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S-Adenosylmethionine: A Novel Factor in the Individualization of Thiopurine Therapy

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1. Introduction

Individualizing drug therapy by the use of pharmacogenomics offers the opportunity to improve drug efficacy, reduce adverse side effects, and provide cost-effective pharmaceutical care. 6-mercaptopurine (6-MP), 6-thioguanine (6-TG) and azathioprine (AZA) are widely prescribed cytotoxic and immunosuppressive drugs used in the therapy of acute leukaemia, inflammatory bowel diseases, allograft rejections and others.

The efficacy and toxicity of thiopurine drugs has been established to correlate with the extent of their deactivation by S-methylation. The discovery that the activity of thiopurine S-methyltransferase (TPMT) in human tissues depends on the presence of germline single nucleotide polymorphisms (SNPs) led to one of the best examples of the successful clinical application of pharmacogenetic studies (Milek et al., 2006; R. Weinshilboum, 2001; R. M. Weinshilboum&Sladek, 1980). TPMT catalyzes the direct S-methylation of 6-MP to produce the inactive metabolite 6-methylmercaptopurine (6-MMP) leading to the lower toxic potential of the drug. Of more than 20 known polymorphisms in the TPMT gene, the most common variant alleles include TPMT*2, *3A and *3C. In Caucasian populations, individuals with homozygous variant and heterozygous genotypes have, respectively, low and intermediate TPMT activity, while individuals carrying the wild-type gene sequence exhibit a very wide range of high activity values. Patients with decreased TPMT activity are, when treated with standard doses of thiopurine medications, at greater risk of developing thiopurine induced toxicities, such as myelosupression, leucopenia and stomatitis (R. Weinshilboum, 2001).

Homozygous patients with low or absent TPMT activity require a reduction to 10% of the standard dose, while heterozygous individuals should be administered 30-70%, depending on the initial treatment response (Relling et al., 2011). Predictive genotyping for the purpose of optimizing thiopurine treatment represents one of the best clinical applications of pharmacogenetic testing.

In addition to being affected by genotype, TPMT activity is also regulated by a complex metabolic network. We and others have reported on the stabilization of TPMT by its co-factor S-adenosylmethionine (SAM), which represents a candidate biomarker affecting TPMT activity and might to some extent explain the discordance between TPMT genotype

and phenotype (Milek et al., 2009; Scheuermann et al., 2004; Tai et al., 1997). In addition, it has been shown that polymorphisms in gene for MTHFR, the enzyme involved in SAM biosynthesis, correlate with the onset of hematotoxic events during the therapy of acute lymphoblastic leukaemia (ALL) (Karas-Kuzelicki et al., 2009).

As the metabolism of SAM is closely related to the methionine cycle and the folate pathway, other endogenous metabolites, such as folates and methionine, as well as enzymes participating in their biosynthesis, might also indirectly influence TPMT activity.

Both the identification and understanding of the factors influencing TPMT activity are crucial for improving the efficacy and safety of thiopurine therapy.

2. Thiopurine drugs

An ingenious idea, the purpose of which was to stop the growth of rapidly growing cells such as bacteria and tumours with modified nucleic acid bases was developed concomitantly with the discovery of DNA structure. A synthetic thiol-analogue of endogenous nucleic bases, thioguanine (6-TG), followed by 6-mercaptopurine (6-MP), and azathioprine (AZA), proved toxic to bacteria and tumours in mice. The initial experiments, conducted in 1948, were followed by clinical trials in 1953 and also present the basis for contemporary thiopurine therapy. Gertrude Elion and George Hitchings were rewarded for this work with the Nobel Prize in Physiology or Medicine in 1988 (Marx, 1988).

2.1 Mode of action

The thiopurines, namely 6-marcaptopurine, azathioprine, and 6-thioguanine are inactive prodrugs which require intestinal absorption, cellular uptake and intracellular metabolism for their cytotoxic activity. The three main metabolic pathways for thiopurines are oxidation by xanthine oxidase (XO), phosphoribosylation by hypoxanthine-guanine phosphoribosyltransferase (HGPRT), and S-methylation by TPMT (R. Weinshilboum, 2001). Oxidation is a purely inactivating pathway, which is relevant only in non-hematopoietic cells, due to the restricted expression of XO in blood cells. Despite the fact that XO activity significantly varies among individuals, a molecular basis has not been completely delineated yet.

The conversion of 6-MP by hypoxanthine phosphoribosyltransferase (HGPRT) yields thioinosine monophosphate (TIMP), which is further metabolized via inosine 5'-monophosphate dehydrogenase (IMPD), guanosine monophosphate synthetase (GMPS), reductase and kinases to active thioguanine nucleotides (TGNs). Alternatively, TIMP can be methylated by thiopurine methyltransferase (TPMT) to 6-methylmercaptopurine ribonucleosides (6-MMPR), namely methylthioinosine monophosphate (MeTIMP), -diphosphate (MeTIDP) and –triphosphate (MeTITP) (Fig.1).

Incorporation of TGNs into DNA and RNA results in S phase arrest and programmed cell death, triggered via the mismatch repair pathway. On the other hand, MeTIMP is a potent inhibitor of *de novo* purine synthesis (DNPS), causing depletion of purine nucleotides, which results in cell growth arrest and cytotoxicity. DNPS inhibition is thought to be responsible for several adverse effects of thiopurines. Nevertheless, the incorporation of TGNs is considered to be the main mode of action of 6-MP (Relling et al., 1999).

Phosphorylation of TIMP by kinases yields 6-thioinosine triphosphate (TITP), which can be dephosphorylated back to TIMP by inosine triphosphatase (ITPA) (Derijks&Wong, 2010).

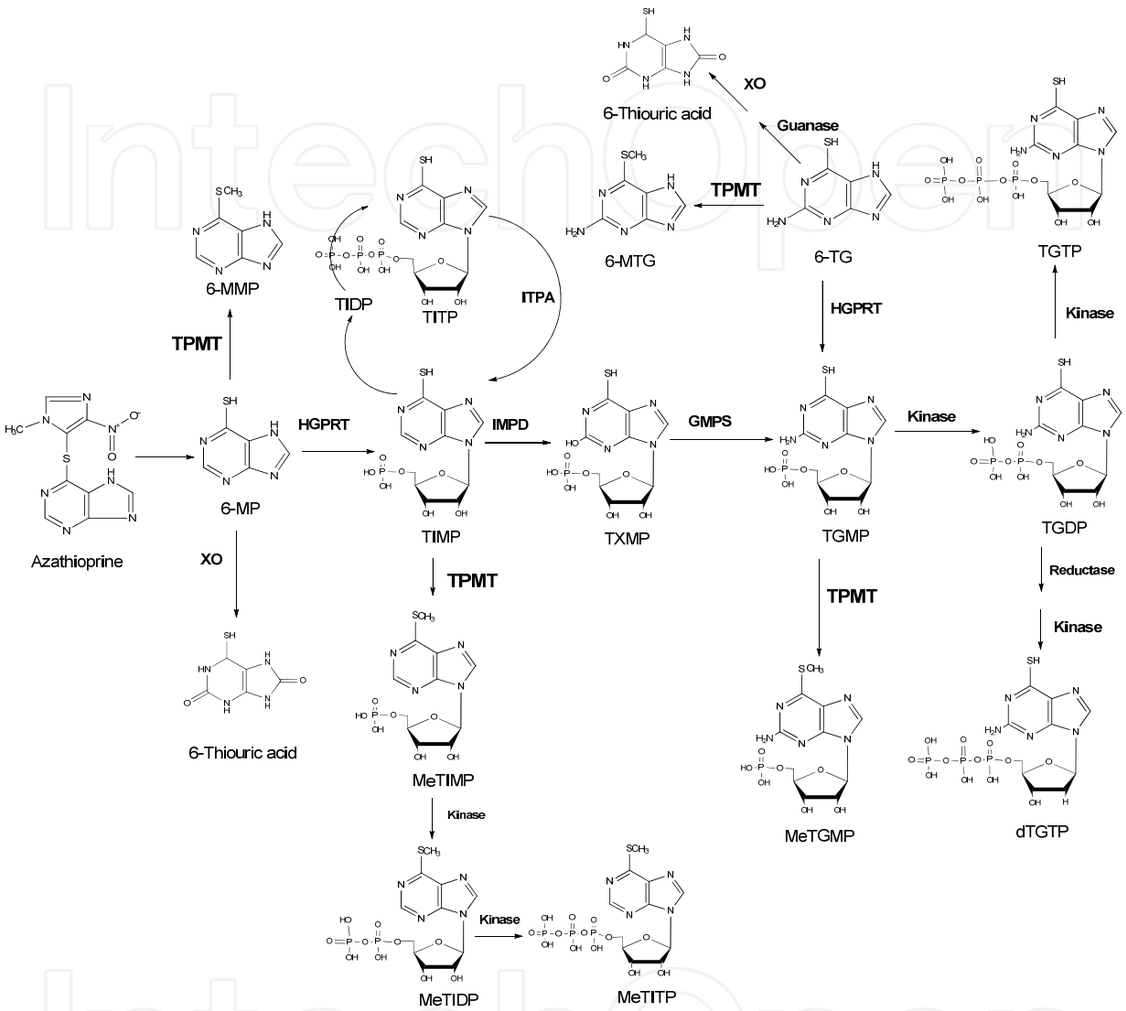

Fig. 1. **Metabolism of thiopurines.** Azathioprine is converted to 6-mercaptopurine (6-MP) by a non-enzymatic process. Both 6-MP and 6-thioguanine (6-TG) are converted by the hypoxanthine–guanine phosphoribosyltransferase (HGPRT) into their respective nucleoside monophosphates (TIMP and TGMP). Thiopurine S-methyltransferase (TPMT) inactivates 6-MP and 6-TG by S-methylation to form 6-methylmercaptopurine (6-MMP) and 6-methylthioguanine (6-TGN), respectively. Xanthine oxidase (XO) inactivates 6-MP by converting it to 6-thiouric acid. TIMP and TGMP are also TPMT substrates, yielding methylated TIMP (meTIMP) and methylated TGMP (meTGMP). TIMP may also be phosphorylated to TIDP and TITP and dephosphorylated to TIMP by ITPA. TIMP that escapes catabolism is further metabolized by inosine monophosphate dehydrogenase (IMPD) and guanine monophosphate synthetase (GMPS) to TGMP. Sequential action of deoxynucleoside kinases and reductase generates the TGTP and dTGTP that are the substrates for incorporation of 6-TG into RNA and DNA, respectively.

The third metabolic pathway, catalyzed by TPMT, is S-methylation of thiopurine to 6-methylmercaptopurine (6-MMP). This pathway is often referred to as being an inactivating pathway, since 6-MMP has no cytotoxic activity (Dervieux et al., 2001) (Fig. 1).

2.2 Efficacy and safety of 6-MP in the treatment of ALL

Acute lymphoblastic leukaemia is the most common malignancy in children. Treatment is stratified on the basis of various combinations of clinical and lymphoblastic characteristics in standard, intermediate and intensive therapy groups. Different therapy protocols have been and continue to be applied, such as USA Pediatric Oncology Group (POG) protocols and German Berlin-Frankfurt-Muenster (BFM) protocols (BFM-83, -86, -90, -95 and IC 2002). Treatment is generally composed of induction, consolidation and maintenance phases along with central nervous system prophylaxis (Moricke et al., 2008).

The induction phase generally lasts 4-6 weeks and involves combinations of drugs including vincristine, prednisone, cyclophosphamide, doxorubicin, and L-asparaginase. This phase is followed by the consolidation phase with multiagent therapy including cytarabine and methotrexate. Maintenance therapy has been included in all protocols. It lasts from 1 to 3 years and consists of 6-MP taken daily per os (50 mg/m^2) and low weekly doses of oral methotrexate (MTX) (20 mg/m^2) (Karas Kuzelicki et al., 2009).

Due to the narrow therapeutic index, a certain level of side effect manifestations is expected in most patients treated with 6-MP. We have investigated the occurrences of side effects in Slovenian pediatric ALL patients, identified through the national oncology patient registry. These patients had been treated with standard protocols at the University Children`s Hospital, University Medical Centre, Ljubljana, Slovenia in the period 1970-2004. The study group consisted of 313 ALL patients. 6-MP and other thiopurines were administered in all phases of ALL treatment. In order to investigate the occurrences of toxic effects and to exclude the influence of other drugs used in ALL treatment, we focused on the maintenance phase of the therapy, because it consisted exclusively of 6-MP and low dose MTX. The doses of 6-MP were calculated on the basis of a patient`s body surface (50 mg/m^2) and adjusted during the treatment according to desired WBC counts, these being 2000 – 3000 WBC/μL.

Therapy data, such as 6-MP dose reduction and the incidence of toxic effects including hematotoxicity, stomatitis, infections, and secondary tumours were obtained from patients' charts for the maintenance phase of treatment protocols consisting exclusively of 6-MP and low dose MTX. 6-MP dose reduction greater than 10 % for a period longer than 3 months was considered significant. The toxic effect was defined as an event causing one of the following: discontinuation of the therapy for longer than one week, a reduction of over 10 % of 6-MP dose of a duration longer than 3 months, or the hospitalization of the patient. Hematotoxicity corresponded to grade 3 and 4 leukopenia, stomatitis to grade 2 and 3, infections to grade 3 and 4 and secondary tumours to grade 4 adverse events of National Cancer Institute Common Toxicity Criteria (version 2.0) (Karas Kuzelicki et al., 2009).

The incidences of undesirable toxic effects are presented in Table 1. Despite the relatively high safety and efficacy of 6-MP, a dose reduction was determined in 20 % of patients, hematotoxicity in 14 %, stomatitis in 5 %, infections in 21 %, and the incidence of secondary tumours in 4 % of patients. Our observations are also in concordance with other published data (Sanderson et al., 2004).

Side effects / % Patients	6-MP DR †	HET ‡	Stomatitis ‡	Infections‡	Secondary TU
Patients with the condition, n (%)	63 (20.2)	44 (14.1)	14 (4.5)	67 (21.4)	13 (4.1)
Patients without the condition, n (%)	249 (79.8)	269 (85.9)	299 (95.5)	246 (78.6)	300 (95.9)

6-MP DR, 6-MP dose reduction; HET, hematotoxicity; TU, tumours; n indicates number of subjects;
† More than 10 % 6-MP dose reduction for a period of more than 3 months.
‡ A 6-MP related toxic effect that caused the discontinuation of the therapy for more than one week, more than 10 % 6-MP dose reduction for a period of more than 3 months or hospitalization of the patient.

Table 1. Analysis of 6-MP related toxic effects in Slovenian ALL patients

2.3 Thiopurines in the immunosuppressive therapy

Azathioprine (AZA) and 6-MP are the most widely used immunosuppressive agents in inflammatory bowel disease (IBD), examples of which being ulcerative colitis and Crohn's disease. AZA is also indicated as an adjunct for the prevention of rejection in renal homotransplantations and for the management of active rheumatoid arthritis. Either alone or, more usually, in combination with corticosteroids and/or other drugs and procedures, AZA has been used in a proportion of patients suffering systemic lupus erythematosus, dermatomyositis and polymyositis, autoimmune chronic active hepatitis, pemphigus vulgaris, polyarteritis nodosa, autoimmune haemolytic anaemia and chronic refractory idiopathic thrombocytopenic purpura (IMURAN® (azathioprine). Product Information). On the other hand, 6-MP has been mostly used in either IBD or acute lymphoblastic leukaemia.

Azathioprine was developed to prolong the half-life of 6-MP; therefore, a 1-methyl-4-nitro-5-imidazole moiety was added to protect the reactive sulphur group from oxidation and hydrolysis. AZA was proven to have better immunomodulatory effects than 6-MP in preventing organ rejection in kidney transplants (Murray et al., 1963). It is postulated that this is associated with an effect of the methyl-nitro-imidazolyl substitute by a currently unknown mechanism. Although both drugs have been extensively used, they have proven ineffective in one-third of patients, while up to one-fifth of patients discontinue thiopurine therapy due to adverse reactions. The observed interindividual differences in therapeutic response and toxicity can, at least partly, be explained by genetic polymorphisms of the genes encoding crucial enzymes in thiopurine metabolism (Derijks&Wong, 2010).

The reported frequencies of dose-dependent and dose-independent adverse effects of AZA and 6-MP are in the ranges of 1.4–5.0 % and 1.0–6.5 %, respectively. Myelotoxicity is considered a dose-dependent adverse effect that can be caused by elevated concentrations of the pharmacologically active 6-TGNs. On the other hand, dose-independent reactions are considered to be immune-mediated, and include rashes, arthralgia, hepatitis, myalgia, flu-like symptoms, gastrointestinal complaints, fever and pancreatitis (de Boer et al., 2007)

3. Individualization of thiopurine therapy

Pharmacogenetic testing has been implemented in clinical practice for selected drugs only. The implementation of pharmacogenetics in the clinical setting was hampered by the

recognition that the metabolism of a given drug does not depend solely on a single drug-metabolizing enzyme, but rather on a complex enzymatic network of competing metabolic pathways. It has become apparent that the identification of relevant pharmacogenetic markers is much more complicated than initially believed.

Upon entering the cell, thiopurines are also subject to a complex metabolic network; their metabolic activation thus depends on genetic predisposition as well as nutritional and other environmental factors.

3.1 Thiopurine S-methyltransferase (TPMT)

Although TPMT pharmacogenetics is addressed in detail in a separate chapter of this book, we need to summarize the most relevant facts, since they are the basis for further elaborations.

TPMT plays a pivotal role in thiopurine drug responses, such that decreased TPMT activity correlates with higher cytotoxic thioguanine nucleotide (TGN) levels which may result in life-threatening toxicity. The distribution of TPMT activity in Caucasian populations is trimodal: approximately 89 % of population has normal to high, 11 % intermediate and 0.3 % low or undetectable TPMT activity. Although numerous alleles have been identified, the most prevalent and clinically significant are TPMT*3A (460G>A and 719A>G), TPMT*3B (460G>A) and TPMT*3C (719A>G). TPMT genotyping prior to the initiation of thiopurine therapy represents a quick and reliable pharmacogenetic test. In accordance with advice provided by the FDA in 2004, the recommendation to perform the test before starting therapy with thiopurines has been included in the Summary of Product Characteristics (SmPC) of Purinethol® (6-MP) and Imuran® to highlight the usefulness of TPMT testing in predicting risk for thiopurine toxicity.

TPMT deficient patients tend to be better responders to 6-MP therapy than wild-type patients- due to higher TGN accumulation in cancer cells- but are at greater risk of developing toxic effects such as hematotoxicity, infections, stomatitis and secondary tumours, as a consequence of their accumulation in normal cells. Conversely, ultra-high enzyme activity can lead to superior 6-MP tolerability but also to an increased risk of relapse and hepatic toxicity, which has been related to methylated metabolites of thiopurines (Evans, 2004).

The clinical relevance of 6-MP dose reduction during maintenance therapy is well defined only in patients homozygous for variant TPMT alleles (TPMT*2, *3A, *3C) who exhibit low TPMT activity. Dosing adjustments based on TPMT status is recommended in thiopurine therapy. In the treatment of malignancies, conventional high doses of thiopurines are recommended for homozygous wild-type TPMT patients, while 30-70 % lower-than-normal starting doses should be used in heterozygous deficient patients, and at least 10-fold reduced doses in homozygous deficient patients (Relling et al., 2011).

Due to an incomplete genotype-to-phenotype correlation in heterozygous individuals with variable intermediate activity, the predictive value of TPMT genotyping for the optimization of thiopurine therapy is limited. Therefore, the identification of novel pharmacogenetic and/or biochemical markers is necessary for high prediction. One such factor is S-adenosylmethionine (SAM), which stabilizes the TPMT protein structure by binding to its active site (Scheuermann et al., 2004). Thus, SAM may also modulate TPMT activity in the

intercellular setting, possibly by post-translational stabilization. Consequently, the endogenous availability of SAM may influence TPMT activity, the formation of 6-MP metabolites, and the toxicity of thiopurine drugs. In addition, endogenous metabolites (e.g. folates, methionine, ATP) and enzymes participating in the biosynthesis of SAM, (e.g. MTHFR, TYMS), could also influence TPMT activity indirectly.

3.2 The role of SAM in cellular metabolism and disease

S-adenosyl-L-methionine (SAM) is one of the most abundant co-factors in eukaryotic cells and has been initially described as "active methionine" (Cantoni, 1951). It participates in many cellular processes and exerts many biological effects. As a pleiotropic molecule, it is the principal methyl donor in processes such as nucleic acid, protein and phospholipid methylation, acting as a co-substrate for many SAM-dependent methyltransferases (P. K. Chiang et al., 1996). In addition, it is an important regulator of replication, transcription and translation, acting on post-transcriptional and post-translational levels and by epigenetic mechanisms (Finkelstein, 2007). SAM is also involved in polyamine synthesis as well as inhibition of DNA demethylation (Detich et al., 2003), and plays an important role in cell growth, cell cycle progression and apoptosis (Loenen, 2006; Nitta et al., 2002).

Cellular methylation is closely connected to the methionine cycle, methionine recycling pathway, folate metabolism, and polyamine synthesis, as well as transsulfuration and glutathione synthesis (Fig. 2) (Hitchler&Domann, 2007). Active metabolic conversions of the folate pathway and methionine (Met) cycle are ubiquitous, while transsulfuration takes place only in the liver, kidney, pancreas, intestinal tract and brain (Finkelstein, 2007). SAM and SAH act as efficient regulatory molecules in these processes, such that their molar ratio (i.e. methylation potential) determines the activity of many methyltransferases and related enzymes.

SAM is synthesized from Met, the availability of which largely depends on folate pools and dietary intake. Tissue SAM levels thus depend on the expression of methionine adenosyltransferase (MAT), which catalyzes the conversion from Met, and 5,10-methylenetetrahydrofolate reductase (MTHFR) that provides the substrate for the remethylation of homocysteine into Met. Mechanisms of SAM-induced metabolic regulation include the modulation of both tissue expression and kinetic properties of metabolizing enzymes as well as the concentrations of their substrates and products. Vice versa, the modified expression or enzyme activity of some methyltransferases has been shown to impact intracellular SAM and SAH levels, which are most notably determined by the expression of glycine N-methyltransferase (GNMT) (Luka et al., 2009). GNMT degrades excess SAM to SAH and decreases cell methylation capacity. Importantly, GNMT has been described as a key regulator of SAM level and methylation capacity in normal livers, while its expression is diminished in tumour tissue and cultured cells such as HepG2 (Martinez-Chantar et al., 2008).

Moreover, the modified activity of enzymes (e.g. methionine adenosyltransferase, S-adenosylhomocystein hydrolase) that catalyse the afore mentioned metabolic conversions significantly influences the dynamics and concentrations of metabolites; most prominently those of SAM and SAH, which have pleiotropic biological effects. In addition, aberrations in methylation and redox homeostasis have been implicated in several pathologies, such as liver carcinogenesis, hepatocellular carcinoma, chronic steatohepatitis and hyperhomocysteinemia-associated cardiovascular diseases (Martinez-Chantar et al., 2002a).

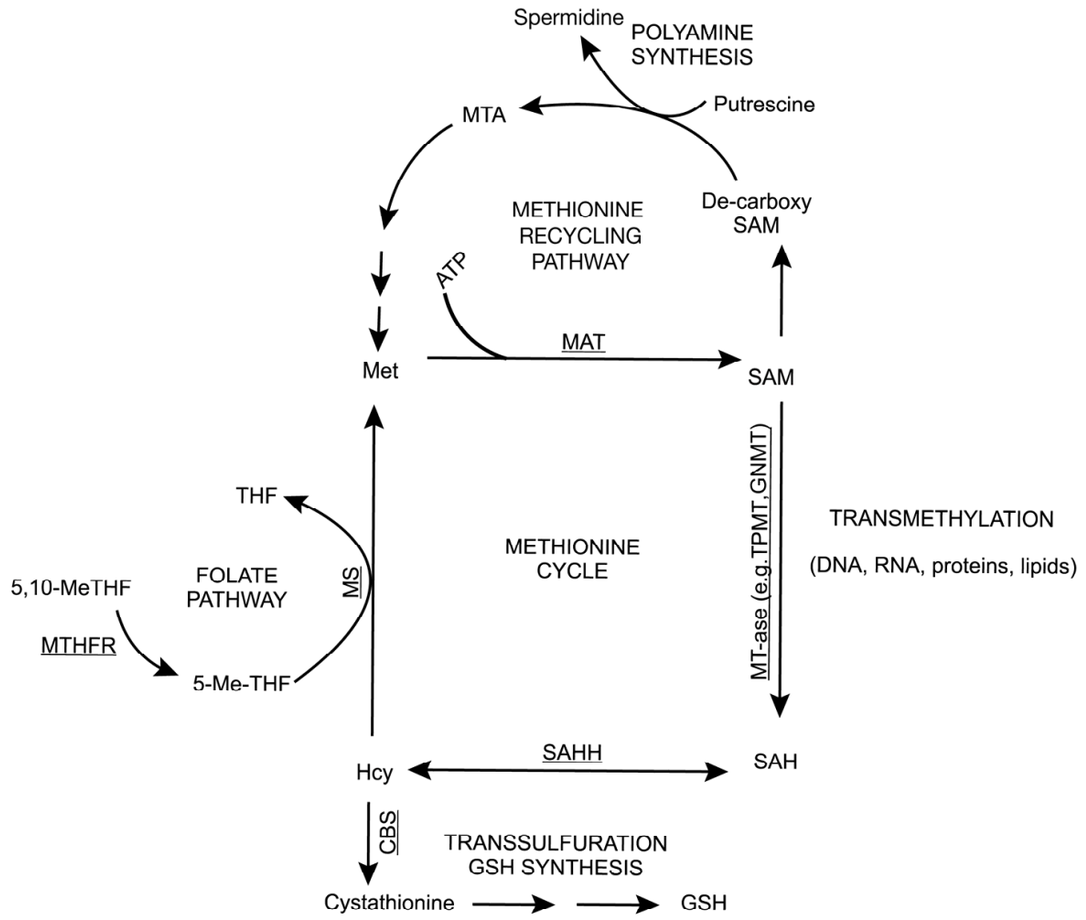

Fig. 2. SAM metabolism, the methionine cycle and related pathways. SAM is consumed in transmethylation reactions catalyzed by SAM-dependent methyltransferases (MT-ases). SAM has many diverse biological effects. Its metabolism is closely connected to homocysteine (Hcy) remethylation and methionine (Met) cycle, the folate pathway, transsulfuration, Met recycling pathway and polyamine synthesis. 5,10-Me-THF, 5,10-methylenetetrahydrofolate; 5-Me-THF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; CBS, cystathionine-β-synthase; Cys, cysteine; GSH, glutathione; SAHH, S-adenosylhomocysteine hydrolase; MAT, methionine adenosyltransferase; MTA, 5'-methylthioadenosine; MTAP, 5'-methylthioadenosine phosphorylase; Cys, cysteine; SAM, S-adenosylmethionine; SAMDC, SAM decarboxylase; TPMT, thiopurine S-methyltransferase, GNMT, glycine N-methyltransferase.

Unbalanced metabolic conversions in the methionine cycle are most frequently a consequence of a low dietary intake of folic acid, high alcohol consumption, poisoning, or genetic abnormalities. Most commonly, this is observed as hyperhomocysteinemia (i.e. elevated plasma homocysteine, Hcy), as well as SAM depletion, and consequently methionine metabolism related pathogenesis. Consequences of inactivated SAM, Met

synthesis and methionine adenosyltransferase activity have been well documented in patients with liver cyrosis and different forms of hyperhomocysteinemia (Martinez-Chantar et al., 2002b).

3.3 The role of SAM in thiopurine metabolism

SAM plays an important role in the intracellular conversions of thiopurines. The metabolism of 6-mercaptopurine and azathioprine results, apart from the synthesis of cytotoxic TGNs, cytosolic, in the production of methylated thiopurine metabolites (methylthioinosine 5'-monofosphate, MeTIMP). These molecules act as antimetabolites by inhibiting phosphoribosyl pyrophosphate amidotransferase, the rate limiting enzyme in *de novo* purine synthesis (DNPS) pathways, which therefore leads to ineffective ATP production. Non-depleted cellular ATP pools are required for SAM biosynthesis from Met catalyzed by methionine adenosyltransferase (MAT).

The relevance of SAM in thiopurine metabolism has been demonstrated by several *in vitro* studies. Decreased SAM recycling via the methionine cycle was observed upon the addition of 6-MP or 6-methylmercaptopurine riboside (6-MMPR) to MOLT cells. Due to DNPS inhibition caused by the metabolite MeTIMP (Vogt et al., 1993), endogenous adenine nucleotide pools were depleted, limiting the ATP-dependent synthesis of SAM from methionine (Stet et al., 1994). The depletion of SAM also resulted in DNA hypomethylation (De Abreu et al., 1995). The inhibitory effect of 6-MMPR on the growth of MOLT lymphoblasts was reversed by supplementing adenine nucleotide pools with exogenous adenosine, adenine and inosine (Stet et al., 1995). Finally, exogenous SAM also prevents 6-MP induced programmed cell death via the reduction of intracellular TGN and MeTIMP levels in MOLT cells (Milek et al., 2009).

3.4 Effect of SAM-TPMT interaction on thiopurine drug action

Since TPMT is a SAM-dependent methyltransferase, components of methionine metabolism are closely connected to thiopurine drug action. As in other reactions catalyzed by SAM-dependent methyltransferases, SAM provides the methyl group in the S-methylation of thiopurines, which is catalyzed by TPMT. As a side product in this process, SAM is converted to S-adenosylhomocysteine (Sahasranaman et al., 2008), a potent methyltransferase inhibitor. Apart from its role as a TPMT cofactor, SAM has been shown to exert additional effects on TPMT, in all probability as an efficient post-translational regulator of its activity.

Non-synonymous amino-acid substitutions resulting from common genetic polymorphisms destabilize TPMT protein structure and increase its susceptibility to proteasomal and autophagy-mediated degradation. The tridimensional structure of the yeast TPMT orthologue revealed that sinefungin, a SAM analogue, stabilizes the protein backbone towards a rigid native conformation, very possibly decreasing its susceptibility to proteolytic degradation (Scheuermann et al., 2004; Tai et al., 1999) (Fig. 3).

A similar effect was observed for catechol-O-methyltransferase (COMT), a SAM-dependent methyltransferase, and for cystathionine β–sythase (CBS), the rate limiting enzyme in the transsulfuration pathway (Prudova et al., 2006). In HepG2 cells SAM was found to modulate

its own production by destabilizing MAT2A mRNA, thus regulating the activity of MAT, the enzyme catalyzing SAM biosynthesis from Met (Martinez-Chantar et al., 2003).

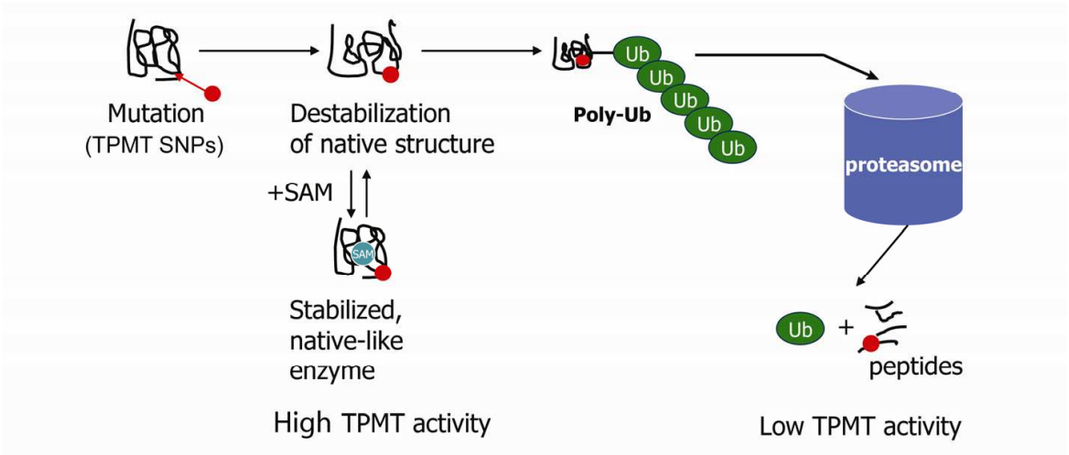

Fig. 3. Role of SAM in TPMT stability and degradation. Decreased tissue TPMT activity is a consequence of the rapid degradation of TPMT variant allozymes via the ubiquitin-proteasome pathway. The binding of SAM to the TPMT protein structure restores intramolecular contacts and shifts the equilibrium towards a highly folded conformation that is less susceptible to intracellular proteolysis. SAM, S-adenosyl-L-methionine; TPMT, thiopurine S-methyltransferase; Ub, ubiquitin.

Molecular and functional studies of TPMT SNPs have shown that non-synonymous amino-acid substitutions in variant TPMT allozymes cause the disruption of intra-molecular van der Waals contacts (Scheuermann et al., 2003). Consequently, such variant proteins are readily degradable via proteasome- and autophagy-mediated proteolysis (Li et al., 2008; Tai et al., 1999). The addition of high concentrations of SAM, the principal cellular methyl donor and a co-substrate in the S-methylation reaction catalyzed by TPMT, resulted in increased TPMT activity in yeast extracts containing recombinant wild-type and TPMT*3C allozymes. The binding of SAM has also been shown to stabilize the 3D structure of the enzyme and shifts the dynamic balance towards the native structure, which prevents the proteolytic degradation of the enzyme. Most recently, exogenous SAM was shown to prevent 6-MP induced programmed cell death via the reduction of intracellular TGN and MeTIMP levels in MOLT cells, possibly by the post-translational stabilisaton of TPMT (Milek et al., 2009). A possible mechanism is indicated in Fig. 4.

The most important evidence of SAM metabolism on TPMT stabilization was presented by two *in vivo* studies, where the presence of low-activity polymorphisms in methylenetetrahydrofolate reductase (MTHFR), the enzyme which catalyzes the formation of 5-methyltetrahydrofolate (5-Me-THF), a rate-determining step in the re-methylation of methionine from homocysteine (Fig. 2), has been found to correlate with decreased TPMT activity in patients with ALL (Arenas et al., 2005). It was postulated that low MTHFR activity results in limited SAM synthesis and, consequently, lower TPMT stability, observed as a modulation of the TPMT phenotype (Karas-Kuzelicki et al., 2009).

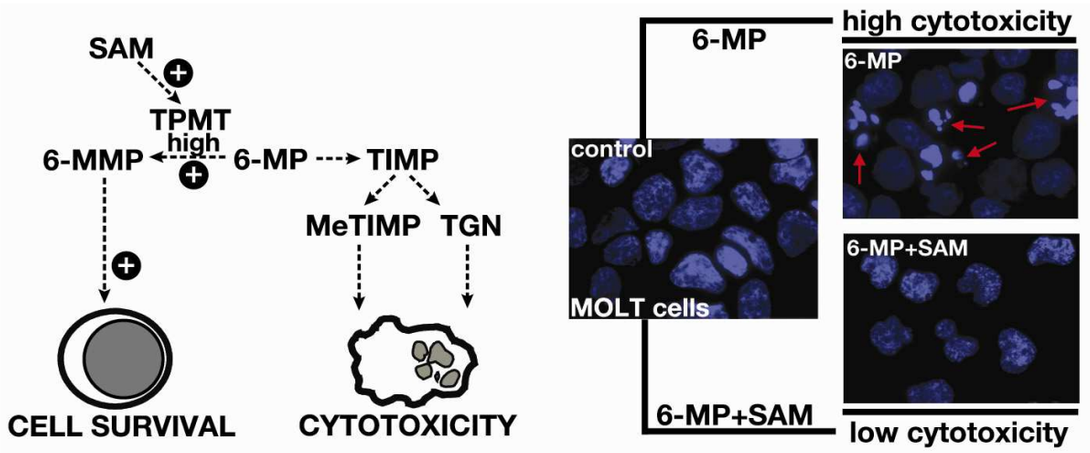

Fig. 4. SAM decreases thiopurine toxicity by stabilizing TPMT protein levels. In TPMT-catalyzed reactions, SAM acts as a methyl donor, but can also stabilize the TPMT 3D structure (indicated by plus, encircled). This results in more extensive deactivation of the drug, i.e. the production of 6-methylmercaptopurine (6-MMP) as opposed to cytotoxic thioguanine nucleotides (TGN) and methylthioinosine monophosphate. Therefore, SAM indirectly decreases the extent of 6-methylmercaptopurine (6-MP) cytotoxicity in MOLT cells. TIMP, thioinosine monophosphate; SAM, S-adenosyl-L-methionine; TPMT, thiopurine S-methyltransferase.

3.5 Synergistic effects of low activity TPMT and MTHFR on 6-MP-induced toxicity

6-MP-induced toxic effects in TPMT heterozygous patients are augmented by a variant methylenetetrahydrofolate reductase (MTHFR) genotype. This is the case as SAM levels depend on the availability of folates, which themselves depend on the activity of MTHFR, the most important folate pathway regulating enzyme.

MTHFR is an enzyme involved in the metabolism of folic acid, through the conversion of 5,10- methylenetetrahydrofolate (5, 10-Me-THF) to 5-methyltetrahydrofolate (5-Me-THF). MTHFR is the rate-determining enzyme of the folate cycle, which plays a major role in methionine and SAM synthesis, and consequently affecting TPMT activity. This is demonstrated in homozygous and heterozygous MTHFR knockout mice (Chen et al., 2001), where decreased MTHFR activity leads to decreased SAM, increased homocysteine and SAH levels, and DNA hypomethylation. In humans, the low activity of MTHFR is coded by two alleles, 677 C>T and 1298 A>C. Homozygosity for 677 C>T and compound heterozygosity for 677 C>T and 1298 A>C is associated with increased blood homocysteine levels, while no apparent discrepancies in biochemical profile are detected in 1298 CC homozygotes (Botto&Yang, 2000; van der Put et al., 1998). These findings reflect enzymatic deficiency due to the presence of polymorphism. MTHFR activity in homozygotes carrying two 677 C>T alleles is 40-50 % of the wild-type enzyme, while in 1298 A>C homozygotes the activity is somewhat higher but still below the normal range. The frequency of 677 T allele is lower in Africans (6-14 %) than in other races (25-43 %), and the highest frequencies have been documented for US Hispanics and Italians. Frequencies of the 1298 C allele are very similar, while the frequencies of compound heterozygosity for both variants range from 15 to 20 % in Caucasian populations (Botto&Yang, 2000).

Individuals with the MTHFR 677 TT genotype have significantly lower serum folate levels (Nishio et al., 2008) and different ratio of methylated to formylated tetrahydrofolates (THF). While only the methylated forms of THF are present in wild-type individuals, up to 59 % of total RBC folates in 677 TT subjects were formyl-THF, as a consequence of lower MTHFR activity and decreased 5,10-Me-THF consumption for the formation of 5-Me-THF (Bagley&Selhub, 1998). Besides genetic predisposition, folate intake is crucial, as sufficient intake may diminish the effect of low-activity alleles. Similarly, the MTHFR 677 TT genotype in transformed human lymphoblasts is most significantly associated with the decreased SAM levels arising from decreased folate-dependent homocysteine re-methylation under conditions of extracellular folate restriction (E. P. Chiang et al., 2007). These data suggest that the effect of genotype-dependent MTHFR status on methionine regeneration and SAM synthesis is also closely related to intracellular folate concentrations.

A correlation between MTHFR and TPMT activity was demonstrated in individuals with intermediate TPMT activity carrying low activity 677 TT MTHFR and wild-type TPMT genotypes (Arenas et al., 2005). The influence of MTHFR activity on TPMT activity is also demonstrated in our recent study addressing the thiopurine toxicity in paediatric ALL patients. The synergistic effect of TPMT and MTHFR variant alleles was observed in patients carrying polymorphisms in both genes, and reflected in severe toxicity. 82 % of these patients experienced hematotoxicity, compared to 4 % of patients with wild-type MTHFR and TPMT genotypes. Similarly, patients carrying polymorphisms in both TPMT and MTHFR genes (59 %) were more likely to have experienced 6-MP dose reductions, as well as stomatitis and infections (Karas-Kuzelicki et al., 2008).

3.6 Other potential pharmacogenetic markers in thiopurine therapy

Individual responses to thiopurine therapy depend beside genetic predisposition, on nutritional and other environmental factors. The genes and their variants identified so far do not suffice to fully justify the variability in drug response. Implementation of novel genetic and metabolomic findings is therefore crucial for the improved prediction of drug efficacy and safety.

Xanthine oxidase (XO) is involved in the first-pass metabolism of 6-MP, and is predominantly expressed in the intestinal mucosa and liver. XO metabolizes 84 % of 6-MP into inactive 6-thiouric acid, resulting in a substantial reduction in 6-MP bioavailability. XO is an alternative name for Xanthine dehydrogenase (XDH), also termed Xanthine oxidoreductase, (XOR). XDH is a molybdenum-containing hydroxylase, readily converted to xanthine oxidase by reversible sulfhydryl oxidation or by irreversible proteolytic modification (http://omim.org/entry/607633). Numerous polymorphisms have been detected either in promoter or coding region of XDH/XO gene. The functional relevance of detected polymorphisms was determined in *in vitro* assays; in the allelic variants tested, a deficiency in enzyme activity was detected in two, low activity in six and high enzymatic activity in two (Kudo et al., 2008). In recent studies, a correlation of two polymorphisms in XO (1936 A>G and 2107 A>G) with the thiopurine therapy outcomes has been addressed; however, due to the small number of patients with myelotoxicity, it was not possible to draw any conclusions (Wong et al., 2007).

Another potential polymorphic enzyme correlating with 6-MP toxicity is inosine triphosphate (ITP) pyrophosphatase (ITPA), which catalyses the pyrophosphohydrolysis of ITP into inosine

monophosphate, thereby preventing the accumulation of ITP in normal cells. Decreased ITPA activity leads to the accumulation of the inosine nucleotide, ITP in the cells. Recent studies have shown that the presence of P32T functional polymorphism in ITPA correlates with unwanted thiopurine toxicity in ALL patients. However, studies performed on patients with inflammatory bowel disease or on liver transplant recipients showed no association. Nevertheless, ITPA is emerging as an interesting candidate biomarker, even more so due to relatively high allele frequencies in some populations (Marsh&Van Booven, 2009).

Thymidylate synthase (TYMS) is an enzyme that catalyzes the conversion of dUMP to dTMP by utilizing 5,10-Me-THF and, as such, constitutes a competing pathway for MTHFR-catalyzed 5-Me-THF synthesis. Due to its considerable effect on 5-Me-THF levels and, consequently, on methionine and SAM synthesis, TYMS might, potentially, influence TPMT activity. There is a common tandem repeat polymorphism in the promoter region of TYMS, with the number of tandem repeats affecting TYMS activity levels, mediated through the effects of the repeats on translation efficiency (Kawakami et al., (1999, 2001)). The double repeat (2R) results in lower gene expression than the triple repeat (3R) (Horie et al., 1995). The 3R/3R genotype and high TYMS activity could lead to low 5-Me-THF, methionine and SAM levels and, consequently, low TPMT activity, resulting in higher TGN concentrations and a better therapy response (Karas-Kuzelicki&Mlinaric-Rascan, 2009).

In addition, several other polymorphic genes encoding crucial enzymes of thiopurine metabolism, such as glutathione S-tranferases, hypoxanthine phosphoribosyltransferase, inosine monophosphate dehydrogenase and multidrug resistance proteins, have been described and represent novel pharmacogenetic markers influencing thiopurine therapy (Derijks&Wong, 2010).

4. Future directions and clinical application

The effective stabilization of TPMT by SAM, which prevents thiopurine toxicity, has several clinical implications. An *in vitro* study showed that in patients receiving 6-MP, a decrease in TPMT activity may be expected after 6-MP administration, due to DNPS inhibition and decreased synthesis of the stabilising factor SAM. In patients with wild-type or heterozygous mutant TPMT genotypes, who exhibit high and intermediate TPMT activities respectively, a decrease in the enzyme activity may result in an overproduction of TGNs, increasing the risk of undesirable toxicity. On the other hand, high levels of endogenous SAM, as well as potential compensatory responses to SAM depletion, may contribute to the detoxification of the drug, and, consequently, lead to the wild-type patients being non-responders, by decreasing the production of cytotoxic TGNs. The availability of folate pools may also significantly influence 6-MP related cytotoxic effects, since the metabolic fluxes of homocysteine remethylation and downstream SAM synthesis are folate dependent. The mechanism described in the present study could, therefore, play an important role in patients receiving folates in dietary supplements during thiopurine treatment, modulating the amount of SAM, and, consequently, TPMT activity. Further *in vivo* studies of the correlation of TPMT with the activity of enzymes involved in SAM metabolism, (e.g. methionine adenosyltransferase, S-adenosylmethionine decarboxylase, 5,10-methylenetetrahydrofolate reductase), could reveal additional factors influencing treatment with 6-MP. Moreover, the presence of activity-modulating genetic polymorphisms in these enzymes could explain the poor TPMT genotype-to-phenotype correlations observed in some individuals.

Detailed and relevant understanding of TPMT regulation by SAM in the context of Met metabolism in several cell lines, primary cells, animal models and human samples is a valuable resource for the improved prediction of clinical outcomes. Further studies will have direct consequences in the clinic, by improving the genotype-to-phenotype correlation in heterozygous and wild-type individuals with unexpectedly low TPMT activity. Furthermore, novel factors influencing TPMT activity and thiopurine drug response will enable a much more realistic implementation of existing genetic and biochemical test(s) in clinical practice. In fact, effective antidotes that rapidly decrease thiopurine toxicity by acting as positive regulators of TPMT levels and thiopurine deactivation would be substantially favourable in the clinical setting.

4.1 Methods for measuring SAM

Given the critical role of SAM in many metabolic pathways and its importance in the diagnosis of various pathological manifestations, as well as its potential implication in individualization of thiopurine therapy, the development of an accurate, sensitive and reproducible method for its quantification is very important.

Various methods for the analysis of SAM in different tissues have been developed in the last two decades. Most of the developed methods are HPLC-based and use UV detection (Bottiglieri, 1990; Molloy et al., 1990; Wise et al., 1997) with either ion-pairing or cation exchange chromatography. Some of them use fluorescent detection after conversion of the analytes to fluorescent analogs (Capdevila&Wagner, 1998; Loehrer et al., 1996), others electrochemical detection (Melnyk et al., 2000).

HPLC methods combined with ultraviolet detection are suitable for measuring the concentrations of SAM in tissues, including red blood cells, where SAM can be found in the micromolar range, whereas methods with fluorescent detection show greater sensitivity and can also be used for the detection of SAM in plasma in nanomolar concentrations. In order to enable even better quantification of SAM presented in plasma or cerebrospinal fluid in low nanomolar concentration, some more sensitive LC–MS (Stabler&Allen, 2004) and LC–MS/MS methods (Gellekink et al., 2005; Struys et al., 2000) have also been developed.

A capillary electrophoresis method has been developed for the determination of SAM and SAH in rat liver and kidney as well as in mouse liver, but it can also be used to determine SAM in whole blood (Uthus, 2003).

The stereospecific colorimetric assay for (S,S)-SAM quantification is based on TPMT-catalyzed thiol methylation. All reagents are commercially available and inexpensive, and the necessary enzymes are robust and readily obtainable in large quantities from recombinant sources. The assay can be carried out on UV–visible spectrometers available in most laboratories and can be adapted for batch assay, for example, in a microplate format. The method is linear from 5 μM to at least 60 μM (S,S)-SAM. The higher limits of the assay are restricted by the linear range of individual spectrophotometers at 410 nm, whereas the lower limits are determined by the sensitivity and precision of the spectrophotometer. Although the method was developed to determine SAM concentration in tablets, it could also be applied to measure SAM concentration in physiological fluids (Cannon et al., 2002).

The commercially available assay for SAM determination is a Mediomics Bridge-It® fluorescence assay based on a combination of fluorescence measurement techniques and an

assay platform design that utilizes DNA-binding proteins as biosensors for their respective ligands. The affinity of the DNA-binding transcriptional repressor MetJ, labelled with fluorophore, for its DNA-binding site is greatly increased in the presence of its ligand, SAM. This method exhibits a high signal to background ratio, a broad linear dynamic range (0.5 μM – 20 μM), and a detection sensitivity of 0.5 μM; therefore, it is useful for the purpose of quantifying SAM in various samples including biological fluids, cell culture and fermentation medium, and extracts of tissues and cells (www.mediomics.com).

5. S-adenosylmethionine (SAM) in therapy

SAM in the form of its stable p-toluensulphonate or butanedisulfonate salt has been used for more than 20 years in the treatment of depression, liver disorders, and musculoskeletal and joint disorders such as osteoarthritis and fibromyalgia. It has been available as a prescription drug marketed under different brand names (Gumbal, Samyr, Adomet, Heptral and Admethionine) in Italy, Spain, Germany, the Czech Republic, Russia, Argentina and Mexico, whereas in the United States and Canada SAM has been available under the Dietary Supplement and Health Education Act as a nutritional supplement under the marketing name SAM-e.

Depression

Although the mechanism of antidepressant action of SAM is not entirely clear, it is thought that its ability to function as a methyl donor increases brain levels of serotonin, dopamine, and norepinephrine. It has been previously reported that serum and cerebrospinal fluid levels of SAM are low in depressed patients (Bottiglieri 1990; Lakhan 2008) and that increases in serum SAM levels correlate with improved treatment response (Bell 1994). Besides the stimulatory effect of SAM on central monoaminergic neurotransmitters, there may exist alternative mechanisms in which increased or restored membrane phospholipid methylation plays a role in the antidepressant effect. SAM may increase the fluidity of cell membranes by stimulating phospholipid methylation, which has previously been linked to an increase in ß receptor and muscarinic (M1) receptor density (Bottiglieri, 2002).

SAM has been studied for use in various depressive disorders for many decades, with the first clinical trials dating back to as early as 1973 (Fazio et al., 1973). The majority of studies performed since then have reported that SAM is effective for treating depression, the conclusion also drawn later by a meta-analysis (Bressa, 1994) and some other systematic reviews (Williams 2005; Papakostas 2003; Mischoulon 2002). However, due to several quality issues and methodological flaws of the individual studies included in these reviews, the findings should be interpreted with caution. Most studies are quite dated (1970s or 1980s), have short treatment duration and are of small sample size ($n < 50$). Furthermore, the most appropriate daily dosage for SAM is also not well established. Due to its low oral bioavailability, many of the earlier SAM studies utilized parenteral formulations (intramuscular or intravenous), which may also limit the clinical relevance of those studies (Carpenter, 2011).

Osteoarthritis

SAM has also been studied extensively in the context of the treatment of osteoarthritis. Experimental studies indicate that SAM increases the chondrocyte proteoglycan synthesis and

proliferation rate. SAM induces the synthesis of polyamines that might stabilize the polyanionic macromolecules of proteoglycans and protect them from attack by proteolytic and glycotic enzymes. Furthermore, *in vitro* studies show that SAM can antagonize the tumour necrosis factor α–induced decreases in synovial cell proliferation and fibronectin mRNA expression. These findings indicate that SAM restores basal conditions in cultured synovial cells after cytokine-induced cell damage (Bottiglieri, 2002). Many trials have demonstrated that SAM reduces the pain associated with osteoarthritis and is well tolerated in this patient population. However, a systematic review (Rutjes et al., 2009) found that available studies were mainly small and of questionable quality, and that, therefore, the routine use of SAM for osteoarthritis of the knee or hip could not be recommended until such time as further evaluation through larger randomised controlled studies has taken place.

Liver Disease

SAM has been used to treat various types of acute and chronic liver diseases. Although the focus of clinical trials in this area has been diffuse, a number of clinical trials have focused on the effect of SAM on cholestasis arising from a variety of causes, including pregnancy (Almasio et al., 1990; Frezza et al., 1990a; Frezza et al., 1990b). SAM may exert beneficial effects on the liver through a variety of mechanisms. Glutathione, the major anti-oxidant in the liver, plays a key role in detoxification and the limiting of oxidative damage. Studies have shown that abnormal SAM synthesis is associated with chronic liver disease, regardless of its etiology. At customary therapeutic doses, SAM has been shown to increase hepatic glutathione concentrations in patients with chronic liver disease (Chawla et al., 1990). Although some studies have demonstrated clinical improvement in patients with intrahepatic cholestasis, hepatic steatosis and alcoholic liver cirrhosis, a systematic review of 9 randomised placebo-controlled studies could not find evidence to support or refute the claim that SAM has a beneficial effect in patients with alcoholic liver disease (Rambaldi&Gluud, 2006).

Neurological Disorders

Several studies indicate that a CNS methyl group deficiency may play a role in the etiology of Alzheimer disease (AD). Hyperhomocysteinemia, often related to folate or vitamin B 12 deficiency, is a common finding in the elderly and is associated with cognitive impairment and cognitive decline. The association between hyperhomocysteinemia and AD is well established; however, the underlying pathophysiology remains unexplained. Studies in cell culture experiments and mouse models have suggested that 2 metabolites of homocysteine, SAM and S- adenosylhomocysteine (Sahasranaman et al., 2008), may be important in Alzheimer pathogenesis, e.g. by influencing the expression of presenilin 1 and β - secretase, leading to an increase in Aβ production (Linnebank et al., 2010). It is important to note that the use of either SAM or alternative methyl group donors (such as betaine or folate and vitamin B-12) might improve measures of cognitive function. These treatments may be able to restore methyl group metabolism and normalize blood homocysteine concentrations (Bottiglieri, 2002).

6. Conclusions

Prediction of TPMT activity and thiopurine drug response based on TPMT genotyping tests represents one of the most relevant applications of pharmacogenetics. Prediction of TPMT activity and treatment response solely on the basis of presence of mutant TPMT alleles is

insufficient, due to the incomplete TPMT phenotype-to-genotype correlation. This problem is most pronounced in heterozygous patients (8-10 % in Caucasian populations), which exhibit a wide range of intermediate enzyme activity, and in those wild-type individuals which do not exhibit high activity. Hence, to improve the prediction of thiopurine therapy outcome, identification of new biomarkers is essential.

One of such candidates is SAM, which, by binding into the active site of TPMT, stabilizes its structure. Several studies suggest that measurement of erythrocyte SAM level, in addition to TPMT genotyping, could serve as an additional predictor of TPMT activity in some thiopurine patient subgroups, and suggest that stabilization of TPMT by SAM has substantial clinical relevance. Some analytical methods for the determination of SAM in biological samples have already been described which are suitable for the implementation into clinical practice.

In addition, SAM, which has been used for more than 20 years in the treatment of depression, liver disorders, and musculoskeletal and joint disorders, may be a promising agent to acutely regulate TPMT activity in order to rapidly decrease excess thiopurine toxicity in some patient subgroups.

In addition to measuring SAM levels in red blood cells, analyses of genes directly or indirectly involved in the folate metabolism (such as MTHFR and TYMS) can add valuable additional information to conventional TPMT genotyping, thus enabling the development of complex diagnostic algorithms, and in turn improving the efficacy and safety of the thiopurine therapy.

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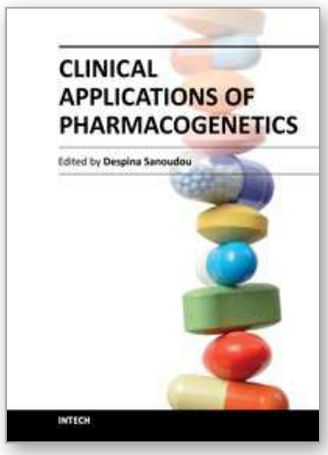

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The rapidly evolving field of Pharmacogenetics aims at identifying the genetic factors implicated in the inter-individual variation of drug response. These factors could enable patient sub-classification based on their treatment needs thus expediting drug development and promoting personalized, safer and more effective treatments. This book presents Pharmacogenetic examples from a broad spectrum of different drugs, for different diseases, which are representative of different stages of evaluation or application. It has been designed so as to serve both the unfamiliar reader through explanations of basic Pharmacogenetic concepts, the clinician with presentation of the latest developments and international guidelines, and the research scientist with examples of Pharmacogenetic applications, discussions on the limitations and an outlook on the new scientific trends in this field.

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