

## New nature-inspired urocanic acid mimic preventing DNA damages and immunosuppressive signalling induced by ultraviolet-B radiations

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### INTRODUCTION

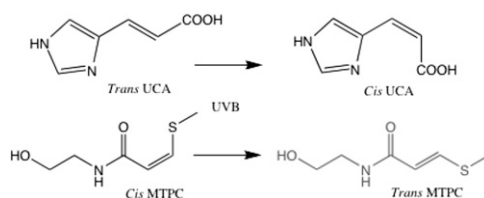
Ultraviolet-B (UVB) absorption by endogenous chromophores such as DNA, tryptophan, or urocanic acid (UCA) initiates mechanisms responsible for the adverse effects of this radiation (local immune suppression and cancer induction, premature aging, inflammation, cell death, etc.). Therefore, the use of exogenous UV-absorbing substances (sun filters) to avoid absorption by endogenous chromophores is a regular and effective strategy for photoprotection of the skin. However, regulatory constraints and inherent limitations of sun filters (solubility, lack of photostability, side effects, etc.) call for the design of effective, stable, and well-tolerated additives.

In the course of our investigations on secondary metabolites found in some tropical plants, we have identified a powerful UVB-absorbing *cis*-methylthiopropenoic acid conjugate (MTPC) (Figure 1). MTPC shares with UCA, a “natural sunscreen” produced in the most superficial layers of the epidermis, the ability to release absorbed photons energy by a mechanism of *trans* to *cis* isomerisation (Figure 1). This is a “nonsacrificial” absorption mechanism (MTPC isomer also absorbs UVB to form the original isomer again) that prevents photodegradation.

MTPC is also endowed with some antioxidant properties, due to the presence of a conjugated thio-ether moiety (Figure 1), and interestingly, some anti-inflammatory properties had been reported for the natural *trans* isomer (1). Therefore, we investigated MTPC photoprotective properties with biochemical and close-to-reality *in vitro* models. Emphasis was placed on UVB-induced immunosuppression as UVB absorption (preventing UCA isomerisation into the immunosuppressive *cis* UCA, Figure 2A), antioxidation (scavenging of ROS involved in the immunosuppressive signalling, Figure 2B), prevention of DNA damages (Figure 2C), and anti-inflammatory properties [tumor necrosis

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**Figure 1.** UVB-induced photoisomerisation of *trans*-UCA and MTPC. *Trans* MTPC is a natural product (Entadamide A) found in tropical plants of the *Entada* genus.

factor  $\alpha$  (TNF $\alpha$ ) also plays a role in UVB-induced immunosuppression, Figure 2D] combine to prevent this detrimental mechanism.

This study highlights the important role of Galectin-7, a  $\beta$ -galactoside-binding lectin naturally expressed in normal skin, in UVB-mediated immunosuppression. Galectin-7 is overexpressed after UVB irradiation and participates to the apoptotic process set up in sunburn keratinocytes (Figure 2E) (3). However, it was recently shown that Galectin-7 overexpression also occurs in the presence of *cis* UCA, and downregulates T lymphocyte functions (Figure 2F) (2), thus pointing to a potential role in immunosuppression.

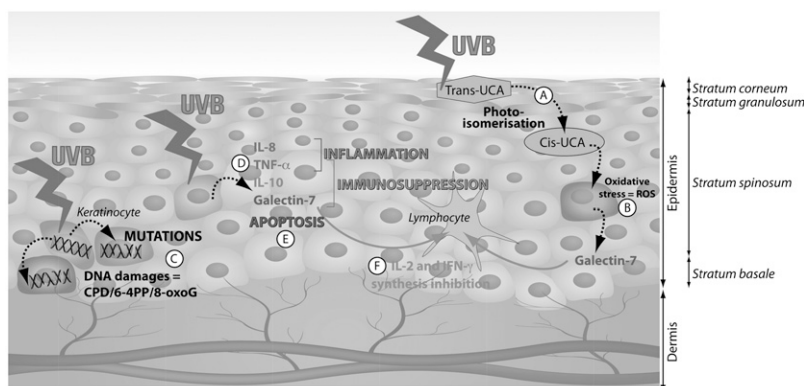
## METHODS

### PHOTOISOMERISATION ASSAYS

*In vitro assays.* Reconstructed human epidermis (RHE) obtained from Skinethic Laboratory (Lyon, France) at day 17 were irradiated at 500 mJ/cm<sup>2</sup> with/without (w/wo) ingredient. Immediately after irradiation, RHEs were cryofreezed before extraction and high-performance liquid chromatography analysis [in collaboration with Synelvia (Toulouse, France)] for *cis*- and *trans*-UCA quantification.

### ANTI-OXIDANT TESTS

The CUPRAC method was used to determine MTPC hydroxyl radical scavenging constant. Cytoprotection was assessed *in vitro*: HaCaT cells were exposed to ultraviolet-A



**Figure 2.** Signalling pathways involved in UVB-induced immunosuppression.

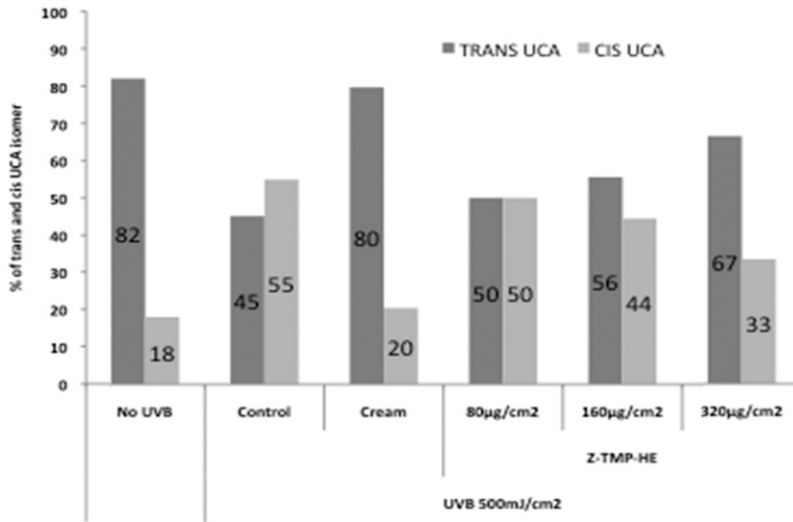


Figure 3. *In vitro* competitive isomerisation assay with human reconstituted epidermis. Dosage of *cis* and *trans* UCA in UVB-irradiated RHE topically treated with the active ingredient MTPC or with a reference photoprotective cream (positive control).

(UVA) irradiation (Waldmann) and generation of intracellular ROS was visualized after incorporation of the 2',7'-dichlorodihydrofluorescein diacetate (CMH<sub>2</sub>-DCFDA) probe.

ASSESSMENT OF PHOTOPROTECTIVE ACTIVITY

**Cell culture.** HaCaT keratinocytes were cultured at 37°C/5% CO<sub>2</sub> in growth media consisting of DMEM [1 g/l glucose, 10% fetal calf serum (FCS)] supplemented by 1% of antibiotic cocktail. All culture media were obtained from Invitrogen. RHEs were supplied from Skinethic laboratory and have been used starting at 17th day of differentiation.

**UVB irradiation.** HaCaT cells were seeded in 6-well plates at 8000 cells/cm<sup>2</sup> 3 days before irradiation (100 mJ/cm<sup>2</sup>, Waldmann lamp) w/wo active ingredient (MTPC) at various

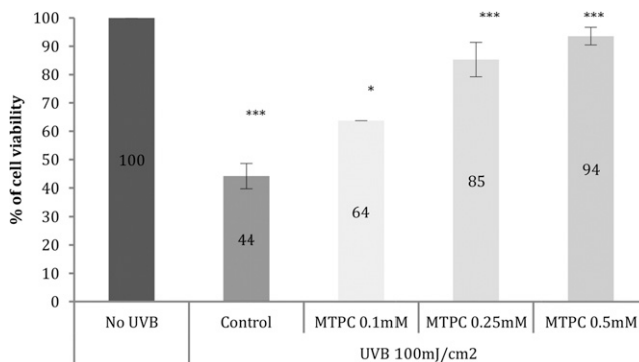
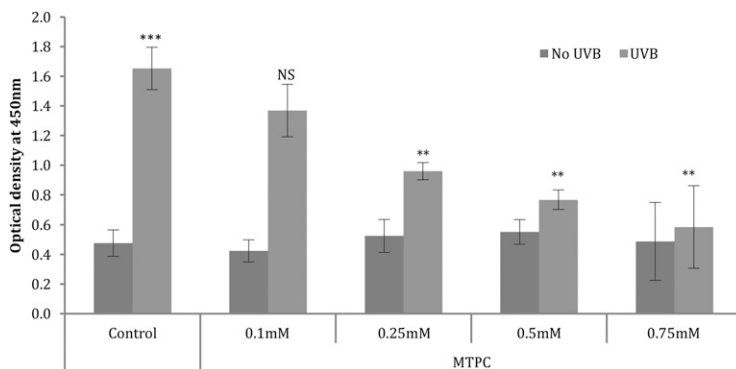


Figure 4. Cell viability monitoring (MTT assay) in UVB-irradiated HaCaT keratinocytes treated with MTPC. All experiments were performed in triplicates. SEM errors bars are represented in black line and *t*-test was used for statistical analysis with \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.



**Figure 5.** Monitoring of CPDs in UVB-irradiated HaCaT keratinocytes treated with MTPC. All experiments were performed in triplicates. *t*-test was used for statistical analysis with \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and NS: nonsignificant.

concentrations. Irradiations were performed in phosphate-buffered saline (PBS) before medium renewing w/wo ingredient for 24 h.

RHEs were irradiated from 300 to 500 mJ/cm<sup>2</sup> w/wo active ingredient in PBS, then replaced in maintenance medium w/wo ingredient for 24 h at 37°C/5% CO<sub>2</sub>.

**Cell viability.** HaCaT cells viability was determined with a specific MTT test, and absorbance was measured using a spectrophotometer equipped with  $\lambda = 570$  nm.

**Dosage of inflammatory and immunosuppressive mediators.** Supernatants collected from HaCaT cells/RHE (stored at -80°C until use) were examined for secreted interleukin (IL)-6, IL-8, TNF-alpha and Galectin-7 protein levels using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biotechne).

**DNA damages.** Cyclobutane pyrimidine dimers (CPDs) were quantified in HaCaT by enzyme immunoassay Oxiselect cellular UV-induced DNA damage ELISA kit (Cell Biolabs). In brief, cells were first seeded in 96-well tissue culture plates before UVB irradiation and CPD revelation. Immunofluorescence assays were performed on RHE cryosections 24 h after UVB irradiation with a specific monoclonal anti-thymine dimer antibody (Sigma, ref.T1192).

**Anti-inflammatory activity.** The catalytic activity of the pro-inflammatory enzyme 5-lipoxygenase (5-LOX) was investigated *in tubo* using the LOX inhibitor screening assay kit (Abcam), and the hydroperoxides produced were detected and measured by a purified LOX. Active ingredient was tested from 1 to 3 g/l and nordihydroguaiaretic acid was used as a reference LOX inhibitor.

#### MONITORING OF IMMUNOSUPPRESSION

Human T-lymphocyte line Jurkat cells were cultured at 37°C/5% CO<sub>2</sub> in suspension in RPMI 1640 medium supplemented with 10% of FCS. Cells were seeded in 24-well



**Figure 6.** Monitoring of CPDs in UVB-irradiated RHE topically treated with MTPC.

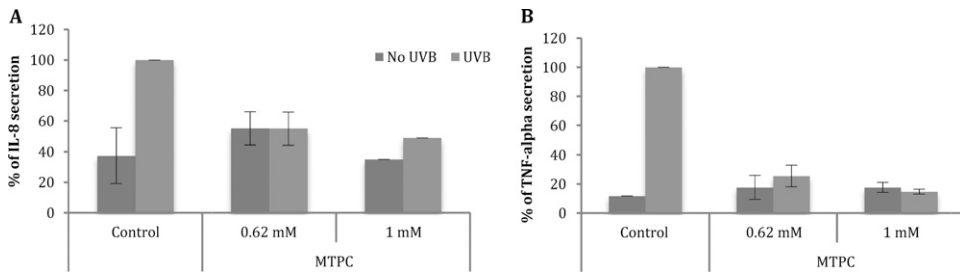


Figure 7. Monitoring of pro-inflammatory cytokines IL-8 (A) and TNF $\alpha$  (B) release from UVB-irradiated RHE topically treated with MTPC.

plates at 150,000 cells/well 2 days before activation with ionomycin and phorbol-12 myristate-13 acetate (Sigma) and stress application: treatment with various concentrations of recombinant human Galectin7 (Biotechne) for 24 h. Specific IL-2 and interferon (IFN)- $\gamma$  cytokines were further monitored by ELISA quantification (Biotechne).

## RESULTS

### UVB-ABSORPTION PROPERTIES OF MTPC

*Absorption spectrum and molar extinction coefficient.* MTPC shows an important absorption in the UVB range [280–320 nm], with a maximum at 284 nm. Its molar extinction coefficient at 290 nm (13,150 L/mol/cm) is comparable to some listed Category I sun filters. Absorption spectrum of *trans* UCA is quite similar to MTPC, indicating that in the skin, these compounds may compete for the absorption of similar UVB wavelengths.

*Limitation of UCA isomerisation.* UCA is produced in the stratum granulosum from the degradation of filaggrin, and accumulates mainly in the stratum corneum. In human epidermis, UCA levels range from 4 to 34 nM/cm<sup>2</sup> (4). Competition for UVB absorption was first examined *in tubo* irradiating equimolar mixtures of UCA and MTPC, a ratio that can easily be attained after topical application of MTPC considering UCA very superficial localization. In such conditions, UCA isomerisation into *cis* UCA was halved (data not shown). *Trans* MTPC (Entadamide A) could also limit UCA isomerisation, but was less potent (data not shown). We further confirmed these observations *in vitro* by the monitoring of UCA isomerisation in UVB-irradiated reconstituted human epidermis (RHE) in the presence of topical MTPC (Figure 3).

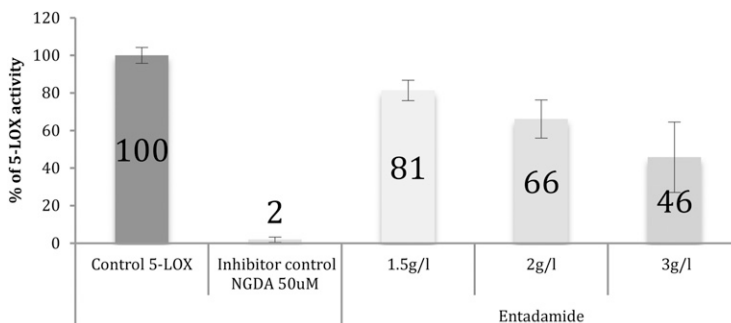
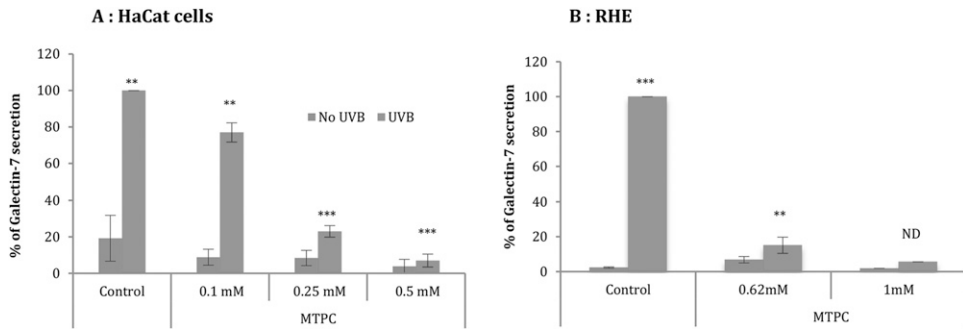


Figure 8. Inhibition of 5-LOX activity by Entadamide A (*trans* isomer of MTPC).



**Figure 9.** Monitoring of Galectin-7 secretion by HaCaT keratinocytes (A) or RHE (B) exposed to UVB. All experiments were performed in triplicates. *t*-test was used for statistical analysis with \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and ND: not determined.

#### ANTIOXIDANT PROPERTIES

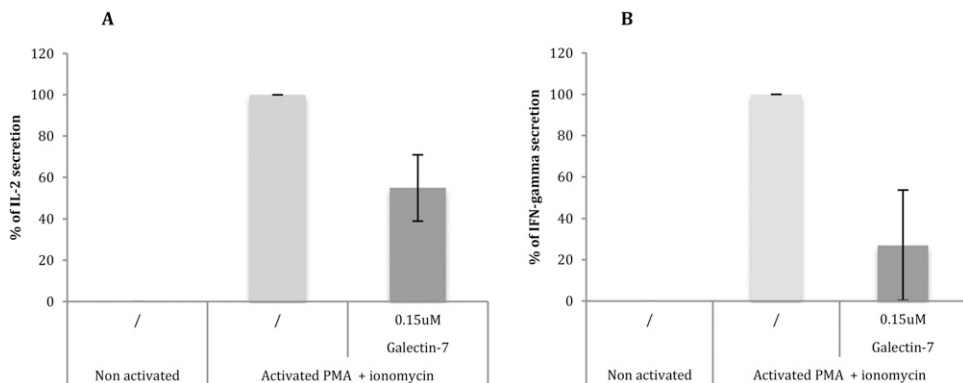
MTPC is a versatile antioxidant, scavenging hydroxyl radicals (CUPRAC method:  $5.72 \times 10^9$  M/s), limiting hydrogen peroxide cell toxicity, and preventing photosensitization reactions. Accordingly, it could limit intracellular oxidative stress induction in UVA-irradiated keratinocytes (monitoring with the CM-DCFDA probe, data not shown).

#### PHOTOPROTECTIVE PROPERTIES

*Cell viability improvement.* MTPC dose-dependently reduces UVB-induced cytotoxicity, when applied during irradiation ( $100 \text{ mJ/cm}^2$ ), as shown in Figure 4.

Improved cell viability was confirmed in RHE experiments by histological analysis (hemotoxylin and eosin staining) that showed that topical application of MTPC preserves epidermis integrity (data not shown).

*Inhibition of UVB-induced DNA damages.* DNA represents the most important cellular chromophore for UVB and absorption causes DNA damage, preferentially CPDs and



**Figure 10.** Monitoring of Galectin-7 effect on the production of IL-2 (A) and IFN- $\gamma$  (B) by activated and nonactivated Jurkat T lymphocytes.

pyrimidine (6-4) pyrimidone photoproducts (6-4PP). We found that MTPC very efficiently prevents UVB-induced DNA damages as revealed by CPD monitoring in irradiated HaCaT keratinocytes (Figure 5) and RHE (Figure 6).

*Inhibition of UVB-induced pro-inflammatory cytokines release.* As UVB is well known to induce pro-inflammatory cytokines release in skin, we measured IL-8, TNF $\alpha$ , and IL-6 secretion from UVB-irradiated HaCaT keratinocytes. We found that MTPC dose-dependently inhibits the production of these three cytokines (data not shown). This inhibition of pro-inflammatory cytokines release was also observed when irradiated RHEs were topically treated with MTPC (Figure 7).

*Anti-inflammatory property of Entadamide A (trans MTPC).* In line with previous *in vitro* results (1) showing that Entadamide A inhibits 5-LOX, a key inflammatory enzyme of the arachidonic acid cascade, we found that the MTPC isomerisation product dose-dependently inhibits 5-LOX catalytic activity (Figure 8).

#### INHIBITION OF UVB-INDUCED IMMUNOSUPPRESSIVE SIGNALLING

The MTPC properties presented above support the hypothesis that MTPC strongly opposes UVB-induced immunosuppression because it interferes with several signalling pathways of this process (Figure 2): *cis* UCA formation (4), DNA damages induction [as CPDs formation is directly involved in immunosuppressive signalling (5)], and TNF $\alpha$  overexpression, which was recently shown to act downstream DNA damage (5). We thus chose to monitor the recently identified mediator Galectin-7 (Gal7), as it is involved in both *cis* UCA and DNA damages immunosuppressive signalling (Figure 2).

In our two-dimensional (HaCaT keratinocytes) or three-dimensional (RHE) *in vitro* models, UVB strongly increases Gal7 expression. Addition of MTPC abrogates this overexpression (Figure 9).

Because UVB also impairs T-cell-mediated immune function, we set up a more specific model. Activated Jurkat T-lymphocyte cells were exposed to Gal7, and we monitored IL-2 and interferon- $\gamma$  (IFN- $\gamma$ , two signalling cytokines of the immune system). We used a recombinant Gal7 as HaCaT cells produce too low amounts, and because some constituents of RHE culture medium interfere with the assay. When exposed to a recombinant Gal7, Jurkat T-lymphocytes produce less IL-2 and IFN- $\gamma$  (Figure 10). This finding shows that Gal7 overexpression can impair T-cell-mediated immunity, and supports an important role for Gal7 in UVB immunosuppressive property.

#### CONCLUSION

Our experiments have shown that MTPC, a nature-inspired molecule, protects the skin from the deleterious effect of UVB thanks to a combination of three mechanisms (UV absorption, antioxidation, and anti-inflammation). These findings support the usefulness of MTPC for sun care or photoaging prevention.

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