxicity; UVA-1; Photobiology; Phototoxicity

1. Introduction

St. John's Wort (*Hypericum perforatum*) has been valued since mediaeval times for its healing properties. Extracts are very commonly used for the treatment of a diverse range of conditions such as burns, bruises,

* Corresponding author. Tel.: +44 1382 496523; fax: +44 1382 646047.
E-mail address: j.woods@dundee.ac.uk (J.A. Woods).

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swelling, anxiety and mild to moderate depression; they are also photoactive. Hypericin and hyperforin are believed to be among the most potent constituents with regard to photosensitivity and anti-depressive effects, respectively and dietary supplements are generally standardized to contain specified levels of each (Soler et al., 2001). Creams containing *Hypericum* extracts are also available.

Hypericin is such an effective photosensitiser that ingestion of large amounts of St. John's Wort by grazing animals can result in a severe photodermatitis (hypericism) (Nomura et al., 2001; Meunier et al., 2002). This has raised concerns that patients ingesting *Hypericum* supplements or using topical *Hypericum* preparations could be at risk of phototoxicity reactions during phototherapy for skin diseases, as the absorption spectrum of hypericin is broad, with peaks in both the UV and visible parts of the electromagnetic spectrum (Fig. 1).

Studies have shown that hypericin may have a long biological half-life. Oral administration of a 0.75 mg dose of hypericin to healthy volunteers resulted in half-lives for absorption, distribution and elimination of 0.6, 6 and 43 h, respectively (Kerb et al., 1996). Moreover, hypericin is retained in, and cleared slowly from, mouse skin suggesting that skin phototoxicity could potentially be a problem (Delaey et al., 2001). However, in humans, plasma and skin hypericin concentrations after oral supplementation appear to be too low to elicit clinical phototoxicity during phototherapy. There is no widespread reporting of phototoxicity with St. John's Wort despite its extensive use; although there is recent evidence that supplementation can reduce the minimal erythemal dose (MED) in patients undergoing high dose UVA-1 phototherapy as described by Beattie et al., where 6/11 subjects (55%) had a reduced MED, three the same MED and two a higher MED after 10 days of 1020 mg St. John's Wort extract daily.

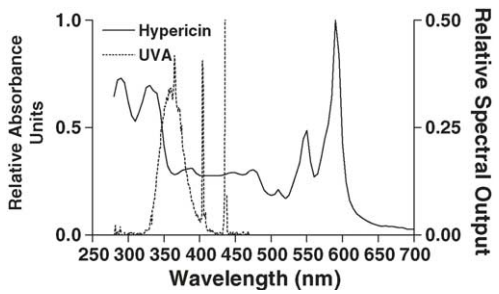

Fig. 1. Spectra. This figure shows the absorption spectrum of hypericin (1 μg/ml) dissolved in ethanol (—), and the relative emission spectrum of the light source after filtering through glass and the plastic lids of the cell culture plates (···).

Although the clinical concern is that *Hypericum* supplementation could result in burning during phototherapy, there may be other risks, such as that of DNA damage caused at sub-toxic and sub-erythemal doses. This sub-clinical damage may not be apparent for many years. The present study used an in vitro human cell culture model (HaCaT keratinocytes) to investigate the clastogenic effect of hypericin when irradiated with UVA. The single cell gel electrophoresis assay (comet assay) was used to measure DNA damage (strand breaks and alkali labile lesions) in individual cells.

2. Experimental methods

2.1. Chemicals

All chemicals and cell culture materials were purchased from Sigma–Aldrich Company Ltd. (Poole, UK) unless otherwise stated. Disposable sterile cell culture plastics were obtained from Costar (Cambridge, UK). A stock solution of hypericin (1 mg/ml) was prepared in dimethyl sulfoxide (DMSO) and further diluted in ethanol (EtOH).

2.2. Cell culture and maintenance

Human HaCaT keratinocytes were kindly provided to the Photobiology Unit by Professor N.E. Fusenig (Heidelberg, Germany). Cells were maintained in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 1% (v/v) non-essential amino acids, 2 mM L-glutamine and 5% (v/v) foetal calf serum (FCS). No antibiotics were used and cells were routinely checked in-house for mycoplasma by staining with Hoechst 33258, and annually by an external testing laboratory (ECACC). Cultures were maintained at 37 °C, 5% CO_2: 95% air in a humidified atmosphere and passaged every 7 days. Cells were seeded the night before experiments at a density of 3×10^4 cells/cm^2. All experiments were carried out under subdued lighting conditions in a specially adapted photobiology laboratory. Ambient light levels were found to be less than 1 lx (Solatell). Incubation with hypericin was in

serum-free medium for 1 h at 37 °C, 5% CO_2: 95% air. Cells were washed with 4 °C phosphate-buffered saline (PBS), PBS added and cells irradiated on ice. Media, ethanol, dark and irradiation-only controls were included. The UVA source used was a bank of two glass-filtered Cosmedico 15500 100 W tubes (Hospital Lamp Supplies, Leicester, UK). The irradiance of the filtered source was monitored with a UV meter (Waldmann, Schwenningen, Germany) calibrated to the source using a double-grating spectroradiometer (Bentham Instruments Ltd., Reading, UK) and was adjusted to 2.8 mW/cm^2 (320–400 nm, centred on 365 nm; Fig. 1). Hypericin accumulation was estimated by spectrofluorometry and expressed as fluorescence units per milligram protein as previously described. Results were expressed as fluorescence units (FU) per milligram of protein (measured by the BCA method).

2.3. Neutral red uptake assay

After irradiation, PBS was replaced with growth media and the cells incubated for a further 24 h. A 0.4% (w/v) solution of neutral red dye was diluted 1:80 in growth medium the night before the experiment and incubated at 37 °C to allow crystals to form. Growth media was removed from the wells and replaced with filtered neutral red dye for 3 h at 37 °C, 5% CO_2: 95% air in a humidified atmosphere. After this time, wells were examined to ensure no crystals had formed. The media was removed from the cells, which were then rapidly washed in a solution of 4% (v/v) formaldehyde containing 1% (w/v) calcium chloride. Intracellular dye was solubilised in a 50% (v/v) solution of EtOH containing 1% glacial acetic acid (v/v). The absorbance was read at 550 nm on a Dynatech MRX plate reader (Dynatech Laboratories, Billinghurst, UK) against a blank of solubilisation solution (Borenfreund and Puerner, 1985).

2.4. Comet assay

The comet assay was performed as previously described (Tice et al., 1990) with some modifications (Woods et al., 1999). Immediately following irradiation, cells were detached from the dishes with 1 ml 4 °C 0.02% ethylenediaminetetraacetic acid (EDTA) for 10 min, 1 ml 4 °C trypsin (0.05%)–EDTA (0.02%) for 10 min, 150 μl 4 °C media followed by gentle scraping. Standard microscope slides were pretreated by spreading 40 μl of 0.3% agarose (Life Technologies Ltd., Paisley, UK) evenly across the slide and allowing them to air dry. After electrophoresis, slides were dried and stained with ethidium bromide (EtBr). From each gel, 50 consecutive nuclei were analysed at a magnification of 250× (Nikon) by image analysis (Komet 3.1, Kinetic Imaging Ltd., UK). Duplicate slides were scored from each sample and each experiment was repeated a minimum of three times. Slides were coded and blinded analysis was performed. Median values of DNA damage in the comet tails from each slide were averaged and the final result expressed as the percentage of DNA damage ± S.E.

2.5. Statistics

Where appropriate, averaged data were analysed using Dunnett's *t*-test (Prism, Graphpad).

3. Results and discussion

Incubating HaCaT cells with 1 μM hypericin for 1 h resulted in a significant uptake of the compound (Table 1). Microscopic analysis revealed a perinuclear intracellular localisation at this dose and at the lower doses. There was no detectable hypericin signal observed in the nucleus. Irradiating the treated cells with

Table 1
Hypericin uptake by HaCaT cells

Incubation (min)	Solvent control	Fluorescence units/mg protein (hypericin, μM)		
		0.1 μM hypericin	0.5 μM hypericin	1 μM hypericin
0	1.8 ± 1.6	1.3 ± 0.4	2.5 ± 0.1	1.7 ± 0.1
30	ND	1.6 ± 0.8	2.5 ± 0.2	3.5 ± 0.4
60	1.1 ± 0.6	2.6 ± 0.1	2.6 ± 0.7	5.3 ± 1.1

Values represent the means ± S.E. of four observations from two independent experiments. ND: not determined.

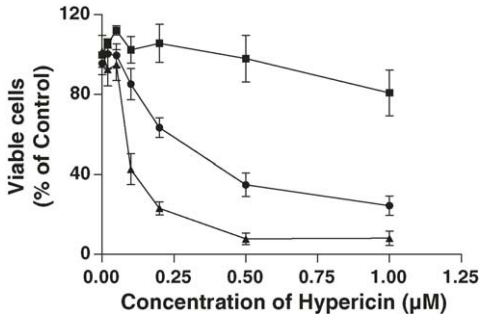

Fig. 2. Phototoxicity of hypericin in HaCaT cells. HaCaT cells were incubated with hypericin for 1 h, and then irradiated with either 0.4 J/cm^2 (●) or 4 J/cm^2 UVA (▲). The sham-irradiated controls are also shown (■). After irradiation, cells were returned to the incubator for 24 h. Phototoxicity was subsequently determined by neutral red uptake as outlined in the experimental procedures. Results represent the mean values ± S.E. of three independent experiments. Viability of UVA-only-treated cells as a percentage of the untreated/dark controls: 110.1 ± 2.9% (0.4 J/cm^2); 97.7 ± 5.9% (4 J/cm^2).

either 4 or 0.4 J/cm^2 UVA resulted in pronounced phototoxicity, with IC_{50} values of 0.06 and 0.36 μM, respectively (Fig. 2).

Irradiating hypericin with 4 J/cm^2 UVA resulted in extensive DNA damage, which decreased as the irradiation dose decreased (Fig. 3). The results suggest a threshold effect, where a sufficient amount of energy must be deposited and/or a sufficiently prolonged triplet state is required to generate enough reactive species to cause DNA damage. DNA damage did not correlate with phototoxicity, as at the respective IC_{50} values of 0.06 and 0.36 μM, the level of damage was approximately 30 and 18%. The occurrence of strand breaks and the apparent absence of a hypericin signal from the nucleus would tend to exclude singlet oxygen as being directly responsible for the detected damage. It is more likely that an intermediate species such as products of singlet oxygen-initiated lipid peroxidation, or the hydroxyl radical produced via a type I photochemical mechanism, are responsible.

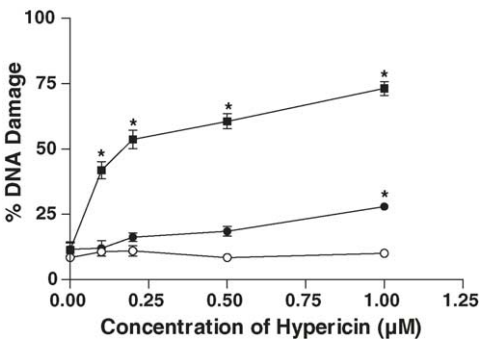

Fig. 3. Photogenotoxicity of hypericin in HaCaT cells. HaCaT cells were incubated with hypericin for 1 h, and then irradiated with either 0.4 J/cm^2 (●) or 4 J/cm^2 UVA (■). The sham-irradiated controls are also shown (○). After irradiation, cells were immediately processed for the comet assay as outlined in the experimental procedures. Results represent the mean values ± S.E. of three/four independent experiments. $^*P < 0.05$, significantly different from negative control. DNA damage for UVA-only-treated cells: 11.5 ± 6.0% (0.4 J/cm^2); 11.5 ± 3.0% (4 J/cm^2); hypericin-only-treated cells: 8.4 ± 1.3%; no treatments: 14.3 ± 0.6% (baseline value).

The comet assay is currently undergoing a formal validation study for inclusion in the test-battery of in vitro genotoxic tests. Recently a report was published by a GUM task force that proposed the comet assay as a method to screen for photogenotoxins, based on the principle that known photogenotoxins are photoclastogenic (Brendler-Schwaab et al., 2004). The present data suggest that most of the DNA damage occurs under conditions that subsequently result in cell lysis. Additionally, the concentration of hypericin detected in blood during supplementation with St. John's Wort extract has been estimated to be around 10 nM, with less being bioavailable to the skin (Staffeldt et al., 1993; Kerb et al., 1996; Schempp et al., 1999). This concentration is therefore 10–100 times less than the IC_{50} values obtained for phototoxicity in the present study. Therefore, any potential risk from genotoxicity is probably low, as in practice, doses of up to 130 J/cm^2 UVA-1 would be administered during a single treatment session. However, the present data, obtained using a UVA source, cannot necessarily be extrapolated to UVA-1. At both doses of UVA, significant DNA damage occurs in cells that do not die in the subsequent 24 h. The fate of these cells and the nature of the DNA damage sustained by them require further investigation. The type of DNA damage measured in the current study (strand breaks) is generally well handled by cells, which are designed to adapt to changing degrees of oxidative attack; we have not explored all the potential forms of damage (e.g. base damage) that could be caused by irradiating hypericin with UVA.

To summarise, the present study shows that combined low doses of UVA and hypericin can result in photogenotoxic damage to human keratinocytes. There are individual and age-related differences in the DNA

repair capacity of skin cells, and reduced DNA repair capacity may be an important risk factor for solar-induced carcinogenesis in susceptible populations.

It remains to be seen whether individuals who are supplemented with oral *Hypericum* extract or use topical *Hypericum* preparations while receiving phototherapy or exposure to solar radiation are subsequently more likely to develop skin cancers, as in vivo, when hypericin is ingested in an extract, the situation may be made more complex by the presence of other polyphenols such as rutin, which is also photoactive, and others that may be photoprotective (Wilhelm et al., 2004).

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