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

# Investigation of UVB-induced cellular mechanisms in human keratinocytes using a novel approach of delivering *in vitro* synthesized mRNA encoding cyclobutane pyrimidine dimer-specific photolyase

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF HEALTH SCIENCES

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| Head of the Examination Committee: | Róza Ádány MD, PhD, DSc |
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| | Zoltán Balajthy PhD |

The Examination takes place at the Conference Room in the Building of the Faculty of Public Health, University of Debrecen 11:00 a.m., December 02, 2015

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The PhD Defense takes place at the Lecture Hall of Building A,
Department of Internal Medicine, Faculty of Medicine, University of Debrecen
13:00 p.m., December 02, 2015

# Introduction

Ultraviolet B (UVB) (280-315 nm) radiation is the main environmental risk factor for sunburn, skin carcinogenesis and premature skin aging. Major biological effects of UVB radiation are attributed to cyclobutane pyrimidine dimers (CPDs), which are the most frequently formed deleterious DNA photolesions. CPDs have been shown to induce inflammation, immune suppression and mutations that may lead to melanoma and non-melanoma skin cancers. However, so far, it has been unclear how CPDs change gene expression and cell activities. Until now, there was no suitable experimental platform to identify directly CPD-responsive genes in human cells, thus distinguish CPD-regulated cellular mechanisms from those mediated by other UVB-induced derivatives, including diverse photoproducts, reactive oxygen species, cross-linked protein-DNA and other damaged macromolecules.

The integrity of DNA can be quickly restored by a light-activated photolyase, which splits the CPDs using the energy of visible light in a process called „photoreactivation". However, this DNA repair enzyme is absent in placental mammals, including humans, that must rely on the slower and less accurate nucleotide excision repair (NER) system to repair UV-induced DNA-lesions. Our scientific idea was to transiently express functional CPD-specific photolyase in cultured human keratinocytes using a novel i*n vitro* transcribed (IVT) mRNA-based gene therapy method, as a platform to distinguish CPD-dependent and -independent events of UVB-induced cellular responses.

## Biological effects of ultraviolet radiation on skin cells

UV radiation is thought to be responsible for many pathological changes in the skin, including sunburn, photoaging and skin cancer. UV radiation induces harmful photochemical reactions causing damage in nucleic acids, lipids, proteins and altering the cell structures. The absorption of UVB photons by genomic DNA is one of the most important cell biological events upon solar radiation, because DNA absorbs UV light very effectively, it carries the genetic information for a cell and it is present the fewest copies in a cell. A variety of DNA photolesions, including cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs) are induced by UVB. The consequence is substantial change in the pattern of actively transcribed genes, including those involved in DNA repair, cell cycle regulation, cell growth, apoptosis and immune responses. UVB-induced DNA damage can also result in mutations thereby playing a critical role in skin tumorigenesis.

## The major UVB-induced DNA damages

*Cyclobutane pyrimidine dimers*

Cyclobutane pyrimidine dimers are the most frequently formed cytotoxic DNA photolesions. It forms in double-stranded DNA when two adjacent pyrimidine bases covalently bind to each other. The formation of CPDs at different dipyrimidine sites depends on the wavelength of UV light. UVA radiation has a poor efficiency in producing CPDs while UVB and UVC readily and effectively induce these photolesions, predominantly at thymine dimers (TTs). CPDs are mainly responsible for the cytotoxic and mutagenic effects of UV radiation in mammalian cells. The removal of CPDs from the mammalian genome is slow and incomplete. Accumulated CPDs induce cell cycle arrest in order to prevent the multiplication of mutated chromosomes, but may lead to induction of apoptosis, genomic instability or skin carcinogenesis. CPDs containing cytosine (CCs or CTs) have the most powerful mutagenic activity. This is primarily due to deamination of cytosine or 5-methylcytosine resulted in

deaminated CPDs which are bypassed by a DNA damage tolerance process.

*Pyrimidine (6-4) pyrimidone photoproducts*

The second most abundant UVB-induced photolesion formed in DNA is the pyrimidine (6-4) pyrimidone photoproduct, briefly called (6-4) photoproduct. These photoproducts produce greater distortion in the structure of DNA double-helix than CPDs. 6-4 PP plays a significant role in the toxic and premutagenic effects of UV radiation. Nevertheless, the removal of 6-4 PPs from the mammalian genome is more efficient compared to the repair rate of CPDs. The reason for this is not completely clear. The more pronounced DNA double-helix distorting effect of 6-4PPs might affect the efficiency of the DNA repair processes.

Timely repair of damaged DNA is critical to prevent the adverse effects of UV radiation. All living organisms have DNA repair mechanisms to eliminate UV-induced DNA photolesions.

**Repair of UVB-induced DNA photolesions**

*Nucleotide excision repair system*

Naturally, UV-induced DNA photoproducts from the human genome are eliminated by the versatile nucleotide excision repair (NER) system. NER is a multistep repair mechanism and more than 30 proteins are involved in the pathway. The main substrates of this complex mechanism are DNA photoproducts caused by UV radiation, however, NER is involved in the repair of numerous structurally different DNA damages indicating its versatility. NER also repairs DNA damage induced by chemical substances including chemotherapeutics that may cause inter– or intrastrand crosslinks of DNA, alkaloids produced by fungi and polycyclic aromatic hydrocarbons. NER plays an important role in removal of UV-induced photolesions and works closely with other proteins involved in DNA replication, transcription, cell cycle progression and alternative repair pathways such as base excision repair.

There are two NER subpathways. Global genome NER (GG-NER) and transcription-coupled NER (TC-NER) basically differ in the initial steps of DNA damage recognition and

recruitment of proteins to the lesions. In GG-NER, first activating enzyme complex, XPC/HR23B does not recognize the type of damage, but perceives distortion of the DNA double-helix. Accordingly, this complex itself is unable to recognize CPDs. 6-4PPs are recognized more efficiently due to the more pronounced DNA-distorting effect of these photolesions. In this subpathway, DNA damage binding (DDB) protein is essential for recognition of CPDs. In TC-NER, the first signal of DNA damage (e.g. CPDs and 6-4PPs) caused by UV radiation is the stalling of RNA polymerase II on the template strand of active genes. This initial step in TC-NER requires the action of CSA and CSB (Cockayne syndrome protein A and B) proteins instead of the combined action of XPC/HR23B. The subsequent actions in NER are the same in both subpathways. NER consists of six main interconnected steps: 1) recognition of DNA damage, 2) verification of DNA lesion, 3) DNA unwinding, 4) dual-strand incision, 5) excision of DNA fragment containing lesion and finally 6) DNA resynthesis and rejoining.

Inherited defects in NER genes result in photosensitive disorders, such as Xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy that are characterized by UV sensitivity of patients with high incidence of skin cancers and/or accelerated aging.

*Photoreactivation*

Photoreactivation is a light-dependent DNA repair mechanism which is carried out by photolyases. Photolyase is a structure-specific DNA repair protein that binds specifically to pyrimidine dimers, either CPDs or 6-4PPs, and converts them back to the original state using the energy of visible light. It contains two chromophores. The first one is a flavin adenine dinucleotide (FAD) which functions as electron donor and catalytic cofactor determining the enzymatic activity of photolyase. The second one is a folate-type (5,10-methylenetetrahydrofolate; MTHF), or a deazaflavin-type (8-hydroxy-5-deazaflavin; 8-HDF) chromophore, depending on the organism. These cromophores act as a light-harvesting

cofactor that increase more than 100-fold the visible light-absorbing capacity of photolyase. All known photolyases rapidly remove UV-induced DNA lesions securing the genomic integrity in a light-dependent cyclic redox reaction. Interestingly, the enzyme has been described in diverse organisms from archaebacteria to marsupials, but seems to be absent in placental mammals, including human.

**Advantages and difficulties of synthetic mRNA-based gene therapy method**

Gene therapy for the replacement of defective genes or for the expression of therapeutic proteins has made great progress in the last decade. After exploring numerous viral and non-viral-based systems for expressing the encoded proteins, *in vitro* transcribed messenger RNA (IVT mRNA) seems to be the most suitable tool for transient protein expression. It has many features that make mRNA-mediated gene transfer especially valuable for functional characterization of encoded protein. The transfected mRNA is translated with high efficiency in any cell, including primary and non-dividing mammalian cells. Importantly, when mRNA is delivered to the cell, only the encoded protein of interest is generated avoiding genomic integration, unlike other vectors, such as plasmids that contain sequences for additional proteins, or viral vectors that not only code for but also carry viral proteins into the cell. In addition, mRNA does not require nuclear localization or transcription to be functional, thus it can be safely used for gene transfer and expression. In spite of these advantageous properties, *in vivo* application of IVT mRNA is limited owing to its labile nature and the fact that it stimulates innate immune system through RNA sensors, including Toll-like receptors (TLR3, TLR7, TLR8). Pseudouridine (Ψ) modifications and HPLC (high-performance liquid chromatography) purification of RNA completely abolished immunogenicity of the RNA and makes it highly translatable. A newly established preparative HPLC purification procedure was critical to obtain IVT mRNA that is free of aberrant transcription products including short RNAs and double stranded RNAs that activate RNA sensors even in the presence of

pseudouridine or other nucleoside modification. IVT mRNA containing nucleoside modifications are currently used in a wide variety of fields, including cancer immunotherapy, vaccines, protein replacement, gene editing and genetic reprogramming.

The lipid-rich nature of the skin, the enhanced secretion of RNases on the skin surface and the *stratum corneum* are important limitations for IVT mRNA to development of *in vivo* skin therapeutic studies. Theoretically, CPD-photolyase mRNA applied topically on the skin might provide a new sun protection strategy with a potential to reduce acute sun damage and could serve as a prototype for expressing other specific proteins related to skin diseases.

# Objectives

It is well known that CPDs are the principal mediators of the harmful effects of UVB radiation. CPD-specific photolyase can rapidly and efficiently repair these DNA photoproducts after photoreactivation, however, this enzyme is absent in humans. Transfection of human keratinocytes with *in vitro* transcribed and nucleoside-modified mRNA encoding a marsupial CPD-specific photolyase seems to be an excellent model to study the cell biological effects of UVB with distinction between CPD-dependent and -independent cellular processes.

Our aims were:

1. To adjust the most appropriate experimental conditions for transfection of cultured human keratinocytes with IVT nucleoside-modified mRNA to achieve high yield translation of CPD-specific photolyase protein.
2. To investigate the effect of UVB pre-exposure on transfection efficiency.
3. To verify that the expressed CPD-specific photolyase is functionally active i.e. to demonstrate the rapid repair of UVB-induced CPDs in mRNA-transfected keratinocytes upon photoreactivation.
4. To determine UVB-induced CPD-dependent and -independent transcriptional responses in human keratinocytes combining the CPD-specific photolyase mRNA delivery with a genomics approach, namely microarray and pathway analysis.
5. To investigate which signalling pathway plays a role in conversion of the cues generated by UVB-induced CPDs into a transcriptional response.

# Materials and Methods

### *In vitro* transcription of pseudouridine-modified mRNA encoding CPD-specific photolyase

A codon-optimized photolyase gene from *Potorous tridactylus* (rat kangaroo) containing GC-rich codons for high translational yields was synthesized by a biotechnology service provider (Entelechon). The optimization increased the GC-content of the photolyase coding sequence (GenBank accession number: D26020) from 51.8% to 65.0%. Messenger RNAs (kindly gifted from Dr. Katalin Kariko, University of Pennsylvania, Philadelphia) encoding CPD-photolyase (CPD-PL Ψ-mRNA) and enhanced green fluorescent protein (eGFP Ψ-mRNA) were generated by *in vitro* transcription from linearized plasmids (pTEV-CPD-PL-A101 and pTEV-eGFP-A101). The Megascript T7 RNA polymerase kit (Ambion) was used for transcription, and UTP was replaced with pseudouridine triphosphate (TriLink). To remove the template DNA, Turbo DNase (Ambion) was added to the reaction mix. Pseudouridine-modified mRNAs were HPLC-purified and provided with cap1 generated by using the m7G capping enzyme and 2′-*O*-methyltransferase, according to the manufacturer's instructions (CellScript). The RNA was transcribed to contain an encoded 101-nt long 3' poly(A) tail, which was extended with ~ 300 nucleotides using poly(A) polymerase (USB). RNA samples were analyzed by agarose gel electrophoresis for quality assurance. Small aliquots of RNA samples were stored in siliconized tubes at −20°C.

### *In vitro* translation assay

To determine protein synthesis from the mRNAs an *in vitro* translation assay (Promega) containing aliquots of rabbit reticulocyte lysates supplemented with $^{35}$S-labeled methionine and cysteine was used. *In vitro* protein synthesis from the mRNAs was carried out by *Karikó Katalin et al.,* according to the manufacturer's instructions.

**Cell culture**

An immortal human keratinocyte cell line (HaCaT) was used in each experiment. HaCaT cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Lonza) with 4.5 g/L glucose and 2 mM L-Glutamine and supplemented with 0.5% antibiotic / antimycotic solution (Sigma-Aldrich) and supplemented with 10% fetal bovine serum (FBS) (Biowest) at 37°C in a 5% $CO_2$ atmosphere.

**Transient transfection of human keratinocytes**

HaCaT cells were seeded into 96-well plates at a density of $2 \times 10^4$ cells per well one day prior to transfection. Aliquots of RNA samples (0.25 µg) were complexed with 1.0 µl Lipofectamine LTX-PLUS (Life Technologies) in a final volume of 100 µl EpiLife (Life Technologies) and the complexed RNA was added to each well. Following a two-hour transfection, the lipofectamine-RNA complex was replaced with fresh culture medium.

**UVB irradiation and photoreactivation**

At 12 h post transfection, cells were covered with 50 µl DPBS (Life Technologies) and subjected to 20 mJ/cm$^2$ UVB using two broadband tubes (Philips). Immediately after UVB treatment, cells were either exposed to photoreactivating light (active CPD-photolyase) using two white fluorescent tubes (Havells-Sylvania) or kept in the dark (inactive CPD-photolyase) for one hour. Cells were further cultured in complete medium before harvest.

To investigate the effect of UVB pre-exposure on the transfection efficiency, HaCaT keratinocytes were first exposed to UVB (20 mJ/cm$^2$), then transfected with lipofectamine-complexed photolyase mRNA ninety minutes later. Immediately after mRNA transfection, cells were either exposed to photoreactivating light (active CPD-photolyase) or kept in the dark (inactive CPD-photolyase) for one hour. Cells were further cultured in complete medium before harvest.

**Fluorescence microscopy**

HaCaT cells were plated on glass coverslips and subjected to 20 mJ/cm$^2$ UVB or left unirradiated prior to transfection of the encoding Ψ-modified mRNA. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 1% Triton X-100 (Amresco). Before immunolabeling, cells were blocked in 20% FBS and then probed with CPD-photolyase-specific polyclonal antibody (kindly gifted from Prof. Gijsbertus van der Horst; Erasmus University Medical Center, Rotterdam) (1:500) at 4°C overnight. Goat anti-rabbit IgG Alexa Fluor 555 secondary antibody (1:2000; Life Technologies) was added at room temperature for 2 h. Nuclei were stained with DAPI (Vector Laboratories) and cells were analyzed by fluorescence microscopy.

For the analysis of CPDs, before blocking, cellular DNA was denatured in 2 M HCl (VWR) at room temperature for 30 min. The samples were probed with a primary anti-CPD monoclonal antibody (TDM-2, Cosmo Bio) at 37°C for 30 min. Alexa Fluor 568-conjugated anti-mouse IgG was used as secondary antibody (1:2000; Life Technologies). Cells were analyzed by fluorescence microscopy.

Expression of eGFP in HaCaT cells transfected with eGFP Ψ-mRNA was documented using an epifluorescent Zeiss Axiovert 100 microscope mounted with a Zeiss AxioCam MRc 5 digital camera with AxioVision imaging system.

In post-UVB mRNA transfection experiment, translated CPD-photolyase protein was monitored after transfection by fluorescence confocal microscopy using an Olympus FV1000S confocal microscope (Olympus Co.) equipped with 60×, oil immersion objective.

**CPD-specific enzyme-linked immunosorbent assay (ELISA)**

Genomic DNA was extracted by the QIAamp DNA mini kit (Qiagen), according to the manufacturer's protocol. Flat-bottom 96-well plates were precoated with 0.003% protamine sulfate (50 µl/well, Sigma-Aldrich) and incubated with denatured DNA (15 ng/well) at 37°C

overnight. The plates were washed with PBS containing 0.05% Tween-20 and incubated with 2% FBS at 37°C for 30 min to prevent non-specific binding of the antibody. Mouse anti-CPD monoclonal antibody (TDM2) diluted in PBS (1:1000) was added to each well and the plates were incubated at 37°C for 30 min. The plates were washed and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (1:3000, BioRad) at 37°C for 30 min. After washing, each sample were equilibrated with 150 µl of citrate-phosphate buffer (51.4 mM $Na_2HPO_4$, 24.3 mM citric acid monohydrate, pH 5.0), then substrate solution was added to each well (100 µl/well) containing 74 nM o-phenylenediamine (OPD) (Sigma-Aldrich) and 60 nM $H_2O_2$ in citrate-phosphate buffer. Fifteen minutes later, 2 M sulfuric acid (VWR) was added (50 µl/well) to stop the reaction and absorbance was measured at 492 nm using an ELISA microplate reader.

**Cell viability assay**

The CPD-photolyase mRNA transfected cells were subjected to 20 and 60 mJ/cm$^2$ UVB, followed by exposure to one hour visible light or darkness. Cell viability was determined by EZ4U assay (Biomedica Gruppe), according to the manufacturer's instructions.

**Microarray analysis**

Total RNA was isolated from HaCaT cells using TRI reagent (VWR) followed by DNase I (Fermentas) treatment. Gene expression profiling of HaCaT cells transfected with CPD-photolyase mRNA was performed using a service provider (ChromoScience, www.chromoscience.hu) using an Agilent 4x44 K Whole Human Genome Oligo microarray platform. Data were normalized by the Feature Extraction software version 9.5 (Agilent Technologies) and then transferred to GeneSpringGX 11.5 software (Agilent Technologies) for further statistical evaluation. Complete raw and normalized microarray data have been deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE65034. Genes showing statistically significant 2-fold change were selected for further study by

Ingenuity Pathway Analysis (IPA, www.ingenuity.com).

**Real-time quantitative RT-PCR analysis**

Total RNA was isolated from HaCaT cells using TRI reagent (VWR) followed by DNase I (Fermentas) treatment. RT was performed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), according to the manufacturer's protocol. RT reactions contained 500-500 ng of RNA. To quantitate the expression of candidate genes, the following TaqMan Gene Expression assays (Life Technologies) were used: *ATF3* (Hs00231069_m1), *CCNE1* (Hs01026536_m1), *CDKN2B* (p15INK4b) (Hs00793225_m1), *EGR1* (Hs00152928_m1), *ID2* (Hs04187239_m1), *IL-6* (Hs00985639_m1), *PTGS2 (*also known as *COX-2)* (Hs00153133_m1), *RUNX1* (Hs00231079_m1), *SNAI2* (Hs00950344_m1). To determine mRNA expression of *SNAI1* the following custom-designed primers and probe set were used: forward primer: 5'-ACT ATG CCG CGC TCT TTC-3'; reverse primer: 5'-GCT GGA AGG TAA ACT CTG GAT-3'; and the probe sequence: 5'-[6-carboxyfluorescein (FAM)] AAT CGG AAG CCT AAC TAC AGC GAG C [tetramethylrhodamine (TAMRA)]-3'. Quantitative PCR was performed on the ABI 7900 HT Sequence Detection System (Life Technologies). Relative RNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method in which expression levels in samples containing active CPD-photolyase were compared to those containing inactive one. *SDHA* (Hs00188166_m1) and *PGK1* (Hs00943178_g1) mRNA levels were used for normalization.

**Western blot**

Cells were lysed in RIPA buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Sigma-Aldrich). Proteins (10 μg) were separated on 10% (CPD-photolyase) or 12% (CCNE1 and p15INK4b) polyacrylamide gels and transferred to nitrocellulose membrane (BioRad). The membranes were blocked in 5% non-fat dry milk and incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-CPD-photolyase

polyclonal antibody (1:500, kindly gifted from Prof. Gijsbertus van der Horst), anti-CCNE1 (1:750, Cell Signaling Technology), anti-CDKN2B (1:750, Thermo Fisher Scientific) and anti-β-actin (1:1000, Cell Signaling Technology). Anti-mouse or anti-rabbit IgG conjugated with HRP was used as a secondary antibody (BioRad). The visualization of proteins was achieved with ECL Prime Western blotting detection system (GE Healthcare).

**Inhibition of protein kinase signalling pathways**

Keratinocytes at 85% confluency were pretreated with specific kinase inhibitors for 1 hour before UVB irradiation. Samples were harvested at 6 and 24 h after UVB exposure. Inhibitors were purchased from the following source: JNK (SP600125), p38 MAPK (SB203580), AKT (MK-2206) (Selleck Chemicals). All chemical agents were dissolved in DMSO (Sigma-Aldrich). Stock solutions were prepared according to the manufacturer's instructions and stored at –20°C.

**Statistical analysis**

Identification of genes differentially expressed in microarray experiments was carried out by the unpaired, Student's *t*-test followed by Benjamini-Hochberg correction. Statistical analysis of qRT-PCR data was performed using GraphPad Prism 5 software (GraphPad Software Inc.). The significance of differences in terms of mRNA expression comparing UVB-irradiated samples to non-irradiated ones, or UVB-irradiated plus photoreactivated (active CPD-photolyase) samples to non-photoreactivated (inactive CPD-photolyase) samples, was determined by the two-tailed, unpaired *t*-test. A *p* value of equal to or less than 0.05 was considered statistically significant. Each experiment was performed in triplicate obtained from three independently biological replicates.

# Results

### Synthesis of CPD-specific photolyase in human keratinocytes transfected with *in vitro* synthesized and pseudouridine-modified mRNA

HaCaT cells were transfected with lipofectamine-complexed pseudouridine-containing mRNA encoding CPD-photolyase or eGFP, which served as a control mRNA. The expression of eGFP was detectable as early as 1 h, peaked between 6 and 12 h, and its level gradually declined, but remained detectable even at 48 h after mRNA transfection. Significant amount of CPD-photolyase synthesized from the transfected CPD-PL Ψ-mRNA could be detected not only in unirradiated HaCaT cells, but also in UVB-pretreated cells at already 1 h after delivery of encoding mRNA. Importantly, the photolyase localized in the nuclei of keratinocytes. Strong expression signal of CPD-specific photolyase was detected at first in the cytoplasm followed by fading of the cytoplasm and increasing fluorescence of the nuclei.

Taken together, these results show that UVB irradiation of keratinocytes did neither reduce the efficacy of transfection nor influence the translation level of *in vitro* synthesized mRNA and, more importantly, confirm that CPD-photolyase accumulated in the nuclei where it can cleave cyclobutane pyrimidine dimers formed on the UV-damaged DNA.

### Accelerated removal of CPD photolesions in keratinocytes transfected with CPD-photolyase mRNA

To determine whether the CPD-photolyase is functional and capable of cleaving CPDs, the mRNA transfected cells were subjected to UVB (20 mJ/cm$^2$) followed by exposure to one hour visible light or darkness. Using CPD-specific antibodies, accelerated repair of these photolesions could be detected in CPD-PL Ψ-mRNA transfected cells that were subjected to photoreactivating light, while those deprived from this energy source or transfected with eGFP Ψ-mRNA contained high levels of CPDs.

To measure the amount of CPDs removed from the genomic DNA of the CPD-PL Ψ-mRNA

transfected keratinocytes immediately and at 5 and 23 h after exposure to visible light, CPD-specific ELISA was used. After UVB irradiation, we detected a 10-fold reduction of CPD lesions in CPD-PL Ψ-mRNA transfected cells subjected to photoreactivating light, as compared to those kept in the dark. A reduced amount of CPD lesions was measured in all irradiated samples at 24 h vs. at immediate and 6 h time points due to slower endogenous NER-mediated DNA repair.

Keratinocytes preexposed to a physiological dose of UVB (20 mJ/cm$^2$) were used to compare the efficacy of CPD removal by the natural nucleotide excision repair mechanism and the photolyase translated from the transfected CPD-photolyase mRNA. At 6 hours following UVB irradiation, CPD lesions were reduced 90% in CPD-photolyase expressing cells in response to photoreactivation. In comparison, it took more than 48 hours for the physiologic NER system to reduce the amount of CPDs by 90% in UVB-irradiated HaCaT cells.

**Rapid repair of CPDs in keratinocytes transfected with CPD-photolyase mRNA improves cell viability**

HaCaT cells were irradiated with 20 and 60 mJ/cm$^2$ UVB 12 h after delivery of Ψ-mRNAs and immediately exposed to photoreactivating light or left in the dark for 1 h. Cell viability, determined 24 h after UVB irradiation, demonstrated that photoreactivation significantly increased cell survival of CPD-PL Ψ-mRNA transfected keratinocytes compared to those that were kept in the dark or transfected with eGFP Ψ-mRNA.

**CPD-dependent changes were distinguished within the UVB-induced alterations of gene expression in human keratinocytes**

To determine the CPD-dependent gene expression profile of human keratinocytes exposed to UVB irradiation, an oligonucleotide microarray analysis was performed in which HaCaT cells transfected with CPD-specific photolyase mRNA were used. Expression level of 2,370 out of 41,000 genes were changed at 6 or 24 h after UVB exposure. Altered expression of 1,334

(56%) of UVB-regulated genes was restored in cells that contained active CPD-photolyase indicating that the changes in the expression of these genes were CPD-dependent. Importantly, more CPD-dependent gene expression changes (1,008 out of 1,334 genes) were observed at 6 h vs. 24 h, potentially due to the higher difference in CPD levels at 6 h comparing cells with active CPD-photolyase to those with inactive one. On the other hand, CPD-dependent genes represented more than half of the UVB-regulated genes (1,008 out of 1,743 genes (58%) and 326 out of 627 genes (52%) at 6 and 24 h after UVB irradiation, respectively) at both time points after the exposure.

**UVB-induced, CPD-dependent genes are associated with cellular stress responses**

To determine possible network interactions and associated biological functions of CPD-regulated genes Ingenuity Pathway Analysis (IPA) online software was used.

Using IPA prediction analysis, we determined that a majority of CPD-dependent gene expression changes at 6 and 24 h after UVB exposure is linked to decreased cell viability and apoptosis induction that is consistent with results obtained from cell viability assay. Interestingly, gene expression changes that were independent of CPDs seemed to influence the cell viability in the opposite direction at 24 h after UVB irradiation.

Analysing network interactions we found that a majority of CPD-dependent genes at 6 h after UVB exposure belongs to the regulatory network of *Gene Expression and Cell Cycle*, while those at the 24 h belong to the network of *Cell death and survival, Cellular Growth and Proliferation*. The two top-rated networks of CPD-regulated genes at 6 and 24 h after UVB irradiation were centered around two transcriptional regulators and proto-oncogenes *c-Myc* and *c-Jun*. In addition, two mediators of signal transduction *Smad4* and *TP53* were central nodes in the top network of CPD-dependent genes at 6 and 24 h after UVB exposure, respectively.

It is noteworthy that the expression of these key regulators, except *c-Jun*, was not changed at

mRNA level after UVB exposure, while the expression of many of their target genes was changed, moreover, in a CPD-dependent manner. Ten target genes were selected for further investigation based on the microarray data and their involvement in cell cycle regulation *(CCNE1, CDKN2B),* transcriptional regulation *(ATF3, EGR1, ID2, RUNX1),* epithelial to mesenchymal transition *(SNAI1, SNAI2)* and inflammation *(IL-6, PTGS2).*

**CPD-dependency of expressional changes of 10 genes selected on the basis of microarray data were confirmed by qRT-PCR**

To validate the microarray data, we employed real time quantitative RT-PCR analyses. We found significant upregulation of the expression of *ATF3, CCNE1, CDKN2B, EGR1, ID2, IL-6, PTGS2, SNAI1* and *SNAI2* and downregulation of the expression of *RUNX1* in response to UVB. Furthermore, presence of active CPD-photolyase significantly prevented the changes in the expression of all tested genes, thereby confirming the results of the microarray analysis.

**Overexpression of cyclin E1 (CCNE1) and p15INK4b (CDKN2B) proteins in response to UVB is dependent on the generation of CPD and activation of JNK pathway**

For further investigation, we selected two cell cycle-regulatory genes that were previously less characterized, related to their involvement in UVB-induced stress response. We have found that the expression of cyclin E1 and p15INK4b proteins were significantly increased at 24 h after UVB irradiation, but this increase was prevented by active CPD-photolyase.

To investigate the signalling pathways involved in modulation of cyclin E1 and p15INK4b expression upon UVB exposure, HaCaT cells were exposed to specific inhibitors of JNK, p38 MAPK or AKT followed by UVB irradiation. We determined the expression of cyclin E1 and p15INK4b proteins by western blot analysis. While suppression of p38 MAPK or AKT had no effect on UVB-induced increase in the levels of these proteins, we found that the treatment of cells with the JNK inhibitor abolished the induction of cyclin E1 and p15INK4b protein expression at 24 h after UVB.

## Discussion

*In vitro* synthesized mRNA has great therapeutic potential to transiently express physiologically important proteins. Here, we applied this novel mRNA-based platform to achieve functional non-human photolyase production and to gain insights into the pathogenesis of UVB-induced DNA damages in cultured human keratinocytes. The CPD-specific photolyase encoded by the mRNA resulted in rapid and efficient repair of UVB-induced CPDs upon photoreactivation regardless of mRNA transfection was applied before or after UVB exposure. We could verify that the encoded protein correctly located to the nucleus of human keratinocytes and was functionally active as it repaired 90% of CPD lesions within 6 hours in response to photoreactivating light. In our study, the physiologic NER required more than 48 hours to reduce the amount of CPDs by 90% in UVB-irradiated HaCaT keratinocytes, which markedly contrasts the rapid CPD removal facilitated by photolyase mRNA delivery. This experimental platform enabled us to distinguish between CPD-dependent and -independent cellular mechanisms induced by UVB irradiation. Transfection of HaCaT cells with CPD-photolyase mRNA led to a significant survival advantage indicating that CPDs are principal contributors to the UVB-induced decrease in cell viability. Furthermore, we confirmed that CPDs are the primary cause of transcriptional changes in response to UVB radiation.

Accumulated CPDs, especially in dividing cells, induce cytotoxic and mutagenic effects contributing primarily to UVB-induced carcinogenesis. Identification of the genes regulated by CPD photolesions might have clinical relevance, as it will facilitate defining novel targets for diagnosis or treatment of UVB-mediated skin diseases. To characterize the CPD-dependent and -independent cellular transcriptional responses to UVB irradiation microarray analysis was performed. Transcriptome analysis identified 1334 CPD-responsive genes, which represent more than 50% of the genes regulated by UVB at 6 and 24 h after the

exposure, indicating that CPDs are major source of altered gene expression induced by UVB irradiation. A significant number of these genes (738 and 250 genes at 6 h and 24 h after the exposure, respectively) were downregulated, most likely due to the stalling of RNA polymerase II and inhibition of transcription elongation caused by UVB-induced DNA damage. Furthermore, we found a markedly higher number of CPD-dependent genes to show altered expression levels at 6 h (1,008), as compared to 24 h (326) owing to the slower naturally-occurring DNA repair mechanism.

Comparative analysis of downstream effects of gene expression changes observed in CPD-dependent and -independent genes revealed that CPDs are key determinants of cell death and survival upon UVB exposure. We found that quick removal of CPDs by photoreactivation resulted in change of the gene expression pattern relating to cell death and survival at 24 h after UVB irradiation. A majority of CPD-dependent gene expression changes after UVB exposure could be linked to decreased cell viability and apoptosis induction, while gene expression changes that were independent of CPDs seemed to influence the cell viability in the opposite direction at 24 h after UVB irradiation. Accordingly, UVB irradiation significantly reduced the cell viability, while photoreactivation significantly increased cell survival of CPD-PL Ψ-mRNA transfected keratinocytes exposed to even high-dose UVB.

Top functional categories were determined among CPD-responsive genes, and we found that CPD-related gene expression changes were mainly associated with regulation of the transcriptional and cell cycle machineries in response to UVB irradiation in human keratinocytes. Recognition of DNA damage, including UVB-induced photolesions, can temporarily halt cell cycle progression, allowing time for cells to repair damages prior to replication. Unsuccessful repair of these photoproducts induces intracellular apoptotic signalling in order to prevent the multiplication of mutated chromosomes, but may lead to a permanent cell cycle block, genomic instability or skin carcinogenesis. Network analysis of

CPD-responsive genes revealed *c-Jun*, *c-Myc*, *Smad4* and *TP53* as the major regulating factors in CPD photolesion-induced cellular stress responses. The importance of these multifunctional transcription regulators in cell cycle progression, apoptosis and cellular transformation is well known. Several studies demonstrated that germline mutations or chemical inactivation of these key mediators, could result in perturbations of cell cycle checkpoint control leading to the transformation of cells and have been associated with a variety of tumours. Based on our microarray data, there was no detectable change in their expression at mRNA level upon UVB exposure, except *c-Jun*, but the expression of many of their target genes has been changed in a CPD-dependent manner. These findings indicate that CPDs significantly contribute to UVB-induced cellular stress responses through regulation of target genes of these key regulatory molecules. We validated the microarray results for six transcriptional factors, *ATF3, EGR1, ID2, RUNX1, SNAI1* and *SNAI2*, and two apoptosis and –inflammation-related genes, *COX-2* and *IL-6*, which could serve as novel biomarkers for evaluation of the involvement of UVB in various photosensitive skin disorders or skin cancers. The most studied genes associated with UVB-mediated cellular processes are cyclooxygenase 2 *(COX-2)* and interleukin 6 (*IL-6*). Their upregulation is described as a diagnostic marker and predictive factor in many tumours and other diseases related to chronic inflammation.

Furthermore, we identified two CPD-dependent cell cycle-regulatory genes, *CDKN2B* and *CCNE1* that are involved in regulation of the G1 phase of cell cycle with less established roles in UVB-mediated cellular damage in keratinocyte biology. We showed that the CPD-photolyase mediated repair of UVB-induced CPDs prevented increased expression of *CDKN2B* (p15INK4b) and *CCNE1* mRNA and protein. It is well known that p15INK4b is a cyclin-dependent kinase inhibitor (CKI) that is related to cell cycle arrest in early to mid G1 phase of the cell cycle, whereas cyclin E1 promotes cell proliferation at the late G1 phase to S

transition. Despite the apparently opposite function of *CDKN2B* and *CCNE1*, we found that UVB irradiation triggered overexpression of both genes in human keratinocytes. However, the effect of a cell cycle-regulatory gene on cell cycle progression is highly dependent on cellular levels of other regulatory molecules and their posttranslational state. Taken together, our data strongly suggest the high relevance of CPDs in disruption of cell cycle progression.

The question of how CPD photolesions activate intracellular signalling and how they modulate the expression of genes in cellular mechanisms (e.g. cell cycle arrest) in response to UVB irradiation has not been answered. UV radiation activates different signal transduction pathways in a wavelength- and dose-dependent manner. These signalling pathways are regulated by specific protein kinases, including mitogen-activated protein kinases (MAPK), protein kinase B (PKB or AKT) and protein kinase C (PKC) that play a crucial role in the response network to skin damage caused by UV exposure. We examined how specific inhibitors of protein kinases, such as AKT, p38 kinase and JNK, affect gene expression changes caused by CPD photolesions. We found that only the JNK-specific inhibitor influenced UVB-induced overexpression of cyclin E1 and p15INK4b proteins. Our findings indicate an important role for JNK in the control of gene expression modulated by CPD photolesions. Activation of JNK is triggered by a variety of extracellular stimuli e.g., by UV radiation, and followed by nuclear translocation of the activated form. Multiple transcription factors including *c-Jun*, *c-Myc*, *Smad4* and *TP53* that were identified as central regulatory molecules in the top network of CPD-dependent genes after UVB exposure are known to be phosphorylated by active JNK. It has been reported that active JNK is mainly associated with keratinocyte proliferation and differentiation. Enhanced JNK activity was observed in psoriatic and wound-healing epidermal cells, as well as in cylindromas and other hair follicle derived tumours.

## Summary

We presented an original approach for studying the cellular mechanisms of UVB-induced DNA damage in cultured human keratinocytes using *in vitro* transcribed and nucleoside-modified mRNA encoding cyclobutane pyrimidine dimer (CPD)-specific photolyase.

Messenger RNA encoding CPD-photolyase of *Potorous tridactylus* was generated by *in vitro* transcription. Superior translation and non-immunogenic nature were achieved by codon-optimization, pseudouridine modification, long polyA-tail and cap1 structure incorporation, and HPLC purification.

It could be proved that the nucleoside-modified mRNA encoding non-human CPD-specific photolyase can be effectively delivered into human keratinocytes, the translated photolyase protein accumulated in the nuclei of cells, and it was functionally active. Rapid and highly efficient repair of UVB-induced CPDs could be demonstrated in transfected cells exposed to UVB and photoreactivating light. Photoreactivation significantly reduced the antiproliferative effect of UVB irradiation.

This experimental platform was suitable for investigating CPD-dependent and -independent cellular processes upon UVB exposure. We found that CPDs are the primary cause of transcriptional changes in response to UVB radiation. In addition, our findings suggested the involvement of JNK signalling pathway in CPD-dependent cellular stress responses upon UVB exposure.

*In vitro* transcribed mRNA can be a new platform for safe and effective delivery of therapeutic proteins opening wide perspectives for dermatological or other medical utilizations. Delivery of *in vitro* synthesized and pseudouridine-modified mRNA encoding CPD-photolyase into keratinocytes *in vivo* offers an innovative solution for skin cancer prevention.

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## List of publications related to the dissertation

1. **Boros, G.**, Mikó, E., Muramatsu, H., Weissman, D., Emri, E., Szegedi, A., Horkay, I., Emri, G., Karikó, K., Remenyik, É.: Identification of Cyclobutane Pyrimidine Dimer-Responsive Genes Using UVB-Irradiated Human Keratinocytes Transfected with In Vitro-Synthesized Photolyase mRNA.
   *PLoS One. 10* (6), e0131141-, 2015.
   DOI: http://dx.doi.org/10.1371/journal.pone.0131141
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2. **Boros, G.**, Mikó, E., Muramatsu, H., Weissman, D., Emri, E., Rózsa, D., Nagy, G., Juhász, A., Juhász, I., van der Horst, G., Horkay, I., Remenyik, É., Karikó, K., Emri, G.: Transfection of pseudouridine-modified mRNA encoding CPD-photolyase leads to repair of DNA damage in human keratinocytes: A new approach with future therapeutic potential.
   *J. Photochem. Photobiol. B. 129*, 93-99, 2013.
   DOI: http://dx.doi.org/10.1016/j.jphotobiol.2013.09.010
   IF:2.803

## List of other publications

3. Emri, E., Mikó, E., Bai, P., **Boros, G.**, Nagy, G., Rózsa, D., Juhász, T., Hegedűs, C., Horkay, I., Remenyik, É., Emri, G.: Effects of non-toxic zinc exposure on human epidermal keratinocytes.
   *Metallomics. 7* (3), 499-507, 2015.
   DOI: http://dx.doi.org/10.1039/C4MT00287C
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4. **Boros G.**, Mikó E., Horkay I., Karikó K., Emri G., Remenyik É.: Az mRNS-alapú génterápia dermatológiai alkalmazásának lehetőségei: Fényvédelem újragondolva = Dermatological application of mRNA-based gene therapy : protection from UV-radiation-caused damages.
   *Bőrgyógyász. Venerol. Szle. 89* (5), 119-122, 2013.
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5. Mikó, E., Czimmerer, Z., Csánky, E., **Boros, G.**, Buslig, J., Dezső, B., Scholtz, B.: Differentially expressed microRNAs in small cell lung cancer.
   *Exp. Lung Res. 35* (8), 646-664, 2009.
   DOI: http://dx.doi.org/10.3109/01902140902822312
   IF:1.177

**Total IF of journals (all publications): 10,799**
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# Presentations

1. **23rd World Congress of Dermatology, Vancouver, Canada,** *Changes of mRNA expression during photoprotection accomplished by mRNA transfection – an in vitro study* (first author)
2. **44th Congress of European Society for Dermatological Research, Copenhagen, Denmark,** *Effect of cyclobutane pyrimidine dimer on gene expression is mediated by activation of c-Jun kinase in UVB irradiated human keratinocytes* (oral presentation)
3. **2nd Experimental Dermatological Conference, Szeged, Hungary,** *Cyclobutane pyrimidine dimer-dependent modulation of mRNA- and protein expression in human keratinocytes following UVB exposure* (oral presentation)
4. **16th Annual Meeting of American Society of Gene and Cell Therapy, Salt Lake City, Utah, USA,** *Transfection of pseudouridine-containing mRNA encoding CPD-photolyase into UVB-irradiated human keratinocytes results in rapid repair of DNA damage* (poster presentation)
5. **43rd Congress of European Society for Dermatological Research, Edinburgh, Scotland,** *Post-UV delivery of CPD-photolyase mRNA leads to repair of DNA damage in human keratinocytes* (poster presentation)
6. **42nd Congress of European Society for Dermatological Research, Venice, Italy,** *CPD-dependent gene expression changes in photolyase mRNA-transfected human keratinocytes irradiated by UVB* (poster walk)
7. **41st Congress of European Society for Dermatological Research, Barcelona, Spain,** *Enhanced repair of UVB-induced DNA lesions in photolyase mRNA-transfected keratinocytes* (poster presentation)

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