Thiopurines, DNA damage, DNA repair and therapy-related cancer

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Aims: The thiopurines, azathioprine, 6-mercaptopurine and 6-thioguanine are one of the success stories of chemotherapy. They are effective immunosuppressants and anti-cancer agents and are prescribed increasingly to treat inflammatory diseases. Although their metabolism has been studied in detail, the optimal use of thiopurines has been guided predominantly by clinical experience and the precise molecular events that underlie their therapeutic activity have remained unclear. The aim of this article is to review some of the properties of the thiopurines and relate them to possible therapeutic mechanisms. In particular, I consider the contribution that DNA substitution by 6-thioguanine makes to their effects as well as some of the possible harmful reactions that DNA 6-thioguanine might undergo.

Conclusions: The increased chemical reactivity of DNA 6-thioguanine underlies its cytotoxic effects and is an important contributor to the anti-leukaemic effects of the thiopurines. The same enhanced reactivity may contribute to the increased risk of acute myeloid leukaemia and skin cancer in thiopurine-treated organ transplant patients.

Keywords: Thiopurines/azathioprine/6-thioguanine/6-mercaptopurine/DNA mismatch repair/oxidative DNA damage/skin cancer/acute myeloid leukaemia

The thiopurines 6-mercaptopurine (6-MP), 6-thioguanine (6-TG), and azathioprine (Aza) (Fig. 1) are used as anti-cancer agents, in the treatment of inflammatory disorders, and as immunosuppressants. 6-MP and 6-TG were identified as growth inhibitory antimetabolites through the pioneering studies of Gertrude Elion and George Hitchins half a century ago (reviewed by Elion).1 They were soon introduced into clinical practice to treat acute leukaemia in children and their use has resulted in a dramatic increase in survival rates for this disease. Both drugs are also effective against colitis, psoriasis and rheumatoid...
Fig. 1 Thiopurines and their metabolism to DNA precursors. 6-MP and 6-TG are converted to TIMP and thioguanosine monophosphate (TGMP) by hypoxanthine phosphoribosyltransferase (HPRT). Both free thiopurines and their respective nucleoside monophosphates are substrates for thiopurine methyltransferase (TPMT) which converts them to S-methylmercaptopurine (me6-MP), S-methylthioguanine (me6-TG), meTIMP and methylTGMP, respectively. Methylation prevents further metabolism but meTIMP is a powerful inhibitor of de novo purine biosynthesis. TGMP is converted directly to the deoxynucleoside triphosphate by the sequential action of deoxynucleotide kinases and reductase. Conversion of TIMP to the 6-TG deoxynucleoside triphosphate involves intermediate conversion to thioxanthine monophosphate (by inosine monophosphate dehydrogenase, IMPD) and to thioguanosine monophosphate (TGMP) by guanine monophosphate synthetase (GMPS). Deoxythioguanine-5′-triphosphate is a substrate for replicative DNA polymerases which incorporate 6-TG into DNA.
arthritis; Aza, a modified 6-MP, is one of the standard immunosuppres-
sants prescribed to prevent graft rejection in organ transplant patients.
Thiopurines are chemically more reactive than the normal DNA bases. This increased reactivity partly underlies their therapeutic effect but it also makes them susceptible to unwanted reactions and, as with many other powerful therapeutics, their long-term use is increasingly associated with malignancy. Here, I review some of the properties of thiopurines with particular emphasis on the mechanisms by which these agents might both cure and cause cancer.

Metabolism of thiopurines

All three thiopurines are prodrugs. Their metabolism culminates in the
formation of TG nucleotides that are precursors of nucleic acid syn-
thesis. This metabolic activation has been reviewed in detail and is
outlined here (Fig. 1). DeoxythiogTP (dthioGTP), the ultimate DNA
precursor, is a good substrate for incorporation by DNA polymerases
and estimates of steady-state levels of DNA 6-TG in patients suggest
that during replication between 0.01 and 0.1% of DNA guanines
(around $10^5$ to $10^6$ per cell) may be substituted by the thiobase.$^{3,4}$
These low levels of DNA 6-TG fall below a threshold for toxicity (see
below) and are tolerated. More extensive substitution is associated
with chromosomal damage and cell death.

Thiopurines are also enzymatically inactivated. Xanthine oxidase—
an important component of general purine catabolism—oxidizes 6-MP
to 6-thiouric acid. A second, more important, pathway is initiated by
thiopurine methyltransferase (TPMT) which converts 6-MP or 6-TG to
inactive S-methyl derivatives. TPMT has received considerable atten-
tion as an example of the potential of pharmacogenomics to provide
individualized therapy (for reviews, see Evans and co-workers)$^{5,6}$ A
number of polymorphic variants of the $TPMT$ gene encode enzymes
with significantly reduced activity. Approximately 0.3% of patients are
homozygous for non-functional $TPMT$ alleles and the consequent
absence of TPMT activity is associated with severe, and potentially
fatal, thiopurine-induced haematological toxicity. Pretreatment screen-
ing for inactive $TPMT$ alleles can identify individuals at risk of severe
bone marrow toxicity. Heterozygosity for a low activity allele identifies
patients in whom lower doses of thiopurine can be used safely. Even
normal TPMT activity does not abolish the risk of myelosuppression,
however, and close monitoring of haematological parameters is still
required. For this reason, whether knowledge of TPMT status provides
significant practical benefits has been questioned.$^7$
How do thiopurines kill cells?

Despite five decades of clinical use, there is still uncertainty about the therapeutic mechanism of thiopurines. Their effectiveness as anti-leukaemic agents testifies to their ability to kill cells and an ability to inhibit the proliferation of activated T cells seems a likely contributor to immunosuppression. Early studies implicated thioguanine nucleotides (usually taken to mean the deoxy- and ribonucleoside triphosphates) and DNA substitution by 6-TG in cytotoxicity.\(^8\) Purine starvation—an alternative mechanism of toxicity that is independent of thioguanine nucleotide formation—is another possible contributor. This is because methylthioinosinemonophosphate (meTIMP) (Fig. 1) is a potent inhibitor of phosphoribosylpyrophosphate amidotransferase\(^9,10\) — the first step of de novo purine biosynthesis. Since thioinosine monophosphate (TIMP) is not produced from 6-TG, purine starvation is a factor in the effects of 6-MP and its precursor Aza. On the other hand, the effects of thioguanine nucleotide formation and DNA substitution are common to 6-MP, Aza and 6-TG. Although thioguanine nucleotide levels have been regarded as predictive for 6-MP cytotoxicity,\(^11\) there appears to be no current consensus as to the relative contributions of purine starvation and DNA substitution to cytotoxicity.

The problem of understanding how thiopurines are cytotoxic and immunosuppressive is compounded by the fact that the presence of 6-TG in DNA is not, in itself, sufficient for its toxic effects. Further modification of DNA 6-TG as well as an active DNA mismatch repair (MMR) system is also required. DNA 6-TG undergoes in situ non-enzymatic methylation—probably by S-adenosylmethionine. This reaction, which is favoured by the increased chemical reactivity of the thiol group, affects an estimated one in \(10^4\) or \(10^5\) incorporated 6-TG bases.\(^12\) Because 6-TG substitution of DNA is so extensive, however, there are several thousand 6-meTG bases in a cell’s DNA at toxic 6-TG doses. DNA 6-meTG preferentially base pairs with thymine during replication. The 6-meTG:T base pairs resemble replication errors and provoke processing by MMR. This results in cell death. An outline of the principal events in MMR is shown in Figure 2A. Briefly, its role is to correct errors committed by DNA polymerases during replication. Incorrect base pairs or supernumerary bases that escape proofreading during replication are recognized and engaged by the MutS\(\alpha\) mismatch binding complex which comprises one subunit each of the MSH2 and MSH6 proteins. Further interaction with a second heterodimer, MutL\(\alpha\) comprising MLH1 and PMS2 leads to recruitment of exonucleases and DNA polymerases that remove the mismatched DNA section and replace it with an accurate copy. A defect in any of these four key
proteins is sufficient to inactivate MMR and prevent the recognition of aberrant base pairs as well as true replication errors. MutSα or MutLα defective cells are therefore very resistant to thiopurines because they are unable to initiate lethal processing. They survive with substantial levels of DNA 6-TG. The resistance conferred by defective MMR is known as methylation tolerance because the same defects also confer substantial resistance to methylating agents that produce DNA methylation.

Fig. 2 DNA MMR and lethal processing of aberrant base pairs (A) MMR. Replication errors in the form of incorrect base pairs, such as G:T, or mispaired bases generated during slippage between template and daughter strands during replication of highly repetitive sequences, are recognized and bound by the MutSα heterodimer. Further interaction with the MutLα heterodimer coordinates the removal of the mismatched segment and its replacement with a correct version by resynthesis using the template DNA strand to define the correct sequence. (B) Formation and lethal processing of aberrant DNA base pairs. Treatment with methylating agents MNU (N-methyl-N-nitrosourea) or MNNG (N-methyl-N’-nitro-N-nitrosoguanidine) produces DNA O6-methylguanine (6-meG). This can be directly demethylated by an enzyme called MGMT which restores DNA G. When MGMT is low or absent, replication of persistent DNA 6-meG generates 6-meG:T pairs that are substrates for recognition and processing by MutSα and MutLα. Processing remains incomplete—possibly because of the persistent presence of the miscoding base in the template DNA strand—and is lethal. Thiopurine treatment causes the accumulation of 6-TG in DNA. A small fraction of incorporated 6-TG is methylated in a chemical methylation reaction with S-adenosylmethionine (SAM) the cellular donor of methyl groups in enzymatic reactions. DNA 6-meTG is not recognized to a significant extent by MGMT. Replication generates 6meTG:T base pairs that are structurally very similar to 6-meG:T pairs and are also substrates for recognition and processing by MutSα and MutLα. Subsequent processing is lethal.
O6-methylguanine. This methylation product miscodes in a similar fashion during replication. O6-methylguanine:T base pairs closely resemble 6-meTG:T base pair (Fig. 2B) and their processing by MMR also triggers cell death.

Does purine starvation contribute to the toxicity of thiopurines? An experimentally induced increase in meTIMP in cultured human cells is associated with increased sensitivity to 6-MP. TPMT overexpression which increases the levels of inhibitory methylthiopurine nucleotides, actually reduces the supply of thioguanine nucleotides for incorporation into DNA. This can increase 6-MP sensitivity but decreases sensitivity to 6-TG. At face value, these findings might suggest that 6-MP and 6-TG cause death by different mechanisms; purine starvation being more important for 6-MP toxicity and DNA substitution the major contributor to killing by 6-TG. These observations appear to be incompatible with a general requirement for active MMR and DNA 6-TG in the toxicity of the thiopurines. Furthermore, clinical findings are more consistent with thioguanine nucleotide formation and DNA 6-TG, rather than purine starvation, as a major determinant of thiopurine cytotoxicity.

Can these apparent contradictions be resolved? With hindsight, it seems that in vitro studies with cultured cells may have exaggerated the contribution of purine starvation. Many involve cell lines in which later work has revealed MMR defects. In the absence of MMR, purine depletion is clearly the most significant factor in toxicity. When MMR is operating, however, processing of 6-meTG base pairs is the major contributor to cell death. This conclusion, which is fully consistent with the clinical effects of different TPMT levels, is supported by observations of the effects of MMR inactivation in mice in which an Msh2 defect effectively abolishes the myelotoxicity associated with 6-MP treatment. Overall, the data suggest the formation of thioguanine nucleotides and subsequent DNA damage represent the major mechanism of thiopurine toxicity. Inhibition of de novo purine biosynthesis appears to play a less significant role. It is, of course, possible that the overt cytotoxic effects and the immunosuppressive effects are mechanistically different and that purine starvation is a significant contributor to the latter. It is noteworthy in this regard that mycophenolate mofetil, one of the later generation immunosuppressants, is an inhibitor of purine biosynthesis.

DNA 6-TG methylation and DNA damage signalling

Although DNA 6-TG is a non-canonical DNA base, it is not strictly DNA damage. The damage is produced by post-replicative processing
and the intervention of MMR is required to initiate DNA damage responses and to trigger DNA damage-related cell cycle checkpoints. Signalling events downstream of MMR recognition of 6-meTG base pairs are being clarified. Prolonged 6-TG treatment induces a sustained cell cycle arrest in G2 phase.\textsuperscript{19} Arrest is correlated with sensitivity. It requires functional MMR and occurs in thiopurine-sensitive repair proficient cells. Unlike most DNA damage checkpoint activation that occurs within hours of treatment, 6-TG-induced G2 arrest is a very late event and is not maximal until day two or three of continuous drug treatment.\textsuperscript{19} The onset of arrest coincides with phosphorylation of the CHK1 and CHK2 checkpoint kinases although the timing appears to correlate better with the former.\textsuperscript{20} CHK1 is the major transducer of signals generated by the ATR DNA damage sensor that is activated preferentially by treatments that interfere with replication fork progression, such as exposure to hydroxyurea or DNA polymerase inhibitors (reviewed in Sancar et al.).\textsuperscript{21} There are sound reasons to believe that ATR/CHK1 signalling is required to impose the G2 arrest after 6-TG treatment. First, reducing the level of ATR or CHK1 by the technique of siRNA silencing abrogates 6-TG-induced G2 arrest. In addition, 6-TG induces G2 arrest in ATM defective cells indicating that the alternative pathway in which CHK2 transduces signals from the ATM DNA damage sensor is less important.\textsuperscript{20} What triggers ATR activation? Analysis of DNA breakage after 6-TG treatment suggests a role for DNA single strand breaks.\textsuperscript{19} Although DNA double-strand breaks are detectable within hours of 6-TG exposure, their appearance is not MMR-dependent and they disappear well before detectable CHK1 activation. They do not seem to contribute significantly to cell death. At later times (from 24 h) an excess of single strand breaks is observed in MMR-proficient cells. Their appearance coincides temporally with CHK1 activation and G2 arrest. Although this might suggest that single-strand breaks trigger checkpoint activation, the presence of double-strand breaks derived from replication of DNA bearing incompletely processed 6-meTG base pairs, is not excluded by these findings. In view of the low number of 6-meTG lesions, any contingent double-strand breaks would probably escape detection by the rather insensitive analysis.

DNA 6-meTG and the DNA O\textsuperscript{6}meG introduced by treatment with methylating drugs are both processed by MMR. Low doses of methylating agents also induce a delayed MMR-dependent G2 arrest that occurs in the second cell cycle after treatment. Arrest is dependent on ATR and CHK1\textsuperscript{22} and requires MMR-dependent processing of O\textsuperscript{6}meG base pairs. Thus, DNA 6-meTG and DNA O\textsuperscript{6}meG ultimately trigger the ATR/CHK1 DNA damage response, albeit indirectly. The timing of checkpoint activation suggests that an inducing signal is
generated late and involves replication of regions of abnormal DNA structure produced when MMR processes DNA containing 6-meTG or O6-meG bases.

It follows from this that MMR proteins themselves are not components, in any meaningful sense, of the ATR/CHK1 G2 checkpoint response. MMR and the G2 checkpoint are best thought of as separate systems. The signalling cascade that results in G2 checkpoint activation (ATR/CHK1 activation, CDC25A destruction) is intact in MMR defective cells and can be triggered by appropriate treatments. Indeed, MMR inactivation and abrogation of the G2 checkpoint have contrasting effects. Whereas the former increases resistance to 6-TG, siRNA-mediated inhibition of CHK1 or ATR expression sensitizes cells to 6-TG-induced apoptosis. MMR and the G2 checkpoint system simply overlap and MMR-mediated processing of 6-meTG (and O6-meG) is required to generate the DNA strand discontinuities or other aberrant DNA structures that trigger activation of the ATR cascade.

**Oxidation of thiopurines**

The increased reactivity of the thiol group means that 6-TG is susceptible to other chemical modification as well as to methylation. In particular, DNA 6-TG is very easily oxidized. As long ago as 1961, DNA 6-TG was identified as an oxygen-dependent radiation sensitizer in bacteria. Later, photochemical studies of thiopurines in solution indicated that the free bases are photodynamically active and undergo light-induced reactions that require molecular oxygen. They are much more susceptible to oxidation than any of the normal DNA bases. Thiopurines have spectral properties that distinguish them from normal DNA bases. The absorbance maxima of adenine and guanine lie in the UVC range (around 260 nm). Replacement of the 6-oxygen atom by sulphur causes a shift in the UV absorbance spectrum and the thiopurines absorb maximally in the UVA region at around 340 nm. In solution both 6-MP and 6-TG are rapidly destroyed by UVA in reactions that are dependent on oxygen. In these reactions, thiopurines (TPs) can act as Type I and Type II photosensitizers. In a Type I reaction, free 6-MP or 6-TG absorbs UVA photons to generate an excited triplet state, (TP)*. This highly unstable compound can interact with molecular oxygen leading to the formation of thiopurine radical species (TP·) and superoxide: (TP)* + O2 → O2·+ TP + H+. Superoxide can be converted into highly reactive hydroxyl radicals (OH·) by Fenton-like reactions. In a Type II reaction, the UVA energy absorbed by the thiopurines is transferred to molecular oxygen (O2) to generate singlet oxygen (O2). Both OH· and O2 are known to
cause DNA damage and the latter is considered to be the predominant source of DNA damage in cells exposed to incident solar UV radiation (>90% of which is UVA). DNA is an important target of these highly unstable reactive oxygen species (ROS). Potentially harmful DNA oxidation products are generated at significant levels during normal oxidative metabolism. Importantly, DNA 6-TG is a significant source of ROS in cells exposed to low UVA doses. Since ROS react very close to their site of formation, the risk of oxidative DNA damage is likely to be particularly high when cells containing DNA 6-TG are exposed to UVA.

6-TG is itself very susceptible to oxidation and is rapidly converted to guanine sulphonate (G$^{SO_3}$). Photochemical G$^{SO_3}$ formation by UVA involves $^1$O$_2$ and the reaction is inhibited in the presence of azide which quenches $^1$O$_2$, and accelerated in deuterium oxide which prolongs the reactive lifetime of $^1$O$_2$. These reactions happen in DNA and moderate UVA doses convert DNA 6-TG to G$^{SO_3}$. Photochemical activation of cellular DNA 6-TG has significant biological consequences and incorporated 6-TG sensitizes cells to the lethal and mutagenic effects of very low UVA doses. $^1$G$^{SO_3}$ has dramatic effects on DNA stability. When placed opposite any of the normal DNA bases in short oligonucleotide duplexes, it is unable to form any kind of stable base pair and destabilizes the double helix more effectively than even a purine:purine or pyrimidine:pyrimidine pair. Consistent with a significant deviation from a normal structure, $^1$G$^{SO_3}$ cannot be bypassed by the Klenow fragment of Escherichia coli DNA polymerase I in primer extension reactions with synthetic substrates. Human DNA polymerase $\eta$ and an archaean Y-family DNA polymerase, both of which have a less stringent fidelity, are able to bypass DNA $^1$G$^{SO_3}$, however. Whether recruitment of reduced fidelity bypass DNA polymerases contributes to the mutagenicity of 6-TG/UVA treatment is unknown. Consistent with the ability of DNA $^1$G$^{SO_3}$ to block DNA polymerases, 6-TG/UVA treatment induces a rapid inhibition of replication in human cells.

### Thiopurines as carcinogens

One consequence of long-term treatment with powerful drugs, many of which are acknowledged carcinogens, is an increased incidence of iatrogenic malignancy. Thiopurines are no exception. Azathioprine is designated as a human carcinogen by the International Association for Research on Cancer. Long-term immunosuppression in organ transplant patients is associated with an increased risk of certain types of cancer. Although several factors including immunosuppression
per se, are likely to contribute to this, there is good reason to believe that Aza treatment may play a significant role.

Leukaemia

The development of drug resistance often limits the success of chemotherapy. 6-MP is widely used in the treatment of acute lymphoblastic leukaemia (ALL) and the emergence of drug-resistant disease contributes to treatment failure in perhaps 25% of cases. The development of resistance is mirrored by results from laboratory studies in which chronic exposure of cultured cells to toxic thiopurine concentrations allows the outgrowth of rare clones with stable thiopurine resistance (Fig. 3A, B). These 6-TG and 6-MP tolerant cells are MMR defective and have the microsatellite instability that is a defining feature of this repair deficiency. The emergence of tolerant clones may have clinical relevance. Studies of MMR protein expression in leukaemic blasts reveal large interindividual variations with frequent undetectable levels. Even a 2-fold reduction in MMR capacity in lymphoblastoid cell lines heterozygous for a mutated MSH2 gene confers a measurable increase in methylating agent resistance. Perhaps, this applies to thiopurines. Heterozygous cells do not exhibit microsatellite instability, retain sufficient capacity to repair replication errors, but have a phenotype that is only evident on drug exposure. In addition, although microsatellite instability is rare in ALL, many established ALL cell lines are MMR-defective. Strikingly, several lines appear to have been established from patients at relapse. It has been pointed out that this is consistent with the clonal expansion of rare MMR-defective 6-MP-resistant leukaemic cells during the development of refractory disease. It is noteworthy in this regard that haematopoietic cells are particularly sensitive to thiopurines. In mouse models this sensitivity is considerably attenuated in MMR-deficient Msh2−/− animals. Thiopurine treatment would provide conditions favouring the selective proliferation of rare MMR-defective variant cells. An example of selective clonal expansion during the development of therapy-related acute myeloid leukaemia (AML) has been described.

Thiopurine treatment is implicated in the development of AML. One study describes five cases of AML among 493 children who received 6-MP during maintenance therapy for ALL. Each of the affected patients had low TPMT activity and/or abnormally elevated thioguanine nucleotide levels. Thoracic organ and kidney recipients have respectively a 5.1-fold and 2.5-fold increased risk of AML (Fig. 3C). The extent of the increased risk is correlated with Aza dosage. MMR defects are generally rare in leukaemia and less than 5% of AML cases
Fig. 3 Thiopurine resistance, DNA MMR defects and immunosuppression-related AML. (A) Cells selected for stable resistance to 6-TG are deficient in MMR. A subclone of the A2780 ovarian carcinoma cell line (SCA5) was grown for several weeks in medium containing toxic concentrations of 6-TG. Several clones, including JA5 and JA8, exhibited >5-fold increased resistance to both 6-TG and 6-MP (not shown). (B) Resistant clones were screened for expression of MMR proteins by western blotting. Neither MLH1 nor PMS2 is detectable in JA5 and JA8. This is due to loss of expression of the MLH1 gene via epigenetic silencing. The PMS2 protein is unstable in the absence of its partner MLH1 and is also not observed. (C) AML in heart and/or lung transplant patients. The expected incidence is derived from a cohort of control subjects matched for age, sex and geographical distribution. The relative risk for transplant patients is 5.3 (P < 0.0001). Cadaver kidney patients also have an increased incidence of AML (RR = 2.1; P < 0.001).
display microsatellite instability. The incidence of microsatellite instability in organ transplant-related tAMLs appears to be significantly higher.\textsuperscript{32} This is again consistent with selection of drug-resistant, MMR-defective myeloid cell clones. Repair defective cells are not, in themselves, malignant but their high rate of spontaneous mutation increases the probability of malignancy development. A high frequency of microsatellite instability has also been reported in lymphomas that arose in immunocompromised organ transplant patients\textsuperscript{37} although the possible contribution of immunosuppressive drugs was not evaluated. Overall, it appears that the relationship between thiopurine resistance and MMR deficiency that has been well-established in laboratory models may have a clinical counterpart.

### Brain tumours

An important study by Relling \textit{et al.}\textsuperscript{38} highlighted a possible interaction between ionizing radiation and thiopurine treatment in a clinical setting. During a series of trials aimed at optimizing treatment regimes for childhood ALL, they noted a remarkable increase in the incidence of brain tumours among a group of patients who had received 6-MP together with prophylactic cranial radiation. A careful analysis of patient groups indicated that the increased risk was particularly associated with long-term 6-MP treatment and high TGN levels. Genetically determined TPMT deficiency was markedly over-represented among the affected individuals. The effects were so striking that subsequent treatment protocols were modified to avoid concurrent antimetabolite and radiation treatment. Although this dramatic synergy still remains unexplained, the susceptibility of thiopurines to oxidation is a possible contributor. As noted above, radiosensitizing effects were among the first properties ascribed to DNA 6-TG. Since 6-MP has a limited distribution in brain, and cell division is largely restricted to glial cells, the contribution of DNA 6-TG may be limited. Is it possible that TGN or other non-DNA thiopurine metabolites might act as a source of ROS in brain cells? In some circumstances, unincorporated thiopurines do promote the formation of oxidative DNA damage in bacteria.\textsuperscript{39} It would certainly be interesting to investigate the chemical and biological consequences of interactions between ionizing radiation thiopurine metabolites, or DNA 6-TG in more detail.

### Skin cancer

Immunosuppressed organ transplant patients have a significantly increased cancer risk. Not all cancers are over-represented, however, and the frequencies of some of the more common tumours are not
greatly different to those of the normal population. Among those that are, many are associated with viruses. There is, however, a dramatic increased risk of non-melanoma skin cancer and the incidence of cutaneous squamous cell carcinoma (SCC) is more than 100-fold higher among organ recipients than in the normal population. Sunshine exposure is an important factor in the increased risk. The possible contribution of viruses to the immunosuppression-related increase in SSC risk has been considered but although some sub-types of human papillomavirus are found more frequently in these tumours, there is no clear evidence for a causative association.

Most transplant patients have been treated with Aza and UVA is the major component (>90%) of incident ultraviolet radiation. The longer wavelengths of UVA penetrate deeper into skin than the more energetic UVB and up to 20% of incident UVA may reach the layers of skin containing the stem cells. As pointed out in a preceding section, DNA 6-TG is highly susceptible to oxidation by UVA in a reaction that generates highly damaging ROS (Fig. 4). The product of DNA 6-TG oxidation, GSO₃ and collateral damage to normal DNA bases from the ROS are both potential contributors to the development of transplant-related skin cancer.

The systemic and long-term administration of 6-MP or Aza favours the accumulation of DNA 6-TG by dividing cells. Peripheral blood lymphocytes of children undergoing 6-MP treatment for ALL and skin cells of transplant patients immunosuppressed with Aza contain measurable DNA 6-TG. The skin of Aza-treated patients is particularly sensitive to UVA. Erythema, the localized reddening of exposed skin is an acute response to sunlight. Aza treatment significantly reduces the minimal erythema dose—the lowest dose of UVA required to provoke perceptible erythema. The same patients’ response to UVB to which DNA 6-TG is not reactive, is unaltered. Erythema is linked to unpaired DNA damage. In cells without DNA 6-TG, UVB induces DNA lesions directly and UVB sunscreens that protect against DNA damage also attenuate erythema. Studies with Xeroderma pigmentosum patients who are unable to excise UVB photoproducts and genetically modified animals with similar DNA repair defects link erythema to the persistence of cyclobutane pyrimidine dimers—one of the most abundant DNA photoproducts of UVB—in transcriptionally active DNA. The reduced MED for UVA in Aza-treated patients implies the photochemical formation (and possible persistence) of DNA lesions via 6-TG. Consistent with this possibility, in laboratory experiments, 6-TG and UVA interact to generate ROS. These cause damage to DNA and other cellular molecules. Oxidation of DNA 6-TG to GSO₃ and the reactions between ROS and
normal DNA bases are all potential sources of mutation and cancer. DNA G\textsuperscript{SO3} is a powerful block to replication in vitro and is bypassed by low fidelity Y-family DNA polymerases. DNA replication in living cells is also rapidly inhibited by the combination of DNA 6-TG and UVA—consistent with the formation of similar photoproducts and possible triggering of error-prone tolerance strategies. Immunosuppression in transplant patients is effectively life-long, and long-term chronic exposure to UVA/DNA 6-TG might result in a sustained mutator phenotype that would accelerate the accumulation of mutations. Fig. 5 outlines how these photochemical reactions of DNA 6-TG might contribute to mutation and the subsequent development of skin cancer.

Fig. 4 UVA generates ROS in cellular DNA. HCT116 cells containing DNA 6-TG were treated with a dye that is enzymatically converted into a sensor of intracellular ROS. Formation of ROS generates a fluorescent product that can be detected by fluorescence activated cell sorting (top) or fluorescence microscopy (bottom). Cells were irradiated with 10 kJ/m\textsuperscript{2} UVA. Untreated (no 6-TG, no UVA), 6-TG treatment alone and UVA treatment alone all generated minimal fluorescence signals. Combining 6-TG and UVA generated significant fluorescence that is seen as a shift along the X-axis. Control cells treated with H\textsubscript{2}O\textsubscript{2}, a known inducer of ROS, were analysed in parallel. Some cells were grown in the presence of 6-TG together with an inhibitor of replication (hydroxyurea, HU). This permits 6-TG incorporation into RNA but excludes it from DNA. Significantly less ROS are generated in 6-TG/HU/UVA treated cells (FACS, top) and all detectable ROS are confined to the cytoplasm (microscopy bottom). These observations identify DNA 6-TG as a significant source of ROS in UVA-treated cells.29
Fig. 5 How thiopurines, UVA and oxygen might combine to accelerate skin cancer development. Thiopurine treatment promotes the incorporation of 6-TG into dividing stem cells of the skin (keratinocytes) DNA during replication. Incident UVA light penetrates to the stem cell layers and in the presence of molecular oxygen, generates ROS including $\cdot O_2$, within DNA itself. These ROS can oxidize DNA 6-TG to the replication blocking $G^{3\text{ox}}$ that may trigger mutagenic bypass. They may also oxidize normal DNA bases to generate known mutagenic products including the directly miscoding DNA 8-oxoguanine shown. The accelerated accumulation of these photodynamic mutations in skin cells would contribute to the development of skin cancer.

Concluding discussion

The biological effects of thiopurines—both desirable and undesirable—reflect the increased reactivity conferred by substitution of the single oxygen atom of guanine by sulphur. The lethal, immunosuppressive and anti-inflammatory properties derive, at least in part, from incorporation into DNA and they favoured *in situ* methylation of DNA 6-TG. This step is essential to trigger the DNA MMR system into generating the ultimately cytotoxic DNA damage. Loss of MMR provides sufficient selective growth advantage and this may account for the development of resistant disease as well as the association of MMR defects with thiopurine treatment-related AML. The biological implications of the increased susceptibility of 6-TG to oxidation remain to be determined. In particular, photochemical formation within DNA itself, of the highly damaging ROS that are implicated in mutagenesis and cancer appears to be a likely hazard. Although ROS are an unavoidable consequence of an oxygen-based metabolism, cells have numerous protective systems that counteract their genotoxicity. Treatments that tip the delicate balance towards an increased burden of oxidative DNA lesions are likely to have adverse long-term consequences. Aza in combination with UVA in transplant patients is likely
to produce conditions of chronic oxidative stress. The idea that Aza might contribute to carcinogenesis in immunosuppressed patients is not new. It was suggested many years ago based on the observation that transplant patients with skin cancer had high levels of thioguanine nucleotides.\textsuperscript{44} More recently, immunosuppression \textit{per se}, rather than any particular drug treatment has generally been regarded as the major contributor.\textsuperscript{45} It is perhaps worth revisiting the epidemiological findings now that there have been several more years of follow-up. There is no doubt that skin cancer remains a huge problem for transplant patients. It seems prudent to at least reconsider a possible role for Aza and the photochemical properties of DNA-6-TG provide a theoretical framework.

I ironically, one obstacle to resolving the separate contributions that overall immunosuppression and Aza make to transplant-associated cancer may soon disappear. Aza is being phased out in favour of newer immunosuppressants like mycophenolate mofetil or sirolimus. These are generally considered to be more effective although this has been questioned.\textsuperscript{46} In time, this will provide a cohort of transplant patients who have undergone many years of immunosuppression without Aza. It will be interesting to review the effect of these changes on transplant-related SCC. Although it is generally considered that more powerful immunosuppression will increase the frequency of transplant-related cancers, this may not prove to be the case if Aza plays a significant role. At the same time, it might be worth keeping a close watch on the increasing number of patients who now receive Aza treatment for chronic inflammatory disorders.

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Thiopurines and therapy-related cancer


