ORIGINAL ARTICLE

Precise determination of the erythema response of human skin to ultraviolet radiation and quantification of effects of protectors

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Summary

Background: We describe highly reproducible methods for quantifying the erythema response of precisely selected areas (spots) of human skin to graded doses of ultraviolet radiation (UVR). These methods have permitted evaluation of the efficacy of protectors, such as sulforaphane from crucifers, that defend cells through induction of cytoprotective (phase 2) genes.

Methods: Spots on the back were precisely located by opaque, adhesive, vinyl templates provided with 16 circular, 2.0 cm diameter occludable windows. Doses (100–800 mJ/cm^2) of narrow-band (311 nm) UVR were administered, and the erythema index (a^*) was measured with a chromometer on treated and control areas, before and after radiation.

Results: Daily variations in basal a^* values of each spot were much smaller than the differences of a^* values among spots of one individual, or those of corresponding spots among different individuals. The increments in erythema responses to UVR (Δa^*) were similar despite large variations of basal a^* of spots. The most appropriate measure of UVR-evoked erythema is therefore the Δa^* value for each spot, which is an independent observational entity. Δa^* was proportional to UVR dose, and independent of spot location. To evaluate effectiveness of protectors against UVR damage we paired horizontally adjacent spots for treatment and controls. Vertical or random spot pairing did not provide significantly higher consistency. Protective efficacy against UVR erythema is appropriately expressed as percent reduction in Δa^* values upon treatment with inducers.

Conclusions: The protection of skin against UVR damage can be quantified precisely from changes in erythema index (Δa^*) obtained with a chromometer.

Extensive epidemiological and biochemical evidence supports the view that ultraviolet radiation (UVR) is the principal contributor to the high and rising global incidence of skin cancers (1), and also plays a major role in aging of the skin. Paradoxically, UV exposure is beneficial in promoting vitamin D biosynthesis. It is also widely used recreationally, and in the phototherapy of skin diseases such as psoriasis. Although erythema is the most easily recognizable effect of UVR on human skin, the precise and repetitive quantitative determination of the erythemic response of selected areas of the skin over time has received limited attention. UVR-evoked erythema arises largely from the inflammation and associated changes in perfusion of the dermal vasculature, but the many associated biochemical, humoral, and cellular processes are only partly understood. Our interest in measuring UVR-evoked skin erythema was motivated by the prospect that precise determinations of erythema could provide a reliable and reproducible index of damage and guide the development of strategies to protect against UVR-induced skin pathology.

This paper describes methods for obtaining highly reproducible repetitive measurements of basal erythema and the response of small, localized regions of human skin to UVR. Conventional methods for determining minimum erythema dose (MED) are inadequate for these purposes. By use of a reflectance instrument, Farr and Diffey (2, 3) established general methods for quantifying the magnitude of erythema of human skin following UVR. Subsequently other investigators have measured the redness of human skin with chromometers (4, 5).

Surprisingly, there is little information on the conditions required for obtaining precise and reproducible measurements over time, on the differences in responsiveness of skin among individuals, or on the variation in basal skin redness and responsiveness to UVR of precisely located skin areas in single individuals. Our goal was to determine the effects of the induction of cytoprotective genes by sulforaphane on UVR-induced skin erythema. This required optimization of methods for obtaining reproducible measurements of erythema and for the statistical analysis of such data. This report describes in detail the experimental conditions required for quantifying the erythemic effects of UVR and for evaluating the magnitude and significance of observed protective effects of intervention. The value of these methods has been recently demonstrated by our study on the protective effects of sulforaphane-rich broccoli sprout extracts against narrow-band UVR (6, 7). Such measurements may be of wider utility for assessing abnormal UVR sensitivity of skin and for evaluating protective methods.

Materials and methods

Clinical studies

Clinical studies were conducted in compliance with ethical and scientific principles and were approved by our Institutional Review Board.

Volunteers

Healthy volunteers, recruited by approved advertising and word of mouth, provided written informed consent. All subjects were light-skinned Caucasians with Fitzpatrick skin phototypes 1, 2, or 3, of both sexes, and ranged in age from 28 to 53 years.

UVR

Narrow band (centered at 311 nm) UVR was delivered to volunteers in a Daavlin Full Body Phototherapy Cabinet (Daavlin, Bryan, OH, USA) equipped with an integrated UVB dosimeter.

Results and discussion

Design of a template for quantifying erythema

The standard device for measuring the MED of UVR is a vest-like nonadhesive cotton girdle with square apertures that can be occluded to control UVR dose exposure. Although qualitatively functional, this device yields diffuse exposure due to variable skin contact, is easily displaced even during slight movements by the subjects, and is difficult to align precisely for UVR and protective treatment at exactly the same positions at multiple time points. We therefore devised an opaque, adhesive, vinyl template (supporting information Fig. S1), with 2.0 cm diameter circular windows that could be occluded individually to adjust UVR doses. These windows were used to align the erythema-measuring chromometer, and to limit the spread of solutions of protective agents that were applied to their centers. The template was positionally aligned reproducibly by means of small registration holes in each corner that were located on the skin with a skin marker. The template remained fixed during slight movements by the subjects, and generated erythema spots with sharply defined margins. Two templates, each with eight 2.0 cm windows about 1.5 cm apart, were placed symmetrically on the mid-back of the chest on either side of the spine about 10 cm below vertebra C7.

Use of chromometry for quantifying skin color

A chromometer (Model CR400 Chroma Meter; Konica-Minolta, Ramsey, NJ, USA) was used to measure the intensity and spectral characteristics of skin reflectivity (8). This instrument is a hand-held, reflectance colorimeter designed to correct for the spectral color sensitivity of the human eye. A xenon arc generates a broad spectrum of visible light, and three independent spectrally-selective photodetectors quantify the fraction of incident red, green, and blue light reflected from the skin. The instrument provides unitless ratios of the intensity of reflected light to that of incident intensity of the xenon arc. Reflected light readings are reported in the $L^*a^*b^*$ system recommended by the Commission Internationale l'Eclairage (CIE). The a^* values represent red–green ratio (red shift, $a^* > 0$). Because skin redness is primarily determined by the presence of hemoglobin and melanin, only a^* values were used to quantify erythema, in agreement with our pilot studies and recommendations of others (2, 3, 9, 10). The a^* value is here referred to as the erythema index. Narrow-band UVR (centered at 311 nm) induces maximal skin erythema in 16–24 h after UVR, and the skin returns to its normal appearance after 3–5 days.

Reproducible determination of changes in skin color

The skin serves many important biological functions including thermoregulation, is under sensitive central and peripheral autonomic control, and is subject to rapid fluctuations in perfusion and consequently color. In order to obtain reproducible determinations of skin erythema, it was therefore necessary to minimize the effects of external factors on skin color during each measuring session. The following protocol was adopted. Subjects were asked to refrain from exercise for several hours before testing. All measurements were made in the same quiet, windowless room in the presence of no more than two observers, and with close control of temperature (23–25 °C). Upon arriving at the clinic, each volunteer was asked to lie in the prone position on the examining table. The skin of the back was exposed and gently wiped with 70% isopropyl alcohol. The arms were abducted and flexed at the elbow with the hands supporting the head, allowing the subject to maintain comfortable balance on the examining table while also providing a relatively flat posterior chest surface for measurement. (A closely similar position of arms was assumed later during UVR treatment in the body cabinet where the subjects were standing.) Each subject was

allowed to rest quietly for 20 min to permit the skin to equilibrate to a normal basal state before measurement. The template was positioned by use of the registration marks generated on the initial visit. The chromometer was placed perpendicular to the skin. The skin was allowed to equilibrate for 20 s under the weight of the chromometer (780 g) before the measurements were started. Eleven repeated readings were then made at each spot in all subjects, at intervals limited by the refractory period of the instrument (about 3–5 s). Room temperature was recorded at the beginning and end of each session. A single operator made all the measurements. On the basis of the a^* values obtained from five subjects, we examined: (1) the reproducibility of repetitive a^* values of single spots as a measure of skin color; (2) the basal variation of skin color among spots of a single subject and among subjects; and (3) methods for quantitative expression of protection against UVR-induced erythema.

Consistency of repetitive measurements of erythema index (a^*)

Repetitive measurements of the erythema index (a^*) of an individual spot at a single session were highly reproducible. Eleven consecutive readings were taken at each spot. Because placement of the chromometer on the skin causes slight physiologic perturbations, readings do not stabilize until after the first two to three readings; we therefore used the mean of the final eight chromometer readings on each spot for analysis. Data for the basal variation in a^* from five subjects were collected on 4 successive days. When analyzed collectively but treated as independent measures, the mean CV of each set of eight repeated basal readings at each spot was 3.79% ($n = 320$), and the CV was 3.33% when analyzed by the random effects model. This indicates that the a^* values obtained for individual spots at any time point are highly consistent and provide more than sufficient precision for the study. Interestingly, the CV of repetitive measurements on 16 individual spots on each of five subjects at 24 h after narrow band UVR (311 nm) was $2.26 \pm 0.19\%$ (SEM; $n = 80$), significantly lower than that observed before UVR ($P < 0.0001$). All a^* values reported in the following studies used only the means of the last eight chromometer readings made on each spot, unless otherwise noted.

Determination of the temporal, spatial, and positional consistency of erythema index (a^*) values under basal conditions

Having evaluated repetitive sequential chromometer readings to establish that a^* values could be reproducibly determined on individual spots, we asked how these values varied over time, between spots, and among individuals. In order to understand the temporal and spatial variations in a^* values under basal conditions, we analyzed a^* values obtained on 16 spots of each of five subjects at 24-h intervals on 4 consecutive days (320 measurements), and interpreted the results with respect to time, location, and subject number. Collectively, all the basal a^* values were normally distributed, with a mean a^* of 4.52 ± 0.11 (SEM; $n = 320$). The basal a^* values varied from 0.59 to 10.17 (over 17-fold) among all spots of all subjects. Notably, the largest range of a^* values observed in a *single* subject during a single visit varied from 1.44 to 9.45 (6.6-fold) (Fig. 1). The reasons for this surprisingly large range of basal erythema a^* values of the spots of a single individual remain unclear. Presumably differences in pigmentation, vascularity, skin thickness, and mosaicism in expression of cytoprotective genes may be contributors to the observed variability.

A random effects model was created into which eight readings per measurement, 4 days of repetitive measurements, 16 spots per subject, and five subjects were factored, giving an overall mean a^* value of 4.52. Using the random effects model, the

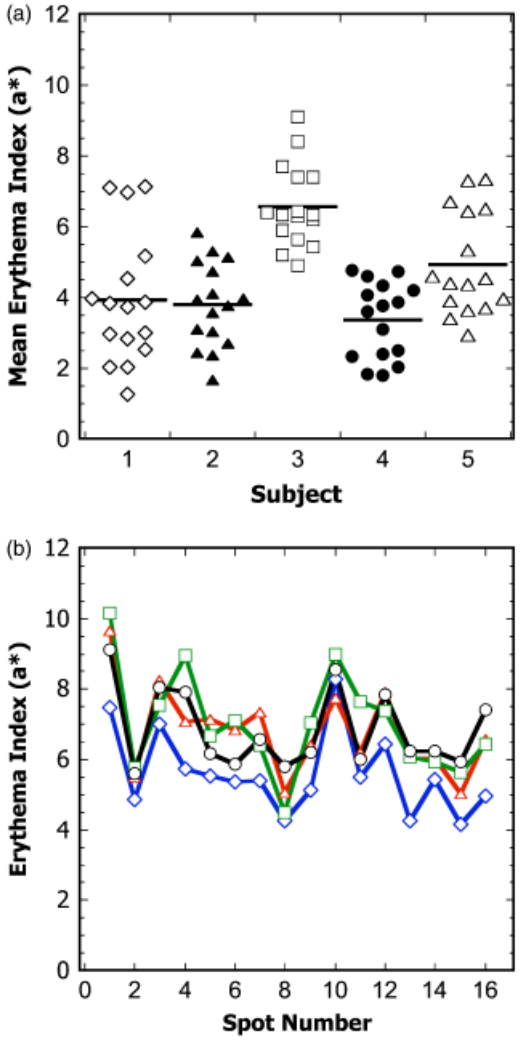

Fig. 1. Basal erythema index (a^*) values. (a) Means of a^* values obtained on 16 spots of each of five subjects on 4 successive days. Each entry represents the mean a^* value of a single spot measured on 4 successive days. The horizontal lines are the mean a^* values for all spots for each subject. (b) Sequential measurements of erythema index (a^*) at 24-h intervals for 4 days of the 16 spots of a single volunteer (Subject 3). ○, first day; □, second day; △, third day; ◇, fourth day.

estimated standard deviations (SDs) were due to temporal variability (SD = 0.80), spatial variability (SD = 1.31), inter-subject variability (SD = 1.09), and multiple chromometer readings (SD = 0.15). Intraclass correlation coefficients were relatively low for the comparison of different subjects ($\rho = 0.333$), moderate for the comparison of day (by subject, without regard to spot number) ($\rho = 0.510$), and very high between subject and spot number ($\rho = 0.817$), and between days of measurement ($\rho = 0.993$). A nested ANOVA (day |spot| subject) was used to evaluate the effects of each variable, and: (a) subject, (b) spot, and (c) day of measurement, were all highly significant ($P < 0.0001$) (see Table 1). The innate variability of subject, spot, and day, is illustrated graphically in Fig. 1. The distribution of basal a^* values among all five subjects varied significantly (Fig. 1a), and might have varied to an even greater degree if we had not utilized only fair-skinned Caucasians since erythema develops more easily in these individuals. The eight readings within each measurement are closely enough clustered so as to be within the confines of the symbols used for plotting. The measurements for a single subject are displayed in Fig. 1b, which illustrates the somewhat greater consistency between four consecutive, daily measurements of individual spots compared with measurement of different spots of the same subject, as previously observed by others (3). In conclusion, there are large local differences in the basal red reflectivity of skin over even very short distances on the same individual and accordingly, to minimize the effects of these factors, each spot of an individual subject must be considered an independent observational unit.

In a prior study, Farr and Diffey (3) observed a decrease in basal a^* values along the back from the cervical to the lumbar/sacral region. Although we noted a similar negative trend in a^* values from the cervical to the thoracic region, this trend was not significant over the more limited region studied by us, and varied greatly from subject to subject. In addition, no significant variation was observed by anatomical position in the coronal plane (i.e., medial vs. lateral). With a better understanding of the basal variation in skin color, we determined the most appropriate methods for quantifying changes in erythema after UVR exposure.

Table 1. Nested ANOVA of basal a^* values (before UVR)

	Statistics		
Factor	F statistic*	P value	Contribution to variance† (%)
Subject	13.9	< 0.0001	36.7
Spot	11.9	< 0.0001	49.4
Day	218	< 0.0001	13.3
Residual‡			0.6

*The F statistic represents a ratio of the variance of a^* values *within* each group to the variances *between* the groups.
†Estimated contribution of each factor to overall variance (from partial sum of squares).
‡Residual term includes variance due to eight repeated chromometer readings.
UVR, ultraviolet radiation.

Analysis of induction of erythema by UVR in human skin: transformation of data

In light of the highly variable nature of the basal a^* values of individual spots even in a single individual, use of absolute a^* values as an index of erythema is inappropriate. Consequently, we normalized the treated measurement for each spot to the basal a^* value determined before UVR. The ratio of a^* values [a^*(post-UVR)/a^*(pre-UVR)] is subject to greater variability because of its dependence on basal a^* values in the denominator. In contrast, the increment in a^* (or $\Delta a^* = [(a^*(\text{post-UVR}) - a^*(\text{pre-UVR})]$) is less subject to variability attributable to basal a^* values, and was therefore the more appropriate transformation. This conclusion is in agreement with Farr and Diffey (3). Consequently, the increment in a^* (Δa^*) was used as the metric for the changes in erythema resulting from UVR for each individual spot. The results of this method of analysis are shown in Fig. 2. The mean a^* values of 16 spots of a single individual averaged over 4 days of observation before UVR (Figs. 2a and d) were compared with the a^* values of the same spots 24 h after treatment with a range of doses of UVR (100–800 mJ/cm^2) (Figs. 2b and e). The increments of a^* values (Δa^*) for each spot are shown in Fig. 2c, and are plotted in Fig. 2f. Trend analysis across all subjects indicates a positive association between the level of UVR and the resultant increment in erythema (Δa^*) in all spots. Linear regression (least-squares) of the UVR-induced increment in a^* (Δa^*) provided the line of best fit ($r^2 = 0.97$; Fig. 2f), for the limited range of UVR doses used in this study. However, for larger UVR doses, S-shaped response curves are observed (3).

Inherent response variability of an individual subject to a single dose of UVR

The variability of erythema response to UVR in a single subject was assessed by measuring the a^* values of all 16 spots of one volunteer on 4 sequential days. After exposure to UVR at 500 mJ/cm^2, the a^* values were remeasured 24 h later. Nonparametric trend analysis indicated a negative association between the mean basal a^* value 24 h before UVR and the observed Δa^* ($P = 0.008$), consistent with the commonly accepted observation that darker skin is more resistant to UVR. Consequently, spots with abnormally high or low basal a^* values may require censoring. In contrast, there was no apparent association between Δa^* and spot location ($P = 0.534$), indicating that Δa^* is a reliable index of erythema across the region selected.

As we proposed to use a paired spot design (i.e., one treated spot paired with one control spot) for protection studies, we next determined the most appropriate pairing of treatment and control spots (i.e., horizontal, vertical, contralateral, or random). The $\Delta\Delta a^*$ [Δa^*(spot A) − Δa^*(spot B)] were calculated for each method of pairing, and are summarized in Table 2. While no pairing method provided a robust statistical advantage, horizontal pairing was experimentally most convenient and has some biological justification; embryological dermatomes develop and are organized in horizontal or transverse sections

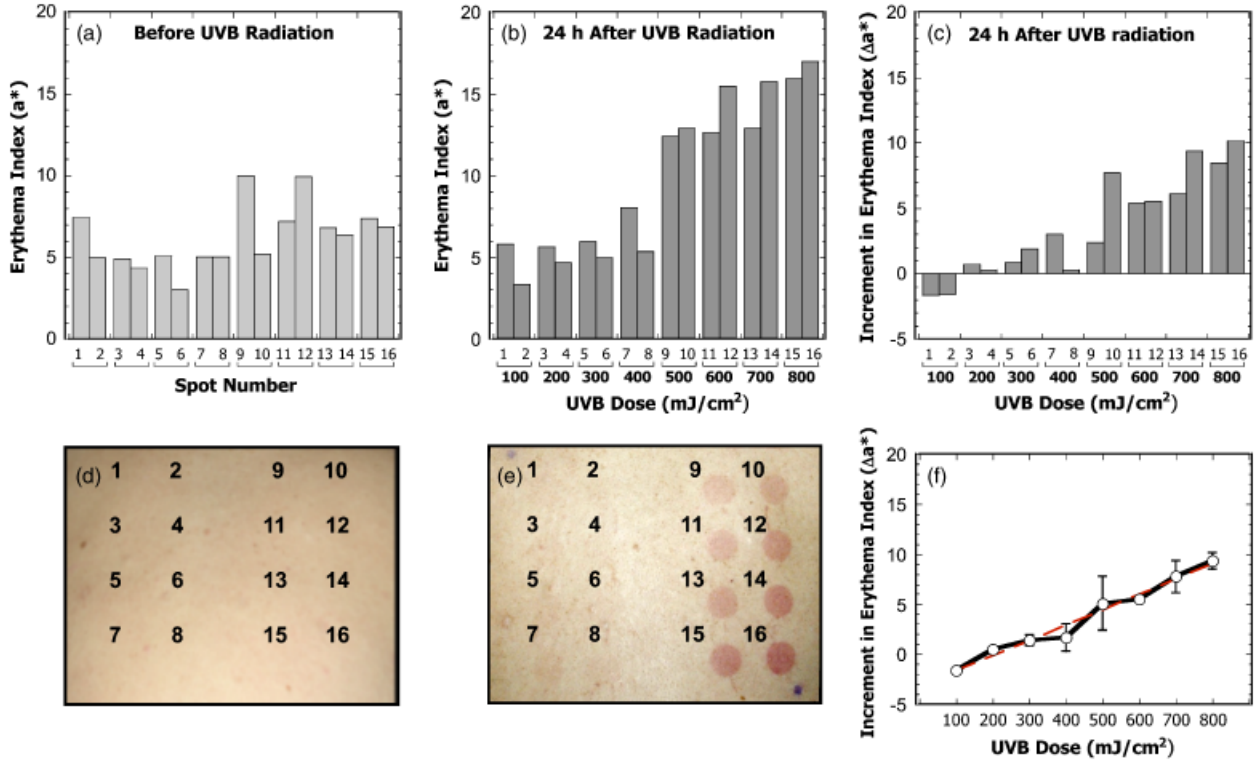

Fig. 2. Effect of a range of doses of ultraviolet radiation (UVR) (100–800 mJ/cm^2) on the erythema index (a^*) and appearance of the skin of a single volunteer. Horizontally paired spots are numbered. (a) Mean a^* values of the 16 spots of a single individual (Subject 3) on 4 successive days before UVR. (b) Mean a^* values of the same 16 individual spots 24 h after UVR at the indicated doses (100–800 mJ/cm^2). Paired spots received the same UVR dose. (c) Increments in erythema index (Δa^*) for each of the 16 spots (i.e. a^* values shown in b minus those in a). (d) Photograph of skin before radiation (corresponding to a). (e). Photographs of spots after UVR (corresponding to b). (f) Increment of erythema index (Δa^*) plotted against UVR dose (corresponding to c). The graph shows the two Δa^* values for each UVR dose, and is drawn through the means of the two values. Linear regression coefficient $r^2 = 0.97$.

Table 2. Analysis of spot-pairing methods for UVR protection studies

Method of spot pairing	Mean difference $\Delta\Delta a^*$†
Horizontal pairing	1.15 ± 0.87
Vertical pairing	1.36 ± 0.91
Random pairing, unilateral	1.45 ± 0.97
Random pairing, bilateral	2.00 ± 1.39
Contralateral pairing	2.43 ± 1.33

†All values are expressed as $\Delta\Delta a^*$ mean ± SD. In the course of this experiment, 14 spots (Spots 2–15 as per supporting information Fig. S1) were irradiated at 500 mJ/cm^2 and Δa^* values were determined for the individual spots. To determine the optimal method of spot pairing, we measured the absolute value of the $\Delta\Delta a^*$ [Δa^*(spot A) − Δa^*(spot B)] for each individual pair. There were six potential unilateral horizontal pairings (e.g., spots 3 and 4) and six potential unilateral vertical pairings (e.g., spots 2 and 4). Random pairing was carried out unilaterally (e.g., spots 2 and 8; n = 41) and bilaterally (e.g., spots 2 and 15, n = 91). Contralateral pairing involves pairing complementary contralateral spots (e.g., spots 2 and 9; n = 6). There were no significant differences among any of the methods of pairing. UVR, ultraviolet radiation.

moving along the back and therefore skin serviced by the same vasculature and autonomic innervation should provide additional consistency. In selecting the pairing of spots for treatment and controls, we therefore used adjacent horizontal spots. The strength of the paired design is that the intersubject variability can be eliminated by examining only the intrasubject differences, reducing the analysis to a one-sample problem (11).

Evaluation of protection against UVR-induced erythema

We have recently reported that treatment of skin areas with a sulforaphane-containing broccoli sprout extract protects against the erythemic response to UVR (6, 7). The protective effects depend on the induction of cytoprotective (antioxidant, anti-inflammatory) phase 2 enzymes. The following describes the rationale and method of analysis applied in this study, which were not fully published previously.

To examine the efficacy of the protective agent against graded dose narrow-band UVR-induced skin erythema, horizontally-paired spots on each of six subjects were treated with either the protective agent or solvent on 3 consecutive days before UVR. On

the fourth day (24 h after the last treatment), the eight pairs of spots were subjected to incremental amounts of UVR (100–800 mJ/cm^2 in 100 mJ/cm^2 increments). Skin color (a^*) was again measured 24 h after UVR exposure.

The protection study was then analyzed with respect to the increment in erythema index (Δa^*) for all six subjects. The observed increments in erythema at 100 and 200 mJ/cm^2 UVR were smaller than the average daily variation in a^*, and were eliminated from the analysis. In both the control and treatment spots, there was a positive association between the level of UVR exposure (300–800 mJ/cm^2) and the measured increment in erythema index (Δa^*). While the mean Δa^*values resulting from UVR were invariably lower in the protector-treated spots than the mean for the vehicle controls at each UVR dose administered, the confidence intervals between sulforaphane-treated and untreated skin areas overlapped at every UVR dose level. In view of the inter-subject variability in Δa^*, we concluded that the pooling of UVR-dependent Δa^* values for different individuals was inappropriate. We therefore compared for each set of horizontally paired spots, the reduction in erythema resulting from protector treatment, expressed as a percentage, according to Eq. 1, as this provides an additional level of internal normalization for each subject:

$$\text{Protection} = \frac{\Delta a^*(\text{vehicle}) - \Delta a^*(\text{protector})}{\Delta a^*(\text{vehicle})} \times 100\% \qquad (1)$$

For each of the effective UVR doses examined (300–800 mJ/cm^2), the mean protection observed for all the six subjects was at least 20%, and the aggregate mean level of protection was $37.7 \pm 11.2\%$ ($\pm$SEM; $n = 6$). Notably, there was no significant association between the UVR dose and the observed percent reduction in erythema resulting from sulforaphane treatment. We therefore pooled the percent reduction in erythema for all doses of UVR to provide more power to the study.

We further analyzed all these measurements cumulatively. The mean protection for all six subjects at all UVR doses was $37.7 \pm 5.61\%$ ($\pm$SEM; $n = 35$; $p < 0.0001$), which was highly significant. The protection data were bimodal with protection being highly significant in three individuals ($56.8 \pm 6.88\%$; $n = 18$, $p < 0.004$) and not significant in the other three individuals ($18.3 \pm 6.09\%$; $n = 17$, $p \in [0.12, 0.21]$). Random spots in the latter subjects were nonresponsive to sulforaphane protection, but the mechanisms underlying this lack of response are unknown. Further insight into these mechanisms will require examination of whether the unresponsive regions are consistently resistant to protection over time, i.e., differ intrinsically (anatomically or biochemically) from responsive regions.

In summary, highly reproducible methods have been developed for the determination of changes in erythema index (a^*) of skin resulting from UVR. When single protector-treated skin areas are compared with paired adjacent controls, the degree of protection can be determined with good precision.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Two-section adhesive vinyl template with sixteen 2.0 cm diameter window template applied to the posterior chest. The windows are 1.5 cm apart and are numbered as shown. Each window can be occluded by a small piece of vinyl to permit exposure to different doses of UVR. The small corner holes are alignment markers.

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