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A clickable psoralen to directly quantify DNA interstrand crosslinking and repair

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Abstract

DNA interstrand crosslinks (ICLs) represent physical obstacles to advancing replication forks and transcription complexes. A range of ICL-inducing agents have successfully been incorporated into cancer therapeutics. While studies have adopted UVA-activated psoralens as model ICL-inducing agents for investigating ICL repair, direct detection of the lesion has often been tempered by tagging the psoralen scaffold with a relatively large reporter group that may perturb the biological activity of the parent psoralen. Here a minimally-modified psoralen probe was prepared featuring a small alkyne handle suitable for click chemistry. The psoralen probe, designated 8-propargyloxypsoralen (8-POP), can be activated by UVA *in vitro* to generate ICLs that are susceptible to post-labeling with an azide-tagged fluorescent reporter via a copper-catalyzed reaction. A modified alkaline comet assay demonstrated that UVA-activated 8-POP proficiently generated ICLs in cells. Cellular 8-POP-DNA lesions were amenable to click-mediated ligation to fluorescent reporters *in situ*, which permitted their detection and quantitation by fluorescence microscopy and flow cytometry. Small molecule DNA repair inhibitors to 8-POP-treated cells attenuated the removal of 8-POP-DNA lesions, validating 8-POP as an appropriate probe for investigating cellular ICL repair. The post-labeling strategy applied in this study is inexpensive, rapid and highly modular in nature with the potential for multiple applications in DNA repair studies.

Graphical Abstract

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Author Contributions

BJE designed the research, performed all experiments except production of 8-POP, analyzed the data, and wrote the manuscript. MLA produced 8-POP. NF conducted the project, analyzed the data, and edited the manuscript. All authors have approved the final version of the manuscript.

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interstrand DNA crosslink; DNA repair; click chemistry; psoralen; imaging; chemical screening

1. INTRODUCTION

DNA interstrand crosslinks (ICLs) are specialized lesions that covalently bridge opposing strands of duplex DNA. Such lesions are generated by agents that occur either endogenously within cells (e.g. aldehydes generated by lipid peroxidation) or exogenously via cellular exposure to various environmental mutagens and certain chemotherapeutic agents [1, 2]. Following their generation within mammalian cells, ICLs are highly lethal to cell viability as they resist DNA strand separation, a process typically required for transcription and DNA replication to proceed. Moreover, these lesions are challenging for mammalian cells to repair and typically represent a greater threat to viability relative to *intrastrand* crosslinks, covalent lesions that link bases within a single DNA strand. Given their lethal nature, agents that induce DNA ICLs occupy an important niche in the repertoire of agents presently available for the treatment of human cancers [2].

Most ICL-inducing agents generate intrastrand crosslinks and monofunctional adducts, with ICLs only occupying a small fraction of all adducts [1-3], a feature that renders them difficult to detect in biological samples. To overcome these limitations, studies have adopted the use of psoralens as model ICL-inducing agents. Psoralens are furocoumarins that exhibit a planar tricyclic ring structure that occur naturally in various plants [3-5]. Following intercalation within duplex DNA, psoralens can be photoactivated by UVA to generate stable monofunctional adducts and ICLs in both naked DNA *in vitro* and in cellular DNA [4]. The psoralens favour intercalation at 5'-TpA dinucleotides of duplex DNA and, following activation by UVA light, covalently bond with a thymine nucleobase in either strand (to generate a monoadduct) or both strands (to produce an ICL) [3, 6]. In comparison to other ICL-inducing agents, the psoralens induce a significantly higher yield of ICLs relative to other lesion types; up to 40% of 8-methoxypsoralen (8-MOP)-induced adducts form ICLs following photoactivation by UVA [3]. Psoralen-induced ICLs within cells have successfully been detected via mass spectrometry, high performance liquid chromatography and alkaline comet assays, although these methods tend to be labor-intensive, time-consuming, technically challenging and often require the use of specialized equipment and materials [1, 5-8]. Direct detection of cellular 8-MOP-DNA adducts can also be achieved by antibodies raised against 8-MOP-DNA photoadducts, however these antibodies are no longer available [9, 10]. Other efforts have been directed at chemically tagging psoralens with various reporter groups including biotin, digoxigenin and fluorescent labels that are

amenable to detection *in situ* [11, 12]. While these psoralen conjugates have yielded valuable insights in ICL repair studies [13, 14], their tags are rather large and bulky that can perturb the molecular nature of native psoralens. Indeed, two of psoralen conjugates demonstrated unusual cellular distribution patterns inconsistent with the native psoralen [12], thereby prohibiting their experimental utility. Electroporation was required to permit sufficient cellular uptake of a biotin-labeled psoralen [11], a condition unnecessary in the case of the native psoralens.

These issues can be circumvented through post-labeling a minimally-modified psoralen probe with a reporter *in situ*. Perhaps the best method presently available to achieve this is through the Cu(I)-catalyzed reaction between an alkyne- and azide-tagged pair of molecules, a bioorthogonal conjugation so-called click chemistry [15, 16]. Given their small steric nature, the introduction of either an alkyne- or azide-handle affords a readily detectable biological probe without grossly affecting the structure of the parent compound [16]. We report herein the preparation and development of a psoralen probe with a small alkyne handle. The probe, termed 8-propargyloxypsoralen (8-POP), can be activated by UVA to generate DNA ICLs and is readily detectable *in situ* by ligation with an azide-tagged fluorescent reporter.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals were sourced from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Sodium ascorbate was from Acros Organics (Geel, Belgium) while hydrogen peroxide (30%) was purchased from Thermo Fisher Scientific (Waltham, MA). Electron microscopy grade paraformaldehyde solution (16%) was obtained from Electron Microscopy Sciences (Hatfield, PA). *NdeI* was purchased from New England Biolabs (Beverly, MA). SYBR green I stain and Alexa fluor azide reagents (488 and 546, US Patents US20080050731 A1) were from Life Technologies (Carlsbad, CA). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA). Propidium iodide was obtained from Roche Life Sciences (Indianapolis, IA). Micro Bio-Spin 6 chromatography columns were purchased from Bio-Rad (Hercules, CA). T2AA ((*S*)-4-(4-(2-Amino-3-hydroxypropyl)-2,6-diiodophenoxy)phenol hydrochloride) was prepared as previously described [17]. Olaparib was obtained from Selleck Chemicals (Houston, TX). A QIAquick PCR Purification Kit was from Qiagen (Valencia, CA). A pEGFP-PCNA plasmid was originally obtained from Daniel Gerlich [18] via Addgene (Cambridge, MA).

2.2. Chemical synthesis

All commercial reagents were used without further purification. Reactions requiring the exclusion of air were carried out under an atmosphere of dry nitrogen in oven dried glassware. All reactions were monitored by thin-layer chromatography (TLC) carried out on EMD Chemicals silica gel 60-F 254 coated glass plates and visualized using UV light (254 nm). Analysis by LC-MS was performed by using an XBridge C₁₈ column run at 1 mL/min and using gradient mixtures of (A) water (0.05% TFA) and (B) methanol. Low-resolution mass spectra (ESI) were collected on a Waters Micromass ZQ in positive-ion mode. Flash

chromatography was performed on a Biotage Isolera chromatography system using Biotage SNAP KP-SIL pre-packed columns, and the solvent mixture in brackets was used as eluent. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance II NMR spectrometer at 400 MHz for ^1H NMR spectra. Chemical shifts (ppm) are reported relative to the solvent peak. Signals are designated as follows: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quadruplet; m, multiplet. Coupling constants (J) are shown in Hertz.

2.2.1. 8-Hydroxypsoralen—A two-neck flask was charged with 8-methoxypsoralen (200 mg, 0.925 mmol) and dichloromethane (6 mL). The flask was flushed with nitrogen and cooled to 0 °C. A solution of tribromoborane in dichloromethane (1 M, 3.7 mL, 3.7 mmol) was added dropwise to the stirring reaction mixture. After 3 h, the reaction mixture was added to water (30 mL) and extracted with ethyl acetate (3×30 mL). The combined organic layers were washed with saturated brine, dried over anhydrous sodium sulfate, filtered, and concentrated. The resulting product was used in the following step without further purification (173 mg, 93% yield). ^1H NMR (400 MHz, DMSO- d_6) δ 10.67 (s, 1H), 8.25 – 7.98 (m, 2H), 7.46 (s, 1H), 7.05 (s, 1H), 6.41 (d, J = 9.7 Hz, 1H).

2.2.2. 8-Propargyloxypsoralen (8-POP)—Propargyl bromide (114 μL , 1.03 mmol) was added to a solution of 8-hydroxypsoralen (173 mg, 0.856 mmol) and potassium carbonate (473 mg, 3.42 mmol) in acetone (4 mL). The reaction mixture was heated to reflux, stirred for 3 h, then cooled and concentrated. Water (20 mL) was added and extracted with ethyl acetate (3×20 mL). The combined organic layers were washed with saturated brine, dried over anhydrous sodium sulfate, filtered, and concentrated. The crude product was purified by flash column chromatography (Biotage Isolera, 25 g SNAP column, eluting with hexanes/ethyl acetate, 0–60% gradient) to produce a white solid (40 mg, 19% yield). ^1H NMR (400 MHz, DMSO- d_6) δ 8.25 – 8.09 (m, 2H), 7.74 (s, 1H), 7.11 (d, J = 2.2 Hz, 1H), 6.45 (d, J = 9.6 Hz, 1H), 5.14 (d, J = 2.4 Hz, 2H), 3.56 (t, J = 2.5 Hz, 1H). MS (ESI) m/z , [M + H] calculated for $\text{C}_{14}\text{H}_9\text{O}_4$, 241.05; observed 241.00.

2.3 *In vitro* crosslinking assay

Supercoiled pEGFP-PCNA plasmid DNA was initially linearized by digestion with *Nde*I using standard procedures and then purified using a QIAquick PCR Purification Kit. Linearized plasmid (40 ng/ μL) was subsequently reacted with 31 μM 8-POP or DMSO vehicle in 35 mM HEPES, pH 7.4 and exposed to UVA light centered at 365 nm at room temperature. Irradiation was delivered using a UVP model UVGL-58 Handheld UV Lamp (Upland, CA) from a distance of 5.08 cm for 0 – 30 min as indicated. Samples were denatured at 80 °C for 5 min in strand separation buffer (30% DMSO, 1 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and subsequently loaded onto a 0.8% agarose gel in $1 \times$ TAE buffer. Control samples that were not thermally denatured were run in parallel and were representative of double stranded DNA. DNA was fractionated by electrophoresis for 2 – 3 h at 80 V and then stained with 0.5 $\mu\text{g/mL}$ ethidium bromide. Agarose gels were destained and then visualized and photographed under UV illumination. Quantitation was performed using ImageJ software (NIH).

2.4 *In vitro* conjugation assay

Supercoiled pEGFP-PCNA plasmid DNA (100 ng/ μ L) was initially incubated with 50 μ M 8-POP in 35 mM HEPES, pH 7.4 and then exposed to UVA irradiation for 30 min at room temperature as described above. To each sample, the following was then added: 20 μ M Alexa fluor 546 azide, 1 mM copper(II) sulfate and 0 – 5 mM sodium ascorbate or TCEP, and the samples incubated for a further 90 min at room temperature. Unreacted fluor azide was removed by gel filtration using Micro Bio-Spin 6 chromatography columns. The contents of each sample were then fractionated by electrophoresis through a 0.8% agarose gel in 1 $\times$ TAE buffer for 90 min at 100 V. Agarose gels were first scanned and visualized on an ImageQuant LAS 4000 instrument (GE Healthcare, Piscataway, NJ) operating in the green epi-illumination mode (520 nm peak excitation wavelength and a 575DF20 emission filter). Gels were subsequently stained with SYBR green I and then visualized again on the ImageQuant LAS 4000 imager using the blue epi-illumination mode (460 nm peak excitation wavelength and a Y515 Di emission filter).

2.5. Cell culture

HeLa and HT1080 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained at sub-confluent levels in Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MD) at 37°C in a humidified atmosphere containing 5% CO₂.

2.6. Alkaline comet assay

HeLa cells (100,000 per well) were seeded into 6-well cluster plates and allowed to settle overnight. Cells were initially treated with DMSO vehicle, 50 μ M 8-MOP or 8-POP for 60 min and then thoroughly washed with PBS prior to irradiation with UVA light (100 J/m²) at 365 nm using a Stratalinker UV Crosslinker 1800 (Stratagene, La Jolla, CA). Following UVA exposure, cell samples were treated with PBS as a control or 200 μ M H₂O₂ for 15 min at 37°C before harvest by trypsinization. Detached samples were subsequently resuspended in molten 1% Type VII low gelling temperature agarose and then allowed to set on glass slides pre-coated with agarose. Cells were then lysed by bathing slides in ice-cold lysis buffer (100 mM Na₂EDTA, 2.5 M NaCl, 10 mM Tris-HCl (pH 10.5), 1% Triton X-100) for 60 min and then subjected to 4 $\times$ 15 min washes with ice-cold MilliQ H₂O. Each slide was then submerged in alkali electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA) for 60 min and then electrophoresed at 30 V for 30 min at 4°C. Samples were neutralized by the addition of neutralization buffer (500 mM Tris-HCl pH 7.5) for 10 min and then allowed to dry overnight at ambient temperature. Comets were stained with SYBR green I for 10 min and then destained using 3 $\times$ MilliQ H₂O washes. Samples were visualized using a Nikon E800 microscope equipped with a 20 $\times$ /0.5 NA Plan Apo objective (Nikon, Tokyo, Japan) and the level of DNA damage assessed using the scoring system detailed by Collins [19]. At least 100 comets were scored per slide.

2.7. *In situ* detection of 8-POP-DNA adducts in cells by microscopy

HeLa cells were initially seeded onto coverslips in 6-well cluster plates at 330,000 cells per well and allowed to attach overnight. The following day, cells were treated with 50 μ M 8-

POP or DMSO vehicle for 60 min, washed with PBS and then exposed to UVA irradiation as described above. Cell samples were then pre-extracted with ice-cold CSK buffer (100 mM NaCl, 10 mM HEPES pH 7.8, 3 mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100) for 2 min on ice to remove non-chromatin bound protein and then fixed with 4% paraformaldehyde in PBS for 12 min at room temperature. Each sample was then washed with 3% FBS in PBS and then incubated with a click reaction mixture (50 μ M Alexa fluor azide 546, 1 mM copper(II) sulfate, 100 mM sodium ascorbate, 100 mM Tris-HCl pH 8.0) at room temperature for 30 min. Cells were then washed extensively using 3 $\times$ 15 min 3% FBS in PBS rinses with mild agitation and then mounted onto glass slides in VectaShield containing 1 μ g/mL DAPI. Stained samples were captured and analyzed on a Nikon E800 microscope equipped with a 20 $\times$ /0.5 NA Plan Apo objective and Nikon NIS elements software (Nikon, Tokyo, Japan).

2.8. Analysis of 8-POP-DNA adduct repair by flow cytometry

HeLa cells (330,000 per dish) were seeded into 6 cm culture dishes and incubated overnight. The following day, samples were treated with 50 μ M 8-POP or DMSO vehicle, PBS washed and UVA irradiated as indicated above. After irradiation, cells were released into media containing DMSO vehicle or 40 μ M of T2AA, olaparib, or aphidicolin for a total of 24 h. At designated time points, samples were harvested by trypsinization and then incubated in hypotonic swelling buffer (10 mM HEPES pH 7.5, 1.5 mM MgCl₂, 10 mM KCl) for 10 min on ice. Cells were then hypotonically lysed by passing each sample through a 27 gauge needle with a total of 3 – 4 repetitions. The integrity of samples was monitored by visual inspection using light microscopy after trypan blue staining. Nuclei were subsequently pelleted by centrifugation and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Samples were subjected to a single wash with 3% FBS in PBS and stored at 4°C in a buffer of the same composition until the day of analysis. On the day of analysis, nuclei were initially incubated in a click reaction buffer (50 μ M Alexa fluor 488 azide, 1 mM copper(II) sulfate, 100 mM sodium ascorbate, 100 mM Tris-HCl pH 8.0) for 30 min at room temperature and then washed extensively using 3 $\times$ 3% FBS in PBS rinses to remove unbound reporter. Samples were then stained with 5 μ g/mL propidium iodide in 3% FBS in PBS and then analyzed using an LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm laser line which was used for sample excitation. Green and red fluorescence was collected using the FL1 and FL3 channels, respectively. Ten thousand events were collected for each sample and gated using forward- versus side-scatter to eliminate cell debris and doublets. The data were analyzed using FlowJo software version 7.6.5 (Tree Star, San Carlos, CA).

2.9. Analysis of 8-POP-DNA adduct repair by chromatin foci quantitation

HT1080 cells were seeded (330,000 per well) onto poly-L-lysine-treated coverslips in 6-well cluster plates and allowed to attach overnight. Samples were treated with 8-POP, rinsed with PBS and irradiated with UVA as described above for the *in situ* detection of 8-POP adducts by microscopy. Following irradiation, cells were released into media containing DMSO vehicle or 63 – 125 nM gemcitabine for 19 h. At designated time points following release, samples were processed as detailed for *in situ* detection of 8-POP lesions by microscopy. Cells were subsequently analyzed on a TE2000 microscope equipped with a C2 confocal

and a 40×/0.8 NA Plan Apo objective (Nikon, Tokyo, Japan). Images were captured and processed using Nikon NIS-elements software. The software was also used to calculate the mean red fluorescence intensity of each nucleus using the DAPI staining pattern to define to perimeter of the nucleus. At least 68 nuclei were scored per sample.

3. RESULTS

3.1. A rationale for the design of 8-POP

Upon NMR solution structures of psoralen-induced DNA monoadducts and ICLs, it was revealed that the 8-position of each psoralen lesion was located in the minor groove of the duplex and was solvent exposed [20]. Accordingly, it was surmised that the 8-position may provide a suitable site for the introduction of an alkyne appendage that may be accessible for conjugation with an azide-tagged reporter (Figure 1A). To this end, the psoralen 8-MOP was adopted as a starting material as the compound was readily amenable to an initial demethylation at the 8-position to yield 8-hydroxypsoralen. The 8-hydroxypsoralen intermediate was subsequently functionalized by a propargyl group to yield 8-POP (Figure 1B). 8-POP does not decompose or self-crosslink under UVA dose being used for crosslinking DNA (Figure S1).

3.2. 8-POP generates stable DNA ICLs *in vitro* following activation by UVA

It was a reasonable concern that the alkyne handle of 8-POP may impair DNA binding and subsequent UVA-mediated adduct formation. To address this concern, an *in vitro* crosslinking assay was applied. In the absence of 8-POP (NT, lanes 0 – 30), linearized pEGFP-PCNA plasmid DNA migrates more rapidly through agarose gels relative to a double stranded control (NT, lane C, Figure 2A). These results suggest that the fragment was successfully denatured into its single stranded components and that UVA light alone was insufficient for duplex stabilization. Upon the introduction of 8-POP (lanes 0 – 30, Figure 2A), there was a clear UVA dose-dependent increase in the fraction of double stranded DNA, a result that is consistent with the generation of a duplex-stabilizing ICL that precludes complete denaturation of linearized plasmid. Quantitation of these bands revealed that approximately 50% of linearized plasmid DNA was stabilized following 30 min of UVA exposure (Figure 2B). Notably, 8-POP failed to stabilize double stranded DNA in the absence of UVA (8-POP, lane 0, Figure 2A), suggesting that intercalated non-covalent 8-POP was insufficient to stabilize the duplex without UVA. Collectively, these results strongly indicate that 8-POP generated stable covalent DNA ICLs *in vitro* following activation by UVA light, much like 8-MOP under similar assay conditions [21].

3.3. 8-POP-DNA adducts can efficiently be labeled with an azide-tagged fluorescent reporter *in vitro* via click chemistry

Next, to establish if 8-POP ICLs and monofunctional DNA adducts were susceptible to conjugation with a reporter, a supercoiled plasmid DNA was initially reacted with 8-POP and UVA to generate 8-POP-DNA adducts. Following ligation with an azide-tagged fluorophore under reducing agent to generate the requisite Cu(I) species, each DNA sample was fractionated by electrophoresis and then analyzed for successful conjugation to the reporter by scanning in-gel for red fluorescence. Figure 3 (top panel) exhibits the staining

pattern of the red fluorescent reporter along with the same gel post-stained with SYBR green (bottom panel) representing the total DNA content of each sample. Figure 3 (bottom panel, lanes 2 – 7) reveals that the application of increasing doses of sodium ascorbate coincides with degradation of plasmid DNA into smaller fragments as indicated by their increased electrophoretic mobility relative to intact plasmid (bottom panel, lane 1, Figure 3), a feature that has been noted previously [22, 23]. The red fluorescent staining profile (top panel, lanes 2 – 7, Figure 3) exhibited essentially the same staining pattern as the DNA content, indicating that the red fluorescent reporter was conjugated to DNA containing covalent 8-POP-DNA adducts. Ligation of the red fluorescent reporter to 8-POP-DNA adducts did not proceed in the absence of copper catalyst (top panel, lane 1, Figure 3). Sodium ascorbate was substituted for TCEP in a parallel series of reactions (lanes 9 – 14, Figure 3) that also yielded an overlapping red fluorescent staining profile (top panel) with total DNA content (bottom panel). The application of TCEP generated no detectable degradation of plasmid DNA (bottom panel, lanes 8 – 14, Figure 3) unlike the use of sodium ascorbate. However, ligation of the fluorescent reporter did not smoothly proceed at 5 mM TCEP (top panel, lane 14, Figure 3). Despite this anomaly, these results collectively suggest that 8-POP-DNA adducts can be successfully post-labeled with a reporter.

3.4. Relative to 8-MOP, 8-POP more readily induces DNA ICLs in HeLa cells following UVA irradiation

A modified alkaline comet assay has previously been applied in the detection of 8-MOP-induced DNA ICLs in human keratinocytes [6]. Similarly, the assay was deployed to test whether 8-POP was proficient at ICL induction within cells. HeLa cells were treated by 8-MOP or 8-POP, UVA irradiated and then exposed to H₂O₂ to generate random single strand breaks. In the absence of UVA and H₂O₂, neither 8-MOP nor 8-POP generated a visual comet score beyond the vehicle-treated control (samples 1 – 3, Figure 4). H₂O₂ exposure induced a relatively high level of DNA strand breakage as reflected by its high visual comet score (sample 4, Figure 4) in the absence of UVA. Neither psoralen alone (samples 5 and 6, Figure 4) influenced the level of damage introduced by H₂O₂ (sample 4, Figure 4), indicating that non-activated, intercalated 8-MOP or 8-POP was insufficient to crosslink DNA. None of the UVA-treated controls in the absence of H₂O₂ (samples 7 – 9, Figure 4) induced strand breakage relative to their non-irradiated counterparts, suggesting that: (a) UVA alone induced no detectable damage and (b) UVA-activated 8-MOP and 8-POP-DNA lesions were not processed by the cell to generate DNA strand breakage in these conditions. While the combination of H₂O₂ and UVA treatment induced an exceptionally high level of strand breakage (sample 10, Figure 4), the introduction of 8-MOP to cells in parallel yielded a large decrease of ~70% in the level of damage (sample 11, Figure 4). Since non-activated, intercalated 8-MOP failed to influence the level of damage generated by H₂O₂ (sample 5, Figure 4), the reversal of damage by activated 8-MOP reflects the formation of duplex-stabilizing 8-MOP ICLs [6]. An equimolar dose of UVA-activated 8-POP yielded an even greater, >99% reversal in the amount of H₂O₂-induced damage (sample 12, Figure 4), indicating that UVA-activated 8-POP was more proficient at ICL generation than 8-MOP.

3.5. 8-POP-DNA lesions can be detected within HeLa cells *in situ*

Having established that the post-labeling protocol was effective in cell-free systems, it was necessary to test whether the strategy could be applied to detect 8-POP-DNA lesions in cells. In a preliminary experiment, sodium ascorbate visualized 8-POP-DNA lesions in cells better than TCEP did (not shown), although TCEP was superior for maintaining DNA integrity than ascorbate *in vitro* (Figure 3). Since DNA integrity is not an absolute requirement in cellular imaging, we used ascorbate afterwards. Cultured HeLa cells were initially exposed to 8-POP or DMSO vehicle and then irradiated with or without UVA. Whole cell samples were immediately pre-extracted to remove non-chromatin bound protein, fixed and then subjected to a click reaction mixture to enable ligation of the azide-tagged red fluorescent reporter to 8-POP-DNA adducts *in situ*. Samples were co-stained with the nuclear dye DAPI, mounted and then analyzed by epi-fluorescence microscopy. Representative images of samples treated with 8-POP are indicated in Figure 5. Control samples exposed to DMSO vehicle consistently demonstrated very low levels of red fluorescence (data not shown), indicating that the fluorescent reporter was not non-specifically ligated to DNA (or other biomolecules) by the click reaction in the absence of 8-POP. Similarly, samples exposed to 8-POP but not UVA irradiation showed comparably low levels of fluorescence (top right panel, Figure 5), evidence that suggests non-covalently bound 8-POP was largely removed by the pre-extraction procedure. Upon the UVA application to 8-POP-treated cells, there was a prominent increase in red fluorescent signal (bottom right panel, Figure 5), indicating that UVA exposure had activated 8-POP to generate covalent DNA adducts and that the post-labeling procedure was effective *in situ*. Co-staining with the nuclear dye DAPI (bottom left panel, Figure 5) revealed that 8-POP lesions were predominantly localized within the nucleus with relatively little detected in cytoplasmic regions. These results suggest that the post-labeling strategy is effective for *in situ* detection of UVA-activated covalent 8-POP DNA lesions.

3.6. The post-labeling strategy can be applied to measure the cellular repair of 8-POP-DNA adducts and its inhibition by small molecules

In an effort to validate the post-labeling strategy for assaying chemical compounds, the cellular repair of 8-POP-DNA adducts was evaluated. HeLa cells were initially treated with 8-POP and then exposed to UVA. Following irradiation, cells were released into media containing DMSO vehicle or a small molecule compound. At designated time points, samples were subsequently assayed for their levels of 8-POP DNA adducts by applying the post-labeling strategy in conjunction with flow cytometry. Figure 6A-B demonstrates that 8-POP DNA adducts were readily detectable by flow cytometry immediately following UVA exposure. Upon their release into media containing DMSO vehicle and 24 h later, there was a notable shift in Alexa fluorescence intensity of the cell population (Figure 6A) with the population demonstrating a reduction of the median fluorescence intensity (Figure 6B). The decrease in Alexa fluorescence signal noted here may reflect the cellular repair of 8-POP DNA adducts with time. The introduction of T2AA, an inhibitor of ICL repair [24], to cells attenuated the decrease in fluorescence intensity relative to the DMSO control (medium grey histogram versus light grey histogram, Figure 6A). The inclusion of aphidicolin, an inhibitor of B-family DNA polymerases [25], or olaparib, a poly(ADP-ribose) polymerase (PARP)

inhibitor [26], also impaired the reduction in fluorescence intensity when compared to the DMSO control (Figure 6B). Given the reputation of these three agents as DNA repair inhibitors, it is likely that the loss of fluorescence with time most likely reflects the repair of 8-POP DNA adducts. The results suggest that the assay may be exploited to identify or further validate small molecule inhibitors of DNA repair.

A ribonucleotide reductase inhibitor gemcitabine potentially inhibited repair of an ICL-containing reporter plasmid in HT1080 cells [27]. The development of 8-POP as a potential probe for evaluating cellular ICL repair provided an opportunity to independently verify whether gemcitabine inhibits ICL repair within genomic DNA of this cell line. In addressing this, HT1080 cells were treated with 8-POP, irradiated with UVA and then released into media containing DMSO vehicle or gemcitabine. At designated time points, samples were processed, clicked and evaluated for their levels of 8-POP-DNA adducts by fluorescence microscopy. In the absence of gemcitabine, the mean nuclear fluorescence intensity significantly decreased 19 h later (Figure 6C), a result consistent with the cellular repair of 8-POP-DNA lesions in this time interval. Under identical conditions, gemcitabine dose-dependently attenuated the reduction in fluorescence, indicating that gemcitabine inhibited the repair of the 8-POP-DNA lesion at this dose and time.

4. DISCUSSION

In the *in vitro* post-labeling protocol, the application of sodium ascorbate to maintain copper in its requisite Cu(I) state was associated with a profound degradation of plasmid DNA (Figure 3). Both DNA and protein are susceptible to cleavage induced by the combination of ascorbate and Cu(II) ions in a series of redox-based reactions [22, 23]. The substitution of sodium ascorbate for TCEP as a reducing agent generated an increase in the yield of clicked product with little detectable damage to plasmid DNA (Figure 3). Interestingly, there was a notable shift in the mobility of plasmid DNA from a supercoiled form to an apparently larger, less mobile species as the TCEP dose increased (lanes 9 – 13, Figure 3). Most likely, this less mobile species is an open circular form of the plasmid that has lost its superhelicity. It has been established that the transition of plasmids from supercoiled to open circular states can occur via reactive oxygen species-mediated “nicking” of the DNA backbone [28-30] and the Cu(II)/TCEP combination may have generated nicks (Figure 3). Although it is unclear why TCEP, but not ascorbate, induces little damage in combination with Cu(II), the use of TCEP may be prudent in contexts where it is necessary to maintain the structural integrity of the clicked DNA product.

Extending on these *in vitro* studies, it was subsequently demonstrated that 8-POP not only formed UVA-dependent DNA ICLs within HeLa cells, but the compound strikingly yielded more of these lesions relative to 8-MOP at equimolar doses (Figure 4). It is conceivable that the terminal alkyne group of 8-POP participates in the crosslinking reaction with DNA following UVA activation. However there is little, if any, precedent for this in the literature at large. There is some evidence in favor of UVA-dependent thiol-yne covalent coupling of alkyne-tagged compounds with thiol-bearing cysteine residues [31, 32], however there are no readily available reports for an equivalent UVA-dependent direct coupling of alkyne functional groups with DNA. It is worth noting that, despite the requirement for UVA in the

induction of 8-POP-DNA ICLs, the alkyne handle remains amenable to conjugation with an azide-tagged fluorescent reporter (Figures 3, 5 and 6), indicating that the alkyne functional group remained intact. Perhaps it is more likely that the alkyne tag of 8-POP engenders the psoralen scaffold with other features that favor greater ICL formation relative to 8-MOP (Figure 4). For instance, initial non-covalent intercalative binding of the psoralens with DNA is significantly influenced by differences in the chemical groups that decorate the central tri-cyclic furocoumarin ring system [4, 33-35]. Although it is beyond the scope of the current study, it would be fascinating to establish a mechanistic basis for the propensity of UVA-activated 8-POP to generate a greater fraction of DNA ICLs relative to 8-MOP and whether this feature may be exploited in a therapeutic context. In any case, this feature is especially favorable in the context of the present study as 8-POP is intended as a tool for use in ICL repair.

The development of 8-POP and its application as a tool for ICL induction and repair within cells now marks the addition of the compound to an expanding list of clickable DNA- and RNA-directed agents originally designed for similar purposes. 8-POP now joins picazoplatin [36, 37] and an azide-appended platinum-acridine hybrid compound [38, 39] as DNA-reactive, click-ready agents designed as tools to better understand the basis of the biological activity of their parent compounds. As analogues of cisplatin, both picazoplatin and the platinum-acridine hybrid likely form a majority of DNA monoadducts that are single stranded in nature (e.g. *intrastrand* crosslinks) [13] as opposed to the psoralens, a group of compounds that typically generate a much greater fraction of DNA ICLs [3, 13, 40]. The introduction of 8-POP as a click-ready tool therefore represents a valuable new addition to these existing platinum-based agents as their differing structures are likely to elicit distinct cellular DNA damage responses.

It is worth highlighting that the *in situ* detection of 8-POP DNA lesions by either microscopy (Figures 5 and 6C) or flow cytometry (Figures 6A and 6B) affords numerous advantages that are contingent on the method of analysis. In preparing samples for microscopy, a pre-extraction procedure using CSK buffer was adopted to facilitate the removal of cellular constituents (such as soluble proteins) while permitting the retention of nuclear chromatin on a solid slide support [41]. Given that pre-extraction is widely applied in immunofluorescence studies and that the procedure is readily compatible with the detection of 8-POP DNA lesions (Figure 5), it is entirely conceivable that the two methods can be coupled to monitor the spatiotemporal dynamics of specific DNA damage response (DDR) proteins to sites of 8-POP-DNA damage [42]. Localized tracts of 8-POP-DNA lesions can potentially be generated by the application of a 364 nm laser to drug-treated cells, as highlighted originally by Thazhathveetil *et al.* [12]. In the context of detecting the lesion by flow cytometry (Figures 6A and 6B), co-staining samples for DNA content using a DNA-specific dye will enable ready quantitation of the lesion within specific stages of the cell cycle. Therefore, by combining microscopic and flow cytometric methods, the assay could be useful to determine role of the recruited DDR proteins for ICL repair in the context of DNA replication.

In summary, we have developed an alkyne-appended psoralen probe termed 8-POP that can be activated by UVA light to generate DNA ICLs *in vitro* and within cells. 8-POP-DNA

lesions were amenable to conjugation with azide-tagged fluorescent reporters via click chemistry, a feature that readily permits their quantitation by microscopy and flow cytometry. The post-labeling strategy applied in this study is rapid and highly modular in nature with the potential for multiple applications in DNA repair studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CSK buffer	cytoskeleton buffer
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
FBS	fetal bovine albumin
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HR	homologous recombination
ICL	interstrand crosslink
8-MOP	8-methoxypsoralen
NMR	nuclear magnetic resonance
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
8-POP	8-propargyloxypsoralen
TAE	tris-acetate EDTA buffer
TLC	thin layer chromatography
TCEP	tris(carboxyethyl)phosphine
TRITC	tetramethylrhodamine isothiocyanate
UVA	ultraviolet A

REFERENCES

1. Clauson C, Schärer OD, Niedernhofer L. Advances in understanding the complex mechanisms of DNA interstrand cross-link repair. *Cold Spring Harb Perspect Biol.* 2013; 5(10):a012732. [PubMed: 24086043]
2. Deans AJ, West SC. DNA interstrand crosslink repair and cancer. *Nat Rev Cancer.* 2011; 11(7): 467–80. [PubMed: 21701511]
3. Dronkert ML, Kanaar R. Repair of DNA interstrand cross-links. *Mutat Res.* 2001; 486(4):217–47. [PubMed: 11516927]
4. Cimino GD, et al. Psoralens as photoactive probes of nucleic acid structure and function: organic chemistry, photochemistry, and biochemistry. *Annu Rev Biochem.* 1985; 54:1151–93. [PubMed: 2411210]
5. Lai C, et al. Quantitative analysis of DNA interstrand cross-links and monoadducts formed in human cells induced by psoralens and UVA irradiation. *Anal Chem.* 2008; 80(22):8790–8. [PubMed: 18947205]
6. Wu JH, et al. Optimization of the comet assay for the sensitive detection of PUVA-induced DNA interstrand cross-links. *Mutagenesis.* 2009; 24(2):173–81. [PubMed: 19147795]
7. Liu S, Wang Y. A quantitative mass spectrometry-based approach for assessing the repair of 8-methoxypsoralen-induced DNA interstrand cross-links and monoadducts in mammalian cells. *Anal Chem.* 2013; 85(14):6732–9. [PubMed: 23789926]
8. Bevilacqua PM, Edelson RL, Gasparro FP. High-performance liquid chromatography analysis of 8-methoxypsoralen monoadducts and crosslinks in lymphocytes and keratinocytes. *J Invest Dermatol.* 1991; 97(1):151–5. [PubMed: 2056186]
9. Santella RM, et al. Monoclonal antibodies to DNA modified by 8-methoxypsoralen and ultraviolet A light. *Nucleic Acids Res.* 1985; 13(7):2533–44. [PubMed: 4000963]
10. Yang XY, DeLeo V, Santella RM. Immunological detection and visualization of 8-methoxypsoralen-DNA photoadducts. *Cancer Res.* 1987; 47(9):2451–5. [PubMed: 2436765]
11. Tsuchida K, Komatsu K. Impaired removal of DNA interstrand cross-link in Nijmegen breakage syndrome and Fanconi anemia, but not in BRCA-defective group. *Cancer Sci.* 2008; 99(11):2238–43. [PubMed: 18771529]
12. Thazhathveetil AK, et al. Psoralen conjugates for visualization of genomic interstrand cross-links localized by laser photoactivation. *Bioconjug Chem.* 2007; 18(2):431–7. [PubMed: 17373769]
13. Huang J, et al. The DNA translocase FANCM/MHF promotes replication traverse of DNA interstrand crosslinks. *Mol Cell.* 2013; 52(3):434–46. [PubMed: 24207054]
14. Muniandy PA, et al. Repair of laser-localized DNA interstrand cross-links in G1 phase mammalian cells. *J Biol Chem.* 2009; 284(41):27908–17. [PubMed: 19684342]
15. Speers AE, Adam GC, Cravatt BF. Activity-based protein profiling in vivo using a copper(i)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J Am Chem Soc.* 2003; 125(16):4686–7. [PubMed: 12696868]
16. Speers AE, Cravatt BF. Profiling enzyme activities in vivo using click chemistry methods. *Chem Biol.* 2004; 11(4):535–46. [PubMed: 15123248]
17. Punchihewa C, et al. Identification of small molecule proliferating cell nuclear antigen (PCNA) inhibitor that disrupts interactions with PIP-box proteins and inhibits DNA replication. *J Biol Chem.* 2012; 287(17):14289–300. [PubMed: 22383522]
18. Steigemann P, et al. Aurora B-mediated abscission checkpoint protects against tetraploidization. *Cell.* 2009; 136(3):473–84. [PubMed: 19203582]
19. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol.* 2004; 26(3):249–61. [PubMed: 15004294]
20. Spielmann HP, et al. Solution structures of psoralen monoadducted and cross-linked DNA oligomers by NMR spectroscopy and restrained molecular dynamics. *Biochemistry.* 1995; 34(40): 12937–53. [PubMed: 7548052]

21. Hartley JA, Souhami RL, Berardini MD. Electrophoretic and chromatographic separation methods used to reveal interstrand crosslinking of nucleic acids. *J Chromatogr.* 1993; 618(1-2):277–88. [PubMed: 8227260]
22. Chiou SH. DNA- and protein-scission activities of ascorbate in the presence of copper ion and a copper-peptide complex. *J Biochem.* 1983; 94(4):1259–67. [PubMed: 6654857]
23. Wang Y, Van Ness B. Site-specific cleavage of supercoiled DNA by ascorbate/Cu(II). *Nucleic Acids Res.* 1989; 17(17):6915–26. [PubMed: 2780314]
24. Inoue A, et al. A small molecule inhibitor of monoubiquitinated Proliferating Cell Nuclear Antigen (PCNA) inhibits repair of interstrand DNA cross-link, enhances DNA double strand break, and sensitizes cancer cells to cisplatin. *J Biol Chem.* 2014; 289(10):7109–20. [PubMed: 24474685]
25. Baranovskiy AG, et al. Structural basis for inhibition of DNA replication by aphidicolin. *Nucleic Acids Res.* 2014; 42(22):14013–21. [PubMed: 25429975]
26. Lee JM, Ledermann JA, Kohn EC. PARP Inhibitors for BRCA1/2 mutation-associated and BRCA-like malignancies. *Ann Oncol.* 2014; 25(1):32–40. [PubMed: 24225019]
27. Fujii N, et al. A novel assay revealed that ribonucleotide reductase is functionally important for interstrand DNA crosslink repair. *Bioorg Med Chem.* 2015; 23(21):6912–21. [PubMed: 26462050]
28. Hoffman M, et al. Generation of DNA-damaging reactive oxygen species via the autoxidation of hydrogen sulfide under physiologically relevant conditions: chemistry relevant to both the genotoxic and cell signaling properties of H(2)S. *Chem Res Toxicol.* 2012; 25(8):1609–15. [PubMed: 22621314]
29. Li Y, Trush MA. DNA damage resulting from the oxidation of hydroquinone by copper: role for a Cu(II)/Cu(I) redox cycle and reactive oxygen generation. *Carcinogenesis.* 1993; 14(7):1303–11. [PubMed: 8392444]
30. Lewis JG, Stewart W, Adams DO. Role of oxygen radicals in induction of DNA damage by metabolites of benzene. *Cancer Res.* 1988; 48(17):4762–5. [PubMed: 2842037]
31. Lo Conte M, et al. Photoinduced addition of glycosyl thiols to alkynyl peptides: use of free-radical thiol-yne coupling for post-translational double-glycosylation of peptides. *J Org Chem.* 2010; 75(13):4644–7. [PubMed: 20527977]
32. Minozzi M, et al. An insight into the radical thiol/yne coupling: the emergence of arylalkyne-tagged sugars for the direct photoinduced glycosylation of cysteine-containing peptides. *J Org Chem.* 2011; 76(2):450–9. [PubMed: 21175145]
33. Gupta M, Ali R. Fluorescence studies on the interaction of furocoumarins with DNA in the dark. *J Biochem.* 1984; 95(5):1253–7. [PubMed: 6746605]
34. Isaacs ST, Wieseahn G, Hallick LM. In vitro characterization of the reaction of four psoralen derivatives with DNA. *Natl Cancer Inst Monogr.* 1984; 66:21–30. [PubMed: 6531031]
35. Wieseahn G, Hearst JE. DNA unwinding induced by photoaddition of psoralen derivatives and determination of dark-binding equilibrium constants by gel electrophoresis. *Proc Natl Acad Sci U S A.* 1978; 75(6):2703–7. [PubMed: 275838]
36. White JD, et al. Picazoplatin, an azide-containing platinum(II) derivative for target analysis by click chemistry. *J Am Chem Soc.* 2013; 135(32):11680–3. [PubMed: 23879391]
37. Osborn MF, et al. Platinum-RNA modifications following drug treatment in *S. cerevisiae* identified by click chemistry and enzymatic mapping. *ACS Chem Biol.* 2014; 9(10):2404–11. [PubMed: 25055168]
38. Ding S, et al. Using fluorescent post-labeling to probe the subcellular localization of DNA-targeted platinum anticancer agents. *Angew Chem Int Ed Engl.* 2013; 52(12):3350–4. [PubMed: 23427109]
39. Qiao X, et al. Investigating the cellular fate of a DNA-targeted platinum-based anticancer agent by orthogonal double-click chemistry. *J Biol Inorg Chem.* 2014; 19(3):415–26. [PubMed: 24407462]
40. Raschle M, et al. DNA repair. Proteomics reveals dynamic assembly of repair complexes during bypass of DNA cross-links. *Science.* 2015; 348(6234):1253671. [PubMed: 25931565]
41. Sawasdichai A, et al. In situ subcellular fractionation of adherent and non-adherent mammalian cells. *J Vis Exp.* 2010; (41)
42. Polo SE, Jackson SP. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev.* 2011; 25(5):409–33. [PubMed: 21363960]

Figure 1.

(A) A schematic representation of the post-labeling strategy adopted in this report. In this scheme, 8-POP, an analogue of the clinically-applied 8-MOP, is activated by UVA to generate covalent 8-POP-DNA adducts. The alkyne-appended 8-POP-DNA lesion may be post-labeled via ligation with an azide-tagged fluorescent reporter (designated here as N₃-linker-FT) using a Cu(I)-catalyzed dipolar cycloaddition. The level of 8-POP-DNA lesions can readily be quantified by measuring fluorescence. (B) The synthetic route used to produce 8-POP.

Figure 2.

8-POP can generate DNA ICLs following activation by UVA light *in vitro*. (A) Linearized pEGFPPCNA plasmid DNA was initially reacted with 0 (NT) or 31 μ M 8-POP and exposed to UVA light for 0 – 30 min as indicated. Each sample was then subjected to denaturation and electrophoresis as described in the Materials and Methods. Controls that were not thermally denatured are denoted by a C and are representative of double stranded linearized plasmid DNA. (B) The fraction of double stranded DNA stabilized by 8-POP represented in (A) was quantitated and is expressed as a function of time of UVA exposure.

Figure 3.

The ligation of 8-POP-modified plasmid DNA with an azide-tagged fluorescent reporter via click chemistry. Supercoiled pEGFP-PCNA plasmid DNA was initially reacted with 50 μ M 8-POP and UVA for 30 min. The contents of each reaction were then subjected to conjugation with Alexa azide fluor 546 via Cu(I)-catalyzed click chemistry using 0 – 5 mM sodium ascorbate or TCEP. Samples were fractionated through agarose gels by electrophoresis and the gels scanned for Alexa fluorescence (top panel). Gels were then counterstained with SYBR green (bottom panel) and scanned to reveal the total DNA content of each sample. M denotes molecular weight markers.

Figure 4. 8-POP generates DNA ICLs in HeLa cells following UVA exposure. HeLa cells were initially treated with DMSO vehicle, 50 μ M 8-MOP or 8-POP as indicated for 60 min and then exposed to 0 or 100 J/m² UVA. Following irradiation, samples were subjected to treatment with 200 μ M H₂O₂ or PBS as a control for 15 min at 37°C. Samples were then processed and assayed for their levels of DNA damage using an alkaline comet assay as detailed in the Materials and Methods section. Each column represents the mean level of DNA damage for each sample as reflected by the visual score in arbitrary units $\pm$ SEM. At least 100 comets were scored per sample.

Figure 5. 8-POP-DNA lesions can be detected within HeLa cells *in situ*. Representative microscopy images of HeLa cells that were treated with 50 μM 8-POP for 60 min and then irradiated with 0 J/m^2 (top panels) or 100 J/m^2 (bottom panels). Samples were subsequently pre-extracted, fixed and subjected to post-labeling with an azide-tagged red fluorescent reporter as described in the Materials and Methods. Samples were counterstained with DAPI, mounted on glass slides and then visualized by epi-fluorescence microscopy using DAPI and TRITC filters to define the nucleus and reveal the red 8-POP adduct staining pattern, respectively.

Figure 6.

The post-labeling strategy of 8-POP can be applied to measure the cellular repair of 8-POP-DNA adducts and its inhibition by small molecules. (**A** and **B**) HeLa cells were exposed to 50 μM 8-POP for 60 min and then irradiated with 100 J/m^2 UVA. Cells were subsequently released into media containing DMSO vehicle or 40 μM aphidicolin, olaparib or T2AA for 24 h. All samples were then hypotonically lysed, fixed, subjected to post-labeling with an azide-tagged Alexa 488 reporter by click chemistry, counterstained with propidium iodide and analyzed by flow cytometry as described in the Materials and Methods. (**A**) A representative histogram displaying the relative levels of 8-POP-DNA adducts as reflected by the Alexa 488 signal immediately following UVA irradiation (“start”, solid black histogram) and 24 h after release into media containing DMSO as a control (light grey histogram) or 40 μM T2AA (medium grey histogram). (**B**) A quantitative representation of the histogram displaying the median ICL (Alexa 488) signal as a function of treatment. (**C**) HT1080 cells were initially treated with 8-POP and UVA as described for (**A** and **B**) and then released into media containing DMSO vehicle or 62.5 – 125 nM gemcitabine for 19 h. Cells were subsequently pre-extracted, fixed and post-labeled with an azide-tagged red fluorescent reporter using click chemistry as detailed in the Materials and Methods. The mean nuclear intensity of the red fluorescent reporter was quantitated by confocal microscopy and is plotted for each cells counted for analysis. “Start” indicates the mean nuclear fluorescence intensity immediately following UVA irradiation. Error bars indicate the standard deviation. Statistics were analyzed by a Student's *t* test (two-tailed, unpaired). Numbers of cells counted for the analysis are indicated at the top for each data.