The effect of thiopurine drugs on DNA methylation in relation to TPMT expression

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ABSTRACT

The thiopurine drugs 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are well-established agents for the treatment of leukaemia but their main modes of action are controversial. Thiopurine methyltransferase (TPMT) metabolises thiopurine drugs and influences their cytotoxic activity. TPMT, like DNA methyltransferases (DNMTs), transfers methyl groups from S-adenosylmethionine (SAM) and generates S-adenosylhomocysteine (SAH). Since SAM levels are dependent on de novo purine synthesis (DNPS) and the metabolic products of 6-TG and 6-MP differ in their ability to inhibit DNPS, we postulated that 6-TG compared to 6-MP would have differential effects on changes in SAM and SAH levels and global DNA methylation, depending on TPMT status. To test this hypothesis, we used a human embryonic kidney cell line with inducible TPMT. Although changes in SAM and SAH levels occurred with each drug, decrease in global DNA methylation more closely reflected a decrease in DNMT activity. Inhibition was influenced by TPMT for 6-TG, but not 6-MP. The decrease in global methylation and DNMT activity with 6-MP, or with 6-TG when TPMT expression was low, were comparable to 5-aza-2′-deoxycytidine. However, this was not reflected in changes in methylation at the level of an individual marker gene (MAGE1A). The results suggest that a non-TPMT metabolised metabolite of 6-MP and 6-TG and the TPMT-metabolised 6-MP metabolite 6-methylthioguanosine 5'-monophosphate, contribute to a decrease in DNMT levels and global DNA methylation. As demethylating agents have shown promise in leukaemia treatment, inhibition of DNA methylation by the thiopurine drugs may contribute to their cytotoxic affects.

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1. Introduction

The methylation of cytosine bases in DNA is an important mechanism regulating gene expression. The discovery that most cancers have aberrant DNA methylation involving hypermethylation of gene promoters, often associated with silencing of tumour suppressor and growth inhibitory genes [1], has led to interest in therapeutic agents which can reduce

DNA methylation [2–5]. The azanucleosides, 5-azacytidine and 5-aza-2′deoxycytidine (5-AdC/decitabine), inhibit the DNA methyltransferases (DNMTs) responsible for methylating DNA [6–8] and have shown particular promise in the treatment of haematological malignancies [9,10]. DNMTs utilise S-adenosylmethionine (SAM) as a methyl donor for the methylation of cytosine bases in DNA, with the subsequent conversion of SAM to S-adenosylhomocysteine (SAH) [11]. Several studies have shown that alterations in the levels of SAM and SAH, and in particular the ratio of SAM to SAH, relate to changes in DNA methylation [12–14], as SAH has an inhibitory affect on DNMT activity [15].

The thiopurine drugs, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are used extensively in the treatment of acute leukaemias [16,17]. Although there are similarities in their metabolism (Fig. 1), the cytotoxicity of these drugs *in vitro* is differentially affected by the level of expression of thiopurine methyltransferase (TPMT) [18,19]. This enzyme has widely- varying activity between individuals, predominantly due to single nucleotide polymorphisms [20,21]. An effect of the thiopurine drug, 6-MP, and 6-methyl-mercaptopurine riboside (ʳMeMP) on DNA methylation has been described in the T-cell acute lymphoblastic leukaemic (T-ALL) cell line MOLT-4 [22–24]. Both 6-MP and ʳMeMP (an analogue of adenosine) inhibited *de novo* purine synthesis (DNPS), via a common metabolite 6-methylthioinosine 5′-monophosphate (MeTIMP) [25,26], and consequently caused a decrease of adenosine triphosphate (ATP). The depletion of ATP, as a precursor of SAM, was thought to result in the depletion of SAM and decreased DNA methylation observed in the MOLT-4 cell line exposed to 6-MP [22].

Since levels of TPMT in cells treated with thiopurine drugs influence the extent of DNPS inhibition [18,19], DNA methylation and changes in the SAM/SAH ratio may also be influenced by TPMT. Given that the TPMT-catalysed metabolic product of 6-TG, 6-methylthioguanine 5′-monophosphate (MeTGMP), is a much weaker inhibitor of DNPS than MeTIMP produced from 6-MP [27], we postulated that 6-TG would have a reduced effect compared to 6-MP on changes in SAM and SAH levels and global DNA methylation. To test these hypotheses, we used a previously generated human embryonic kidney cell line in which human TPMT cDNA is inducibly expressed under the control of an ecdysone promoter [19]. We show that both 6-MP and 6-TG reduced global DNA methylation in these cells and that decreases in DNMT activity, rather than changes in SAM and SAH levels, may be the overriding mechanism. Alterations in global DNA methylation and DNMT activity were TPMT dependent only when the cells were treated with 6-TG.

2. Materials and methods

2.1. Chemicals

Chloroacetaldehyde, SAM, SAH, 6-MP, 6-TG, ʳMeMP, Nuclease P_1, ribonuclease A (from bovine pancreas), deoxyribonuclease 1 (DNase 1), and propidium iodide were purchased from Sigma–Aldrich (Poole, Dorset, UK). All other chemicals were purchased from Sigma–Aldrich, Fischer Scientific UK Ltd. (Leicestershire, UK), VWR International (Leicestershire, UK) or Roche (Lewes, UK) unless otherwise stated.

2.2. Cell culture and induction of TPMT

Human embryonic kidney cells containing the pVgRXR vector (EcR293) were transfected with pIND vector containing human TPMT cDNA (EcR293-TPMT/20) and maintained as previously described within our laboratory [19]. The EcR293 cells were
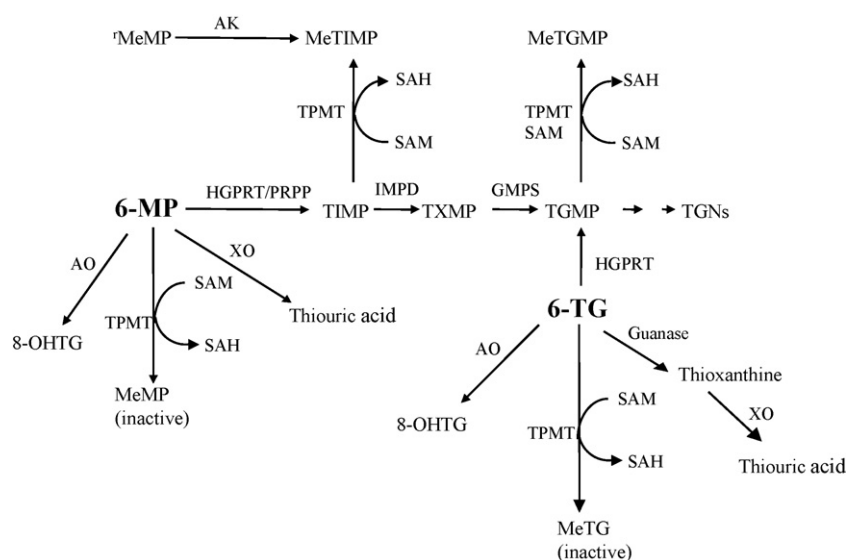
Fig. 1 – Metabolism of 6-MP and 6-TG in human ALL cells. PRPP, 5′-phosphoribosyl-1-pyrophosphate; GMPS guanosine monophosphate synthase; HGPRT, hypoxanthine guanine phosphoribosyltransferase; IMPD, inosine monophosphate dehydrogenase; SAM, S-adenosine methionine; TPMT, thiopurine methyltransferase; AO, aldehyde oxidase; 8-OHTG, 8-hydroxythioguanine; XO, xanthine oxidase; TGN, thioguanine nucleotides; TIMP, thioinosine 5′-monophosphate; TXMP, thioxanthine monophosphate; TGMP, thioguanosine monophosphate; MeTG, methylthioguanine; MeMP, methylmercaptopurine; ʳMeMP, methylmercaptopurine riboside; AK, adenosine kinase.

maintained in a similar way, but medium was supplemented only with the antibiotic for maintaining selection of the pVgRXR vector. TPMT expression in EcR293-TPMT/20 cells was induced with 3 μM Muristerone A (MA) (Invitrogen Ltd., Paisley, UK) dissolved in ethanol, for 24 h prior to 48 h exposure of cells to 6-MP, 6-TG, ʳMeMP or 5-AdC. Control cells were treated with 3 μL/mL ethanol alone. For *MAGE1A* gene promoter analysis cells were treated with drugs for six doubling times (174 h for MA-induced cells and 126 h for ethanol-treated control cells). The parental EcR293 cells were treated with MA or ethanol control in the same way. After drug treatments cells were harvested, washed twice with PBS and cell pellets snap frozen in liquid nitrogen and then stored at −80 °C prior to analysis.

2.3. MeTIMP levels

MeTIMP levels were measured as described by Coulthard et al. [19]. Briefly, pellets of 5×10^6 cells were re-suspended in 500 μL Tris–EDTA buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and sonicated for 20 s. A 100 μL aliquot was removed for total protein estimation using the BCA kit as described in the manufacturer's instructions (Perbio Science Ltd., Cramlington, UK). Remaining lysate was filtered using a Centrikon-3K filter (Millipore, Bedford, MA) at $20,800 \times g$ for 45 min and MeTIMP measured in the filtrate by HPLC, as described by Krynetski et al. [28]. MeTIMP concentration was expressed per mg total protein.

2.4. SAM and SAH levels

Sample preparation for the measurement of SAM and SAH levels was based on a method by Shugart [29]. Pellets of approximately 5×10^6 cells were lysed in 0.3 mL ice-cold water by sonication for 5 s at 10 μm. To 180 μL of lysate or SAM/SAH standards (4–0.03 μM) prepared in water, 20 μL of 40% TCA was added, vortexed, and incubated on ice for 30 min; the remaining lysate was used for total protein estimation as described above. After 30 min the samples and standards were centrifuged at $20,800 \times g$ for 10 min at 4 °C. To 150 μL of cell lysates and standards, 150 μL of 2 M sodium acetate buffer pH 4.5 and 120 μL of 12% (v/v) chloroacetaldehyde was added, followed by incubation at 60 °C for 1 h. Samples were then centrifuged at $20,800 \times g$ for 10 min at room temperature and fluorescent SAM and SAH chloroacetaldehyde derivatives were analysed by HPLC. One hundred microliters of sample was injected into a Waters HPLC system (Waters, Elstree, UK) equipped with a Synergi 4 μm Hydro RP 80 Å 250 mm × 4.6 mm column (Phenomenex, Macclesfield, UK) with an AQ-C18 4 mm × 3 mm guard column (Phenomenex) maintained at 30 °C, a refrigerated (4 °C) sample chamber, and a 980 programmable fluorescence detector (Applied Biosystems, Warrington, UK) set at an excitation wavelength of 270 nm with a 389 nm emission cut-off filter. The mobile phase consisted of an isocratic phase over 0–10 min of 0.1 M sodium acetate pH 4.5 with 7% acetonitrile followed by a linear gradient of 7–45% acetonitrile for 10 min, returning to initial conditions over 2 min and continued isocratically for 8 min, with a total run time of 30 min and a flow rate of 1 mL/min. SAM and SAH levels were calculated as μmol per mg total

protein. Levels of SAM, SAH and SAM/SAH ratio in drug-treated cells were then calculated as a percentage of control, drug vehicle treated cells.

2.5. Global DNA methylation assay

Measurement of global methylation of newly synthesised DNA was based on a method by Lambooy et al. [24]. Briefly, cells were pulse labelled for 4 h with 165 nM L-[methyl-3H] Methionine (Amersham Biosciences, Chalfont St. Giles, UK; 3.07 TBq/mmol) and 344 nM [2-14C] Thymidine (Amersham Biosciences; 2.15 GBq/mmol). The DNA was extracted from 5×10^6 cells, using a QIAamp DNA Mini Kit (QIAGEN Ltd., Crawley, UK) according to the manufacturer's instructions. Purified DNA was eluted with 200 μL 10 mM Tris–HCl, 0.1 mM EDTA, pH 9.0 and radioactivity measured in a liquid scintillation counter. As SAM levels are affected by 6-MP, 6-TG and ʳMeMP, the global methylation results for these agents were calculated to take into account the differences in specific activity of SAM. The amount of endogenous SAM and radiolabelled methionine added to cells were calculated to be of similar concentrations per mg of cellular protein, hence the global methylation results were multiplied by the percentage decrease in endogenous SAM divided by 100 to correct for percentage change in specific activity of radi-olabelled SAM derived from the radiolabelled methionine.

2.6. DNMT activity

The measurement of DNMT activity was based on a method by Fiala et al. [30], with modifications. Cell pellets were lysed in 50 mM Tris/HCl pH 7.8, 1 mM EDTA, 10% glycerol, 1% Tween, 0.2 mM phenylmethylsulfonyl fluoride and 0.1 mg/mL (69 U/mg) ribonuclease A by passing through a $25\frac{1}{4}$ gauge needle three times followed by three freeze/thaw (liquid nitrogen/37 °C) cycles. Cell lysates containing 8 μg of protein were incubated with 1.5 μg (2.5 μL) poly(dI-dC)-poly(dI-dC) duplex (Amersham Biosciences) and 1.9 μM, 111 kBq [methyl-3H]-SAM (Amersham Biosciences; 2.89 TBq/mmol) in a total volume of 20 μL lysis buffer at 37 °C for 4 h. After stopping the reaction and adding 640 μL ice-cold ethanol the DNA was allowed to precipitate overnight at −20 °C. Digestion of the DNA was carried out as in the original method except that 20 units (4 μL) of DNase 1 with 20 μL of DNase 1 buffer (100 mM Tris/HCl pH 7.5, 25 mM MgCl₂, 5 mM CaCl₂) was added prior to adding 4 U (4 μL) of nuclease P₁. Two units (2 μL) of alkaline phosphatase (Roche) were used. One hundred microliters of sample were run using the same column type as for SAM/SAH analysis but maintained at 25 °C, a Waters 996 PDA detector and Flow Scintillation Analyzer 500TR Series (PerkinElmer, Beacons Field, UK). Standards of deoxyinosine (dI) (Sigma–Aldrich) prepared in water were also run on the HPLC, to determine the fraction of dI in samples which became methylated by [methyl-3H]-SAM, as a determinant of DNMT activity. The mobile phase consisted of an isocratic phase from 0 to 12 min of 25 mM sodium acetate, 12.5 mM sodium citrate, pH 5.1 with 8% methanol followed by a linear gradient of 8–20% methanol for 3 min, held isocratically at 20% methanol for 5 min and returning to initial conditions over 3 min and continued isocratically for 7 min, with a total run time of

30 min and a flow rate of 1 mL/min. DNMT activity was calculated as pmol of ^{3}H methyl groups transferred to deoxycytidine/μg dI and levels for drug-treated cells quoted as a percentage of control, drug vehicle treated cells.

2.7. Methylation status of CpG sites in MAGE1A promoter by pyrosequencing

One microgram of DNA, isolated from cell pellets using the QIAamp DNA Mini Kit (QIAGEN), was bisulphite-modified using the Epitect Bisulfite Kit (QIAGEN) and eluted into 40 μL TE buffer. The MAGE1A region ($-90/+209$) was amplified using 5 μL-modified template DNA, 300 ng of each primer (TAGN, Gateshead, United Kingdom), 0.2 mM deoxynucleotide triphosphates (Applied Biosystems, Warrington, UK), and 2U FastStart Taq (Roche) in a total volume of 50 μL. Primers 5′-TTTTTATTTTTATTTAGGTAGGAT-3′ and 5′-biotin-TCTAAAAACAACCCAAACTAAAAC-3′, forward and reverse respectively, were used. Pyrosequencing was performed using a PSQ 96MA Pyrosequencer (Biotage, Hertford, UK) and the sequencing primer 5′-TGTTGTTAGTTTTGGTT-TAT-3′ according to the manufacturer's protocol.

2.8. Western blotting

Cells were lysed in RIPA buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing EDTA-free protease inhibitor cocktail (Roche) for 10 min on ice, vortexing for 3 s every minute. Debris was removed by centrifugation at 20,800 × g at 4 °C for 10 min. Protein concentration of the cell lysate was quantified using the BCA kit (as described above), and lysates diluted with 2× Laemmli sample buffer (4% SDS, 40% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue and 0.125 M Tris–HCl, pH 6.8). Twenty micrograms of lysate was separated on 4–12% Tris/Glycine gels (Invitrogen, Paisley, UK) and blotted onto polyvinylidene difluoride (BioRad, Hemel Hempstead, UK). Membranes were blocked for one hour in blocking buffer (100 mM Tris, pH 7.5, 0.9% NaCl, 0.05% Tween 20 and 5% non-fat dried milk) and probed with 1/2000 dilution of rabbit polyclonal DNMT1 amino-terminal antibody (New England Biolabs, Hitchen, UK) in blocking buffer overnight at 4 °C. After stripping the blots with 2% SDS in 62.5 mM Tris buffer pH 6.7 for 30 min, at 50 °C they were re-probed with 1/2000 dilution of mouse mono-clonal vinculin antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 1 h, as a loading control. Visualisation was achieved using a 1/2000 dilution of HRP-conjugated goat anti-rabbit or goat anti-mouse antibody secondary antibodies (Dako, Ely, UK) in blocking buffer for 45 min at room temperature followed by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions and exposure to X-ray film.

2.9. Cell cycle

Cells were harvested, washed in PBS and then fixed in 1 mL 70% ethanol:30% PBS and stored at 4 °C for up to 1 month. For cell cycle analysis, cells were washed and then rehydrated in 1 mL PBS for 15 min at room temperature. Cells were pelleted

and resuspended in PBS with 10% (v/v) 1 mg/mL RNAse and 10% (v/v) 0.4 mg/mL of propidium iodide and incubated for 30 min in the dark. A single cell suspension was made by passing the cells through a 25-gauge needle. Cell cycle analysis was performed on a FACScan Flow Cytometer (Becton Dickinson, Warwickshire, UK) at an excitation wavelength of 488 nm and fluorescence detection at 530 nm using CellQuest Software.

2.10. Statistical analysis

Experiments were performed using the parental EcR293 cells and the EcR293-TPMT/20 derivative. Assay data were analysed with General Linear Models (GLM) with log 10 drug dose, cell type (parental EcR293 cells or the EcR293-TPMT/20 derivative), inducer (MA or ethanol control) and independent repeat as independent factors using Systat version 10 (SPSS Inc., Chicago, IL). Each experiment was repeated independently on at least three occasions and each of these repeats (with samples in duplicate at each point) was coded as a factor in the analysis to take account of experimental variation between repeats. In the GLM, interaction between cell type and TPMT induction status was also included as an effect, and hypothesis tests used to compare effects of TPMT induction on the response by EcR293-TPMT/20 cells when TPMT induction and interaction terms were significant. A GLM was also used to analyse pyrosequencing data, with drug type (5-AdC, 6-TG, 6-MP), drug treatment (drug or vehicle control), TPMT status, and CpG island as main effects, and with drug treatment × CpG island, drug type × drug treatment, TPMT status × drug type, TPMT status × drug treatment, and drug type × drug treatment × TPMT as interaction terms.

3. Results

3.1. MeTIMP levels after exposure of cells to 6-MP and ^{r}MeMP

To determine doses of 6-MP and ^{r}MeMP which produced comparable amounts of MeTIMP, the levels of MeTIMP produced after exposure of EcR293-TPMT/20 cells, with and without induction of TPMT expression, to these agents were measured (Fig. 2). In response to ^{r}MeMP the MeTIMP levels increased, producing 10–14 nmol MeTIMP/mg cellular protein at doses of 4–16 μM ^{r}MeMP (Fig. 2A); there was no significant difference in MeTIMP levels between EcR293-TPMT/20 cells with or without induction of TPMT expression ($F_{1,33} = 1.46$, $P = 0.23$) and this is consistent with the production of MeTIMP by adenosine kinase and not TPMT (see Fig. 1). In contrast, after treatment with 6-MP, cells induced to express TPMT produced increased levels of MeTIMP ($F_{1,21} = 27.3$, $P < 0.00004$; Fig. 2B); MeTIMP levels increased to a plateau of 4 nmol MeTIMP/mg cellular protein at 6-MP doses >20 μM in the absence of TPMT induction but to 12 nmol MeTIMP/mg cellular protein at the same 6-MP doses after induction of TPMT expression. Thus, under conditions of TPMT induction, 0.5–16 μM ^{r}MeMP and 1.8–80 μM 6-MP produced comparable levels of MeTIMP.
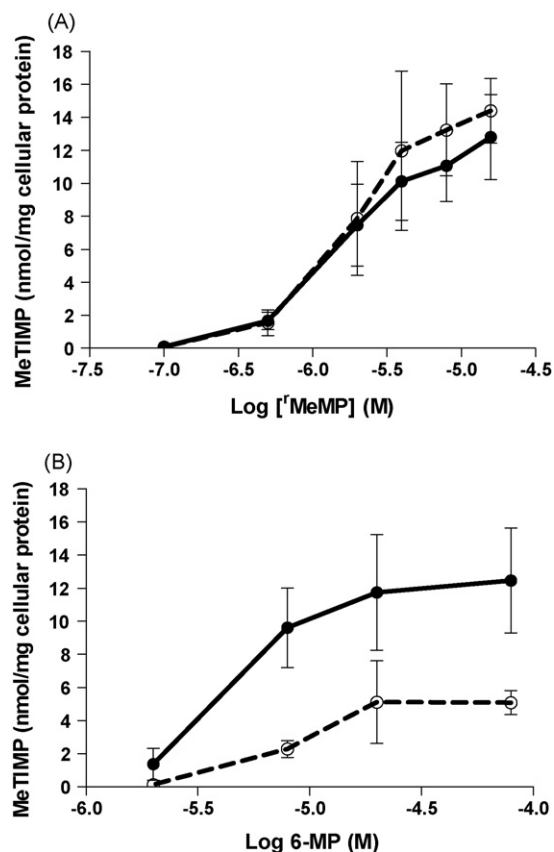
Fig. 2 – The levels of MeTIMP after 48-h exposure of EcR293-TPMT/20 cells to ^{r}MeMP (A) and 6-MP (B), with low (○) and high TPMT (●) expression. Error bars represent the S.D. from three separate experiments.

3.2. The effect of TPMT induction on drug-induced changes in SAM and SAH levels

The effect of TPMT expression on the level of SAM and SAH levels and the SAM/SAH ratio after exposure of EcR293-TPMT/20 cells to 6-MP, ^{r}MeMP or 6-TG for 48 h is shown in Fig. 3. After 6-MP treatment, EcR293-TPMT/20 cells, induced to produce TPMT, showed a statistically-significant increase in SAH compared to cells without TPMT induction. At doses of 1.8–80 μM, SAH increased 1.2–1.9-fold, respectively ($F_{1,38} = 23.68$, $P < 0.0001$; Fig. 3A). The inducing agent, MA, had no significant effect on SAH levels in parental EcR293 cells exposed to 6-MP ($F_{1,38} = 0.024$, $P > 0.8$), data not shown.

To determine if the increase in SAH with 6-MP in high TPMT-expressing cells was caused by the 6-MP metabolite MeTIMP, parental EcR293 cells and EcR293-TPMT/20 cells were treated with ^{r}MeMP, which is converted directly to MeTIMP intracellularly (Fig. 1). The amount of ^{r}MeMP used in these assays (4–16 μM) was chosen to produce similar amounts of MeTIMP as formed with exposure of cells to 6-MP (Fig. 2) in TPMT-induced cells. There were no dose effects of ^{r}MeMP on SAH levels (Fig. 3A, $F_{1,29} = 0.019$, $P > 0.893$) and no effect of TPMT induction ($F_{1,29} = 0.307$, $P > 0.58$).

As with exposure to 6-MP, TPMT induction significantly increased SAH levels in response to 6-TG (Hypothesis test; $F_{1,57} = 9.8$, $P = 0.003$), but the effect was small compared to the TPMT-induced increase in SAH levels obtained with 6-MP (Fig. 3A). There was no effect of the inducing agent, MA, on SAH levels in parental EcR293 cells treated with 6-TG ($F_{1,57} = 0.03$, $P = 0.863$), data not shown. With respect to SAM levels (Fig. 3B), there was no effect of TPMT induction in response to 6-MP ($F_{1,39} = 0.157$, $P = 0.694$) or ^{r}MeMP ($F_{1,41} = 1.4$, $P = 0.24$). However, TPMT expression reduced the extent of SAM depletion in response to 6-TG (Hypothesis test for EcR293-TPMT/20 cells, $F_{1,57} = 8.251$ $P = 0.006$). In contrast to the apparent lack of effect of TPMT induction on SAM levels in EcR293-TPMT/20 cells treated with ^{r}MeMP, the addition of MA significantly reduced the extent of SAM depletion in ^{r}MeMP-treated parental EcR293 cells ($F_{1,29} = 40.2$, $P = 0.000001$). This implies that MA affected the response to ^{r}MeMP which was counteracted by TPMT induction in the EcR293-TPMT/20 cells.

The changes in SAM and SAH levels in these experiments were reflected in the SAM/SAH ratio (Fig. 3C). For 6-MP, the increased SAH levels while SAM remained unchanged resulted in a decreased SAM/SAH ratio in response to TPMT induction. With 6-TG there was no overall effect of TPMT on SAM/SAH ratio since reduction in depletion of SAM and elevation of SAH occurred in response to TPMT induction.

3.3. The effect of TPMT induction on drug-induced changes in global DNA methylation

The effects of 6-MP, 6-TG and ^{r}MeMP on global DNA methylation in EcR293-TPMT/20 cells, corrected for the effects of TPMT induction on SAM levels, are shown in Fig. 4A. 6-MP and 6-TG inhibited the methylation of newly-synthesised DNA in a dose-dependent manner, but there was no statistically-significant dose effect of ^{r}MeMP ($F_{1,13} = 1.127$, $P = 0.308$).

The induction of TPMT had no significant effect on inhibition of DNA methylation induced by treatment with 6-MP ($F_{1,51} = 1.91$, $P = 0.17$). The extent of inhibition of DNA methylation in response to ^{r}MeMP was significantly increased after induction of TPMT ($F_{1,13} = 9.77$, $P = 0.008$. However, MA significantly decreased the extent of inhibition of DNA methylation in response to ^{r}MeMP in parental EcR293 cells ($F_{1,13} = 20.7$, $P = 0.00055$). As was found with the SAM measurements and ^{r}MeMP treatment, MA appeared to affect the response of global methylation to ^{r}MeMP which was counteracted by TPMT induction in the EcR293-TPMT/20 cells. The induction of TPMT expression with MA significantly decreased the extent of inhibition of DNA methylation in response to increasing doses of 6-TG relative to un-induced cells ($F_{1,61} = 28.6$, $P < 0.0001$).

As a positive control for effects on DNA methylation, cells were also treated with 5-AdC. The reduction in DNA methylation with increasing dose of 6-MP, ^{r}MeMP and 6-TG when TPMT expression was low was comparable to that observed with 5-AdC. For 5-AdC, induction of TPMT with MA had a small but statistically-significant effect on increasing the extent of de-methylation, but there was no effect of MA on the parental cells (data not shown).
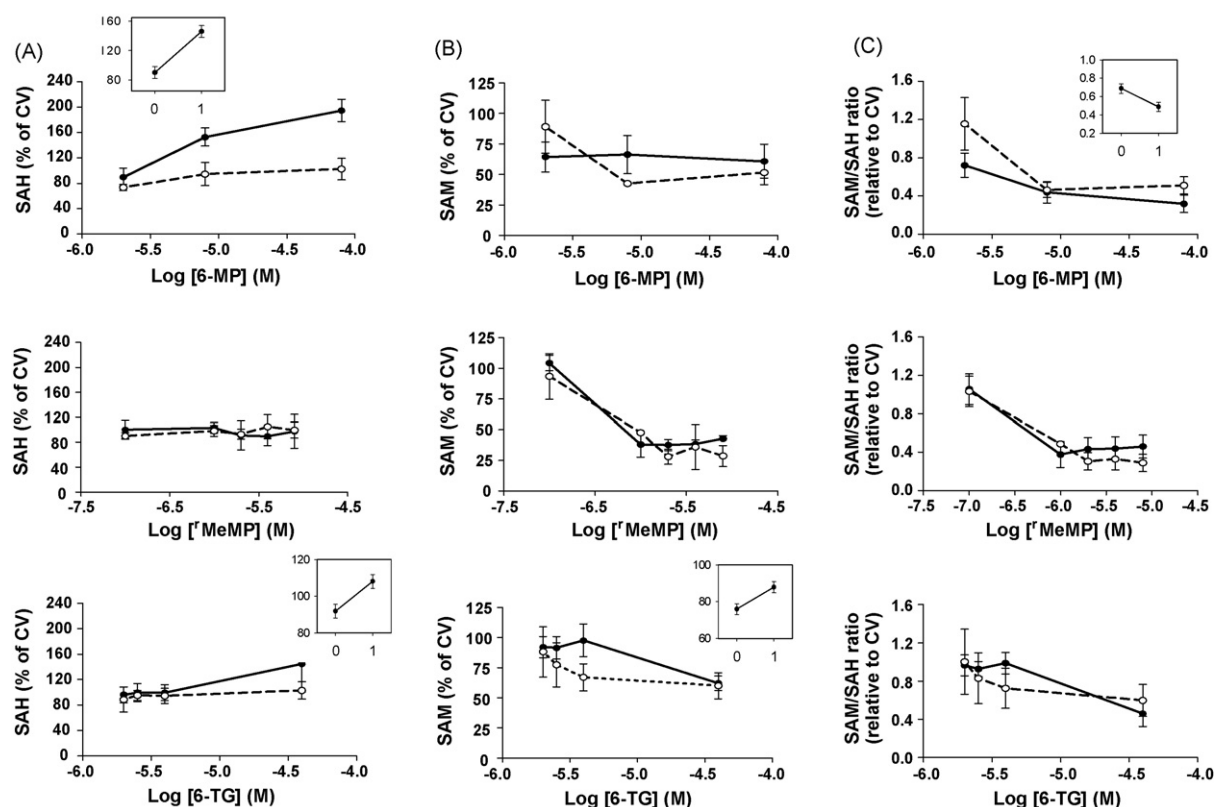
Fig. 3 – The effect of low (○) compared with high TPMT (●) expression on SAH (A) and SAM (B) levels and the SAM/SAH ratio (C) after 48-h exposure of EcR293-TPMT/20 cells to 6-TG, 6-MP and ^{r}MeMP. Error bars represent the S.D. of duplicates from three separate experiments. Inset graphs show where there is a significant effect of TPMT induction (P < 0.05) on global methylation with exposure to drug (see text for details). The points are the least-squares means ± S.E. from GLM analysis. Ordinate: global methylation (% control); abscissa: TPMT induction (1) or no TPMT induction (0, ethanol control).

### 3.4. The effect of 6-TG, 6-MP, ^{r}MeMP and 5-AdC on gene-specific DNA methylation

Patterns of gene-specific methylation vary between cell types but since the MAGE1A gene is methylated in normal tissues [31] and is demethylated by dAdC [32] this gene is a good candidate for studying the effects of the thiopurine drugs on gene-specific methylation in a line derived from normal embryonic cells. To study the effects of 6-MP, 6-TG and 5-AdC on the methylation of MAGE1A, methylation at three CpG sites in the promoter was measured in EcR293-TPMT/20 cells expressing low and high TPMT (Table 1). For this experiment, the concentration of each drug used was the dose showing the maximum decrease of global DNA methylation and DNMT activity (Fig. 4A and B) at the start of the plateau where further increase in dose did not cause a significantly-greater decrease in DNA methylation and DNMT activity. In the analysis by GLM, the three CpG sites differed in the extent of demethylation in response to drug and was taken into account by including this effect in the analysis; there was no differential effect of TPMT between CpG sites (P = 0.36). There was a significant decrease in CpG-site methylation in response to drug treatment ($F_{1,92} = 42.3$; $P < 0.00001$), but this varied depending on drug type: treatment with 5-AdC produced a marked decrease in methylation from 67.5% to 43% (averaged across the three CpG sites, least-squares means; Hypothesis

test: $F_{1,94} = 368$; $P < 0.0001$); conversely, treatment with 6-TG or 6-MP did not decrease methylation, but instead gave evidence of slightly increased methylation from 67% to 72% (Hypothesis tests: $F_{1,94} > 12$; $P < 0.001$). Overall, TPMT had no statistically-significant effect on methylation (TPMT main effect: $F_{1,92} = 1.7$; $P = 0.193$).

3.5. The effect of 6-TG, 6-MP, ^{r}MeMP and 5-AdC on DNMT levels

The effect of TPMT on DNMT activity after 48-h exposure of EcR293-TPMT/20 cells to drug is shown in Fig. 4B. The inhibition of DNMT activity largely reflected the changes in global DNA methylation obtained with each drug. For 6-MP, ^{r}MeMP and 5-AdC there was no difference in magnitude of DNMT activity between low and high TPMT-expressing cells ($F_{1,19} \leq 2.65$, $P > 0.119$) however, after 6-TG treatment cells expressing high TPMT showed a statistically-significant elevation in DNMT activity ($F_{1,25} = 18.86$, $P = 0.0002$). The effect of 6-TG, 6-MP, ^{r}MeMP and 5-AdC on expression of soluble DNMT1 protein is shown in Fig. 5A. All drugs decreased DNMT1 protein expression and this reflected the changes in the DNMT activity.

To determine whether changes in global methylation and DNMT activity related to extent of drug-induced growth
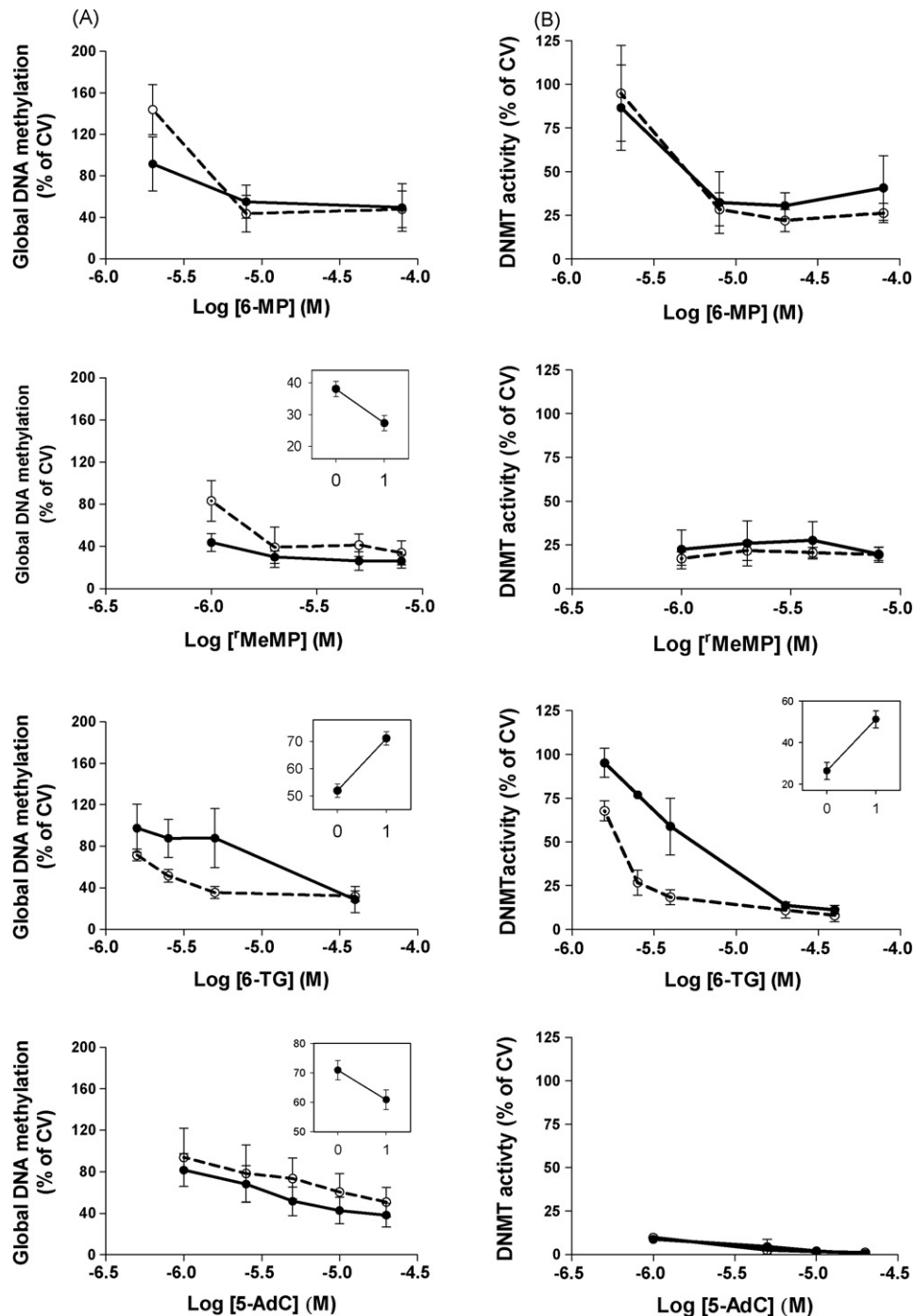
Fig. 4 – The effect of low (○) compared with high TPMT (●) expression on global DNA methylation (A) and DNMT activity (B) after 48-h exposure of EcR293-TPMT/20 cells to 6-TG, 6-MP, ᵀMeMP and 5-AdC. Error bars represent the S.D. of duplicates from three separate experiments for DNMT activity and at least three separate experiments for global DNA methylation. Inset graphs show where there is a significant effect of TPMT induction ($P < 0.05$) on global methylation with exposure to drug (see text for details). The points are the least-squares means ± SE from GLM analysis. Ordinate: global methylation (% control); abscissa: TPMT induction (1) or no TPMT induction (0, ethanol control).

inhibition, the effects of equitoxic doses (EC₅₀) of 6-MP and 6-TG, for high and low TPMT expression, on global methylation and DNMT activity were compared. These drugs are cytostatic and did not cause apoptosis at time points studied; therefore, EC₅₀ values represent inhibition of proliferation by 50%. For 6-

MP, equivalent growth inhibition with low and high TPMT expression showed statistically-different levels of global DNA methylation and DNMT activity (Fig. 5B), suggesting that inhibition of global DNA methylation and DNMT activity were not directly related to growth inhibition. Similar levels of

Table 1 – Comparison of the effect of 6-MP, 6-TG and 5-AdC on the percentage methylation of three CpG sites of the *MAGE1A* promoter						
	CpG#1		CpG#2		CpG#3	
	Mean	±S.D.	Mean	±S.D.	Mean	±S.D.
4 μM 6-TG □	92.0	1.0	57.7	2.5	68.7	1.5
4 μM 6-TG ■	87.7	0.6	56.0	3.5	66.0	1.7
8 μM 6-MP □	91.7	2.9	62.0	3.5	70.3	3.5
8 μM 6-MP ■	88.3	1.2	58.3	4.0	68.3	1.2
CV (for 6-MP and 6-TG) □	87.3	3.8	52.3	2.5	64.7	1.5
CV (for 6-MP and 6-TG) ■	85.3	6.4	52.0	1.7	62.3	6.1
5 μM 5-AdC □	54.3	2.5	27.7	3.1	47.7	4.6
5 μM 5-AdC ■	51.3	6.7	31.3	1.2	46.0	2.6
CV (for 5-AdC) □	83.3	1.5	51.0	2.0	62.0	1.0
CV (for 5-AdC) ■	87.3	0.6	54.3	0.6	67.0	1.5
IVM	87.3	3.2	43.3	2.5	61.7	3.2
N	88.0	1.0	47.7	3.1	62.3	4.0

Low and high TPMT expressing EcR293-TPMT/20 cells were exposed to 6-MP, 6-TG, 5-AdC or drug control vehicle (CV) for six doubling times, replacing with fresh drug and TPMT inducing agent (MA, ■) or MA control vehicle (ethanol, □) after three doubling times. *In vitro* methylated DNA (IVM) and human whole blood DNA (N) were used as controls for each experiment. The mean and S.D. of three individual measurements, of percentage methylation of each CpG site, for each treatment is shown.

global DNA methylation and DNMT activity were observed at equitoxic doses of 6-TG for high and low TPMT expression.

It has been reported that DNMT levels and global methylation alter during different phases of the cell cycle [32]. Hence, the effects of 6-MP and 6-TG on the fraction of cells in G_1, S and G_2 phases were measured to determine if changes in DNMT activity could be related to changes in the cell cycle. Both 6-MP and 6-TG appeared to cause S-phase arrest with a concomitant decrease of cells in the G_2 phase, in TPMT un-induced cells (Fig. 5C). However, this did not quite reach statistical significance when compared to TPMT un-induced control (non-drug treated) cells (fraction of cells in G2; 8 μM 6-MP $P = 0.07$, 2.8 μM 6-TG $P = 0.08$). As DNMT and DNA methylation have been shown to peak in the S phase, 6-MP- and 6-TG-induced changes in the cell cycle are unlikely to be responsible for the 6-MP- and 6-TG-induced decrease in DNMT and global DNA methylation.

4. Discussion

We originally postulated that TPMT levels would differentially affect global DNA methylation changes mediated by 6-MP and 6-TG. This hypothesis was based on the knowledge that TPMT metabolises 6-MP to MeTIMP, a strong inhibitor of DNPS, which decreases ATP and leads to a concomitant reduction in SAM levels [22]. Utilisation of SAM by TPMT-mediated metabolism of the thiopurine drugs has also been postulated to contribute to reduction in SAM with increased SAH levels in the T-ALL cell line Molt-4 [23]. As SAM is required as a substrate for DNA methylation by DNMTs, increased cellular TPMT would be expected to be associated with a greater decrease in global DNA methylation on exposure of cells to 6-MP. However, this was not the case in the studies presented here. Global DNA methylation of EcR293-TPMT/20 cells was inhibited by 6-MP to a similar extent in conditions of low and high TPMT expression. Also, as 6-TG is not metabolised to MeTIMP, but to MeTGMP, a weak inhibitor of DNPS in contrast to MeTIMP [27], this drug would be expected to have less effect

on DNA methylation than 6-MP. This was only the case in high TPMT expressing cells, as 6-TG in low TPMT expressing cells inhibited DNA methylation to a similar extent to 6-MP, at equivalent drug doses. These results suggest that a non-TPMT metabolised metabolite of 6-MP and 6-TG, possibly thioguanine nucleotide (TGN), contributes to a decrease in global DNA methylation, as illustrated in Fig. 6.

Interestingly, induction of TPMT increased the extent of global DNA de-methylation with 5-AdC. It is possible that there is increased utilisation of SAM by increased levels of TPMT resulting in decreased SAM available for methylation of 5-AdC incorporated into DNA by DNMTs. Although MA, used in the induction of TPMT, was originally thought to have no effects on mammalian cells and the expression of methyl-transferases is apparently not affected by MA (M. Zornig, personal communication), it has been reported that cytokine signalling in a murine pre-B cell line and the expression of apoptosis-related genes in a human colon carcinoma cell line can be affected by MA [33,34]. We found that MA reduced the extent of SAM depletion and global DNA methylation by ᵗMeMP in the parental EcR293 cells. However, this was not apparent in the EcR293-TPMT/20 cells, in which these effects were reversed by TPMT induction. The mechanism for these effects is unclear.

For both 6-MP and 6-TG, SAH levels were increased in response to TPMT induction, as would be expected if higher TPMT levels increased the methyl transfer rate from SAM. However, this was not reflected by changes in SAM levels, implying that the production of SAM was not rate-limiting. The increase in SAH observed when TPMT was induced was greater with 6-MP than with 6-TG, possibly due to increased utilisation of SAM in the production of methylated metabolites of 6-MP, or because a metabolite of 6-MP is inhibiting the breakdown of SAH. However, increase in SAH was not associated with MeTIMP levels *per se*, as equivalent levels of MeTIMP produced on exposure of cells to ᵗMeMP, compared to 6-MP, were not associated with increased SAH.

Several studies have shown that alterations in the ratio of SAM to SAH, rather than SAM or SAH levels alone, relate to
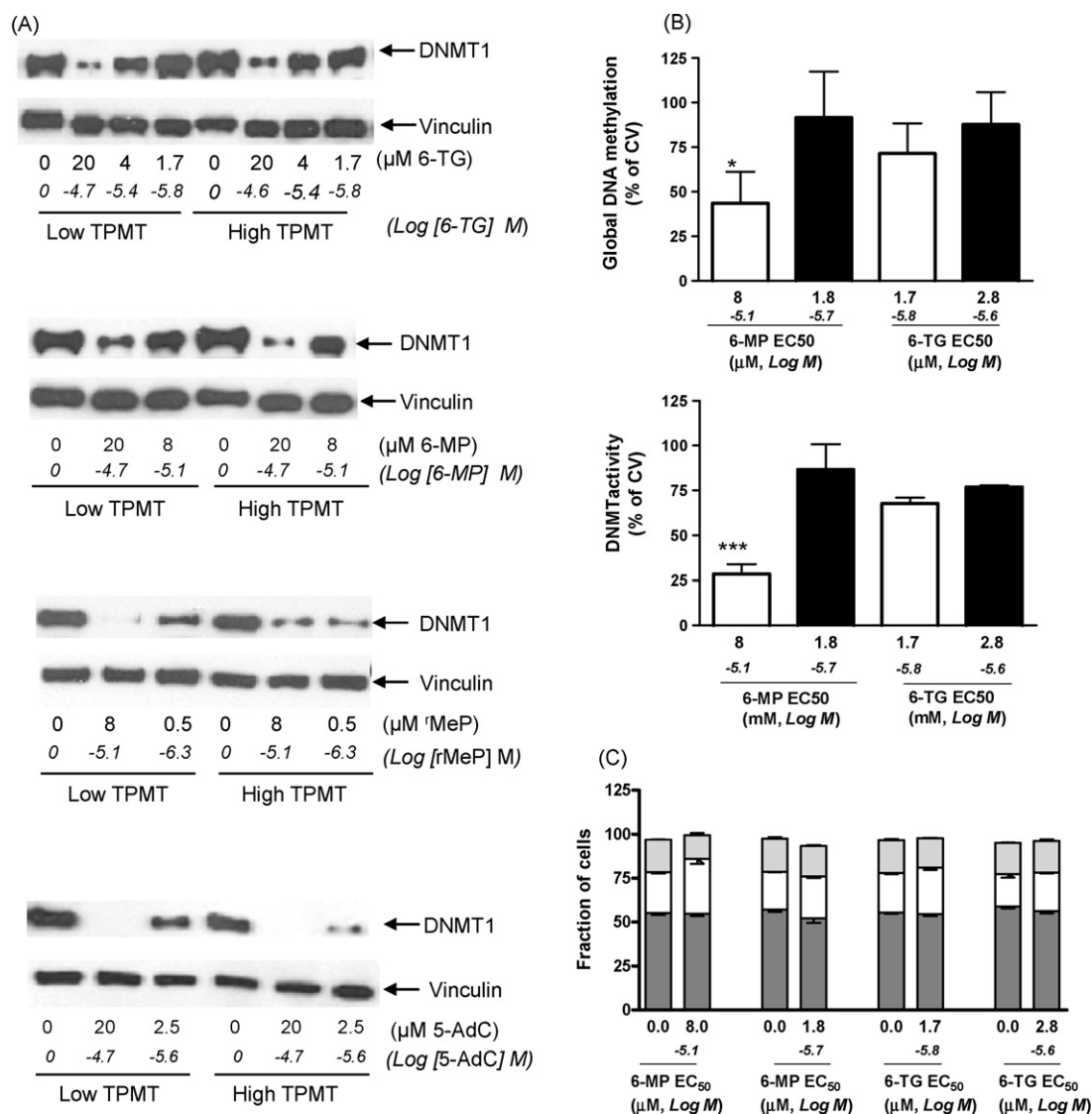
Fig. 5 – Western blots showing the effect of low compared with high TPMT expression on DNMT1 protein expression after 48-h exposure of EcR293-TPMT/20 cells to 6-TG, 6-MP, ʳMeMP and 5-AdC (A). The effect of equitoxic 6-MP and 6-TG doses on global DNA methylation, DNMT activity (B) and cell cycle (C) in TPMT low (□) and TPMT high (■) expressing EcR293-TPMT/20 cells. Error bars represent the mean ± S.D. from three separate experiments for DNMT activity (*P < 0.05, comparing EC$_{50}$ 6-MP doses) and at least three separate experiments for global DNA methylation (*P < 0.001, comparing EC$_{50}$ 6-MP doses). Cell cycle graph shows cells in G$_1$ (dark grey bar), S (white bar) and G$_2$ (light grey bar).**

changes in DNA methylation [12–14], as SAH has an inhibitory affect on DNMT activity [15]. With 6-TG there was a statistically-significant difference in global DNA methylation in the presence of induced compared to uninduced TPMT, which was not reflected by a difference in the SAM/SAH ratio. Also, in comparison to 6-MP and ʳMeMP, 6-TG with low TPMT reduced the SAM/SAH ratio to a lesser extent but DNA methylation to a similar extent. This suggests that factors other than SAM/SAH levels influence methylation, particularly with 6-TG. This led to the investigation of the effect of these drugs on DNMT activity.

As changes in DNA methylation were mirrored by changes in DNMT activity, for all drugs studied, inhibition of this enzyme is likely to play a major role in reduction of DNA methylation. The

decrease in DNMT activity was also associated with a decrease in soluble DNMT1 protein expression. The mechanism by which 6-TG decreases DNMT levels is unclear, but is more prominent with non-TPMT related metabolism, as low TPMT levels were associated with significantly greater inhibition of DNMT activity. Unlike 6-TG, 6-MP showed an equivalent inhibition of global DNA methylation and DNMT activity under conditions of low and induced TPMT expression. When TPMT is induced this inhibition is likely to be due to the 6-MP metabolite MeTIMP, as exposure of cells to ʳMeMP also resulted in a significant decrease in global DNA methylation and DNMT activity and protein expression.

During the cell cycle, DNMT1 expression is significantly reduced in the G$_0$- and G$_1$-phases and peaks during S-phase
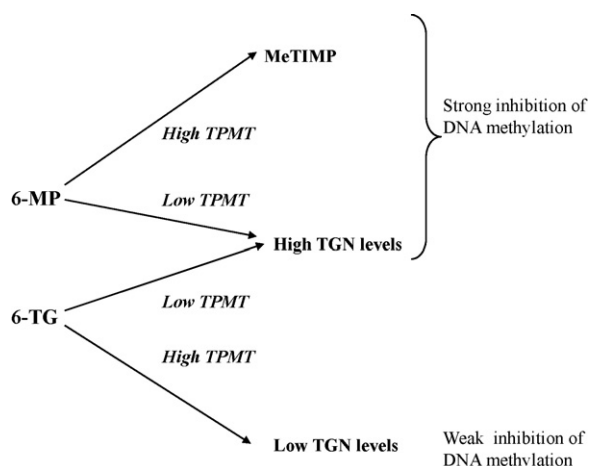
Fig. 6 – Hypothesis for inhibition of DNA methylation by 6-MP and 6-TG in relation to TPMT status.

(reviewed in [35]). The thiopurine-induced reduction in global DNA methylation and DNMT levels was not associated with statistically-significant changes in the cell cycle phases or with the observed trend for an increase in the fraction of cells in S-phase and decrease of cells in G_2. This suggests that inhibition of DNMT by 6-TG and 6-MP is not due to changes in the fraction of cells in specific cell-cycle phases.

Although 6-TG and 6-MP produced changes in global demethylation, it is not clear if this is also reflected at the level of individual genes. In contrast to global demethylation, decrease in methylation of the MAGE1A promoter was detected with 5-AdC but not with the thiopurine drugs. However, single-gene analyses require a longer-term exposure to drug than the global methylation assays. Since 6-TG and 6-MP cause extensive cell kill after long-term exposure at the concentrations necessary to observe a decrease in global DNA methylation, this will mitigate against identifying changes in methylation status of individual genes. Furthermore, 5-AdC caused a greater decrease in DNMT activity and DNMT1 protein levels than 6-MP and 6-TG. The biological significance of the apparent slight increase in methylation at the MAGE1A locus in response to 6-TG or 6-MP is not clear, but may relate to the properties of surviving cells resistant to the drug treatment.

These results do not negate the possibility of gene-specific demethylation effects at other genetic loci in cancer cells by the thiopurine drugs. It has been reported that DNMT1 and DNMT3b cooperate to silence genes and that knockout of both these genes results in a significantly greater reduction in methylated cytosines compared to the additive effect of knocking out each DNMT alone [36] and this substantial loss of methylation occurs in the promoter regions of tumour suppressor genes in cancer cell lines [37]. It has also been reported that DNMT3b may play a compensatory role in maintaining methylation patterns [36], which is possibly cell type dependent [38]. As 5-AdC has been shown to form DNMT3-DNA adducts to a similar extent to DNMT1-DNA adducts [39], this may contribute to the demethylating effect of 5-AdC at the MAGE1A promoter. It is unknown if the thiopurines affect DNMT3b expression in these cells as the

endogenous levels were too low to be detected using three commercially available antibodies (Hogarth and Coulthard, unpublished data).

It is important to note that DNMT1 knockdown causes effects which are independent of DNA methylation and histone deacetylation, including induction of genes involved in cell cycle arrest and stress response, and that these effects may have important therapeutic implications [40]. These authors point out that inhibition of DNA methylation can induce genes that promote metastasis and cause unwanted side effects and therefore targeting methylation-independent effects of DNMT1 may be therapeutically advantageous. Further investigations are required to determine if the thiopurines mediate methylation-dependent and/or methylation-independent effects of DNMT inhibition.

In summary, we have shown that 6-MP and 6-TG, at concentrations shown to be achievable in the plasma of patients administered these drugs [41–43], cause inhibition of global DNA methylation and that the extent of this inhibition is influenced by TPMT for 6-TG, but not for 6-MP. Although it has been suggested that inhibition of global DNA methylation by 6-MP is due to changes in SAM and SAH levels [23,24] our results suggest that a reduction in DNMT activity, in association with decreased DNMT1 protein levels, also has a prominent role for both 6-MP and 6-TG. These observations should be confirmed using in vivo leukaemic models as there are insufficient blast cells to be able to determine methylation changes in patients treated with thiopurines during remission. As demethylating agents are known to be active in leukaemia [9,10], it is possible that inhibition of DNA methylation by the thiopurine drugs may also contribute to their cytotoxic affects.

Acknowledgements

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