SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

PARylation independent effects of PARP inhibitors in UVB and UVA-induced cell death

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The Examination took place at 2.306 office at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen at 11 AM, 24th of June, 2015.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1 PM, 14th of March, 2017.

1. INTRODUCTION

1.1. Ultraviolet radiation from the Sun

The human skin is exposed to solar ultraviolet radiation which contributes to the increasing incidence of skin tumors such as basal and squamous cell carcinoma and melanoma. Whereas the short wavelength UVC is effectively filtered out by the ozone layer, UVB and UVA reach the Earth surface and get absorbed in the skin. The biological effects of UV radiation in the skin depend on the type and dose of UV radiation and range from the induction of pigmentation, vitamin D synthesis and immunosuppression to photoaging and carcinogenesis.

1.2. Characteristics of UVC/UVB

The absorption spectrum of DNA includes wavelengths from 240 to 310 nm, so the UVC and the UVB irradiations are the most potentional DNA damiging effects. The most common DNA lesions induced by UV irradiation are cyclobutane-pirimidine-dimers (CPD) and pirimidine-pirimidone-photoproducts ((6-4)-PP).

UVC and UVB irradiations are able to induce different modifications in the structure of RNAs. These structural changes could be lethal, mostly in case of mRNA strucutre, because the modification in the sequence of mRNA can result unfunctional proteins.

Absorption of UVB, in some special cases, can result increased reactive oxygen species (ROS) production. Tryptophan or thyrosine amino acids in triplett state reduce the disulfides priducing disufides-anions which can react with molucular oxygen resulting superoxide-anions. Superoxide-anion is one of the most dangerous to the lipid membrans or the proteins.

1.3. Characteristics of UVA

The direct harmful effect, like causing CPDs or (6-4)-PP, of UVA irradiation to the DNA stucture is very weak. Whereas energy of more than 300 nm wavelength irradiation can lead the formation of Dewar isomers from CPDs, which are more stabile than the original DNA lesions and almost unrepairable by DNA repair mechanisms.

The main effect of UVA irradiation is the induction of ROS production by damge of plasmamembrane’s lipids or compartments of cytoplasm inside the cells. The long half-life species can enter to the nucleus and there they can cause oxidative damages in the DNA. 8-OH-dG is the most common oxidative damage by the UVA irradiaition.

1.4. Effects of UV irradiation induced DNA damages

Nucleotid excision repair (NER) is the most important mechanism in the repair of UV induced DNA photolesions like CPD sor (6-4)-PP. The proteins of NER system try to eliminate the photolesions before the arriving replication fork, but this mechanism can lead to DNA breaks which can induce cell death, in most of time apoptosis. Generally it is a fact that the high dose of UV or long – term UV irradiaiton induces necrosis.

The apoptotic cells have special morfology in the skin, they are called „sunburn" cells. In this cells the main inductor of the apoptosis is the DNA damage but a cell death can caused by p53 independent pathway or cell death receptors mediated or mitocondrial disfunction induced apoptotic pathway as well, or these combinations.

1.5. Phototherapy, photodynamic therapy, photochemotherapy

Phototherapy, photochemotherapy and photodynamic therapy are efficient treatment used to treat various different types of diseases ranging from psoriasis, acne to certain forms of cancer.

Phototherapy is the therapeutic use of ultraviolet light to improve conditions and diseases of the skin. Ultraviolet radiation can be UVA, UVB, and UVC light.

Photodynamic therapy (PDT) is based on the combination of a photosensitizing compound with irradiation using a light source emitting at or near the absorption maximum of the photosensitizer. The excited photosensitizer promotes the generation of oxygen radicals by directly (type I reaction) or indirectly (via intracellular substrates; type II reactions) manner reacting with molecular oxygen. Reactive oxygen species (ROS) formed in these reactions and responsible for cytotoxic effects of PDT.

Photochemotherapy (PCT) is a combination of psoralen with suberythemogenic ultraviolet A (PUVA). Psoralens are tricyclic furocoumarins, 8-methoxypsoralen (8-MOP) is the most commonly used sensitizers. Photochemical interaction between psoralens and DNA may follow three pathways: a) intercalation of psoralen into DNA (it may also take place without UV radiation); b) formation of 3,4-monoadducts or 4,6-monoadducts with the latter being capable of crosslinking psoralen with DNA following the absorption of a second photon; c) excited psoralen may directly react with molecular oxygen leading to the generation of singlet oxygen as in PDT.

1.6. Biological role of PARPs

1.6.1. The poly(ADP-ribose)polymerase -1 enzyme

PARPs catalyze the ADP-ribosylation reactions of acceptor proteins. PARP-1 is the most well known member of the enzyme familiy and the human cells contain it in high amount.

The main intiatior of PARP-1 activation is the DNA damage, mostly DNA single breaks. The enzyme consists three main domains: DNA-binding domain, automodification-domain and the catalitic domain. The N-terminal DNA binding domain conatins three Zn-finger domains, a nuclear localisation signal domain and a caspase-3 cleavage site. The most important region of the automodification doamin is the BRCT regio, which has an importnt role in the protein-protein interaction between DNA rapair enzymes or their proteins. On the C- terminus there is a WGR domain, it has a role in the development of the interactions between domains. Moreover there is the catalitic region of the enzyme, which is responsible for the binding of NAD^+ and producing the PAR polimer.

1.6.2. Mechanism of poly(ADP-ribose)polimer synthesis

Poly-ADP-ribosylation (PARylation) is a reversible posttranslational modification catalyzed byPARPs. The activated PARPs use NAD^+ as a substrate and cleave it to nicotinamide and ADP-ribose, then use the ADP-ribose units for polymer synthesis which are covalently attached to acceptor proteins. PAR chains can be attached, inter alia, to glutamate, aspartate or lysine residues of acceptor proteins. The most abundant PARylated protein i*n vivo* is PARP-1 itself, which automodification leads to the inhibition of its enzymatic activity. Due to the size and negative charge of ADP-ribose units, PARylation influences the physico-chemical properties, stability, activity and interactions of proteins.

Poly(ADP-ribosyl)ation is a reversible, dynamic process, PAR has a short half-life, degradation of polymers starts immediately after synthesis. Degradation of polymers are catalyzed by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl hydrolase 3 (ARH3).

More than 200 nuclear target of PARP has been identified. They are histons, transcription factors, DNA repliacation or repair proteins, cell signaling proteins for expmple NFkB, topoisomerase I II, DNA dependent protein kinases.

1.6.3. Role of PARP-1 in different types of DNA reapair mechanism

1.6.3.1. Base excision repair (BER)

The basis of DNA strand can be modified by deamination, alkilation or oxidative effects. Base excision repair is the main mechanism in DNA repair to elimination of modificated DNA bases and in case of single DNA breaks. PARP-1 enzyme can interact with many poriteins in the BER system for example XRCC or aprataxin. PARP-1 enzyme activity influences the efficiency of BER by regulation of DNA polimerase -β, DNA-ligase III, condensin or 8-osoguanin DNA glicosolase (OGG1).

1.6.3.2. Homologous recombination (HR) and non-homologous end joining (NHEJ)

Double DNA breaks can be repaired by homolog recombination or non-homologous end joining. PARP-1 has role in both of mechanism. In case of HR the accumulated PAR polimers can increase the activity of ATM or influence the function of ATM.

In classic NHEJ mechanism the Ku protein and PARP-1 enzyme are competing against each other for the free DNA ends. If the Ku or other NHEJ proteins value are limited, the PARP-1 became the main protein. The mechanism is called alternative NHEJ pathway. In this mechanism DNA ligase III or DNA dependent protein kinase are PARilated.

1.6.3.3. Nucleotid excision repair (NER)

The nukleotid excision reapir is the most important mechanism of the DNA photolesion's elimination. The role of PARP –1 enyme in NER mechanism have been researched very well in last years. PARilation of XPA and XPC proteins have been identified by more than one research groups. DDB-2 protein also interacts with PARP-1 moreover the interaction of the PARP-1 and DDB-2 is significantly stronger in presence of an UVC induced damaged DNA. Inhibiton of PARP-1 activity suppresses the efficiency of NER mechanism.

1.7. Connection of PARP-1 and the different types of cell death

After the DNA damage and PARP activation there are three options depending on the intesity of DNA damage. In case of mild DNA damage, poly(ADP-ribosyl)ation facilitates DNA repair which leading to cell survival.

More intense genotoxic stimuli activates apoptotic pathway, where PARP-1 is cleaved by caspase 3 or 7. These fragments are 24 kDa and 89 kDa. The cleavage inactivates the enzyme, which prevents ATP depletion and necrosis during DNA fragmentation. The role of PARP-1 in apoptosis is passive, but its cleavage is necessary for the process.

Excessive DNA damage may cause excessive PARP activation, depleting cellular NAD^+/ATP stores leading to necrosis.

Inhibition of PARP activity in case of mild or excessive DNA damage lead to apoptotic cell death instead od cell survival or necrosis.

1.8. Role of PARP inhibitors in chemotherapy

About ten years ago was demonstrated that breast cancer type 1 susceptibility protein and breast cancer type 2 susceptibility protein (BRCA1/2) dysfunction results in a loss of HR and significantly sensitized cells to inhibition of PARP enzymatic activity. PARP inhibition lead to chromosomal instability, cell – cycle arrest and apoptosis which probably caused by unrepaired DNA lesions. This process is a type of the synthetic lethality which arises inhibition or depletion of two cellular pathway in same time leads to cell death whereas inhibiton of either one of the two pathway alone does not. In this case exist BRCA1/2 mutation and exogen PARP-1 inhibition can lead to increased genotoxic effect of many chemotherapeutic agent.

Most of PARP inhibitors under clinical investigation inhibit the PARP-1 or -2. The key mechanism in synthetic lethality is the role of PARP-1 in HR, but as secondary effect very important the regulatory role of PARP-1 in BER and NHEJ. PARP inhibitors have different specificity anf effectivity but the inhibitoty mechanism is usually same. They are NAD^+ analoge and can bind to the catalitic domain of the enzyme, this is the way of inhibition.

In recent work three different PARP inhibitors were used:

<u>3-aminobenzamide (3-AB):</u>

This is one of the oldest PARP inhibitor. Compare to the new generation inhibitors 3-AB has a very high effective concetration (3-5 mM) and it is not so specific. Nonetheless it is a popular inhibitor in our days, mostly in aimal experiments.

PJ-34:

It is a more specific and potent PARP inhibitor than 3-AB. It has lower effective dose dependeng on the cell type (1-20 uM). PJ-34 by a PARP independent mechanism can activate the expression of p21 which lead to mitotic arrest in some investigeted cell lines.

Veliparib:

Veliparib is also called ABT-888. It is an usable oral PARP1 and 2 inhibitor. Clinical studies have shown Veliparib well tolerable for the body with good bioavailability and clearence. Preclinical models had demonstrated that Veliparib potentiated the anti-cancer effect of severeal cytotoxic agents and ionizing radiation.

2. AIMS OF THE STUDY

I.

1. Comparsion the UVB sesitivity of HaCaT cell line and normal human epidermal keratinocytes (NHEK).

2. Investigation the effect of PARP-1 silencing during UVB induced cell death.

3. Observation the effects of different PARP inhibitors in UVB induced cell death on NHEKs.

II.

1. Observation the effect of PARP-1 silencing on UVA irradiated A431 cell line.

2. Investigation the role of PARP-1 silencing in PUVA induced phototoxicity.

3. Using different PARP inhibitors in our PUVA model.

3. MATHERIALS AND METHODS

3.1. Cell culture

Cryopreserved normal human keratinocytes were cultured in Epidermal Keratinocyte Medium (low bovine pituitary extract and low calcium formulation) supplemented with penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere (5 % CO2), and were passaged at 80-90 % confluency after trypsinization with trypsin-EDTA solution. Second and third passage cells were used for experiments.

HaCaT spontaneously immortalized keratinocyte cells were cultured in DMEM supplemented with 10 % heat-inactivated fetal bovine serum, 5 % penicillin-streptomycin and 5 %, L-glutamine.

The human epidermoid carcinoma cell line A431 was cultured in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% heat-inactivated fetal bovine serum, 5% penicillin streptomycin and 5%, L-glutamine.

HaCaT and A431 cells were maintained at 37°C in a humidified atmosphere (5% CO2), and were split at 70-80% confluency after trypsinization with trypsin-EDTA solution. Cultures at 80-95% confluency were used in all experiments.

3.2. Cell treatment and UV irradiation

In first part of experiments cells were pretreated for 30 min with two different PARP inhibitors, 10 μM PJ34 and 3 mM 3-AB. In one experiment other potent PARP inhibitors (TIQ-A, DPQ) were also used in 10 μM concentration. The primary keratinocytes were irradiated in the culture medium with different UVA/UVB doses. Irradiation of HaCaT cells was carried out in phenol red free medium.

In second part of experiments cells were pretreated for 50 min with the photosensitizer 0.8 μM methoxypsoralen (8-MOP) or with PARP inhibitors, 10 μM PJ-34 or 10 μM Veliparib. In assays involving antioxidants, 200 μM Trolox or 500 μM ascorbic acid were added to the cultures together with the PARP inhibitors. Following pretreatment the cells were irradiated with UVA in the presence or absence of PARP inhibitors in phosphate-buffered saline (PBS).

The cells ain all experiments were irradiated with a Bio-Sun microprocessor-controlled UV irradiation system. After irradiation PBS was replaced with fresh cell culture medium.

3.3. Spheroid culture

We grew spheroids from A431 cells in 96 well plates pre-coated with 0.5% agar-water solution. Cells were seeded onto plates at $1x10^4$ cells/ml density in 50 µl cell suspension per well. The cells were allowed to clump together for 48 h and were then pretreated with PARP inhibitors (PJ-34 and Veliparib) for 50 min. Cells were irradiated with UVA (2.5 J/cm2). Immediately after irradiation, the medium was changed to fresh cell culture medium and spheroids were incubated for 16 h. Pictures of spheroids were taken with a Leica confocal microscope system. Diameter of spheroids was measured with Image J software.

3.4. Cell viability and proliferation assay

After treatments and incubation, MTT solution was added to each well at a final concentration of 0.5 mg/ml. Following incubation the medium was aspirated and formazan crystals were dissolved in dimethyl sulfoxide. Optical density was measured at 590 nm using a Multiskan MS plate reader.

For the cell proliferation assay keratinocytes were grown to 70-80% confluency. Cells were then trypsinized, resuspended in the media, and counted. The cells were reseeded into 6-well tissue culture plates (2500 per well). After the pretreatment with PJ-34 and 3-AB and irradiation with UVB, the cells were incubated for 10 days. Fresh medium was added immediately after the irradiation, and on day 5. On day 10, medium was removed from the wells and cells were washed once with PBS. Half of the wells were stained with hematoxilin for microscopic evaluation and the other half was stained for viability with MTT. Hematoxillin-stained samples were photographed whereas MTT-stained samples were used for the quantification of cell proliferation.

3.5. Detection of reactive oxygen species

A431 cells were seeded into 96 well plates one day before the experiments. Cells were incubated for 50 min with 10 µM H2DCFDA, Fluorescence was detected with a Fluoroskan Ascent FL plate reader immediately after UVA irradiation (ex. 480 nm, em. 590 nm).

3.6. Hydrigen-peroxide production

NHEKs were first irradiated with UVB followed by the addition of Amplex Red reagent (50 µM) and horse radish peroxidase (0.1 U/ml). After incubation (30min, 37ºC), the fluorescence was measured with a fluorescence spectrophotometer (ex 530 nm; em 590 nm).

3.7. Gene silencing

SMARTpool siRNA was specific for human PARP-1 sequence and SiGLO RISC-Free siRNA was used as control. HaCaT cells were seeded into 12-well plates (2×10^5 cells/well) and into 96 well plates (5000 cells/ well) the day before transfection in DMEM containing 10% FBS without antibiotics. DharmaFECT 2 transfection reagent was diluted 1:100 in serum free DMEM and incubated at room temperature for 5 min. In parallel, 10 μM siRNA in 1×siRNA buffer was diluted 1:1 in serum-free DMEM and were incubated for 20 min at room temperature prior to addition to cells with a final siRNA concentration of 50 nM. After 24 h, the medium was replaced with complete growth medium. After another 24 h, efficiency of silencing was determined from 12-well plates by Western blotting and cells in 96-well plates were used for the viability assay in paralel with the UV experiments.

A431 cells were transfected in suspension (after trypsinization and prior to seeding). SiGENOME SMARTpool siRNA specific for human PARP-1 sequence, ON-TARGET plus Control siRNA and DharmaFECT 2 transfection reagent were obtained from Dharmacon Research, Inc. and silencing was carried out according to the manufacturer's instruction. After 48 h, efficiency of silencing was determined from 24-well plates by Western blotting and cells in 96-well plates were used for the viability assay 16 h after irradiation with different doses of UVA (2.5-10 J/cm2).

3.8. Western-blotting

PAR (primary antibody: clone 10H), PARP-1, H2AX/gamma-H2AX, cleaved-PARP-1 were detected by Western blotting. Cells were washed with cold PBS and harvested by scraping in lysis buffer. After sonication, cell lysates boiled, and then equal amounts of protein were separated by SDS-PAGE. After electrophoresis, samples were transferred onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked with 5% non-fat dry milk or 5 % BSA in TBS-Tw. Membranes were incubated with primary antibody diluted in 1% milk in PBSTw or 5 % BSA in TBS-Tw for O/N at 4º C, which was followed by incubation with peroxidase-conjugated secondary antibodies. To visualize the antibody reactions, chemiluminescent substrates were added and chemiluminescent signals were detected using a FluorChem FC2 Imager.

3.9. Comet assay

Single-stranded DNA breaks were detected by alkaline comet assay. Slides were coated with 1% normal melting point agarose. A single-cell suspension was prepared and the cells were treated with PARP inhibitors and UVA. Immediately and 1 h after irradiation the cell suspension was mixed with 0.5% low melting point agarose and layered on coated slides. After lysis, electrophoresis was carried out in alkaline buffer, slides were neutralized in 0.4M Tris buffer and nuclei were stained with TO-PRO. CometScore software was used for quantitative analysis.

3.10. Detection of caspase activation

Cells were harvested 4, 8, 16 h after irradiation, washed 1x with PBS and were lysed in lysis buffer. Cell lysate and fluorogenic substrates [aminomethylcoumarin-conjugated caspase-3- and caspase–8- specific peptides (Ac-DEVD-AMC for caspase-3 and Ac-VETD-AMC for caspase-8) were mixed in caspase reaction buffer. Aminomethylcoumarine (AMC) liberation was detected after 1 h incubation at 37°C using a Fluoroskan Ascent FL plate reader with excitation at 355nm and emission at 460 nm.

3.11. DNA fragmentation

Oligosomal DNA fragmentation was detected by agarose gel electrophoresis. After pretreatment and UV irradiation cells were centrifuged and pellets resuspended in sample buffer. Agarose (2%) was made in a horisintal gel suppurt, and after solidification the top of part was repleaced with 1% agarose contained SDS and proteinase K. Cells were loaded in 15 µl sample buffer. Electrophoresis was carried out at 35 V for O/N and the gel was stained by ethidium-bromid. After washing the DNA signals were detected using a FluorChem FC2 Imager.

3.12. LDH release assay

Lactate dehydrogenase released from damaged cells was measured with a commercial kit. Briefly, supernatants were transferred to 96-well flat bottom plates. Detection reagent (100 µl) was added, plates were incubated (30 min) and the absorbance of samples was measured at 490 nm using a Multiskan MS plate reader.

3.13. Immunocytochemistry

PAR polimer and cleaved PARP-1 were detected by immunocytochemistry. Cells were fixed in ice-cold methanol for 10 minutes at -20°C and were rehydrated with 4 washes in PBS. After blocking endogenous peroxidase with 0.5 % H_2O_2 in methanol, non-specific binding was blocked by incubating coverslips in 1% BSA/PBS for 1 h. The cells were incubated for 1.5 h at room temperature with the primary antibodies: a monoclonal anti-PAR antibody (clone 10H, in 1:500 dilution) and a monoclonal cleaved hPARP-1 (in 1:100 dilution). After washes in PBS, coverlips were incubated for 30 minutes at room temperature with peroxidase-conjugated rabbit/mouse secondary antibody. After washes, the peroxidase reaction was developed with 3,3-diaminobenzidine (DAB) substrate. Coverslips were viewed with a Zeiss Axiolab microscope. Pictures were taken with a Zeiss Axiocam digital camera.

3.14. May-Grünwald-Giemsa staining

NHEKs were washed with phosphate-buffered saline (PBS), and fixed with prechilled methanol at -20ºC for 20 min. After being washed with distilled water, the cells were incubated for 30 min with freshly diluted May-Grünwald-Giemsa solution and then washed with distilled water. After drying, coverslips were mounted in Mowiol and viewed with a Zeiss Axiolab microscope.

3.15. Mitochondrial membrane potential

The cells were seeded into black 96-well plates. After the pretreatment and UVB irradiation, the incubation time was 8 hours. Then the cells were loaded with 100 nM tetramethylrhodamine ethyl ester for 20 min at 37° C. Cells were then washed with PBS, and TMRE fluorescence was measured with a fluorescence spectrophotometer (ex 535 nm; em 590 nm).

3.16. ^{3}H-NAD$^+$ incorporation Assay

PARP activity was determined by an assay based on incorporation of isotope ^{3}H-NAD$^+$ from cell lysate and TCA precipitate. After H_2O_2 treatment cell culture medium was repleaced by assay buffer that contained the isotop. After 30 min incubation cells were collectet and ice-cold TCA was added. Pellets were solubilized in SDS/NaOH at 37º C. Radioactvity was determined using liquid scintillation counter.

3.17. Spectroscopy

Absorption spectrum of PJ-34 (dissolved in PBS) was measured with a Thermo Scientific Multiskan GO UV/VIS spectrophotometer. The NMR measurements were performed at 298 K on a Bruker AVANCE II spectrometer operating at 500.13 MHz proton resonance frequency. PJ34 (1 mg) was dissolved in 500 µl DMSO-d6 in order to make all proton signals - including those of exchangeable NH- and OH-protons as well - detectable.

3.18. Statistical analysis

All experiments were performed at least three times on different days. All the values shown in the figures are expressed as mean±S.E.M. of n observations where n represents the number of observations. Intergroup comparisons involved one-way ANOVA followed by Tukey's test. A p-value less than 0.05 was considered as significant.

4. RESULTS

4.1. The PARP indepentent protective effect of 3-AB against UVB or UVA induced cell death

4.1.1. Silencing of PARP-1 sensitizes HaCaT cells to UVB induced cell death

PARP-1 was silenced by siRNA in HaCaT cell line. PARP-1 expression and activation after H_2O_2 treatment was detected by Western blot analysis. Based on the results of two experiments we accepted the efficiency of the silencing so the following effects caused by PARP-1 depletion in our opinion.

0,6 J/cm^2 UVB irradition was the lowest dose which significantly decreased the viability of HaCaT cells. The PARP-1 silencing significantly increased this phototoxic effect, so the viability was lower in PARP-1 depleted cells comapred to siCTL cells.

4.1.2. Opposing effect of PJ-34 and 3-AB on UVB -induced toxicity

First we demonstrated that low dose of UVB casused significant cytotoxicity in NHEKs as determined with MTT viability assay. We set out to investigate the role of PARylation in UVB induced cell death. Only the weak PARP inhibitor 3-AB but not the the more potent PJ-34 provided protection from UVB induced suppression of viability. At the dose of 0.1 J/cm^2 PJ-34 slightly sensitized the NHEKs to UVB induced cell death similarlly to PARP-1 silencing.

10 days after UVB irradiation we determined the proliferation capacity of the NHEKs: (i) the proliferation capacity at 0.025 J/cm^2 was significantly decreased. (ii) this decreasing was stronger in PJ-34 treated cells (iii) 3-AB had a protective effect against all of UVB doses.

4.1.3. 3-AB inhibits both apoptotic and necrotic cell death in UVB -irradiated kertinocytes

Morphology of May-Grünwald–Giemsa stained cells indicated that 0.2 J/cm^2 UVB irradiation caused apoptosis as indicated by smaller, condensed, picnotic nuclei. PJ-34 sensitized cells to UVB-induced cell death as morphological alterations such as condensed apoptotic cells and also swollen necrotic cells appeared at lower UVB dose (0.1 J/cm^2). Pretreatment of cells with 3AB prevented these morphological alterations. Activity of caspases-3-like proteases, general marker of apoptosis could be clearly demonstrated by immunodetection of cleaved PARP-1. PJ-34 sensitized cells to apoptosis, whereas 3-AB prevented it. Increased plasma membrane permeability as indicated for example by LDH release is a sign of necrotic cell death. UVB treatment caused a dose-dependent LDH release

from keratinocytes, which showed an overlapping pattern with the loss of viability. Whereas PJ-34 had no effect on LDH release, pretreatment with 3-AB completely abolished plasma membrane disruption.

4.1.4. 3-AB prevents mitochondrial and decreased the H_2O_2 production in UVB-irradiated kertinocytes

UVB induces ROS production leading to mitochondrial dysfunction. Here we showed out that UVB radiation triggered the production of H_2O_2 as determined by Amplex red assay.

Moreover, UVB also induced mitochondrial depolarization. Both H_2O_2 production and the mitochondrial alterations were effectively blocked by 3-AB but were not (or only very slightly) affected by PJ-34.

4.1.5. The photoprotective effect of 3-AB is not restricted to UVB radiation

In order to find out whether our findings are restricted to UVB-irradiated cells, we have also investigated the effect of 3-AB in the UVA response of keratinocytes.

The protective effect of 3-AB was determined in UVA induced cell death as well. Morphological alterations such as cell compaction or swelling were detected at higher UVA doses, but 3-AB treatment enhanched the viability and supressed these morfological alterations. Significantly higher apoptoic (cleaved PARP-1) and necrotic (LDH release) parameters could be detected only at higher UVA dose, but 3-AB treatment significantly decreased these parameters levels. The H_2O_2 level was significantly high in all of UVA doses, but 3-AB slightly decreased them.

4.1.6. The protective effect of 3-AB is probably a PARP independent mechanism

In order to elucidate the mechanism for the differential effects of the two PARP inhibitors we have also tested the effects of two additional potent and specific PARP inhibitors TIQ-A and DPQ. Similarly to PJ34, TIQ-A and DPQ also failed to protect keratinocytes from UVB-induced toxicity. In fact all three potent PARP inhibitors slightly but significantly sensitized cells to UVB-induced cell death. These data indicate that the photoprotective effects of 3-AB are not due to PARP inhibition.

4.2. Caracteristics of photogenotoxic effect of PJ-34

4.2.1. Low dose of UVA does not decreases the viability of A431 cells

In order to investigate the role of PARP-1 in photochemotherapy, we chose A431 skin carcinoma cells (a common model cell line in PCT research). In first step we determined the non-lethal dose of UVA in case of A431 cell line. Using increasing doses of UVA (2.5 – 10 J/cm^2) and different incubation times (16-24-48 h) after the irradiation we showed out that the 2.5 J/cm^2 UVA dose had no significant toxic effect on the cells even after 48 h incubation. So we choosed it for further experiments in our PCT model.

4.2.2. PARP-1 is not a key regulator in our PCT model

PARP-1 expression was silenced using by siRNAs in A431 cells. The silencing efficiency of siRNAs in A431 cells was confirmed by a dual approach. On the one hand, we have detected PARP-1 protein levels by Western blotting. We have also used a functional test by treating cells with H_2O_2 in order to induce DNA breakage and detected PAR polymer formation as a sign of PARP-1 activation. This latter approach also confirmed that our siRNA treatment was effective as PAR polymers could not be detected in H2O2-treated cells when PARP-1 was silenced. In order to set up a photodynamic model we chose the photosensitizer compound 8-methoxypsoralen (8-MOP) and pretreated the cells with the photosensitizer prior to irradiation with the non-toxic UVA dose (2.5 J/cm^2). The combination of photosensitizer and UVA irradiation caused a near 50% drop in viability. Interestingly, silencing PARP-1 had no significant effect on cell viability in this PCT cell death model.

4.2.3. PJ-34 and Veliparib PARP inhibitors was used in same concentrations in our PCT model

In order to find the most effective dose of the two PARP inhibitors we measured the PARP enzyme activity by ^{3}H-NAD incorporation assay. H_2O_2 treatment causes PARP activation and PAR polimer accumulation which was failed completly by 10 μM of PJ-34 and 1 μM Veliparib. We got similar results with Western blot analysis after H_2O_2 treatment. Due to the comparability is importnt the two PARP inhibitors was used in same concetration (10 μM) in our experiments.

4.2.4. The PARP inhibitor PJ-34 acts as a photosensititzer and increases the phototoxicity of PCT treatment

In order to further confirm that PARP-1 is not an important regulator of cell death in PUVA-induced cytotoxicity, we also used two common PARP inhibitors PJ-34 and Veliparib.

Cells were pretreated with PJ-34 (10 μM) or Veliparib (10 μM) followed by either the standard irradiation protocol with 2.5 J/cm^2 UVA or combined by 8-MOP. Interestingly, PJ-34 sensitized cells to low dose of UVA and also to UVA+8-MOP treatment. It is noteworthy that the photosensitizing effect of PJ-34 was as potent as that of 8-MOP. In the dark control samples (cells kept in the dark without UVA radiation) PJ-34 had no toxic effect. In contrast to PJ-34, Veliparib a same potent PARP inhibitor had no sensitizing effect to UVA. From the data obtained with the PARP-1 silenced A431 cells and those treated with Veliparib we concluded that PARP-1 is not a major player in PCT, but the PARP-1 independent photosensitizing effect of PJ-34 is worthy of investigation.

4.2.5. The photosensitizing effect of PJ-34 can also be detected in shperoids

In order to test whether or not the photosensitizing effect of PJ-34 is restricted to 2D cultures, we managed to grow spheroids from A431 cells and repeated the photosensitization experiments. We found that under special culture conditions A431 cells form spheroids PJ-34 sensitized cells to otherwise non-toxic UVA doses, while the Veliparib had no such effect.

4.2.6. The phototoxic effect of PJ-34 is not supressed by antioxidants

We have investigated whether or not PJ-34 raise ROS production and to what extent this may explain the photosensitizing effect of the compound. The A431 cell were pretreted with PARP inhibitors and with two different antioxidants, trolox and ascorbic acid. We detected ROS production using the ROS-sensitive fluorescent dye immediately after the UVA irradition. 16 h after UVA irradiation the cell vibility was meaured by MTT assay.

We found that the antioxidants Trolox and ascorbic acid could not prevent phototoxicity of PJ-34+UVA despite efficiently scavenging UVA or PJ-34+UVAinduced ROS production. Moreover, the fact that ROS production was significantly but not dramatically higher in PJ-34 UVA-treated cells compared to UVA-irradiated cells may also raise the possibility that the photosensitizing effect of PJ-34 is not due to increased ROS production.

4.2.7. DNA damage accompanies the photogenotoxic effect of PJ-34

In order to better understand the mechanism of the photosensitizing effects of PJ-34 in UVA-irradiated A431 cells, next we determined DNA damage in the irradiated cells. We found that at this subtoxic UVA dose no obvious sign of DNA strand breakage could be revealed with the alkaline single cell gel electrophoresis assay (comet assay). However, in the presence of PJ-34, marked DNA breakage could be observed both immediately and 1 h after UVA treatment, which was absent in Veliparib-pretreated cells. Moreover, detection of phosphorylated H2AX histone variant (γH2AX) is a commonly used indicator of DNA damage triggered by photosensitizers. We found γH2AX accumulation in PJ-34 pretreated and UVA-irradiated cells while the applied UVA dose alone or in combination with Veliparib had no such effect. In summary, of these two experiments it can be stated that DNA damage occurs in the presence of PJ-34 if cells are exposed to a subtoxic dose of UVA. In the timeframe, the investigated DNA damage proved stable.

4.2.8. UVA – irradiated A431 cells die by apoptosis in the presence of PJ-34

In order to characterize the cell death induced by PJ-34+UVA treatment, apoptotic parameters were investigated. We found that 4 h after irradiation caspase-3 which is a marker of apoptosis is activated in UVA irradiated cells if PJ-34 is present, but Veliparib had no such effect. In the PJ-34+UVA-treated samples caspase activity was further enhanced at 8 h and the activity was same high even 16 h after irradiation. Increased caspase-3-like activity was also evidenced by detecting PARP-1 cleavage. The 89 kDa cleavage product of PARP-1 was detectable at 4 h after irradiation with its abundance increasing even further at 8 h and 16 h.

Activation of caspase-8, an initiator caspase of the extrinsic apoptotic pathway could also be detected as early as 4 h after irradiation with further significant increases at 8 h and 16 h. Internucleosomal DNA fragmentation could also be detected in our UVA irradiation model but only in the presence of PJ-34 and not in the presence of Veliparib. A marked necrotic component could not be seen to accompany apoptosis.

4.2.9. PJ-34 + UVA induced cell death can be suppressed by different caspase inhibitros

Similarly to caspase- 3 increased caspase-8 activity was measured 4 h after PJ-34 +UVA treatment. In case of dark control cells, only UVA irradiated and Veliparib+ UVA treated cells there was no detectable significant caspase – 8 activity. The active role of caspases in the cell death process initiated by PJ-34+UVA treatment is supported by our observations that the pan-

caspase inhibitor z-vad-fmk, the caspase-3 inhibitor z-DEVD-fmk as well as the caspase-8 inhibitor z-IETD-fmk protected A431 cells from the toxicity.

4.2.10. PJ-34 and Veliparib do not lose their PARP inhibitor ability under the UVA irradiation

Inhibition of PAR accumulation by PARP inhibitors was measured with Western blot analysis after H_2O_2 treatment. There was maintained either by PJ-34 and Veliparib in both UVA irradiated and not irradiated cells. Based on the result of this experiment we can conclude: (i) low dose of UVA does not cause PARP activation or single DNA breaks. (ii) PARP inhibitors in same concentration effectively decreased the PARP activation and PAR polimer accumulation following the H_2O_2 treatment in the UVA irradiated cells as well.

4.2.11. UVA irradiation induces structural change in the PJ-34 molecule

Moreover, it appears likely that UVA irradiation triggers a structural change in the PJ-34 molecule as indicated by the altered absorption spectrum and NMR spectrum of the PARP-independent photosensitization by PJ-34 compound recorded before and after UVA irradiation. Based on the new resonance signals appearing in the aromatic (7-9 ppm) and phenolic -OH (11-11.5 ppm) regions, tautomers (ca. 20-30% of PJ-34) with extended conjugated pi-electron system are likely to be formed.

5. DISCUSSION

5.1. The protective effect of 3-AB against UVB and UVA irradiation is a PARP independent mechanism

The original aim of our first project was to investigate the possible role of PARP-1 in UVB induced cell death. UVB usually cause DNA damage, which is a well known PARP enzyme activator effect so it was a logical hypothesis that PARP-1 has a role in the reapair of UVB induced photolesion furthermore in cell death or in cell survivial.

Silencing of PARP-1 by siRNA was significantly increased the UVB photoxicity on HaCaT cell line. For further experiments was used normal human epidermal keratinocytes cells. In this case the PARP-1 depletion was reached by inhibitors. The two PARP inhibitors what was used in our experiments were the 3-AB and the more potent PJ-34. Their mechanism is same, so they are NAD^+ anlogues so can bind to the catalitic domain of enzyme. Similarly to the result of the previous experiment on HaCaT cells PJ-34 sensitized the NHEKs to UVB irradiation in viability and in proliferation assay as well. However 3-AB protected very well the NHEK cells against UVB induced cell death and significantly suppressed the irradiation induced proliferation rate decreasing. Not only PARP-1 silencing or PJ-34 had a photosenstizing effect but two other specific PARP inhibitors (TIQ-A, DPQ) also increased the photoxic effect of UVB irradiation. We conclude that 3-AB had a PARP independent protective effect. Appearance of Cleaved PARP-1 fragement was detected in UVB irradiated cells but also could be detected in UVB + PJ-34 treated cells at lower doses. In 3-AB treated cells there was no detectable PARP-1 fragment, so 3- AB absolutely inhibeted the UVB induced apoptotic cell death.

In case of necrosis we got a similar results. PJ-34 had no effect to this parameter's changes, but in the 3-AB pretreated cells the LDH release was significantly lower compare to the only UVB irradiated cells. We can conlude that 3-AB has a protective effect against UVB induced necrosis as well. PARP inhibition by PJ-34 had no effect to the UVB induced mitocondrial depolarisation, but 3-AB siginificantly decreased this photoeffect. Similarly to hydrogen-peroxid production after UVB irradiation. Based on the literature it is possible that 3-AB can act as free radical scvanger. However in our experiments we did not find any evidence of it, but this is the most likely explanation.

3-AB had protective effect on NHEKs after UVA irradiation as well. It increased significantely the viabilty, inhibited the apoptosis and necrosis and suppressed the hydrogen-peroxide production.

In our experiments we found that PARP -1 depletion and farmakological inhibition by PJ-34 sensitized the HaCaT or NHEKs to UVB induced cell death, 3-AB PARP inhibitor had a protective effect against UVB or UVA induced cell death as well, on both of skin cell type.

3-AB can absorb UVB and lesser extent UVA light, so theoretically it can be a physical sunscreener. There are some information that 3-AB can act like a scavanger or antioxidant. These data and our findings indicate that 3-AB can be an effective sunscreener, which works by a PARP independent mechanism.

5.2. PARP independent phosensitizing effect of PJ-34

The role of PARP-1 in PCT induced cell death is not well known yet. Our PCT model was PUVA treatment which is a combination of a psoralen and UVA irradiation on A431 cell line.

Our hypothesis was that PARP inhibition would increase the cytotoxicity of PUVA treatment by suppression of DNA repair. This theorie was faild in the early stage of our project, because PARP-1 depletion by siRNA on A431 cell line did not sensitize the cells to different doses of UVA irradiation. Lack of PARP-1 did not enhance the 8MOP+UVA induced loss of viability. We conclude that PARP-1 is not a key regulator of this process. To confirm these result we used farmakological inhibition of PARP by two different compound: PJ-34 and Veliparib. Similarly to PARP – 1 scilencing Veliparib did not sensitized the A431 cells to low dose of UVA irradiation. Surprisingly PJ-34 had an opposite effect so increased the phototxicity of PUVA treatment and it had photsensitizing effect combined by UVA irradiation.

To understand this mechanism, important to note that two inhibitors do not lose their PARP inhibitor ability during UVA irradiation. PAR polimer accumulation following the hydrogen-peroxide treatment was suppressed by both of PARP inhibitors presence or absence of UVA irradiation as well.

We showed out that the PJ-34 photosensitizing effect is not limitied only at monolyer cell cultur but it work on 3D spheroid culture as well.

It is a well known data that UVA irradiation induces ROS production, in our experiments this ROS level was increased by PJ-34 significantly but Veliparib had no effect to this parameter. The trolox and ascorbic acid suppressed the ROS level in only UVA irradiatiated and Veliparib/PJ-34+UVA treated cells very well, but they had not effect to the PJ34+UVA induced loss of viability. We conclude that PJ-34+UVA induced cell death is probably an oxidative stress independent process. In the literature we can find some example that antioxidants were uneffective in a ROS dependent PDT agent induced photoxicity.

Single DNA breaks could be detected immediately after irradiation but only in the PJ-34 pretreated cells. Furthermore these DNA single breaks were detectable 1 h later after irradiation. H2AX phosphorilated proteins were only in the PJ-34 +UVA treated cells. Only UVA irradiation or Veliparib + UVA did not induce DNA breaks. There are several publication about H2AX phosphorilation where this postranslational proten modfication was used as a marker of DNA double strand breaks caused by a photosensitizator. There is an other possible background of H2AX phosphorilation during a PDT or PCT treatment. γ-H2AX could be an indirect marker of DNA double strand breaks because the by the place of the photolesion in DNA the replication fork arrested and this step induce the phosphorilation of histon.

In our model PJ-34 +UVA caused in a short time DNA breaks or damage, which are not repaired even 8 h after the irradiation. We can not know this is depend on the level of DNA damage or is it in context the PARP inhibitiory ability of PJ-34.

Apoptotic parameters was unchanged in our model in only UVA irradiated or Veliparib+UVA treated cells. PJ-34 +UVA irradiation signficantly increased the caspase-3 effetor caspase enzyme activity, so celaved PARP-1 fragments could be detected as well.
Moreover DNA fragmentation was identified in these sample 4 hours after UVA irradiation. It can indicate this DNA fragmentation was made due to increased activity of caspase-3 but it can caused by DNA break inducing effect of PJ-34 + UVA treatment. One of iniciator caspase the caspase -8 activity was significantly increasing after UVA+PJ34 treatment. It can indicate that cell deth receptor mediated apoptosis is a part of cell death pathway. The used specific caspase inhibitors protected signifcantly but not cempletely against PJ-34 +UVA induced cell death. This means that probably more than one or two cell death pathway is swiched on in this process. There are many study where have shown that a PDT o PCT treatment can activate different cell death pathways.

As we showed out by NMR spectroscopy the UVA irradiation induces modification in stucture of PJ-34. We suggest that this modofication cause that PJ-34 act as a photosensitizer combined with UVA. Based on our finding it is an PARP independent mechanism, but we suggest that PJ-34 PARP inhibitory ability is contribute to the serius phototxic effect of this compound by the inhibiton of DNA repair. Several open questions remain to be answered about the photosensitization effect of PJ-34 must be characterized in more detail and various different types of cancer cell lines should be tested for sensitivity to PJ-34+UVA treatment. Furthermore, the in vivo efficiency of the compound should also be verified.

6. CONCLUSIONS

6.1. The PARP indepentent protective effect of 3-AB against UVB or UVA induced cell death

1. UVB irradiation increases the viability of HaCaT and NHEK cells by dose dependent manner.

2. The scilencing of PARP-1 in HaCaT cells and farmakological inhibition of the enzyme activity in NHEKs sensititised them to UVB irradiation.

3. 3-AB has a protective effect against UVB and UVB probably by a PARP independent mechanism.

4. 3-AB treatment prevent the apoptotic and necrotic cell death as well. Morover can decrease the UV induced H_2O_2 production.

6.2. Caracteristics of photogenotoxic effect of PJ-341

1. PARP-1 scilencing or inhibition by Veliparib do not sensitize the A431 cells to low dose of UVA or not influence the phototoxic effect of PUVA treatment.

2. PJ-34 PARP inhibitor triggers the cytotoxic effect of 8-MOP+ UVA but it acts as a photosensitizer combined by low dose of UVA.

3. PJ-34+UVA causes DNA breaks and damages probably directly manner not due to reactive oxigen species.

4. PJ-34 + UVA treatment induces apoptosis, which can be suppressed by caspase inhibitors.

5. UVA irradiation induces modification in stucture of PJ-34 as we showed out by NMR spectroscopy.

7. SUMMARY

Poly(ADP-ribose) polymerase – 1 (PARP-1) has diverse biological functions including has an important role in different types of DNA repair mechanisms: base excision repair, nucleotid excision repair, homologous recombination and in non-homologous recombination system. In these mechanisms the role of PARP has been investigated, but it is still not fully elucidated.

The sunlight of different wavelength can cause many types of DNA damage in the exposed cells. These DNA photolesions can be starting points of different types of skin cancer if the DNA repair is not complete. In the first part of the project we aimed to investigate the possible role of PARP-1 or PARylation in UVB-induced cell death. We found that specific silencing or pharmakological inhibition of PARP-1 sensitized HaCaT cells or normal human epidermal keratinocytes to UVB irradiation. This sensitizing effect likely stems from the decreased DNA repair caused by PARP silencing or inhibition. We showed that the PARP inhibitor 3-aminobenzamide (3-AB) has a PARP inpendent protective effect against UVB or UVA irradiaton. It provides short- and long-term protection to primary keratinocytes against UV-induced necrosis and apoptosis as well. Moreover, our findings indicate that the protective effect of 3-AB has a PARP independent mechanism and data suggest that a sunscreen-like effect or presumable radical scavenger activity of 3-AB contributes to the protection.

A combination of a psoralen (P) with light of matching wavelength (UVA) is a common treatment modality in various diseases including psoriasis. PUVA treatment can be mutagenic due to PUVA-induced DNA damage. The scilencing of PARP-1 and pharmacological inhibition by Veliparib had no significant effect to the cell viability during UVA irradiation or PUVA treatment. Surprisingly, PJ-34 triggered the phototoxic effect of PUVA and it acts as a photosensitizer when combined with low dose of UVA. This phototoxic effect includes DNA single and double strand breaks and induction of apoptosis. Specific caspase inhibitors significantly but not completely decreased the phototxic effect of PJ-34+UVA treatment. Irradiation of PJ-34 with UVA caused changes both in the UV absorption and in the 1H NMR spectra of the compound. These structural changes do not influence the PARP inhibitory ability of PJ-34 but probably it increases its photoxic effect.

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List of publications related to the dissertation

1. **Lakatos, P.**, Hegedűs, C., Salazar Ayestarán, N., Juarranz, Á., Kövér, K. E., Szabó, É., Virág, L.: The PARP inhibitor PJ-34 sensitizes cells to UVA-induced phototoxicity by a PARP independent mechanism.
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