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# Reviewing the mechanism of action of thiopurine drugs: toward a new paradigm in clinical practice

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## Summary

The precise mechanism of action of thiopurine drugs remains unclear despite more than 40 years of use. Recent knowledge in the field of apoptosis and a better insight into, as well as a rapid increase in, their use in several important areas of clinical medicine justify this appraisal. This is a review of the recent advances in the knowledge of their mechanism of action and is primarily intended to help clinicians understand the pharmacological properties of these drugs adequately and to find ways to improve their use in clinical practice.

The parent compound is azathioprine (AZA), which is rapidly reduced in the presence of glutathione to 6-mercaptopurine (6-MP) and then metabolized into active metabolites with immune-modifier activity. Recent observations and new data indicate that AZA/6-MP could be considered as a "two-in-one" drug, providing a source of 6-thioguanine nucleotides (6-TGNs) and methylated metabolites, and that both compounds could contribute to its antiproliferative effects. This review will also focus on mechanisms that may help to explain a number of recent observations showing that myelotoxicity may occur in patients with high TPMT level or low 6-TGN rate. Our final proposal suggests that the immunosuppressive effects of these drugs are due to a balanced combination of antimetabolic and pro-apoptotic actions.

**key words:** **apoptosis • azathioprine • mechanism of action • thioguanine • TPMT**

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## BACKGROUND

Azathioprine and other thiopurine drugs have been administered in the treatment of a number of autoimmune conditions, such as lupus erythematosus, pemphigus, and myasthenia gravis. More recently their use has been extended in the management of patients with chronic inflammatory bowel diseases (IBDs, including Crohn´s disease and ulcerative colitis) and multiple sclerosis. Their precise mechanism of action remains unclear despite more than 40 years of use as well as *in vivo* and *in vitro* investigations [1,2]. Recently several papers have suggested that the traditional considerations about thiopurine drugs must be revisited [3,4].

The parent compound is AZA, which is rapidly reduced to 6-mercaptopurine (6-MP) and then metabolized by three competitive enzymatic pathways [5]: thiopurine methyl transferase (TPMT) catalyzes S-methylation of 6-MP to 6-methylmercaptopurine (6-MMP), xanthine oxidase (XO) catalyzes the oxidation of 6-MP to biologically inactive metabolites and finally hypoxanthine phosphoribosyl transferase followed by inosine monophosphate dehydrogenase, and guanosine monophosphate synthase convert 6-MP to 6-TGNs, largely considered the active metabolites with immune-modifier activity (Figure 1). Incorporation of 6-TGNs into lymphocyte DNA results in DNA damage and has been considered the main mechanism of AZA cytotoxicity [6].

### Genetic variability and functional TPMT expression

The level of thiopurine methyltransferase (TPMT) activity is controlled by a common genetic codominant polymorphism and is responsible for the inter-individual differences observed in the metabolism, toxicity, and therapeutic efficacy of thiopurine drugs [7]. At present the monitoring of AZA/6-MP treatment is based primarily on the study of TPMT genetic polymorphism or in the corresponding activity in erythrocytes. In Spain, red blood cell (RBC) TPMT phenotype is routinely monitored in patients entering AZA therapy. Not yet published figures in our database include, at the moment of this communication, 11,337 individuals, of whom 4,518 correspond to patients affected by Crohn´s disease, 1,783 to ulcerative colitis, 752 to multiple sclerosis, and 4,284 to several autoimmune diseases apart from those mentioned (Figure 2). In a previously reported series of TPMT phenotyping [8] (measured by a radiochemical method based on the conversion of 6-MP to 6-MMP, using $^{3}$H-S-adenosyl-L-methionine as methyl donor [9]), it was demonstrated that approximately 89.4% of the population showed high TPMT activity (13.8–25.1 U/ml RBC); 9.7% had intermediate activity (5.0–13.7 U/ml RBC); and only 0.9% of the subjects showed low TMPT activity (<5.0 U/ml RBC).

As mentioned above, the level of TPMT activity has been considered responsible for the variation in the therapeutic efficacy and toxicity of thiopurine drugs [10]. Patients with either inherited TPMT deficiency or low enzyme activity have a pronounced risk of potentially life-threatening hematopoietic toxicity when treated with conventional doses of these medications. In those patients, aza/6-MP metabolism is shunted towards excessive production of active 6-TGNs, leading to toxic accumulation after standard dosages. Using this simplified model, the patients with higher levels of TPMT activity should present lower 6-TGN levels and lack of response to therapy.

### Toward a new paradigm

In a recent study focused on patients with inflammatory bowel disease treated with AZA or 6-MP [11], no correlation between whole blood 6-TGN concentrations and remission was found, suggesting that the AZA and 6-MP doses used and whole blood 6-TGN concentrations achieved were sufficient to reach the desired clinical effect in most of the patients. In this IBD patient group established on AZA, TPMT activity was not predictive of clinical response or drug toxicity. Another recent study, using 6-TGN metabolite levels to optimize AZA therapy in patients with IBD, showed a subset of individuals unresponsive to therapy despite, previously considered, adequate 6-TGN levels [12]. Finally, Dubinsky et al. [13] pointed out that when IBD patients were treated with 6-TG, a 6-TGN level of at least 1,300 pmol/8×10$^{8}$ RBC, which is much higher than the 235 pmol/8×10$^{8}$ RBC cutoff suggested when using 6-MP or AZA, may be the required therapeutic target level necessary to optimize the therapeutic efficacy of this thiopurine. As seen in leukemia, the therapeutic range of 6-TGNs appears to be much higher when treating with 6-TG than with 6-MP/AZA. All these data suggest that the predictive value of TPMT activity in the monitoring of thiopurine treatment is scarce because there are other mechanisms involved in the immunosuppressive properties of thiopurines than the regulation of the availability of 6-TGNs by TPMT and their incorporation into DNA.

## MECHANISM OF ACTION

The precise mechanism of thiopurine action in lymphocytes is still a matter of debate. Incorporation of thiopurines (AZA, 6-MP, and 6-TG) as well as several other antileukemia drugs into DNA seems to be essential for their cytotoxic effects [14]. However, the widely held view that these agents are cytotoxic by impairing DNA polymerization is probably an oversimplification [15], because incorporation of many nucleoside analogs does not abrogate DNA elongation. In this review, a new model explaining the mechanism of action of thiopurines is proposed.

### Nucleotides and beyond

The assumption that 6-TGN levels are a direct measure of cytotoxicity of AZA, 6-MP, and 6-TG ignores the significant differences that exist among the intracellular metabolisms of these thiopurine drugs. These thiopurines are prodrugs that require activation by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) to exert a cytotoxic effect [16]. Metabolism of 6-TG by HGPRT produces 6-thioguanosine 5´-monophosphate (TGMP), which is further metabolized by a series of kinases and reductases to produce deoxy-6-thioguanosine 5´triphosphate (dG$^{s}$). Incorporation of dG$^{s}$ into DNA has been shown to trigger cell-cycle arrest and apoptosis by a process that involves the mismatch repair pathway [17]. The metabolism of 6-MP to TGMP is less direct than that of 6-TG, involving two additional enzymes, inosine monophosphate dehydrogenase and guanosine monophosphate synthase. This difference is potentially important because the intermediate of the AZA/6-MP pathway, thioinosine monophosphate, can act as a substrate for TPMT, leading to the production of S-me-

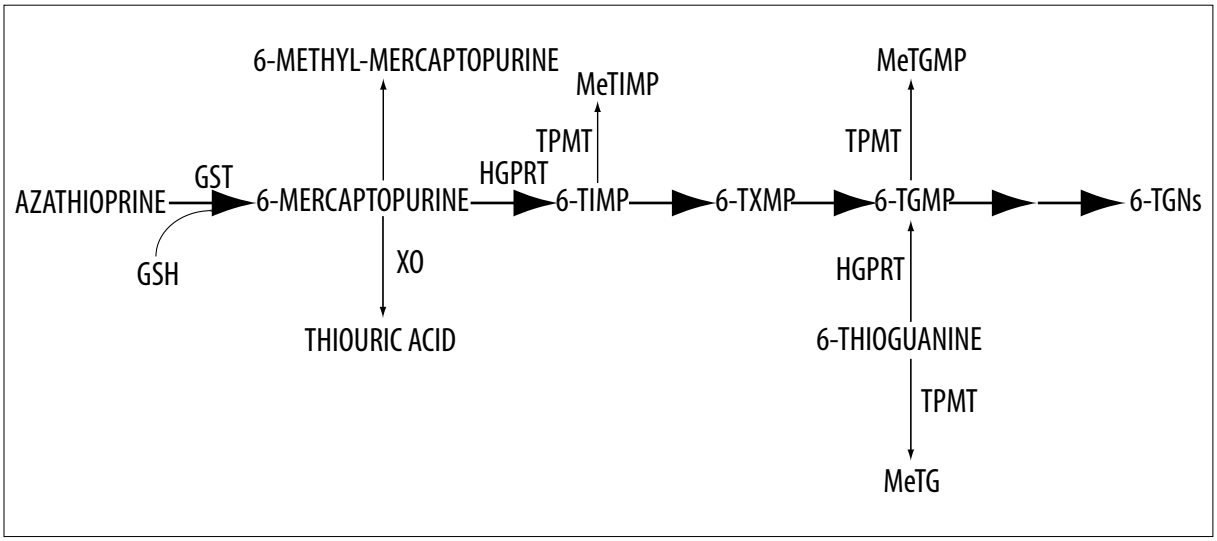


**Figure 1.** Thiopurines (azathioprine, 6-mercaptopurine and 6-thioguanine) metabolic pathway, delivering 6-thioguanine nucleotides, largely considered the active metabolite.GST – glutathione-S-transferase; GSH – glutathione; XO – xanthine oxidase; TPMT – thiopurine methyl transferase; 6-MeMP – 6-methylmercaptopurine; HGPRT – hypoxanthine guanine phosphoribosyl transferase; 6-TIMP – 6-Thioinosine monophosphate; MeTIMP – methyl Thioinosine monophosphate; 6-TXMP – 6-Thioxanthine monophosphate; 6-TGMP – 6-Thioguanine monophosphate; MeTGMP – methyl Thioguanine monophosphate; 6-TGN's – 6-thioguanine nucleotides; MeTG – methyl Thioguanine.

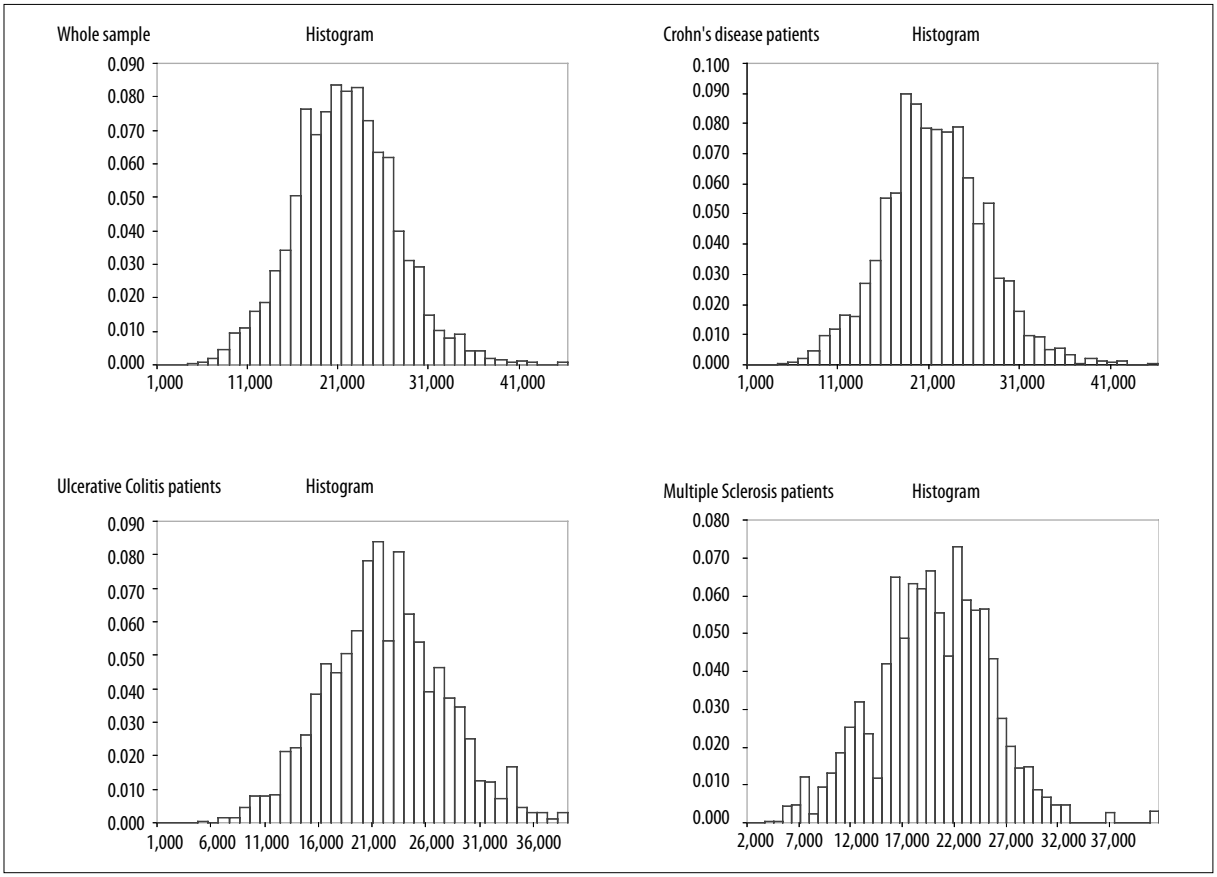


**Figure 2.** Red blood cell (RBC) TPMT activity (IU/ml packed RBC) frequency distribution histograms in a Spanish patient series. Charts include: whole sample (21.83±5.59 IU/ml pRBC; mean ±SD) and subgroups suffering from Crohn's disease (21.68±5.54 IU/ml pRBC; mean ±SD), ulcerative colitis (22.23±5.45 IU/ml pRBC; mean ±SD) and multiple sclerosis (20.04±5.46 IU/ml pRBC; mean ±SD). The multiple sclerosis group differs significantly from the Crohn's Disease ($p<0.0001$) and ulcerative colitis ($p<0.0001$) groups. Student's test was used in the study of significance.

thyl-thioinosine 5´-monophosphate (MeTIMP), a strong inhibitor of purine *de novo* synthesis (PDNS) [18,19]. Inhibition of PDNS is a well-established protocol to achieve immunosuppression and to block proliferation of various types of lymphocyte lines [20]. Thus it seems likely that significant PDNS inhibition by MeTIMP can be achieved *in vivo* after oral thiopurines and can contribute to the cytotoxic action of AZA/6-MP [21]. In this context, AZA/6-MP could produce immunosuppression in two ways; a) generation of 6-TGNs (with pro-apoptotic effects) and b) production of 6-MeTIMP (with antimetabolic effect) (Figure 3). Previous studies have demonstrated that in patients with wild-type TPMT activity who received mercaptopurine [22], MeTIMP is produced in vast excess compared with 6-TGNs. Although TPMT-deficient patients must be treated with markedly lower doses of thiopurines to avoid toxic concentrations of 6-TGNs [23], these patients do not form 6-MeTIMP and can tolerate considerably higher 6-TGN concentrations than patients with high TPMT activity [24]. Interestingly, patients receiving 6-TG tolerate much higher 6-TGN concentrations than patients receiving AZA/6-MP [22]; patients treated with 6-TGNs form no 6-MeTIMP and the ratio of 6-MTIMP to 6-TGNs is lower compared with patients treated with AZA/6-MP [21]. Together, these data indicate that AZA/6-MP could be considered as a "two-in-one" drug [25], providing a source of 6-TGNs and 6-MeTIMP, and both types of compounds could contribute to the immunosuppressive effects.

These results obtained in patients have been corroborated by studies performed *in vitro* [26] Cells expressing high TPMT were more sensitive to the effects of 6-MP than 6-TG; in contrast, cells expressing low TPMT activity were more sensitive to the effects of 6-TG than 6-MP. Because near 90% of patients phenotypically show high TPMT activity, the administration of AZA/6-MP could represent an advantage compared with 6-TG, because AZA/6-MP provides a source of active 6-MeTIMP, which could compensate for the lower 6-TGN levels observed in patients with high TPMT.

## THIOPURINE DRUGS AND APOPTOSIS

### Apoptosis: a review

Programmed cell death, or apoptosis, is a physiological process which selectively deletes cells whose function is no longer required or whose continued presence may have deleterious consequences in the host tissue [27]. It plays indispensable roles in embryogenesis, adult tissue homeostasis, the regulation of the immune system, and the development of the nervous system, but it can also contribute to the pathogenesis of a number of human diseases when dysregulated [28]. Apoptosis involves shrinkage, nuclear disassembly, and fragmentation of the cell into discrete bodies with intact plasma membranes. Neighboring cells rapidly phagocytize these bodies. An important feature of apoptosis is the requirement for ATP (adenosine triphosphate) to initiate the execution phase [29]. In contrast, cell swelling and lysis characterize necrotic cell death. As a consequence, lytic release of cellular constituents promotes a local inflammatory reaction, whereas the rapid removal of apoptotic bodies minimizes such a reaction.

For apoptosis, three phases of cell death can be distinguished: initiation, decision, and execution. Initiators of

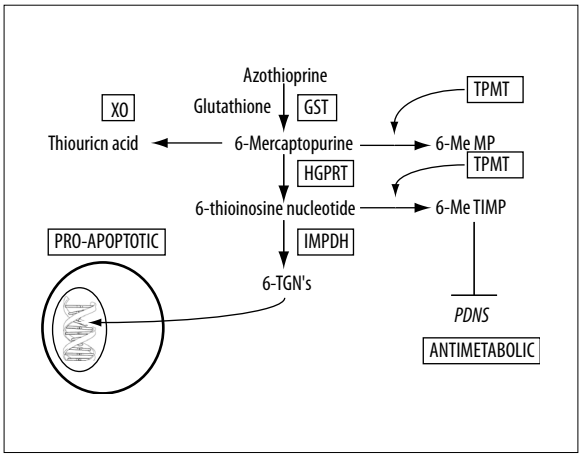


**Figure 3.** Proposed thiopurine metabolic pathway and mechanisms of action. Incorporation of 6-TGN into DNA induces morphological distortions and alterations in the mismatch repair system with consequent apoptosis. Inhibition of purine *de novo* synthesis by methylated ribonucleotides exerts an antimetabolic effect. XO – xanthine oxidase; GST – glutathione-S-transferase; TPMT – thiopurine methyl transferase; 6-MeMP – 6-methylmercaptopurine; HGPRT – hypoxanthine guanine phosphoribosyl transferase; 6-MeTIMP – 6-methyl Thioinosine monophosphate; IMPDH – inosine monophosphate dehydrogenase; 6-TGN's – 6-thioguanine nucleotides; PDNS – purine *de novo* synthesis.

apoptosis include anticancer drugs, gamma and ultraviolet irradiation, deprivation of survival factors such as interleukin-1, and various other cytokines that activate "death receptors", such as Fas and TNF receptors. Two major signaling pathways control apoptosis initiation in mammals, and both involve the participation of specialized proteolytic enzymes, caspases, which contain cysteine at the active sites and cleave the aspartate [30]. The cell-extrinsic pathways involve engagement of cell-surface death receptors by ligands that belong to the tumor necrosis factor (TNF) superfamily and consequent activation of caspase-8 [31]. The cell-intrinsic pathway involves mitochondrial membrane disruption by pro-apoptotic Bcl-2 family members and consequent release of factors, such as cytochrome c, that promote activation of caspase-9 [32]. These caspase activations culminate in cleavage of a set of proteins, resulting in a characteristic pattern of gene expression and cell disassembly.

Selective mitochondrial membrane permeabilization (MMP) is an important mechanism underlying cell death [3]. Mitochondria are organelles with two well-defined compartments: the matrix, surrounded by the inner membrane (IM), and the intermembrane space, surrounded by the outer membrane (OM). The IM contains the protein complexes from the electron transport chain, the ATP synthase and the adenine nucleotide translocator (ANT). To function properly, the IM is almost impermeable in physiological conditions, allowing the respiratory chain to create an electrochemical gradient, indispensable for driving ATP synthesis. Mitochondrial disruption culminates in a rapid loss of this mitochondrial membrane potential and cytochrome c release. Members of the Bcl-2 family act in the mitochondrial membrane to regulate mitochondrial integrity [34].

The members of this protein family can be divided into anti-apoptotic proteins (such as Bcl-2 and Bcl-$X_L$) and pro-apoptotic proteins (e.g. Bax and Bad). Members that inhibit apoptosis prevent mitochondrial damage, whereas members that promote cell death act by inducing mitochondrial membrane permeabilization. The balance between both proteins determines whether the cell lives or dies.

Recent work has identified Bax protein as a trigger or amplifier of cell death, and Bcl-2 appearing to regulate Bax [35]. By suppressing Bax activity, its function is down-regulated and neutralized, hence cells are protected from cell death. It is therefore likely that expression of Bcl-2/Bax protein ratios may be important factors in the regulation of apoptosis. The control of lymphocyte death/apoptosis is clearly vital to the regulation of the immune system. Too much apoptosis may result in immunodeficiency and a risk of infections, too little in autoimmune disease.

Apoptosis has been claimed as the final mechanism by which thiopurines exert their immunosuppressive effect [36]. Very recently, the mechanism to induce apoptosis in CD28 co-stimulated CD4+ lymphocytes has been fully explained.

## A TALE OF TWO SITES

### Alterations in DNA

It was recently reported that the DNA mismatch repair system (MMR) participates in the recognition and processing of deoxythioguanosine ($dG^S$) incorporated into DNA [37] and that mismatch repair complex (Mut Sα)-deficient cell lines are more resistant to thiopurines [38]. Although thioguanine incorporation into DNA is considered mandatory for thiopurine cytotoxicity, it has been shown that the level of $dG^S$ incorporation is not directly related to the extent of cytotoxicity in human lymphoblastic leukemia cells [39]. Previous findings indicate that the alterations in DNA structure caused by $dG^S$ incorporation into DNA likely contribute to the pharmacological effects of thiopurines [40]. Therefore it has been hypothesized that specific cellular proteins detect subtle changes in DNA structure, thereby triggering a chain of biochemical events that culminates in cytotoxic-insensitive cells. In contrast to previous reports that MMR-deficient colorectal carcinoma, endometrial carcinoma, and Burkitt's lymphoma cell lines are resistant to thioguanine [41], it has been shown that two of the most purines-sensitive human lymphoblastic leukemia cell lines, CEM and Nalm6, lack one or more components of MMR [42]. Very recently it has been found that MSH2 protein (a major component of the MMR system) deficiency attenuates but does not abolish hematopoietic toxicity *in vivo* after thiopurine therapy [43]. These findings indicate that MMR protein expression is not the only determinant of thiopurine sensitivity in human cells, and that MMR-deficient cells can be highly sensitive to thiopurines.

Moreover, electrophoretic mobility shift assay (EMSA) experiments with nuclear extracts from human leukemia cells have revealed protein complexes distinct from the MMR complex that interact with duplex DNA containing either $dG^S$.T- or $dG^S$.C-pairs. This complex is distinct from MutSα (fraction B) because it is present in MMR-deficient as well as MMR-proficient cells, and its interaction with DNA is not sensitive to ATP. Subsequent biochemical isolation of this new protein complex identified GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as one of its components. GAPDH is a well-known example of a multifunctional enzyme, with DNA repair as one of its functions [44]. Previous reports demonstrated intranuclear translocation of GAPDH and its participation in apoptotic pathways after cytotoxic treatment [45]. Consistent with this report, Krynetski et al. observed translocation of GAPDH into the nucleus after 6-MP treatment; their work establishes GAPDH as a part of the protein complex interacting with DNA modified by thioguanine incorporation, indicating a role of this protein in a broader spectrum of cytotoxic agents. More recently, Krynetski et al. hypothesized that this new multiprotein nuclear complex. containing HMGB1, HMGB2, HSC70, Erp60 and GAPDH, binds $dG^S$-modified DNA with substantially (4–5 fold) higher affinity than DNA containing only natural nucleotides, recognizing DNA distortion rather than formation of specific mispairs [46]. In addition, the activities of Rnase H [47], DNA ligase [48], and topoisomerase II [49], key enzymes involved in DNA repair and replication, have been shown to be significantly altered in the presence of nucleic acid substrates containing a single thioguanine modification opposite cytosine.

### The mitochondrial pathway

Current knowledge about the molecular sites of action of AZA in nucleic acid modifications cannot fully explain the effects of the drug on the immune system.

CD28 co-stimulation during T-cell activation results in increased proliferation [50], decreased activation-induced cell death (AICD) [51], and improved long-term lymphocyte survival [52], based on a peak of Bcl-XL expression at 24–48 h with a subsequent decline [53].

A recent, very elegant paper has demonstrated that AZA and its metabolite 6-MP and 6-TG induce apoptosis of human CD4+ T lymphocytes only when the cells are co-stimulated via CD28 [54]. Functional studies showed that AZA uses a caspase-9-dependent mitochondrial pathway that involves down-regulation of Bcl-xL and suppression of the mitochondrial membrane potential. Caspase 9 activation was essential for AZA-induced apoptosis, as blockade of caspase-9 blocked AZA-induced apoptosis. Finally, it has been observed that the AZA metabolite 6-thio-GTP binds to Rac1, a Rac GTPase crucial in MEK/NF-κB activation in T cells upon CD28 co-stimulation. Further studies showed that Bcl-xL down-regulation was due to suppression of NF-κB p65 subunit activity. These results suggest that Rac-dependent activation of the MEK kinase pathway is the molecular target mechanism of AZA. Dahl et al. [55] demonstrated that, in the absence of CD28, Bcl-XL expression prolonged lymphocyte survival, but did not restore normal proliferation or effector cell development. This suggests that the proliferation and survival signals generated by ligation of CD28 are separated and remained consistent with the hypothesis that specific induction of Bcl-XL by CD28 co-stimulation results in enhanced activated T-cell survival.

## THE MODEL

A significant group of IBD patients respond adequately to immunosuppressant therapy with AZA/6-MP, while others

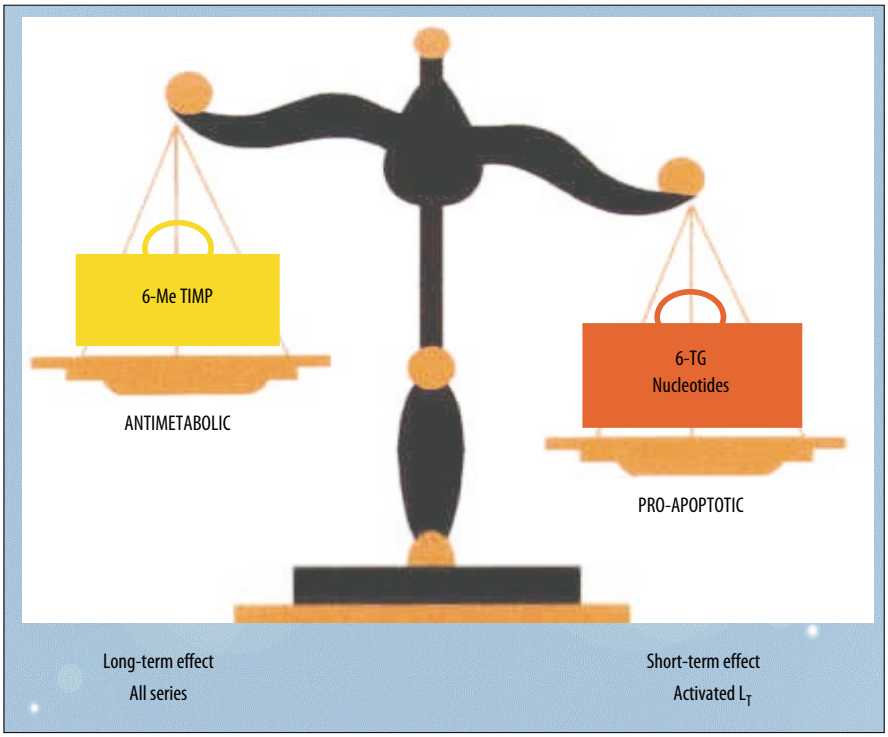


**Figure 4.** The balanced contribution of different metabolites to the immunosuppressive effects of thiopurine derivatives. Pro-apoptosis is a short-term effect induced by 6-TGN's, mainly in activated T cells. In an anti-metabolic manner, methylated ribonucleotide (6-MeTIMP) production acts by blocking, in the long--term, cell proliferation through inhibition of ATP and GTP *de novo* biosynthesis. The lower the antimetabolic pathway is, the higher the apoptotic way should be in order to keep the thiopurine immunosuppressive effect operational.present with several small gallstones (arrow).

Table 1. Thiopurine optimization: suggested schedule. Expected clinical pharmacology outcomes from our model, based on pre-treatment TPMT monitoring. Azathioprine recommended dosage is included for every TPMT activity sub-group. Due to lack of evidence in some areas, this schedule should be only considered as a proposal.

| TMPT activity | Aza recommended dose | Major active metabolite | Mechanism of action | Therapeutic 6-TGN levels | Potential nielotoxicity | Malignancy risk |
|---|---|---|---|---|---|---|
| Very high (>26.1 IU/ml) | 3.0 mg/kg/day | Methylated ribonucleotides | Antimetabolic | Low | Delayed | Low |
| High (18.1–26 IU/ml) | 2.5 mg/kg/day | Methylated ribonucleotides | Antimetabolic | Low | Delayed | Low |
| Intermediate (13.8–18 IU/ml) | 1.5 mg/kg/day | | | | | |
| Low (5.1–13.7 IU/ml) | 0.5 mg/kg/day | 6-TGN | Apoptotic | High | Acute | High |
| Very low (<5 IU/ml) | 0.125 mg/kg/day | 6-TGN | Apoptotic | High | Acute | High |

with apparently similar patterns do not improve. As mentioned above, no correlation between 6-TGN levels, TPMT activity, AZA/6-MP dose, white blood cells count, and disease activity or remission has been recently shown by some authors [11]. In order to provide a full explanation for the range of results obtained after AZA/6-MP administration, a molecular mechanism for their action and metabolism is needed. Therefore, further to our review, a triple mechanism to explain the immunosuppressive effect of AZA is proposed: a) the induction of a very specific apoptotic pathway in the CD4+ subset of CD28 co-stimulated lymphocytes. In this group of cells the impairment of the Rac-1 cascade by 6-Thio-GTP binding produces Bcl-xL down-regulation and subsequent apoptosis; b) the distortion in DNA and the impairment in its repair system produced by incorporation of deoxythioguanosine ($dG^s$) induces the activation of non-specific apoptotic pathways in proliferating lymphocytes and, finally, c) the inhibition of ATP and GTP *de novo* biosynthesis by methylmercaptopurine nucleotides (mainly MeTIMP), affecting non-stimulated cells, contributes to the immunosuppressive properties of AZA/6-MP.

Based on this model we can hypothesize that the immunosuppressive effects of AZA/6-MP are achieved from a balanced contribution of pro-apoptotic (6-TGN's) and antimetabolic (methylated ribonucleotides) pathways (Figure 4). This model clarifies why such different 6-TGN levels can be found in thiopurine-responsive patients, and this probably depends of how important TPMT activity is in providing anti-metabolic derivatives to collaborate with pro-apoptotic 6-TGN's. The lower the TPMT activity, the higher the 6-TGN levels needed to reach clinical response. Also, this

proposal fits with the delayed leukopenia observed in patients receiving thiopurines for a long time, with no hematological events in the near-term. In these individuals, 6-TGN levels do not reach the point to reduce autoreactive lymphocytes, but accumulation of methylmercaptopurine nucleotides induces a chronic depletion of *de novo* purines, able to stop leukocyte proliferation.

From our findings [56] we routinely recommend TPMT activity monitoring to initiate the dosing of AZA, mainly due to a safety issue, to rule TPMT-deficient patients out from purine therapy (Table 1). Only in non-responders to traditional doses of AZA in clinical trials (2–3 mg/kg/day) is the monitoring of 6-TGN and methylmercaptopurine nucleotide levels advisable to identify the resistant phenotype to AZA/6-MP characterized by a high ratio of 6-MeTIMP to 6-TGNs. In this case a 6-TG treatment seems advisable to reach a therapeutic response.

## CONCLUSIONS

In conclusion, our review provides new insights into the cytotoxicity of thiopurines and suggests a rationale for the contradictory results obtained with the use of different thiopurine pro-drugs (AZA, 6-MP, or 6-TG) and recent monitoring methods (TPMT activity, 6-MMP, and 6-TGN level) intended to optimize these therapies.

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