OXIDATION-MEDIATED DNA CROSSLINKING CONTRIBUTES TO THE TOXICITY OF 6-THIOGUANINE IN HUMAN CELLS

Reto Brem & Peter Karran

Cancer Research UK London Research Institute,
Clare Hall Laboratories,
South Mimms,
Herts. EN6 3LD, UK

1Corresponding author: Tel. + 44 1707625870
Fax + 44 1707625812
email peter.karran@cancer.org.uk

Running head: Thioguanine toxicity in human cells
Keywords: thioguanine, reactive oxygen species, DNA damage, DNA crosslinks, Fanconi anemia proteins

This work was supported by Cancer Research UK
The authors state there is no actual or potential conflict of interest
Word count: 3824 Figures 7 Tables 0
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 utilized 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 crosslinks. 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 (2)).

The most widely prescribed thiopurines, 6-MP and its prodrug azathioprine, are converted to TGN in several steps (for review see (3)). Conversion is counteracted by the activities of three enzymes: thiopurine methyltransferase (TPMT) which inactivates thiopurines by methylation (4), xanthine oxidase (XO) which catabolises 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 methylTIMP, 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 (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$^{303}$) (11) and -sulfinate (G$^{302}$) (13), DNA interstrand crosslinks (ICLs) (14), and DNA-protein crosslinks (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 defences 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 (17)). FA-deficient cells are typically hypersensitive to killing, chromosome breakage and particularly to radial chromosome induction by agents that cause DNA interstrand crosslinks (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 mismatch-repair defective human leukaemia cell line CCRF-CEM was grown in RPMI, all other cells in DMEM. Media were supplemented with 10% FCS. The Fanconi anemia (FANCA−/−) and wildtype (FANCA+/+) MEFs 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. Non-transfected HeLa, MLH1-deficient colon cancer cells HCT116 were obtained from Cancer Research UK Central Cell Services. Their identity was confirmed by isoenzyme analysis and STR profiling (March 2011) and skin fibroblasts derived from a Lesch-Nyhan syndrome patient (GM03467) (isoenzyme analysis November 2010) were obtained from The Coriell Institute.

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

Immunoblotting
Whole cell extracts were prepared using RIPA buffer. Proteins (50 µg) were separated on 3-8% Tris-Acetate polyacrylamide gels (Invitrogen). After transfer, membranes were probed with antibodies against FANCD2 (Novus Biologicals), MSH2 (BD Pharmingen) or Xanthine Oxidase (abcam). Antigen-antibody complexes were visualized using ECL blotting detection agent (GE Healthcare).

RNA interference
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 nM. Cells were subcultured into normal medium 24 h after transfection.
**Cell survival, Comet Assay, ^3^H-thymidine incorporation**

To determine cell survival, treated cells were seeded into 96-well plates (1000 cells/well) in normal medium. Viability was assayed 5 days later using the MTT assay. For clonal survival, 500 cells/well were seeded into 6-well plates and colonies were counted 7-10 days later. Each analysis was performed in triplicate.

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

DNA replication was assessed by measuring [^3^H]-thymidine incorporation. Cells were treated with 6-TG and then pulsed with [5' ^3^H]thymidine (1 µCi/ml, 511 GBq/mmol) in normal medium for 30 min. Trichloroacetic acid insoluble radioactivity in duplicate samples of 2x10^6 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 CHO 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\(^{-/-}\) mouse embryonic fibroblasts (MEFs) which were more than 4-fold more sensitive to 6-TG than their FANCA\(^{+/+}\) counterparts (Figure 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 (Figure 1B). Importantly, *FANCD2* silencing in MMR-deficient HCT116 cells also resulted in 6-TG hypersensitivity (Figure 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 -defective cells. FANCD2 monoubiquitination was apparent in MMR-defective HeLa-MSH2 and HCT116 cells following treatment with 6-TG concentrations of 0.8 \(\mu\)M and above - approximately 2-3-fold higher than the concentrations required to trigger FANCD2 activation in MMR-proficient HeLa-SX cells (Figure 2). We conclude that 6-TG treatment activates the FA pathway and that activation is partly independent of MMR-mediated processing of DNA 6-TG.

**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 (Figure 3A). The purine catabolising enzyme xanthine oxidase (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 (Figure 3B). The lower ROS levels in allopurinol-treated HCT116 cells were
associated with a significantly reduced 6-TG sensitivity. Whereas treatment with 0.8 µM 6-TG for 48 h reduced cell viability to < 50%, inclusion of allopurinol reversed this toxicity and cell survival was comparable to that following treatment with allopurinol alone (Figure 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 µM 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 µM 6-TG (Figure 3D). Allopurinol also protected MSH2-deficient CCRF-CEM cells against FANCD2 activation (data not shown).

Allopurinol is a powerful XO inhibitor at µM concentrations. In our experiments, however, protection against 6-TG toxicity and FANCD2 modification required its inclusion at mM 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 µM 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 Figure 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 ability to act as a ROS scavenger.

Since 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. Glutathione (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 co-treatment 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 (Figure 4B). It was associated with FANCD2 monoubiquitination at lower 6-TG concentrations (Figure 4C) and an enhanced sensitivity to killing by 6-TG (Figure 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 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 LN75 cells also induced ROS (Figure 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 (Figure 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 (Figure 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 [\(^3\)H]-thymidine incorporation into nascent DNA (Figure 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 analysed 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 crosslinks. 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 two pathways contribute to 6-TG toxicity in MMR-proficient cells. They are illustrated schematically in Figure 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: (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 demonstrated 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 xrcc2 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 two 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, both 6-MP and azathioprine have been shown to deplete reduced glutathione 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 -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 crosslinking occurs even in unirradiated cells treated with 6-TG is surprising but is, however, consistent with our previous observations 6-TG-induced chromosome aberrations. FA cells are hypersensitive to the induction of chromosome 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 -independent mechanisms. In our experiments, 6-TG-induced ICLs were detected by the comet assay and inferred from FANCD2 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 appears 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 G\textsuperscript{SO2}, G\textsuperscript{SO3} 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 (IBD). 6-TG has been less frequently prescribed. Its limited use seems to stem from its liver toxicity (30) although this appears somewhat controversial (discussed in (31). 6-TG is now considered a viable alternative for IBD 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 might 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 two principal metabolites, the TGNs and methylmercaptopurine ribonucleotide (MMP). 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 appear 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 sub-therapeutic TGN, high MMP levels and severe hepatotoxicity.

The effectiveness of azathioprine or 6-MP is improved by co-administration 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 might be a reason for the less severe adverse side effects of combined treatment. Co-administration 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 might 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 mismatch repair 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 might 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.
ACKNOWLEDGEMENTS

This work was supported by Cancer Research UK.
REFERENCES
12. Cooke MS, Duarte TL, Cooper D, Chen J, Nandagopal S, Evans MD. Combination of azathioprine and UVA irradiation is a major source of cellular 8-oxo-7,8-dihydro-2'-deoxyguanosine. DNA Repair 2008;7: 1982-1989.


LEGENDS TO FIGURES

Figure 1. 6-TG cytotoxicity.
A) MEFs FANCA^{+/+} and FANCA^{−/−} MEFs were grown in the presence of 6-TG at the concentrations indicated for 16 h. They were then returned to normal medium and survival was assessed by clonal assay 7 days later.
B) and C) Human cells FANCD2 was depleted by siRNA transfection of HeLa (B) and HCT116 (C) cells. Three days after transfection, cells were seeded into 96-well plates (1000/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 three experiments.
FANCD2 levels were analyzed by western blotting (inserts) 4 days after transfection.

Figure 2.Mismatch repair-independent activation of FANCD2.
Mismatch repair-proficient (HeLa-SX) or -defective (HeLa-MSH2 and HCT116) cells were grown in the presence of the indicated concentrations of 6-TG for 48 h. Western blots of extracts were probed with antibodies against FANCD2. The positions of unmodified (FANCD2) and activated FANCD2 (FANCD2-ubiq) are arrowed. The MSH2 deficiency of HeLa-MSH2 cells was confirmed by reprobing the blots with anti-MSH2. β-actin served as a loading control.

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 h with 0 (black line), 0.4 µM (blue), 0.8 µM (grey) or 1.2 µM (purple) 6-TG. 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 µM 6-TG for 48 h in the presence or absence of 500 µM allopurinol. ROS production was analysed as described in A).
C) Allopurinol protection against 6-TG toxicity. Cells were grown in the presence of 0.8 µM 6-TG ± 500 µM allopurinol for 48 h and seeded into 96-well plates. Survival was determined 6 days later by MTT assay. The data are means of three independent experiments.

D) Prevention of FANCD2 activation by allopurinol. Cells were treated for 48 h with the concentrations of 6-TG indicated in the presence or absence of 500 µM allopurinol. Western blots of extracts were probed for FANCD2. Unmodified FANCD2 and the activated form (FANCD2-ubiq) are arrowed. β-actin was included as a loading control.

Figure 4. 6-TG and glutathione. Glutathione depletion and potentiation of 6-TG toxicity by BSO.

A) Glutathione depletion. HCT116 or HeLa-MSH2 cells were treated with 6-TG at the concentrations indicated for 48 h. Extracts were prepared and glutathione levels were measured. Values are expressed as percentage of those from untreated cells. Values are the mean of two independent determinations.

B) The effects of BSO on 6-TG induced ROS. HeLa-MSH2 cells were treated for 48 h with 0.8 µM 6-TG in the presence or absence of 200 µM BSO. ROS production was analysed by FACS. Untreated cells (black line), 6-TG alone (blue), BSO alone (red), 6-TG + BSO (purple).

C) Potentiation of FANCD2 activation by BSO. Extracts of HeLa-MSH2 cells treated for 48 h with the concentrations of 6-TG shown were analysed 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 h with the concentrations of 6-TG shown in the presence or absence of 200 µM BSO. Survival was determined by MTT assay. Data are the means of three experiments.
Figure 5. The effects of 6-TG in LN75 Lesch-Nyhan cells.

A) ROS levels: GM03467 cells were grown in the presence of 4 μM 6-TG for 72 h. ROS were analysed by FACS.
B) FANCD2 monoubiquitination. GM03467 cells were treated for 72 h with the indicated doses of 6-TG or with 30 ng/ml mitomycin C. FANCD2 activation was analysed by western blotting.
C) The effects of FANCD2 depletion in Lesch-Nyhan cells. 48 hrs after transfection of GM03467 with FANCD2 or control RNAi, cells were seeded into 96-well plates in the presence or absence of 4 μM 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 crosslinking.

A) HeLa-MSH2 Cells treated with the indicated concentrations of 6-TG for 48 h in the presence or absence of 500 μM allopurinol were pulse labelled for 20 min 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 (500μM) for 48 hrs. 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 two experiments.

Figure 7. Mismatch repair-independent and -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.

Figure S1   Xanthine oxidase levels in cell lines
Western blots prepared from the cell extracts shown were probed with antibody against xanthine oxidase. Tubulin served as a loading control.
Figure 1

A

B

C

Survival (%) vs. 6-TG (µM)

HCT116

FANC D2

FANC D2

FANC D2

β-actin

β-actin

β-actin
Figure 3

A

ROS fluorescence (arbitrary units)

Untreated
0.4 μM 6-TG
0.8 μM 6-TG
1.2 μM 6-TG

1

B

ROS fluorescence (arbitrary units)

Untreated
6-TG
allop.
+ 6-TG

1

C

% survival

6-TG
allop.
- - -

100

D

6-TG [μM]

0 0.4 0.8 1.2

allop.
- - -

FANC2-ubiq.
FANC2

β-actin
Figure S

A

B

C

Downloaded from cancerres.aacrjournals.org on January 30, 2016. © 2013 American Association for Cancer Research.
Figure 7

6-THIOGUANINE (6-TG)

HPRT (1) → INCORPORATION → DNA 6-TG

S-adenosylmethionine (2) → DNA DAMAGE

ROS (2) → DNA ox6-TG DNA ICL (3)

Replication → FA/HR (4) → Survival Cell Death

MMR-independent

MMR-dependent

DNA me6-TG

Replication → MMR (5) → HR (6) → Survival Cell Death

DNA DAMAGE PROCESSING

OUTCOME
OXIDATION-MEDIATED DNA CROSSLINKING CONTRIBUTES TO THE TOXICITY OF 6-THIOGUANINE IN HUMAN CELLS

Peter Karran and Reto Brem

Cancer Res Published OnlineFirst July 20, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-1278
Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/07/20/0008-5472.CAN-12-1278.DC1
Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2012/07/20/0008-5472.CAN-12-1278. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.