Published in final edited form as:

Science. 2005 September 16; 309(5742): 1871–1874. doi:10.1126/science.1114233.

Azathioprine and UVA Light Generate Mutagenic Oxidative DNA Damage

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Abstract

Oxidative stress and mutagenic DNA lesions formed by reactive oxygen species (ROS) are linked to human malignancy. Clinical treatments inducing chronic oxidative stress may therefore carry a risk of therapy-related cancer. We suggest that immunosuppression by azathioprine (Aza) may be one such treatment. Aza causes the accumulation of 6-thioguanine (6-TG) in patients' DNA. Here we demonstrate that biologically relevant doses of ultraviolet A (UVA) generate ROS in cultured cells with 6-TG-substituted DNA and that 6-TG and UVA are synergistically mutagenic. A replication-blocking DNA 6-TG photoproduct, guanine sulfonate, was bypassed by error-prone, Y-family DNA polymerases in vitro. A preliminary analysis revealed that in five of five cases, Aza treatment was associated with a selective UVA photosensitivity. These findings may partly explain the prevalence of skin cancer in long-term survivors of organ transplantation.

The thiopurines azathioprine (Aza), 6-mercaptopurine (6-MP), and 6-thioguanine (6-TG) are cancer therapeutic and immunosuppressive agents. They are all prodrugs (compounds that the body converts into active drugs) requiring metabolic activation into the thioguanine nucleotides (TGNs) that are precursors for 6-TG incorporation into DNA (1).

Experimentally, 6-TG is a surrogate for Aza because it bypasses many of the activation steps and is directly converted to TGN. The normal DNA bases do not absorb significantly at ultraviolet A (UVA) wavelengths (320 to 400 nm), whereas thiopurines do, and 6-TG has an absorbance maximum at 342 nm. 6-MP generates ROS when exposed to UVA (2). ROS are pernicious DNA-damaging agents (3), and although cells are equipped to deal with them, abrupt increases in ROS cause oxidative stress and produce mutagenic DNA lesions (4). The possibility that DNA 6-TG might act as an endogenous UVA chromophore and provide a source of promutagenic oxidative DNA damage prompted us to investigate the

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Supporting Online Material

www.sciencemag.org/cgi/content/full/309/5742/1871/DC1

photochemical properties of 6-TG and the biological consequences of the interaction between DNA 6-TG and UVA.

HCT116 human colorectal carcinoma cells are mismatch repair-defective and tolerant of high levels of DNA 6-TG (5). We found that UVA generated intracellular ROS in 6-TG-treated HCT116 cells in which 6-TG replaced approximately 0.2% of DNA guanine. After uptake of CM-H2DCFDA dye and irradiation with UVA (3 kJ/m^2) [approximately equivalent to 1 to 2 min of exposure around noon in England at midsummer (6)], the cells emitted a green fluorescence indicating the formation of ROS. This was detected by fluorescence-activated cell sorting and by microscopy (Fig. 1A). Because ROS are highly unstable, they tend to react close to their site of formation. When the thiopurine was selectively excluded from DNA by carrying out 6-TG treatment in the presence of the DNA replication inhibitor hydroxyurea (HU), UVA-induced fluorescence was markedly reduced and nuclear fluorescence was abolished (Fig. 1A). This indicates that DNA 6-TG is a major source of intracellular ROS, and DNA is likely to be a significant target for oxidative damage. 6-TG-treated HCT116 cells were killed by the UVA doses that generated ROS (Fig. 1B), consistent with the formation of lethal DNA damage. UVA was also cytotoxic to 6-TG-treated, mismatch repair-proficient, A2780 human ovarian carcinoma (7) and CHOD422 cells (Fig. 1B). These cells are not tolerant of 6-TG but grow normally when 6-TG replaces $\sim 0.05\%$ of DNA guanine.

To investigate whether 6-TG plus UVA was mutagenic, we examined induction of adenine phosphoribosyltransferase (*aprt*) mutations in CHOD422 cells. In cells containing $\sim 0.01\%$ DNA 6-TG irradiated with a nontoxic UVA dose of 1 kJ/m^2 , there was a threefold ($P=0.005$) increase in *aprt* mutation frequency (Fig. 1C). Neither 6-TG nor UVA alone was detectably mutagenic. Without 6-TG treatment, 500 kJ/m^2 of UVA was required to induce a similar increase in CHO *aprt* mutation frequency (8). The susceptibility of 6-TG-treated cells to UVA-induced mutation and killing reveals DNA 6-TG to be a potent photosensitizer that increases the biological effectiveness of UVA by two orders of magnitude.

We also examined the photochemical properties of 6-TG both as a free base and in DNA. Like 6-MP (2), free 6-TG was destroyed by UVA in an oxygen-dependent reaction that generated free radicals (Fig. 2A). 6-TG was converted into a single fluorescent product (excitation maximum 324 nm, emission maximum 410 nm) that was resolved from the parent compound by reverse-phase high-pressure liquid chromatography (HPLC) (Fig. 2B). The same fluorescent photoproduct was formed in irradiated DNA. It was acid-labile and was destroyed under the conditions used for DNA depurination, but was recovered as the deoxynucleoside after enzymatic digestion (Fig. 2C). The fluorescent 6-TG photoproduct was identified as 2-aminopurine-6-sulfonate [guanine-6-sulphonate (G-6-SO₃)] (fig. S1). Its absorbance and fluorescence spectra and acid lability were identical to G-6-SO₃ prepared by alkaline permanganate treatment of 6-TG (9-11) and authenticated by ¹H nuclear magnetic resonance and mass spectroscopy [see supporting online material (SOM)]. This authentic G-6-SO₃ also coeluted with the fluorescent 6-TG photoproduct on HPLC. In addition, the fluorescent deoxynucleoside from digests of UVA-irradiated 6-TG DNA coeluted with the single fluorescent photoproduct of UVA-treated 6-TGdR (fig. S2).

To examine the effects of UVA 6-TG photoproducts on DNA replication, we carried out in vitro primer extension assays with Klenow fragment (KF). A 22-mer oligonucleotide containing a single 6-TG (fig. S3A) was irradiated with 5, 20, or 100 kJ/m^2 of UVA. HPLC analysis indicated that the lowest dose converted around 70% of the 6-TG to G-6-SO₃. At 20 and 100 kJ/m^2 , 90% of the 6-TG was destroyed. Irradiated oligonucleotides were used as templates for KF-mediated extension of a 14-mer primer that terminated immediately 3' to

the 6-TG. An irradiated 22-mer oligonucleotide in which G replaced 6-TG was used as a control.

With the control or UVA-irradiated G template, KF extended all primer molecules to full-length 22-mers within 60 s (Fig. 3A). As reported (12), 6-TG slightly inhibited replication. In contrast, UVA-irradiated 6-TG was a powerful replication block. Thus, 5 kJ/m² significantly impaired primer elongation, and inhibition was almost complete (90%) at the higher UVA doses (Fig. 3A). The inhibitory effects were targeted to the photoproduct, and no other polymerase arrest or pause sites were evident. Thus, although KF replicated unmodified 6-TG with reasonable facility in this assay, it did not bypass G-6-SO₃ efficiently.

Y-family DNA polymerases bypass replication-blocking lesions in a DNA damage tolerance strategy. This process is potentially mutagenic owing to their low replication fidelity (13). We found that two representative Y-family DNA polymerases, human DNA polymerase η (14) and Sso P2Y1 polymerase (SsoY1pol) of *Sulfolobus solfataricus*, a hyperthermophilic archaeon (15), replicated a heavily UVA-irradiated (100 kJ/m²) 6-TG template (Fig. 3B and fig. S3B). Coding by the photoproduct was ambiguous; both DNA polymerase η and SsoY1pol inserted T or C opposite the lesion with about equal frequency (Fig. 3C and fig. S3C). Thus, in vitro, Y-family DNA polymerases overcome the G-6-SO₃ replication block in a potentially mutagenic manner.

To extend these findings to a clinical setting, we measured the amount of 6-TG in DNA extracted from the normal skin of three Aza-treated [1 to 2 mg per kilogram of body weight (mg/kg) daily] patients undergoing surgical excision of squamous cell skin carcinoma (SCC). All three samples contained 6-TG representing around 0.02% substitution of DNA guanine (table S1). Similar levels are present in lymphocyte DNA of thiopurine-treated patients (16, 17). As expected, no 6-TG was detected in skin DNA of patients who were not taking Aza. In a further five patients, we measured the effect of 1 to 2 mg/kg daily Aza on the minimal erythema dose (MED). This is the lowest dose of radiation required to produce just perceptible erythema 24 hours after the irradiation of skin not normally exposed to sunlight. In each patient, Aza treatment caused a significant reduction in the MED for UVA ($P = 0.025$ by paired t test as compared to the pretreatment value) (Fig. 4). The MED for solar-simulating radiation was also reduced, but there was no concomitant sensitization to UVB (fig. S4, A and B). Erythema is associated with replication- and transcription-blocking DNA photodamage in mouse skin models (18) and is regarded as a surrogate indicator of persistent DNA damage in human skin (19). The selective UVA sensitivity associated with Aza treatment is consistent with the production of 6-TG DNA photoproducts.

Our findings indicate that normal exposure to sunlight may induce chronic oxidative stress and increase the levels of oxidative DNA lesions in the skin of patients taking Aza. A defect in processing the highly mutagenic oxidation products of normal DNA bases is associated with human cancer (20). Sustained generation of ROS-induced DNA lesions might represent a similar carcinogenic hazard. The susceptibility of DNA 6-TG itself to oxidation and the formation of DNA G-6-SO₃ has additional implications. Bypass of replication-blocking G-6-SO₃ by error-prone Y-family DNA polymerases represents another potential source of mutation.

The photochemical reactions of DNA 6-TG have implications for skin cancer. In a clinical setting, DNA 6-TG and UVA are likely to interact in the skin of organ transplant patients. Around 25,000 solid organ transplants are performed annually throughout Europe and in North America. SCC is 50 to 250 times more common among transplant patients than in the general population (21, 22), and 20 years after transplant, between 60 and 90% of patients

are affected (23). Transplant-related SCC develops primarily on chronically sun-exposed skin, and sunlight plus the duration of treatment with immunosuppressive drugs are acknowledged risk factors. Until recently, most transplant patients have been treated with Aza. UVA is the major component of solar radiation, and a high fraction of incident UVA penetrates to the basal layers of the skin containing the stem cells. To date, epidemiological studies have not identified the contributions of individual immunosuppressive agents to transplant-related SCC (23, 24). The photochemical properties of DNA 6-TG described here indicate how UVA and an immunosuppressive drug might contribute to post-transplant SCC: a significant cause of morbidity in this group of patients.

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27. We thank M. Yamada for her kind gift of the SsoP2Y1 DNA polymerase and the staff of the Oligonucleotide Synthesis Laboratory, Cancer Research UK, for oligonucleotides. The work was supported in part by a Royal Society K. C. Wong Fellowship to X.Z. while on leave from Shanghai Jiao Tong University. We also acknowledge support from the Spanish Ministerio de Educacion y Ciencia (to B.M.) and from the Association for International Cancer Research. All

studies involving patients were carried out with prior local ethics committee approval. The procedures involved and their implications were fully explained to the patients who gave informed consent.

Fig. 1.

Biological consequences of UVA irradiation of DNA 6-TG. (A) UVA generates intracellular ROS in cells with DNA 6-TG. HCT116 cells were grown for 24 hours in medium containing 1 μ M 6-TG in the presence or absence of 10 mM HU. In the absence of HU, 6-TG replaced approximately 0.2% of DNA guanine. After thorough washing with phosphate-buffered saline, 6-TG-treated cells were incubated with CM-H2DCFDA and irradiated with 3 kJ/m² of UVA as described (25). Green fluorescence generated by the reaction between CM-H2DCFDA and oxygen free radicals was analyzed by FACS or fluorescence microscopy. HCT116 cells grown without 6-TG and treated with H₂O₂ served as a control for ROS generation. Representative photomicrographs of fluorescent cells are shown. Scale bar, 20 μ m. Note the absence of nuclear fluorescence in cells treated with HU. (B) 6-TG sensitizes cells to UVA. HCT116 (circles), A2780 (squares), or CHO422 (triangles) cells were grown for 48 hours in medium containing 1, 0.1, or 0.1 μ M 6-TG, respectively. Treated cells were irradiated with the UVA doses shown and replated in medium without 6-TG. Clonal survival was determined after 10 days. Open symbols: Survival after 30 kJ/m² of UVA administered to the same cells grown in the absence of 6-TG. (C) Mutagenesis by 6-TG/UVA. CHO422 cells were grown for 48 hours in 0.1 μ M 6-TG, washed, and irradiated with 1 kJ/m² of UVA. The *aprt* mutation frequency was calculated by determining the number of 8-azaadenine-resistant colonies (26). Results are the means of five independent determinations $\pm$ SD. The mean spontaneous mutation frequency was 4.1×10^{-6} (range: 2.1×10^{-6} to 6.0×10^{-6}), and the mean for 6-TG+UVA was 12.6×10^{-6} (range: 7.1×10^{-6} to 19.4×10^{-6}).

Fig. 2. Photochemical reactions of 6-TG. **(A)** Conversion of free 6-TG into a fluorescent product by UVA. An aqueous solution of 6-TG (0.1 mM) was irradiated with UVA at a dose rate of 0.1 kJ/m²/s. The progress of the reaction was monitored simultaneously by A_{342} (solid circles) and by fluorescence (open circles). Excitation was at 324 nm and emission at 410 nm. The same reaction was carried out in the presence of the free-radical scavenger *N*-acetyl-L-cysteine (triangles) and monitored by fluorescence. **(B)** UVA irradiation converts 6-TG into a fluorescent product. 6-TG was irradiated in solution with 10 kJ/m² of UVA, and the products were analyzed by reverse-phase HPLC. Column eluates were monitored simultaneously by A_{342} (left panel) and fluorescence (right panel). 6-TG is converted from a UVA-absorbing compound with minimal fluorescence into an earlier-eluting highly fluorescent product (arrowed) that does not absorb at 342 nm. (solid line, unirradiated; dashed line, irradiated). **(C)** The fluorescent 6-TG photoproduct is also formed in DNA. Unirradiated (left panel) or UVA-irradiated (50 kJ/m², right panel) 18-mer oligonucleotides that contained a single 6-TG were digested to deoxynucleosides with P1 nuclease and acid phosphatase. These were separated by HPLC. Eluates were monitored by A_{342} and fluorescence at 410 nm. (Left) In digests of unirradiated oligonucleotides, 6-TGdR elutes at

21 min. It is detected by A_{342} but has minimal fluorescence. (Right) After irradiation, no 342-nm-absorbing material is detectable, and a major fluorescent product elutes coincident with the fluorescent UVA photoproduct of authentic 6-TGdR (fig. S2) at 12 min (arrow). (solid line, unirradiated; dashed line, irradiated).

Fig. 3.

Primer extension by KF and photoproduct bypass by a Y-family DNA polymerase. **(A)** 22-mer templates containing G (left) or a single 6-TG (right) were irradiated with the UVA doses indicated. They were annealed to a ³²P end-labeled 14-mer primer that terminated immediately 3' to the site-specific 6-TG. The primer/templates were used to direct replication by KF. Polymerization was for 60 s at 37°C. Products were analyzed by denaturing gel electrophoresis. The arrow indicates the full-length 22-mer product. **(B)** Photoproduct bypass by a Y-family DNA polymerase. Primer and templates containing unirradiated template G (control), unirradiated template 6-TG (6-TG), or irradiated (100 kJ/m²) template 6-TG (6-TG + UVA) were supplemented with all four deoxynucleoside triphosphates (dNTPs) and increasing amounts of purified human DNA polymerase η. After 10 min of incubation at 37°C, products were separated by denaturing gel electrophoresis. **(C)** Nucleotide insertion opposite 6-TG and the 6-TG photoproduct by a Y-family DNA polymerase. Primer and templates containing irradiated template 6-TG were incubated with human DNA polymerase η in the presence of a single dNTP as indicated. After 10 min of incubation at 37°C, products were separated by denaturing polyacrylamide gel electrophoresis. The positions of unaltered primer (-1) and the product that is two nucleotides longer (+1) are indicated.

Fig. 4.

Skin photosensitivity in patients treated with Aza. The MED for UVA was determined in five patients who were about to begin a course of Aza treatment for polymorphic light eruption, Crohn's disease, ulcerative colitis, pemphigus vulgaris, or recurrent erythema multiforme. MED measurements were repeated 3 months after beginning Aza treatment (1 to 2 mg/kg/day). Before treatment, black bars; during treatment, white bars.