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

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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
Skin Cancer chemoprevention strategy through mutant p53 reactivation

Chemical synthesis of SLMP53-2

(S)-tryptophanol + 2-Benzoylbenzoic acid

Toluene, Reflux

1

DMF, r.t.
Methyl Iodide, NaH

Tryptophanol-derived oxazoloindolinone
European patent EP3013833 and US patent 20160347765

Mutant p53 reactivator

In vitro studies for evaluation of SLMP53-2 preventive effect

IC$_{10}$ = 3.5 ± 0.5 µM

SLMP53-2

24h pre-treatment, before UVB

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

Enhanced p53 binding to DNA

Increased cell survival

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.

PS3 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).

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 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-κB nuclear translocation and DNA binding ability**

NF-κB p65 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).

**Reduction in inflammatory markers expression levels**

Protein levels were analyzed by WB for 20h after UVB (6h for NF-κB p65). Representative blots (n=3) GAPDH - loading control.

**Increased expression of differentiation markers**

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).

Immunofluorescence of p65 NF-κB 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).

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).
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 16h 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**

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 γH2A histone 1 h after UVB. Data are means ± SEM (n=3). *p < 0.05 (Two-Way ANOVA test, Bonferroni post-test).
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).

**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).
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, 2 weeks. 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 = x200.

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 = 50 μm; magnification = 200x.

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 = 200x.
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/cm²).

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**

Evaluation of 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×.
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.
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