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. Together, our findings provide mechanistic evidence to support the proposed use of thiopurines to treat HR-defective tumors and for the coadministration of 6-TG and allopurinol as an immunomodulation strategy in inflammatory disorders.

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 catalyzes 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 (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 (GSO3; ref. 11) and guanine-sulfinate (GSO2; ref. 13), DNA interstrand cross-links (ICL; ref. 14), and DNA-protein...
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 determined by isoenzyme analysis and short tandem repeat profiling (March 2011) and skin fibroblasts derived from a Lesch-Nyhan syndrome patient (GM03467) (sozyme 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 (Trevigen) 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 [5-3H]thymidine (1 μCi/mL, 511 GBq/mmol) in normal medium for 30 minutes. Trichloroacetic acid insoluble radioactivity in duplicate samples of 2 × 10^6 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 FANCID2, a key component of the FA pathway, significantly increased 6-TG sensitivity of HeLa cells (Fig. 1B). Importantly, FANCID2 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 FANCID2 modification in both MMR-proficient and MMR-defective cells. FANCID2 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 FANCID2 activation in MMR-proficient HeLa-SX cells (Fig. 2). We conclude that 6-TG treatment activates the FA pathway and that activation is
catalyzing 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 FANCD2 ubiquitination. In the absence of allopurinol, monoubiquitinated FANCD2 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, FANCD2 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 FANCD2 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 FANCD2 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. FANCD2 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 FANCD2 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 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

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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-H2DCF staining and FACS analysis indicated that 6-TG treatment of GM03467

![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.](image-url)

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

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-H2DCF staining and FACS analysis indicated that 6-TG treatment of GM03467
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 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.

Figure 4. 6-TG and GSH. GSH depletion and potentiation of 6-TG toxicity by BSO. A, GSH depletion. HCT116 or HeLa-MSH2 cells were treated with 6-TG at the concentrations indicated for 48 hours. Extracts were prepared and GSH levels were measured. Values are expressed as percentage of those from untreated cells. Values are the mean of 2 independent determinations. B, the effects of BSO on 6-TG induced ROS. HeLa-MSH2 cells were treated for 48 hours with 0.8 μmol/L 6-TG in the presence or absence of 200 μmol/L BSO. ROS production was analyzed by FACS. C, potentiation of FANCD2 activation by BSO. Extracts of HeLa-MSH2 cells treated for 48 hours with the concentrations of 6-TG shown were analyzed by Western blotting. The positions of unmodified (FANCD2) and activated (FANCD2-ubiq) forms of FANCD2 are arrowed. β-Actin was included as a loading control. D, 6-TG cytotoxicity enhancement by BSO. HeLa-MSH2 cells were treated for 48 hours with the concentrations of 6-TG shown in the presence or absence of 200 μmol/L BSO. Survival was determined by MTT assay. Data are the means of 3 experiments.
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 FANCD1) 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,
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 methylmercapturine (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, biostatistics, 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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