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Chemoprevention of ultraviolet B radiation-induced skin cancer with the mutant p53 reactivator SLMP53-2

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Chemoprevention of ultraviolet B radiation-induced skin cancer with the mutant p53 reactivator SLMP53-2

Graphical Abstract
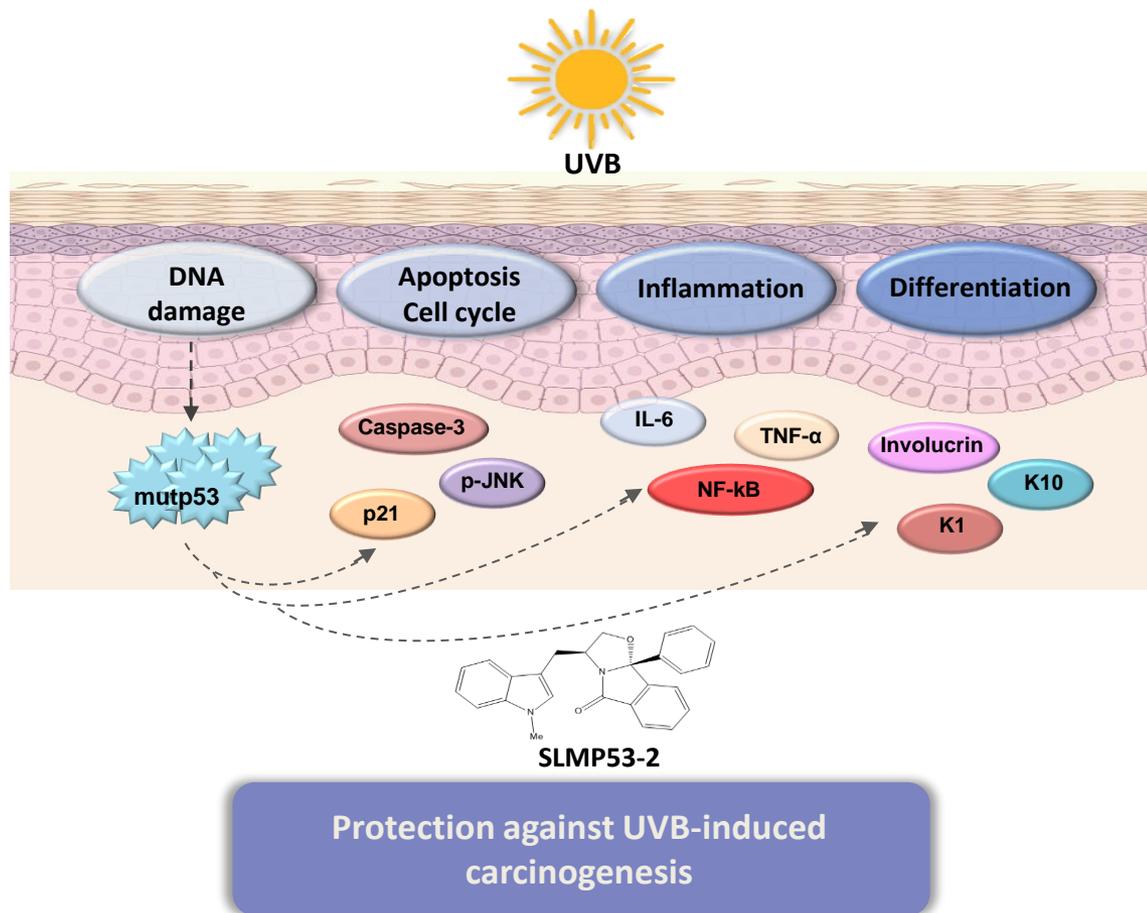
Abstract

Skin cancer (SC) is one of the most common cancers in fair-skinned population. To counteract this public health concern, additional preventive approaches to sunscreen is needed. Mutant p53 (mutp53) is an appealing target for SC prevention given its crucial role in ultraviolet B (UVB) radiation-induced skin carcinogenesis. Therefore, the reduction of mutp53 protein levels by its reactivation would constitute a valuable preventive strategy. Herein, we investigated the potential of our recently identified mutp53 reactivator SLMP53-2, as a SC chemopreventive agent. The SLMP53-2 pre-treatment of keratinocyte HaCaT cells, before UVB exposure, reduced mutp53 protein levels with restoration of its wild-type-like p53 activity. Subsequently, SLMP53-2 pre-treatment increased cell survival, by promoting G1-phase cell cycle arrest, while reducing UVB-induced apoptosis through inhibition of c-Jun N-terminal kinase activity. UVB-induced reactive oxygen species and oxidative damages were also reduced by SLMP53-2. Furthermore, it protected from UVB-induced DNA damage, through increased DNA repair via nucleotide excision repair pathway. Also, by decreasing the nuclear translocation and DNA binding ability of NF-κB, it reverted UVB-induced inflammation, and stimulated the expression of keratinocytes differentiation markers. Consistently, the topical application of SLMP53-2 in mice, previous to UVB irradiation, also inhibited cell death, DNA damage and expression of inflammatory-related proteins, while promoting cell differentiation, without displaying signs of skin toxicity. Collectively, the results reveal a promising application of SLMP53-2 in UVB-induced SC prevention.

Keywords: Chemoprevention; p53; skin cancer; tryptophanol-derived oxazoloisoindolinone; UVB radiation

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Skin Cancer chemoprevention strategy through mutant p53 reactivation
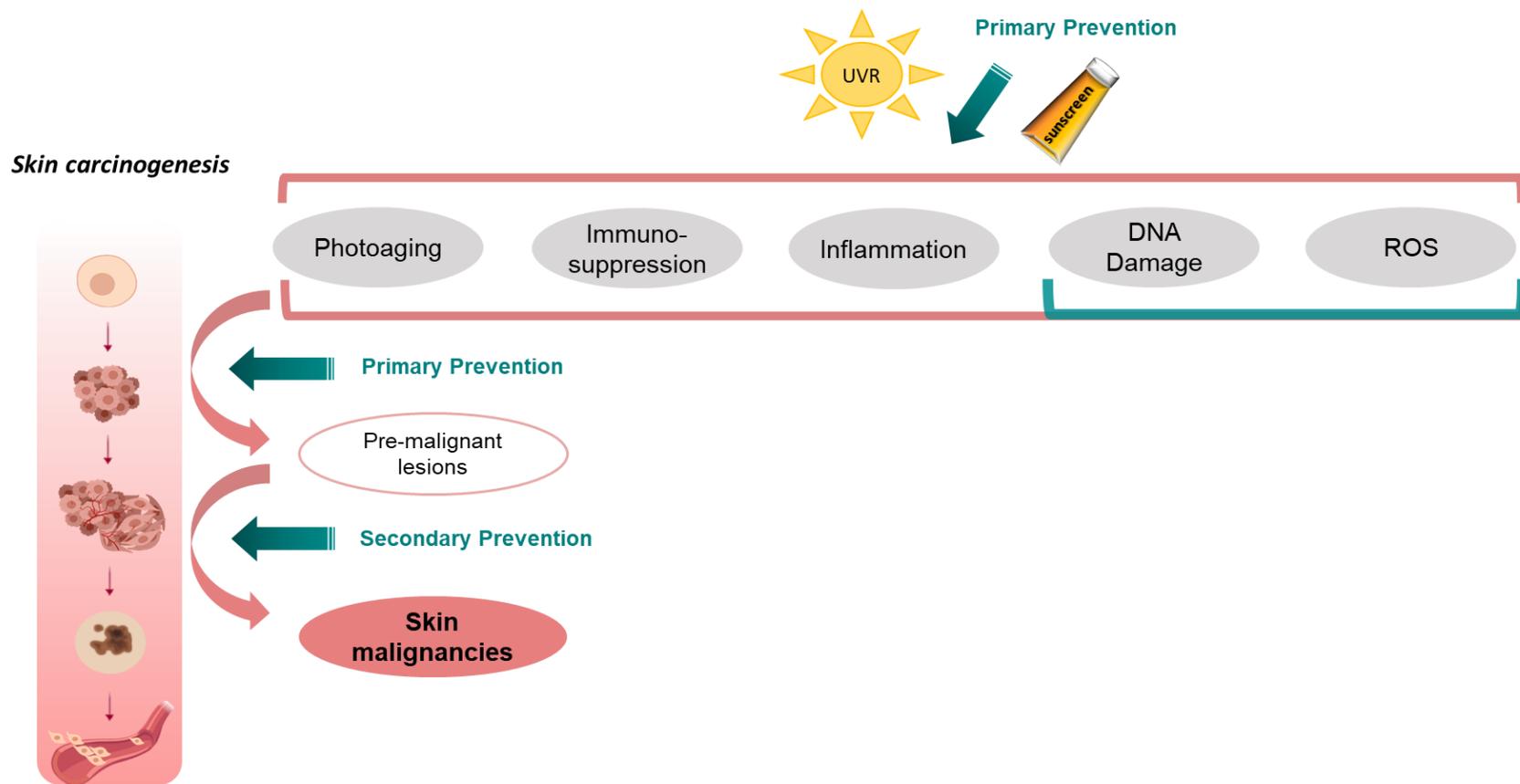
Loureiro JB, et al. *Biochim Biophys Acta Rev Cancer.* 2020;1874(2):188438.

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Chemical synthesis of SLMP53-2

(S)-tryptophanol + 2-Benzoylbenzoic acid

Toluene Reflux

1

DMF, r.t.

Methyl Iodide, NaH
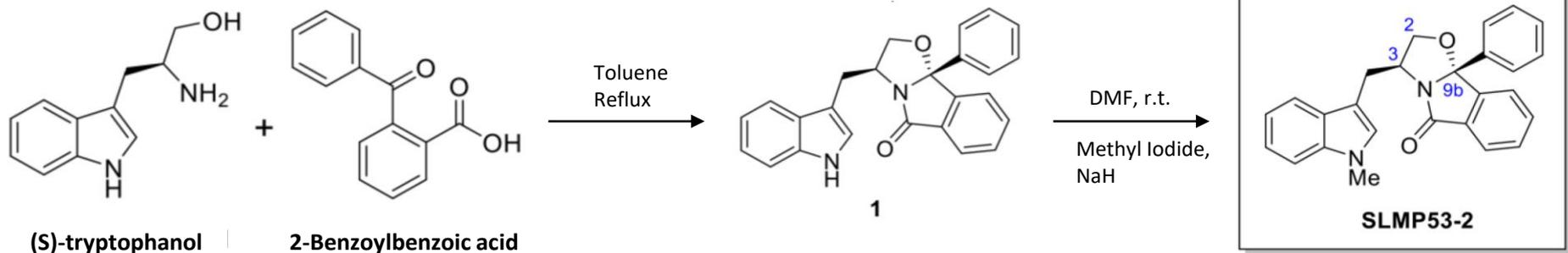
SLMP53-2

Tryptophanol-derived oxazoloindolinone

European patent EP3013833 and
US patent 20160347765

Mutant p53 reactivator

Gomes, S. et al. Cancers 2019, 11, 1151.

In vitro studies for evaluation of SLMP53-2 preventive effect

SLMP53-2
24h pre-treatment, before UVB

$IC_{10} = 3.5 \pm 0.5$ μM

HaCaT cells
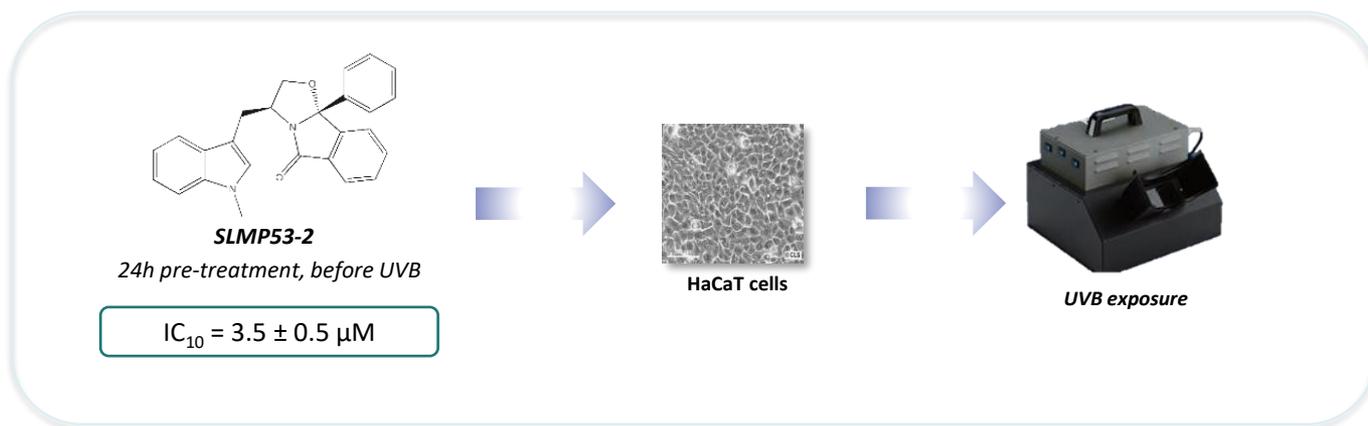
UVB exposure

SLMP53-2 promotes cell survival after UVB and reduces mutp53 levels with increase of DNA binding ability

Reduction of mutp53 expression levels, even upon UVB exposure
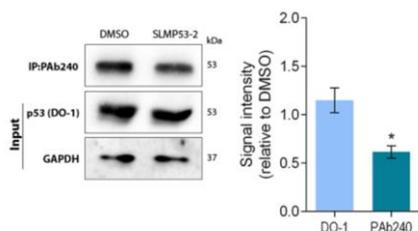
Immunoprecipitation assay was performed in HaCaT cells treated with SLMP53-2 (3.5 µM) for 24 h. p53 from IP - loading control. Data shown are means ± SEM (n=4). * p < 0.05, unpaired Student's t-test.
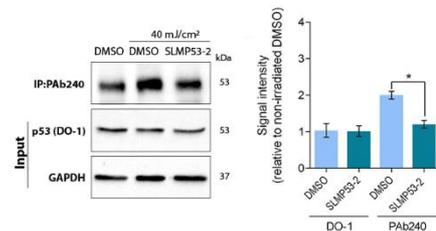
Immunoprecipitation assay was performed in HaCaT cells pre-treated with SLMP53-2 (3.5 µM) for 24 h, exposed to UVB and then collected 20h after. p53 from IP - loading control. Data shown are means ± SEM (n=5). * p < 0.05, unpaired Student's t-test.

Enhanced p53 binding to DNA
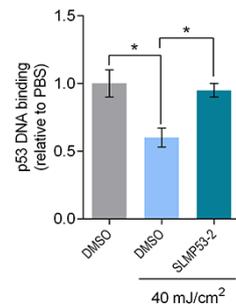
P53 binding ability to DNA (by TransAM kit) analyzed for 20h after UVB. Data are mean ± SEM (n=3); *p < 0.05 (One-Way ANOVA Tukey's test).

Increased cell survival
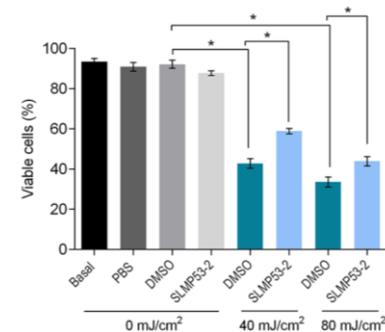
Cells viability determined by trypan blue assay 20 h after UVB. Data are mean ± SEM (n=4). *p < 0.05 (One-Way ANOVA Tukey's test).

Cell cycle arrest with increase of p21 expression
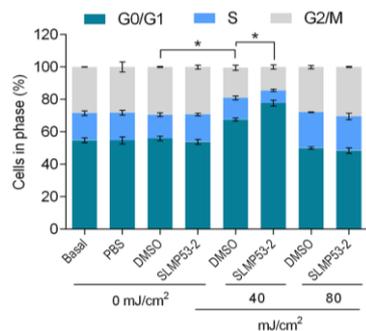
Cell cycle phases analyzed by flow cytometry using PI, 24h after UVB. Data are mean ± SEM (n=4); *p < 0.05 (One-Way ANOVA Tukey's test).
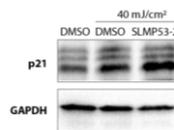
Analysis of protein levels by WB of in HaCaT cells exposed to UVB, 20h after exposure. Representative blots (n=3); GAPDH was used as loading control.
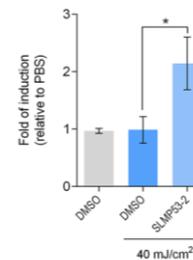
mRNA levels (RT-qPCR) of *CDKN1A* (p21), 6 h after UVB; Data are mean ± SEM (n=4); *p < 0.05 (One-Way ANOVA Tukey's multiple test).

SLMP53-2 counteracts UVB-induced inflammation and promotes cells differentiation

Decreased NF-kB nuclear translocation and DNA binding ability

Reduction in inflammatory markers expression levels
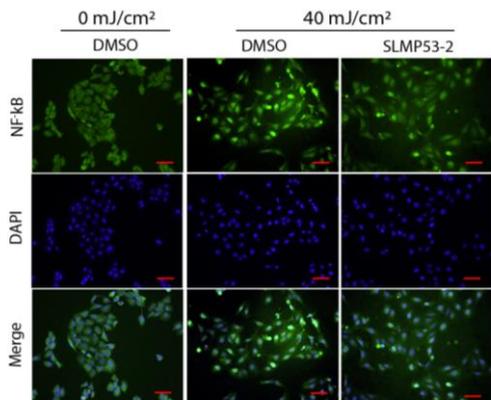
Immunofluorescence of p65 NF-kB for 6h post UVB exposure of cells. Scale bar = 50 μm; magnification = ×200. Data are mean ± SEM (n=4); *p < 0.05 (One-Way ANOVA Tukey's est).
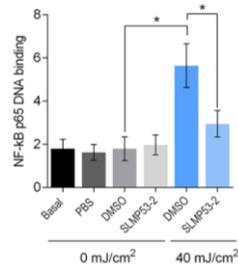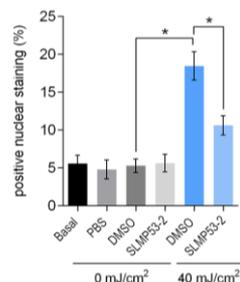
NF-kB p6 DNA binding ability evaluated for 6h post UVB. Data correspond to OD 450 nm. Data are mean ± SEM (n=3); *p < 0.05 (One-Way ANOVA Tukey's multiple test).
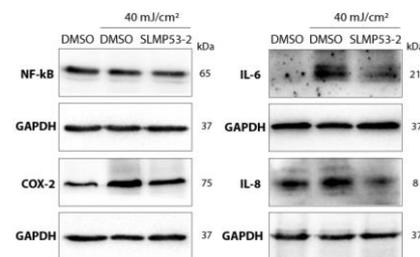
Protein levels were analyzed by WB for 20h after UVB (6h for NF-kB p65). Representative blots (n=3) GAPDH - loading control.
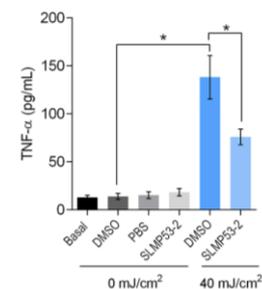
TNF-α secretion (by ELISA) analyzed for 20h after UVB. Data are mean ± SEM (n=5); *p < 0.05 (One-Way ANOVA Tukey's test).

Increased expression of differentiation markers
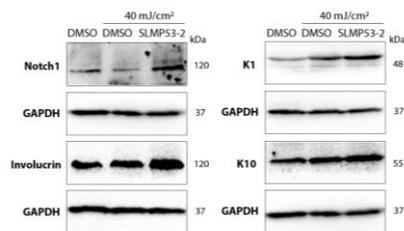
Notch1

Protein levels were analyzed by WB for 20h after UVB irradiation. Representative immunoblots (n=3); GAPDH - loading control.
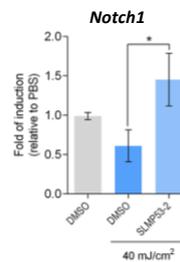
mRNA levels (RT-qPCR) of *Notch1*, 6 h after UVB; Data are mean ± SEM (n=4); *p < 0.05 (One-Way ANOVA Tukey's multiple test).

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SLMP53-2 protects cells from UVB-induced apoptosis, ROS and DNA damage

Suppression of UVB-induced apoptosis, reduction of JNK activity
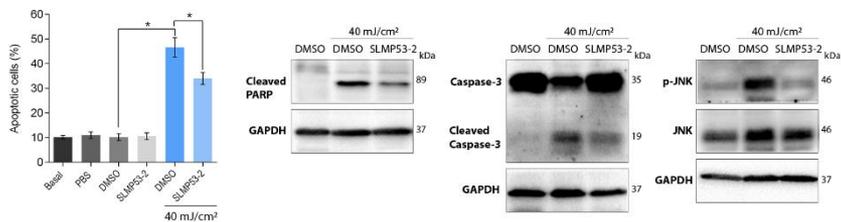
Annexin-positive cells (%) determined for 10h after UVB. Data are mean ± SEM (n=5); *p < 0.05 (One-Way ANOVA Tukey's).

Protein levels were analyzed by WB for 6h (p-JNK/JNK) or 20h (PARP, Caspase-3) after UVB. Representative immunoblots (n=3); GAPDH - loading control.

Reduction of UVB-induced ROS and of oxidative damages in lipids and proteins
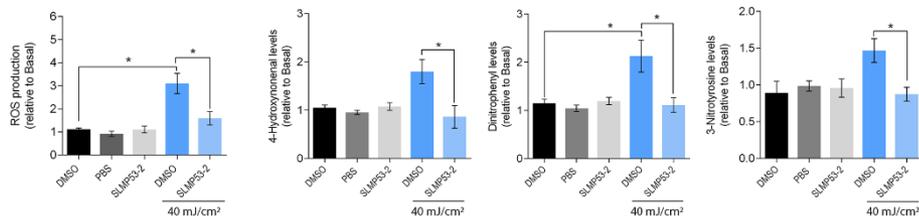
ROS production measured by DCF fluorescence, 24h after UVB. Results are mean ± SEM (n=4). *p < 0.05 (One-Way ANOVA Tukey's test).

Measurement of lipid peroxidation and of proteins carbonylation and nitration, respectively, by slot-blot. Results are mean ± SEM (N=4-8). *p < 0.05 (One-Way ANOVA Tukey's test).

Reduction of UVB-induced DNA damage

Representative images (n=3); Scale bar = 10 µm; magnification = ×400.
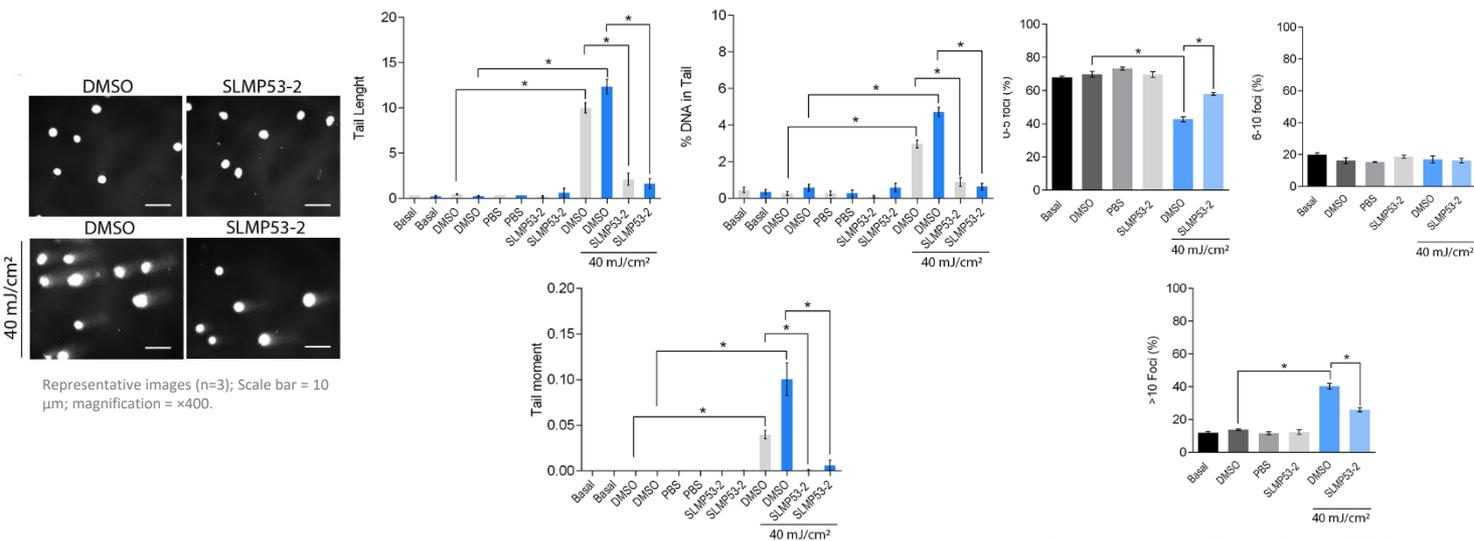
T4 endonuclease-modified alkaline comet assay immediately after UVB. Results are mean ± SEM (n=3). *p < 0.05 (Two-Way ANOVA test, Bonferroni post-test).

Immunostaining of γH2AX histone 1 h after UVB. Data are means ± SEM (n=3). *p < 0.05 (Two-Way ANOVA test, Bonferroni post-test).
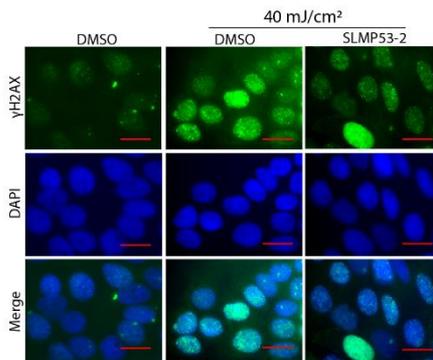
Representative images (n=3); Scale bar = 5 µm; magnification = ×1000.

6

SLMP53-2 promotes DNA repair (NER pathway) reducing UV-induced CPD lesions

Reduction of UVB-induced CPD
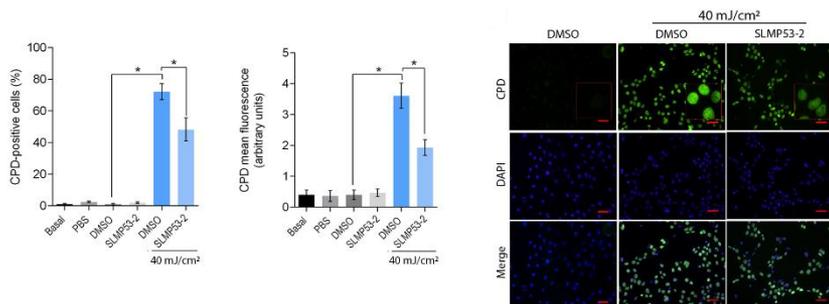
Fluorescent staining of CPD (cyclobutane pyrimidine dimers) in DNA, 6h after UVB. Data are means ± SEM (n=5). *p < 0.05 (One-Way ANOVA Tukey's multiple comparisons test)

Scale bar = 50 µm; magnification = ×200.

Enhanced DDB-2 expression
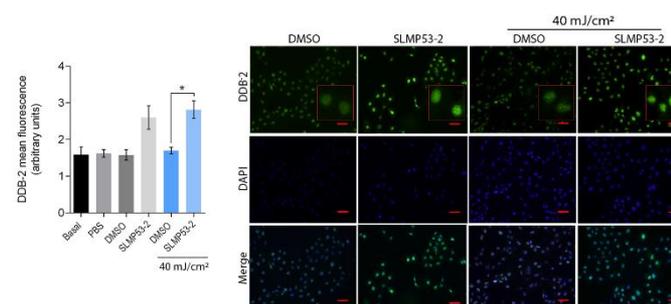
Evaluation of the DDB-2 fluorescence staining, performed 1h after UVB. Data are presented as mean fluorescence of DDB-2 and are means ± SEM (n=3). *p < 0.05 (One-Way ANOVA Tukey's multiple comparisons test); scale bar = 50 µm; magnification = ×200.

Enhanced XPC expression
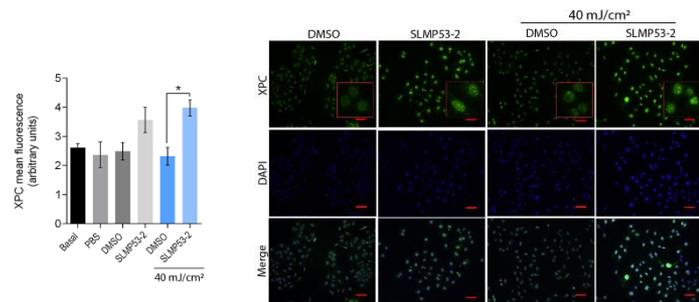
Evaluation of the XPC fluorescence staining, performed 1h after UVB. Data are presented as mean fluorescence of XPC and are means ± SEM (n=3). *p < 0.05 (One-Way ANOVA Tukey's multiple comparisons test); scale bar = 50 µm; magnification = ×200.

Increased DDB-2 and XPC, mRNA and protein levels
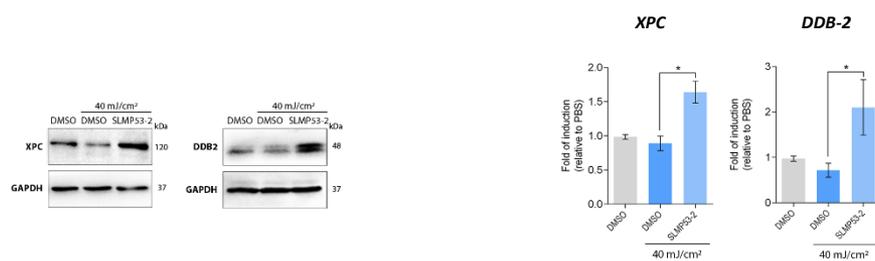
Expression levels of proteins involved in NER-pathway, by WB, in UVB-irradiated keratinocytes. Protein levels were analyzed for 1h after UVB irradiation. Representative blots (n=3); GAPDH - loading control.

mRNA levels (RT-qPCR) of *XPC and DDB-2*, 1 h after UVB. Data are mean ± SEM (n=4); *p < 0.05 (One-Way ANOVA Tukey's multiple comparisons test).

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SLMP53-2 did not induce skin histological alterations or inflammation

In vivo **toxicological evaluation**

FVB/N female mice were topically treated with **1.5 mg SLMP53-2** applied for 2x/week, 2weeks. Vehicle: acetone.

No histological alterations
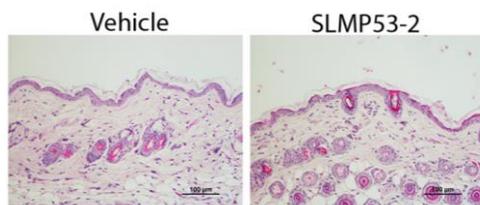
H&E staining of mice skin from toxicological evaluation; mice were topically treated for 2 weeks/twice a week with 1.5 mg SLMP53-2. Scale bar = 100 µm; magnification = ×200.

No alterations in proliferation index
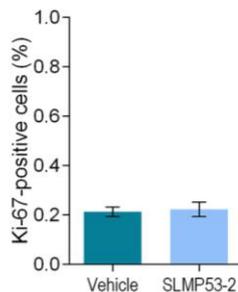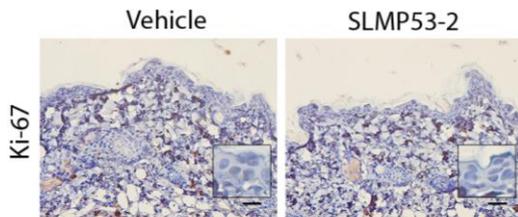
Evaluation of Ki-67 expression levels in mice skin from the toxicological study; Data are means ± SEM (n=5); p > 0.05, unpaired Student's t-test. Scale bar = 10 µm; magnification = 200×.

No alterations in COX-2 expression levels

Analysis of COX-2 expression by immunofluorescence in mice skin from the toxicological evaluation. Data are means ± SEM (n=5); p > 0.05, unpaired Student's t-test. Scale bar = 50 µm; magnification = 200×.

8

SLMP53-2 reverts UVB-induced histological alterations *in vivo*

Chemopreventive activity evaluation

FVB/N female mice were pretreated with SLMP53-2, **1.5 mg topically** applied for **1h** before UVB irradiation (180 mJ/cm^2).
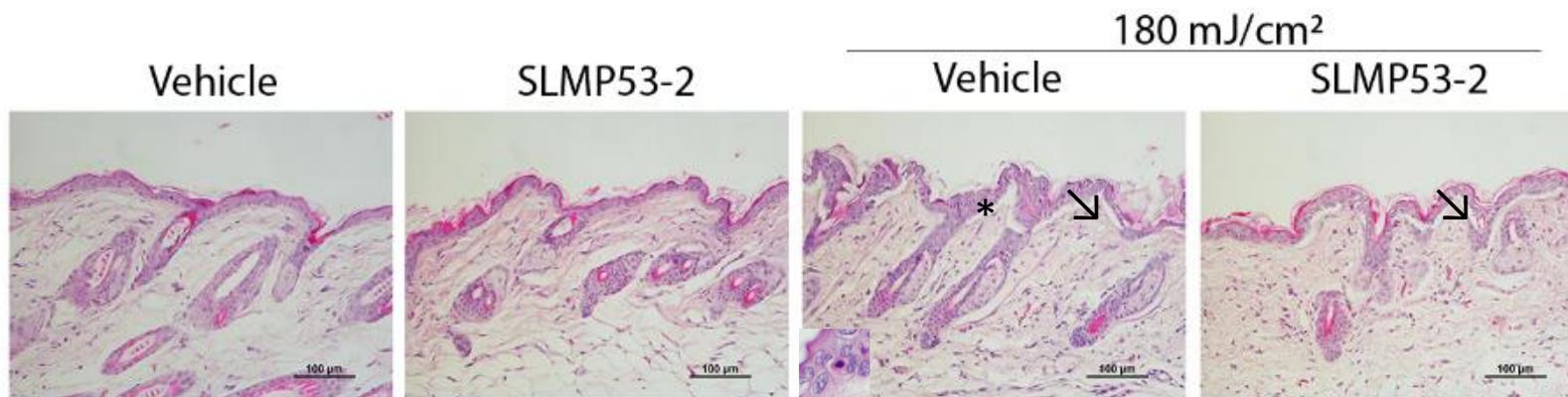
H&E staining of non-irradiated and UVB-exposed mice skin; main histological alterations included mild epidermal hyperplasia (*), multifocal dermo-epidermal separation (↘) and the presence of sunburn cells (inset).

SLMP53-2 protects from cell death, repairing DNA damage

Decreased UVB-induced cell death and DNA Damage
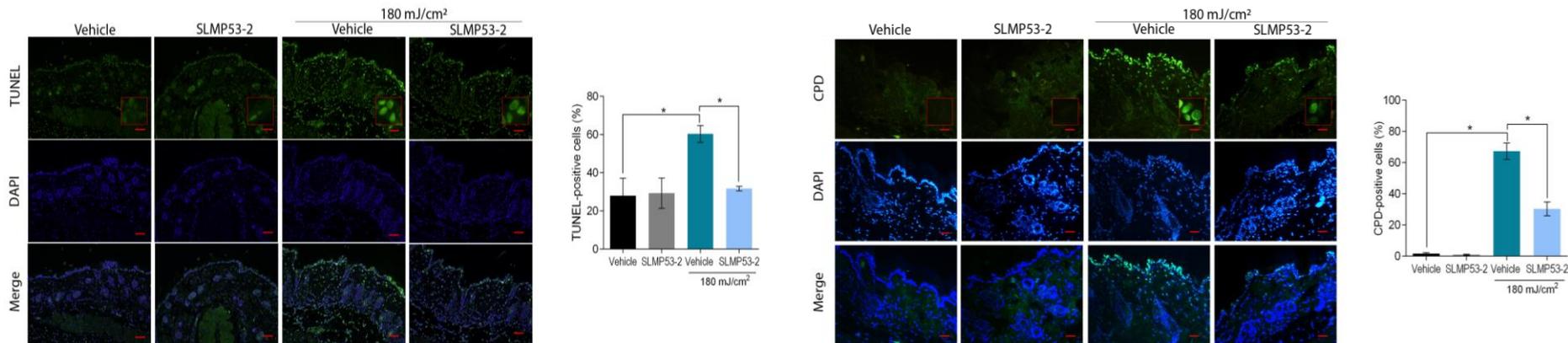
Evaluation of TUNEL labeling and of CPD (cyclobutane pyrimidine dimers) levels by immunofluorescence in mice skin. Quantification of TUNEL- and CPD-positive cells; data are means ± SEM (n=5); *$p < 0.05$; One-Way ANOVA Tukey's multiple comparisons test. Representative images; scale bar = 50 μm; magnification = 200×.

Enhanced DNA Repair, via NER, by DDB-2 and XPC
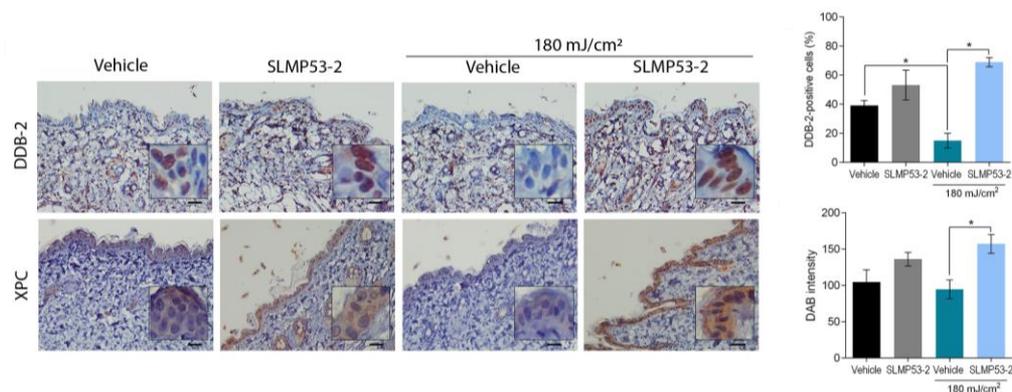
Evaluation of DDB-2 and XPC protein levels by DAB staining in mice skin. Quantification of DDB-2-positive cells and XPC DAB intensity ; data are means ± SEM (n=5); *$p < 0.05$; One-Way ANOVA Tukey's multiple comparisons test. Scale bar = 10 μm; magnification = 200×.

SLMP53-2 promotes cell differentiation and reduces UVB-induced inflammation

Decreased expression of inflammatory proteins IL-6 and COX-2
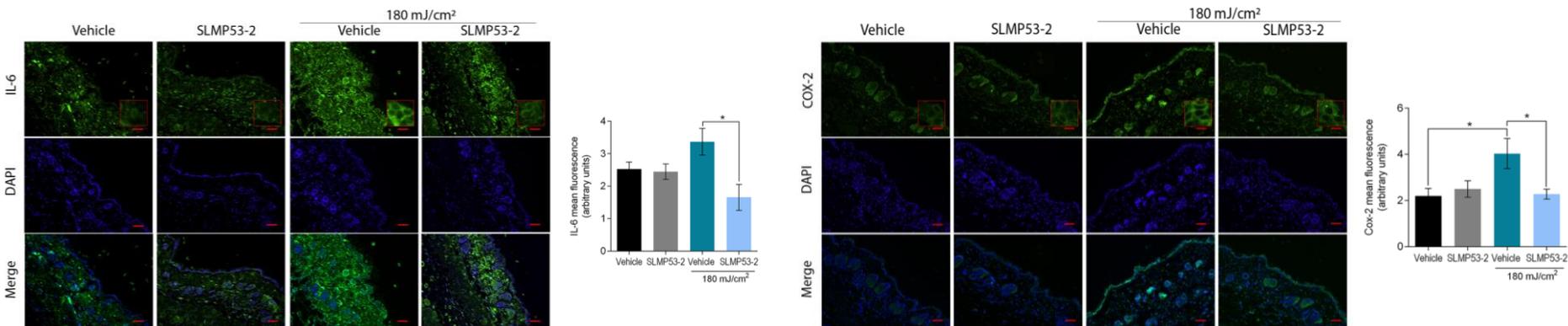
Evaluation of IL-6 and Cox-2 protein levels by immunofluorescence in mice skin. Quantification of mean fluorescence of IL-6 and COX-2 signal; data are means ± SEM (n=5); *p < 0.05; One-Way ANOVA Tukey's multiple comparisons test. Representative images; scale bar = 50 µm; magnification = 200×.

Increased expression of differentiation markers
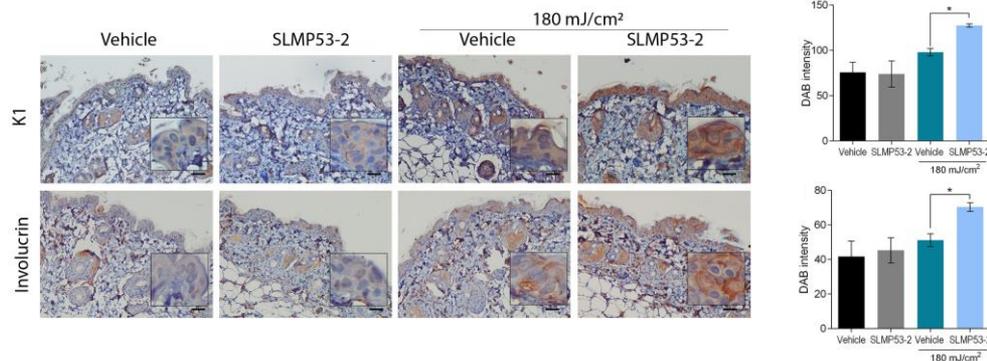
K1 and involucrin protein levels by DAB staining in mice skin. Quantification of DAB intensity ; data are means ± SEM (n=5); *p < 0.05; One-Way ANOVA Tukey's multiple comparisons test. Scale bar = 10 µm; magnification = 200×.

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Conclusions

SLMP53-2 **reactivates mutp53**, making exposed cells more prone to counteract UVB damages

SLMP53-2 **promotes cell survival** upon UVB exposure, leading to cell cycle arrest or apoptosis, in a dose-dependent manner

SLMP53-2 promotes **DNA repair by NER-pathway**, markedly reducing UVB-induced damages

SLMP53-2 lead to decreased UVB-induced **inflammation** and promoted cell **differentiation**

SLMP53-2 showed promising *in vivo* **protective** profile against UVB, with no signs of skin toxicity

Acknowledgments

This work received financial support from PT national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior), through LAQV/REQUIMTE (UID/QUI/50006/2020), and PTDC/QUI-QOR/1304/2020. We also thank FCT for the fellowship SFRH/BD/128673/2017 (J. B. Loureiro) and SFRH/BD/137544/2018 (E. A. Lopes).

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