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Topical Nutlin-3 Potentiates the UVB-induced p53 Response and Reduces DNA Photodamage and Apoptosis in Mouse Epidermal Keratinocytes *in Vivo*

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Abstract

Background/Aims: (-)-Nutlin-3 (nutlin) is a cis-imidazoline analog, which activates p53 by antagonizing murine double minute (Mdm2) protein. To our knowledge, no studies have assessed the effect of topical nutlin on cyclobutane pyrimidine dimers (CPD) repair and apoptosis. We therefore conducted a study in hairless mice to investigate the effect of topical nutlin on murine epidermis after UVB-radiation.

Methods: Female C3.Cg/TifBomTac immunocompetent mice were treated with 100 µl 43 mM nutlin 30 min before and after irradiation with 3 SED (100 mJ/cm^2) UVB. Animals were euthanized 24 hours after irradiation, the dorsal, treated skin was biopsied and fixed in 4% formalin. Sections were incubated with the antibodies against p53, thymine dimers and terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling (TUNEL).

Results: We showed here that nutlin was active after topical application in hairless mice and potentiated the p53 nuclear translocation in epidermal keratinocytes after ultraviolet B irradiation. Moreover, topical treatment with nutlin resulted in a decrease in the number of keratinocytes exhibiting positive nuclear staining for thymine dimers ($P<0.05$ compared with the vehicle control) and decreased the frequency of apoptotic, TUNEL-positive cells ($P=0.02$).

Conclusion: We hypothesize that nutlin stimulates DNA photodamage repair in the epidermis and may prove useful for the chemoprevention of skin cancer.

Keywords: Nutlin-3; p53; Apoptosis; Thymine dimer; Hairless mice

Background

(-)-Nutlin-3 (nutlin) is a cell-permeable antagonist of the murine double minute (Mdm2) p53 binding protein [1,2] . Nutlin activates p53 by inhibiting the Mdm2-p53 interaction and is under development for the treatment and prevention of cancer [1,2]. Nutlin increases apoptosis in many types of cells, but this effect in not universal. For example nutlin blocks UV-induced apoptosis in the osteosarcoma cell line U2OS and in keratinocytes via a pathway involving $p21^{cip/WAF}$ and kinase JNK [3]. Apoptosis in keratinocytes is mainly caused by cyclobutane pyrimidine dimers (CPD). In vivo studies on human cancer xenografts showed that nutlin inhibits tumor growth and activates the p53-dependent apoptosis after oral administration [1,4]. In view of the role of p53 in DNA repair and apoptosis in keratinocytes it was conceivable that nutlin is able to stimulate repair of CPD and possibly affect apoptosis in epidermis exposed to UVB. We tested this hypothesis in hairless mouse model after topical application of nutlin.

Questions Addressed

Can topically applied nutlin affect CPD repair and apoptosis in murine epidermis after UVB irradiation?

Experimental Design

Animals

Female C3.Cg/TifBomTac immunocompetent mice (age 15 weeks) were purchased from Taconic (Ry, Denmark). The mice were kept in an animal facility on a 12 h light/dark cycle at 23 - 24°C. Animal care and treatment followed Danish national guidelines.

Drug treatment, ultraviolet source and experimental design

Mice were sedated with 0.05 ml HypDorm (fentanyl citrate 0.158 mg/ml, fluanisone 5mg/ml, midasolam 2.5mg/ml) and treated on one of the lateral halves of the back skin with 100 µl isopropanol solution of 43 mM nutlin [(-) 4-(4,5-bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxyphenyl)-4,5-dihydro-1H-imidazole-1-carbonyl)piperazin-2-one] (Cayman Chemical) 30 min before irradiation. The other half was treated with the same volume of isopropanol. The mice were then irradiated with 3 SED (100 mJ/cm^2) UVB from a light source comprising an array of 6 TL12 tubes (Philips, Eindhoven, The Netherlands) and re-treated with nutlin or isopropanol, as above. Control mice were not irradiated. Animals were euthanized 24 hours after irradiation because studies in vitro in human skin and in vivo in mice skin suggest that after 24 hours about half of the CPD are repaired [5,6]. The treated dorsal skin was biopsied and fixed in 4% formalin.

Immunohistochemistry

4 µm sections were cut from the paraffin embedded skin biopsies, deparaffinised with xylene, re-hydrated and incubated with the antibodies against p53 (rabbit, polyclonal, Novocastra Newcastle upon Tyne, UK) or peroxidase-conjugated monoclonal anti-thymine dimer antibody (Kamiya Biomedical, Seattle, WA). The antibodies were visualised using the LSAB+ System-HRP (Dako,

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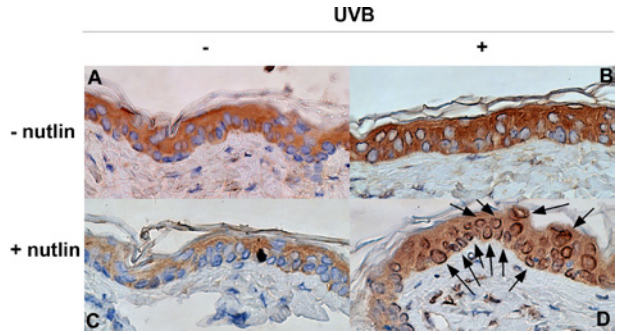

Figure 1: Induction of p53 by nutlin and UVB irradiation in murine epidermis. Mice (n=15) were treated on the counterlateral halves of dorsal skin with nutlin (C, D) or the isopropanol vehicle (A, B). They were un-irradiated (A, C) or irradiated with 100 mJ/cm^2 UVB (B, D), as described in Methods. Skin samples were taken 8 h after irradiation and stained with anti-p53 antibody with peroxidase visualisation (brown color). Arrows show p53-positive nuclei in mice treated with UVB and nutlin. Magnification x400.

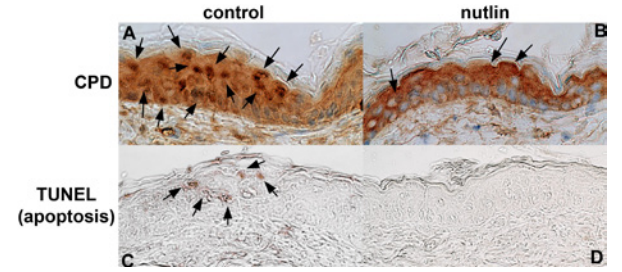

Figure 2: Nutlin reduces DNA damage and keratinocyte apoptosis in UVB-irradiated skin. Mice (n=10) were treated on the counterlateral halves of dorsal skin with nutlin (B, D) or the isopropanol vehicle (A, C) and irradiated with 100 mJ/cm^2 UVB. Skin samples taken 24 h after irradiation were stained with anti-thymine dimer or with TUNEL technique (see Methods). Arrows show CPD- or TUNEL-positive nuclei. Magnification x400.

Glostrup, Denmark). Terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling (TUNEL) was performed using the DeadEnd Colorimetric TUNEL system (Promega, Madison, WI). Apoptotic, TUNEL-positive cells were counted in a 3 mm long section of epidermis. The extent of thymine-dimer positivity was assessed on an ordered categorical interval scale: 1) 0% stained nuclei, 2) 1-25%, 3) 26-50%, 4) 51-75% positive nuclei. In no samples the frequency of positive nuclei exceeded 75%.

Results

As shown in Figure 1, the p53 staining of unexposed epidermis revealed moderate immunoreactivity in the cytoplasm but not in the nuclei of epidermal keratinocytes. After UVB irradiation the cytoplasmic staining was more intense and single cell nuclei were stained positive for p53. As expected, the UVB-induced nuclear translocation of p53 was markedly potentiated by topical treatment with nutlin. In non-irradiated skin nutlin did not induce p53.

Since it is known that p53 is induced by DNA photoproducts and p53 is involved in the repair of CPD [7] we stained skin biopsies with the antibody against thymine dimers. Topical treatment with nutlin resulted in an overall decrease in the number of keratinocytes exhibiting positive nuclear staining for thymine dimers ($P<0.05$, Wilcoxon signed-rank sum test) (Figure 2 A-B). The decrease in CPD reflects probably repair, since no apoptotic CPD-expressing cells have been detected.

Nutlin treatment significantly inhibited the UVB-induced apoptosis (Figure 2C-D). TUNEL staining revealed 9.0 ±2.6 (mean ±standard deviation) TUNEL-positive cells / mm epidermal length in UVB-irradiated, vehicle-treated skin versus 1.7 ±0.5 TUNEL-positive cells /mm in the nutlin-treated skin ($P=0.02$, paired t-test).

Conclusions

This study shows that the Mdm2 inhibitor, nutlin, activates p53 in the epidermis in UVB-irradiated mice. This was accompanied by a significant decrease in the frequency of the cells harbouring thymine dimers and diminished keratinocyte apoptosis. We suggest that the decreased apoptosis is caused by enhanced CPD repair due to p53 activation by topically applied nutlin. It is conceivable that nutlin may be used for chemoprevention of squamous cell carcinoma in humans.

Conflicts of Interest

This study was financed solely by the Bispebjerg University Hospital and has not been supported by any pharmaceutical company.

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