# Similar Mutagenicity of Photoactivated Porphyrins and Ultraviolet A Radiation in Mouse Embryonic Fibroblasts: Involvement of Oxidative DNA Lesions in Mutagenesis†

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ABSTRACT: Ultraviolet A (UVA) radiation is implicated in the etiology of human skin cancer. However, the underlying mechanism of carcinogenicity for UVA is not fully delineated. A mutagenic role for UVA has been suggested, which involves activation of endogenous photosensitizers generating oxidative DNA damage. We investigated the mutagenicity of UVA alone and in combination with δ-aminolevulinic acid (δ-ALA), a precursor of the intracellular photosensitizers porphyrins, in transgenic Big Blue mouse embryonic fibroblasts. A significant generation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), a typical promutagenic oxidative DNA lesion, was observed in cells treated with a combination of δ-ALA (1 mM) and UVA (0.06 J/cm$^2$) as quantified by high-pressure liquid chromatography−tandem mass spectrometry ($p < 0.001$; relative to the control). The steady-state level of 8-oxo-dG, however, remained unchanged in cells irradiated with UVA or treated with δ-ALA alone. Other photolesions including cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts were not detectable in cells treated with δ-ALA and/or irradiated with UVA as determined by terminal transferase-dependent polymerase chain reaction assay. Mutation analyses of the *cII* transgene in cells treated with a combination of δ-ALA and UVA showed an approximately 3-fold increase in mutant frequency relative to the control ($p < 0.008$), as well as a unique induced mutation spectrum as established by DNA sequence analysis ($p < 0.005$; 95% CI, 0.002−0.009). No mutagenic effects were observed in cells irradiated with UVA or treated with δ-ALA alone. The spectrum of mutations produced by δ-ALA plus UVA was characterized by a significantly increased frequency of G → T transversions ($p < 0.0003$; relative to the control), which are the hallmark mutations induced by 8-oxo-dG. Notably, the 8-oxo-dG-mediated mutagenicity of UVA plus δ-ALA is similar to that established previously for UVA alone at a mutagenic dose of 18 J/cm$^2$. We conclude that, in the presence of exogenous photosensitizers, UVA at a nonmutagenic dose induces mutations through the same mechanism as does a mutagenic dose of UVA per se.

The most well-characterized physical carcinogen in our environment is solar ultraviolet (UV)[1] radiation, which has long been linked to basal and squamous cell carcinomas and malignant melanoma of the skin in humans (*1*−*3*). Although the UV portion of the sunlight includes UVC (<280 nm wavelength), UVB (280−320 nm wavelength), and UVA (>320−400 nm wavelength), only UVA and UVB are etiologically relevant for human skin cancer. To date, the involvement of UVB in skin cancer is mostly ascribed to its induction of promutagenic *cis-syn* cyclobutane pyrimidine dimers (CPDs), pyrimidine (6-4) pyrimidone photoproducts [(6-4)PPs], and Dewar valence photoisomers (*2*, *4*, *5*). UVA is widely believed to contribute to skin carcinogenesis via indirect photosensitizer-mediated induction of DNA damage and mutagenesis (*2*, *5*−*8*). Also, it is postulated that UVA directly causes genetic alterations, e.g., by introducing DNA strand breaks (*9*, *10*). Most recently, UVA has been shown to induce CPDs in rodent cells as well (*10*−*12*). For the most part, the poor absorbance of UVA by DNA favors the idea that photosensitization reactions occurring within the cells are somehow responsible for UVA carcinogenicity. Presumably, the UVA-generated reactants give rise to a variety of DNA lesions some of which have the potential to trigger mutagenesis (*13*). A relevant class of endogenous photosensitizers for such reactions is the cellular chromophores "porphyrins" (*14*). δ-Aminolevulinic acid (δ-ALA) is a precursor of the active photosensitizer protoporphyrin IX (Pp-IX) (*15*). Upon UVA irradiation, δ-ALA has been shown to produce 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG),

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[1] Abbreviations: (6-4)PP, pyrimidine (6-4) pyrimidone photoproduct; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; CPD, *cis-syn* cyclobutane pyrimidine dimer; δ-ALA, δ-aminolevulinic acid; DMEM, Dulbecco's Modified Eagle's Medium; HPLC−MS/MS, high-pressure liquid chromatography/tandem mass spectrometry; mCpG, methylated CpG dinucleotide; MPC, magnetic particle concentrator; PBS, phosphate-buffered saline; Pp-IX, protoporphyrin IX; TD-PCR, terminal transferase-dependent polymerase chain reaction; UV, ultraviolet.

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a typical promutagenic oxidative DNA lesion, in murine leukemia cells (*16*). Also, multiple abnormalities and deficiencies in heme biosynthesis, which regulates the generation of porphyrins via inhibition of δ-ALA synthase, are observed in human cancers. The latter regulatory process is the basis for photodynamic diagnosis/therapy, in which administration of δ-ALA results in a selective accumulation of porphyrins in cancerous tissues, thereby providing the opportunity to localize/destroy the neoplastic tissues by a subsequent photoactivation (*15*, *17*−*21*).

In the present study, we explored the biological consequences of UVA-activated porphyrins in Big Blue mouse embryonic fibroblasts. This transgenic modality offers a chromosomally integrated target gene, *cII*, which can easily be recovered and screened for DNA damages as well as mutagenesis at the level of nucleotide resolution (*22*). Here, the δ-ALA-treated and/or UVA-irradiated cells were assessed for the formation of 8-oxo-dG by high-pressure liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) (*23*) of dimeric photoproducts, i.e., CPDs and (6-4)-PPs and/or of DNA strand breaks by terminal transferase-dependent polymerase chain reaction (TD-PCR) (*24*). Also, the induction of *cII* mutations was determined by a λ phage-based mutation detection system (Stratagene, La Jolla, CA).

## MATERIALS AND METHODS

*Cell Culture and Treatment.* Early passage Big Blue mouse embryonic fibroblasts (prepared from 13.5-day old embryos) were grown to monolayer confluence (∼70%) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. The confluent cells were kept in phenol red and serum free medium, Opti-MEM I (Invitrogen Co., Carlsbad, CA), at least 12 h prior to treatments. The cells were treated with 1 mM δ-ALA hydrochloride (Sigma−Aldrich Inc., Saint Louis, MO) or diluent double-distilled water for 24 h in the dark. After multiple washes with phosphate-buffered saline (PBS), the cells were irradiated (in Opti-MEM I) with doses of 0.06, 0.12, or 0.18 J/cm$^2$ UVA. The UVA source was a Sellas Sunlight System (Medizinische Geräte GmbH, Gevelsberg, Germany) with an average fluence rate of 60 mW/cm$^2$ emitting wavelengths 340−400 nm. For homogeneous irradiation of the cells, the culture Petri dishes were placed directly under the source at a distance of 1 cm and were rotated during the course of irradiation. No excessive heat was generated in the medium throughout the irradiation. After irradiation, the cells were immediately harvested by trypsinization for determination of cell survival and for detection of DNA damages. Alternatively, the cells were cultured in complete growth medium for an additional 8 days and afterward were analyzed for mutant frequency and mutational spectrum of the *cII* transgene. The 8-day growing period is essential for the fixation of all mutations into the genome. The cells were passed once during the 8-day culturing. All experiments were conducted 3 times.

*Genomic DNA Isolation for HPLC−MS/MS Analysis.* The cell pellets were washed twice with prewarmed PBS and lyzed with 4 mL of a solution containing 0.5 M Tris-HCl at pH 8.0, 20 mM EDTA at pH 8.0, 10 mM NaCl, 1% SDS, and 0.5 mg/mL proteinase K at 37 °C overnight. Subsequently, 2 mL of saturated NaCl (∼6 M) was added to each sample, and the samples were incubated at 56 °C for 10 min. After centrifugation at 5000*g* for 30 min, the supernatant containing DNA was mixed with 2 volumes of prechilled ethanol (100%) and the DNA was spooled by gently inverting the mix. The DNA was washed thoroughly with 70% ethanol, air-dried, and subsequently dissolved in TE buffer (1 mM EDTA and 10 mM Tris-HCl at pH 7.5). Aliquots of 50 μg of DNA were dried in a Speed-Vac concentrator and then dissolved in 100 μL of a buffer solution containing 10 mM Tris-HCl at pH 7.5 (98 μL) and 1 M sodium acetate at pH 6.0 (2 μL). Each DNA aliquot was digested with 10 units of nuclease P1 (Sigma−Aldrich Inc.) for 1 h and afterward with 0.004 unit of phosphodiesterase 1 (Sigma−Aldrich Inc.) together with 34 units of alkaline phosphatase (Roche, Indianapolis, IN) for 2 h at 37 °C. The samples were filter-centrifuged (Millipore Co., Bedford, MA) at 6000*g* for 30 min. The eluents were mixed with an equal volume of methanol and dried with the Speed-Vac concentrator.

*Genomic DNA Isolation for TD-PCR and Mutation Analyses.* Genomic DNA was isolated using a standard phenol and chloroform extraction and ethanol precipitation protocol (*25*). The DNA was dissolved in TE buffer and preserved at −80 °C until further analysis.

*HPLC−MS/MS for Quantification of 8-oxo-dG.* Quantification of 8-oxo-dG was done using the previously published method of Singh et al. (*23*) with some modifications. Briefly, analytical-grade 8-oxo-dG was purchased from Cayman Chemical (Ann Arbor, MI), and mass-labeled 8-oxo-dG was kindly provided by Dr. Miral Dizdaroglu of the National Institute of Standards and Technology. Instrumentation consisted of an Agilent 1100 Capillary LC system (Agilent Technologies, Palo Alto, CA) in line with a Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer (Micromass, Inc., Beverly, MA). The detector settings were as follows: capillary voltage = 2.20 kV, cone voltage = 16 V, collision cell voltage = 13 V, source temperature = 125 °C, desolvation temperature = 260 °C, cone gas flow = 130 L/h, and desolvation gas flow = 500 L/h. The mass transitions monitored for 8-oxo-dG and internal standard were 284 → 168 and 286 → 170, respectively. Chromatographic conditions consisted of isocratic separation across a Synergi C18 4μ 150 × 2.0-mm analytical column (Phenomenex, Torrance, CA) using a mobile phase of 8% methanol in water with 0.1% formic acid. The flow rate was 0.2 mL/min, with a total run time of 30 min and a retention time of 8-oxo-dG of 14.9 min. The lower limit of quantitation for 8-oxo-dG was 0.1 ng/mL in the starting solution or 0.2 pg on the column. Both precision and accuracy of the assay are within ±10% of the target values, respectively. Simultaneous quantification of 2′-deoxyguanosine was performed to normalize the results.

*TD-PCR for Mapping of Photoinduced DNA Lesions.* The entire length of *cII* transgene was subjected to TD-PCR as described earlier with some modifications (*24*). Briefly, genomic DNA (100 ng) was used as a template, and single-stranded products were made by repeated primer extensions. The extension protocol consisted of a custom-made biotinylated primer, U1: 5′-AATCGAGAGTGCGTTGCTT-3′, $T_m$ = 49.9 °C in a mixture of Vent$^{(exo-)}$ DNA polymerase (New England Biolabs Inc., Beverly, MA) in a thermocycler setting of 2 min at 95 °C, 2 min at 61 °C, 3 min at 72 °C, 9 cycles (in which 1 cycle consisted of 45 s at 95 °C, 2 min

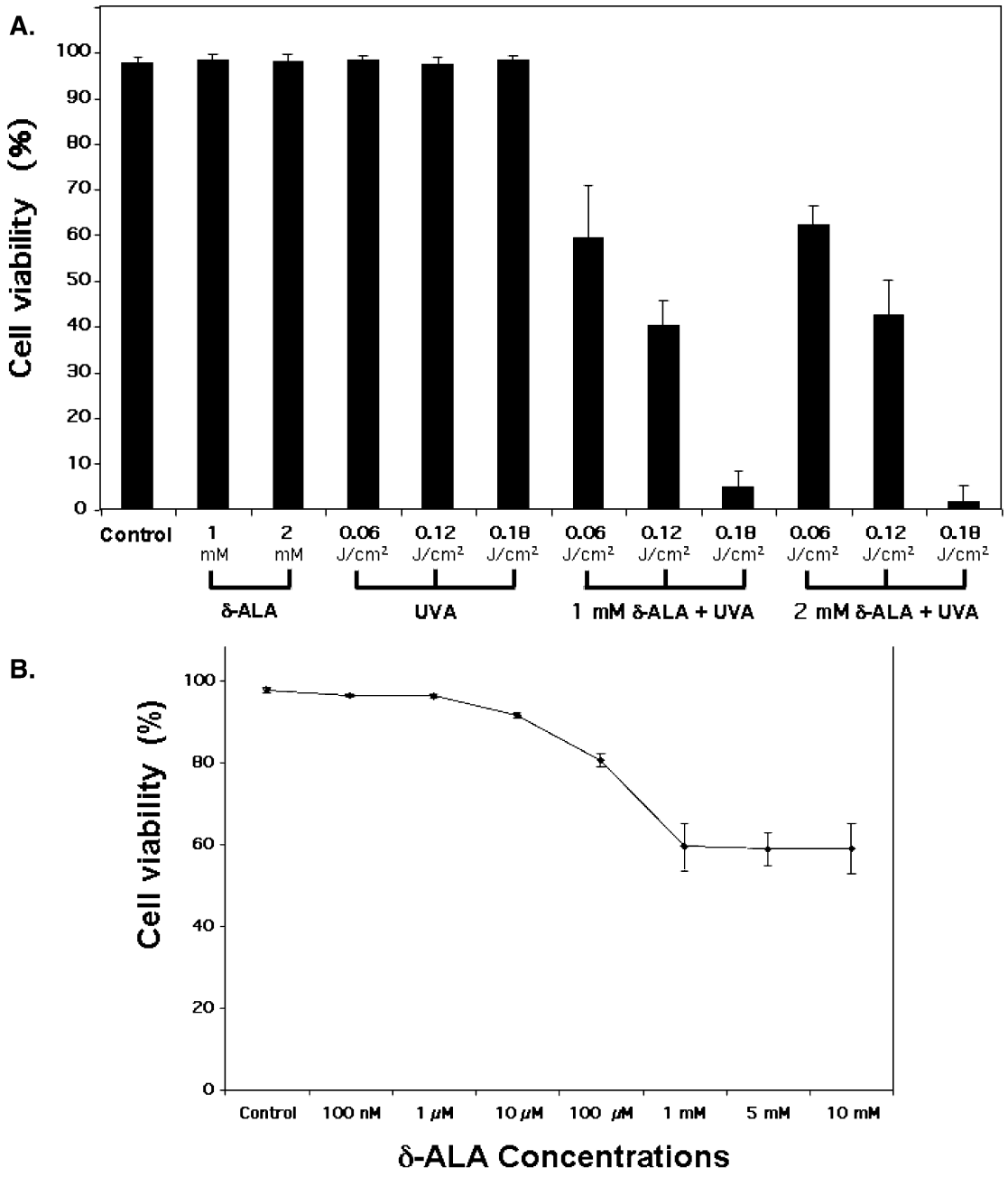


FIGURE 1: (A) Cytotoxicity of δ-ALA and/or UVA in Big Blue mouse embryonic fibroblasts. Confluent cell cultures were treated with δ-ALA (1 mM, for 24 h) and irradiated with UVA (0.06, 0.12, and 0.18 J/cm$^2$) individually and combined, and cell viability was determined immediately afterward by the trypan blue exclusion assay. Viability is expressed as a percentage of the total cell number. Results are expressed as medians of three independent experiments, with each experiment run 3 times. Error bars = SE. (B) δ-ALA concentration-dependent cytotoxicity of combined δ-ALA and UVA in Big Blue mouse embryonic fibroblasts. Confluent cell cultures were treated with δ-ALA (100 nM, 1 μM, 10 μM, 100 μM, 1 mM, 5 mM, and 10 mM, for 24 h) and subsequently irradiated with UVA (0.06 J/cm$^2$), and cell viability was determined 24 h afterward by the trypan blue exclusion assay. Viability is expressed as a percentage of the total cell number. Results are expressed as medians of three independent experiments, with each experiment run 2 times. Error bars = SE.

at 61 °C, and 3 min at 72 °C), 45 s at 95 °C, 2 min at 61 °C, and 10 min at 72 °C. The resulting product was mixed with Streptavidin-coupled magnetic beads (Dynal ASA, Oslo, Norway), and binding was achieved by gently rotating the mixture for 45 min at room temperature. The Streptavidin-bound DNA was incubated with 0.15 M NaOH at 37 °C for 10 min. After multiple washes with 1× TE at pH 7.5, in a Magnetic Particle Concentrator (MPC) (Dynal ASA), the single-stranded DNA was resuspended in 0.1× TE at pH 7.5 and afterward was subjected to homopolymeric ribotailing and adapter ligation (*26*). The ligation product was washed with 1× TE at pH 8.0, in the MPC, resuspended in 0.1× TE at pH 8.0, and amplified in a PCR using primer U2: 5′-GCGTTGCTTAACAAAATCGCAATGCT-3′, $T_m$ = 63.1 °C and a custom-made LP25 primer (*26*) in the presence of Expand Long Polymerase (Roche). The thermocycler settings were as follows: 2 min at 95 °C, 2 min at 62 °C, 3 min at 72°C, 18 cycles (in which 1 cycle consisted of 45 s at 95 °C, 2 min at 62 °C, and 3 min at 72 °C), 45 s at 95 °C, 2 min at 62 °C, and 10 min at 72 °C. The PCR product was labeled by a fluorescence infrared dye primer [IRD-700] (LI-COR Inc., Lincoln, NE) U3: 5′-GCAATGCTTGGAACT-GAGAAGACAGC-3′, $T_m$ = 61.4 °C in a thermocycler setting of 2 min at 95 °C, 2 min at 66 °C, 3 min at 72 °C,

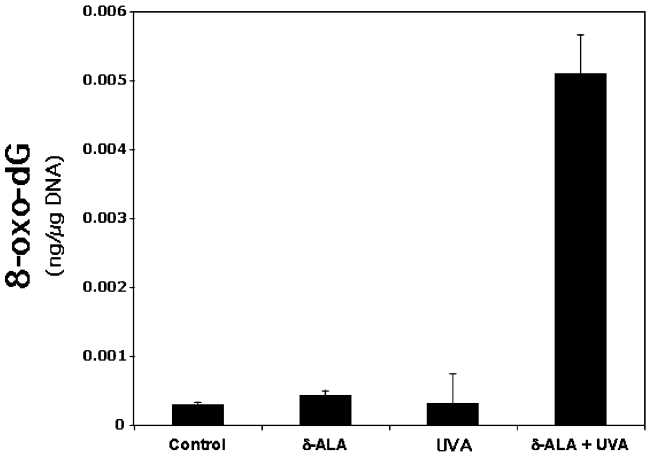


FIGURE 2: Determination of 8-oxo-dG generation in Big Blue mouse embryonic fibroblasts treated with δ-ALA and/or UVA. Confluent cell cultures were treated with δ-ALA (1 mM, for 24 h) and irradiated with UVA (0.06 J/cm$^2$) individually and combined, and 8-oxo-dG levels were quantified using a standard HPLC−MS/MS (*23*). Results are expressed as medians of three independent experiments, with each experiment run 3 times. Error bars = SE.

1 cycle (in which 1 cycle consisted of 45 s at 95 °C, 2 min at 66 °C, and 3 min at 72 °C), 45 s at 95 °C, 2 min at 66 °C, and 10 min at 72 °C. The labeled products were loaded onto a 5% acrylamide/urea gel for electrophoresis and simultaneous quantification by an IR$^2$ Long Ranger 4200 system (LI-COR Inc.). As a standard control, known dimeric photoproduct-containing DNA isolated from UVB-irradiated cells was run in all analyses. The sites of DNA lesion formation were identified as the locations at which the presence of the lesions stopped the DNA polymerase from progressing, resulting in an intense dark band (dependent on the lesion frequency) in the sequencing gel. A nontreated control DNA sample was included in all runs to correct for nonspecific background bands.

*cII Mutant Frequency Analysis.* The *cII* mutant frequency was quantified by the λ select-*cII* mutation detection system for Big Blue rodents (Stratagene). The assay system is based on the ability of the λ phage to multiply either lytically or lysogenically in *Escherichia coli* host cells (*22*). The commitment of the λ phage to lysis or lysogeny upon infection of the host depends on a chain of events, of which *cII* transcription is a determiner (*27*). The cII protein activates the transcription of the cI repressor and λ integrase, both of which obligate the phage to undergo lysogenization (*27*). Only the λ phages carrying a mutant *cII* can enter the lytic pathway and form visible plaques on an *E. coli* lawn (*22*). The λ LIZ vector, however, harbors a *cI857* temperature sensitive (ts) mutation, which makes the $cI_{(ts)}$ protein labile at temperatures over 32 °C (*28*). Thus, all λ LIZ phages regardless of their *cII* mutant/nonmutant status multiply lytically in the host *E. coli* at incubating temperatures exceeding 32 °C (nonselective condition) (*22*).

Briefly, the λ LIZ shuttle vectors were recovered from the genomic DNA (∼5 μg) and packaged into viable phage particles by Transpack packaging extract according to the instructions of the manufacturer (Stratagene). The phages were preadsorbed to G1250 *E. coli*, and the bacterial culture was plated on TB1 agar plates. The plates were incubated for 48 h at 24 °C or overnight at 37 °C (regarded as selective and nonselective conditions, respectively). The *cII* mutant

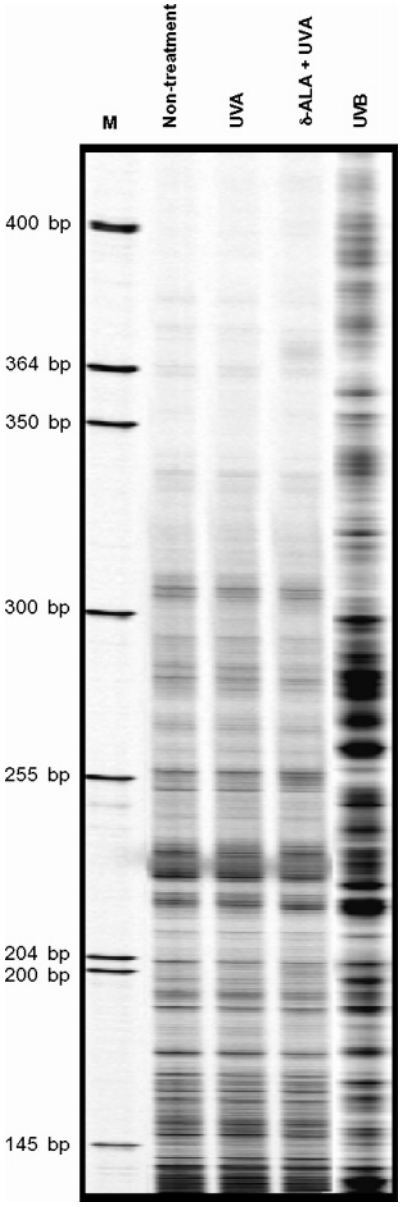


FIGURE 3: Mapping of photoinduced DNA lesions in the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with a combination of δ-ALA (1 mM, for 24 h) and UVA (0.06 J/cm$^2$) or irradiated with UVA alone. Genomic DNA was extracted and subjected to TD-PCR (*24*). Isolated DNA from UVB-irradiated cells was used as a positive control. A nontreated control DNA sample was used to correct for nonspecific background bands. M = sizing standard.

frequency was expressed as the ratio of the number of plaques formed on the selective plates to that formed on the nonselective plates. As recommended by the manufacturer (Stratagene), a minimum of $3 \times 10^5$ rescued phages was screened for each experimental condition. For quality assurance, control phage solutions containing a mixture of λ *cII*$^{(+)}$ and λ *cII*$^{(-)}$ (Stratagene) with known mutant frequencies were assayed in all runs.

*cII Mutation Spectrum Analysis.* The putative *cII* mutant plaques were all verified after being replated under the selective conditions on a second TB1 agar plate. The verified plaques were subsequently amplified in a PCR by the λ select-*cII* sequencing primers according to the recommended protocol of the manufacturer (Stratagene). The PCR products were purified with the QIA quick (PCR) purification kit

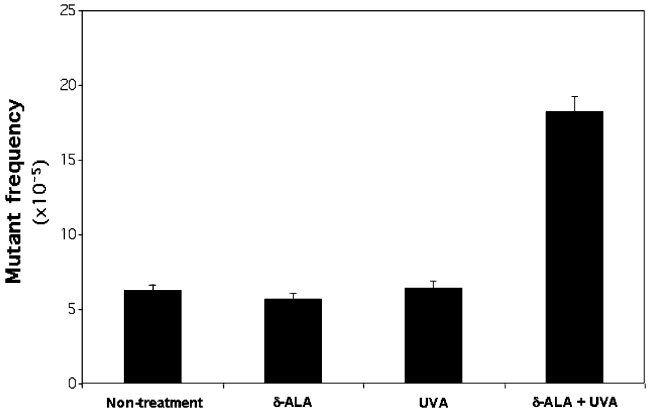


FIGURE 4: Mutant frequency of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with δ-ALA (1 mM, for 24 h) and irradiated with UVA (0.06 J/cm$^2$) individually and combined. Quantification was done 8 days after treatments using the λ select-*cII* mutation detection system for Big Blue rodents (Stratagene), a phage-based assay that permits detection of mutations within the transgene on the basis of plaque formation. Mutant frequency was determined from a minimum of $3 \times 10^5$ plaques. Results are expressed as medians of three independent experiments, with each experiment run 3 times. Error bars = SE.

(QIAGEN GmbH, Hilden, Germany) and sequenced by a Big Dye terminator cycle sequencing kit and an ABI-377 DNA Sequencer (ABI Prism, PE Applied BioSystems, Foster City, CA).

*Statistical Analysis.* Results are expressed as medians ± standard error (SE). All variables in treated versus control groups were compared by the Wilcoxon signed rank test. The entire mutation spectra and the specific types of mutation in the treated versus control groups were compared by the hypergeometric test of Adams and Skopek (*29*) and $\chi^2$ test, respectively. Values of $p \leq .05$ were considered statistically significant.

## RESULTS

*Cytotoxicity Examination.* Neither UVA irradiation nor δ-ALA treatment alone at any of the administered doses was cytotoxic in mouse embryonic fibroblasts (Figure 1A). In combination with UVA irradiation, however, the δ-ALA treatment was highly cytotoxic. The severity of cytotoxicity in the combination treatment was dependent on both the UVA dose and δ-ALA concentration (parts A and B of Figure 1). The δ-ALA concentration-dependent lethality of the combined treatment reached a plateau in the millimolar dose range of δ-ALA (Figure 1B).

*Quantification of 8-oxo-dG and Mapping of Photoinduced DNA Lesions.* δ-ALA treatment (1 mM) in combination with UVA irradiation (0.06 J/cm$^2$) resulted in the formation of 8-oxo-dG in mouse embryonic fibroblasts (17-fold over the background; $p < 0.001$). Treatment with δ-ALA or irradiation with UVA alone, however, did not increase the level of 8-oxo-dG relative to the background (Figure 2). As shown in Figure 3, there was no detectable formation of dimeric photoproducts and/or DNA strand breaks in mouse embryonic fibroblasts treated with a combination of δ-ALA and UVA or irradiated with UVA alone. However, the presence of photoadducts was readily detectable in control UVB-irradiated cells.

*cII Mutant Frequency and Mutation Spectrum Analyses.* The combined treatment with δ-ALA (1 mM) and UVA (0.06 J/cm$^2$) was mutagenic in mouse embryonic fibroblasts because it significantly increased the *cII* mutant frequency relative to the background ($18.2 \pm 1.1 \times 10^{-5}$ versus $6.2 \pm 0.4 \times 10^{-5}$; $p < 0.008$). Treatment with δ-ALA or irradiation with UVA alone, however, did not appreciably affect the *cII* mutant frequency relative to the background (Figure 4).

For mutation spectra analyses, DNA sequencing was performed on the *cII* plaques formed in samples treated with a combination of δ-ALA and UVA or in the nontreated control (number of sequenced plaques = 192 and 173, respectively). Overall, DNA sequencing confirmed a mutated *cII* in over 96% of all analyzed plaques. Of these, the vast majority were single-base substitutions and less frequently insertions, deletions, and multiple-base substitutions/deletions, respectively (parts A and B of Figure 5). In both induced and spontaneous mutation spectra, there were four "jackpot" mutations at nucleotide positions 179−184 [G insertion/deletion], 196 [G → A transition], 211 [G → C transversion] and 221 [T → G transversion] (parts A and B of Figure 5). These jackpot mutations are common phenomena in transgenic model systems and have been consistently reported by us (*24*, *30*, *31*) as well as by others (*32*, *33*). Presumably, jackpot mutations occur in the early development of the transgenic rodents and undergo clonal expansion such that many cells from various tissues harbor the same type of mutations. Alternatively, they might represent actual hotspots of spontaneous mutagenesis (*34*, *35*). Methodologically, therefore, it is appropriate to exclude all jackpot mutations from the comparative spectra analyses. When the jackpot mutations were excluded, the spectrum of mutations induced by δ-ALA plus UVA was significantly different from that derived spontaneously ($p < 0.005$; 95% CI, 0.002−0.009).

The *cII* transgene in the Big Blue rodent system is not transcribed after being integrated into the genome (*36*). Thus, the "strand-dependent mutagenesis", a phenomenon caused by transcription-coupled DNA repair in mammalian endogenous genes (*37*−*39*), is an unlikely event in this model system. Accordingly, it is justified to combine the strand mirror counterparts of all transitions (e.g., G → A + C → T) and transversions (e.g., G → T + C → A and G → C + C → G) when comparing the specific types of mutation between different treatment groups. As shown in Figure 6, the spectrum of mutations induced by δ-ALA plus UVA was different from that derived spontaneously; i.e., the relative frequency of G → T + C → A transversions was significantly increased after the treatment with δ-ALA and UVA combined ($p < 0.0003$). The combined treatment with δ-ALA and UVA also produced base substitutions more significantly at G/C residues ($p < 0.0001$) and less frequently at A/T residues ($p < 0.002$) relative to the control. However, the relative frequency of C → T transitions at "dipyrimidine sites", indicative of dipyrimidine photoproduct-induced mutations, was not different between the induced and spontaneous mutational spectra ($p = 0.12$).

## DISCUSSION

Despite the wealth of information available on the involvement of solar UV in human skin cancer, the contribution of the UVA fraction to the carcinogenic process as well as the underlying mechanism of action for UVA remain

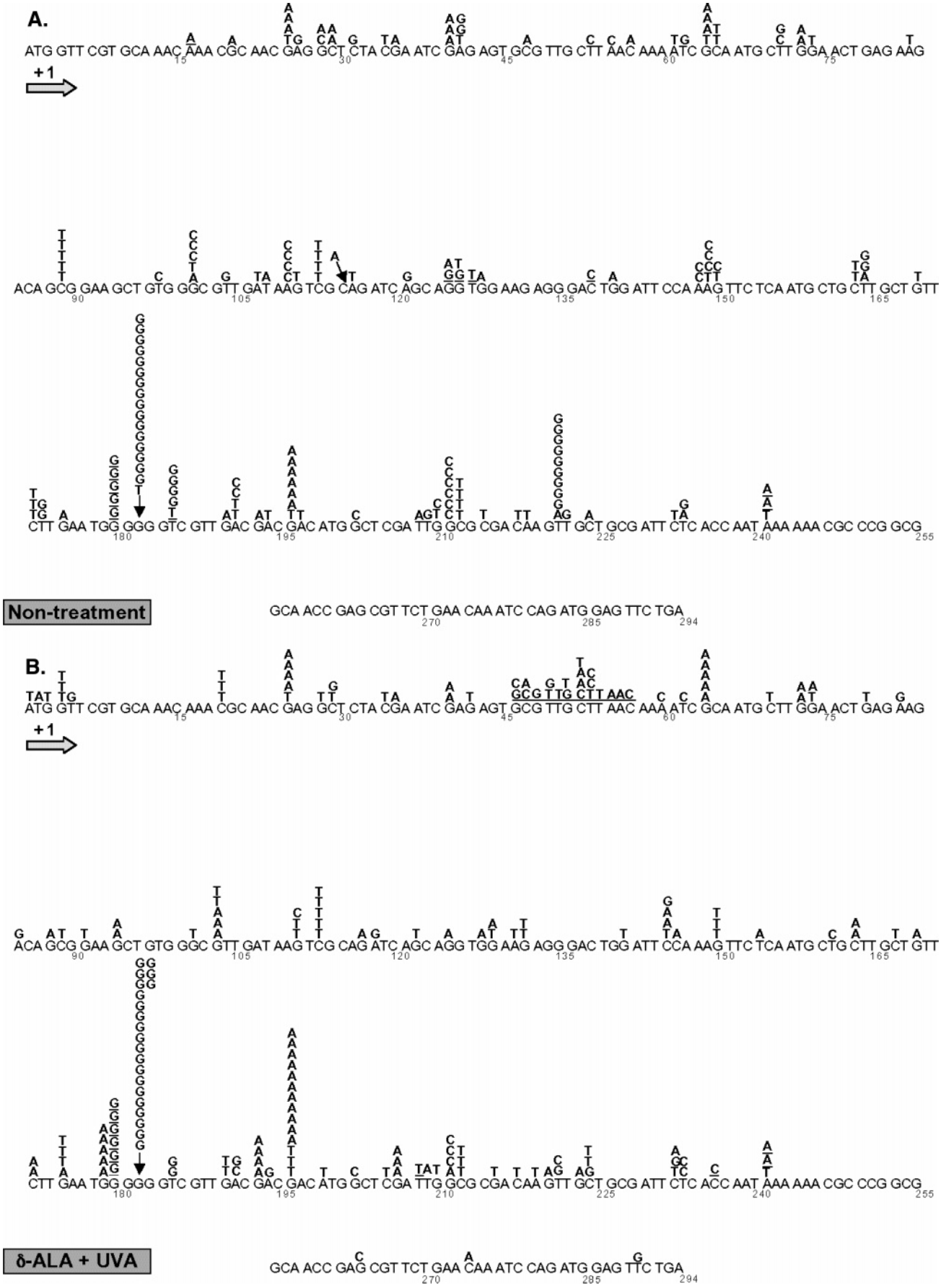


FIGURE 5: Detailed mutation spectra of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with a combination of *δ*-ALA (1 mM, for 24 h) and UVA (0.06 J/cm$^2$). (A) Control solvent (double-distilled water) treated cells. (B) *δ*-ALA plus UVA treated cells. (total number of sequenced mutant plaques = 173 and 192, respectively). Substituted bases are in bold; deleted bases are underlined; and multiple deletions are continuously underlined. Inserted bases are shown with an arrow. Numbers below the bases are the nucleotide positions.

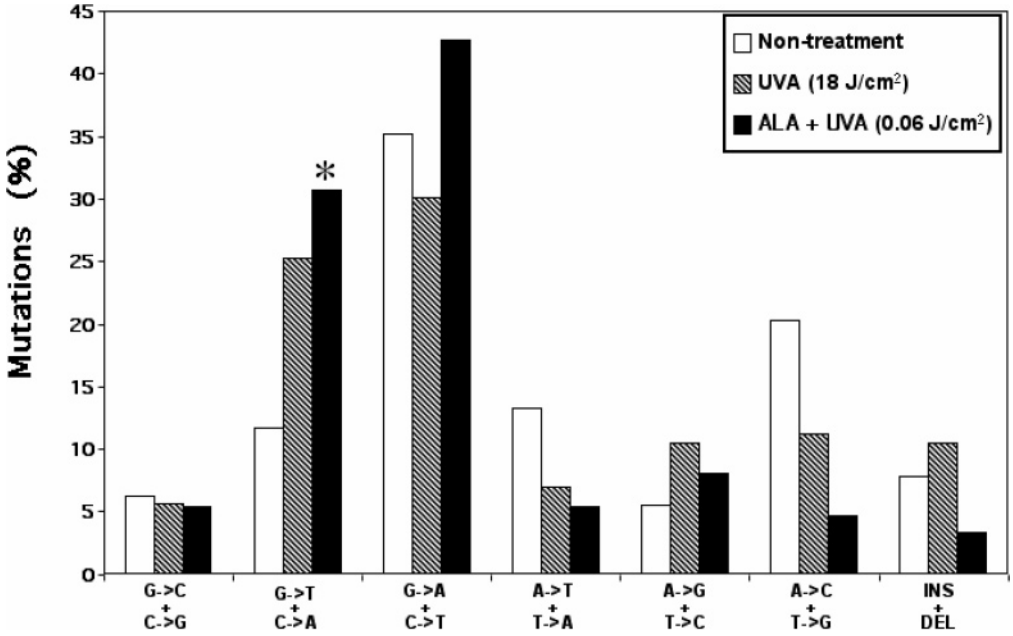


FIGURE 6: Mutation spectra of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with a combination of δ-ALA (1 mM, for 24 h) and UVA (0.06 J/cm$^2$) or irradiated with a mutagenic dose of 18 J/cm$^2$ UVA (total number of sequenced mutant plaques = 192 and 161, respectively). For comparison, the strand mirror counterparts of all transitions (e.g., G → A + C → T) and transversions (e.g., G → T + C → A and G → C + C → G) were combined for each treatment group. Ins = insertion; Del = deletion. The asterisk indicates the same value as compared with the "nontreated control", $p < 0.0003$.

elusive (*1*−*3*). In rodents, UVA is proven to be a complete carcinogen (*5*). Such carcinogenicity of UVA is partially attributed to its tumor promoting effects, which might involve immunosuppression and/or stimulation of cell proliferation. UVA carcinogenicity is also ascribed to its mutagenicity; however, the exact mechanism of mutagenesis is unclear (*1*−*3*, *5*, *8*). UVA is believed to cause gross genomic instability, e.g., by introducing deletion events resulting from DNA strand breaks (*9*). UVA is also thought to promote genetic alterations, e.g., by inducing promutagenic photoadducts such as CPDs (*10*−*12*). In addition, UVA can activate endogenous photosensitizers thereby, giving rise to a variety of DNA reactants some of which could trigger mutagenesis (*2*, *5*−*8*). Here, we investigated the mutagenicity of UVA per se and in combination with δ-ALA, a precursor of the intracellular photosensitizers Pp-IX (*15*).

Our cytotoxicity examinations revealed that neither UVA irradiation per se nor δ-ALA treatment alone had any detrimental effects. However, δ-ALA treatment in combination with UVA irradiation showed extreme cytotoxicity chiefly dependent on the UVA dose and to some extent on the δ-ALA concentration (parts A and B of Figure 1). The δ-ALA concentration-dependent lethality of the combined treatment was saturated at a millimolar dose of δ-ALA (Figure 1B). This might reflect a saturable cellular uptake of δ-ALA in mouse embryonic fibroblasts. Overall, the lethality of the combined treatment is exploited for photodynamic therapy/diagnosis in which the combination of a photosensitizer that preferentially accumulates in neoplastic cells and photoactivation by UVA or visible light helps destroy/identify the malignant cells (*18*). The increasingly popularized porphyrin-based photodynamic therapy utilizes excess exogenous porphyrins such as δ-ALA, which induces, via the heme synthesis pathway, a buildup of the endogenous photosensitizer Pp-IX, followed by light sensitization (*15*, *18*).

Our HPLC−MS/MS analyses of the cells treated with a combination of δ-ALA and UVA showed a substantial generation of promutagenic 8-oxo-dG, a prototype lesion for oxidative DNA damage. However, the steady-state level of 8-oxo-dG was not affected in cells irradiated with UVA or treated with δ-ALA alone (Figure 2). In determining the levels of 8-oxo-dG, the possibility of an artifact must be taken into account given the large discrepancies that have been reported when oxidative DNA damage has been measured using different techniques (*40*−*42*). Our measurements of 8-oxo-dG are more in line with the lower levels that have been reported; i.e., the steady-state level of 8-oxo-dG was >0.3 pg/μg of DNA. At the same time, there was no detectable level of UV-associated dimeric photoproducts, i.e., CPDs and (6-4)PPs, in cells treated with δ-ALA and/or irradiated with UVA as determined by TD-PCR (Figure 3).

Our mutagenicity experiments showed that the combined treatment with δ-ALA and UVA was significantly mutagenic because it elevated the *cII* mutant frequency approximately 3-fold over the background (Figure 4) and produced a unique mutation spectrum (Figure 5B). However, the δ-ALA treatment or the UVA irradiation per se did not have any mutagenic effects. Mutation spectrometry in cells treated with a combination of δ-ALA and UVA showed a hallmark of G → T transversions (Figure 6). This spectrum of induced mutations is in good agreement with the above-mentioned pattern of 8-oxo-dG generation. Experimentally, 8-oxo-dG is known to induce G → T transversions in a variety of test systems (*43*−*48*). Furthermore, the observed mutation spectrum corroborates the nondetectability of the photolesions mentioned earlier. Accordingly, the characteristic mutation signature of dimeric photolesions, i.e., C → T and CC → TT transitions at "dipyrimidine sites", was not discernible in the induced mutation spectrum (*49*−*54*) (Figure 5B). In fact, the relative frequency of C → T transitions at "dipyrimidine sites" was not different between the induced and

spontaneous mutation spectra ($p = 0.12$) nor was there any occurrence of CC → TT transitions in either spectrum of mutations.

Recently, we have shown that UVA at a biologically relevant dose of 18 J/cm$^2$ [corresponding to a single session exposure in a UVA tanning parlor or 10 min of sun exposure in summer at noon at 45° latitude (*8*)] is significantly mutagenic in transgenic mouse embryonic fibroblasts (*55*). The spectrum of UVA-induced mutations was characterized by pronounced frequencies of G → T transversions and small tandem base deletions together with an absence of signature mutations of dimeric photolesions (Figure 6). Of notice was a concurrent elevated level of 8-oxo-dG (5.4-fold over the background) consequent to UVA irradiation. Such 8-oxo-dG-mediated mutagenicity of UVA closely resembles that observed here produced by δ-ALA plus UVA treatment. For the most part, the spectra of mutations induced by the respective treatments are similar; however, G → T transversions are more frequent after treatment with δ-ALA plus UVA than after irradiation with UVA alone (30.7% versus 25.2%). The latter correlates with the significant generation of 8-oxo-dG after δ-ALA plus UVA treatment (17-fold over the background). It appears that in the absence of exogenous photosensitizers, UVA at a dose of 18 J/cm$^2$ induces mutations through the same mechanism yet somewhat less intensively, as does a nonmutagenic dose of UVA in the presence of δ-ALA. With a minimum UVA dose, i.e., 0.06 J/cm$^2$, the induced mutagenesis is dependent upon the presence of exogenous photosensitizers; however, at high doses of UVA, e.g., 18 J/cm$^2$, the endogenous photosensitizers seem to play a more crucial role. For now, the exact nature of UVA-reactive endogenous photosensitizers remains unknown. As to which reactive species of δ-ALA is responsible for the observed photosensitized-based mutagenesis, further research is needed. Potential candidates must be indeed capable of efficiently absorbing UVA. Of relevance is Pp-IX that constitutes >95% of the endogenous porphyrins induced by δ-ALA and absorbs long wavelength UV (*56*, *57*). The δ-ALA-induced Pp-IX is synthesized inside mitochondria and accumulates preferentially in these organelles. The Pp-IX has also been localized at the plasma membrane and perinuclear regions as well as inside lysosomes but to a lesser extent (*17*, *58*). The genotoxic effects of Pp-IX might be ascribed to its generation of singlet oxygen and other reactive oxygen species (*59*). The singlet oxygen is known to cause single-strand DNA breaks and produce various promutagenic species (*60*, *61*). It can directly interact with guanine residues in the DNA molecule and promote single-base deletions (*62*). It can also give rise to other reactive species that induce mutations, e.g., hydroxyl radicals that may cause base substitutions especially G → T transversions (*63*). In the plasma membrane, singlet oxygen can initiate lipid peroxidation, whose products may also be mutagenic (*17*, *64*). The intracellular trafficking of Pp-IX can determine the contribution of each of the above-mentioned promutagenic pathways to the overall genotoxicity of Pp-IX (*17*).

Presumably, the UVA-activated photosensitizers generate more cytotoxic reactive species at the higher radiation dose. This is supported by our observation that the severity of cytotoxicity in the combination treatment was determined by the UVA dose (Figure 1A). At the same time, the extent of mutagenicity was not dose-dependently related to UVA because an excessive radiation dose (36 J/cm$^2$) did not enhance the mutagenicity but only caused severe cytotoxicity (data not shown). *In vivo*, the biological effects of UVA could be modulated by the existence and amount of endogenous photosensitizers. Dependent upon the cell type and content of endogenous photosensitizers, a finely tuned interplay between UVA and photosensitizers can determine the consequent cytotoxicity and/or mutagenicity.

In summary, we provide mechanistic data showing a similar pattern of mutagenicity for UVA-photoactivated porphyrins and UVA alone based on the induction of oxidative DNA damage, i.e., 8-oxo-dG. The signature of induced mutations is the characteristic G → T transversions specific for 8-oxo-dG. To our knowledge, this is the first time that not only a direct link between UVA-induced DNA damages and mutagenesis is established but also the underlying mechanism of action is delineated.

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