Guanine sulphinate is a major stable product of photochemical oxidation of DNA 6-thioguanine by UVA irradiation

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ABSTRACT

The DNA of patients taking the immunosuppressant and anticancer drugs azathioprine or 6-mercaptopurine contains 6-thioguanine (6-TG). The skin of these patients is selectively sensitive to ultraviolet A radiation (UVA) and they suffer an extremely high incidence of sunlight-induced skin cancer with long-term treatment. DNA 6-TG interacts with UVA to generate reactive oxygen species, which oxidize 6-TG to guanine sulphonate (G^{SO3}). We suggested that G^{SO3} is formed via the reactive electrophilic intermediates, guanine sulphenate (G^{SO}) and guanine sulphinate (G^{SO2}). Here, G^{SO2} is identified as a significant and stable UVA photoproduct of free 6-TG, its 2'-deoxyribonucleoside, and DNA 6-TG. Mild chemical oxidation converts 6-TG into G^{SO2}, which can be further oxidized to G^{SO3}—a stable product that resists further reaction. In contrast, G^{SO2} is converted back to 6-TG under mild conditions. This suggests that cellular antioxidant defences might counteract the UVA-mediated photooxidation of DNA 6-TG at this intermediate step and ameliorate its biological effects. In agreement with this possibility, the antioxidant ascorbate protected DNA 6-TG against UVA oxidation and prevented the formation of G^{SO3}.

INTRODUCTION

Reactive oxygen species (ROS) are hazardous to cells. They cause damage to DNA, proteins and membranes. Cells are well adapted to the levels of ROS produced in the normal course of aerobic metabolism and are equipped with powerful antioxidant defences and repair systems to counteract the effects of inevitable damage to DNA. Exogenous sources of ROS can perturb this homeostasis and excess ROS create a condition of oxidative stress in which cellular defences are overwhelmed and cellular constituents are damaged. Potentially damaging ROS are produced when cells are exposed to ultraviolet A (UVA, wavelengths 320–400 nm) radiation. This reaction, which occurs at high ($>100 \, kJ/m^2$) UVA doses, involves the absorption of UVA energy by cellular chromophores (1). DNA, a major target for damage by ROS, is a UVC and UVB chromophore but does not absorb the longer wavelength UVA to a significant degree. UVA comprises >90% of incident ultraviolet radiation at the earth's surface and the inability of DNA to absorb UVA, and thereby generate ROS, is an important factor in minimizing sunlight-induced damage to DNA (2).

The thiopurines azathioprine (Aza), 6-mercaptopurine (6-MP), and 6-thioguanine (6-TG) are immunosuppressant, anti-inflammatory, and anticancer drugs (3). They are all metabolized to 6-TG nucleotides (TGNs) and cause the incorporation of 6-TG into DNA (4). Unlike the canonical DNA bases, 6-TG is a strong UVA chromophore. When 6-TG is irradiated with UVA (340 nm) reactive oxygen species (ROS) and, in particular, singlet oxygen ($^1O_2$) are generated (5,6). DNA 6-TG and the TGN pool are both significant sources of ROS when thiopurine-treated cells are UVA irradiated (7). The formation ROS from 6-TG within DNA itself is potentially extremely hazardous and $^1O_2$ is an acknowledged source of oxidized DNA bases, DNA strand breaks (8) and oxidation of DNA associated proteins (9). In addition to this danger to normal cellular constituents, the low oxidation potential of 6-TG makes it a preferred target for oxidation and this compounds the hazard of photochemically generated ROS. We have previously shown that this

favoured oxidation of DNA 6-TG generates guanine sulphonate (G^{SO3}) a highly effective block to replication and transcription (5,6,10).

Long-term therapy with thiopurines, for example, in immunosuppressed organ transplant recipients treated with Aza, is associated with a very high incidence of skin cancer for which sunlight exposure is a contributory factor (11). The skin of patients undergoing Aza treatment contains DNA 6-TG and, as a consequence, is selectively sensitive to the induction of erythema by UVA (12). This UVA-selective photosensitivity suggests that photochemically induced DNA damage is physiologically relevant. In view of the huge incidence of skin cancer in this patient group, we have examined in more detail the photochemistry of 6-TG and DNA 6-TG in order to define the DNA lesions likely to be present in the skin of Aza-treated patients exposed to sunlight. The UVA doses we use in these *in vitro* studies fall well within the range to which patients might be exposed on an averagely sunny summer day in Northern Europe (13). DNA guanine sulphinate (G^{SO2}) as a quantitatively major product of the photochemical oxidation of DNA 6-TG and an intermediate in the formation of the previously described DNA G^{SO3} (5). We demonstrate that G^{SO2} is converted back to 6-TG under relatively mild conditions, suggesting that cellular reducing agents may also provide some protection against sunlight by reversing photochemical damage to DNA 6-TG. In view of the involvement of ROS in the formation of these DNA lesions, we also examined the protective potential of the dietary vitamin ascorbate (vitamin C)—an acknowledged antioxidant in skin (14). We report that ascorbate prevents DNA damage and protects 6-TG, and DNA 6-TG, from UVA-induced oxidation.

EXPERIMENTAL PROCEDURES

Chemicals

6-thioguanine (6-TG), deoxyguanosine (dG), ascorbate (vitamin C), sodium sulphide ($Na_2S \cdot 9H_2O$), hydrogen peroxide 30%, acetic acid, magnesium monoperoxyphthalate (MMPP), $Fe(NH_4)_2(SO_4)_2$, Rose Bengal and iodine (I_2) were obtained from Sigma Aldrich (Dorset, UK). Oligodeoxyribonucleotides were from Oligos Etc., Inc. (Wilsonville, USA). G^{SO3} was prepared as described (5). G^{SO2} was prepared according to a published protocol (15). Guanine-6-thioguanine (G^SG) was prepared as before (6).

Chromatography

Four different systems of reverse-phase analyses for modified 6-TG and 6-TGdR were used.

System 1. Bases were separated by high-performance liquid chromatography (HPLC) on a Waters dC_{18} (Atlantis 3 μm, 150×2.1 mm) column using a Waters 2695 Alliance system equipped with photodiode array and Waters 474 dual monochromator fluorescence detector. Column eluates were monitored simultaneously by absorbance and fluorescence. Elution was with

three solvents: A = methanol; B = water; C = 100 mM KH_2PO_4, pH 6.7. Flow rate was increased from 0.2 to 0.25 ml/min during the first 23 min, and at 0.25 ml/min thereafter. Solvent C was kept constant at 5% for 23 min and 0% thereafter. A gradient 0–20% Solvent A was applied during the first 10 min. Solvent A was increased to 80% over the next 10 min and to 90% over the next 3 min and maintained at 90% for 7 min.

System 2. Bases were separated by HPLC on a Waters C_{18} column (Xterra MS 3.5 μm, 150×2.1 mm) equipped and monitored as System 1. Solvents A and B were as System 1, Solvent C = 10 mM KH_2PO_4, pH 6.5. Flow rate was constant at 0.2 ml/min. Solvent C was kept at 20%. A gradient 0–20% Solvent A was applied during the first 10 min. Solvent A was increased to 80% over the next 10 min and maintained at 80% for 5 min.

System 3. Column, solvents A, B and C and eluates monitored were as System 1. Flow rate was 0.2 ml/min. Solvent C was kept constant at 5%. Solvent A was increased from 0% to 20% during the first 10 min, then increased to 80% during the next 10 min and maintained at 80% for 5 min.

System 4. 2-deoxyribonucleosides were separated by HPLC on a Waters Symmetry C_{18} Reversed Phase column as described (5). 6-TGdR and dG^{SO2} were quantified by A_{342}, dG by A_{260} and dG^{SO3} by its fluorescence (Ex = 320 nm; Em = 410 nm). Elution was with two solvents: A = 10 mM KH_2PO_4 pH 6.7; B = methanol. Flow rate was constant at 0.5 ml/min. A gradient 100–90% Solvent A was applied during the first 10 min, then 90–60% during the next 10 min, maintained at 60% for 2.5 min. This was followed by a sharp gradient 60–100% Solvent A for 0.5 min and constant at 100% for 17 min.

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) experiments were conducted at 300 MHz from JOEL (JNM-LA 300, FT NMR) and 400 MHz from JOEL (JNM-EX 400, FT NMR). Samples were dissolved in DMSO-d6 and chemical shifts were adjusted with reference to an internal standard tetramethylsilane (TMS).

Mass spectroscopy

Mass spectroscopy (MS) analysis was performed on a Fisons Instruments (VG BioTech, Altrincham, UK) Quattro Electrospray ionization mass spectrometer. Samples were dissolved in 50:50 MeOH:Water (ESI negative mode). High-resolution mass spectra (ESI negative mode) were obtained by the EPSRC Mass Spectrometry Service Centre, University of Swansea.

Fluorescence spectrometry

Fluorescence emission and excitation spectra were obtained using a FLUOROMAX-P spectrofluorometer, Jobin Yvon Horiba Inc. UK.

UVA irradiation

6-TG, 6-TGdR, oligonucleotides and genomic DNA were irradiated at a dose rate of $0.07\,kJ/m^2/s$ (for 6-TG) or $0.1\,kJ/m^2/s$ (for all other samples) using a UVH 254 lamp (UV Light Technology, wavelength range 320–400).

Digestion of DNA to 2′-deoxyribonucleosides

Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) from cells grown in medium containing $1\,\mu M$ 6-TG for 24 h. Standard digestion to nucleosides for single-stranded oligonucleotides and genomic DNA was with nuclease P1 at pH 4.7 followed by shrimp alkaline phosphatase as described (10).

RESULTS

6-TG photoproducts

UVA irradiation of 6-TG in 0.1 mM aqueous solution causes a rapid loss of its characteristic A_{342} (5). HPLC separation of the 6-TG photoproducts after $21\,kJ/m^2$ revealed four novel peaks with absorbance at 320 nm eluting at 2.8, 3.5, 6.5 and 14.8 min (Figure 1A, ii). The small amount of unchanged 6-TG eluted at 9 min. The compounds eluting at 2.8 and 14.8 min were the previously identified as the highly fluorescent ($\lambda_{ex} = 324$ nm, $\lambda_{em} = 408$ nm) G^{SO3} and the 6-TG dimer, G-S-G, respectively. Their identities were confirmed by co-elution with the authentic compounds. A small peak at 6.5 min was identified as guanine by its UV spectrum.

The formation of these photoproducts was examined over a range of UVA doses. HPLC analysis indicated that the photoproduct eluting at 3.5 min was generated rapidly and that its formation preceded that of detectable amounts of G^{SO3} and G-S-G (Figure 1B). Comparison of its absorbance spectrum with published data (15) suggested that this initial product was guanine-6-sulphinate, G^{SO2}. Authentic G^{SO2} was prepared by I_2-mediated oxidation of 6-TG according to a published protocol (15) and crystallized. The product was not fluorescent, as expected, and its absorbance spectrum together with mass and NMR spectrometry confirmed that it was G^{SO2} (Supplementary Figure S1). Authentic G^{SO2} co-eluted from HPLC exactly coincident with the 3.5-min 6-TG photoproduct (Figure 1A, iii and iv). Together, the four identifiable UVA photoproducts: G^{SO2}, G^{SO3}, G, and G-S-G accounted for >90% of the 6-TG that was destroyed by UVA. G^{SO2} was the most abundant initial product. These findings indicate that G^{SO2} is a relatively stable intermediate in UVA-mediated 6-TG oxidation. It can be further oxidized to G^{SO3}. Guanine and the G-S-G dimer are also generated as minor photoproducts.

Sequential oxidation of 6-TG was investigated further using MMPP which oxidizes 6-TG to G^{SO3} (6). Figure 1C shows that when 6-TG was treated for 10 min at room temperature (RT) with equimolar MMPP, some 6-TG remained unchanged and G^{SO2} was the only product detectable by A_{320}. When MMPP was in 2-fold molar excess over 6-TG, approximately equal amounts of G^{SO2} and G^{SO3} were produced. Under stronger oxidising conditions (6-TG:MMPP = 1:3), 6-TG was stoichiometrically converted to G^{SO3}. Purified G^{SO2} was quite stable and remained unchanged by overnight incubation at 20° in aqueous solution (data not shown). It could be, however, oxidized by MMPP. A 10-min treatment at RT with a limiting MMPP concentration (G^{SO2}:MMPP = 1:0.5) converted about 80% to G^{SO3}. No other oxidation products were detected and higher MMPP concentrations (G^{SO2}:MMPP = 1:1), induced a stoichiometric conversion to G^{SO3} (Figure 1D).

We conclude that G^{SO2}—which has absorbance at 320 nm but is not fluorescent (Supplementary Figure S1)—is the first stable product of 6-TG photo-oxidation. G^{SO2}, G^{SO3}, guanine and G-S-G together account quantitatively for all the 6-TG destroyed by UVA. G^{SO2} is produced by chemical oxidation under relatively mild conditions and, although stable, it easily undergoes further oxidation to G^{SO3}.

The stoichiometry of oxidation of the 6-TG 2′-deoxyribonucleoside (6-TGdR) was similar and dG^{SO2} was the major UVA photoproduct when an aqueous solution of 6-TGdR (20 μM) was irradiated. Approximately 50% was destroyed by $15\,kJ/m^2$ and 90% by $45\,kJ/m^2$. Figure 2 shows that at $15\,kJ/m^2$, dG^{SO2} accounted for around 90% of the destroyed 6-TGdR. At higher doses, both dG^{SO3} and dG were formed. These minor products were present in similar amounts and each accounted for 10–20% of the destroyed 6-TGdR at $45\,kJ/m^2$ UVA. There was no indication of the formation of other significant photoproducts at least up to $135\,kJ/m^2$, the highest dose used (data not shown). dG^{SO3}, dG^{SO2} and dG together accounted for ≥90% of the 6-TGdR destroyed by UVA. The minor fraction of input 6-TGdR that was unaccounted for was most likely converted to the nucleoside dimer analogous to G-S-G as we also observed a late-eluting product with A_{342} which might be consistent with this compound (Supplementary Figure S2). A final confirmation and quantification of this structure awaits synthesis of the authentic nucleoside dimer.

To approach the identification of DNA 6-TG photoproducts, we first examined the effect of UVA on single-stranded oligonucleotides containing a single 6-TG. Since our previous findings indicated that dG was a potential photoproduct, we first examined the effect of UVA on an 8-mer comprising 7As and a single 6-TG (A_7TG_1). This permitted the accurate quantitation of any dG formed. Irradiated single-stranded oligos were digested with nuclease P1 followed by alkaline phosphatase and the 2′-deoxyribonucleosides were quantified as above. In an attempt to minimize losses due to the known acid lability of the photoproducts (15), nuclease digestion of irradiated 8-mers was first carried out at pH 7. Under these conditions, photoproduct recovery was poor and over a range of UVA doses (5–45 kJ/m²) only around 30% of destroyed 6-TG could be accounted for by dG^{SO3}, dG^{SO2} and dG. Previously unidentified photoproducts were generated in UVA-dependent fashion (Supplementary Figure S3). This appeared to be the
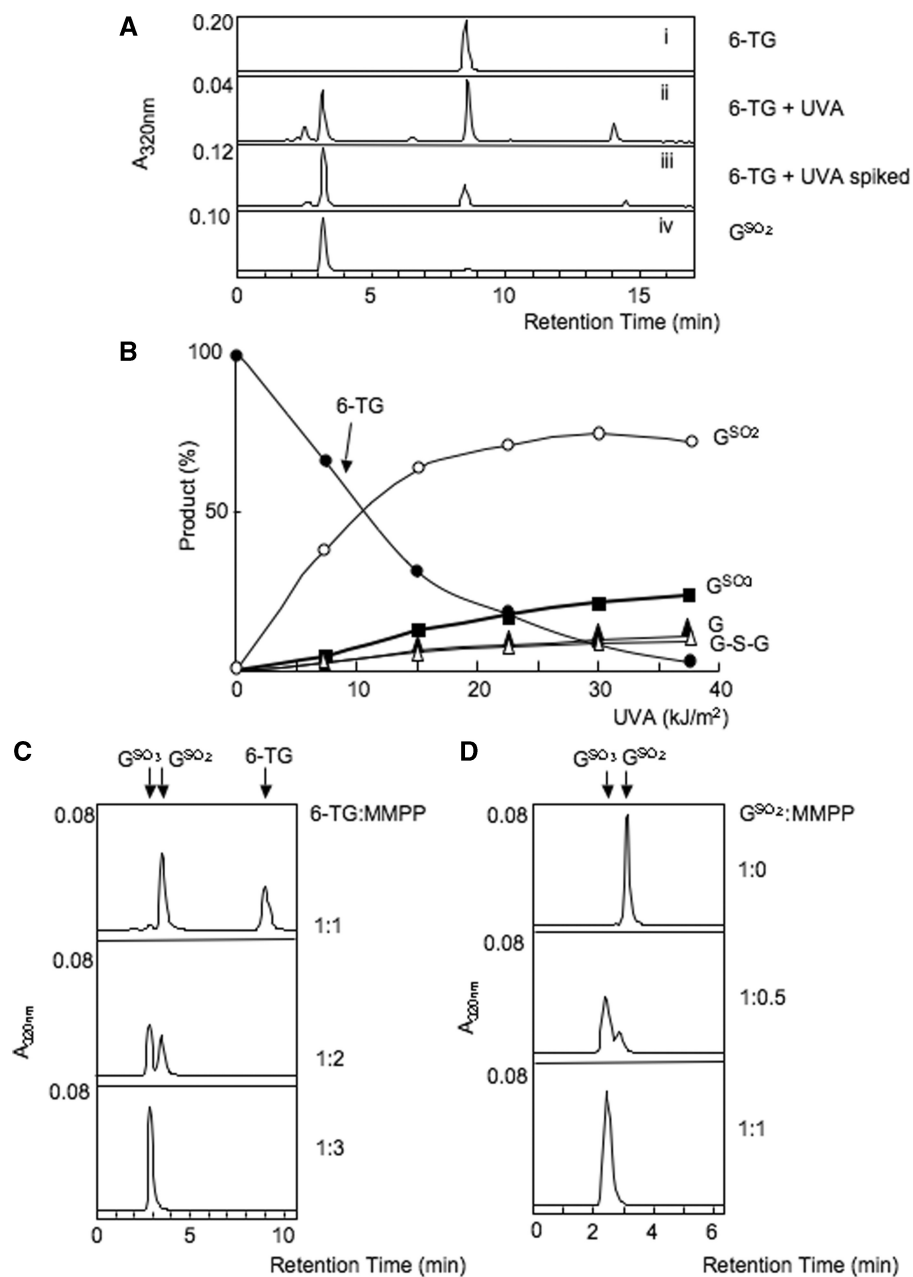
Figure 1. 6-TG/UVA photoproducts. (**A**) Identification of G^{SO2} as a major photoproduct. 6-TG (0.1 mM in aqueous solution) was irradiated with 21 kJ/m^2 UVA. Products were separated by HPLC System 1 as described in 'Experimental Procedures' section and the eluate monitored at 320 nm. (Top panel) Unirradiated 6-TG. (Second panel) 6-TG after 21 kJ/m^2 UVA. (Third panel) As second panel, with authentic G^{SO2} added before HPLC. (Bottom panel) Authentic G^{SO2} (prepared by mild I_2 oxidation of 6-TG) alone. (**B**) Quantitation of photoproducts. 6-TG [as in (A)] was irradiated with UVA at a dose rate of 0.07 kJ/m^2/s. Samples were removed and analysed on HPLC. The four peaks of 320 nm absorbance with retention times of 2.8 min (filled square), 3.5 min (open circle), 6.5 min (filled triangle) and 14.8 min (open triangle) were identified as G^{SO3}, G^{SO2}, G and G-S-G as described in the text. Unaltered 6-TG (filled circle) eluted at 9 min. Quantitation was at the absorbance maximum for each product and by comparison to authentic standard compounds. G^{SO3} (A_{325}), G^{SO2} (A_{320}), G (A_{273}), 6-TG (A_{340}) and G-S-G (A_{331}). Products are expressed mole % of unirradiated 6-TG. A representative of three independent experiments is shown. (**C**) 6-TG oxidation by MMPP. 6-TG (0.1 mM) was treated for 10 min at 20°C with MMPP at the final molar ratio indicated. Products were separated by HPLC System 2 and eluates monitored at 320 nm. Unchanged 6-TG elutes at 9 min, G^{SO2} at 3.5 min and G^{SO3} at 3.0 min. (**D**) G^{SO2} oxidation by MMPP. G^{SO2} (0.1 mM) was treated for 10 min at 20° with MMPP at the molar ratios shown. Products were separated by HPLC System 3.

product of incomplete P1 nuclease digestion under these sub-optimal conditions. Digestion was also incomplete when the irradiated oligo was incubated under optimal conditions (6 h at pH 8.0) for benzonase (Sigma), phosphodiesterase I and alkaline phosphatase

(data not shown). These findings suggest that some 6-TG photoproducts are somewhat resistant to enzymatic digestion.

Under conditions that were optimal for nuclease P1, pH 4.7 and 50°C, digestion of irradiated (A_7TG_1) was

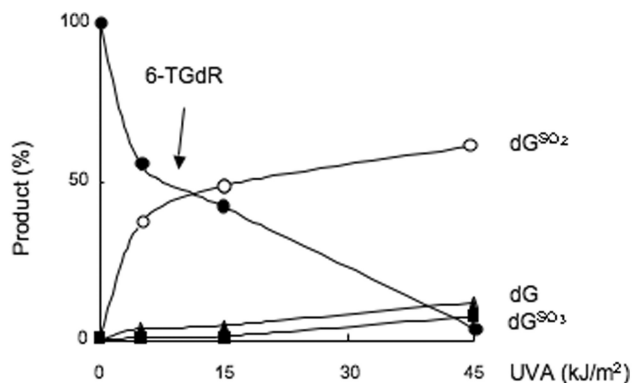**Figure 2.** UVA oxidation of 6-TG 2′-deoxyribonucleoside. 6-TGdR (20 µM aqueous solution) was irradiated with UVA in neutral conditions (10 mM Tris–HCl pH 7.5). Products were immediately separated by HPLC System 4. Quantitation of unaltered 6-TGdR (filled circle) and dG^{SO2} (open circle) by their A_{342}, dG^{SO3} (filled square) by its fluorescence at 410 nm, dG (filled triangle) by its A_{260} were by comparison to authentic standard compounds. Products are expressed mole % of unirradiated 6-TG. A representative of three independent experiments is shown.

complete and all of the destroyed 6-TG was accounted for by dG^{SO3}, dG^{SO2} and dG. Under these conditions, dG appeared to be the major product (Figure 3A). In view of the known lability of G^{SO2} under acid conditions, it seemed likely that some of the dG had been generated from dG^{SO2} during DNA digestion at pH 4.7. To investigate this, UVA-irradiated 6-TGdR was subjected to the same conditions of temperature and pH. Figure 3B shows that this treatment resulted in loss of a significant fraction of dG^{SO2}. The major part of the missing dG^{SO2} was recovered as dG with a small amount converted to 6-TGdR. dG^{SO2} formation in UVA irradiated oligos was corrected for this artefactual destruction of dG^{SO2} (see legend to Figure 3C). When this correction for artefactual conversion of dG^{SO2} to dG was applied, dG^{SO2} and dG^{SO3} together accounted for ≥95% of the destroyed 6-TG. dG^{SO2} was the major photoproduct (75–90% of the total) (Figure 3C).

dG^{SO2} was also the major photoproduct in double-stranded DNA (Table 1). DNA uniformly substituted with 6-TG to ~2% of G, was purified from cells that had been grown for 24 h in the presence of 6-TG. After UVA irradiation, heat denaturation, digestion with nuclease P1 under standard conditions (pH 4.7, 1 h, 50°) and treatment with alkaline phosphatase, dG^{SO3} appeared to be the predominant photoproduct detected by HPLC (it was not possible to quantify any increase in dG). The two oxidized forms, dG^{SO3} and dG^{SO2}, together accounted for ~20% of the starting DNA 6-TG and no other significant peaks of absorbance at 260, 320 or 342 nm were observed. When corrected for losses of labile dG^{SO2} under the conditions of enzyme digest, however, around 90% of input DNA 6-TGdR was accounted for and dG^{SO2} was again the major photoproduct. These data indicate that dG^{SO3} and dG^{SO2} comprise almost all of the UVA photoproducts of DNA 6-TGdR. At the relatively low doses of UVA
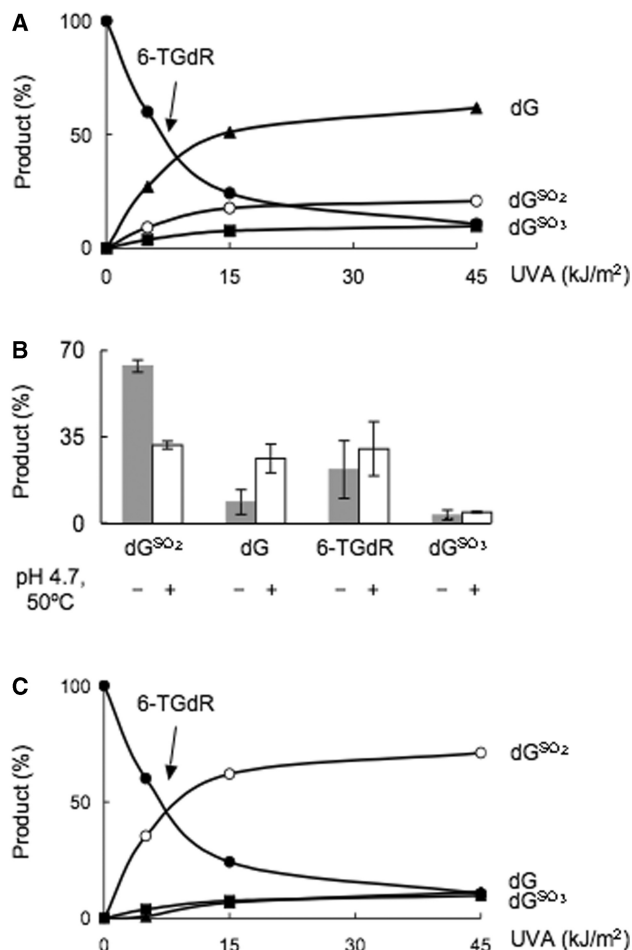
Figure 3. UVA oxidation of 6-TG in oligonucleotide. (**A**) Single-stranded 8-mer oligo A_7TG_1 (AAAA**X**AAA where $\mathbf{X} = $ 6-TG; 50-µM aqueous solution) was irradiated with UVA, then digested to 2′-deoxyribonucleosides by nuclease P1 (1 h, 50°, pH 4.7) followed by alkaline phosphatase as described in 'Experimental Procedures' section. Products were separated and quantitated as described in legend to Figure 2. Products are expressed mole % of unirradiated 6-TG. A representative of three independent experiments is shown. (**B**) 6-TGdR (20 µM aqueous solution) was irradiated with 15 kJ/m² UVA, half the sample was immediately analysed by HPLC. The other half was subjected to the same conditions of temperature and pH as for nuclease P1 (pH 4.7, 1 h at 50°C) and alkaline phosphatase digestion before HPLC analysis. The means and SD of three independent experiments are shown. (**C**) Corrected photoproduct yield. The amount of dG^{SO2} destroyed under acidic conditions was calculated based on yield of dG from the irradiated A_7TG_1 and the known dG formation after UVA irradiation of 6-TGdR at pH 7. The total yield of $dG^{SO2} = dG^{SO2}$ measured + dG^{SO2} calculated from (dG measured in A_7TG_1 oligo digested at pH 4.7—dG measured from irradiated 6-TGdR at neutral pH from Figure 2).

that we use (5–15 kJ/m²), dG^{SO2} accounts for essentially all of the destroyed 6-TGdR and that dG^{SO3} and dG are formed to a minor extent. At higher doses, a measurable fraction (10–20% of the total) is oxidized to dG^{SO3} and a similar fraction of dG is formed. These photochemical reactions are largely independent of the 6-TG context and the proportions of photoproducts are similar for the base, the 2′-deoxyribonucleoside and for 6-TG in single- or double-stranded DNA.

Table 1. 6-TG/UVA photoproducts in genomic DNA

UVA (kJ/m^2)	Measured 6-TGdR (pmole)	Total dG^{SO2} (pmole)	Measured dG^{SO3} (pmole)	Estimated dG (pmole)	Recovery (%)
0	510	0	0	0	100
5	282	158	30	7	94
15	158	230	53	26	92
45	65	271	78	54	92

DNA containing 6-TG (2% of guanine) was irradiated with UVA, and digested to nucleosides which were separated and quantified. Data are corrected for destruction of dG^{SO2} as in the legend to Figure 3.

Mechanism of 6-TG photooxidation

6-TG is a Type II UVA photosensitizer (6). We therefore investigated whether $^1O_2$ generated photochemically by Rose Bengal and visible light mediated the stepwise conversion of 6-TG to G^{SO3} via G^{SO2}. When a mixture of 6-TG (0.1 mM) with Rose Bengal (0.5 mM) was irradiated with 360-kJ visible light, ≥90% of the 6-TG was oxidized. Both G^{SO2} and G^{SO3} were produced (Figure 4A).

Rose Bengal plus visible light treatment of authentic G^{SO2} provided further evidence for stepwise oxidation from 6-TG to G^{SO3}. When combined with a 5-fold molar excess of Rose Bengal and irradiated with different doses of visible light, G^{SO2} was oxidized to G^{SO3} (Figure 4B, i–iii). In contrast, G^{SO3} was stable and remained unaltered when it was irradiated with 360-kJ visible light (Figure 4B, iv). Thus, oxidation of 6-TG to G^{SO2} in a Type II photochemical reaction can be recapitulated using Rose Bengal and visible light as a source of $^1O_2$. G^{SO3}, in which the S atom is in the highest oxidation state, is refractory to further oxidation by $^1O_2$.

Direct chemical oxidation of 6-TG also proceeded via a reactive G^{SO2} intermediate to the ultimate product, G^{SO3}, which resisted further oxidation. When 6-TG was treated under mild oxidizing conditions, G^{SO2} was formed as a stable product. Thus, oxidation with I_2 is the synthetic route for G^{SO2} (15) (Figure 1A, iv). Under more stringent conditions, G^{SO2} was susceptible to oxidation and the pure compound was converted to G^{SO3} in the presence of Fe^{2+} in the form of $Fe(NH_4)_2SO_4$ in a concentration-dependent manner (Figure 4C). These findings are consistent with the favourable oxidation of 6-TG to G^{SO2} as the first stable reaction product. G^{SO2} is further oxidized to G^{SO3} in a reaction that requires more rigorous conditions. G^{SO3} is refractory to further oxidation.

Ascorbate protects 6-TG against UVA-mediated oxidation

Ascorbate (vitamin C, Vc), an acknowledged antioxidant, prevented the UVA-mediated destruction of 6-TG. Solutions of 6-TG (0.1 mM) and ascorbate were UVA irradiated and the loss of A_{342} was monitored spectrophotometrically. In the absence of ascorbate, a dose of 11 kJ/m^2 UVA destroyed >80% of the 6-TG (Figure 5A). A 12-fold molar excess of ascorbate during irradiation conferred significant protection and >80% and >30% of the 6-TG remained unchanged after UVA doses
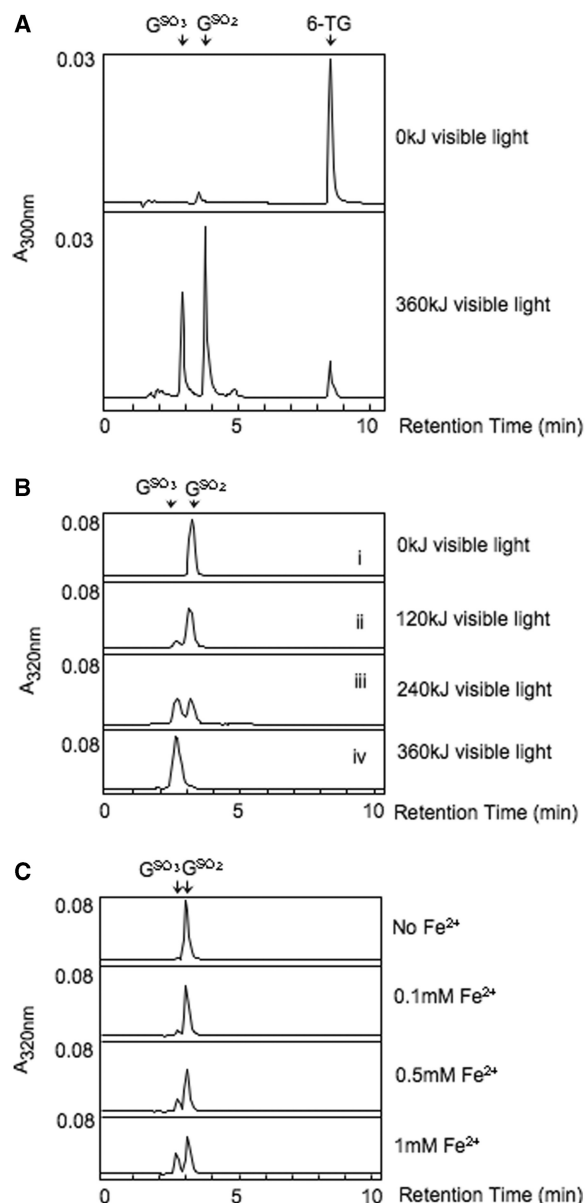

Figure 4. Chemical oxidation of 6-TG, G^{SO2} and G^{SO3}. (**A**) Rose Bengal and light treatment of 6-TG. Aqueous 6-TG (0.1 mM) was irradiated with visible light (200 W) at a dose of 360 kJ in the presence of 0.5 mM Rose Bengal. Products were separated by HPLC System 1. The relevant part of the absorbance (300 nm) trace is shown. (**B**) Rose Bengal and light treatment of G^{SO2} and G^{SO3}. Aqueous G^{SO2} (upper panels) or G^{SO3} (lowest panel) (both 0.1 mM) were irradiated with visible light for the indicated times in the presence of 0.5 mM Rose Bengal. Products were separated by HPLC System 3. (**C**) Oxidation of G^{SO2}. Aqueous G^{SO2} (0.1 mM) was incubated with the indicated concentrations of $Fe(NH_4)_2SO_4$, and products analysed immediately by HPLC System 3.

of 11 and 23 kJ/m^2, respectively (Figure 5A). Higher ascorbate concentrations were even more protective and approached completion at Vc:6-TG ratios of ≥25, at which <20% of the 6-TG was destroyed by 30 kJ/m^2 UVA. Experiments conducted in parallel confirmed that neither G^{SO2} nor G^{SO3} reacted with ascorbate (data not shown). Ascorbate only protected against 6-TG oxidation
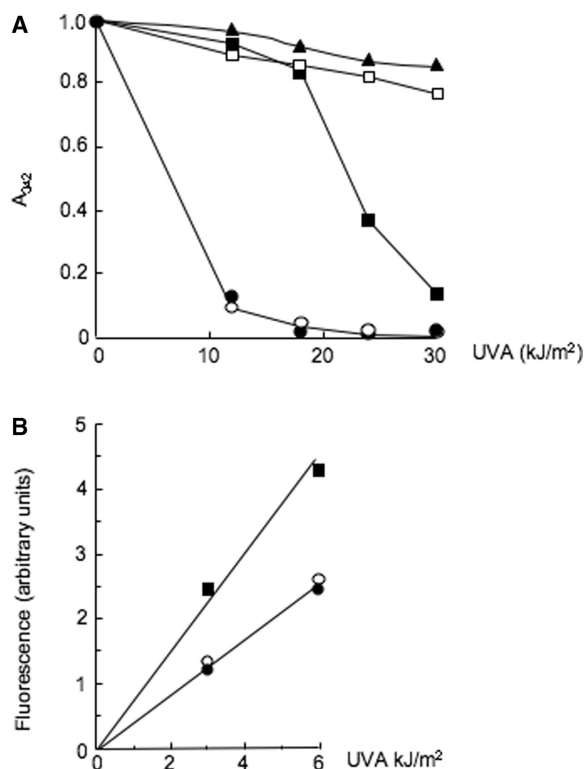
Figure 5. Ascorbate protects against oxidation. (**A**) Protection of 6-TG against photochemical destruction. The 6-TG (0.1 mM in aqueous solution) was irradiated with the doses shown in the presence of different concentrations of ascorbate. Photochemical destruction of 6-TG was monitored by the reduction in A_{342} spectrophotometrically. (open circle) no ascorbate (filled circle) ascorbate:6-TG 3:1 (filled square) 12:1 (open square) 25:1 (filled triangle) 100:1. (**B**) Protection of DNA 6-TG. A 11-mer oligonucleotide (1 μM) (CAG**X**AATTCGC where **X** = 6-TG) was UVA irradiated in the presence of ascorbate as indicated; 0 μM ascorbate (filled square), 10 μM (open circle) or 20 μM (filled circle). Conversion of 6-TG to G^{SO3} in the intact oligonucleotide was monitored fluorimetrically (λ_{ex} 320 nm; λ_{em} 410 nm).

if it was present during the irradiation. When a 100-fold excess of ascorbate was added to 6-TG that had been previously irradiated with 21 kJ/m^2, there was no change in the amounts of photoproducts and 6-TG was not regenerated (data not shown).

Ascorbate also prevented oxidation of 6-TG in an oligodeoxynucleotide. A 10- or 20-fold molar excess of ascorbate reduced by ~2-fold the rate of UVA-induced conversion from 6-TG to G^{SO3} as monitored by spectrophotofluorimetry (Figure 5B).

Reversal of 6-TG oxidation by Na$_2$S

Na$_2$S converts G^{SO3} to 6-TG in a slow reaction that takes several days at RT (6). When G^{SO2} (0.1 mM aqueous solution) was combined with Na$_2$S at RT and the mixture analysed immediately by HPLC, there was a significant Na$_2$S concentration-dependent conversion of G^{SO2} to 6-TG (Figure 6). A 20-fold excess of Na$_2$S, converted ~90% of the input G^{SO2} to 6-TG. This rapid and efficient reaction is in contrast to the slow generation of 6-TG from G^{SO3}.

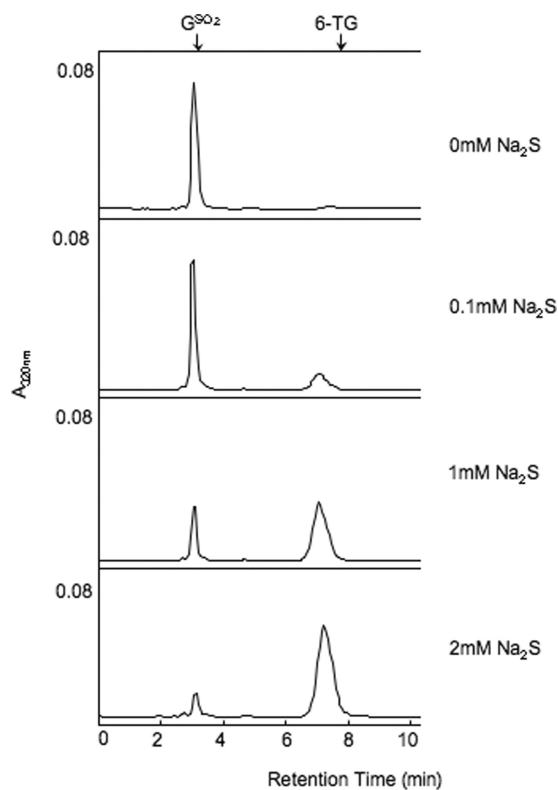**Figure 6.** Reversion of G^{SO2} by Na$_2$S. Aqueous G^{SO2} (0.1 mM) was mixed with Na$_2$S at RT to the final concentration indicated and the sample was immediately analysed by HPLC System 3. Products were detected by $A_{320\,nm}$. The known position of elution of 6-TG is shown arrowed.

DISCUSSION

We previously reported that 6-TG is labile to UVA irradiation both as a free base and when incorporated into DNA (5,6). Exposure of 6-TG to UVA in the presence of oxygen causes the formation of $^1O_2$. 6-TG is particularly vulnerable to oxidation and is easily oxidized by Rose Bengal plus visible light, an acknowledged $^1O_2$ source. The highly fluorescent DNA G^{SO3} was previously identified as a significant product of $^1O_2$-mediated oxidation (5,6). 6-TG has a low oxidation potential and under conditions in which normal DNA bases are invulnerable, 6-TG is quantitatively oxidized to G^{SO3} by treatment with the mild oxidizing agent MMPP. We proposed that, on UVA exposure, G^{SO3} was formed via the partially oxidized intermediates guanine sulfenate (G^{SO}) and G^{SO2}, both of which are reported to be unstable (16). Figure 7 summarizes these reactions. In this report, we identify G^{SO2} as a major stable photoproduct of the free 6-TG base, its 2′-deoxyribonucleoside, and of 6-TG in DNA. Under mild conditions, G^{SO2} is also the major stable product of MMPP oxidation of 6-TG. The initial oxidation of 6-TG to G^{SO2} is more favourable than the subsequent oxidation to G^{SO3}. G^{SO3} itself is refractory to further oxidation. UVA irradiation of 6-TG or its 2′-deoxyribonucleoside also generated small amounts of G or dG. We did not observe any photoproduct,
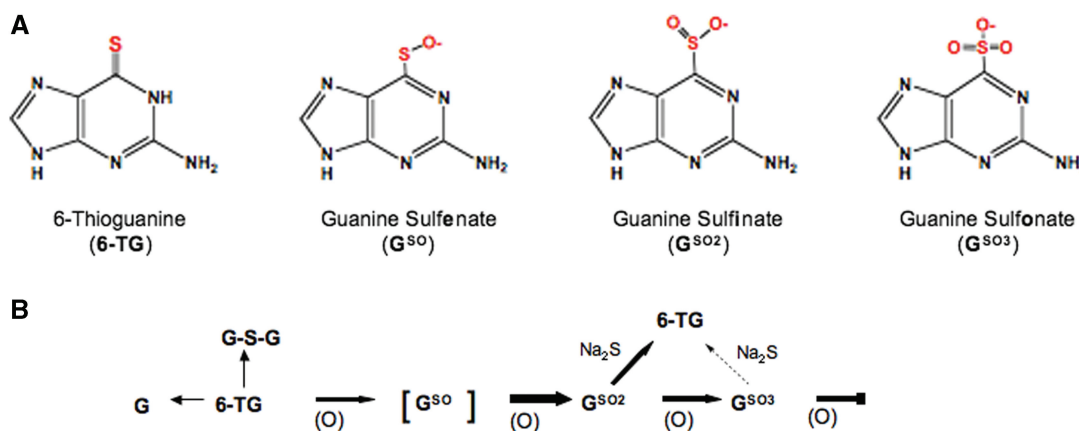
Figure 7. The 6-TG Oxidation: products and reactions. (**A**) Structures of 6-TG and oxidation products. (**B**) Reaction scheme for 6-TG. (O) represents oxidizing treatment and, in particular the $^1O_2$ that is generated by the interaction of 6-TG with UVA. The more favourable reactions are shown with bold arrows.

either from the free base or from DNA 6-TG, with properties expected of guanine sulfenate (G^{SO}). This likely intermediate is possibly too unstable to withstand our analysis procedure. In a description of the properties of sulfenate, Abraham *et al.* (16) reported that it was not possible to isolate free sulphenic acids, and instead purified the silver salt of purine-6-sulfenate from which purine sulphenic acid could be released by acidification. The free acid had a relatively short half-life in aqueous solution, particularly at neutral pH. Under acidic conditions, purine-6-sulphenic acid was shown to break down to 6-thiopurine and hypoxanthine via purine-6-sulphenic acid. In the case of G^{SO}, the analogous products would be 6-TG and G produced via G^{SO2}.

This skin of patients taking thiopurines contains DNA 6-TG and is selectively hypersensitive to erythema induction by UVA (5,12). This is consistent with the formation of replication- and transcription-blocking DNA lesions (17). UVA treatment of cells containing DNA 6-TG generates $^1O_2$ in DNA and this is also associated with severe inhibition of transcription and replication (6,10). The formation of replication- and transcription-blocking DNA lesions can be demonstrated in biochemical assays using purified enzymes and UVA- or MMPP-treated synthetic DNA substrates containing 6-TG. One aim of this study was to characterize the 6-TG photoproducts in DNA. In general, the DNA photoproducts qualitatively and quantitatively mirrored those formed by irradiation of free 6-TG or 6-TGdR in solution. The major exception is formation of the G-S-G dimer. This would be unlikely in DNA with a relatively low level of 6-TG substitution. We confirmed the formation of G^{SO3} and showed further that G^{SO2} is the major stable DNA photoproduct. Since together these two lesions account for >90% of the destroyed DNA 6-TG, at least at lower doses (45 kJ/m^2), they are likely to be the ones with the biggest impact on biological processes.

In biochemical assays of DNA replication and transcription using template oligos containing a single 6-TG, the inhibition caused by a quantitative destruction of

template 6-TG by UVA irradiation or by MMPP are closely similar (6,10). Our measurements reported here indicate that in the former case, >80% of the photoproducts will be G^{SO2}, whereas MMPP treatment results in 100% conversion to G^{SO3}. The similar effects of these DNA lesions on DNA and RNA polymerases indicated that DNA G^{SO2} and G^{SO3}, which contain large, negatively charged substituent groups, are both very effective inhibitors of these enzymes.

The generation of damaging ROS is one of the inescapable hazards of aerobic metabolism and oxidation is a constant threat to DNA. Cellular antioxidant defences protect key cellular macromolecules from damage by normal levels of ROS. In addition to being a photochemical source of $^1O_2$, DNA 6-TG is an important target for damage by cellular ROS, including H_2O_2 (18). We observed that ascorbate, an acknowledged dietary antioxidant and a significant intracellular consumer of $^1O_2$, protected 6-TG and DNA 6-TG against photooxidation. These findings suggest that antioxidant defences may protect thiopurine-treated patients by preventing the oxidation of DNA 6-TG. Additional protection might arise via reversion of the major photoproduct, G^{SO2}, back to 6-TG. Although G^{SO3} is stable and its reversion requires quite stringent conditions, the reaction of G^{SO2} is relatively favourable. Conversion of this potentially replication- and transcription-blocking DNA lesion back to the relatively innocuous 6-TG would ameliorate the UVA-mediated biological effects of DNA 6-TG. Overall, the oxidation of 6-TG via the reactive intermediates guanine sulfenate (G^{SO}) and G^{SO2}, and the persistence of the relatively reactive G^{SO2} is, however, likely to be hazardous. Both G^{SO} and G^{SO2} are much more reactive towards nucleophiles than G^{SO3} the final oxidation product. Their reactivity raises the possibility that intermediate DNA G^{SO} or accumulated G^{SO2}, which forms in the skin of UVA-exposed patients taking thiopurines, may interact with cellular nucleophiles to form complex addition products that might compromise cellular DNA transactions and contribute to the extremely high

incidence of sun-exposure-related skin cancer in this patient group. The possible reactions of G^{SO2} (and the highly unstable guanine sulfenate, G^{SO}) with cellular reducing agents and nucleophiles are currently under investigation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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