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Synergistic cytotoxicity and DNA strand breaks in cells and plasmid DNA exposed to uranyl acetate and ultraviolet radiation

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Abstract

Depleted uranium (DU) has a chemical toxicity that is independent of its radioactivity. The purpose of this study was to explore the photoactivation of uranyl ion by ultraviolet (UV) radiation as a chemical mechanism of uranium genotoxicity. The ability of UVB (302 nm) and UVA (368 nm) radiation to photoactivate uranyl ion to produce single strand breaks was measured in pBR322 plasmid DNA, and the presence of adducts and apurinic/apyrimidinic sites that could be converted to single strand breaks by heat and piperidine was analyzed. Results showed that DNA lesions in plasmid DNA exposed to UVB- or UVA-activated DU were only slightly heat reactive, but were piperidine sensitive. The cytotoxicity of UVB-activated uranyl ion was measured in repair-proficient and repair-deficient Chinese hamster ovary cells and human keratinocyte HaCaT cells. The cytotoxicity of co-exposures of uranyl ion and UVB radiation was dependent on the order of exposure and was greater than co-exposures of arsenite and UVB radiation. Uranyl ion and UVB radiation were synergistically cytotoxic in cells, and cells exposed to photoactivated DU required different DNA repair pathways than cells exposed to non-photoactivated DU. This study contributes to our understanding of the DNA lesions formed by DU, as well as their repair. Results suggest that excitation of uranyl ion by UV radiation can provide a pathway for uranyl ion to be chemically genotoxic in populations with dermal exposures to uranium and UV radiation, which would make skin an overlooked target organ for uranium exposures.

Keywords

Depleted uranium; DNA damage; DNA repair; plasmid DNA; cell culture

Introduction

Uranium is an emerging toxicant on the global stage, with large-scale contamination resulting from the mining and processing of natural uranium and from the use of depleted uranium (DU) in the military. Recently, worldwide uranium mine tailings in the top 15 uranium producing countries have been estimated at 2300 metric tons; a consequence of

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Conflict of Interest

The Authors did not report any conflict of interest.

three decades of uranium mining and processing (Dinis and Fiúza, 2013). In the United States, the four corners region of the Colorado Plateau contributed heavily to uranium mining and production from World War II into the early 1980's (Brugge and Goble, 2002), with the Navajo reservation producing 13 million tons of uranium ore from more than 1000 mines, now abandoned (Dawson and Madsen, 2011). It has been estimated that 321 tons of DU were used in the Gulf War, 3 tons of DU were used in Bosnia-Herzegovina and 10 tons of DU were used in Kosovo (Bleise *et al.*, 2003). The extensive production and use of both natural and DU has resulted in multiple situations for occupational and environmental exposures (Handley-Sidhu *et al.*, 2010), which has subsequently created concern regarding health effects that may include cancer and other chronic diseases.

The health risks from exposure to natural uranium are well established, but those alleged for DU exposure are more controversial. One of the earliest known occupational diseases was the lung cancer found in the uranium miners of central Europe, attributed to the ^{222}Rn and other alpha emitters formed in the decay process of radioactive ^{238}U and ^{234}U isotopes (Těšínská, 2009). Cohorts of uranium miners have since been followed in Europe and the Southwestern USA (Gilliland *et al.*, 2000; Hunter *et al.*, 2013; Tirmarche *et al.*, 2012). Advocates for civilians in Iraq allege that DU is causing increases in birth defects (Busby *et al.*, 2010), Kaposi sarcoma (Shelleh, 2012), as well as leukemia, lymphoma, and brain, breast, lung, bladder, skin and stomach cancers (Aitken, 1999; Fathi *et al.*, 2013). However, authors associated with government agencies contend that exposure levels are too low to cause any significant adverse effects (Bogers *et al.*, 2013; Brown, 2006; Hines *et al.*, 2013; Marshall, 2008; McDiarmid *et al.*, 2013).

Understanding the cellular and molecular mechanisms underlying the genotoxic endpoints of DU exposure will contribute to the evaluation of chemical risks and to identification of biomarkers of DU exposure that may relate to the alleged adverse health effects. DU has a chemical genotoxicity that is independent of its radioactivity. It forms U-DNA adducts and DNA single strand breaks (SSB) (Yazzie *et al.*, 2003; Stearns *et al.*, 2005; Wilson *et al.*, 2014), and apurinic/apyrimidinic (AP) sites (Yellowhair, 2011) in cultured cells and in isolated DNA. It is mutagenic (Miller *et al.*, 1998; Stearns *et al.*, 2005) and aneugenic (Darolles *et al.*, 2010), but only weakly clastogenic (LaCerte *et al.*, 2010). It produces transformation in cell culture (Miller *et al.*, 1998; Xie *et al.*, 2010; Yang *et al.*, 2002) and is tumorigenic when implanted in rats (Hahn *et al.*, 2002).

We have recently proposed that another mechanism of uranium toxicity may stem from its ability to be photoactivated by ultraviolet (UV) radiation (George *et al.*, 2011). Uranyl acetate dihydrate or uranyl ion (UA) is known to be photoactivated by the absorption of UV radiation (300–420 nm) into the $\text{U}=\text{O}$ bond, which excites the electron pair and forms an excited state described as U^{5+} bound to an oxygen radical (Wang *et al.*, 1995). First described by Buchholz in 1805 (described in Fay, 1896), photoactivated UA has been used as a chemical catalyst (reviewed in Balzani *et al.*, 1978; Wang *et al.*, 1995), as well as a laboratory tool for DNA footprinting (Nielsen, 2009).

The purpose of the current study was to characterize the stability of DNA lesions caused by photoactivated UA *in vitro*, and to begin to assess the toxicity of photoactivated UA in

cultured cells. Using methods we have previously developed (Wilson *et al.*, 2014), pBR322 plasmid DNA was exposed to DU as UA followed by exposure to UVB (302 nm) or UVA radiation (368 nm). The generation of DNA SSB was measured, and the presence of DNA adducts and AP sites were probed by post-treatment exposures to heat and piperidine, which convert these lesions to SSB (Hejmadi *et al.*, 1994). Chinese hamster ovary (CHO) cells, either proficient in DNA repair, deficient in base excision repair (BER), or deficient in nucleotide excision repair (NER), and human keratinocyte HaCaT cells were exposed to UA and UVB radiation to measure the effect of DNA repair proficiency on cell survival.

Materials and Methods

Chemicals

UA (CAS 6159-44-0) was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA, USA) and used as received. The $^{234}\text{U}/^{238}\text{U}$ atom ratio was $7.6 \pm 2 \times 10^{-6}$, consistent with the depleted form of uranium (Wilson *et al.*, 2014). Cis-diamminedichloro-platinum (II) (CAS 15663-27-1, cisplatin) was obtained from Selleck Chemicals (Houston, TX, USA); potassium dichromate (CAS 7778-50-9) was obtained from Mallinckrodt Baker, Inc. (Paris, KY, USA) and sodium (meta)arsenite (CAS 7784-46-5) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). All were used as received. *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (VWR International, Radnor, PA, USA), was prepared as 33.0 mM or 50.0 mM stock solutions, and the desired pH was obtained by addition of NaOH or HCl at 37 °C. Buffers were treated with Chelex[®] 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) followed by 0.2 µm filtration to remove trace metals. All metal solutions were prepared immediately before use. The final pH values of reaction solutions were measured in mock 2 ml reaction solutions. Reactions with UA in pH 7.44 ACES buffer yielded a final reaction pH 7.40 ± 0.05 at 37 °C. The pBR322 plasmid was obtained from New England Biolabs (Ipswich, MA, USA) and diluted in water and reacted at final concentrations of 0.30 ± 0.08 mM DNA-P.

Incubations of pBR322 DNA with Uranyl Ion and Ultraviolet Radiation

UA (500 µM) was preincubated with pBR322 plasmid DNA in 25.0 mM ACES buffer (pH 7.4, 37 °C) for 30 min in the dark at 37 °C. Exposures to UVB radiation were carried out under a 15 W Blak-Ray Model XX-15 M bulb (mid-range of 302 nm) (UVP Inc., Upland, CA, USA) at a fixed distance of 31.0 ± 0.1 cm with a measured irradiance of 590 ± 4 µW cm⁻². UVB intensity was measured with a UVB Model UVX-31 Digital Radiometer (UVP Inc.). Exposures to UVA radiation were carried out under a 15 W USHIO BLB UVA fluorescent lamp (peak emission 368 nm) (USHIO America, Cypress, CA, USA) at a fixed distance of 31.0 ± 0.1 cm with a measured irradiance of 120 ± 5 µW cm⁻². UVA intensity was measured with a Blak-Ray Model J-221 Longwave UV meter (UVP Inc.). Control reactions consisted of untreated pBR322 DNA, and pBR322 DNA reacted individually with UA, UVA or UVB radiation.

Detection of Heat-Sensitive and Piperidine-Sensitive Sites in pBR322 Plasmid DNA

Samples of pBR322 DNA exposed to combinations of UA and UV radiation, or control reactions, were carried out as described above in triplicate. After the UA incubations and

UV radiation irradiation, three different post-treatment exposures were carried out to measure the effect of heat and piperidine on the reaction-induced DNA lesions. One set of samples serving as the control was placed in an unheated (room temperature, RT) heat block for 30 min after receiving a volume of water equivalent to that for piperidine exposures. The second set of samples, representing the heat treatment, was incubated with an equal volume of water in the absence of piperidine for 30 min in a heat block at 60 °C. The third set of samples was incubated for 30 min at 60 °C with 30 μ M piperidine. Samples were then treated with 2 μ l loading dye and analyzed by gel electrophoresis.

Gel Electrophoresis

Relaxation of supercoiled pBR322 plasmid DNA was observed by gel electrophoresis on 1% agarose gels with $0.5 \times$ TBE running buffer (45 mM Tris base, 45 mM boric acid and 1 mM EDTA) at 120 V for 90 min. Gels were stained with ethidium bromide, destained with water and images were digitally captured on a FluoroChem SP camera (Cell Biosciences, Inc., Santa Clara, CA, USA). The percentage plasmid relaxation relative to supercoiled DNA was quantified from the original digital images using UN-SCAN-IT gel software, Macintosh version 5.3 (Silk Scientific Inc., Orem, UT, USA). Gel images are presented with inverted intensity. Combined data are presented as mean $\pm$ SEM for $n = 4$ –45 independent experiments.

Cell Culture

The CHO cell lines AA8, EM9, UV5, UV20, UV41 and UV135 were obtained from the American Type Culture Collection (Manassas, VA, USA), and were cultured as adherent monolayers in α MEM (Mediatech Inc., Manassas, VA, USA) supplemented with 10% (v/v) FetalClone II Serum (Hyclone Laboratories, Logan, UT, USA), antibiotic/antimycotic (100 U ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin (Hyclone) and 0.25 $\mu\text{g ml}^{-1}$ amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), and 1% L-alanyl-glutamine (Mediatech, Inc.). Human keratinocyte HaCaT cells were obtained from GT Bowden, University of Arizona, and were cultured as adherent monolayers in Dubecco's modified Eagle medium (Mediatech, Inc.) supplemented with 10% (v/v) FetalClone II Serum, 1% penicillin/streptomycin and 1% L-alanyl-glutamine.

Cells were maintained in a 37 °C humidified incubator with 5% carbon dioxide/ambient air, which was calibrated with a Fryrite analyzer (Bacharach Co., Pittsburgh, PA, USA). For cell survival experiments, cells were seeded in 100 mm plates at a density of 8×10^5 cells per plate (CHO AA8), and 0.9 – 1.0×10^6 cells per plate (repair-deficient CHO or HaCaT) cells per plate, allowed to adhere for over ~ 24 h, and treated with sterile-filtered aqueous solutions of UA, sodium arsenite, potassium dichromate or cisplatin. Cisplatin solutions were solubilized in a small volume of DMSO (1% final v/v) and diluted in water before sterile filtration. All treatments with metal solutions lasted 24 h. For UV treatments, after UA or sodium arsenite treatments and just before harvesting, adherent cells were rinsed three times with $1 \times$ phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 12.0 mM NaH_2PO_4 , 1.76 mM KH_2PO_4 , pH 7.4), PBS was removed, and cells were exposed to UVB radiation (302 nm) at a fixed distance of 30.3 ± 0.1 cm and a fluency rate of 650 ± 3 $\mu\text{W cm}^{-2}$. UVB radiation intensity was quantified using a UVB Model UVX-31 Digital

Radiometer (UVP Inc.). Treatment order was also reversed, for which cells were allowed to adhere for 24 h, rinsed with PBS, exposed to UVB, and medium was replaced, followed by UA treatments for 24 h. For colony formation assays, cells were harvested with 0.05% trypsin/0.02% EDTA (Sigma Aldrich) in PBS; cell counts were determined with a Z1 Coulter particle counter (Beckman Coulter, Inc., Miami, FL, USA), and reseeded in quadruplicate at a density of 200 (CHO AA8 and CHO UV20), 250 (CHO EM9 and CHO UV5), 400 (CHO UV41 and CHO 135) or 1500 (HaCaT) cells per 60 mm dish with 5 ml complete medium. After 8 days, colonies were rinsed with PBS and stained with crystal violet. CHO colonies were counted by hand and images of HaCaT colonies were captured with a FluoroChem SP camera (Cell Biosciences), and colony number was quantified with Image-J software and averaged for quadruplicate doses. Data are presented as percentage of controls, mean $\pm$ SEM for $n = 3$ –14 independent experiments. Plating efficiencies were $86 \pm 2\%$ (AA8), $77 \pm 1\%$ (UV20), $74 \pm 1\%$ (EM9), $73 \pm 1\%$ (UV5), 49 ± 1 (UV135), $33 \pm 2\%$ (UV41) and $29 \pm 1\%$ (HaCaT).

Statistical Analyses

The statistical significance for levels of plasmid relaxation and for cell survival doses was evaluated by ANOVA with a Tukey honestly significant difference *post hoc* test. The statistical significance of the effect of piperidine, mannitol or catalase on plasmid relaxation, and of the synergistic effect of combined exposures on cell survival was evaluated by a Student's *t*-test. Differences were considered significant at $P < 0.05$. Statistical outliers were verified by Grubbs' test (extreme studentized deviate method), $P < 0.05$.

Results

Effect of Heat and Piperidine on pBR322 Plasmid DNA/Exposed to Uranyl Acetate and Ultraviolet Radiation

Previous work in this laboratory has shown that reactions of UA and ascorbate with pBR322 DNA produced uranyl–DNA adducts that converted to SSB upon heating, and that piperidine decreased the formation of SSB by removing uranyl–DNA adducts (Wilson *et al.*, 2014). The purpose of the current work was to apply these methods to photoactivated UA by comparing the ability of UVB (302 nm) and UVA (368 nm) radiation to activate UA to produce DNA damage, and to compare the effects of heat and piperidine on the DNA lesions formed from these reactions.

Plasmid DNA was incubated with 500 μ M UA for 30 min at 37 °C before exposure to UVB radiation (18, 35 or 53 mJ cm^{-2}) (Fig. 1A) or UVA radiation (18, 35 or 53 mJ cm^{-2}) (Fig. 1B). Control reactions consisted of DNA alone, uranyl alone, or exposure to UVB radiation in the absence of UA. All experiments were run in triplicate, then subjected to three different post-treatment exposures. After exposure to UA $\pm$ UV radiation, one set of reactions was incubated for 30 min with 30 μ M aqueous piperidine at 60 °C, one set was incubated with an equivalent volume of water at 60 °C and one set received an equivalent volume of water at RT. All samples were then subjected to gel electrophoresis.

Reactions of plasmid DNA with UA alone were consistent with previous experiments (Wilson *et al.*, 2014; Fig. 1A,B). Incubations that did not receive additional heat or piperidine produced an insignificant 1.1-fold plasmid relaxation above background (Fig. 1A,B, lane 2); however, UA incubations that were subjected to additional heat produced an average 1.4-fold higher plasmid relaxation than UA incubations that were not heated ($P < 0.01$) (Fig. 1A,B, lane 10 vs. lane 2). The effect was lost upon incubation with piperidine, which produced an averaged plasmid relaxation equivalent to background levels (Fig. 1A,B, lane 18 vs. lane 2).

Reactions of UA-exposed plasmid with UVB radiation (302 nm) that were not subjected to heat or piperidine showed an increase in plasmid relaxation (Fig. 1A, lanes 6–8) that was not observed for control reactions (Fig. 1A, lanes 1–5). Equivalent reactions that were subjected to heat showed the same increase in plasmid relaxation for UA $\pm$ UVB exposures (Fig. 1A, lanes 14–16) and the same lack of increase in control reactions (Fig. 1A, lanes 9–13). Equivalent reactions that were subjected to heat and piperidine also showed more relaxation for reactions (Fig. 1A, lanes 22–24) than controls (Fig. 1A, lanes 17–21); however, there was less plasmid relaxation overall compared to the –heat and + heat exposures. In addition, the decrease in supercoiled DNA bands observed for UA $\pm$ UVB reactions (Fig. 1A, lanes 22–24) was reproducible for replicate experiments.

Results for the reactions of UA-exposed plasmid with UVA radiation (368 nm) were similar to those with UVB radiation, in that DNA exposed to UA followed by UVA (Fig. 1B: lanes 6–8; 14–16; 22–24) had more plasmid relaxation than control plasmid (Fig. 1B: lanes 1–5; 9–13; 17–21).

Quantification of the amounts of plasmid relaxation represented in Fig. 1 showed differences among post-treatment exposures (Fig. 2). Within each experiment, percentage plasmid relaxation for untreated DNA was subtracted from that for treated DNA, and results were averaged for replicate experiments. Data are shown for reactions not subjected to heat or piperidine (Fig. 2A), for those subjected to heat (Fig. 2B) and for those subjected to heat plus piperidine (Fig. 2C). Four comparisons were made within each post-treatment exposure: (1) percentage plasmid relaxation for reactions was compared to that for controls; (2) plasmid relaxation was compared to that predicted for an additive response; (3) the dose–response, in other words the effect of energy density on percentage plasmid relaxation was compared for each wavelength of UV radiation; and (4) the effect of UV wavelength (302 vs. 368 nm) on plasmid relaxation was compared for equivalent energy densities. For comparisons among post-treatment exposures, the effect of heated vs. unheated or heat $\pm$ piperidine were compared across equivalent reaction conditions.

For experiments not subjected to heat or piperidine (Fig. 2A), photoactivation of UA by either UVB or UVA radiation resulted in statistically greater plasmid relaxation than was measured for DNA treated with UA or UV radiation individually. Furthermore, exposure to photoactivated UA caused significantly higher plasmid relaxation than would be predicted for additive exposures, defined as a synergistic response (Berenbaum, 1981). There was a slight dose–response, in terms of the effect of energy density on percentage plasmid relaxation; however, it was not statistically significant for the three radiant exposures (18, 35

or 53 mJ cm^{-2}) regardless of wavelength of UV radiation. For reactions with equivalent energy densities, for example, UA-treated DNA exposed to 18 mJ cm^{-2} UVB vs. UA-treated DNA exposed to 18 mJ cm^{-2} UVA, there was a trend for slightly more plasmid relaxation with UVB vs. UVA; however, the effect was not statistically significant. This result was consistent with our working hypothesis that photoactivated UA is the agent responsible for generation of SSB (*vide infra*).

Post-treatment exposure to heat was used to analyze the extent to which DNA adducts could be converted to SSB, as had previously been shown for uranyl–DNA adducts at the phosphate backbone (Wilson *et al.*, 2014). For the current experiments, reactions that received subsequent heat exposures were similar to those that did not, in that the percentage plasmid relaxation for each reaction was also statistically greater than controls and statistically greater than the additive (Fig. 2B). The only significant dose–response for heat-treated samples was the greater relaxation for the radiant exposure of 53 mJ cm^{-2} UVB vs. 18 mJ cm^{-2} UVB ($P < 0.05$). The wavelength for photoactivation had no significant effect on plasmid relaxation within heat-treated samples.

The effect of heat on DNA lesions generated by UA with UVA exposures was slightly stronger than that for UA with UVB exposures (Fig. 2B vs. Fig. 2A). For photoactivation of UA by three different exposures to UVB radiation, the effect of additional heat caused minor increases of 1.1–1.3-fold more plasmid relaxation than treatments that did not receive heat. However, for the exposures of UA-treated DNA to three different levels of UVA, the increase in plasmid relaxation was 1.5–1.9-fold higher, which was consistent with the DNA lesions expected for UVA vs. UVB radiation that may be produced in addition to those from photoactivated UA (*vide infra*). The effect of heat on lesions induced by photoactivated UA was less than that for UA in the absence of UV radiation. DNA exposed to UA alone followed by heating produced 3.8-fold more plasmid relaxation than exposures to UA with no extra heat (Fig. 2B vs. Fig. 2A). The observation that reaction of UA with DNA produced heat-labile lesions was consistent with previous results (Wilson *et al.*, 2014).

Post-treatment exposure to warm piperidine was used to analyze the extent to which any photoactivated UA-induced lesions, or UV-induced alkali-labile lesions, for example pyrimidine(6-4)pyrimidone photoproduct ([6-4]PP), Dewar isomers, adenine and thymine products, and other abasic sites, could be converted to SSB (Hejmadi *et al.*, 1994). For reactions that received subsequent heat and piperidine exposures (Fig. 2C), the loss of relaxed plasmid was apparent for reactions with both wavelengths of UV radiation. DNA samples exposed to UA and the two highest energy densities of UVB radiation had plasmid relaxation levels that were statistically greater than controls, and just significantly greater than the additive; however, plasmid relaxation for reactions with UVA radiation were not significantly greater than controls. Neither wavelength produced a significant dose–response, nor was there a statistically significant effect of UV wavelength.

The post-treatment exposure of DNA samples to warm piperidine resulted in two-to fivefold lower levels of measureable plasmid relaxation for UVB reactions than those exposed to heat without piperidine, and analogous reactions with UVA produced five-to sixfold less plasmid relaxation than those exposed only to heat (Fig. 2C vs. Fig. 2B). Warm piperidine

exposure produced two-to fourfold less plasmid relaxation than RT exposures for both UVB and UVA (Fig. 2C vs. Fig. 2A). Results are consistent with the presence of AP sites for reactions of UA with both UVB and UVA (*vide infra*).

Effect of Mannitol and Catalase on pBR322 Plasmid DNA Exposed to Uranyl Acetate and Ultraviolet Radiation

Both UA and UV radiation have the potential to involve reactive oxygen species in their mechanisms of DNA damage formation, UA through possible redox chemistry (Taqui Khan and Martell, 1969), and UV radiation through its generation of reactive oxygen species (Cadet *et al.*, 2012). Therefore, the presence of reactive oxygen species was probed by carrying out reactions in the presence of mannitol and catalase, which represent scavengers for hydroxyl radical (HO[•]) and H₂O₂, respectively. For reactions with either UVB or UVA radiation, addition of 500 μM mannitol had no statistical effect on plasmid relaxation for either reactions or controls, except for a slight increase in plasmid relaxation observed for DNA alone (Supplementary Fig. S1B,1E). Similarly, uranyl photoactivation with either UVB or UVA carried out in the presence of 70 U ml⁻¹ catalase had no statistical effect on plasmid relaxation for any treatment (Supplementary Fig. S1C,1 F). Thus, reactions carried out under the current conditions could not be inferred to include reactive oxygen species as a major mechanism of DNA strand break formation, in agreement with previous studies (Nielsen *et al.*, 1992).

In summary, experiments measuring SSB formation in plasmid DNA showed that both UVB and UVA could photoactivate UA to cause strand breaks in an equally synergistic manner, DNA lesions formed in the presence of UVA-activated UA were slightly more heat labile than those formed with UVB-activated UA, and postexposure incubations with warm piperidine degraded the relaxed plasmid to control levels, with the observed effect stronger for UVA than UVB.

Cytotoxicity of Uranyl Acetate and Ultraviolet B Radiation in Repair-Proficient Chinese Hamster Ovary Cells

The cytotoxicity of photoactivated UA was measured in cultured cells. Three categories of experiments were carried out. (1) Cytotoxicity was measured in DNA repair-proficient and repair-deficient CHO cell lines exposed individually to known genotoxic metals and to UA. Comparisons of established genotoxins to UA gave insight into the pathways for DNA repair used for UA-induced DNA damage. (2) The CHO AA8 line was used to determine the importance of the order of exposure for UA and UVB treatments, and to compare the effects of UVB radiation on UA and on sodium arsenite exposures. These comparisons further tested the proposed mechanism of photoactivation as the mode of action. (3) Uranyl photoactivation was explored in NER- and BER-deficient CHO cells, and in human HaCaT keratinocytes to determine the extent to which synergistic toxicity could be observed across cell lines.

Cytotoxicity was measured in both repair-proficient and repair-deficient CHO cell lines. The cell lines are described in Table 1. The overall hypothesis guiding these experiments is that if a test agent causes DNA damage, then cell lines deficient in the repair of that category of

lesions should be more sensitive to that test agent and should show decreased cell survival relative to a DNA repair-proficient cell line if the impaired enzyme is needed for repair of the produced DNA lesions.

Cisplatin and potassium dichromate were chosen as examples of known mutagenic metals for comparison to UA and photoactivated UA. Both of these metals have been previously tested in some of the repair-deficient CHO cell lines with various exposure times; however, experiments were repeated here because there were discrepancies in the literature, and not all of the previous studies were carried out for 24 h exposures. Cytotoxicity was measured as a reduction in colony forming ability for treated groups relative to untreated controls (Fig. 3), using dose ranges that produced an observable range of cell death.

For cisplatin, the parental AA8 line showed a dose–response range of 100% to ~3% survival for a dose range of 0–2 μM after 24 h exposures (Fig. 3A). All repair-deficient lines were more sensitive to cisplatin than the AA8 line. The UV5, EM9 and UV135 lines displayed moderately decreased cell survival relative to the AA8 line. For the 0.8 μM dose, UV5 had 4.5-fold lower survival, EM9 had 14-fold lower survival and UV135 had 40-fold lower survival than the equivalent dose in AA8. The UV20 and UV41 lines displayed the strongest sensitivity to cisplatin within the tested series. A 50 nM dose in the UV135 line yielded $94 \pm 7\%$ survival, and in comparison the UV20 and UV41 lines were ~2-fold and 14-fold more cytotoxic, respectively. The approximate LC_{50}s calculated from linear curve fits were 1, 0.5, 0.4, 0.4, 0.05 and 0.02 μM for AA8, UV5, EM9, UV135, UV20 and UV41, respectively.

Potassium dichromate treatment resulted in a different relative order of sensitivity for the repair-deficient CHO lines than for the cisplatin treatment (Fig. 3B) and smaller differences in sensitivities. The approximate LC_{50}s calculated from linear curve fits were 36, 25, 21, 21, 20 and 18 μM for AA8, UV41, UV20, UV135, EM9 and UV5, respectively, giving a range of relative sensitivities of only ~1.4- to 2-fold compared to AA8. In summary, both metals were more sensitive in the repair-deficient lines than the parental line. These data suggest that both Pt(II) and Cr(VI) produce DNA lesions that can be targeted by both NER and BER (*vide infra*).

Studies with UVB radiation and UA were carried out in the same six CHO cell lines. For exposures to UVB radiation alone, all repair-deficient lines were more sensitive than the parental AA8 line (Fig. 4A). However, two repair-deficient lines, the EM9 and UV5, were more resistant to UVB radiation than the UV20, UV41 and UV135 lines. The EM9 line was only ~1.4-fold more sensitive than the AA8 line, whereas the UV5 line was only ~3-fold more sensitive, based on curve fitting approximations. This is consistent with the dominant lesions for UVB being cyclobutane pyrimidine dimer (CPD) photoproducts, which would require NER, and minor lesions, including oxidative damage, which would require BER (Cadet *et al.*, 2012).

In contrast to results with platinum, chromium and UVB radiation, UA only decreased cell survival in the XRCC1-deficient EM9 line and the XPG/ERCC5-deficient UV135 line, and showed no decrease in cell survival in the other NER-deficient CHO lines relative to the parental AA8 line at exposures up to 200 μM (Fig. 4B). However, expanding the UA dose to

300 μM and the treatment time to 48 h did produce a slightly increased cytotoxicity in the UV5 line relative to the AA8 line (Supplementary Fig. S2), which may reflect the accumulation of NER-dependent lesions with more extended treatment conditions. The results in the EM9 line were consistent with previous studies from this laboratory (Stearns *et al.*, 2005), and likely reflect a different spectrum of DNA damage than is produced by cisplatin and potassium dichromate, in that uranyl-induced DNA damage is more dependent on BER than NER (Wilson *et al.*, 2014, *vide infra*). The results for the UV135 line were also consistent with this interpretation as, although XPG is known to be involved with NER, it also serves as a cofactor for DNA glycosylase-AP lyase hNth1, which has been shown to be involved in the initiation of BER (Klungland *et al.*, 1999).

Experiments measuring the cytotoxicity of photoactivated uranium in repair-proficient CHO AA8 cells showed that activity was dependent on the order of exposures, and was stronger than the cytotoxicity of combined exposures of sodium arsenite and UVB radiation (Fig. 5). The hypothesis guiding these experiments was that if UA binds to DNA and becomes photoactivated, it would produce more DNA damage and cell death than individual exposures. CHO AA8 cells that were exposed to UA for 24 h, then washed with PBS and exposed to UVB radiation, showed increased cytotoxicity relative to cells exposed to UVB alone, and were more cytotoxic than what would be calculated for an additive response to UA and UVB (Fig. 5A). For example, the 200 μM dose of UA resulted in $90 \pm 4\%$ survival, the 4.5 mJ cm^{-2} exposure to UVB yielded $35 \pm 4\%$ survival, and the combined exposure gave $6 \pm 1\%$ survival, compared to a calculated additive response of 31%.

It was further hypothesized that if the observed cytotoxicity was a result of UA binding to DNA and undergoing photo-activation, then reversing the treatments, exposing cells to UVB radiation then to UA, should not have a synergistically toxic effect. Experiments in which AA8 cells were rinsed with PBS, exposed to UVB radiation, and then exposed to UA for 24 h showed that the synergistic cytotoxicity was lost, and cells displayed no more decreased survival than that resulting from UVB exposures alone (Fig. 5B); for example, UVB exposure followed by 200 μM UA exposure resulted in $36 \pm 7\%$ survival vs. the 31% expected.

The activity of UA in the presence of UVB radiation was compared to that for sodium arsenite in AA8 cells. Arsenite is a well-established systemic human carcinogen, with mechanisms of action that do not require direct reactions with DNA, but rather involve interference with signal transduction pathways and tumor suppressor systems, and inhibition of DNA repair (Hartwig, 2013). Arsenite is also a known co-carcinogen with solar UV radiation (Rossman *et al.*, 2004) that may enhance UV-induced tumorigenicity by perturbing DNA damage or oxidative stress responses (Komissarova and Rossman, 2010; Qin *et al.*, 2012; Sun *et al.*, 2011; Zuo *et al.*, 2012). Unlike the case of UA, the direct photo-oxidation of arsenite by wavelengths of UV radiation above 254 nm is negligible (Ryu *et al.*, 2013). With conditions identical to those for UA and UVB radiation (Fig. 5A), AA8 cells were exposed to 0–20 μM sodium arsenite in the presence and absence of UVB radiation (Fig. 5C). The highest dose of As (III) tested, 20 μM , resulted in $59 \pm 1\%$ survival, and the highest UVB radiant exposure, 4.5 mJ cm^{-2} , resulted in $34 \pm 3\%$ survival. The theoretical additive response would be 20% survival, and $18 \pm 3\%$ was observed, which was not a

statistically significant difference. Thus, the effects of combined exposures of UA and UVB radiation are not modeled by combined exposures of arsenite and UVB radiation, even at what are considered relatively high doses of arsenite.

Cytotoxicity of Uranyl Acetate and Ultraviolet B Radiation in Repair-Deficient Chinese Hamster Ovary Cells and Human Keratinocyte Skin Cells

The ability of photoactivated UA to be synergistically cytotoxic was measured in NER-deficient and BER-deficient CHO cells, and in human keratinocyte HaCaT cells. Experiments were carried out in the repair-proficient AA8 line, and the repair-deficient EM9 and UV5 lines as the other repair-deficient CHO lines were already so sensitive to UVB radiation that additional toxicity from uranyl exposures would not be detectable under the treatment conditions available. In NER-deficient CHO UV5 cells, the highest dose of UA tested, 200 μM for 24 h, resulted in $90 \pm 3\%$ survival.

The two doses of UVB radiation, 0.66 and 1.3 mJ cm^{-2} , resulted in 32 ± 5 and $3.6 \pm 0.4\%$ survival respectively, and the combined treatments for 200 μM UA and the low and high exposures of UVB resulted in 5 ± 1 and $0.4 \pm 0.2\%$ survival respectively, which were statistically lower than the theoretical additive responses of 29% and 3%, respectively (Fig. 6A). Thus, UA was capable of potentiating the cytotoxicity of UVB radiation in cells deficient in NER.

In BER-deficient EM9 cells, the highest dose of UA tested, 200 μM for 24 h, resulted in $48 \pm 5\%$ survival. The two doses of UVB radiation, 2.5 and 5.0 mJ cm^{-2} , resulted in 58 ± 2 and $13 \pm 2\%$ survival respectively, and the combined treatments for 200 μM UA and the low and high exposures of UVB resulted in $10 \pm 3\%$ and $1.1 \pm 0.1\%$ survival respectively, which were statistically lower than the theoretical additive responses of 28% and 6%, respectively (Fig. 6B). Thus, UA was also capable of synergistically increasing the cytotoxicity of UVB radiation in cells deficient in BER.

In the human keratinocyte HaCaT cells, which are spontaneously immortalized but non-tumorigenic (Boukamp *et al.*, 1988), and have p53 mutations but otherwise normal DNA repair (Lehmann *et al.*, 1998), the highest dose of UA tested, 200 μM for 24 h, resulted in $91 \pm 5\%$ survival. The two highest doses of UVB radiation, 5.9 and 7.9 mJ cm^{-2} , resulted in 59 ± 4 and $31 \pm 5\%$ survival respectively, and the combined treatments for 200 μM UA and the lower and higher exposures of UVB resulted in $33 \pm 9\%$ and $11 \pm 4\%$ survival respectively, which were statistically lower than the theoretical additive responses of 54% and 28%, respectively (Fig. 6C). Thus, UA was also capable of potentiating the cytotoxicity of UVB radiation in human keratinocyte cells.

In summary, UA alone displayed a different cytotoxicity than UVB radiation, cisplatin and potassium dichromate. The cytotoxicity of co-exposures of UA and UVB radiation was dependent on the order of reaction, and greater than exposures to arsenite and UVB radiation, and UA and UVB radiation were synergistically cytotoxic in DNA repair-deficient and DNA repair-proficient cells.

Discussion

The current work has explored the ability of UA and photo-activated UA to cause DNA strand breaks, adducts and AP sites in isolated plasmid DNA, and to cause cytotoxicity in cultured cells. The dose range for cell culture experiments consisted of 50–200 μM UA (12–48 ppm U), which is lower than that reported in contaminated groundwater from mill tailings sites in Grants, NM (USA) (up to 50 ppm U, or 210 μM UA) (Abdelouas *et al.*, 2000), and in contaminated groundwater in Oak Ridge, TN (USA) (up to 60 ppm U, or 250 μM UA) (Cardenas *et al.*, 2008). The dose of 500 μM UA used for the plasmid relaxation experiments is also below the recommended limits for genotoxicity testing of 1 mM or 500 ppm (Parry *et al.*, 2010). Therefore, although these *in vitro* experiments have been carried out to probe the mode of action underlying uranium genotoxicity, they are pertinent to possible high ranges of human exposures.

The UV radiation levels in these experiments are also physiologically relevant. Using the definition of a standard erythral dose (SED) of 10 mJ cm^{-2} (or 100 J m^{-2}) (Diffey, 2002), the maximum doses of UVB radiation used in cell culture experiments (8 mJ cm^{-2}), and in the plasmid DNA experiments (53 mJ cm^{-2}) are equivalent to 0.8–5.3 SED. Using Diffey's estimates of ambient erythral UV exposure (Diffey, 2009), a value of 5.3 SED would be equivalent to ~ 1 h of June sunlight for a UV index of 12 in Flagstaff, AZ (USA).

UA is known to be activated by UV radiation (300–420 nm) to form what is interpreted as uranium 5+ and a uranium-bound oxygen radical (Nielsen, 2009; Wang *et al.*, 1995). The overall hypothesis guiding the current work is that if UA is photoactivated in the presence of DNA, then the nature or levels of DNA damage should be different than that produced by UA or UVB radiation independently, and that this may serve as a chemical mechanism for uranium toxicity. Initial support for this hypothesis was found for reactions of UA and UVB radiation (302 nm) in the presence of plasmid DNA, which produced DNA strand breaks in a synergistic manner (George *et al.*, 2011).

The first of the current experiments compared the ability of three radiant exposures of UVB (302 nm) and three radiant exposures of UVA (368 nm) to photoactivate 500 μM UA to produce SSB in pBR322 plasmid DNA. Both wavelengths of UV radiation produced synergistic levels of SSB in the presence of UA. This is consistent with the interpretation that SSB were produced by photoactivated UA, because UA is known to be photo-activated by radiation in the range of UVB to UVA radiation, 300–420 nm (Nielsen, 2009); and the levels of SSB were found to be independent of radiant exposure, and independent of the wavelength of UV radiation, but equivalent for equivalent concentrations of UA.

DNA lesions formed in the presence of photoactivated UA were less heat labile than those formed by non-photoactivated UA, and DNA lesions formed in the presence of UVA-activated uranyl were slightly more heat labile than those formed with UVB-activated UA. The observation of heat sensitivity of uranyl-induced DNA damage was consistent with previous results, and interpreted to suggest that uranyl binding to the phosphate backbone produced hydrolysis of the backbone to form a SSB, and that this process was augmented by heat (Wilson *et al.*, 2014). The observation that photoactivated UA did not produce

significant levels of heat-sensitive DNA damage was consistent with the interpretation that the majority of lesions consisted of SSB, which would be formed before the post-treatment exposure to heat.

For photoactivated UA, activation by UVA produced slightly more heat-sensitive lesions than activation by UVB. This may reflect the presence of UV-induced damage in addition to that caused by photoactivated UA. For UVB exposures, the dominant form of damage consists of CPDs and [6-4]PPs (Cadet *et al.*, 2012) that are not expected to be heat labile (Rydberg 2000). For UVA exposures, DNA damage would be expected to include not only CPDs and [6-4]PPs but also radical-mediated damage (Cadet *et al.*, 2012; Lehmann *et al.*, 1998) and oxidative damage (von Sonntag and Schulte-Frohlinde, 1978) that would likely include H-atom abstraction from ribose, which would be heat labile (Rydberg, 2000).

Postexposure incubations with warm piperidine degraded the relaxed plasmid to near control levels, with the observed effect stronger for UVA-activated UA than UVB-activated UA. This may reflect the presence of more oxidative damage for UVA radiation than UVB radiation (Cadet *et al.*, 2012), for which nucleobase modification would be piperidine labile (Burrows and Muller, 1998). Piperidine exposure also degraded supercoiled plasmid DNA that was exposed to UVB-activated UA, but not to UVA-activated UA. This may reflect the presence of the [6-4]PP or the Dewar isomer, which would be formed to a greater extent with UVB radiation than UVA radiation (Cadet *et al.*, 2012), would not produce a gel shift in electrophoresis of the plasmid, but would be cleaved by piperidine treatment (McHugh and Knowland, 1995). The observation that post-treatment exposure to piperidine caused a loss of plasmid relaxation for DNA exposed to UA has been interpreted to suggest that piperidine served to remove uranium–DNA adducts (Wilson *et al.*, 2014). Thus, results are consistent with a range of lesions being produced in plasmid DNA exposed to UA in the presence of UV radiation; however, levels of UV-specific DNA lesions were not quantified in this study.

The second set of experiments measured the cytotoxicity of photoactivated UA in a series of cell lines that displayed proficient and deficient DNA repair, and compared results to those from individual exposures of UA and UVB radiation, and to results with the known genotoxic metals cisplatin and potassium dichromate. UA was observed to be less cytotoxic than UVB radiation, cisplatin and potassium dichromate, which gives insights into the types of DNA lesions formed, and their required DNA repair.

Cisplatin is known to form DNA intrastrand crosslinks as the predominant lesion (Eastman, 1986), but also forms DNA interstrand crosslinks and DNA–protein crosslinks (Wynne *et al.*, 2007). The relative repair of the different lesions contributes to the efficacy of cisplatin as a chemotherapeutic agent and to the acquired resistance of a tumor cell. The current results were consistent with previous studies reporting a ~3-fold increased sensitivity for cisplatin in UV135 and ~1.3-fold sensitivity for an XPD-deficient line (comparable to UV5) (De Silva *et al.*, 2002) and ~3- to 4-fold increased sensitivity in XRCC1-deficient CHO lines (comparable to EM9) (Abdel-Fatah *et al.*, 2013). The highest sensitivity observed for the UV20 and UV41 lines was consistent with previous work (De Silva *et al.*, 2002) and with the observation that the level and efficiency of the ERCC1/XPF dimer responsible for the 5'

incision of cisplatin–DNA adducts is a crucial determinant of the efficacy of cisplatin chemotherapy in patients with cancer (Rosell *et al.*, 2005). Four other studies reported similar sensitivities for UV5, higher sensitivities for UV20 and UV41, and lower sensitivities for EM9 (Caldecott and Jeggo, 1991; Hoy *et al.*, 1985; Meyn *et al.*, 1982; Sorenson and Eastman, 1988) than were measured here. The observed sensitivity of EM9 to cisplatin is also consistent with the report that XRCC1 was involved in the repair of interstrand crosslinks produced by cisplatin (Zhu and Lippard, 2009), and that BER may be invoked in the repair of interstrand crosslinks for a variety of agents (Wilson and Seidman, 2010).

Chromate ion is known to cause a range of DNA lesions, including Cr–DNA adducts, SSB, DNA–DNA crosslinks, DNA–protein crosslinks, alkali-labile sites and oxidative damage (Jomova and Valko, 2011; McCarroll *et al.*, 2010). The relative amounts of each type of lesion depend on the relative amounts of enzymes or biological molecules in each tissue or cell line that can donate electrons to Cr(VI) to form the ultimate reactive metabolites of Cr(V), Cr(IV), Cr(III) and free radicals. The current results agree most closely with previous studies of 24 h doses of sodium chromate in UV5 and UV41 showing ~2-fold increased sensitivity relative to AA8 (Brooks *et al.*, 2008; O’Brien *et al.*, 2005). However, a previous study of 24 h treatments of potassium chromate in EM9 showed a greater sensitivity relative to AA8 than observed currently (Messer *et al.*, 2006), and a different study reported no increased sensitivity between EM9 and AA8 for 24 h sodium chromate treatments (Grlickova-Duzevik *et al.*, 2006).

The DNA lesions produced by UA are less well characterized than those produced by cisplatin or potassium dichromate. Nevertheless, DU has been reported to produce U-DNA adducts and DNA SSB (Stearns *et al.*, 2005; Wilson *et al.*, 2014), and AP sites (Yellowhair, 2011) in cultured cells and in isolated DNA. This chemical genotoxicity is likely to contribute to a mode of action through which DU is mutagenic, aneugenic, clastogenic, transforming and tumorigenic (vide supra). The observation in the current study, that UA up to 200 μ M for a 24 h exposure is cytotoxic in BER-deficient cells but not NER-deficient cells (other than the XPG/ERCC5-deficient UV135 line), is consistent with uranyl interacting at the phosphate backbone either as an adduct or crosslink to form a relatively non-distorting lesion that does not require NER for repair beyond the crossover function of XPG/ERCC5 (Klungland *et al.*, 1999). We have previously proposed a mechanism through which a uranyl-phosphate adduct serves as a “parent lesion” to be further converted to a strand break or AP site (Wilson *et al.*, 2014).

This is the first report of the cytotoxicity of photoactivated UA in cultured cells. Consistent with the observation of the synergistic formation of SSB in plasmid DNA *in vitro*, photoactivated UA was synergistically cytotoxic in repair-proficient and repair-deficient CHO cells and human HaCaT cells. These data support the working hypothesis that photoactivated UA may provide a new mechanism for toxicity of UA, and further testing of this hypothesis is in progress.

The photoactivation of UA would be most relevant for dermal exposures. Although the lung has been the most widely recognized target organ for uranium exposures, evidence does

exist for skin being a target for cancers in uranium miners, although adverse effects were historically ascribed to radiological rather than chemical modes of action. For example, basal cell and squamous cell carcinomas were documented in Czech uranium miners (Sevc *et al.*, 1988; Sevcová *et al.*, 1978) and in miners and quarrymen in Italy, France and Spain (Suárez *et al.*, 2007). Malignant melanoma has also been observed in a cohort of Czech miners (Kulich *et al.*, 2011; Tomásek *et al.*, 1993).

Previous risk assessments for both natural uranium and DU have ignored skin as a target organ. For example, the National Research Council was recently charged with evaluating the data related to the risks to military personnel exposed to uranium. Their report did not mention skin as a target organ for military exposures to DU, but only analyzed data “representative of the risk factors associated with common routes of exposure” specifically kidney, lung, bone and liver. Furthermore, that report did not supply cancer risk estimates for soldiers who had embedded DU fragments (National Research Council, 2008). Thus, the importance of skin as a target organ for uranium exposure has been overlooked rather than disproven.

In spite of the lack of attention paid to skin as a route or target organ for uranium exposure in epidemiological studies, the dermal uptake of uranium is well established. Animal studies have shown that uranium passes through both intact and injured skin to reach other target organs (de Rey *et al.*, 1983; López *et al.*, 2000; Petitot *et al.*, 2010; Spagnul *et al.*, 2011). Monitoring of Gulf War veterans with uranium-containing shrapnel embedded in their skin has determined that the shrapnel dissolves and the uranium migrates to systemic organs and is excreted in urine (Leggett and Pellmar, 2003; McDiarmid *et al.*, 2000). Dermal exposure to uranium and other nuclides remains a major concern in the nuclear industry (Phan *et al.*, 2013; Spagnul *et al.*, 2011; Weber *et al.*, 2012).

Lastly, UA was found to be capable of potentiating the cytotoxicity of UVB radiation in cells deficient in NER, illustrated here in the XPD-deficient CHO UV5 line. This may have possible relevance to the increased incidence of xeroderma pigmentosum (XP) in the Navajo people of the southwestern USA. XP is a hereditary disease in which people with mutations in one of eight genes coding for NER proteins develop non-melanoma skin cancer at rates of ~1000-fold higher than the general population (Nikolaou *et al.*, 2012). The increased incidence of XP and other hereditary diseases in Navajo has been attributed to a “genetic bottleneck,” specifically, the severe population decline that was a consequence of the traumatic “long walk,” or forced relocation of the Navajo people from Arizona to New Mexico in the 1860 s (Erickson, 2009). The Navajo reservation, which spans the four corners region of the southwestern USA, is also the site of historically high levels of uranium mining and processing (Brugge and Goble, 2002) and the subsequent contamination and pollution that accompanies those industries (Abdelouas *et al.*, 2000; Brugge *et al.*, 2007; Dawson and Madsen, 2011). Our observation that uranium potentiates the toxicity of UV radiation in NER-deficient cells may have implications for a population with relatively high exposures to uranium, solar radiation, and with both homozygous and heterozygous carriers of XP gene mutations.

Conclusions

This is the first study to build on previous knowledge of UA photoactivation to show that excitation of UA by UV radiation can provide a mechanism for phototoxicity in cells, and thus may provide another pathway for UA to be chemically toxic for populations with dermal exposures. The doses of UA used for cell culture experiments were lower than uranium levels in contaminated groundwater. The current study contributes to our understanding of DNA lesions formed by DU, as well as their repair. Characterizing the types of DNA lesions formed by DU under different conditions will be necessary to understand fully the extent to which the DNA lesions may or may not be formed and repaired *in vivo*, how these DNA lesions may be converted to the mutations that have been observed in cultured cells and, ultimately, if any uranium-specific biomarkers of exposure exist in humans exposed to uranium, either occupationally or environmentally. The National Research Council's Report on cancer risk assessment for DU exposure was admittedly based on the radioactive, not chemical, properties of DU, and the report concluded: "the committee recommends research into whether there is a chemical mechanism of uranium carcinogenesis" (National Research Council, 2008). We propose that skin has not been ruled out as a target organ for uranium exposure, but rather may have been simply overlooked. Therefore, we recommend that future risk assessment regarding the chemical carcinogenicity of uranium includes the possibility of photoactivation in skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representative gels illustrating DNA single strand breaks (Form II) produced in pBR322 DNA after exposure to UA and UV radiation, and effects of heat and piperidine on DNA lesions. (A) Reactions of pBR322 DNA with UA (500 μ M) and UVB radiation (302 nm, 18, 35, or 53 mJ cm⁻²) followed by 30 min post-treatment exposure to water at room temperature (lanes 1–8); water at 60 °C (lanes 9–16) or 30 μ M piperidine (aq) at 60 °C (lanes 17–24). (B) Reactions of pBR322 DNA with UA (500 μ M) and UVA radiation (368 nm, 18, 35 or 53 mJ cm⁻²) followed by 30 min post-treatment exposure to water at room temperature (lanes 1–8); water at 60 °C (lanes 9–16) or 30 μ M piperidine (aq) at 60 °C (lanes 17–24). UA, uranyl acetate; UV, ultraviolet.

Figure 2.

Quantification of percentage DNA migrating as Form II (Relaxed) for reactions of UA and UV radiation and the effect of post-treatment exposures. Data represent % plasmid relaxation in treated samples – % plasmid relaxation in untreated DNA. pBR322 DNA was exposed to UA (500 μ M) and either UVB (302 nm) or UVA (368 nm) radiation for 30 min at 37 $^{\circ}$ C as in Fig. 1. Post-treatment exposures consisted of incubation with (A) water at RT; (B) water at 60 $^{\circ}$ C; or (C) 30 μ M piperidine (aq) at 60 $^{\circ}$ C. Data represent mean $\pm$ SEM for $n = 4$ experiments. Statistical significance of plasmid relaxation for combined UA and UV exposures vs. individual exposures was determined by ANOVA (*ns* not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$). Statistical significance of plasmid relaxation for combined UA and UV exposures vs. relaxation calculated for additive response was determined by Student's *t*-test (*ns*, not significant; $\#P < 0.05$; $\##P < 0.01$; $\###P < 0.001$; $\####P < 0.0001$). PIP, piperidine; RT, room temperature; Rxn, reactions; UA, uranyl acetate; UV, ultraviolet.

Figure 3.

Cytotoxicity in Chinese hamster ovary cell lines measured by a colony formation assay after exposures to (A) cisplatin; (B) potassium dichromate. Exposure times were 24 h. Data represent mean $\pm$ SEM for $n = 4-6$ experiments. Repair-proficient (AA8) and repair-deficient (UV5, EM9, UV135, UV20, UV41) cell lines are described in Table 1. Cr(VI), potassium dichromate; Pt, cisplatin.

Figure 4.

Cytotoxicity in Chinese hamster ovary cell lines measured by a colony formation assay after exposures to (A) UVB radiation (302 nm); or (B) UA for 24 h. Data represent mean $\pm$ SEM for $n = 3-8$ experiments. Repair-proficient (AA8) and repair-deficient (EM9, UV5, UV20, UV41, UV135) cell lines are described in Table 1. For graph B, equivalent doses of UA were determined as statistically more toxic in the UV135 and EM9 lines than in the repair-proficient AA8 line by a Student's t -test ($##P < 0.01$). UA, uranyl acetate; UV, ultraviolet.

Figure 5.

Cytotoxicity in repair-proficient Chinese hamster ovary AA8 cells measured by a colony formation assay. (A) Cells were exposed to UA for 24 h followed by UVB radiation. (B) Cells were exposed to UVB radiation followed by exposure to UA for 24 h. (C) Cells were exposed to NaAsO₂ for 24 h followed by UVB radiation. Data represent mean $\pm$ SEM for $n = 3$ –14 experiments. For graphs A–C, the percentage survival for combined exposures was compared to the calculated additive response by a Student's t -test (ns, not significant; ## $P < 0.01$; #### $P < 0.0001$). (A,B) UA treatments yielded a survival of $> 90\%$ in CHO AA8 cells and combined exposures were determined as statistically more toxic than UVB exposure alone by ANOVA when UA exposure preceded UVB exposure, but not when UVB exposure preceded UA exposure (ns, not significant; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). NaAsO₂, sodium arsenite; UA, uranyl acetate; UV, ultraviolet.

Figure 6.

Cytotoxicity measured as colony formation in three cell lines exposed to UA for 24 h followed by UVB radiation (302 nm). (A) Repair-deficient CHO UV5 cells; (B) repair-deficient CHO EM9 cells; (C) repair-proficient human HaCaT cells. Data represent mean $\pm$ SEM for $n = 4-14$ independent experiments. (A–C) Combined exposures were determined as statistically more toxic than the calculated additive response by a Student's t -test ($\#P < 0.05$; $\#\#P < 0.01$; $\#\#\#P < 0.0001$). (A,C) UA treatments yielded a survival of $> 91\%$ in CHO UV5 and HaCaT cells and combined exposures were determined to be statistically more toxic than UVB exposure alone by ANOVA ($*P < 0.05$; $****P < 0.0001$). CHO, Chinese hamster ovary; UA, uranyl acetate; UV, ultraviolet.

Table 1

Chinese hamster ovary (CHO) lines used in this study

CHO line	Gene deficiency	Protein function impaired	Reference
AA8	None	–	
EM9	XRCC1	Scaffold protein, ligation, gap filling	Caldecott <i>et al.</i> , 1994
UV20	ERCC1 (dimer with XPF)	5' incision	Murray and Rosenberg, 1996
UV5	ERCC2 (XPD)	5'–3' DNA helicase	Cullinane <i>et al.</i> , 1997
UV41	ERCC4 (XPF)	5' incision	Murray and Rosenberg, 1996
UV135	ERCC5 (XPG)	3' incision	Thompson <i>et al.</i> , 1987