Oxidation-Mediated DNA Cross-Linking Contributes to the Toxicity of 6-Thioguanine in Human Cells

Reto Brem and Peter Karran

Abstract

The thiopurines azathioprine and 6-mercaptopurine have been extensively prescribed as immunosuppressant and anticancer agents for several decades. A third member of the thiopurine family, 6-thioguanine (6-TG), has been used less widely. Although known to be partly dependent on DNA mismatch repair (MMR), the cytotoxicity of 6-TG remains incompletely understood. Here, we describe a novel MMR-independent pathway of 6-TG toxicity. Cell killing depended on two properties of 6-TG: its incorporation into DNA and its ability to act as a source of reactive oxygen species (ROS). ROS targeted DNA 6-TG to generate potentially lethal replication-arresting DNA lesions including interstrand cross-links. These triggered processing by the Fanconi anemia and homologous recombination DNA repair pathways. Allopurinol protected against 6-TG toxicity by acting as a ROS scavenger and preventing DNA damage.

Introduction

The clinical effectiveness of the immunosuppressant, anti-inflammatory and anticancer thiopurines: azathioprine, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) relies on their ability to selectively kill dividing immune effector or cancer cells. Surprisingly, despite successful use for over 50 years (1), the mechanisms by which they cause the death of their target cells are still incompletely understood. Thiopurines are prodrugs. They are metabolized to the 6-TG nucleotides (TGN) that early studies identified as important determinants of toxicity. TGN are substrates for incorporation into DNA and the accumulation of DNA 6-TG is a major factor in thiopurine toxicity. Other contributors include the formation of toxic thiopurine metabolites and oxidation of DNA 6-TG by exogenous chemicals or ultraviolet A (UVA) radiation (for review see ref. 2).

The most widely prescribed thiopurines, 6-MP and its prodrug azathioprine, are converted to TGN in several steps (for review see ref. 3). Conversion is counteracted by the activities of 3 enzymes: thiopurine methyltransferase (TPMT), which inactivates thiopurines by methylation (4), xanthine oxidase (XO), which catabolizes 6-MP and azathioprine to inactive thiouric acid, and the MRP4 transporter protein that exports thiopurine mononucleotides from cells (5). TPMT expression is an important determinant of the clinical effectiveness of thiopurines. Polymorphic TPMT variants with significantly reduced activity are associated with high TGN levels and extreme, potentially lethal, thiopurine toxicity. High TPMT activity is associated with lower intracellular TGN concentrations and reduced clinical efficacy (6). Prevention of cell proliferation by limiting the supply of purine nucleotides that are essential for DNA and RNA synthesis is another potential contributor to thiopurine toxicity. In this case, TPMT methylates thioinosine monophosphate (TIMP), a metabolite of azathioprine and 6-MP, to generate methyl-TIMP, a powerful inhibitor of the first enzyme in the de novo pathway of purine nucleotide biosynthesis. Purine nucleotide depletion does not explain all thiopurine cytotoxicity, however, (7) and the potently cytotoxic 6-TG is metabolized to TGN by a different route that does not generate TIMP.

The DNA mismatch repair (MMR) system is also a major contributor to thiopurine toxicity. DNA 6-TG deceives MMR into a potentially lethal intervention and, as a consequence MMR-defects confer significant thiopurine resistance (for review see ref. 8). MMR-deficient cells do, however, retain a susceptibility to killing by high thiopurine concentrations, indicating the existence of MMR-independent thiopurine cytotoxicity (9).

DNA 6-TG is also implicated in a cytotoxic pathway, which involves oxidative DNA damage. Cells containing DNA 6-TG are hypersensitive to reactive oxygen species (ROS) generated chemically, biologically (10), or photochemically by UVA (11). ROS inflict widespread DNA damage. In addition to the well-characterized DNA 8-oxoGuanine (12), they cause oxidation of DNA 6-TG itself. Among these oxidation products, guanine-6 sulfonate (G=S03; ref. 11) and guanine-sulfinate (G=S02; ref. 13), DNA interstrand cross-links (ICL; ref. 14), and DNA-protein cross-links (DPC; ref. 15) are all potential contributors to 6-TG toxicity.
cross-links (15) have been identified as replication-blocking and potentially cytotoxic DNA lesions.

Here we describe 6-TG-mediated cytotoxicity that requires the incorporation of 6-TG into DNA but is independent of MMR, exogenous sources of ROS, and of UVA. It does require an oxidizing environment, however, and we show that 6-TG itself provides this by depleting endogenous antioxidant defenses and thereby increasing steady-state ROS levels. Cell killing reflects the formation of potentially lethal DNA lesions that inhibit replication. Its effects are particularly marked in cells with defects in the Fanconi anemia (FA) and homologous recombination (HR) pathways and cells with defects in either pathway are extremely sensitive to 6-TG (9, 14, 16). Together the FA and HR pathway protect cells against DNA damage that arrests replication (reviewed in ref. 17). FA-deficient cells are typically hypersensitive to killing, chromosome breakage and particularly to radial chromosome induction by agents that cause DNA ICLs. They exhibit a similar pattern of sensitivity to 6-TG (14), and we show that ICLs are among the DNA lesions that efficiently block DNA replication in cells treated with 6-TG.

Materials and Methods

Cell culture

The MMR-defective human leukemia cell line CCRF-CEM was grown in RPMI, all other cells in Dulbecco’s Modified Eagle’s Medium. Media were supplemented with 10% fetal calf serum. The Fanconi anemia (FANCA−/−) and wild-type (FANCA+/+) mouse embryonic fibroblasts (MEF) have been described (18). Their status was confirmed by isoenzyme analysis and DNA fingerprinting (January 2010). MSH2-defective (HeLa-MSH2) and their control transfectant HeLa SilenciX (HeLa-SX) cells (tebu-bio Cat Nr 01-00023) were cultured in the presence of hygromycin B. Nontransfected HeLa and MLH1-deficient colon cancer cells HCT116 were obtained from Cancer Research UK Central Cell Services. Their identity was confirmed by isoenzyme analysis and short tandem repeat profiling (March 2011) and skin fibroblasts derived from a Lesch-Nyhan syndrome patient (GM03467) (isozyme analysis November 2010) were obtained from The Coriell Institute (Camden, NJ).

ROS and glutathione detection

To measure ROS, trypsinized cells were washed once in PBS and incubated in 5 μmol/L CM-H2DCFDA (Invitrogen) in PBS for 20 minutes at 37°C. They were then washed twice in PBS and green fluorescence was analyzed by flow cytometry. Total and oxidized levels of glutathione (GSH) were measured using the Glutathione Assay ( Treviggen) according to the manufacturer's protocols.

Immunoblotting

Whole cell extracts were prepared using radioimmunoprecipitation assay buffer. Proteins (50 μg) were separated on 3% to 8% Tris-acetate polyacrylamide gels (Invitrogen). After transfer, membranes were probed with antibodies against FANCD2 (Novus Biologicals), MSH2 (BD Pharmingen), or XO (Abcam). Antigen–antibody complexes were visualized using ECL blotting detection agent (GE Healthcare).

RNA interference

The siRNA duplex smart pools were purchased from Dharmacon. Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) or Dharmafect (Dharmacon) according to the manufacturers’ instructions, with a final siRNA concentration of 50 nmol/L. Cells were subcultured into normal medium 24 hours after transfection.

Cell survival, comet assay, 3H-thymidine incorporation

To determine cell survival, treated cells were seeded into 96-well plates (1,000 cells per well) in normal medium. Viability was assayed 5 days later using the MTT assay. For clonal survival, 500 cells per well were seeded into 6-well plates and colonies were counted 7 to 10 days later. Each analysis was carried out in triplicate.

DNA interstrand cross-linking was determined by the comet assay. Cells were grown in the presence of 6-TG for 48 hours. They were then irradiated with 5 Gy IR and lysed. Following 2 hours digestion at 37°C with 1 mg/mL Proteinase K (Roche) they were analyzed by the alkaline comet assay as described (19).

DNA replication was assessed by measuring [3H]-thymidine incorporation. Cells were treated with 6-TG and then pulsed with [3H]thymidine (1 μCi/mL, 511 GBq/mmol) in normal medium for 30 minutes. Trichloroacetic acid insoluble radioactivity in duplicate samples of 2 × 10⁶ cells was determined by scintillation counting.

Results

MMR-independent 6-TG sensitivity

Cells derived from FA patients are surprisingly sensitive to thiopurines (16). An early study (20) reported that FANCG-defective Chinese hamster ovary cells are even more sensitive to 6-TG than to acknowledged ICL-inducing agents such as mitomycin C and diepoxybutane. This extreme 6-TG sensitivity was also apparent in FANCA−/− MEFs that were more than four-fold more sensitive to 6-TG than their FANCA+/+ counterparts (Fig. 1A). FA-defective human cells were also hypersensitive to 6-TG. siRNA-mediated depletion of FANCD2, a key component of the FA pathway, significantly increased 6-TG sensitivity of HeLa cells (Fig 1B). Importantly, FANCD2 silencing in MMR-deficient HCT116 cells also resulted in 6-TG hypersensitivity (Fig. 1C), indicating that the FA pathway provides protection against the cytotoxicity of 6-TG that is partly independent of MMR.

MMR-independent activation of the FA pathway by 6-TG

Monoubiquitination of the FANCD2 protein is a sensitive indicator of FA pathway activation by replication-arresting DNA damage. Western blotting revealed that 6-TG treatment induced this FANCD2 modification in both MMR-proficient and MMR-defective cells. FANCD2 monoubiquitination was apparent in MMR-defective HeLa-MSH2 and HCT116 cells following treatment with 6-TG concentrations of 0.8 μmol/L and above—approximately 2- to 3-fold higher than the concentrations required to trigger FANCD2 activation in MMR-proficient HeLa-SX cells (Fig 2). We conclude that 6-TG treatment activates the FA pathway and that activation is
analyzed by Western blotting (inserts) 4 days after transfection.

FANCD2 levels were increased by cotreatment with buthionine sulfoxide (BSO), a GSH synthesis inhibitor that prevents GSH replenishment. BSO and allopurinol had opposite effects. In HeLa-MSH2 cells, BSO significantly reduced 6-TG sensitivity. Whereas treatment with 0.8 μmol/L 6-TG for 48 hours reduced cell viability to <50%, inclusion of allopurinol reversed this toxicity and cell survival was comparable to that following treatment with allopurinol alone (Fig. 3C). Allopurinol also significantly increased the concentration of 6-TG required to trigger MMR-independent FANC2D ubiquitination. In the absence of allopurinol, monoubiquitinated FANC2D was detectable in extracts of HCT116 cells treated with 0.4 μmol/L 6-TG. When 6-TG treatment was carried out in the presence of allopurinol, FANC2D was not detectably ubiquitinated even after treatment with 1.2 μmol/L 6-TG (Fig. 3D). Allopurinol also protected MSH2-deficient CCRF-CEM cells against FANC2D activation (data not shown).

Allopurinol is a powerful XO inhibitor at micromoles per liter concentrations. In our experiments, however, protection against 6-TG toxicity and FANC2D modification required its inclusion at millimoles per liter levels. This suggested that its effect was not a consequence of XO inhibition. To examine this possibility, cells were treated with 6-TG in the presence or absence of an alternative XO inhibitor, febuxostat (22). Febuxostat concentrations up to 200 μmol/L had no detectable effect on ROS levels, FANC2D ubiquitination or HCT116 cell survival (data not shown), confirming that XO was not the source of ROS. In addition, Western blotting and direct assays of XO activity indicated that, with the exception of HeLa cells, XO was undetectable in cultured cells including HCT116, CCRF-CEM and several lymphoblastoid cell lines (Supplementary Fig. S1). These findings indicate that allopurinol protection against 6-TG is independent of its ability to inhibit XO. It therefore most likely reflects the ability of allopurinol to act as a ROS scavenger.

Because 6-TG metabolism by XO was excluded as a possible source of ROS, we examined whether 6-TG increased ROS levels by depleting antioxidant protection. GSH is one of the most important cellular antioxidants. Figure 4A shows that 6-TG induces a dose-dependent reduction of GSH levels in both HCT116 and HeLa-MSH2 cells. The depletion was exacerbated by cotreatment with buthionine sulfoxide (BSO), a GSH synthesis inhibitor that prevents GSH replenishment. BSO and allopurinol had opposite effects. In HeLa-MSH2 cells, BSO potentiated 6-TG mediated ROS formation (Fig. 4B). It was associated with FANC2D monoubiquitination at lower 6-TG concentrations (Fig. 4C) and an enhanced sensitivity to killing by 6-TG (Fig. 4D). Similar results were obtained in HCT116 cells (data not shown). We conclude that 6-TG treatment increases steady-state ROS levels by depleting the levels of protective GSH. The ensuing oxidative stress results in the formation of potentially lethal DNA lesions that activate the FA pathway.

6-TG cytotoxicity. A, MEFs, FANCA−/− and FANCA+/+. MEFs were grown in the presence of 6-TG at the concentrations as indicated for 16 hours. They were then returned to normal medium and survival was assessed by clonal assay 7 days later. B and C, human cells. FANC2D was depleted by siRNA transfection of HeLa (B) and HCT116 (C) cells. Three days after transfection, cells were seeded into 96-well plates (1,000 per well) in medium containing the indicated doses of 6-TG. Two days later, the medium was replaced with normal medium and growth continued for a further 3 days. Survival was determined by MTT assay. Data are means of at least 3 experiments. FANC2D levels were analyzed by Western blotting (inserts) 4 days after transfection.

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6-TG sensitivity and FA activation: the role of ROS

FACS analysis of cells stained with CM-H2DCFDA, a reporter for ROS, revealed that 6-TG treatment induced a dose-dependent increase in intracellular ROS (Fig. 3A). The purine catabolizing enzyme XO detoxifies mercaptopurine in a reaction that generates ROS, although its role in 6-TG catabolism is less clear. We investigated whether XO was the source of the ROS produced by 6-TG. When HCT116 cells were treated with 6-TG in the presence of allopurinol, an acknowledged XO inhibitor (21), ROS levels declined to those of untreated cells (Fig. 3B). The lower ROS levels in allopurinol-treated HCT116 cells were associated with a significantly reduced 6-TG sensitivity. Whereas treatment with 0.8 μmol/L 6-TG for 48 hours reduced cell viability to <50%, inclusion of allopurinol reversed this toxicity and cell survival was comparable to that following treatment with allopurinol alone (Fig. 3C). Allopurinol also significantly increased the concentration of 6-TG required to trigger MMR-independent FANC2D ubiquitination. In the absence of allopurinol, monoubiquitinated FANC2D was detectable in extracts of HCT116 cells treated with 0.4 μmol/L 6-TG. When 6-TG treatment was carried out in the presence of allopurinol, FANC2D was not detectably ubiquitinated even after treatment with 1.2 μmol/L 6-TG (Fig. 3D). Allopurinol also protected MSH2-deficient CCRF-CEM cells against FANC2D activation (data not shown).

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6-TG sensitivity and FA activation: the role of DNA 6-TG

The observations described above firmly implicate increased ROS levels in MMR-independent 6-TG cytotoxicity. To address whether the ROS generated from 6-TG are sufficient for toxicity or whether incorporation of 6-TG into DNA was also a requirement, we examined the effects of 6-TG on Lesch-Nyhan GM03467 fibroblasts. These HPRT-negative cells are extremely resistant to 6-TG and do not incorporate 6-TG into DNA. CM-H2DCFDF staining and FACS analysis indicated that 6-TG treatment of GM03467

![Graph showing ROS generation by 6-TG and the protective effects of allopurinol in HCT116 cells.](image)

**Figure 3.** ROS generation by 6-TG and the protective effects of allopurinol in HCT116 cells. A, 6-TG dose dependence of ROS production. Cells were harvested after treatment for 48 hours with 0, 0.4 μmol/L, 0.8 μmol/L, or 1.2 μmol/L 6-TG as indicated. ROS levels were determined by FACS. The fluorescence intensity is expressed relative to that of untreated cells. B, protection by allopurinol against ROS. Cells were treated with 1.2 μmol/L 6-TG for 48 h in the presence or absence of 500 μmol/L allopurinol. ROS production was analyzed as described in A. C, allopurinol protection against 6-TG toxicity. Cells were grown in the presence of 0.8 μmol/L 6-TG ± 500 μmol/L allopurinol for 48 hours and seeded into 96-well plates. Survival was determined 6 days later by MTT assay. The data are means of 3 independent experiments. D, prevention of FANCD2 activation by allopurinol. Cells were treated for 48 hours with the concentrations of 6-TG as indicated in the presence or absence of 500 μmol/L allopurinol. Western blots of extracts were probed for FANCD2. Unmodified FANCD2 and the activated form (FANCD2-ubiq) are arrowed. β-Actin served as a loading control.
cells also induced ROS (Fig. 5A). The survival of GM03467 cells was completely unaffected by 6-TG concentrations that induced ROS at levels that caused significant lethality in HCT116 cells indicating that 6-TG-induced oxidative stress is insufficient in itself to cause cell death. Importantly, 6-TG treatment did not induce detectable activation of FANCD2 in GM03467 cells—even at extremely high concentrations. The FA pathway was functional in these cells, however, and FANCD2 ubiquitination was detectable following mitomycin C treatment (Fig. 5B). Confirmation that DNA damage derived from incorporated 6-TG is required to trigger the FA pathway and is responsible for 6-TG induced cytotoxicity, was provided by GM03467 cells in which FANCD2 was down-regulated by RNA interference. Abrogation of the FA pathway did not detectably alter their 6-TG sensitivity. It did, however, significantly sensitize them to mitomycin C (Fig. 5C). Taken together, these findings indicate that both DNA 6-TG and ROS are required to generate potentially lethal DNA lesions that activate the FA pathway.

The lethal DNA 6-TG lesions

The FA pathway is activated by replication-arresting DNA lesions. 6-TG treatment induced a dose-dependent inhibition of DNA replication as assessed by [³H]-thymidine incorporation into nascent DNA (Fig. 6A). Inhibition occurred in a MMR-independent manner and was observed in HCT116 and HeLa-MSH2 cells. Replication arrest was significantly alleviated by treatment with allopurinol indicating that it was dependent on ROS and not simply a consequence of 6-TG incorporation into DNA.

Defects in the FA pathway are particularly associated with sensitivity to DNA interstrand crosslinking agents. In a previous publication (14) we reported that the ROS produced when DNA 6-TG is activated by UVA induce ICLs and the chromosome aberrations that are typically associated with these DNA lesions. To investigate whether 6-TG induced ICLs independently of UVA activation, cells were treated with 6-TG and the introduction of ICLs was analyzed by the Comet assay. By comparing ICL formation in cells treated with 6-TG in the presence or absence of allopurinol, the contribution of ROS was also assessed. Cell lysates were extensively digested with proteinase K before electrophoresis to remove any DNA–protein cross-links. Figure 6B shows that 6-TG treatment reduced the comet tail moment produced by IR consistent with the formation of ICLs. ICL induction was 6-TG dose dependent and was largely abolished when allopurinol was present during incubation with 6-TG. We conclude from these data that 6-TG incorporated into DNA is a target for damage by ROS induced by the 6-TG treatment itself and that oxidation of DNA 6-TG results in the formation of ICLs.
Discussion

Our findings define a novel mechanism of 6-TG toxicity that does not rely on MMR. Both pathways require incorporation of 6-TG into DNA and its post-incorporation modification. One important difference is that MMR intervention is triggered by S-methylation of DNA 6-TG rather than the oxidation that is implicated in MMR-independent cell killing. In both cases, toxicity is counteracted by HR. The 2 pathways contribute to 6-TG toxicity in MMR-proficient cells. They are illustrated schematically in Fig. 7.

The FA pathway comprises 14 known FANC proteins that coordinate the sensing and repair of DNA lesions, including ICLs, that arrest replication (for review see ref. 17). Current models of lesion processing invoke damage recognition and recruitment of a ubiquitinated FANCD2:FANCI heterodimer to sites of DNA damage by the FA “core” complex. Subsequent nuclease-mediated incisions generate DNA double-strand breaks that are then directed towards HR for resolution. The FA pathway provides significant protection against the lethal effects of agents that cause replication stalling and FA-defective cells are very sensitive indicators of the presence of a variety of DNA lesions that block advancing replication forks. Some time ago, FANCG-defective hamster cells were reported to be unusually sensitive to 6-TG (20). We confirmed this hypersensitivity in FANCA-deficient MEFs and showed that abrogation of the FA pathway also confers 6-TG sensitivity in human cells. Importantly, this sensitivity is independent of MMR indicating that it represents a novel mechanism of 6-TG toxicity. Taken together with a previously noted hypersensitivity of HR-defective xrc2 cells (14) and the recently reported 6-TG sensitivity of cells deficient in the BRCA2 (also known as FANCID1) or BRCA1 proteins (9), these findings firmly implicate the FA and HR pathways in preventing some of the potentially lethal effects of 6-TG.

The observations reported here define 2 properties of 6-TG that are necessary and sufficient for MMR-independent cytotoxicity. The first is its ability to increase intracellular ROS by depleting antioxidant levels. This is probably a general property of thiopurines. Consistent with our observations, 6-TG toxicity is counteracted by HR.

Figure 5. The effects of 6-TG in Lesch-Nyhan cells. A, ROS levels: GM03467 cells were grown in the presence of 4 μmol/L 6-TG for 72 hours. ROS were analyzed by FACS. B, FANCD2 monoubiquitination. GM03467 cells were treated for 72 hours with the indicated doses of 6-TG or with 30 ng/mL mitomycin C. FANCD2 activation was analyzed by Western blotting. C, the effects of FANCD2 depletion in Lesch-Nyhan cells. Forty-eight hours after transfection of GM03467 with FANCD2 or control RNAi, cells were seeded into 96-well plates in the presence or absence of 4 μmol/L 6-TG or 15 ng/mL MMC. Three days later the medium was replaced by drug-free medium and survival was determined by MTT assay after a further 6 days. FANCD2 silencing was confirmed by Western blotting (insert).

Figure 6. DNA synthesis and interstrand cross-linking. A, HeLa-MSH2 cells treated with the indicated concentrations of 6-TG for 48 hours in the presence or absence of 500 μmol/L allopurinol were pulse labeled for 20 minutes with [3H]-thymidine and incorporation of radioactivity into TCA-insoluble material was determined. Values are expressed as percentage of the incorporation of untreated cells. B, FANCA−/− MEFs were grown in the presence of 6-TG at the concentrations shown in the presence or absence of allopurinol (500 μmol/L) for 48 hours. Cells were irradiated with 10 Gy ionizing radiation, DNA damage was analyzed by alkaline comet assay after digestion with proteinase K and is expressed as comet tail moment. The data shown are representative of 2 experiments.
Figure 7. Mismatch repair-independent and repair-dependent pathways of 6-TG cytotoxicity. Purine salvage by HPRT (1) leads to the incorporation of 6-TG into DNA. DNA 6-TG is a substrate for damage (2) by methylation or by ROS generated from 6-TG-mediated depletion of cellular antioxidants. DNA 6-TG oxidation causes DNA replication-blocking lesions, including ICLs (3). These are lethal unless correctly processed by the FA and HR DNA repair pathways (4). Base pairs containing methylated DNA 6-TG are recognized by MMR, which processes them into potentially lethal DNA lesions (5). Survival may be increased by HR processing of DNA breaks resulting from the intervention of MMR.

Both 6-MP and azathioprine have been shown to deplete reduced GSH in cultured human cells (23). ROS scavenging abrogated both 6-TG-mediated replication inhibition and FA pathway activation. The second essential property for toxicity is the provision, in the form of DNA 6-TG, of a DNA target for damage by ROS. Our findings indicate that the FA and HR pathways process the potentially lethal oxidized DNA 6-TG lesions that are generated by these reactions.

HR is also implicated in reversing MMR-dependent 6-TG toxicity (9). In that case, by analogy to MMR involvement in methylating agent toxicity (24), HR processing lies downstream of MMR-induced DNA breakage and replication fork disruption most likely after incision at me6-TG:T mismatches (8, 25). TGN are good substrates for incorporation into DNA (26) and unmodified DNA 6-TG is not a replication block in vitro (27). The strict requirement for both 6-TG and ROS indicates that replication disruption in the MMR-independent pathway is caused by oxidized DNA 6-TG lesions.

The complete absence of detectable toxicity in L-N cells is eloquent testimony to the requirement for DNA substitution by 6-TG in both MMR-dependent and MMR-independent killing. The oxidation potential of 6-TG is lower than that of canonical DNA bases (28) and the well-documented oxidation of DNA 6-TG to lesions that inhibit replication is consistent with involvement of the FA and HR pathways. The extreme vulnerability of FA cells to crosslinking drugs reflects their inability to process ICLs correctly (17). UVA activation of DNA 6-TG produces ROS that contribute to the formation of ICLs (14). The finding that ROS-dependent DNA cross-linking occurs even in unirradiated cells treated with 6-TG is surprising but is, however, consistent with our previous observations of 6-TG-induced chromosome aberrations. FA cells are hypersensitive to the induction of chromosomal aberrations, breaks and radials, by UVA activated DNA 6-TG (14). Importantly, that study also revealed that 6-TG induced the same spectrum of aberrations in unirradiated cells. This pattern of chromosomal damage—generally associated with ICLs—is consistent with ICL formation by UVA-dependent and UVA-independent mechanisms. In our experiments, 6-TG-induced ICLs were detected by the comet assay and inferred from FANCID2 activation. These are both extremely sensitive indicators of DNA damage and probably reflect relatively rare DNA lesions. It is also important to note that the FA pathway is activated as a general response to stalled replication and its function seems to be to direct processing of replication-related DNA breaks away from the alternative nonhomologous end joining pathway (29). It is likely that other DNA 6-TG oxidation products such as GSO2, GSO3, or DNA-protein crosslinks also engage the FA pathway in 6-TG-treated cells.

Our experimental findings have implications for the clinical use of thiopurines. Azathioprine and 6-MP are widely used to treat leukemia and, increasingly, inflammatory bowel disease. 6-TG has been less frequently prescribed. Its limited use seems to stem from its liver toxicity (30) although this seems somewhat controversial (discussed in ref. 31). 6-TG is now considered a viable alternative for inflammatory bowel disease patients who fail to respond to azathioprine or 6-MP (32), however. Indeed, in view of its more direct metabolism to TGN, appropriate doses of 6-TG may offer a more predictable and generally better treatment option than 6-MP or azathioprine. The effectiveness of thiopurines is thought to reflect the ratio between 2 principal metabolites, the TGNs and methylmercaptopurine (MMP) ribonucleotide. TGNs, the precursors of DNA 6-TG, are regarded as the pharmacologically active metabolites whereas the therapeutically inactive TPMT-derived MMP contributes to the dose-limiting hepatotoxicity (30). Direct conversion of 6-TG to TGN avoids the generation of 6-MMP and would therefore seem to be therapeutically advantageous. Indeed, 6-TG is particularly efficacious in a subgroup of patients for whom 6-MP or azathioprine treatment is associated with subtherapeutic TGN, high MMP levels and severe hepatotoxicity.

The effectiveness of azathioprine or 6-MP is improved by coadministration of the XO inhibitor allopurinol (33). By preventing the catabolism of a significant fraction of these drugs to inactive thiouric acid, XO inhibition increases the availability of thiopurines for conversion to TGN. When combined with an appropriately reduced thiopurine dose, allopurinol permits the attainment of therapeutic TGN levels. This is accompanied by a surprising and dramatic reduction in the levels of methylated intermediates (33), which may be a reason for the less severe adverse side effects of combined treatment. Coadministration of allopurinol also alleviates some of the side
effects of 6-TG treatment. Unlike 6-MP, 6-TG is not a substrate for XO and it can be administered with allopurinol without dose reduction. In our experiments, allopurinol protected cells against 6-TG toxicity by acting as a ROS scavenger and preventing DNA 6-TG oxidation. It is possible that these scavenging properties contribute to ameliorating toxic side effects in patients and that some of the beneficial effects of allopurinol in combination with azathioprine and 6-MP may also stem from its antioxidant properties.

In summary, we show that the cytotoxicity of 6-TG partly reflects its accumulation in DNA where it serves as a target for the intracellular ROS that are present at increased levels because 6-TG also depletes antioxidant defences. The cytotoxic effects of oxidized DNA 6-TG are independent of MMR and reflect the formation of DNA lesions, including ICLs that require processing by the FA and HR DNA repair pathways. These properties of 6-TG suggest that it may be particularly efficient in the treatment of tumors with inactive BRCA1 or BRCA2 (9) that are defective in these pathways. Our findings also provide support, and a mechanistic rationale, for the possible clinical effectiveness of combined 6-TG and allopurinol in certain patient groups.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P. Karran, R. Brem
Development of methodology: R. Brem
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Brem
Analysis and interpretation of data (e.g., statistical analysis, biosciences, computational analysis): P. Karran, R. Brem
Writing, review, and/or revision of the manuscript: P. Karran, R. Brem
Study supervision: P. Karran

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