Thiopurine Therapies

Problems, Complexities, and Progress With Monitoring Thioguanine Nucleotides

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Abstract: Metabolism of thiopurine drugs—azathioprine, 6-mercaptopurine, and 6-thioguanine—has provided a powerful pharmacogenetic model incorporating polymorphism of the enzyme thiopurine methyltransferase (TPMT) and the primary active metabolite, thioguanine nucleotide (TGN). However, a sense of uncertainty about the usefulness of TGNs and other thiopurine metabolites has appeared.

This review critically appraises the basis of thiopurine metabolism and reveals the problems and complexities in TGN research. Erythrocyte TGN is used in transplantation medicine and in chronic inflammatory conditions such as Crohn's disease, as a "surrogate" pharmacokinetic parameter for TGN in the target cells: leukocytes or bone marrow. It is not generally appreciated that erythrocytes do not express the enzyme IMP dehydrogenase and cannot convert mercaptopurine to TGN, which explains some of the confusion in interpretation of erythrocyte TGN measurements. TGN routinely measured in erythrocytes derives from hepatic metabolism. Another concern is that TGN are not generally assayed directly: most methods assay the thiopurine bases. Ion-exchange HPLC and enzymatic conversion of TGNs to nucleosides have been used to overcome this, and may reveal undisclosed roles for an unusual cytotoxic nucleotide, thioinosine triphosphate, and methylated thiopurines. There appear to be additional interactions between xanthine oxidase and TPMT, and folate and TPMT, that could predict leukopenia. Difficult questions remain to be answered, which may be assisted by technological advances. Prospective TGN studies, long overdue, are at last revealing clearer results.

Key Words: azathioprine, 6-mercaptopurine (6MP), thioguanine, thiopurine methyltransferase (TPMT), inosine monophosphate (IMP), inosine triphosphate pyrophosphohydrolase (ITPase), xanthine oxidase, folate, S-adenosylmethionine (SAM)

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Therapeutic drug monitoring of thiopurine therapy was initially limited to plasma 6MP and urinary excretion of the xanthine oxidase metabolite thiouric acid.[1–3] The latter was useful for compliance assessment but did not provide any indication of therapeutic response. Development of an assay of thioguanine nucleotides (TGN) in red blood cells was an advance in monitoring thiopurine therapy.[4–6] This arose from an appreciation of the metabolic pathways for thiopurines, which utilize the salvage pathways of endogenous purines.[7]

Coincident with the assay of TGN were the first descriptions of polymorphic variation in the activity of thiopurine methyltransferase (TPMT), which inactivates thiopurines, and its role in life-threatening leukopenia experienced by some patients on thiopurines.[8–10] An inverse relationship was demonstrated between erythrocyte TPMT activity and TGN levels, and, importantly, high erythrocyte TGN levels correlated with bone marrow toxicity in TPMT deficient patients receiving thiopurines. This demonstrated a direct link between TGN and therapeutic effect.[8,11,12] With subsequent descriptions of TPMT mutations and their effects on drug response, thiopurines have provided an important model for pharmacogenetics, with TPMT and TGN representing primary parameters to establish the viability of this model.[13–17] As a result, TGN has been studied by many laboratories worldwide and extensively reviewed.[18–29] Assay of erythrocyte TGN has become commonly used as a "surrogate" pharmacokinetic parameter for intracellular levels of TGN in target cells, ie, peripheral blood leukocytes or bone marrow precursors.[15,30] At the very least, this has proved useful for assessing thiopurine compliance and confirming severe thiopurine toxicity in cases of complete TPMT deficiency.[31,32] But although it has been suggested by some groups that TGN can be used to monitor response reliably,[19,33–35] it has become increasingly apparent that erythrocyte TGN levels are highly variable, even in the same patient, and that they do not reliably provide sufficient information to predict efficacy or toxicity.[28,36–38] A sense of uncertainty about differences between TGN methodologies and applications for measuring erythrocyte TGNs and other thiopurine metabolites has arisen.[39–43]

ERYTHROCYTE NUCLEOTIDE SYNTHESIS IS LIMITED TO SALVAGE PATHWAYS

Before speculating on possible clinical roles for erythrocyte TGN and other thiopurine metabolites, an appreciation of

purine metabolic pathways and the unusual nature of erythrocyte nucleotide synthesis is essential (Fig. 1).

Purine nucleotides are synthesized in nucleated cells by an energetically expensive de novo pathway to form the mono-nucleotide inosine monophosphate (IMP). But nucleotide levels can also be maintained by salvage of the endogenous purine bases, hypoxanthine and guanine, via phosphoribosyl-transferase activity (HPRT) to form IMP and guanosine mono-phosphate (GMP), respectively. IMP is a central nucleotide. It is converted to the adenosine nucleotides (AMP/ADP/ATP) via succinyl-AMP synthase and adenylosuccinate lyase, and to the guanosine nucleotides (GMP/GDP/GTP) via IMP dehy-drogenase and GMP synthase, and to their deoxy equivalents via ribonucleotide reductase. Adenosine (and deoxyadenosine) can be salvaged by adenosine kinase or degraded by adenosine deaminase, and deoxyguanosine can be utilized by mitochon-drial kinases. The base adenine, a by-product of polyamine synthesis, is salvaged by adenine phosphoribosyl-transferase (APRT). Nucleotides are broken down to the bases by nucle-otidases and phosphorylases. Guanine and hypoxanthine bases that are not salvaged are catabolized by guanase and xanthine oxidase to form uric acid as an endpoint of purine metabolism in humans.

On the other hand, the mature erythrocyte, lacking a nucleus, does not have full purine functionality. The de novo synthetic pathway is not present. Nucleotide salvage is also limited: erythrocytes provided with radiolabeled hypoxanthine under physiological conditions in vitro salvage it to form ra-diolabeled IMP but are unable to further metabolize this to AMP or GMP because of absence of adenylosuccinate synthase and IMP dehydrogenase, respectively.[44,45] Erythrocytes also do not produce deoxy nucleotides and lack xanthine oxidase to pro-duce uric acid. Despite these metabolic deficiencies, the erythrocyte maintains high levels of ATP, GTP, and other nucleotides. Erythrocyte ATP does not rely on adenine salvage via APRT because patients with inherited APRT deficiency exhibit normal erythrocyte ATP levels. In contrast, patients with inherited HPRT deficiency show reduced levels of eryth-rocyte guanosine nucleotides.[46] The principal mechanisms left for maintaining purine nucleotides in erythrocytes are thus salvage of adenosine (for ATP) and guanine (for GTP) (Fig. 1): these 2 purines are presumably absorbed by the erythrocyte during its circulation through other tissues. Surprisingly, IMP does not accumulate in erythrocytes in vivo,[47] leading to the hypothesis that the erythrocyte may act as a "carrier" for hypoxanthine.[48] This may be directly relevant to TGN levels, as explained below.

SOME PARADOXES EXPLAINED AND PREDICTIONS MADE

Monitoring TGN and Why Erythrocytes Are Not an Ideal Cell for This

6TG is a guanine analogue, and erythrocyte purine sal-vage pathways can readily convert this to thio-GMP and TGN via HPRT (Fig. 2).[49] On the other hand, 6MP is a hypoxanthine analogue. Although it is "salvaged" by HPRT to form thio-IMP, IMP dehydrogenase is required to form thio-GMP and TGN. (Deoxythioguanosine and perhaps thioguanosine may also be salvaged by deoxyguanosine kinase.)

Monitoring leukocyte TGN in patients on 6MP/AZA makes metabolic sense because there is an active route, via IMP dehydrogenase, for TGN synthesis in these cells (Fig. 2). But the serious flaw in the concept of using erythrocyte TGN as a "surrogate" marker for 6MP/AZA drug monitoring is that the erythrocyte lacks constitutive IMP dehydrogenase and can-not convert 6MP to TGN. The erythrocytes of patients on 6MP/AZA must therefore make TGN indirectly, by absorbing 6TG from tissues that are capable of converting 6MP to 6TG via the circuitous nucleotide route shown in Figure 2.[50] It has been previously suggested that erythrocyte TGN may be formed from thiopurine "metabolites" formed in hepatic and nonhepatic tissues,[51] and 6TG has been shown to accumulate in vitro following incubation of liver extracts with 6MP.[52] Despite these problems with the origins and control of eryth-rocyte TGN, some authors have proposed (and patented) a closely defined "normal" therapeutic range for erythrocyte TGN.[53,54] We suggest, however, that erythrocyte TGN levels are more likely to reflect integration of hepatic metabolism rather than target white cells, and thus, any "normal" result does not necessarily reflect the immunomodulated status of the patient.
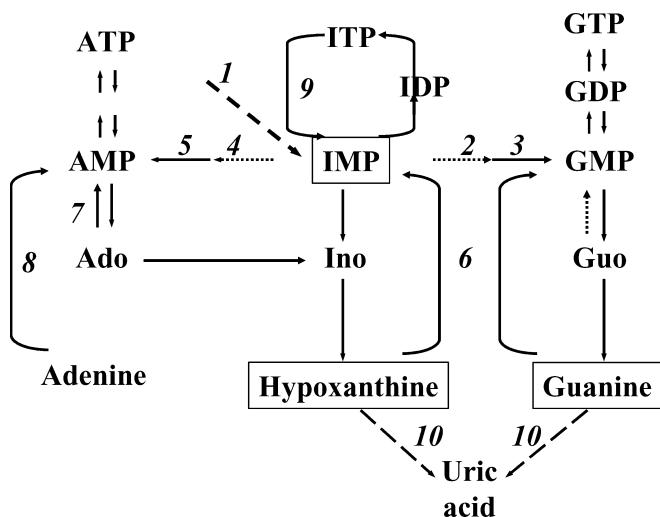

FIGURE 1. Synthesis and degradation of endogenous purine nu-cleotides. (1) De novo synthesis of IMP; (2) IMP dehydrogenase and (3) GMP synthase convert IMP to GMP, or (4) succinyl-AMP synthase and (5) adenylosuccinate lyase convert IMP to AMP. (Steps 1, 2, and 4 are normally absent in erythrocytes). Salvage occurs via (6) hypoxanthine-guanine phosphoribosyl-transferase/HPRT, (7) adenosine kinase and (8) adenine phosphoribosyl-transferase/APRT; kinases convert AMP, GMP, and IMP to di- and tri-phosphates. (9) ITPase returns ITP to IMP; nucleotidases and phosphorylases catabolize nucleotides to bases. (10) Guanase and xanthine oxidase then break down hypoxanthine and guanine; deoxynucleotides are produced via ribonucleotide reductase (not shown). Abbreviations: AMP/ADP/ATP, adenosine mono-/di-/tri-phosphate nucleoti-des; GMP/GDP/GTP, guanosine nucleotides; IMP/IDP/ITP, inosine nucleotides; Guo, guanosine; Ado, adenosine; Ino, inosine.
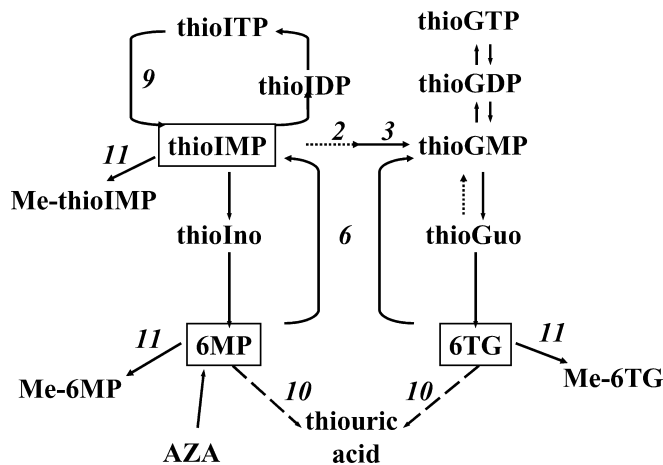
FIGURE 2. Synthesis and degradation of thiopurine nucleotides. 6MP and 6TG are "salvaged" by (6) HPRT to thio-IMP and thio-GMP; thio-IMP is converted to thio-GMP via (2) IMP dehydrogenase and (3) GMP synthase; kinases convert thio-GMP and thio-IMP to di- and triphosphates; thio-ITP may be a substrate for (9) the futile ITPase cycle; deoxythionucleotides are produced via ribonucleotide reductase (not shown); 6MP and 6TG are also catabolized via (10) xanthine oxidase or (11) thiopurine methyltransferase/TPMT; thio-GMP may also be methylated by TPMT (not shown); (deoxy)thioguanosine may be salvaged by deoxyguanosine kinase or catabolized to 6TG. Abbreviations: AZA, azathioprine; 6MP, 6-mercaptopurine; 6TG, 6-thioguanine; thio-GMP/GDP/GTP, thioguanosine mono-/di-/triphosphate; thio-IMP/IDP/ITP, thioinosine nucleotides; thio-Ino, thio-Inosine; thio-Guo, thioguanosine; Me, methyl-.

Why TPMT Heterozygosity May Cause Early Nausea and Late-Onset Leukopenia

The conversion of 6MP to TGN in erythrocytes by a more circuitous metabolic route explains the poor correlation seen among 6MP dosage, erythrocyte TGN, and clinical effects, particularly bone marrow toxicity.[51] This would also explain why HPRT has not been found to correlate with erythrocyte TGN levels because the rate-limiting steps would occur in the conversion of 6MP to 6TG.[55] Nonetheless, it has been clearly established that in the extreme case of completely TPMT-deficient patients given normal-dose 6MP/AZA, there is a good correlation between high erythrocyte TGN and severe bone marrow suppression.[18,31,32,43]

An early study reported an inverse relationship of TPMT activity versus TGN levels and clinical response.[8] In keeping with this putative relationship, some studies have found heterozygous or intermediate TPMT levels associated with higher TGN levels and improved response.[56,57] Other studies have failed to find any significant correlation between heterozygous TPMT levels and TGN levels or outcome.[34,56,58,59]

Toxicity will be increased in a tissue when and where TGN levels are raised. Thus, the mode of drug delivery should determine the side effects experienced. Early studies concentrated on leukemic patients who received intravenous 6MP, for which the most common side effect was bone marrow suppression. Because first-pass metabolism is important, we

would predict that heterozygous patients receiving full-dose oral thiopurine would be at higher risk of initially developing gastrointestinal toxicity and nausea. This appears to be the case for Crohn's disease and rheumatoid arthritis patients, where it has been reported that nausea is a primary side effect experienced by patients with reduced TPMT activity.[28,60–63] From this, we would predict that heterozygotes would be more likely to withdraw from therapy, but those who overcome the nausea and persist with a full-dose regimen may develop late-onset bone marrow suppression and other side effects.[29,56,60] Oral (versus intravenous) delivery of 6MP/AZA would also be predicted to be associated with increased risk of hepatotoxicity, and this has also been reported in at least 1 study (see below: ITPase metabolites).

Why Erythrocyte TGN Is Much Higher in Patients on 6TG Without Causing Leukopenia

The scheme shown in Figure 2 also explains the differences in TGN profiles between patients on 6TG and those on 6MP. It has been recommended that patients who prove intolerant to 6MP may benefit from 6TG therapy.[49,64,65] Erythrocytes of patients on 6TG accumulate far higher apparent levels of TGN than for corresponding doses of 6MP.[38,49,51,65,66] We propose that this is because erythrocytes are able to directly incorporate 6TG into TGN, in contrast to the indirect route for TGN incorporation with 6MP. This also means that to achieve equivalent levels of TGN with 6MP, the tissues converting 6MP to 6TG must themselves be subjected to very high TGN levels and will thus be prone to toxicity.

Leukocytes are capable of making TGN directly from 6MP, and this fact is supported by the observation that equivalent doses of 6MP and 6TG produce similar TGN levels in lymphoblastic leukocytes. Thus, peripheral blood leukocytes more closely reflect the TGN exposure in other tissues including bone marrow stem cells.

PROBLEMS WITH MEASURING TGN

The serious weakness in the methods for assaying TGN by most laboratories is that they do not directly measure the thiopurine nucleotides. Most "TGN" studies measure the thiopurine bases derived from TGN by reversed-phase HPLC technology but report these as the nucleotides. There are a number of technical concerns about the validity of these methods.

First, it is presumed that the breakdown of the nucleotides by acid hydrolysis—a common procedure in the methods—has a high efficiency. But this does not appear to be confirmed against known nucleotide standards in many laboratories because of the lack of commercially synthesized TGN and of standardization of locally synthesized TGN. Second, concern has been raised[67] about differences among methodologies used to assay "TGN" by pointing to a lower recovery from the original acid hydrolysis method of Lennard compared with that of Dervieux and Boulieu.[4,68] This concern has been answered in depth.[69] However, the problem remains that the actual TGN level is unknowable by either acid hydrolysis method. Third, a serious concern also arises when these methods report "TGN" in patients who are receiving

6TG because there can be no discernment between unmetabolized drug being carried in erythrocytes or leukocytes and the hydrolysis derivatives of TGN. Values for "TGN" reported in patients on 6TG using acid hydrolysis methods must therefore be regarded as contentious until proved otherwise.

Two methodologies have attempted to overcome these weaknesses. An ion-exchange HPLC method capable of direct assay of a range of thiopurine nucleotides has been used to describe TGN distribution in leukemic lymphocytes.[70] This used locally synthesized nucleotide standards for the three TGN forms, thio-GTP/GDP/GMP. The method was replicated in the Purine Research Laboratory at Guy's Hospital, London, and results were reported.[71] The clinical significance of this assay is that it can discern (1) TGN from 6TG, and so it can be used for patients receiving 6TG drug; and (2) thio-ITP from 6MP, and may thus be useful to study toxicity in ITPase-deficient patients (see below). But the method is not simple to maintain and requires nucleotide standards that are not easily obtainable.

More recently, a method has been described for indirectly assaying thiopurine nucleotides by enzymatically converting the TGNs to nucleosides rather than bases, which are then analyzed by reverse-phase HPLC.[72] This method has been established at our laboratory in Brisbane. Although this does not differentiate between thio-GTP/GDP/GMP, which merely reflect the energy charge of the cell, it does provide more direct evidence of TGN and other thionucleotide levels. In particular, the erythrocyte level of thioinosine can be taken as a measure of thio-ITP (because thio-IMP, like IMP, does not normally accumulate in erythrocytes in vivo) and as such may be the best hope for confirming the presence of this unusual nucleotide (see below). The method also discerns methyl-thio-IMP from methyl-6MP and methyl-thio-GMP from methyl-6TG (see "Methylated Thiopurines").

From a laboratory viewpoint, it is currently unattractive to measure TGN in the target cells, ie, leukocytes because their purification is tedious and requires a greater volume of blood—a practical consideration with pediatric patients. However, ideally TGN levels should be assayed directly in nonstimulated peripheral leukocytes or leukemic cells.[72]

Related to this, a fourth and unresolved problem with measuring TGN arises from the preparation and identity of the cells used for assay. Red and white cell nucleotides are labile and subject to degradation soon after a blood sample is taken,[47] the degree of degradation being a function of factors such as time, the anticoagulant used, and temperature of storage. One group has made a study of this,[73] but in the absence of any standard procedure for blood collection and handling, TGN ranges between laboratories will not necessarily be comparable. In addition, laboratory preparations of "erythrocytes" typically contain highly varying levels of contamination by leukocytes, and this could significantly affect TGN assays. We have found that in vitro incubation of "erythrocytes" with 6MP yielded primarily methyl-thio-IMP, but a small amount of TGN was also found, revealing the contribution of leukocyte contamination (unpublished data). In contrast, leukocyte fractions, though tedious to prepare, are usually relatively free of erythrocytes, although again the level of contamination may vary significantly between laboratories depending on the methods used.

CLINICAL SIGNIFICANCE OF OTHER THIOPURINE NUCLEOTIDES

Methylated Thiopurines

Although early pharmacogenetic research demonstrated the relationship among low TPMT, high TGN, and bone marrow toxicity, some studies also linked higher TPMT with nonresponsiveness to AZA[65,74] and from this association evolved the concept that higher methylation might correlate with nonresponse.[75]

Subsequently it has been reported that non-responsive patients who were dose escalated had a higher incidence of hepatotoxicity accompanied by high red cell "methyl-thio-IMP," a putative product of erythrocyte TPMT.[62,76] However, as with TGN, doubts have arisen concerning this interpretation. A relationship among dose escalation, increased erythrocyte methyl-thio-IMP, and hepatoxicity has not been confirmed by other key studies.[57,77,78] The usefulness of measuring red cell methyl-thio-IMP must also be questioned because "surrogacy" of erythrocyte methylated thiopurines has not been established for liver thiopurine toxicity. In fact, erythrocytes of patients receiving AZA/6MP appear to preferentially accumulate methyl-thio-IMP compared with other cells: this is 1 thiopurine metabolite that they do make efficiently, compared with leukocytes or liver.[38,52,66,79,80]

Interest in methyl-thio-IMP arose because of a possible immunosuppressive role: the molecule has been shown to inhibit purine de novo synthesis in vitro.[81,82] This effect, however, has not been demonstrated in vivo for patients receiving thiopurines (this could be achieved by measuring purine production as uric acid excretion). But a question also remains over which is the principal methylated metabolite of 6MP. Nearly all workers have used acid hydrolysis to produce methyl-6MP but report this as "methyl-thio-IMP." Only one report[79] has directly compared levels of methyl-thio-IMP (using enzymatic digestion of the methylated nucleotide to its nucleoside equivalent) with levels of the methyl-6MP base (assayed by the usual acid hydrolysis method). Surprisingly, this report concluded that methyl-thio-IMP was the only TPMT product in erythrocytes, and this conclusion has been widely quoted since. However, the reported relationship between methyl-thio-IMP and methyl-6MP was significantly different from equivalence, with the majority of patients having methyl-6MP levels higher than methyl-thio-IMP (in some patients this was almost double). This suggests that erythrocytes accumulate varying proportions of methyl-6MP and its nucleotide, methyl-thio-IMP, perhaps in response to genetic or environmental factors. This may be significant for leukocyte toxicity in view of the putative inhibition of de novo purine synthesis by methyl-thio-IMP rather than methyl-6MP.

Methylated thiopurines may have other clinical significance. "Methyl-thio-IMP" is produced by lymphoblastic cells during in vitro incubation with 6MP,[80,83] but as predicted by Figure 2, methyl-thio-GMP is not found. Although nodular regenerative hyperplasia, or veno-occlusive disease, is rarely observed with AZA/6MP therapy, it is a common enough side effect of 6TG therapy to cause concern.[64,84–86] We speculate that methyl-6TG (or methyl-thio-GMP), as a major metabolite of 6TG,[40] may be a causative agent or marker of this serious side effect.

ITPase Metabolites

The importance of developing a more direct assay for thiopurine nucleotides has been highlighted by the recent finding that polymorphic deficiency of the enzyme inosine triphosphate pyrophosphohydrolase (ITPase) is associated with significant side effects from AZA/6MP therapy, including pancreatitis, rash, and flu-like symptoms.[63] It was hypothesized that the side effects associated with ITPase deficiency result from accumulation in tissues of an unusual cytotoxic nucleotide, thio-ITP (Fig. 2). One retrospective study has failed to confirm this association,[87] and proof of this theory will depend on further prospective studies demonstrating thio-ITP accumulation. ITPase is also important because it provides a model for nucleotide analogues having organ-specific toxicity. This may explain the variety of side effects to thiopurines, including "allergic" pancreatitis observed in some individuals.

UNRESOLVED ISSUES AND FURTHER RESEARCH

The Physiological Role of TGN

Despite over 30 years of research, there is still a paucity of evidence for the physiological targets of TGN in vivo. A quoted range for therapeutic efficacy of erythrocyte TGNs in patients taking 6MP/AZA is 230–400 pmol/8 $\times$ 10^8 cells. Assuming an average erythrocyte volume of 90 fL, this equates to an intracellular concentration up to 6 μmol/L. TGN concentrations in erythrocytes of patients on 6TG have been reported to have medians of 650–1400 pmol/8 $\times$ 10^8 cells, ie, up to 20 μmol/L. In leukocytes of patients on 6MP/AZA or 6TG, TGN concentrations are typically reported to range from 1200 to 1500 pmol/8 $\times$ 10^8 cells, or up to 10 μmol/L assuming an average lymphocyte volume of 200 fL.

TGN has been shown to incorporate thiopurine bases into DNA,[88] which itself carries an increased risk of carcinogenesis,[34,89,90] and inhibition of DNA synthesis has been shown to be an effect of 6TG incorporation.[91] More recently, G-proteins such as Rac-1 have also been implicated in down-regulating immune function in the presence of TGN.[92] Significantly, the affinity constant (K_m) values for these proteins for guanosine nucleotides (for which TGNs are the drug analogues) are in the low micromolar range, compatible with observed concentrations in vivo calculated above.[93] Other reported gene expression targets include tumor necrosis factor-related apoptosis-inducing ligand, tumor necrosis factor receptor superfamily member 7, and α4-integrin.[94]

The target cells for TGN action have also surprisingly defied discovery. Thiopurine therapy was initially shown to reduce T-cell suppressor cells in a mouse model,[95] but a subsequent study in Crohn disease patients has found azathioprine produces an overall, less specific decrease in peripheral blood leukocytes and neutrophils,[96] as predicted by other mouse studies.[50] Apoptosis of proliferating lymphocyte clones would explain why intravenous loading of 6MP/AZA does not accelerate the pharmacodynamic effect of these drugs,[97] because the pharmacodynamic effect should be determined by which lymphocytes are activated at any particular time as well as by the level of TGN in the target cell.

The Enigma of Xanthine Oxidase

Other non-TGN mechanisms may also be at work. One possibility is a cytotoxic role for oxidative radicals. Xanthine oxidase/dehydrogenase and aldehyde oxidase provides additional pathways for 6MP/AZA breakdown.[50] While the role of aldehyde oxidase has not been studied, xanthine oxidase converts 6MP (and 6TG following guanase conversion to thioxanthine) to thiouric acid (Fig. 2) in human liver and gut (and to a lesser extent in the kidney). Allopurinol inhibits xanthine oxidase, thus theoretically increasing the conversion efficiency of 6MP to TGN. Bone marrow toxicity arising from co-administration of allopurinol and 6MP/AZA is well documented and this apparent increased efficacy has even been used as a basis for improving azathioprine response.[98] Furthermore, raised erythrocyte 'TGN' has been demonstrated in the patients receiving allopurinol.[99] The recommended 'rule of thumb' is to reduce 6MP/AZA dosage to a third or less of normal for a patient also receiving allopurinol.[100]

However, this rule does not fit quantitatively with the observation that patients with complete TPMT deficiency are extremely sensitive to thiopurines, showing excellent therapeutic response and high TGN levels on about 5% of normal dose. This predicts that about 95% of 6MP is normally catabolized by TPMT and contradicts the experience with allopurinol, which suggested that at least two-thirds of a normal dose is catabolized by xanthine oxidase.

Complex interactions may be at work. Using cultured liver cells, two independent groups have shown that thiopurine hepatotoxicity may be attributable to mitochondrial damage by superoxide free radicals generated by xanthine oxidase.[101,102] Consequent cytotoxic mechanisms include activation of cell apoptosis and immunogenic mechanisms including heat stress proteins. Interestingly, a role for oxidative damage by thiopurines was initially suggested from clinical studies using azathioprine in combination with allopurinol,[98] where increased bone marrow toxicity was found to be accompanied by decreased renal graft toxicity.

The tissue distribution of xanthine oxidase may also explain this effect. In liver, gut, and kidney (where xanthine oxidase activity is high) allopurinol may primarily protect cells from oxidative damage. But in bone marrow (where xanthine oxidase is very low or absent), the increased cytotoxicity of thiopurines in the presence of allopurinol may relate to reduction of first-pass metabolism resulting in improved efficiency of bone marrow production of TGN. In addition, it may be significant that oxypurinol—the active form of allopurinol—is a hypoxanthine analogue that also forms a nucleotide analogue of IMP.[103] We suggest that an area for further research might include the possibility that allopurinol metabolites could produce an in vivo inhibition of TPMT and/or ITPase.

Unexplained Leukopenia

Although TPMT is important to thiopurine response, it is not the only factor causing bone marrow suppression. Between half and three-quarters of all cases of leukopenia arising during thiopurine therapy appear to have a normal TPMT genotype and phenotype.[60,104] This suggests that other pharmacological factors or metabolic pathways may be relevant to thiopurine therapy. These include imidazole sensitivity,[105] drug

interaction (eg, aminosalicylates, allopurinol, co-trimoxazole, and diuretics),[26,29,74] and intercurrent illness (eg, parvovirus).[29] The genetics of drug transporter polymorphisms (eg, MDR gene) is poorly understood for thiopurines and may be another aspect waiting to be discovered.

An interesting model for unexplained leukopenia can be hypothesized for the effect of folate metabolism on TGN. Polymorphism of a key folate gene, *MTHFR,* which regulates supply of the S-adenosyl-methionine (SAM) cofactor for TPMT activity, has recently been implicated in thiopurine response.[106] Previous studies have noted that an antifolate drug, methotrexate, increases thiopurine sensitivity and raises TGN levels.[42,107–109] An explanation for this effect has been previously hinted.[82] We suggest that folate depletion reduces intracellular levels of SAM, the cofactor of TPMT, causing a reduction of TPMT activity and higher thiopurine bioavailability to form TGN. A normal activity assay may not necessarily demonstrate this loss of TPMT activity because the assay substrates, 6MP and SAM, are usually provided in excess.

We predict that low-dose methotrexate may thus provide an ideal TPMT antagonist for unresponsive patients who have documented low TGN levels and are considered to have a "fast TPMT" phenotype. Conversely, folate-depleted individuals on thiopurines, for example Crohn's disease patients with low folate or with an adverse *MTHFR* genotype, may be predicted to suffer unexplained leukopenia as a result of raised TGN levels.

CONCLUSIONS

We conclude that thiopurines have served as an excellent model for the emerging science of pharmacogenetics, both in terms of providing clear correlations between gene defects versus drug response and in demonstrating that interacting genes (eg, *TPMT, ITPA, MTHFR*) may produce a range of discrete clinical responses. But complex issues remain. Although it is clear that a relationship between erythrocyte TGN and clinical effect does exist in some form, all other relationships are in question. If there is to be a better understanding of the mechanisms of thiopurine efficacy and side effects, then TGN and methylated thionucleotides need to be measured more rigorously, preferably in the target cell. In particular, for research into TGN interactions with G-proteins such as Rac-1, it will be essential to distinguish thioguanine nucleotides from the drug base. Technological advances may assist. At Guy's Hospital, London, a semiautomated assay of TPMT based on tandem mass spectrometry to measure methyl-6MP assay product has been in routine use for over three years. Such advanced technologies may also assist more direct TGN assays. Improved measurement of thiopurine nucleotide metabolites will permit more accurate clinical correlations with genotype and therapeutic effect as well as those side effects that have a typical dose–response character.

On the other hand, elucidation of the poorly understood but all-too-common hypersensitivity and allergic side effects associated with 6MP/AZA may require further insight into molecular and immunogenic mechanisms. Prospective studies, long overdue for such a well-studied model, are at last revealing clearer results. But the persistence of poorly conceived analytic methodology and incomplete concepts will only result in the literature continuing to be muddied by publications purporting clinical correlations that cannot be critically upheld.

REFERENCES

1. Feagan BG. Maintenance therapy for inflammatory bowel disease. *Am J Gastroenterol.* 2003;98:S6–S17.
2. Elion GB. Historical background of 6-mercaptopurine. *Toxicol Ind Health.* 1986;2:1–9.
3. Hamilton L, Elion GB. The fate of 6-mercaptopurine in man. *Ann N Y Acad Sci.* 1954;60:304–314.
4. Lennard L, Maddocks JL. Assay of 6-thioguanine nucleotide, a major metabolite of azathioprine, 6-mercaptopurine and 6-thioguanine in human red blood cells. *J Pharm Pharmacol.* 1983;35:15–18.
5. Lennard L, Keen D, Lilleyman JS. Oral 6-mercaptopurine in childhood leukemia: parent drug pharmacokinetics and active metabolite concentrations. *Clin Pharmacol Ther.* 1986;40:287–292.
6. Lennard L, Singleton H. High-performance liquid chromatographic assay of the methyl and nucleotide metabolites of 6-mercaptopurine: quantitation of red blood cell 6-thioguanine nucleotide, 6-thio-inosinic acid and 6-methylmercaptopurine metabolites in a single sample. *J Chromatogr.* 1992;583:83–90.
7. Zimmerman TP, Chu LC, Bugge CJ, et al. Formation of 5′-nucleotides of 6-methylmercaptopurine ribonucleoside in human tissues in vitro. *Biochem Pharmacol.* 1974;23:2737–2749.
8. Lennard L, Lilleyman JS, Van Loon J, et al. Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukaemia. *Lancet.* 1990;336:225–229.
9. Weinshilboum RM, Raymond FA, Pazmino PA. Human erythrocyte thiopurine methyltransferase: radiochemical microassay and biochemical properties. *Clin Chim Acta.* 1978;85:323–333.
10. Weinshilboum RM, Sladek SL. Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity. *Am J Hum Genet.* 1980;32:651–662.
11. Lennard L, Van Loon JA, Lilleyman JS, et al. Thiopurine pharmacogenetics in leukemia: correlation of erythrocyte thiopurine methyltransferase activity and 6-thioguanine nucleotide concentrations. *Clin Pharmacol Ther.* 1987;41:18–25.
12. Lennard L, Van Loon JA, Weinshilboum RM. Pharmacogenetics of acute azathioprine toxicity: relationship to thiopurine methyltransferase genetic polymorphism. *Clin Pharmacol Ther.* 1989;46:149–154.
13. Krynetski EY, Tai HL, Yates CR, et al. Genetic polymorphism of thiopurine S-methyltransferase: clinical importance and molecular mechanisms. *Pharmacogenetics.* 1996;6:279–290.
14. Krynetski EY, Schuetz JD, Galpin AJ, et al. A single point mutation leading to loss of catalytic activity in human thiopurine S-methyltransferase. *Proc Natl Acad Sci USA.* 1995;92:949–953.
15. McLeod HL, Relling MV, Liu Q, et al. Polymorphic thiopurine methyltransferase in erythrocytes is indicative of activity in leukemic blasts from children with acute lymphoblastic leukemia. *Blood.* 1995;85:1897–1902.
16. Yates CR, Krynetski EY, Loennechen T, et al. Molecular diagnosis of thiopurine S-methyltransferase deficiency: genetic basis for azathioprine and mercaptopurine intolerance. *Ann Intern Med.* 1997;126:608–614.
17. Loennechen T, Yates CR, Fessing MY, et al. Isolation of a human thiopurine S-methyltransferase (TPMT) complementary DNA with a single nucleotide transition A719G (*TPMT*3C*) and its association with loss of TPMT protein and catalytic activity in humans. *Clin Pharmacol Ther.* 1998;64:46–51.
18. Weinshilboum R. Thiopurine pharmacogenetics: clinical and molecular studies of thiopurine methyltransferase. *Drug Metab Dispos.* 2001;29:601–605.
19. Armstrong VW, Shipkova M, von Ahsen N, et al. Analytic aspects of monitoring therapy with thiopurine medications. *Ther Drug Monit.* 2004;26:220–226.
20. Cara CJ, Pena AS, Sans M, et al. Reviewing the mechanism of action of thiopurine drugs: towards a new paradigm in clinical practice. *Med Sci Monit.* 2004;10:RA247–RA254.

21. Clunie GP, Lennard L. Relevance of thiopurine methyltransferase status in rheumatology patients receiving azathioprine. *Rheumatol.* 2004;43:13–18.

22. Evans WE. Pharmacogenetics of thiopurine S-methyltransferase and thiopurine therapy. *Ther Drug Monit.* 2004;26:186–191.

23. Dubinsky MC. Optimizing immunomodulator therapy for inflammatory bowel disease. *Curr Gastroenterol Rep.* 2003;5:506–511.

24. Keuzenkamp-Jansen CW, Leegwater PA, De Abreu RA, et al. Thiopurine methyltransferase: a review and a clinical pilot study. *J Chromatogr B Biomed Appl.* 1996;678:15–22.

25. Krynetski EY, Evans WE. Genetic polymorphism of thiopurine S-methyltransferase: molecular mechanisms and clinical importance. *Pharmacology.* 2000;61:136–146.

26. Lennard L. Clinical implications of thiopurine methyltransferase—optimization of drug dosage and potential drug interactions. *Ther Drug Monit.* 1998;20:527–531.

27. McLeod HL, Siva C. The thiopurine S-methyltransferase gene locus—implications for clinical pharmacogenomics. *Pharmacogenomics.* 2002;3:89–98.

28. Sandborn WJ. Pharmacogenomics and IBD: TPMT and thiopurines. *Inflamm Bowel Dis.* 2004;10:S35–S37.

29. Sanderson J, Ansari A, Marinaki T, et al. Thiopurine methyltransferase: should it be measured before commencing thiopurine drug therapy? *Ann Clin Biochem.* 2004;41:294–302.

30. Lancaster DL, Patel N, Lennard L, et al. Leucocyte versus erythrocyte thioguanine nucleotide concentrations in children taking thiopurines for acute lymphoblastic leukaemia. *Cancer Chemother Pharmacol.* 2002;50:33–36.

31. Schutz E, Gummert J, Mohr FW, et al. Should 6-thioguanine nucleotides be monitored in heart transplant recipients given azathioprine? *Ther Drug Monit.* 1996;18:228–233.

32. Lennard L, Gibson BE, Nicole T, et al. Congenital thiopurine methyltransferase deficiency and 6-mercaptopurine toxicity during treatment for acute lymphoblastic leukaemia. *Arch Dis Child.* 1993;69:577–579.

33. Hindorf U, Lyrenas E, Nilsson A, et al. Monitoring of long-term thiopurine therapy among adults with inflammatory bowel disease. *Scand J Gastroenterol.* 2004;39:1105–1112.

34. Relling MV, Hancock ML, Boyett JM, et al. Prognostic importance of 6-mercaptopurine dose intensity in acute lymphoblastic leukemia. *Blood.* 1999;93:2817–2823.

35. Derijks LJ, Gilissen LP, Engels LG, et al. Pharmacokinetics of 6-mercaptopurine in patients with inflammatory bowel disease: implications for therapy. *Ther Drug Monit.* 2004;26:311–318.

36. Lennard L, Lilleyman JS. Variable mercaptopurine metabolism and treatment outcome in childhood lymphoblastic leukemia. *J Clin Oncol.* 1989;7:1816–1823.

37. Lowry PW, Franklin CL, Weaver AL, et al. Measurement of thiopurine methyltransferase activity and azathioprine metabolites in patients with inflammatory bowel disease. *Gut.* 2001;49:665–700.

38. Mardini HE, Arnold GL. Utility of measuring 6-methylmercaptopurine and 6-thioguanine nucleotide levels in managing inflammatory bowel disease patients treated with 6-mercaptopurine in a clinical practice setting. *J Clin Gastroenterol.* 2003;36:379–381.

39. Wright S, Sanders DS, Lobo AJ, et al. Clinical significance of azathioprine active metabolite concentrations in inflammatory bowel disease. *Gut.* 2004;53:1123–1128.

40. Herrlinger KR, Fellermann K, Fischer C, et al. Thioguanine-nucleotides do not predict efficacy of tioguanine in Crohn's disease. *Aliment Pharmacol Ther.* 2004;19:1269–1276.

41. Reuther LO, Sonne J, Larsen NE, et al. Pharmacological monitoring of azathioprine therapy. *Scand J Gastroenterol.* 2003;38:972–977.

42. Pettersson B, Almer S, Albertioni F, et al. Differences between children and adults in thiopurine methyltransferase activity and metabolite formation during thiopurine therapy: possible role of concomitant methotrexate. *Ther Drug Monit.* 2002;24:351–358.

43. Lennard L. TPMT in the treatment of Crohn's disease with azathioprine. *Gut.* 2002;51:143–146.

44. Guicherit OM, Cooper BF, Rudolph FB, et al. The muscle and nonmuscle isozymes of adenylosuccinate synthetase are encoded by separate genes with differential patterns of expression. *Adv Exp Med Biol.* 1994;370:585–590.

45. Montero C, Duley JA, Fairbanks LD, et al. Demonstration of induction of erythrocyte inosine monophosphate dehydrogenase activity in ribavirin-treated patients using a high performance liquid chromatography linked method. *Clin Chim Acta.* 1995;238:169–178.

46. Simmonds HA, Fairbanks LD, Morris GS, et al. Altered erythrocyte nucleotide patterns are characteristic of inherited disorders of purine or pyrimidine metabolism. *Clin Chim Acta.* 1988;171:197–210.

47. Simmonds HA, Duley JA, Davies PM. Analysis of purines and pyrimidines in blood, urine and other physiological fluids. In: Hommes FA, ed: *Techniques in Diagnostic Human Biochemical Genetics.* NewYork: Wiley-Liss, 1990:397–424.

48. Berman PA, Black DA, Human L, et al. Oxypurine cycle in human erythrocytes regulated by pH, inorganic phosphate, and oxygen. *J Clin Invest.* 1988;82:980–986.

49. Lennard L, Davies HA, Lilleyman JS. Is 6-thioguanine more appropriate than 6-mercaptopurine for children with acute lymphoblastic leukaemia? *Br J Cancer.* 1993;68:186–190.

50. Kurowski V, Iven H. Plasma concentrations and organ distribution of thiopurines after oral application of azathioprine in mice. *Cancer Chemother Pharmacol.* 1991;28:7–14.

51. Lancaster DL, Lennard L, Rowland K, et al. Thioguanine versus mercaptopurine for therapy of childhood lymphoblastic leukaemia: a comparison of haematological toxicity and drug metabolite concentrations. *Br J Haematol.* 1998;102:439–443.

52. Rowland K, Lennard L, Lilleyman JS, et al. In vitro metabolism of 6-mercaptopurine by human liver cytosol. *Xenobiotica.* 1999;29:615–628.

53. Seidman EG. Clinical use and practical application of TPMT enzyme and 6-mercaptopurine metabolite monitoring in IBD. *Rev Gastroenterol Dis.* 2003;3:S30–S38.

54. Seidman EG, Ernest G, Theoret Y. Methods of optimizing drug therapeutic efficacy for treatment of immune-mediated gastrointestinal disorders. *US Patent.* 2004;6,680,302.

55. Lennard L, Hale JP, Lilleyman JS. Red blood cell hypoxanthine phosphoribosyl-transferase activity measured using 6-mercaptopurine as a substrate: a population study in children with acute lymphoblastic leukaemia. *Br J Clin Pharmacol.* 1993;36:277–284.

56. Relling MV, Hancock ML, Rivera GK, et al. Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J Natl Cancer Inst.* 1999;91:2001–2008.

57. Hindorf U, Peterson C, Almer S. Assessment of thiopurine methyltransferase and metabolite formation during thiopurine therapy: results from a large Swedish patient population. *Ther Drug Monit.* 2004;26:673–678.

58. Regueiro M, Mardini H. Determination of thiopurine methyltransferase genotype or phenotype optimizes initial dosing of azathioprine for the treatment of Crohn's disease. *J Clin Gastroenterol.* 2002;35:240–244.

59. Evans WE, Hon YY, Bomgaars L, et al. Preponderance of thiopurine S-methyltransferase deficiency and heterozygosity among patients intolerant to mercaptopurine or azathioprine. *J Clin Oncol.* 2001;19:2293–2301.

60. Ansari A, Hassan C, Duley J, et al. Thiopurine methyltransferase activity and the use of azathioprine in inflammatory bowel disease. *Aliment Pharmacol Ther.* 2002;16:1743–1750.

61. Stolk JN, Boerbooms AM, de Abreu RA, et al. Reduced thiopurine methyltransferase activity and development of side effects of azathioprine treatment in patients with rheumatoid arthritis. *Arthritis Rheum.* 1998;41:1858–1866.

62. Dubinsky MC, Lamothe S, Yang HY, et al. Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. *Gastroenterology.* 2000;118:705–713.

63. Marinaki AM, Ansari A, Duley JA, et al. Adverse drug reactions to azathioprine therapy are associated with polymorphism in the gene encoding inosine triphosphate pyrophosphatase (ITPase). *Pharmacogenetics.* 2004;14:181–187.

64. Cheung TK, Florin TH. 6-thioguanine: a new old drug to procure remission in inflammatory bowel disease. *Intern Med J.* 2003;33:44–46.

65. Lennard L, Lilleyman JS. Individualizing therapy with 6-mercaptopurine and 6-thioguanine related to the thiopurine methyltransferase genetic polymorphism. *Ther Drug Monit.* 1996;18:328–334.

66. Erb N, Harms DO, Janka-Schaub G. Pharmacokinetics and metabolism of thiopurines in children with acute lymphoblastic leukemia receiving 6-thioguanine versus 6-mercaptopurine. *Cancer Chemother Pharmacol.* 1998;42:266–272.

67. Shipkova M, Armstrong VW, Wieland E, et al. Differences in nucleotide hydrolysis contribute to the differences between erythrocyte 6-thioguanine nucleotide concentrations determined by two widely used methods. *Clin Chem*. 2003;49:260–268.

68. Dervieux T, Boulieu R. Simultaneous determination of 6-thioguanine and methyl 6-mercaptopurine nucleotides of azathioprine in red blood cells by HPLC. *Clin Chem*. 1998;44:551–555.

69. Lennard L. Commentary on: Differences in nucleotide hydrolysis contribute to the differences between erythrocyte 6-thioguanine nucleotide concentrations determined by two widely used methods. *Clin Chem*. 2003;49:1551.

70. Keuzenkamp-Jansen CW, De Abreu RA, Bokkerink JP, et al. Determination of extracellular and intracellular thiopurines and methylthiopurines by high-performance liquid chromatography. *J Chromatogr B Biomed Appl*. 1995;672:53–61.

71. Ansari A, Arenas M, Lindsay J, et al. Prospective study of thioguanine nucleotide measurement during azathioprine therapy for inflammatory bowel disease (abstract). American Gastroenterologic Association New Orleans, LA, 2004.

72. Dervieux T, Chu Y, Su Y, et al. HPLC determination of thiopurine nucleosides and nucleotides in vivo in lymphoblasts following mercaptopurine therapy. *Clin Chem*. 2002;48:61–68.

73. Welch J, Lennard L, Morton GC, et al. Pharmacokinetics of mercaptopurine: plasma drug and red cell metabolite concentrations after an oral dose. *Ther Drug Monit*. 1997;19:382–385.

74. Chocair PR, Duley JA, Simmonds HA, et al. The importance of thiopurine methyltransferase activity for the use of azathioprine in transplant recipients. *Transplantation*. 1992;53:1051–1056.

75. Chocair PR, Duley JA, Sabbaga E, et al. Fast and slow methylators: do racial differences influence risk of allograft rejection? *Q J Med*. 1993;86:359–363.

76. Dubinsky MC, Yang H, Hassard PV, et al. 6-MP metabolite profiles provide a biochemical explanation for 6-MP resistance in patients with inflammatory bowel disease. *Gastroenterology*. 2002;122:904–915.

77. Lennard L, Welch J, Lilleyman JS. Mercaptopurine in childhood leukaemia: the effects of dose escalation on thioguanine nucleotide metabolites. *Br J Clin Pharmacol*. 1996;42:525–527.

78. Lennard L, Welch JC, Lilleyman JS. Thiopurine drugs in the treatment of childhood leukaemia: the influence of inherited thiopurine methyltransferase activity on drug metabolism and cytotoxicity. *Br J Clin Pharmacol*. 1997;44:455–461.

79. Giverhaug T, Bergan S, Loennechen T, et al. Analysis of methylated 6-mercaptopurine metabolites in human red cells: comparison of two methods. *Ther Drug Monit*. 1997;19:663–668.

80. Dervieux T, Blanco JG, Krynetski EY, et al. Differing contribution of thiopurine methyltransferase to mercaptopurine versus thioguanine effects in human leukemic cells. *Cancer Res*. 2001;61:5810–5816.

81. Vogt MH, Stet EH, De Abreu RA, et al. The importance of methylthio-IMP for methylmercaptopurine ribonucleoside (Me-MPR) cytotoxicity in Molt F4 human malignant T-lymphoblasts. *Biochim Biophys Acta*. 1993;1181:189–194.

82. De Abreu RA, Bokkerink JP, Keuzenkamp-Jansen CW, et al. Thiopurine treatment in childhood leukemia. Metabolic aspects and sensitivity. *Adv Exp Med Biol*. 1998;431:687–692.

83. Krynetski EY, Krynetskaia NF, Yanishevski Y, et al. Methylation of mercaptopurine, thioguanine, and their nucleotide metabolites by heterologously expressed human thiopurine S-methyltransferase. *Mol Pharmacol*. 1995;47:1141–1147.

84. Shrastri S, Dubinsky MC, Poordad F, et al. Early nodular hyperplasia of the liver occurring with inflammatory bowel diseases in association with thioguanine therapy. *Arch Pathol Lab Med*. 2004;128:49–53.

85. Stoneham S, Lennard L, Coen P, et al. Veno-occlusive disease in patients receiving thiopurines during maintenance therapy for childhood acute lymphoblastic leukaemia. *Br J Haematol*. 2003;123:100–102.

86. Dubinsky MC, Vasiliauskas EA, Singh H, et al. 6-Thioguanine can cause serious liver injury in inflammatory bowel disease patients. *Gastroenterology*. 2003;125:298–303.

87. Gearry RB, Roberts RL, Barclay ML, et al. Lack of association between the ITPA 94C>A polymorphism and adverse effects from azathioprine. *Pharmacogenetics*. 2004;14:779–781.

88. Yen Y, Grill SP, Dutschman GE, et al. Characterization of a hydroxyurea-resistant human KB cell line with supersensitivity to 6-thioguanine. *Cancer Res*. 1994;54:3686–3691.

89. Bo J, Schroder H, Kristinsson J, et al. Possible carcinogenic effect of 6-mercaptopurine on bone marrow stem cells: relation to thiopurine metabolism. *Cancer*. 1999;86:1080–1086.

90. Relling MV, Yanishevski Y, Nemec J, et al. Etoposide and antimetabolite pharmacology in patients who develop secondary acute myeloid leukemia. *Leukemia*. 1998;12:346–352.

91. Carrico CK, Sartorelli AC. Effects of 6-thioguanine on macromolecular events in regenerating rat liver. *Cancer Res*. 1977;37:1868–1875.

92. Tiede I, Fritz G, Strand S, et al. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4⁺ T lymphocytes. *J Clin Invest*. 2003;111:1133–1145.

93. Kenakin T. Efficacy in drug receptor theory: outdated concept or undervalued tool? *Trends Pharmacol Sci*. 1999;20:400–405.

94. Thomas CW, Myhre GM, Tschumper R, et al. Selective inhibition of inflammatory gene expression in activated T lymphocytes: a mechanism of immune suppression by thiopurines. *J Pharmacol Exp Ther*. 2004;312:537–545.

95. Medzihradsky JL, Hollowell RP, Elion GB. Differential inhibition by azathioprine and 6-mercaptopurine of specific suppressor T cell generation in mice. *J Immunopharmacol*. 1981;3:1–16.

96. Cattan S, Lemann M, Thuillier F, et al. 6-Mercaptopurine levels and study of blood lymphocyte subsets during azathioprine treatment of Crohn's disease. *Gastroenterol Clin Biol*. 1998;22:160–167.

97. Sandborn WJ, Tremaine WJ, Wolf DC, et al. Lack of effect of intravenous administration on time to respond to azathioprine for steroid-treated Crohn's disease. North American Azathioprine Study Group. *Gastroenterology*. 1999;117:527–535.

98. Chocair P, Duley JA, Simmonds HA, et al. Low-dose allopurinol plus azathioprine/cyclosporin/prednisolone, a novel immunosuppressive regimen. *Lancet*. 1993;342:83–84.

99. Chrzanowska M, Krzymanski M. Determination of 6-thioguanine and 6-methylmercaptopurine metabolites in renal transplantation recipients and patients with glomerulonephritis treated with azathioprine. *Ther Drug Monit*. 1999;21:231–237.

100. EBPG EGoRT. European best practice guidelines for renal transplantation. Section IV: Long-term management of the transplant recipient. IV.9.2. Haematological complications. Leukopenia. *Nephrol Dial Transplant*. 2002;17:S49.

101. Menor C, Fernandez-Moreno MD, Fueyo JA, et al. Azathioprine acts upon rat hepatocyte mitochondria and stress-activated protein kinases leading to necrosis: protective role of N-acetyl-ʟ-cysteine. *J Pharmacol Exp Ther*. 2004;311:668–676.

102. Tapner MJ, Jones BE, Wu WM, et al. Toxicity of low dose azathioprine and 6-mercaptopurine in rat hepatocytes. Roles of xanthine oxidase and mitochondrial injury. *J Hepatol*. 2004;40:454–463.

103. Krenitsky TA, Elion GB, Strelitz RA, et al. Ribonucleosides of allopurinol and oxoallopurinol. Isolation from human urine, enzymatic synthesis, and characterization. *J Biol Chem*. 1967;242:2675–2682.

104. Colombel JF, Ferrari N, Debuysere H, et al. Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. *Gastroenterology*. 2000;118:1025–1030.

105. McGovern DPB, Travis SPL, Duley JA, et al. Azathioprine intolerance in patients with IBD may be imidazole-related and is independent of TPMT activity. *Gastroenterology*. 2002;122:838–839.

106. Ansari A, Marinaki A, Arenas M, et al. Polymorphism in the *MTHFR* gene affects TPMT activity. British Society for Gastroenterology Annual Meeting, Birmingham, UK, 2002.

107. Andersen JB, Szumlanski C, Weinshilboum RM, et al. Pharmacokinetics, dose adjustments, and 6-mercaptopurine/methotrexate drug interactions in two patients with thiopurine methyltransferase deficiency. *Acta Paediatr*. 1998;87:108–111.

108. Giverhaug T, Fuskevaag OM, Aarbakke J. Methotrexate increases red blood cell concentrations of 6-methylmercaptopurine ribonucleotide in rats in vivo. *Cancer Chemother Pharmacol*. 1997;40:367–370.

109. Giverhaug T, Loennechen T, Aarbakke J. Increased concentrations of methylated 6-mercaptopurine metabolites and 6-thioguanine nucleotides in human leukemic cells in vitro by methotrexate. *Biochem Pharmacol*. 1998;55:1641–1646.