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DNA breakage and cell cycle checkpoint abrogation induced by a therapeutic thiopurine and UVA radiation

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Abstract

The frequency of squamous cell skin carcinoma in organ transplant patients is around 100-fold higher than normal. This dramatic example of therapy-related cancer reflects exposure to sunlight and to immunosuppressive drugs. Here we demonstrate that the interaction between low doses of UVA, the major ultraviolet component of incident sunlight, and 6-TG, a UVA chromophore that is introduced into DNA by one of the most widely-prescribed immunosuppressive drugs, causes DNA single- and double strand breaks (DSB). S phase cells are particularly vulnerable to this DNA breakage and cells defective in rejoining of S-phase DSB are hypersensitive to the combination of low dose UVA and DNA 6-TG. 6-TG/UVA-induced DNA lesions provoke canonical DNA damage responses involving activation of the ATM/Chk2 and ATR/Chk1 pathways and appropriate cell cycle checkpoints. Higher levels of photochemical DNA damage induce a proteasome-mediated degradation of Chk1 and checkpoint abrogation that is consistent with persistent unrepaired DNA damage. These findings indicate that the interaction between UVA and an immunosuppressant drug causes photochemical DNA lesions, including DNA breaks, and can compromise cell cycle checkpoints. These two properties could contribute to the high risk of sunlight-related skin cancer in long-term immunosuppressed patients.

Keywords

Thiopurines; UVA; skin cancer; DNA breakage; cell cycle checkpoints

Introduction

The thiopurines azathioprine, 6-thioguanine (6-TG) and 6-mercaptopurine are anti-cancer, anti-inflammatory and immunosuppressant drugs whose effectiveness requires their enzymatic conversion and incorporation into DNA as 6-TG (Karran and Attard, 2008). Treatment with azathioprine, until recently the most widely-prescribed immunosuppressant for organ transplant patients, is associated with measurable DNA 6-TG in lymphocytes and skin (Warren *et al.*, 1995, O'Donovan *et al.*, 2005). The patients' skin is hypersensitive to erythema induction by ultraviolet A (UVA, 320-400nm), but not UVB radiation (Perrett *et*

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Conflict of Interest

All authors declare there is no conflict of interest.

al., 2008) and immunosuppression following organ transplantation is associated with high rates of skin cancer (Penn, 1994, Euvrard *et al.*, 2003). Duration of immunosuppressive treatment and exposure to sunlight are acknowledged contributors to this cancer susceptibility.

UVA comprises 95% of the UV radiation of incident sunlight. It is generally considered to be less carcinogenic than UVB which is directly absorbed by DNA nucleobases and induces photochemical DNA lesions. UVA is absorbed very poorly by DNA and its genotoxicity is mainly mediated by non-DNA chromophores (Cadet *et al.*, 2005). Unlike the canonical DNA nucleobases, 6-TG is a strong UVA chromophore with maximum absorbance at 340nm. The UVA energy absorbed by DNA 6-TG is transferred to molecular oxygen to generate singlet oxygen ($^1\text{O}_2$), a form of reactive oxygen species (ROS) (Zhang *et al.*, 2006). The unprecedented generation of highly damaging ROS within DNA itself has dramatic consequences. DNA 6-TG and UVA are synergistically cytotoxic and mutagenic in cultured cells (O'Donovan *et al.*, 2005) and their interaction creates a genotoxic hazard that might contribute to the acknowledged carcinogenicity of immunosuppressive thiopurines. 6-TG itself is highly susceptible to damage by ROS (Daehn and Karran, 2008). Its UVA-induced oxidation products are blocks to replication (Zhang *et al.*, 2006) and transcription (Brem *et al.*, 2008) *in vitro* and *in vivo*, indicating that they contribute to the genotoxicity of DNA 6-TG. At least one photoproduct, guanine sulfonate (G^{SO_3}), is bypassed by error-prone DNA polymerases *in vitro* and represents a possible promutagenic DNA lesion (O'Donovan *et al.*, 2005). Importantly, potentially lethal DNA 6-TG photoproducts are not subject to removal by excision repair (Brem *et al.*, 2008). The normal DNA nucleobases and DNA-associated proteins are also at risk of damage from ROS generated in DNA. DNA base damage, including 8-oxo-7,8-dihydroguanine, the principal mutagenic base lesion induced by ROS (Kasai and Nishimura, 1984, Barnes and Lindahl, 2004), is generated when human cells are treated with 6-TG and UVA (Cooke *et al.*, 2008). Protein damage is exemplified by the covalent crosslinking of PCNA subunits (Montaner *et al.*, 2007) and it seems likely that other proteins close to the replication fork are similarly vulnerable. ROS induce direct DNA breaks via hydrogen abstraction within the sugar - a significant threat to genome stability (reviewed in Demple and DeMott, 2002). It has been known for some time that low UVA doses can combine with a DNA-intercalating UVA chromophore to induce DNA breaks in cultured cells (Limoli and Ward, 1993).

Because oxidation is a ubiquitous hazard, most DNA oxidation products can be repaired. Oxidized nucleobases are removed by base excision repair that also processes some DNA single-strand breaks (Caldecott, 2003). DNA double-strand breaks (DSBs) are repaired either by homologous recombination (HR) or non-homologous end-joining (NHEJ). The choice of mechanism for DSB repair is governed by the cell cycle (Yun and Hiom, 2009): HR is the predominant pathway in the S and G2 phases of the cell cycle when identical sister chromatids are available. The NHEJ pathway predominates in G1 phase cells (reviewed in Hoeijmakers, 2001).

DSB repair pathways are intimately connected with other facets of the DNA damage response in which the ATM protein senses DNA damage and regulates downstream signalling pathways for checkpoint activation and cell cycle arrest (Shiloh, 2001). Inactivating *ATM* mutations in the genetic disorder ataxia telangiectasia (AT) cause genetic instability, severe sensitivity to agents that cause DNA breakage and an increased cancer risk.

AT links DNA damage, genetic instability and cancer predisposition. The chromosomal aberrations that characterize cancer often reflect unrepaired or misrepaired DNA strand breaks. Thiopurine immunosuppressants are unequivocally linked to skin cancer. In view of

the properties of DNA 6-TG, we investigated photochemical DNA breakage, repair, and checkpoint activation. Our findings indicate that even low doses of UVA are particularly hazardous for S phase cells containing DNA 6-TG. Persistent photochemical DNA damage induces degradation of the Chk1 protein accompanied by abrogation of the damage-induced G₂/M cell cycle checkpoint.

Results

Induction of DNA breaks

Single-strand breaks—We examined whether ¹O₂ generated by the interaction between DNA 6-TG and UVA causes DNA strand breaks. MRC5VA cells were grown in the presence of 6-TG to allow incorporation of the thiopurine into DNA and irradiated with UVA (10 kJ/m²). DNA breakage was analysed immediately after irradiation by the alkaline comet assay to which detects the presence of DNA single-strand breaks (or alkali-labile sites). UVA induced DNA breaks in a DNA 6-TG-dependent manner. The comet distribution was heterogeneous and only a subset of irradiated cells sustained significant DNA breakage (Figure 1A), suggesting a non-random introduction of single-strand lesions. In contrast, comets induced by ionizing radiation, which causes random DNA breakage, were homogeneous. No DNA damage was detected after 6-TG or UVA alone. Similar results were obtained with the HaCaT keratinocyte cell line (Supplementary Figure 1A) and HCT116 colorectal carcinoma cells (data not shown).

The asymmetric DNA damage distribution prompted us to examine whether DNA breakage was related to cell cycle phase. Since it was not possible to synchronize MRC5VA cells with sufficient precision, we used HCT116 cells synchronized by a double thymidine block in these experiments. Cells were allowed to incorporate 6-TG for 4 h immediately after release into S phase after which they were returned to 6-TG-free medium. They were irradiated with UVA (10 kJ/m²) either 9h after the release, when around 90% had passed into the G1 or G2 phases of the cell cycle as determined by FACS analysis, or at 16 h, when the majority of cells had progressed into a second S phase (Figure 1B, upper panels). Alkaline comet assays performed immediately after irradiation revealed that DNA strand breakage, expressed as comet tail moment, was largely confined to cells that had been irradiated in S phase. The extent of DNA damage in G1- or G2-irradiated cells was only slightly higher than that in untreated control cells (Figure 1B, lower panel). DNA breakage was independent of ongoing replication and the distribution of comet tails was similar when the same synchronized S phase cells were UVA irradiated following treatment with hydroxyurea which inhibits replication leaving the arrested replication forks in an open configuration (data not shown). These findings indicate that treatment with 6-TG and UVA induces single-strand DNA breaks/alkali labile sites and that S phase cells are particularly vulnerable to this type of DNA damage.

Double-strand breaks—We also investigated DNA double strand breakage by 6-TG/UVA. 6-TG-treated MRC5VA cells were UVA- irradiated and analysed immediately by the neutral comet assay which detects DSBs. Figure 2A shows that whereas 10 Gy ionizing radiation induced detectable DNA breakage (Distribution 3), 6-TG in combination with UVA appeared ineffective in this regard (Distribution 5). A significant increase in comet tail moment was, however, observed when cells were digested with proteinase K prior to electrophoresis. DSBs were confined to cells that had received both UVA and 6-TG (Distribution 6). They were present immediately following irradiation indicating that they are introduced directly and not via advancing replication forks encountering template strand damage. The requirement for protease digestion to reveal DSBs by the comet assay indicates

that the presence of these lesions is masked by the simultaneous formation of DNA-protein crosslinks.

To address DSB induction in S phase, cells were treated with 6-TG for 2 h to substitute DNA in active replicons and comets analysed immediately after UVA treatment. As we observed with uniformly 6-TG substituted DNA, DSB formation was dependent on both 6-TG and UVA and its detection required protease treatment (compare Distributions 5 & 6, Figure 2B). Because the 6-TG was incorporated into DNA as a short pulse, these findings confirm the particular susceptibility of DNA 6-TG close to replication forks to photochemical damage which results in DNA double strand breaks.

The formation of nuclear γ H2AX foci provides an alternative indication of DNA DSB (reviewed in (Fernandez-Capetillo *et al.*, 2004)) and this analysis is less susceptible to interference by DNA-protein crosslinks. 6-TG-treated cells were irradiated with UVA and stained with anti- γ H2AX antibody one hour after irradiation. Fluorescent microscopy revealed distinct γ H2AX foci, indicating DSBs, or pan nuclear γ H2AX staining, a sign of stalled replication, in about 40% of the cells treated with both 6-TG and UVA (Figure 2C). No staining was observed in cells treated with either 6-TG or UVA alone. In contrast, 10 Gy IR, induced DSBs in all cells. A similar pattern of mixed pan- and focal staining affecting approximately half the cells was observed with HaCaT keratinocytes treated under similar conditions (Supplementary Figure 1B). These findings provide an independent demonstration that 6-TG and UVA cause DNA DSBs that are confined to a subpopulation of cells.

Biological effects of 6-TG/UVA-mediated DNA breaks: the influence of DSB repair

DSBs that arise in S phase are substrates for homologous recombination (HR) and cells defective in this pathway are hypersensitive to agents that induce DSB. Since our findings suggested a particular vulnerability of S phase cells to DNA strand breakage by 6-TG/UVA, we examined the possible involvement of HR in mitigating the biological consequences of photochemical damage. The *xrcc2* mutant *irs1* cells are defective in HR. Clonal assays of cell survival revealed that, at similar levels of DNA 6-TG (0.02% of DNA G), *irs1* cells are more sensitive to UVA than their *xrcc2*-corrected counterparts (Figure 3A). In contrast, no significant differences in survival were noted in 6-TG/UVA treated NHEJ-defective XRS6 cells compared to their parental repair-proficient K1 cells (Supplementary Figure 2). These findings indicate the involvement of HR, rather than the alternative NHEJ, in protecting against the lethal effects of 6-TG/UVA. They are consistent with the selective introduction of DSBs in S phase.

UVA and 6-TG at replication forks

The findings presented above indicate that cells in S phase are particularly susceptible to DNA breakage by 6-TG/UVA. To investigate their vulnerability further, we examined the effects of 6-TG proximal or distal to replication forks. Synchronized HCT116 cells released into S phase from a double-thymidine block were treated with 6-TG for 2 h and irradiated with UVA either immediately or after additional incubation in 6-TG-free medium for 2 or 5 h to allow the forks to advance away from the incorporated 6-TG. DNA breakage was assessed by the alkaline comet assay and the cell cycle distribution was determined by flow cytometry. Figure 3B shows that irradiation immediately after the 6-TG treatment (0 h chase) caused more DNA damage than irradiation after a 2h chase in 6-TG-free medium (compare distributions 2 and 4). Flow cytometry performed at the time of UVA irradiation revealed that in both cases, 70-80% of the cells were in S phase (Figure 3B, lower panels). When the chase time was extended to 5 h, when only 20% of the cells remained in S phase and 70% had reached G₂ phase, the comets distribution in irradiated cells was similar to that

after a 2 h chase (compare distributions 4 and 6). As expected, in the absence of UVA irradiation, 6-TG treatment did not induce measurable DNA damage at any time. The observation that the extent of DNA breakage is reduced by a 2 or 5 h chase, during which time replication forks have moved away from incorporated 6-TG, indicates that DNA 6-TG at, or very close to, replication forks is a target for photochemical damage that generates DNA strand breaks (or alkali labile sites).

The 6-TG nucleotide pool can also be a source of DNA damaging ROS following UVA irradiation (Cooke *et al.*, 2008). A chase in non-6-TG containing medium also depletes 6-TG nucleotides in the nucleotide pools, thereby removing an alternative source of ROS and DNA damage. This non-DNA 6-TG made only a minor contribution to DNA breakage, however. UVA irradiation of 6-TG treated HCT116 cells, held in early S phase by double-thymidine block but non-replicating, induced only a low level of DNA breakage (Figure 3B, Distribution 8). 70% of the cells displayed strand breakage comparable to that in non-irradiated control cells. Around 30% of cells, possibly a minority that had escaped the thymidine block and incorporated some 6-TG, sustained higher levels of damage. Taken together, the findings indicate that DNA at active replication forks is highly susceptible to photochemical damage to generate DNA breaks.

6-TG/UVA and the DNA damage response

The presence of DSB is signalled by the ATM protein which, *via* phosphorylation of downstream targets, controls DNA repair, cell cycle arrest and apoptosis. ATM deficient cells are characteristically sensitive to treatments that induce DSB. At comparable levels of DNA 6-TG substitution (0.04% of DNA G), ATM-deficient AT5BIVA fibroblasts were more sensitive to UVA than ATM-proficient MRC5VA cells (Figure 4A).

To investigate the DNA damage response further, we examined activation of the Chk2 and Chk1 proteins - the respective downstream targets of the ATM and ATR DNA damage sensors. Figure 4B shows that both the Chk1 and Chk2 signal transducers were phosphorylated following UVA irradiation of 6-TG-treated MRC5VA cells. One hour after irradiation, a 6-TG dose-dependent phosphorylation of Chk2 on Tyr68 was observed. By 4 h, activation was reversed and Chk2 phosphorylation had returned to pre-irradiation levels. Throughout this time, the amount of total Chk2 protein recovered in the extracts remained unchanged.

The behaviour of Chk1 differed significantly from that of Chk2. One hour after irradiation, Chk1 phosphorylation on Ser317 increased with 6-TG doses up to 0.4 μ M. At higher 6-TG concentrations, Chk1 activation appeared to be diminished. This biphasic dose response was more pronounced four hours after irradiation. Examination of the overall levels of Chk1 protein indicated that diminished phosphoChk1 reflected a 6-TG dose-dependent reduction in the recovery of Chk1 protein rather than its reduced activation. Four hours after irradiation, Chk1 was barely detectable in extracts of cells treated with 1.6 μ M 6-TG. A similar 6-TG/UVA damage-dependent reduction in Chk1 recovery was observed in both HCT116 and CCRF-CEM leukaemia cells.

The reduced recovery of Chk1 suggested that it was targeted for degradation. In response to persistent replicative stress, Chk1 is degraded *via* the ubiquitin-proteasome pathway (Zhang *et al.*, 2005). We investigated whether proteasomal activity contributed to the reduced recovery of Chk1 after 6-TG/UVA. 6-TG treated MRC5VA cells were maintained in the presence or absence of the proteasome inhibitor MG132 for 4 h after UVA irradiation and Chk1 levels were monitored by western blotting. Figure 4C shows that MG132 significantly enhanced recovery of Chk1. We conclude that 6-TG/UVA causes persistent DNA damage

and that failure to process this damage appropriately induces a proteasome-dependent degradation of Chk1.

Since Chk1 activation triggers cell cycle checkpoints, we investigated the effect of 6-TG/UVA on checkpoint induction and maintenance. 6-TG-treated MRC5VA cells were irradiated with UVA and 8 h and 24 h later, the cell cycle distribution was analysed by FACS. In parallel, cell extracts were prepared 24h post-irradiation for analysis of Chk1 by western blotting. Figure 5A shows that at lower 6-TG concentrations (0.8 μ M), UVA provoked a robust checkpoint response and >50% of the cells accumulated in G₂/M 24h after irradiation. In contrast, Chk1 was barely detectable in irradiated cells that had been treated with 1.6 μ M 6-TG. In this case, we noted an almost complete abrogation of the cell cycle arrest and only around 25% of cells were in G₂/M 24 h after UVA irradiation.

For the above experiments, cells were grown for 48 h in the presence of 6-TG and UVA irradiation performed immediately after transfer to PBS. Under these conditions, 6-TG is present in both newly-synthesized daughter and template DNA strands. In order to minimize the effects of daughter-strand 6-TG, cells grown for 48h in 6-TG were cultured for a further 2 h in 6-TG-free medium before UVA irradiation. Figure 5B shows that the 2 h incubation in drug-free medium prior to UVA irradiation significantly reduced UVA-induced Chk1 degradation. Concomitant with this increased Chk1 recovery, UVA irradiation elicited a robust G₂/M checkpoint response even in cells treated with the highest 6-TG concentration. These observations indicate that persistent DNA damage that causes Chk1 degradation is significantly reduced when 6-TG is not present in daughter DNA strands at the replication fork. As the extent of template DNA 6-TG substitution is unaffected by incubation in 6-TG-free medium, we conclude that Chk1 degradation predominantly reflects UVA-induced events in daughter DNA strands at active replication forks.

Because comet analysis after a 6-TG pulse demonstrated that DNA in the region of incorporated 6-TG is particularly vulnerable to breakage, we examined the effects on Chk1 stability of 6-TG incorporated during a short pulse. MRC5VA cells were treated with 6-TG for 2 h to label exclusively daughter DNA and irradiated with UVA either immediately or after an additional incubation (chase) in 6-TG-free medium. Figure 6A shows that delaying irradiation and allowing the replication fork time to move away from the incorporated 6-TG, affected Chk1 stability. A chase of 30 min in drug-free medium resulted in increased recovery of Chk1. If irradiation was delayed by 2 h, Chk1 levels were similar to those of unirradiated control cells. Deferring the UVA irradiation also had a dramatic effect on cell viability. Cells irradiated after a 2 h chase were significantly more resistant to killing by UVA. The 2 h chase was sufficient to restore clonal survival to levels similar to those of UVA irradiated cells that had not been treated with 6-TG (Figure 6B). Taken together, these observations are consistent with DNA 6-TG close to active replication forks being particularly hazardous. This can be seen as the introduction of persistent DNA damage, an abrogated cell cycle checkpoint response, and reduced cell survival.

DISCUSSION

Therapy-related cancer is a serious clinical problem

Immunosuppression carries a significant cancer risk and organ transplantant patients suffer a high incidence of cancer. The squamous cell skin carcinomas that are particularly frequent in this patient group are less prevalent among HIV immunosuppressed patients (Grulich *et al.*, 2007). This suggests a causative role for long-term pharmacological immunosuppression. Azathioprine, a mutagen in mice (Smith *et al.*, 1999), is designated a human carcinogen by the IARC (IARC, 1987). Its potential carcinogenicity is particularly important for organ transplant patients who may require decades-long immunosuppression.

The contribution of DNA 6-TG to the effects of thiopurines has long been recognized (Lennard *et al.*, 1985). The unusual ability of DNA 6-TG to absorb energy from UVA and to act as a source of ROS, led us to investigate whether photochemical DNA damage might contribute to the development of skin cancer in patients taking azathioprine. The finding that the skin of azathioprine patients contains DNA 6-TG and is selectively sensitive to doses of UVA that are well within the range of normal sunlight exposure is consistent with a photochemical etiology (O'Donovan *et al.*, 2005). We have previously shown that UVA and DNA 6-TG interact to produce DNA lesions that block DNA and RNA polymerases *in vitro* and inhibit replication and transcription in cultured cells (O'Donovan *et al.*, 2005, Brem *et al.*, 2008). Importantly, some DNA 6-TG photoproducts are highly persistent and are not repaired by DNA excision repair (Brem *et al.*, 2008). In the present study, we have extended these findings to show that low doses of UVA cause both single- and double-strand breaks in DNA containing 6-TG. We also provide the first indication of the formation of UVA-mediated DNA-protein crosslinks. Covalent binding of free 6-TG to proteins and crosslinks between oligonucleotides containing 6-TG and proteins have been demonstrated *in vitro* (Cahill *et al.*, 1996). Covalent protein-DNA lesions are likely to be particularly difficult for the replication and transcription machineries to deal with. The aim of this study was to determine whether combined DNA 6-TG/UVA has the potential to cause DNA breakage and to affect the cell cycle. Although it was not our main objective to examine the responses of specific cell types, our observations of 6-TG/UVA related DNA breakage and toxicity were generally replicated in keratinocytes which would be a potentially important target for UVA in patients receiving thiopurines.

The effects of UVA and DNA 6-TG are largely mediated by ROS, particularly by $^1\text{O}_2$ that is generated within DNA (Zhang *et al.*, 2006). $^1\text{O}_2$ damages cellular macromolecules and causes single- (Kumar *et al.*, 2000) and double-strand (Toyooka *et al.*, 2006) breaks as well as DNA base damage. HR-defective cells, but not cells with defects in the alternative non-homologous end joining pathway of DSB repair, were particularly sensitive to UVA activation of DNA 6-TG. As DSBs which arise in S phase are substrates for HR, this finding is consistent with the demonstration that 6-TG/UVA treatment induces DSBs predominantly in S phase. DNA DSBs occur frequently in S phase when active replication forks encounter a template strand interruption or a lesion that blocks replication. 6-TG/UVA-induced DNA breaks were at least partly independent of active replication. Breakage did require the presence of 6-TG in either template or daughter strand in the vicinity of replication forks, however. These observations suggest that replicating DNA is intrinsically more sensitive to damage by $^1\text{O}_2$ generated locally through the interaction between DNA 6-TG and UVA. This increased vulnerability most likely reflects the particular chromatin architecture of replicating DNA. Chromatin compaction and the presence of histones protect against damage by OH radicals generated by ionizing radiation (Ljungman *et al.*, 1991, Ljungman and Hanawalt, 1992), and it seems reasonable to expect that these factors confer a similar protection against $^1\text{O}_2$ mediated DNA damage. The vulnerability of replicating DNA 6-TG to $^1\text{O}_2$ may therefore reflect its more open structure and a reduced level of protective proteins that can quench $^1\text{O}_2$. In this regard, histidine, cysteine, and the aromatic amino acids are known to be particularly reactive towards $^1\text{O}_2$ (Davis, 2004). One corollary of the heightened sensitivity of replicating DNA is that dividing skin stem cells – the targets for malignant transformation – are likely to be most vulnerable to DNA breakage by azathioprine and UVA.

Photochemical DNA 6-TG damage also provoked canonical DNA damage responses and checkpoint activation. ATR and ATM are the proximal proteins involved in triggering cell cycle checkpoints *via* their respective signal transducers, the Chk1 and Chk2 proteins. ATM responds directly to the presence of DSB and, consistent with our observation of direct DSB induction, AT cells were hypersensitive to killing by 6-TG/UVA. Although low levels of

damage provoked both ATR and ATM-mediated responses with robust activation of both Chk1 and Chk2, higher levels caused Chk1 depletion. Chk1 is indispensable for checkpoint induction. Failure of DNA damage-related checkpoint responses and inappropriate processing of DNA lesions are hallmarks of cancer. Chk1 is activated by replication stress through ATR and also required to elicit the ATM-dependent G₂/M checkpoint in response to IR. Its inhibition potentiates the cytotoxicity of DNA strand-breaks (Koniaras *et al.*, 2001). 6-TG/UVA-induced degradation of Chk1 was mediated by the proteasome. This mode of Chk1 destruction reflects persistent DNA damage (Zhang *et al.*, 2005) and is consistent with irreparable replication- and transcription-blocking photochemical DNA lesions. Chk1 degradation was accompanied by abrogation of the G₂/M cell cycle checkpoint. Complete Chk1 deficiency is embryonic lethal but there are indications that a reduced level of Chk1 may have important consequences. Thus, conditional *Chk1* heterozygosity is associated with an accumulation of spontaneous DNA damage and inappropriate entry into mitosis (Lam *et al.*, 2004). Both of these factors are likely to contribute to genetic instability and malignancy.

Persistent oxidative DNA lesions are associated with cancer and their removal plays a key role in preventing the malignant transformation of keratinocytes (Al-Tassan *et al.*, 2002, Bickers and Athar, 2006). Organ transplant patients require lifelong immunosuppression. If that immunosuppression involves azathioprine, their inescapable exposure to UVA radiation puts this patient group at risk of chronic damage to skin DNA. Our findings indicate that some photochemically-induced DNA lesions are likely to be persistent. An increased burden of DNA lesions is analogous to ineffective DNA repair: both result in a high steady-state level of DNA base damage and breakage. Defective DNA repair is inextricably linked to genome instability. Human chromosome breakage syndromes caused by malfunction of factors involved in genome maintenance and DSB repair, such as DNA Ligase IV, ATM, NBS or BRCA1 are characterized by chromosomal aberrations and a high cancer risk (Kerzendorfer and O'Driscoll, 2009). The chronic DNA breakage and induction of persistent DNA lesions that may compromise a DNA damage checkpoint provide a plausible mechanism by which patients on thiopurines might be susceptible to sunlight-related skin cancer. In view of this possibility, it seems prudent to consider alerting azathioprine patients to their, possibly unique, susceptibility to the potential carcinogenicity of UVA.

Materials and Methods

Cell culture

Cells were grown in DMEM containing 10% fetal calf serum. HCT116 colon cancer cells, SV40-transformed AT5BIVA (*ataxia telangectasia* mutated), SV40-transformed MRC5VA normal human fibroblasts, and HaCaT keratinocytes were from Cancer Research UK Central Cell Services. CHO irs1 (*xrcc2* mutant) and irs1/XRCC2 (*xrcc2* complemented irs1) cells (Cartwright *et al.*, 1998) were provided by Dr. John Thacker and CHO XRS6 (*Ku80* mutant) and its *Ku80*⁺wildtype parent K1 by Dr. Mark O'Driscoll. HCT116 cells were synchronized by double thymidine (2.5 mM) block.

Irradiation

UVA - filtered to remove wavelengths below 320 nm - was delivered from a UVH 253 lamp (UV light Technology, Oldbury, UK) at a dose rate of 0.1 kJ/m²/s. IR (2.2 Gy/min) was from a ¹³⁷Cs γ -radiation source with an IBL 437C irradiator.

Cell survival

6-TG-treated cells were irradiated in PBS and seeded in 96-well plates (1000 cells/well). Viability was determined 5 days later by MTT assay. For clonal survival, cells were replated

in 6-well plates (250 fibroblasts/well, 500 CHO cells/well). Colonies were counted 10 d later. Data are based on triplicate samples from 2 experiments.

Immunoblotting

Cell extracts (25 μ g protein) were separated on 4-20% Tris-Glycine (Invitrogen, Carlsbad, CA) or 10% Bis-Tris (Criterion XT, Hercules, CA) gels. Following transfer, membranes were probed with antibodies against Chk1 (1:1000 dilution, Sigma, St. Louis, MO, or 1:750 dilution, Cell Signaling, Beverly, MA), Chk2, Chk1-Ser317, Chk2-Thr68 (all 1:1000 dilution, Cell Signaling) and actin (1:5000 dilution, abcam, Cambridge, UK). Antigen-antibody complexes were detected by ECL western blotting detection reagent (GE Healthcare, Little Chalfont, UK).

Immunofluorescence

MRC5VA cells grown on culture slides (BD Falcon, San Jose, CA) in 1 μ M 6-TG were irradiated with 10 kJ/m² UVA. Control cells were irradiated with 10 Gy IR. Irradiated cells were returned to normal medium for 4 h and then fixed (3.7% formaldehyde, 100mM PIPES, pH6.8, 5mM MgSO₄, 10mM EGTA, 2mM DTT; 20 min at RT), followed by 100% ice-cold methanol for 1 min. Cells were washed in PBS, permeabilized (0.3% Triton X-100 in PBS, 10 min, 0°C) followed by a PBS wash. After blocking (10% FCS, 0.05% Tween20 in PBS, 1 h, RT) cells were stained with γ H2AX antibody (Upstate, Temecula, CA) for 2 h at RT and with Alexa488-coupled anti-mouse secondary antibody (Alexa Fluor 488, Invitrogen) for 1 h at RT. Cells were then washed and mounted (Immu-Mount with DAPI (1.5 μ g/mL), Thermo Scientific, Pittsburgh, PA). Pictures were taken using a Zeiss Axioplan microscope and a Hamamatsu Digital camera (C4742-95).

Quantitation of DNA 6-TG

DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI). DNA (40 μ g) was digested with nuclease P1 (Sigma, 10 units, 1 h at 50°C) and alkaline phosphatase (2 units, 1 h at 37°C). 2'-deoxyribonucleosides were separated by HPLC (O'Donovan *et al.*, 2005). 2'-deoxyguanosine and 6-TG 2'-deoxyribonucleoside were detected and quantified by A_{260nm} and A₃₄₂ respectively.

Flow cytometry

Cells were processed using BrdU/Propidium Iodide as described (Ormerod, 2000). BrdU was detected with a FITC-coupled mouse antibody (BD Pharmingen, San Diego, CA). Analysis was carried out using a Fascalibur (Becton Dickinson, Franklin Lakes, NJ), cell-cycle distribution was assessed using the Flowjo (Tree Star, Ashland, OR) software. Each experiment was done at least twice.

Comet assay

Comet assays were performed using the Single Cell Gel Electrophoresis Assay kit (Trevigen, Gaithersburg, MD) according to the manufacturer's protocol. Alkaline comets detect predominantly SSBs and alkali-labile sites. For this purpose, 5000 treated cells were mixed with 60 μ l 0.5% low melting agarose, placed on slides and lysed for at least 1 h in pre-chilled 2.5 M NaCl, 100mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH10 at 4°C in the dark. DNA was allowed to unwind in alkaline solution (300 mM NaOH, 1 mM EDTA, pH>13) for 1 h before electrophoresis at 0.86 V/cm for 20 min at 4°C. Probes were then neutralized in 0.4 M Tris, pH 7.5, for 15 min, fixed in 100% ethanol for 10 min and air-dried.

DNA double-strand breaks were detected under neutral conditions. Cells were irradiated on ice, lysed immediately, washed with PBS and incubated for 2 h at 37°C in TE ± proteinase K (Roche, Basel, Switzerland: 1 mg/mL). Slides were equilibrated in electrophoresis buffer (100 mM Tris, 300mM NaAc, pH 8.3) for 1 h and electrophoresis carried out at 0.5 V/cm for 1 h at 4°C. Neutralization and fixation was as described above. Images of 100 ethidium bromide-stained (10 µg/mL) cells were analyzed from duplicate slides by Comet IV software (Trevigen) for both assays. To minimize the generation of replication-induced breaks or SSBs which arise as repair intermediates, cells were irradiated on ice and lysed immediately afterwards. All steps were performed in the dark.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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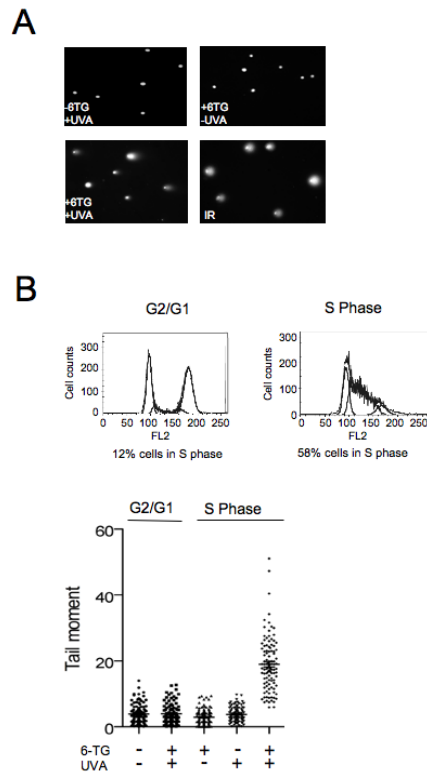
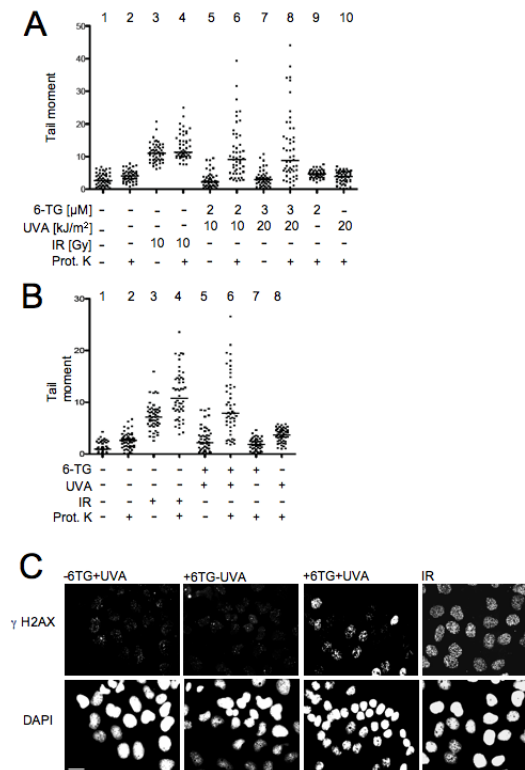


Figure 1.

S phase dependent DNA breakage by 6-TG/UVA

A) MRC5VA cells were grown in medium containing 1 μM 6-TG for 24 h and irradiated with 10 kJ/m^2 UVA. DNA damage was assayed immediately after irradiation by alkaline single-cell gel electrophoresis. Control cells grown in the absence of 6-TG were irradiated with 10 Gy IR. Representative images of ethidium bromide-stained slides are shown for each condition.

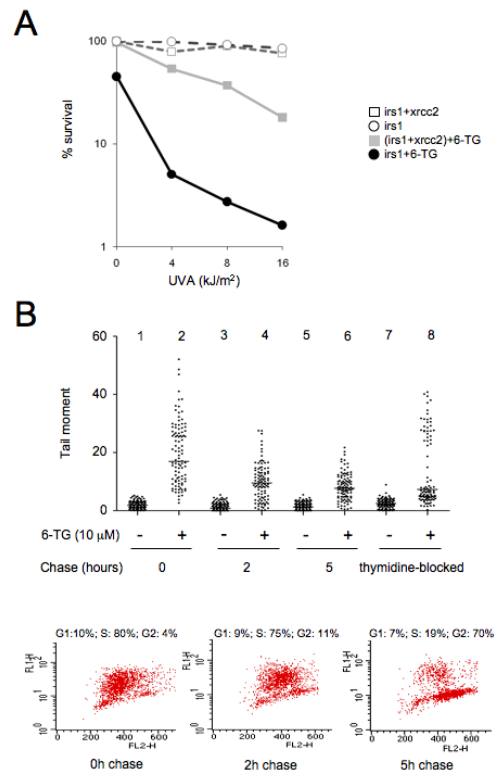
B) Synchronized HCT116 cells were allowed to incorporate 6-TG (1 μM) during the first S phase for 4 h immediately after release from the double thymidine block. Cells were irradiated with 10 kJ/m^2 UVA either 5 h (upper left panel) or 12 h (upper right panel) after 6-TG treatment. FACS analysis was performed on cells at the time of irradiation. DNA damage was assessed immediately after irradiation by alkaline comet analysis (lower panel). Damage is expressed as comet tail moment and each data point represents the tail moment of one cell.

**Figure 2.****DNA double-strand break analysis**

A) Neutral comet – uniform 6-TG label MRC5VA cells were grown for 48 h in medium containing 6-TG at the indicated concentrations, washed and irradiated with indicated doses of UVA. DNA breakage was analysed by neutral comet assay immediately after irradiation and following digestion with Proteinase K as indicated. Control cells received 10 Gy ionizing radiation. Damage is expressed as comet tail moment and each data point represents the tail moment of one cell.

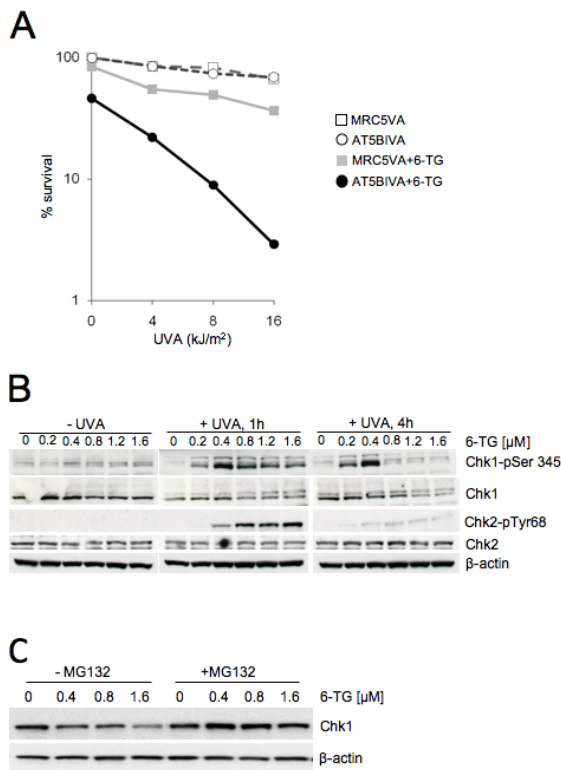
B) Neutral comet – S phase cells MRC5VA cells were treated with 6-TG (20 μM) for 2 h to label S phase cells. They were irradiated with 20 kJ/m^2 UVA and analysed by neutral comet assay after Proteinase K digestion as indicated. Control cells received 10 Gy ionizing radiation.

C) γ H2AX focus formation MRC5VA cells grown in the presence of 1 μM 6-TG for 48 hours were irradiated with 10 kJ/m^2 UVA. Control cells grown in the absence of 6-TG were irradiated with 10 Gy IR. γ H2AX foci were visualized 1 h after irradiation using anti- γ H2AX primary and Alexa488-conjugated secondary antibody. DNA was stained with DAPI. Scale bar 20 μm .

**Figure 3.****Sensitivity of S phase cells to 6-TG/UVA**

A) Survival of HR-defective cells Xrcc2-deficient *irs1* cells and *irs1* cells with reconstituted wildtype *xrcc2* (*irs1+xrcc2*) were grown in the presence of 0.05 μM or 0.025 μM 6-TG, respectively, for 48 h to achieve similar 6-TG incorporation levels. Cells were then irradiated with the indicated doses of UVA and survival was assessed by clonogenic assay.

B) DNA damage from 6-TG in the vicinity of replication forks Synchronized HCT116 cells were released from a double-thymidine block into S phase and incubated in medium containing 10 μM 6-TG for 2h. They were then returned to 6-TG free medium and 0, 2 or 5 h later, the cell cycle distribution of part of the population was analysed by BrdU/PI FACS (lower panels). A fraction of the remaining cell population was irradiated with 15 kJ/m² UVA and DNA damage was measured immediately after irradiation by alkaline comet analysis (upper panel). Each data point represents the tail moment of one cell.

**Figure 4.****DNA damage responses and 6-TG/UVA**

A) Sensitivity of AT cells MRC5VA and AT5BIVA cells were grown for 48 h in the presence of 0.2 or 0.1 μ M 6-TG, respectively, to achieve similar 6-TG substitution of DNA. Cells were then irradiated with the indicated doses of UVA and survival was assessed by clonogenic assay 10 days later.

B) Chk1 and Chk2 phosphorylation MRC5VA cells were grown for 48 h in medium containing 6-TG at the concentrations shown and irradiated with 10 kJ/m² UVA. Cell extracts prepared 1 h and 4 h after irradiation were analyzed by Western blotting.

C) Proteasome-dependent Chk1 degradation MRC5VA cells were grown for 48h in the presence of 6-TG as indicated. The MG132 proteasome inhibitor (5 μ g/mL) was added to the growth medium and 1 h later cells were irradiated with 10 kJ/m² UVA. Irradiated cells were grown for a further 4 h in the presence or absence of MG132 before protein extraction and immunoblotting.

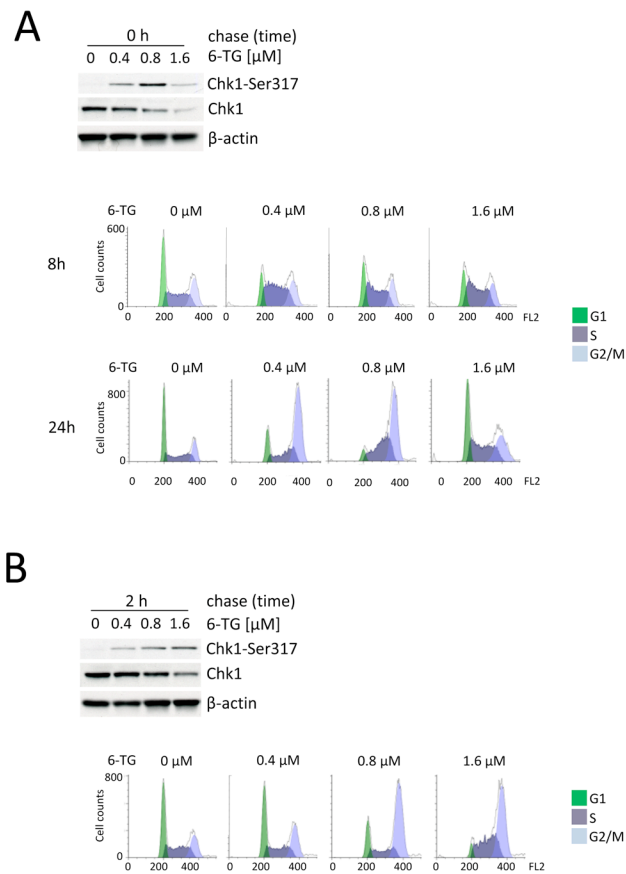
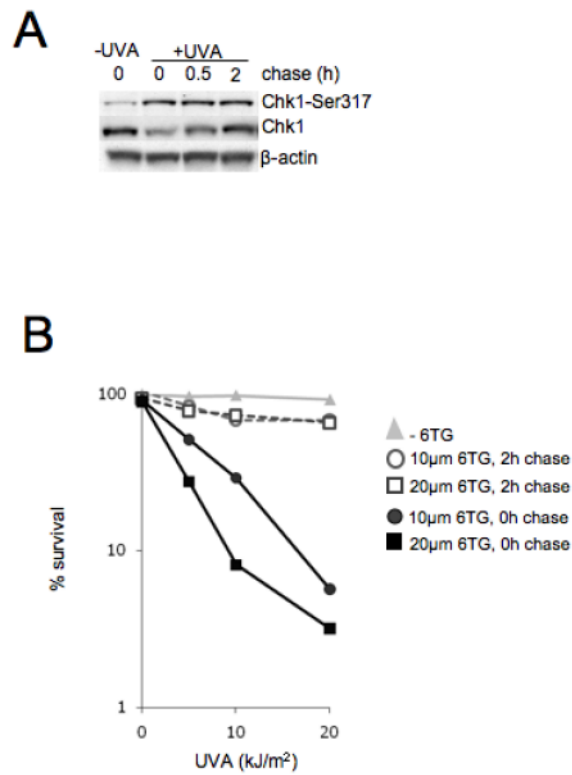


Figure 5.

Chk1 degradation and the 6-TG/UVA-induced G₂/M checkpoint

A) MRC5VA cells grown for 48 h in the presence of indicated concentrations of 6-TG were irradiated with 10 kJ/m² UVA. 8 h and 24 h later, the cell cycle distribution was determined by PI staining and flow cytometry. Total Chk1 levels and phosphorylation at Ser317 was analyzed by Western blotting of protein samples extracted 24 h after irradiation. Shown are the results of one representative experiment.

B) Exclusion of 6-TG from the replication fork. Cells were treated and processed as above except that they were returned to growth medium containing no 6-TG for 2 h before UVA irradiation. Cell cycle distribution and Chk1 were analyzed 24 h after irradiation

**Figure 6.****6-TG pulse chase**

A) MRC5VA cells were pulse labelled with 20 μM 6-TG for 2 h and returned to normal medium. Cells were then irradiated with 15 kJ/m^2 UVA after the indicated times of growth in normal medium. Cell extract were prepared 24 h later and analyzed by Western blotting.

B) MRC5VA cells were treated with 6-TG (10 or 20 μM) for 2 h and UVA irradiated either immediately or following a further 2h incubation in 6-TG-free medium. Cell survival was measured by clonal assay.