

The Effect of Thiopurine Methyltransferase Expression on Sensitivity to Thiopurine Drugs

SALLY A. COULTHARD, LINDA A. HOGARTH, MARGARET LITTLE, ELIZABETH C. MATHESON, CHRISTOPHER P. F. REDFERN, LYNNE MINTO, and ANDREW G. HALL

Leukaemia Research Fund Molecular Pharmacology Specialist Programme, Cancer Research Unit (S.A.C., L.A.H., M.L., E.C.M., L.M., A.G.H.), and Medical Molecular Biology Group (C.P.F.R.), Medical School, Newcastle University, Newcastle-upon-Tyne, UK

Received November 9, 2001; accepted March 25, 2002

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Although the thiopurine drugs 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are well established agents for the treatment of leukemia, controversies remain regarding their main mode of action. Previous evidence has suggested that although 6-TG exerts a cytotoxic effect through incorporation of 6-thioguanine nucleotides into newly synthesized DNA (DNA-TGN), an important component of the mode of action of 6-MP is inhibition of purine de novo synthesis (PDNS) through the production of S-methyl-thioinosine 5'-monophosphate (MeTIMP), not formed in cells exposed to 6-TG. We have shown that thiopurine methyltransferase (TPMT) modulates this effect. By transfection of the human *TPMT* gene using an inducible system to produce a 3.8-fold increase in TPMT activity in the ecdysone receptor 293 embryonic kidney cell line, we demon-

strated a 4.4-fold increase in sensitivity to 6-MP. This was associated with a rise in intracellular levels of MeTIMP but a decrease in levels of DNA-TGN. In contrast, induction of TPMT produced a 1.6-fold decrease in sensitivity to 6-TG, a decrease in levels of DNA-TGN, and an increase in levels of methylated thioguanosine monophosphate. Exposure of cells to equitoxic doses of drug showed similar incorporation of DNA-TGN for 6-TG but for 6-MP significantly reduced DNA-TGN in TPMT-induced compared with uninduced cells. For equitoxic doses of 6-MP, equivalent levels of MeTIMP correlated with equivalent amounts of PDNS. These observations suggest that intracellular TGN levels do not give an accurate reflection of cytotoxic potential in patients treated with 6-MP, because different levels of DNA-TGN may be associated with equitoxic effects.

Since their introduction into clinical practice more than 4 decades ago, the purine analogs 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) have been used extensively in the treatment of acute leukemia. 6-MP has been preferred over 6-TG (mostly for reasons of custom and practice) during continuing (maintenance) therapy in childhood acute lymphoblastic leukemia (ALL), whereas 6-TG has been used during consolidation and in remission induction in acute myeloid leukemia. However, several recent trials have sought to establish whether 6-TG might be a more effective agent than 6-MP during continuing therapy (Erb et al., 1998; Lancaster et al., 1998), including the present Medical Research Council ALL 97 trial. One rationale for these studies

has been the observation that treatment with 6-TG results in higher levels of thioguanine nucleotides (TGNs) within erythrocytes, which are used as a surrogate for levels in leukemic cells. It has been assumed that the TGN level correlates with higher levels of incorporation of fraudulent nucleotides into DNA and therefore higher levels of cell kill (Maddocks et al., 1986).

The assumption that TGN levels are a direct measure of cytotoxicity ignores the significant differences that exist between the intracellular metabolism of the thiopurine drugs. Both 6-MP and 6-TG are prodrugs that require activation by hypoxanthine-guanine phosphoribosyl transferase (HGPRT; Fig. 1) to exert a cytotoxic effect (Bertino, 1991). Competing with HGPRT for the metabolism of 6-MP are three enzymes: thiopurine methyltransferase (TPMT), aldehyde oxidase, and xanthine oxidase. In the case of 6-TG, xanthine oxidase can metabolize the drug only after prior conversion by guanase. The products of these competing reactions produce metabo-

This work was supported by grants from the Leukemia Research Fund and the North of England Children's Cancer Research Fund.

S.A.C. and L.A.H. contributed equally to the completion of this study. This work was previously presented in part in the Ph.D. dissertation of S.A.C., entitled "The role of thiopurine methyltransferase in the metabolism of cytotoxic drugs".

ABBREVIATIONS: 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; ALL, acute lymphoblastic leukemia; TGN, thioguanine nucleotide; HGPRT, hypoxanthine guanine phosphoribosyltransferase; TPMT, thiopurine methyltransferase; TGMP, 6-thioguanosine 5'-monophosphate; MeTIMP, S-methyl-thioinosine 5'-monophosphate; PDNS, purine de novo synthesis; EcR, ecdysone receptor; MA, muristerone A; dG^s, deoxythioguanosine; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; MeMP, S-methyl mercaptopurine; MeTG, S-methyl thioguanine; 'MeMP, S-methyl mercaptopurine riboside; MeTGMP, S-methyl-thioguanosine 5'-monophosphate; SRB, sulforhodamine B; CI, confidence interval; ANOVA, analysis of variance; MMR, mismatch repair.

lites with little or no cytotoxic activity. Metabolism of 6-TG by HGPRT produces 6-thioguanosine 5'-monophosphate (TGMP), which is further metabolized by a series of kinases and reductases to produce deoxy-6-thioguanosine 5'-triphosphate. Incorporation of deoxy-6-thioguanosine 5'-triphosphate into DNA has been shown to trigger cell cycle arrest and apoptosis by a process that involves the mismatch repair pathway (Swann et al., 1996). The metabolism of 6-MP to TGMP is less direct than that of 6-TG, involving two additional enzymes, inosine monophosphate dehydrogenase and guanosine monophosphate synthetase. This difference is potentially important as the first intermediate in this pathway, thioinosine monophosphate can act as a substrate for TPMT, leading to the production of *S*-methyl-thioinosine 5'-monophosphate (MeTIMP), a strong inhibitor of purine de novo synthesis (PDNS) (Tay et al., 1969).

It has been suggested that PDNS inhibition may make a significant contribution to the cytotoxic action of 6-MP (Allan and Bennett, Jr., 1971; Erb et al., 1998; Lancaster et al., 1998). We postulated that the relative contribution to cell kill made by TGMP incorporation into DNA and PDNS inhibition by MeTIMP may depend on the level of TPMT expression. As the level of expression of TPMT varies greatly between individual persons, partly because of genetic polymorphisms that produce inactive forms of the enzyme (Szumlanski et al., 1996; Tai et al., 1996; Krynetski et al., 1997; Otterness et al., 1997; De la Moureyre et al., 1998; Spire-Vayron et al., 1999; Colombel et al., 2000; McLeod et al., 2000; Seki et al., 2000), this has important clinical significance, both for the selection of the agents to use in clinical practice and in the propensity of the drugs to generate DNA mutations (Relling et al., 1998, 1999; Thomsen et al., 1999; Pui and Relling, 2000). To explore the effect of changes in TPMT expression on the cytotoxic effect of 6-MP and 6-TG, we have transfected cDNA for the human *TPMT* gene under the control of an inducible promoter into human embryonic EcR293 cells and provide detailed evidence for the role of TPMT in the modulation of thiopurine activity.

Materials and Methods

Cell Lines and Transfection. Human TPMT cDNA was subcloned into the pIND vector of the ecdysone-inducible expression system (Invitrogen, Paisley, UK) from a T84 clone kindly provided by Dr. R. Weinshilboum (Department of Pharmacology, Rochester, MN) (Szumlanski et al., 1996). The linearized pIND vector was transfected into embryonic kidney EcR293 cells pretransfected with the pVgRXR vector, as provided by Invitrogen.

TPMT-transfected cells were selected and maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 0.11 g/l sodium pyruvate (Invitrogen, Paisley, UK), 100 IU/ml penicillin, 100 µg/ml streptomycin, 10% heat inactivated fetal bovine serum (Invitrogen), 400 µg/ml Zeocin (Invitrogen), and 500 µg/ml Geneticin (Invitrogen). TPMT expression was induced with 3 µM muristerone A (MA) (Invitrogen) in ethanol.

TPMT Assay. TPMT activity in transfected cell lines was determined in cell lysates using a radiochemical assay, as described previously (Weinshilboum et al., 1978). Lysate protein concentration was estimated using a commercially available kit (BCA Protein Assay; Perbio Science UK Ltd, Cheshire, UK) with bovine serum albumin as a standard. Results were expressed as units per milligram of cellular protein, where one unit is the amount of activity required to catalyze the formation of 1 nmol of 6-methylmercaptapurine per hour.

Western Blotting. Western blotting was performed using a rabbit polyclonal antibody raised against human recombinant TPMT. Cell pellets were re-suspended in 500 µl of ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride in isopropanol and a protease inhibitor cocktail of 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucylamide(4-guanido)butane, bestatin, leupeptin, and aprotinin (Sigma, Poole, Dorset, UK) and disrupted by sonication. Lysates were centrifuged at 7000 g for 5 min at 4°C, heated at 100°C for 5 min and stored at -20°C before analysis.

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using precast gradient gels [4–20%; Novex (Invitrogen)] with 7.5 µg of protein per well. Enhanced chemiluminescence molecular weight markers (5 µl; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and 8 ng of recombinant TPMT were run as control tracks.

Immunoblotting was performed using nitrocellulose membranes (0.45 µm; Hybond-C; Amersham Biosciences). Nonfat dried milk (5%) in TBS/Tween (0.1 M NaCl, 0.05% Tween 20, 0.01 M Tris-HCl,

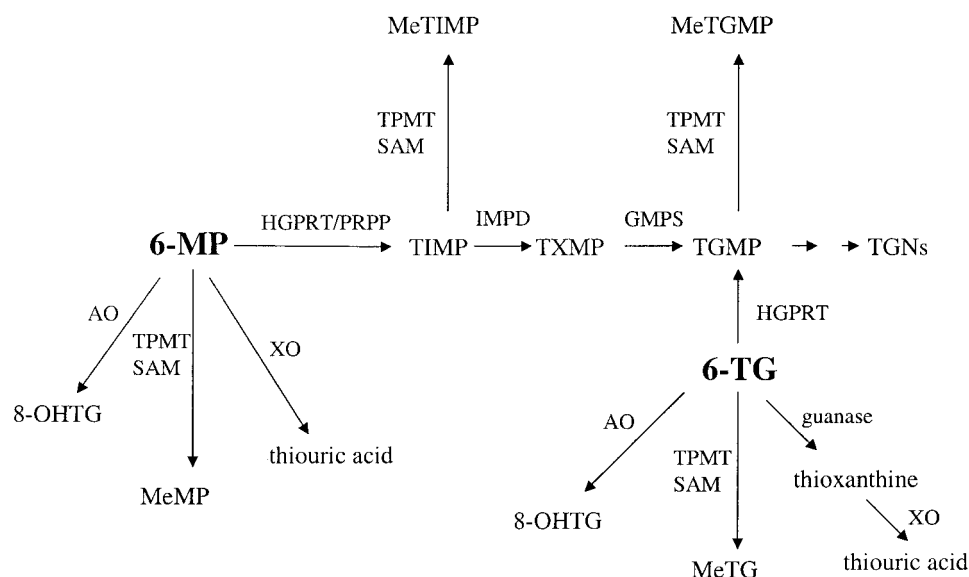


Fig. 1. Metabolism of 6-MP and 6-TG in human ALL cells. PRPP, 5'-phosphoribosyl-1-pyrophosphate; GMPS, guanosine monophosphate synthetase; IMPD, inosine monophosphate dehydrogenase; SAM, *S*-adenosine-L-methionine; AO, aldehyde oxidase; 8-OHTG, 8-hydroxythioguanine; XO, xanthine oxidase; TIMP, thioinosine 5'-monophosphate; TXMP, thioxanthine monophosphate.

pH 7.5) was used to block nonspecific binding. Membranes were exposed to TPMT primary antibody (diluted 1000-fold in blocking buffer) for 1 h followed by biotinylated swine anti-rabbit immunoglobulins (Dako, Ely, Cambridgeshire, UK) (1/2000 in TBS/Tween) for 30 min and Streptavidin-horseradish peroxidase (1/5000 in TBS/Tween) for 30 min. Membranes were washed thoroughly in TBS/Tween between incubations. Immune complexes were detected using an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham Biosciences).

Measurement of Deoxythioguanosine Incorporation into DNA. Deoxythioguanosine (dG^s) incorporation into DNA was measured using an adaptation of the method described by Warren et al. (1995). DNA was extracted from 5×10^6 cells suspended in 200 μ l of phosphate-buffered saline (PBS) using a commercially-available spin column method according to the manufacturer's instructions (QIAamp DNA Mini Kit; QIAGEN, Valencia, CA). Purified DNA was eluted with 200 μ l of 10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0. Before digestion and derivatization, DNA samples were denatured at 100°C for 5 min followed by rapid chilling on ice. Ten microliters of digestion buffer (500 mM sodium acetate buffer, 10 mM MgCl₂, pH 4.5) and 20 μ l of 25 μ g/ml P₁ nuclease (Roche Diagnostics Ltd, Lewes, UK) in 50 mM sodium acetate buffer, pH 4.5, containing 1 mM MgCl₂, were added to 100 μ l of spin column eluent. After incubation at 42°C for 1 h, 20 μ l of 1 M Tris, pH 8.0, and 1 μ l (1 unit) of calf intestinal alkaline phosphatase (Roche Diagnostics) were added and the mixture incubated at 37°C for 30 min before the addition of 10 μ l of 400 mM formic acid and 60 μ l of methanol. Samples were derivatized overnight in the dark at room temperature using 5 μ l of a 1 mM solution of *N*-[6-(7-amino-4 methylcoumarin-3-acetamido) hexyl]-3'-(2'-pyridyldithio)propanamide (Pierce Chemical Company, Rockford, IL) in dimethyl formamide.

High-performance liquid chromatography (HPLC) was performed as described by Warren et al. (1995), except that each run included an isocratic phase from 0 to 10 min of 10% methanol, 90% 0.2 M formate buffer, pH 4.0, for the separation of DNA nucleosides followed by a linear gradient over 2 min to 40% methanol and 60% 0.2 M formate, which was held isocratically for 25 min. Thymidine was detected by absorbance at 461 nm. Compounds were identified on the basis of retention time and quantified using calibration curves (Warren and Stordal, 1993). Because dG^s is unavailable commercially, thioguanosine was used as a standard for this compound. A 10 mM solution of thioguanosine was made in 20 mM sodium hydroxide and further dilutions were made in 10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0. Thymidine standards were prepared in 10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0. dG^s incorporation into DNA was expressed as the number of dG^s residues per 100 thymidine residues (dG^s/100 T).

Measurement of Methylmetabolites of 6-Mercaptopurine and 6-Thioguanine. Methylmetabolite standards were prepared as follows. Stocks of 20 mM 6-MP, 6-TG, *S*-methyl mercaptopurine (MeMP), *S*-methyl thioguanine (MeTG) and methylmercaptapurine riboside (³MeMP) (Sigma) were prepared in 0.1 N NaOH. MeTIMP and *S*-methyl thioguanosine monophosphate (MeTGMP) were prepared from the incubation of freshly prepared erythrocytes with ³MeMP or MeTG, respectively. Fresh blood collected into lithium heparin was centrifuged, the plasma and buffy coat layer was removed, and the erythrocytes were washed twice with PBS before resuspension at 25% (v/v) in 50 mM potassium phosphate buffer, pH 7.4, containing 75 mM NaCl, 2 mM MgSO₄, 10 mM glucose, and 1 mM ³MeMP or MeTG. Suspensions were incubated for 22 h in an orbital shaker at 37°C. Perchloric acid (70%) was added to a concentration of 10%, incubated on ice for 30 min, and then centrifuged. The supernatant was removed and the pH adjusted to 8.0 with 10 M KOH. The sample was centrifuged to remove precipitated salt and the supernatant concentrated approximately 15-fold by centrifugation under vacuum. The concentrate was centrifuged at 14,000g for 10 min and 150- μ l aliquots were separated by HPLC using the separation procedure described by Krynetski et al. (1995). Peaks corresponding to MeTIMP and MeTGMP were collected, freeze-

dried, and resuspended in 1 M Tris-HCl, pH 8.0. Confirmation that the peaks collected were the correct compounds was shown by heating MeTIMP and MeTGMP for 1 h at 100°C in 10% perchloric acid, which gave a peak (by HPLC analysis) at the same retention time as MeMP and MeTG treated in the same way, respectively. Removal of a phosphate group from MeTGMP was also confirmed by treatment with alkaline phosphatase resulting in a change in retention time by HPLC. Treatment of MeTIMP with alkaline phosphatase produced a compound with the same retention time as ³MeMP. The correct mass for MeTIMP and MeTGMP was confirmed by mass spectrum analysis (data not shown).

Methylmetabolites were measured using an adaptation of the reversed-phase HPLC method described by Krynetski et al. (1995). Cells were washed twice in PBS after drug exposure and stored as pellets of approximately 5×10^6 cells at -80°C. For analysis, cells were resuspended in 500 μ l of Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and sonicated for 20 s. A 100- μ l aliquot was removed for protein measurement as described above. Remaining lysate was filtered using Centricon-3K filters (Millipore, Bedford, MA) in a microcentrifuge for 45 min at 20,800g and metabolites were measured in the filtrate. Metabolite concentrations were expressed per milligram of protein. Standards were diluted in the Tris-EDTA buffer and 500 μ l of each was added to a pellet of 5×10^6 untreated cells and prepared as described for samples.

PDNS Assay. The rate of PDNS was measured as described by Masson et al. (1996). Cells were incubated for 2 h with [¹⁴C]formate (Amersham Biosciences) to a final specific activity of 127 d.p.m./pmol, concentration 0.5 mM. PDNS was expressed as the amount of radiolabeled purine base (adenine and guanine) relative to the respective nonradiolabeled base and the amount of PDNS in experimental treatments calculated as a percentage of the control cells.

Estimation of Doubling Times and Drug Sensitivity. Cell doubling times were determined by the sulforhodamine B (SRB) assay (Skehan et al., 1990) and drug sensitivity was determined using both the SRB and clonogenic assays. For the determination of drug sensitivity, 96-well plates were seeded with 3×10^3 cells per well and TPMT expression was induced using 3 μ M MA for 24 h the day before drug or ethanol (control vehicle) was added. Cells were incubated for 2.5 culture doublings each. Percentage survival was calculated from the absorbance measurements at 530 nm, using the no-drug control as 100%.

For the determination of clonogenic survival, adherent cells at approximately 60% confluence were treated with either MA to a final concentration of 3 μ M or with the equivalent volume of ethanol and incubated for 24 h before harvesting. Duplicate 90-mm dishes were seeded with single-cell suspension of 1.5×10^3 in 10 ml of medium containing either 6-TG or 6-MP for 10 doubling times. Colonies were stained with 0.4% crystal violet (Sigma). Numbers of colonies were expressed as a percentage of control vehicle.

Statistical Methods. Drug sensitivity assays were analyzed using PRISM software (GraphPad, San Diego, CA) in which a sigmoidal dose-response curve (variable slope) or the spline LOWESS (point to point) curve analysis was fitted to all data for which drug concentrations were logged and the log IC₅₀ measured from the curve automatically. Mean log IC₅₀ values from separate experiments were compared by two-tailed unpaired *t* test and expressed as a mean + 95% CI in the original (un-logged) scale. Drug metabolite levels and PDNS measurements were compared, and hypotheses tested, by two-way ANOVA using Systat software (version 10.0; SPSS Inc., Chicago, IL).

Results

Characterization of TPMT Transfectants. The clone showing the highest level of induction, clone 20 (EcR293-TPMT/20), was selected for further study. The optimum concentration required for TPMT induction was determined by

performing Western blotting for TPMT and TPMT activity measurements after exposure for 24 h to 0 to 5 μM MA (Fig. 2). Western blotting confirmed induction of TPMT protein. TPMT activity rose from a baseline of 0.69 U/mg of protein to reach a plateau of 2.67 U/mg. To determine the stability of TPMT induction, EcR293-TPMT/20 cells were treated with 3 μM MA and TPMT activity was measured at 24, 48, and 72 h (Fig. 2C). There was a continued increase in TPMT activity after addition of MA that began to reach a plateau after 72 h. The doubling time of EcR293-TPMT/20 cells treated with MA was 29 h compared with 21 h for uninduced cells.

The Effect of Increased TPMT Expression on Sensitivity to Thiopurine Drugs. To compensate for the lengthening of doubling times after MA induction, cells were treated for an equivalent number of cell doublings in both SRB and clonogenic assays. For the SRB assays, IC_{50} values (the drug concentration required to inhibit growth by 50%) were assessed from growth inhibition curves. There was a small but statistically significant decrease in sensitivity to 6-TG with IC_{50} values rising on induction of TPMT from 1.74 μM (95% CI, 1.65 to 1.83 μM) to 2.84 μM (95% CI, 2.25 to 3.59 μM , $t_4 = 3.73$, $P < 0.01$) (Fig. 3A). Conversely, when the cells were treated with 6-MP, there was an almost 4-fold increase in the sensitivity to 6-MP in cells with induced TPMT with IC_{50} values for the TPMT induced cells decreasing to 1.78 μM (95% CI, 1.43 to 2.20 μM) from 8.01 μM for the uninduced cells (95% CI, 3.30 to 19.42 μM , $t_5 = 5.34$, $P < 0.01$) (Fig. 3B).

In contrast to the cytotoxicity assays, assessment of clonogenicity revealed no statistically significant difference be-

tween those cells expressing high levels of TPMT or the uninduced cells with either 6-TG or 6-MP. With 6-TG, IC_{50} values were 1.40 (95% CI, 0.75 to 2.05 μM) and 1.46 μM (95% CI, 1.11 to 1.91 μM) for induced and uninduced cells, respectively ($t_3 = 0.88$, $P > 0.05$) (Fig. 4A). IC_{50} values for 6-MP treated cells were 0.66 μM (95% CI, 0.59 to 0.77 μM) and 0.68 μM (95% CI, 0.60 to 0.78 μM) for induced cells uninduced cells, respectively ($t_4 = 0.94$, $P > 0.05$) (Fig. 4B).

The Effect of TPMT Expression on dG^{s} Incorporation into DNA. An HPLC technique was used to determine whether alteration in TPMT expression led to a change in levels of incorporation of dG^{s} into DNA. Comparisons were made after 24-h exposure to a range of concentrations of either 6-TG or 6-MP in cells with or without induced TPMT (Fig. 5). For both 6-TG and 6-MP there was a dose-dependent increase in dG^{s} incorporation into DNA. dG^{s} levels were significantly lower in TPMT induced compared with uninduced cells using 6-TG at concentrations of 1.7 to 40 μM (two-way ANOVA on data for drug-treated cells, effect of TPMT induction $F_{1,16} = 25.578$, $P < 0.001$) and 6-MP at concentrations of 1.8 to 80 μM (two-way ANOVA, effect of TPMT induction $F_{1,12} = 15.577$, $P < 0.01$). At equitoxic concentrations of 6-TG (Fig. 5A) in TPMT-induced (2.8 μM 6-TG) and uninduced (1.7 μM 6-TG) cells there was no significant difference in the incorporation of dG^{s} into DNA (hypothesis test, $F_{1,16} = 0.111$, $P > 0.7$). In contrast, using equitoxic doses of 6-MP (Fig. 5B), dG^{s} levels were markedly lower in cells with induced TPMT (1.8 μM 6-MP) compared

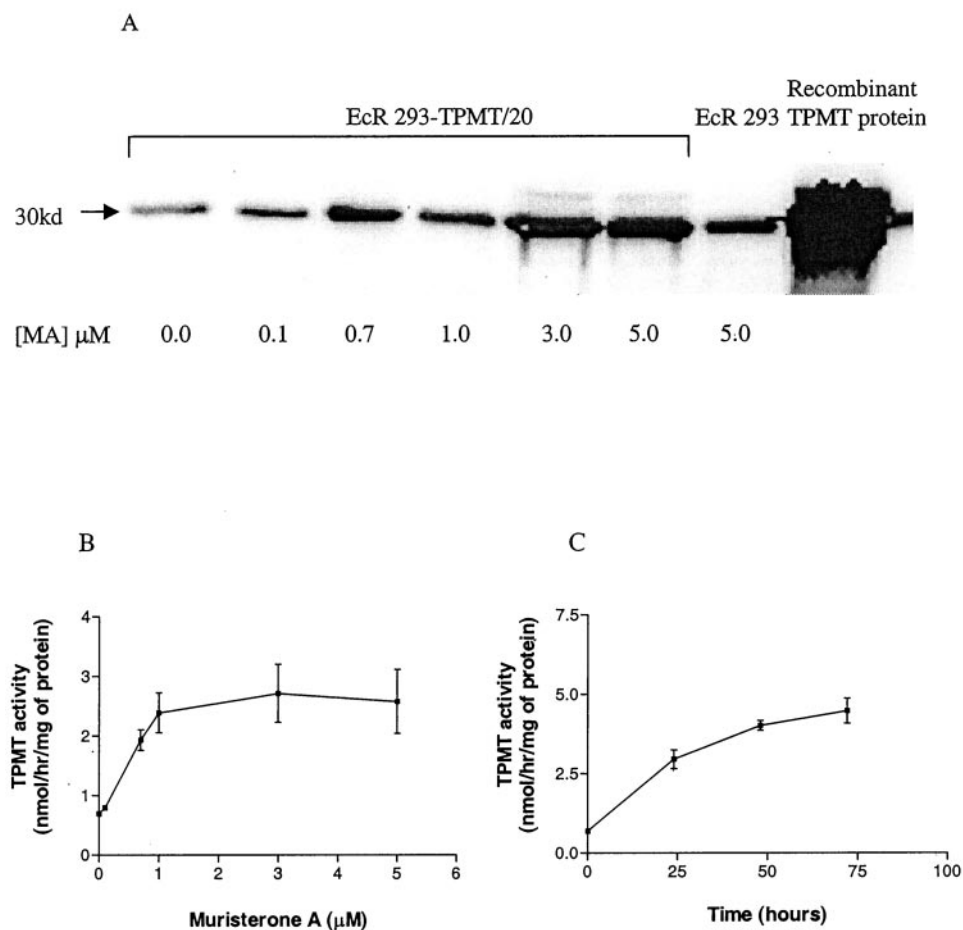


Fig. 2. Effect of muristerone A for 24 h on TPMT activity and stability of expression in EcR293-TPMT/20 cells. The effect of increasing MA concentration on TPMT protein expression by Western blot (A) and activity (B). Stability of TPMT activity over time after addition of 3 μM MA (C). Error bars represent the S.E.M. of three replicates from three separate experiments.

with uninduced cells ($8 \mu\text{M}$ 6-MP) (hypothesis test, $F_{1,12} = 11.073$, $P < 0.01$).

Measurement of Levels of Methylated Thiopurine Metabolites. The effect of TPMT expression on the extent of methylated thiopurine metabolite production is shown in Fig. 6. Cells were treated with 40, 4, 2.8, and $1.7 \mu\text{M}$ 6-TG or 80, 8, and $1.8 \mu\text{M}$ 6-MP, and levels of MeTG, MeMP, MeTIMP, and MeTGMP were measured after 24-h drug exposure. Irrespective of TPMT status, MeTG and MeMP were not detected in cells exposed to 6-TG and 6-MP, respectively. With $40 \mu\text{M}$ 6-TG, increased TPMT expression led to a significant increase in MeTGMP [0.25 ± 0.05 (S.D.) versus 0.04 ± 0.03 nmol/mg protein, $t_4 = -6.609$, $P < 0.01$] (Fig. 6A). This metabolite was not detected in cells exposed to lower concentrations of 6-TG or in cells exposed to 6-MP. With 80, 8, and $1.8 \mu\text{M}$ 6-MP, induction of TPMT led to a significant increase in the level of MeTIMP (two-way ANOVA, effect of TPMT induction $F_{1,9} = 25.845$, $P < 0.001$) (Fig. 6), but MeTIMP

levels at equitoxic doses were similar (two-way ANOVA, hypothesis test $F_{1,9} = 0.542$, $p = 0.48$).

PDNS Measurements. At equitoxic doses of 6-MP, similar amounts of PDNS were observed (two-way ANOVA, $F_{1,18} = 0.02$, $P > 0.8$ for adenine and $F_{1,18} = 0.08$, $P > 0.7$ for guanine; $1.8 \mu\text{M}$ 6-MP for TPMT induced versus $8 \mu\text{M}$ 6-MP for uninduced). However, for 6-TG, equitoxic doses produced a marked difference in PDNS, with TPMT-induced cells having higher PDNS than those not expressing TPMT (two-way ANOVA, $F_{1,24} = 19.1$, $P < 0.001$ for adenine and $F_{1,24} = 20.67$, $P < 0.001$ for guanine; $2.8 \mu\text{M}$ 6-TG for TPMT induced versus $1.7 \mu\text{M}$ 6-TG for uninduced).

Discussion

The level of activity of the enzyme TPMT varies greatly between individual subjects in part because of the presence of polymorphisms in the TPMT gene that may affect either the

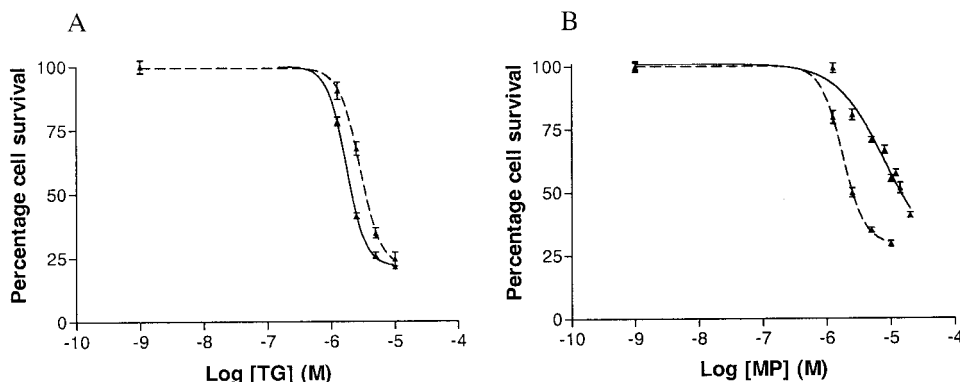


Fig. 3. The effect of TPMT induction on sensitivity to 6-TG and 6-MP as assessed by SRB assay. Broken line represents EcR293-TPMT/20 induced with $3 \mu\text{M}$ MA solid line represents EcR293-TPMT/20 treated with ethanol [control vehicle (CV)]. Cells were exposed to either 6-TG (A) or 6-MP (B) for 2.5 doubling times. For each experiment, six replicate wells were used to calculate the mean response at each dose and the error bars represent the S.E.M. of the means for three separate experiments.

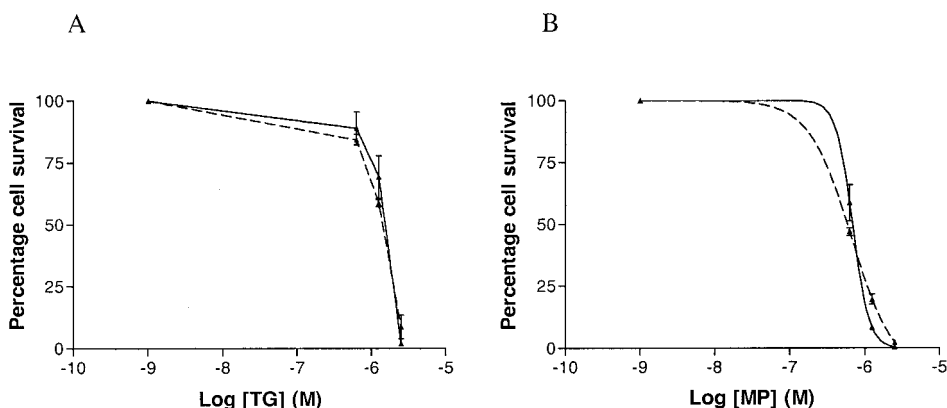


Fig. 4. The effect of TPMT induction on sensitivity to 6-TG and 6-MP as assessed by clonogenic assay. Broken line represents EcR293-TPMT/20 induced with $3 \mu\text{M}$ MA solid line represents EcR293-TPMT/20 treated with ethanol (CV). Cells were exposed to either 6-TG (A) or 6-MP (B) for 10 doubling times. Error bars represent the S.E.M. of duplicates from three separate experiments.

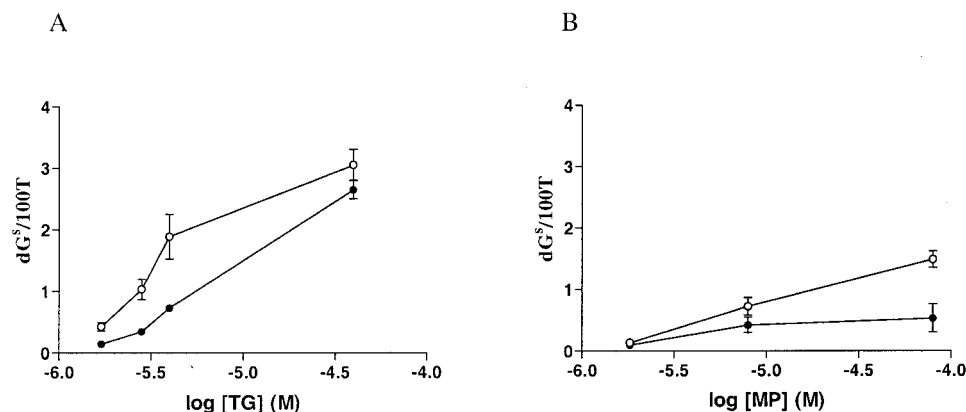


Fig. 5. Deoxythioguanosine incorporation into genomic DNA of EcR293-TPMT/20 cells after exposure to drug. EcR293-TPMT/20 cells exposed to $3 \mu\text{M}$ MA (●) or ethanol (CV) (○) treated for 24h with various concentrations of either 6-TG (A) or 6-MP (B). Error bars represent the S.E.M. of duplicates from three separate experiments.

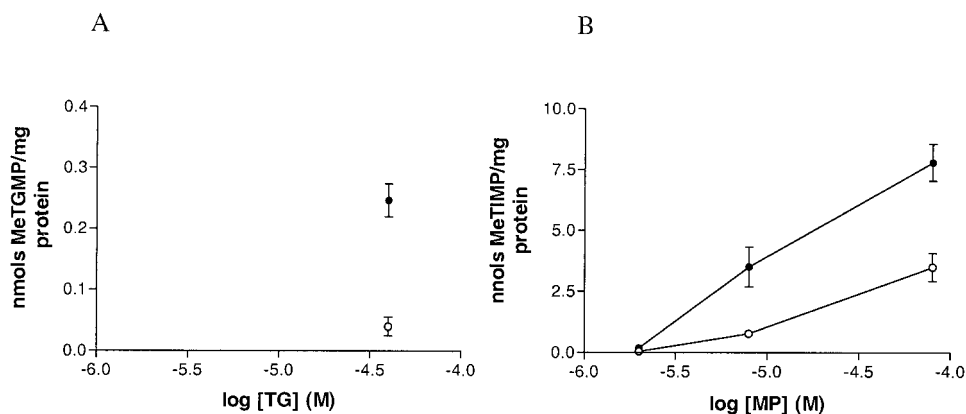


Fig. 6. Intracellular methylated metabolite levels in EcR293-TPMT/20 cells after exposure to drug. EcR293-TPMT/20 cells exposed to 3 μ M MA (●) or ethanol (CV) (○) treated for 24h with various concentrations of either 6-TG (A) or 6-MP (B). Error bars represent the S.E.M. from three separate experiments.

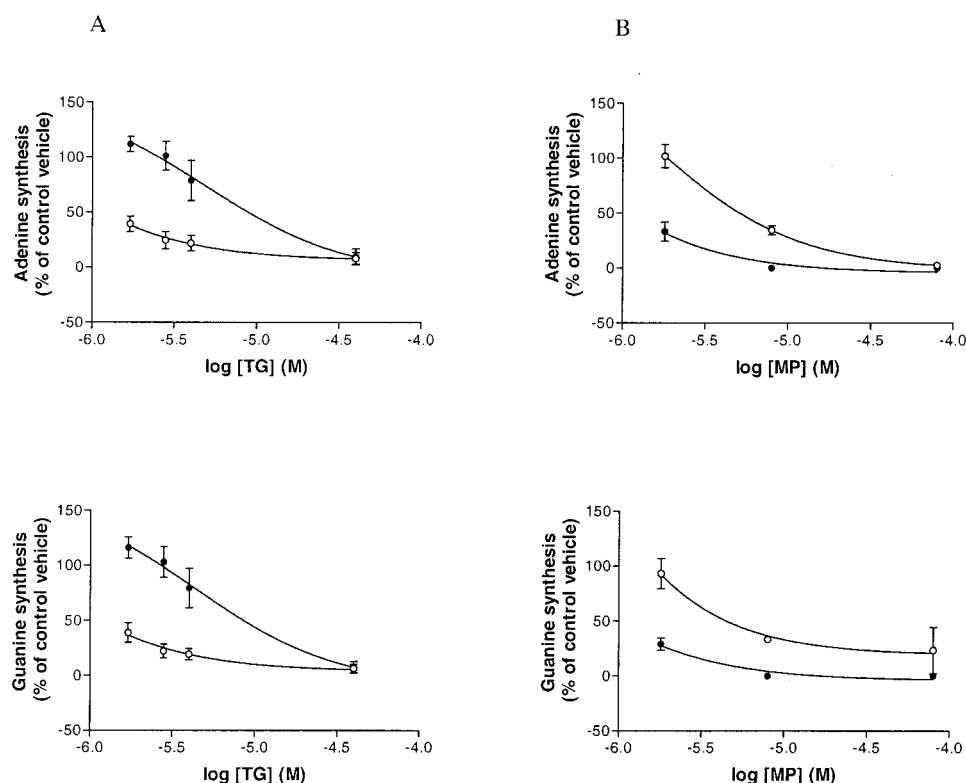


Fig. 7. PDNS in EcR293-TPMT/20 cells after exposure to drug. EcR293-TPMT/20 cells exposed to 3 μ M MA (●) or ethanol (CV) (○) treated for 48 h with various concentrations of either 6-TG (A) or 6-MP (B). Error bars represent the S.E.M. from four separate experiments.

enzymic activity or protein stability (Szumlanski et al., 1996; Tai et al., 1996; Krynetski et al., 1997; Otterness et al., 1997; De la Moureyre et al., 1998; Spire-Vayron et al., 1999; Colombel et al., 2000; McLeod et al., 2000; Seki et al., 2000). Approximately 10% of white persons carry a polymorphic *TPMT* gene and biallelic polymorphisms occur in about 1 in 300 persons. (Otterness et al., 1997; Yates et al., 1997). Subjects with this genotype have very low levels of TPMT activity; if they are treated with standard doses of thiopurine drugs, they experience profound myelosuppression. This is associated with greatly elevated intracellular TGN levels, as determined in red blood cells.

By considerably reducing the dose used and carefully monitoring the white blood cell count, it has been possible to reintroduce 6-MP in patients with TPMT deficiency (Lennard et al., 1993; 1997a,b; Evans et al., 1991; McLeod et al., 1993; Andersen et al., 1998). Interestingly, it has been noted that the red blood cell TGN levels in these cases are above the range seen in cases with normal TPMT activity (Erb et al.,

1998), suggesting that in the absence of TPMT, higher TGN levels are required to produce equitoxic effects. One possible explanation for this observation is that 6-MP exerts a cytotoxic effect by a mechanism independent of TGN production, for example through inhibition of PDNS by MeTIMP.

Treatment of transfected cells with 3 μ M MA caused a 4-fold increase in TPMT activity, comparable with the range we reported previously in leukemic blasts (Coulthard et al., 1998).

The effect of enhanced TPMT expression on drug sensitivity, MeTIMP production, DNA-TGN incorporation, and PDNS was found to differ markedly for 6-MP and 6-TG. In the case of 6-TG, induction of TPMT led to a 1.6-fold decrease in cytotoxic sensitivity as assessed by the SRB assay. This was mirrored by a decrease in the level of DNA-TGN incorporation. At equitoxic doses, the level of incorporation was similar, supporting the hypothesis that for 6-TG, the chief mode of cytotoxic action is incorporation of TGNs into DNA. MeTGMP, a weak inhibitor of PDNS, was detectable only

with 40 μM 6-TG but not at the equitoxic doses. At equitoxic doses of 6-TG, there was a greater than 2-fold increase in the amount of PDNS in those cells with TPMT induction. At equimolar doses, our results are comparable with those of Dervieux et al. (2001), who showed that PDNS was greater when TPMT was induced. These results, in combination with the finding of equivalent amounts of DNA-TGNs at equitoxic doses, support the historical belief that the mechanism of cytotoxicity for 6-TG is dependent on DNA-TGN incorporation rather than inhibition of PDNS.

In marked contrast with the results obtained with 6-TG, induction of TPMT led to an increase in sensitivity to 6-MP of more than 4-fold as measured by the SRB assay. However, this was accompanied by a decrease in the level of DNA-TGN incorporation similar to that seen for 6-TG. At equitoxic doses of 6-MP, DNA-TGN levels fell to below the level of detection when TPMT expression was induced. After equimolar doses of 6-MP treatment, the level of MeTIMP showed a significant increase with a decrease in PDNS after TPMT induction.

However, at equitoxic doses of 6-MP in cells expressing high and low TPMT, there were similar levels of PDNS and MeTIMP. Because MeTIMP is a strong inhibitor of PDNS (Allan and Bennett, 1971), the fact that there were similar levels of PDNS and MeTIMP at equitoxic doses suggests that inhibition of PDNS is the main mechanism of cytotoxicity in these cells. Reduced TGN incorporation at equitoxic doses is consistent with diversion of 6-MP metabolism away from TGMP.

TPMT induction had no effect on the percentage of viable cells able to form colonies after exposure to 6-MP or 6-TG. In response to drug exposure, the total number of cells measured by SRB assay was affected by TPMT induction, with increase in cell number for cells treated with 6-TG and the converse for 6-MP. This would imply that TPMT alters the rate of proliferation without detectable effects on clonogenic ability at the doses used.

During the preparation of this article, Dervieux et al. (2001) published an article reporting similar effects of TPMT transfection on thiopurine sensitivity in the T-cell leukemic cell line CCRF-CEM; these cells have been reported to have a defect in the MMR pathway (Taverna et al., 2000), which may be expected to influence their sensitivity to thiopurines, whereas the EcR293-TPMT/20 cells are MMR-proficient (data not shown). A comparison of the level of TGN incorporation into DNA after 24 h exposure to 6-TG indicates that for equivalent levels of cytotoxicity CCRF-CEM cells tolerate nearly 10 times the level of DNA-TGN incorporation than MMR-proficient EcR293 cells. For transfected CCRF-CEM (3.1 dG^s/100 T) at 1 μM TG (IC₅₀ = 1.1 μM), 0.39 dG/100T at IC₅₀ for uninduced EcR293-TPMT/20 cells (IC₅₀ = 1.7 μM). These results indicate that the CCRF-CEM cells exhibit tolerance resistance to thiopurines, an effect that may complicate the analysis of the results.

One important conclusion from our results is that, in cells with high levels of TPMT, 6-MP can exert a cytotoxic effect without measurable levels of TGN incorporation into DNA and, therefore, with reduced risk of mutagenesis posed by TGN incorporation. This may help to explain why, in contrast to 6-TG, secondary malignancy has not been associated with chronic exposure to 6-MP or to the 6-MP prodrug azathioprine in clinical practice. In cells with low TPMT, however,

levels of TGN incorporation in the DNA are much higher for a given level of cytotoxicity. These results may provide a mechanistic explanation for the recent observation that the rates of secondary malignancy are raised in patients with congenitally reduced TPMT levels (Relling et al., 1998, 1999; Thomsen et al., 1999; Pui and Relling, 2000). The possible association between thiopurine treatment and mutagenic affects needs further clarification to support these clinical findings. This is currently being investigated using this model system. The results of these experiments will help confirm if the lack of clinical benefit associated with 6-TG, plus the additional risk of mutagenesis and secondary malignancy, would preclude the use of this drug in preference to 6-MP.

Acknowledgments

We thank Dr. A. Boddy for the mass spectrum analysis and Marian Case for proofreading this manuscript.

References

- Allan PW and Bennett LL Jr (1971) 6-Methylthioguanilic acid, a metabolite of 6-thioguanine. *Biochem Pharmacol* **20**:847–852.
- Andersen JB, Szumlanski C, Weinshilboum RM, and Schmiegelow K (1998) Pharmacokinetics, dose adjustments and 6-mercaptopurine/methotrexate drug interactions in two patients with thiopurine methyltransferase deficiency. *Acta Paediatr* **87**:108–111.
- Bertino JR (1991) Improving the curability of acute leukemia: pharmacologic approaches. *Semin Hematol* **28**:9–11.
- Colombel JF, Ferrari N, Debuysere H, Marteau P, Gendre JP, Bonaz B, Soule JC, Modigliani R, Touze Y, Catala P, et al. (2000) Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. *Gastroenterology* **118**:1025–1030.
- Coulthard SA, Howell C, Robson J, and Hall AG (1998) The relationship between thiopurine methyltransferase activity and genotype in blasts from patients with acute leukemia. *Blood* **92**:2856–2862.
- De la Moureyre CSV, Debuysere H, Sabbagh N, Marez D, Vinner E, Chevalier ED, Lo GJM, and Broly F (1998) Detection of known and new mutations in the thiopurine S-methyltransferase gene by single-strand conformation polymorphism analysis. *Hum Mutat* **12**:177–185.
- Dervieux T, Blanco JG, Krynetski EY, Vanin EF, Roussel MF, and Relling MV (2001) Differing contribution of thiopurine methyltransferase to mercaptopurine versus thioguanine effects in human leukemic cells. *Cancer Res* **61**:5810–5816.
- Erb N, Harms DO, and Janka-Schaub G (1998) Pharmacokinetics and metabolism of thiopurines in children with acute lymphoblastic leukemia receiving 6-thioguanine versus 6-mercaptopurine. *Cancer Chemother Pharmacol* **42**:266–272.
- Evans WE, Horner M, Chu YQ, Kalwinsky D, and Roberts WM (1991) Altered mercaptopurine metabolism, toxic effects and dosage requirement in a thiopurine methyltransferase-deficient child with acute lymphocytic leukemia. *J Pediatr* **119**:985–989.
- Krynetski EY, Fessing MY, Yates CR, Sun D, Schuetz JD, and Evans WE (1997) Promoter and intronic sequences of the human thiopurine S-methyltransferase (TPMT) gene isolated from a human *PacI* genomic library. *Pharm Res* **14**:1672–1678.
- Krynetski EY, Krynetskaia NF, Yanishevski Y, and Evans WE (1995) Methylation of mercaptopurine, thioguanine and their nucleotide metabolites by heterologously expressed human thiopurine S-methyltransferase. *Mol Pharmacol* **47**:1141–1147.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227**:680–685.
- Lancaster DL, Lennard L, Rowland K, Vora AJ, and Lilleyman JS (1998) Thioguanine versus mercaptopurine for therapy of childhood lymphoblastic leukaemia: a comparison of haematological toxicity and drug metabolite concentrations. *Br J Haematol* **102**:439–443.
- Lennard L, Gibson BES, Nicole T, and Lilleyman JS (1993) Congenital thiopurine methyltransferase deficiency and 6-mercaptopurine toxicity during treatment for acute lymphoblastic leukaemia. *Arch Dis Child* **69**:577–579.
- Lennard L, Lewis IJ, Michelagnoli M, and Lilleyman JS (1997a) Thiopurine methyltransferase deficiency in childhood lymphoblastic leukaemia: 6-mercaptopurine dosage strategies. *Med Pediatr Oncol* **29**:252–255.
- Lennard L, Welch JC, and Lilleyman JS (1997b) Thiopurine drugs in the treatment of childhood leukaemia: the influence of inherited thiopurine methyltransferase activity on drug metabolism and cytotoxicity. *Br J Clin Pharmacol* **44**:455–461.
- Maddocks JL, Lennard L, Amess J, Amos R, and Thomas RM (1986) Azathioprine and severe bone marrow depression [letter]. *Lancet* **1**:156.
- Masson E, Synold TW, Relling MV, Schuetz JD, Sandlund JT, Pui CH, and Evans WE (1996) Allopurinol inhibits de novo purine synthesis in lymphoblasts of children with acute lymphoblastic leukemia. *Leukemia* **10**:56–60.
- McLeod HL, Krynetski EY, Relling MV, and Evans WE (2000) Genetic polymorphism of thiopurine methyltransferase and its clinical relevance for childhood acute lymphoblastic leukemia. *Leukemia* **14**:567–572.
- McLeod HL, Miller DR, and Evans WE (1993) Azathioprine-induced myelosuppression in thiopurine methyltransferase deficient heart transplant recipient. *Lancet* **341**:1151.
- Otterness D, Szumlanski C, Lennard L, Klemetsdal B, Aarbakke J, Park-Hah JO,

- Iven H, Schmiegelow K, Branum E, O'Brien J, et al. (1997) Human thiopurine methyltransferase pharmacogenetics: gene sequence polymorphisms. *Clin Pharmacol Ther* **62**:60–73.
- Pui CH and Relling MV (2000) Topoisomerase II inhibitor-related acute myeloid leukaemia. *Br J Haematol* **109**:13–23.
- Relling MV, Rubnitz JE, Rivera GK, Boyett JM, Hancock ML, Felix CA, Kun LE, Walter AW, Evans WE, and Pui C-H (1999) High incidence of secondary brain tumours after radiotherapy and antimetabolites. *Lancet* **354**:34–39.
- Relling MV, Yanishevski Y, Nemec I, Evans WE, Boyett JM, Behm FG, and Pui C-H (1998) Etoposide and antimetabolite pharmacology in patients who develop secondary acute myeloid leukemia. *Leukemia* **12**:346–352.
- Seki T, Tanaka T, and Nakamura Y (2000) Genomic Structure and multiple single-nucleotide polymorphisms (SNPs) of the thiopurine S-methyltransferase (TPMT) gene. *J Hum Genet* **45**:299–302.
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, and Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82**:1107–1112.
- Spire-Vayron de la Moureyre C, Debuysere H, Fazio F, Sergeant E, Bernard C, Sabbagh N, Marez D, Lo Guidice JM, D'halluin JC, and Broly F (1999) Characterization of a variable number tandem repeat region in the thiopurine S-methyltransferase gene promoter. *Pharmacogenetics* **9**:189–198.
- Swann PF, Waters TR, Moulton DC, Xu YZ, Zheng Q, Edwards M, and Mace R (1996) Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. *Science (Wash DC)* **273**:1109–1111.
- Szumslanski C, Otterness D, Her C, Lee D, Brandriff B, Kellsell D, Spurr N, Lennard L, Wieben E, and Weinshilboum R (1996) Thiopurine methyltransferase pharmacogenetics: human gene cloning and characterization of a common polymorphism. *DNA Cell Biol* **15**:17–30.
- Tai HL, Krynetski EY, Yates CR, Loennechen T, Fessing MY, Krynetskaia N, and Evans WE (1996) Thiopurine S-methyltransferase deficiency: two nucleotide transitions define the most prevalent mutant allele associated with loss of catalytic activity in Caucasians. *Am J Hum Genet* **58**:694–702.
- Taverna P, Liu L, Hanson AJ, Monks A, and Gerson SL (2000) Characterization of MLH1 and MSH2 DNA mismatch repair proteins in cell lines of the NCI anticancer drug screen. *Cancer Chemother Pharmacol* **46**:507–516.
- Tay BS, Lilley RM, Murray AW, and Atkinson MR (1969) Inhibition of phosphoribosyl pyrophosphate amidotransferase from Ehrlich Ascites-tumour cells by thiopurine nucleotides. *Biochem Pharmacol* **18**:936–938.
- Thomsen JB, Schroder H, Kristinsson J, Madsen B, Szumlanski C, Weinshilboum R, Andersen JB, and Schmiegelow K (1999) Possible carcinogenic effect of 6-mercaptopurine on bone marrow stem cells—relation to thiopurine metabolism. *Cancer* **86**:1080–1086.
- Warren DJ, Andersen A, and Slordal L (1995) Quantitation of 6-thioguanine residues in peripheral blood leukocyte DNA obtained from patients receiving 6-mercaptopurine-based maintenance therapy. *Cancer Res* **55**:1670–1674.
- Warren DJ and Slordal L (1993) A high-performance liquid chromatographic method for the determination of 6-thioguanine residues in DNA using precolumn derivatization and fluorescence detection. *Anal Biochem* **215**:278–283.
- Weinshilboum RM, Raymond FA, and Pazmino PA (1978) Human erythrocyte thiopurine methyltransferase: radiochemical microassay and biochemical properties. *Clin Chim Acta* **85**:323–333.
- Yates CR, Krynetski EY, Loennechen T, Fessing MY, Tai HL, Pui CH, Relling MV, and Evans WE (1997) Molecular diagnosis of thiopurine S-methyltransferase deficiency: genetic basis for azathioprine and mercaptopurine intolerance. *Ann Intern Med* **126**:608–614.

Address correspondence to: Dr. Andrew Hall, The LRF Molecular Pharmacology Specialist Programme, Cancer Research Unit, Medical School, Framlington Place, Newcastle Upon Tyne, NE2 4HH, UK. E-mail: a.g.hall@ncl.ac.uk
