Increased sensitivity to thiopurines in methylthioadenosine phosphorylase-deleted cancers

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Abbreviations

6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; ALL, acute lymphoblastic leukaemia; dFCS, dialysed foetal calf serum; dG₈, deoxythioguanosine nucleotides; MeTIMP, methylthioinosine monophosphate; MMPR, methyl mercaptopurine riboside; MMR, mismatch repair; MTA, methylthioadenosine; MTAP, methylthioadenosine phosphorylase; TGN, thioguanine nucleotides; TIMP, thionosine 5’-monophosphate; TPMT, thiopurine methyltransferase; TXMP, thioxanthine monophosphate; DNPS, de novo purine synthesis; SAM, S-adenosyl methionine; TGMP, thioguanine monophosphate; TGDP, thioguanine diphosphate; TGTP, thioguanine triphosphate; MeTGMP, methylthioguanosine monophosphate; DTT, dithiothreitol

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Abstract

The thiopurines, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are used in the treatment of leukaemia. Incorporation of deoxythioguanosine nucleotides (dG\textsuperscript{5}) into the DNA of thiopurine-treated cells causes cell death but there is also evidence that thiopurine metabolites, particularly the 6-MP metabolite methylthioinosine monophosphate (MeTIMP), inhibit \textit{de novo} purine synthesis (DNPS). The toxicity of DNPS inhibitors is influenced by methylthioadenosine phosphorylase (MTAP), a gene frequently deleted in cancers. Since the growth of MTAP-deleted tumour cells is dependent on DNPS or hypoxanthine salvage, we would predict such cells to show differential sensitivity to 6-MP and 6-TG. To test this hypothesis, sensitivity to 6-MP and 6-TG was compared in relation to MTAP status using cytotoxicity assays in two MTAP-deficient cell lines transfected to express MTAP: the T-cell acute lymphoblastic leukaemic cell line, Jurkat, transfected with MTAP cDNA under the control of a tetracycline-inducible promoter, and a lung cancer cell line (A549-MTAP\textsuperscript{ve}) transfected to express MTAP constitutively (A549-MTAP\textsuperscript{ve}). Sensitivity to 6-MP or methyl mercaptopurine riboside, which is converted intra-cellularly to MeTIMP, was markedly higher in both cell lines under MTAP\textsuperscript{ve} conditions. Measurement of thiopurine metabolites support the hypothesis that DNPS inhibition is a major cause of cell death with 6-MP, whereas dG\textsuperscript{5} incorporation is the main cause of cytotoxicity with 6-TG. These data suggest that thiopurines, particularly 6-MP, may be more effective in patients with deleted MTAP.
Introduction

Several drugs used in cancer treatment inhibit *de novo* purine synthesis (DNPS), including methotrexate, L-alanosine and pemetrexed. The toxicity of DNPS inhibitors is influenced by expression of methylthioadenosine phosphorylase (MTAP) (EC2.4.2.28), an enzyme catalysing the phosphorolysis of 5'-deoxy-5'-methylthioadenosine (MTA), a by-product of polyamine synthesis, to adenine and 5’-methylthioribose-1-phosphate. The *MTAP* gene is located on chromosome 9p21, 100 kb telomeric to the *p15^INK4B* and *p16^INK4A* genes. MTAP is expressed ubiquitously in haematopoietic cells (1) but deletion of the *MTAP* gene is frequent in several cancer types (2), including haematological malignancies (3). In cancer cells not expressing MTAP, purine synthesis is entirely dependent on DNPS or the salvage of extracellular purines such as hypoxanthine; thus, *in vitro*, MTAP-deleted cells are hypersensitive to inhibitors of the DNPS pathway (4) in hypoxanthine-depleted media. This property has been exploited by using drugs such as L-alanosine (5) or pemetrexed (ALIMTA) (6) to target MTAP-deleted tumour cells. Cells expressing MTAP can be rescued from the toxicity of these agents by the addition of MTA (4, 7) or 9-beta-D-erythrofuranosyladenine (EFA) (8).

The thiopurines 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) have been used extensively for treatment of leukaemia. 6-MP is used in the current UKALL2003 trial (9). These drugs are metabolised extensively to cause their cytotoxic effect reviewed by Fotoohi et al. (10) (Fig 1). The main mechanism of cytotoxicity was initially thought to be via incorporation of deoxythioguanosine nucleotides (dG5) into DNA and in this context cytotoxicity varies in relation to MMR status (11, 12). Studies within the last decade have revealed additional mechanisms of cytotoxicity, particularly inhibition of DNPS (13, 14), interference of the rac1-vav signalling pathway (15) and alteration of the methylation status of DNA (16). Although 6-MP via methylthioinosine monophosphate, and to a lesser extent 6-TG via methylthioguanosine monophosphate, inhibit DNPS, the influence of MTAP on the
cytotoxic action of the thiopurines is unknown. This could be an issue of considerable importance in leukaemia since 9p abnormalities occur in 14-33% of patients (17-19).

We have previously reported that the metabolism of thiopurines and extent of DNPS is differentially affected by the activity of the enzyme thiopurine methyltransferase (TPMT) (14), which is significantly reduced in 10% of Caucasians (20). In cells with wild-type TPMT levels 6-MP is a more effective inhibitor of DNPS than 6-TG due to the differential efficacy of their TPMT-mediated metabolites methylthioinosine monophosphate (MeTIMP) and methylthioguanosine monophosphate (MeTGMP), respectively (14). The present study was undertaken to test the hypothesis that MTAP activity differentially affects sensitivity to 6-MP and 6-TG using two different cancer cell types as a model, a T-cell leukaemia and a lung cancer cell line, both with TPMT activity in the normal range.

Materials and methods

Cell culture

The MTAP-deleted T-cell leukaemic line, Jurkat-MTAP−ve (Clontech™ Saint-Germain-en-Laye, France), which expresses wild-type TPMT (21), were stably transfected with MTAP cDNA under the control of a tetracycline promoter. Jurkat cells used for this study were obtained from the American Type Culture Collection, and no subsequent authentication was done by our laboratory. The Jurkat-MTAP−ve parental cells were maintained in RPMI containing 10% dialysed foetal calf serum (dFCS) (Cambrex™, Milan, Italy) and 100 μg/mL Geneticin® (Invitrogen™, Paisley, UK) Jurkat-MTAP+ve transfected cells were maintained in the same medium supplemented with 200 μg/mL Hygromycin B (Invitrogen™). To induce expression of MTAP in the Jurkat-MTAP+ve cells 2 μg/mL of doxycycline was added to these cells and to the negative control parental Jurkat-MTAP−ve cells.

The MTAP+ve lung cancer cell line, A549, and its paired counterpart, A549-MTAP+ve cells transfected with antisense or sense MTAP cDNA, respectively (22), were a gift from Prof.
T. Nobori, Mei University, Japan. These cell lines were not authenticated by our laboratory after receiving them from Mei University. However, the expression status of MTAP was checked at intervals in all cell lines during these experiments. These cells were maintained in RPMI containing 10% dFCS supplemented with 200 µg/mL of Geneticin® and were checked regularly for mycoplasma contamination.

Cellular drug metabolite levels, MeTIMP, free thioguanine nucleotides (TGNs) and deoxythioguanosine (dG₃) incorporated into the DNA, were determined in cells exposed to 6-MP or 6-TG, for 3-doubling times (72 hours). Each experiment was repeated in triplicate on three separate occasions. Cells were washed twice in PBS and 5 X 10⁶ cells pelleted and stored at -80°C prior to analysis.

**MTAP activity assay**

For MTAP activity assays, viable PBS-washed cells (5 X 10⁶) were snap frozen in liquid nitrogen and pellets stored at -80°C. Just prior to analysis cells were thawed on ice and re-suspended in 200 µL of 50 mM K₂PO₄ pH7.4, 0.1 mM DTT, sonicated on ice for 3 seconds, 3 times, at an amplitude of 12 µm and centrifuged at 20800 g for 30 min at 4°C. Protein concentration was measured on an aliquot of the supernatant using the Pierce BCA kit (Thermo Scientific, Cramlington, UK), as described according to the manufacturer’s instructions. Measurement of MTAP activity (23) was based on a method by Savarese et al.(7).

**TPMT activity assay**

TPMT activity in Jurkat-MTAP⁺ve and Jurkat-MTAP⁻ve cells was measured as described (23) using an adaptation of the method originally published by Weinshilboum et al.(24). TPMT activity was measured after induction with doxycycline for 96 h in the presence of drug control vehicle and in the A549 cell lines 96 h after seeding in the presence of drug control vehicle i.e. at the same time point that cells were harvested after drug treatment for drug metabolite
measurements. Each of these induction and activity assays were done on three separate occasions.

**Immunoblotting**

To confirm the expression of MTAP in the transfected A549-MTAP\textsuperscript{+ve} cells 5 × 10\textsuperscript{6} cells were lysed in 100 µL of 2 X Laemmli buffer (8% SDS, 40% glycerol, 20% 2-mercaptethanol, 0.04% bromophenol blue and 0.25 M Tris HCl, pH 6.8) and heated for 7 min at 95°C and centrifuged at 20800 g, 4°C, for 10 min. Protein concentration of the cell lysate was quantified using the Pierce kit. Ten µg of lysate was separated on 4-12% Tris/Glycine gels (Invitrogen, Paisley, UK) and blotted onto polyvinylidene difluoride (Bio-Rad, 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 containing 5% non-fat dried milk) and probed either 1/4000 dilution of mouse polyclonal MTAP antibody (Salmidix Inc., San Diego, USA) in blocking buffer overnight at 4°C or 1/80000 dilution of mouse monoclonal α tubulin antibody (Sigma-Aldrich, Poole, Dorset, UK) for 1 hour, as a loading control and detected using HRP-conjugated goat anti-mouse secondary antibody. Visualisation was achieved with 1/2000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Dako, Ely, UK) for 45 min at room temperature followed by enhanced chemiluminescence using ECL Plus (GE Healthcare (Amersham) Ltd., Little Chalfont, UK), according to the manufacturer’s instructions, and exposure to Kodak Medical X-ray film (GRI Medical Products Inc., Arizona, USA).

**Drug sensitivity assays**

Jurkat and A549 paired cell lines were treated with 6-MP, 6-TG, etoposide, or methyl mercaptopurine riboside (MMPR), in the presence of 40 µM or 16 µM MTA to rescue MTAP expressing cells, for Jurkat and A549 cells, respectively. The drug MMPR is converted directly by adenosine kinase to the 6-MP metabolite MeTIMP, which is a potent inhibitor of DNPS.
(25). For drug sensitivity analysis, Jurkat cells were seeded at 1 X 10^4/100 µL (in the presence of 2 µg/mL of doxycycline to induce expression of MTAP) and A549 cells at 1.5 X 10^3/100 µL in a 96-well plate. After 24h each drug was titrated in varying concentrations in the presence of the appropriate concentration of MTA and incubated with cells for 3-doubling times (approximately 72 hours) before cell viability was determined using the MTS assay (CellTiter 96® AQueous Assay (Promega™, Southampton, UK) according to the manufacturer’s protocol. Cells were treated with 6-MP, 6-TG (with and without 1 µM hypoxanthine), etoposide or MMPR and each experiment was repeated at least twice more.

**Drug metabolite measurement assays**

Cellular drug metabolite levels, MeTIMP, and free TGNs (consisting of thioGTP, thioGDP and thioGMP) were determined as described (23). Deoxythioguanosine (dG') incorporation into DNA was measured as described previously (14, 23, 26). Briefly, DNA was extracted from 5x10^6 cells re-suspended in 200 µL of PBS using a commercially-available spin column method according to the manufacturer’s instructions (QIAamp DNA Mini Kit, Qiagen™, Crawley, UK). Purified DNA was eluted with 200 µL of 10 mM Tris-HCl, 0.1 mM EDTA (pH 9.0) and stored at -20°C prior to analysis (23). All metabolite assays were done in triplicate on three separate occasions.

**Statistical analysis**

Each experiment was repeated independently on at least three occasions. Drug sensitivity assays were analyzed using GraphPad PRISM software (San Diego, CA, USA) in which sigmoidal dose-response curves (variable slope) were fitted to all data; drug concentrations were logged and the log EC_{50} measured from the curve automatically. Other comparisons for treatments on MTAP-expressing and non-expressing cell lines were carried
out using one- and two-way ANOVA, as appropriate, with contrasts to compare treatment categories, using Systat version 15 (SPSS Inc, Chicago, IL). Where appropriate, log transformation was used to equalize variances.

Results

MTAP and TPMT activity in cell line models

The impact of MTAP status on sensitivity to thiopurine drugs was determined in vitro using two MTAP-deleted cell types, transfected to express MTAP cDNA. A549 cells, transfected with sense (A549-MTAP<sup>+</sup>) and antisense (A549-MTAP<sup>-</sup>) MTAP cDNA have been characterised previously (22); the expression of MTAP at the protein level in the A549-MTAP<sup>+</sup> and A549-MTAP<sup>-</sup> cell lines was confirmed by Western blot (data not shown). As an additional model, MTAP cDNA under the control of a tetracycline-inducible promoter was transfected into Jurkat cells and a stably-transfected clone selected and characterised. MTAP activity in the transfected Jurkat cells was measured after 24, 48, 72 and 96 h in the presence and absence of 2 µg/mL of doxycycline. Doxycycline increased MTAP activity from 0.83 to 1.87 U/mg protein/min between 24 and 96 h and therefore there was a clear correlation between MTAP activity and protein expression (Fig 2). However, MTAP expression was leaky in the absence of doxycycline and uninduced cells had MTAP activity of 0.24 U/mg protein/min. Therefore, the parental Jurkat cells, which showed no detectable MTAP activity with or without doxycycline (data not shown), were used as the negative control and are referred to as Jurkat-MTAP<sup>-</sup> cells. MTAP-transfected cells treated with doxycycline are referred to as Jurkat-MTAP<sup>+</sup> cells.

Since thiopurine metabolism and the extent of DNPS is differentially affected by TPMT (14), the activity of TPMT was measured in the presence of drug control vehicle and MTA in both cell types and also in the presence of doxycycline for the Jurkat cells. TPMT activity of the Jurkat-MTAP<sup>-</sup> and Jurkat-MTAP<sup>+</sup> was 3.03±0.33 and 2.50±0.23 nM/µg protein/h.
respectively and 1.94+/− 0.09 and 1.62+/− 0.21 nM/µg protein/h for the A549-MTAP−ve and A549-MTAP+ve cells, respectively. The difference between cell lines and the effect of MTAP status independently of cell type was statistically significant (Two-way ANOVA on log-transformed data, cell type: F1,8=62.8, P<0.001; MTAP status: F1,8=11.5, P=0.01; interaction term not significant P>0.9). These results show that increased expression of MTAP reduced the activity of TPMT.

**Drug sensitivity to 6-MP in relation to MTAP expression**

The sensitivity of the Jurkat and the A549 cell lines to 6-MP and 6-TG were compared in relation to MTAP status. In these experiments inhibition of DNPs in response to thiopurines was inferred on the basis of rescue by hypoxanthine supplementation. MMPR was used as a positive control because it is converted to MeTIMP, a metabolite of 6-MP and a known DNPS inhibitor. As an additional control, the sensitivity of the cells to etoposide was also compared in relation to MTAP status; etoposide is not a DNPS inhibitor and does not produce fraudulent bases within the DNA, and was used to exclude the possibility that MTAP expression altered sensitivity to chemotherapeutic drugs in general, rather than specifically to DNPS inhibitors. With respect to these control experiments, there was no significant difference between Jurkat-MTAP−ve and Jurkat-MTAP+ve cells (P>0.05; Fig. 2A) or between the A549-MTAP−ve and A549-MTAP+ve cells (P>0.05; Fig 2B) in sensitivity to etoposide. Conversely, with MMPR there was a highly-significant reduction in viability of Jurkat-MTAP+ve compared to Jurkat-MTAP−ve cells (P<0.05; Fig 2C) and also with the A549-MTAP+ve compared to the A549-MTAP−ve cells (P<0.05; Fig 2D). These data indicate that both cell lines had increased sensitivity to a DNPS inhibitor in the absence of MTAP expression and that this was not due to an altered sensitivity to chemotherapeutic drugs in general.
Cells were tested for drug sensitivity to both 6-MP alone and 6-MP supplemented with hypoxanthine; under conditions of DNPS inhibition, cells are expected to be rescued by the addition of hypoxanthine via conversion to inosine monophosphate. Jurkat-MTAP-ve cells were more resistant to 6-MP than Jurkat-MTAP+ve cells (P<0.05; Fig 3A). The sensitivity of MTAP-deficient Jurkat cells to 6-MP was significantly decreased by supplementation with hypoxanthine (P<0.05; Fig 3A). Similar results were obtained with the A549-MTAP+ve cells which were more resistant to 6-MP than the MTAP-deficient A549 cells (P<0.05; Fig 3B black lines); this difference was also significantly decreased by supplementation with hypoxanthine (P<0.05; Fig 3B grey lines), producing an increase in 6-MP EC50 from 64.3 µM to 123.0 µM.

Since MeTIMP is a metabolite of 6-MP that inhibits DNPS, MeTIMP levels were measured in all cell lines after treatment with equimolar doses of 6-MP. The doses of 6-MP used for these experiments were 65 µM for the Jurkat cells and 200 µM for A549 cells, the approximate EC50 doses for 6-MP in MTAP-deficient Jurkat and A549 cells, respectively (EC50 doses were not reached for the MTAP-expressing cells). At equimolar doses of 6-MP, MeTIMP levels were one and a half-fold lower in MTAP-expressing Jurkat cells compared to the Jurkat-MTAP-ve cells (65 µM 6-MP, P<0.0001 Fig 4A) and three-fold lower in MTAP-expressing A549 cells compared to the A549-MTAP+ve cells (200 µM 6-MP, P=0.001 Fig 4A). The lower MeTIMP levels in MTAP-expressing, compared to non-expressing cells were consistent with the observed decrease in TPMT activity in cells expressing MTAP.

Differences in levels of free thioguanine nucleotides (TGNs) could contribute to differences in cell viability between MTAP-expressing and non-expressing cells after 6-MP treatment; at equimolar doses of 6-MP, there were significantly higher levels of free TGNs in the MTAP-ve cells compared to paired MTAP+ve cells for Jurkat (65 µM 6-MP, P<0.0001 Fig 4B) and A549 cells (200 µM 6-MP, P<0.0001 Fig 4B).

Drug sensitivity to 6-TG in relation to MTAP expression
Jurkat-MTAP\textsuperscript{+ve} and Jurkat-MTAP\textsuperscript{-ve} cells were equally sensitive to 6-TG (P>0.05; Fig 5A) and there was no effect of hypoxanthine supplementation in either cell line (P>0.05; Fig 5A). In contrast, the A549 cell pairs showed a statistically-significant difference in 6-TG toxicity (P<0.05; Fig 5B). In the absence of hypoxanthine, A549-MTAP\textsuperscript{+ve} cells were considerably more sensitive to 6-TG than A549-MTAP\textsuperscript{-ve} cells. Hypoxanthine supplementation had no effect on the sensitivity of A549-MTAP\textsuperscript{+ve} cells to 6-TG (P<0.05; Fig. 5B), in contrast to the A549-MTAP\textsuperscript{-ve} cells where the addition of hypoxanthine resulted in a significant decrease in sensitivity (P>0.05; Fig 5B) from an EC\textsubscript{50} of 2.56 to 52.11 µM.

To assess the contribution of TGN to drug toxicity, free TGNs were measured at equimolar (3 µM) and equitoxic (3.4 µM and 2 µM for MTAP\textsuperscript{+ve} and MTAP\textsuperscript{-ve} respectively) concentrations of 6-TG. For Jurkat cells with equimolar doses of 6-TG, MTAP-expressing cells had significantly lower levels of free TGNs (2-way ANOVA on log-transformed data, effect of MTAP F\textsubscript{1, 20}=56.7, P<0.001) from 3.7 to 2.7 pmol/10\textsuperscript{6} cells, but no effect of drug dose (Dose or dose*MTAP interaction, F\textsubscript{1, 20}<0.6, P>0.4; Fig. 6A). However, there were no differences between Jurkat-MTAP\textsuperscript{-ve} and Jurkat-MTAP\textsuperscript{+ve} exposed to either equitoxic or equimolar 6-TG treatments in the levels of dG\textsuperscript{3} incorporated into DNA (One-way ANOVA, F\textsubscript{3, 7}=0.6, P=0.62; Fig. 6C).

In the A549 cells, free TGNs were measured at equitoxic doses (170 µM and 4 µM for MTAP\textsuperscript{+ve} and MTAP\textsuperscript{-ve}, respectively) and at doses of 170 µM for MTAP\textsuperscript{-ve} and 4 µM for MTAP\textsuperscript{+ve} to allow equimolar comparisons (Fig 6B). There was no significant difference in free TGN levels between MTAP\textsuperscript{+ve} and MTAP\textsuperscript{-ve} A549 cells after treatment with equitoxic concentrations of 6-TG (one-way ANOVA, contrasts t\textsubscript{7}=-0.04, P>0.9). Maximal levels of free TGNs appeared to be obtained with 4 µM 6-TG, since these were not increased with 170 µM 6-TG (contrasts t\textsubscript{7}=-1.1, P=0.323). However, TGN levels were much lower in the MTAP\textsuperscript{+ve}, compared to the MTAP\textsuperscript{-ve}, cells with 4 µM 6-TG (one-way ANOVA, contrasts t\textsubscript{7}=3.9,
As observed with the Jurkat cells, there was no difference between A549-MTAP<sup>−ve</sup> and A549-MTAP<sup>+ve</sup> cells at either equitoxic or equimolar doses of 6-TG in the incorporation of dG<sup>+</sup> into DNA (One-way ANOVA contrasts |t|<2.4, P≥0.128; Fig 6D). Higher levels of dG<sup>+</sup> incorporation into DNA were obtained with the Jurkat cells treated with 2.0-3.4 µM 6-TG (Fig 6), compared to A549 cells treated with up to 170 µM 6-TG.

**Discussion**

This study shows that loss of MTAP resulted in increased sensitivity to MMPR, the precursor of the known DNPS inhibitor MeTIMP, which is also a metabolite of 6-MP. That increased sensitivity is due to DNPS inhibition was supported by rescue of purine synthesis using hypoxanthine in combination with MMPR. The effect of MTAP status on MMPR is consistent with previous studies which also show that drug sensitivity in cell lines with altered MTAP status differ when treated with inhibitors of DNPS (17, 22, 27). Furthermore, in both cell models used for this study, the absence of MTAP expression was associated with increased sensitivity to 6-MP. This was probably due, at least in part, to inhibition of DNPS by the thiopurine methylated metabolite MeTIMP, which was increased in MTAP-deficient cells in combination with reduced purine salvage as a result of lack of MTAP activity. The higher MeTIMP levels in cells lacking MTAP are consistent with the higher levels of TPMT activity in the absence of MTAP expression.

Drug sensitivity to 6-MP was partially decreased by the addition of hypoxanthine, supporting the hypothesis that the availability of purines is critical for cell survival and that sensitivity to 6-MP was at least partly due to inhibition of DNPS. Rescue of cells by the addition of hypoxanthine was greater in the A549-MTAP<sup>−ve</sup>, compared to Jurkat-MTAP<sup>−ve</sup>, cells perhaps due to greater inhibition of DNPS as a result of higher MeTIMP levels in the A549-MTAP<sup>−ve</sup> cells. A major component of 6-MP cytotoxicity is believed to be the formation of free...
TGNs for incorporation into DNA. Note that the Jurkat cells had higher TGN and lower MeTIMP levels than the A549 cells; this may result from higher inosine monophosphate dehydrogenase activity in Jurkat cells (Sofie Haglund, personal communication). After 6-MP treatment, free TGN levels were greater in both cell types in the absence of MTAP expression; this might have resulted from reduced salvage of endogenous nucleotides in MTAP -ve cells, allowing greater relative contribution of free drug-derived TGNs to the nucleotide pool.

In contrast to treatment with 6-MP, the Jurkat cells did not show MTAP-dependent differential sensitivity to 6-TG and there was no evidence from the hypoxanthine-rescue experiments that DNPS was involved in the response of these cells to 6-TG. These conclusions are supported by our previous data suggesting that inhibition of DNPS is not a major mechanism of cytotoxicity of 6-TG (14). Since the levels of dG\textsuperscript{5} incorporated into Jurkat DNA did not differ in response to MTAP status, thiopurine incorporation is likely to be the main mechanism of 6-TG toxicity in these cells. Although the Jurkat cells (regardless of MTAP status) and the MTAP\textsuperscript{ve} A549 cells had similar sensitivity to 6-TG, Jurkat cells had greater levels of dG\textsuperscript{5} incorporated into the DNA. These cells are MMR-deficient (28) whereas A549 cells are MMR proficient (29) which may contribute to their resistance (12). However the role of MMR in thioguanine cytotoxicity will have to be addressed in isogenic cell lines as other proteins distinct from the MMR complex have also been shown to be involved in thioguanine cytotoxicity (30). Furthermore, other studies suggest a lack of correlation between MMR proficiency and dGs incorporation in response to thiopurines (31). As there was no dose-dependent effect of 6-TG on dG\textsuperscript{5} levels in A549 cells regardless of MTAP status or the differential sensitivity to 6-TG, therefore it is possible that resistance to 6-TG imparted by MTAP expression is mediated by mechanisms other than changes in the levels of dG\textsuperscript{5} incorporation. A549 cells (which are 9p deleted and lack endogenous MTAP) have been shown previously to be sensitive to 6-TG (32). For the paired MTAP\textsuperscript{-ve} and MTAP\textsuperscript{+ve} A549 cells used for this study, the A549-MTAP\textsuperscript{-ve} cells were significantly more resistant to 6-TG than the
A549-MTAP<sup>+</sup> parental cells, and the addition of hypoxanthine did not have a significant effect on sensitivity. In contrast, the presence of hypoxanthine increased the resistance of A549-MTAP<sup>+</sup> cells to 6-TG; we speculate that this increased resistance to 6-TG may result from the metabolism of hypoxanthine to IMP with a consequent dilution of the relative TGN content in endogenous nucleotide pools. Similarly, the lower free TGNs at 4 μM 6-TG in the A549-MTAP<sup>+</sup> cells compared to A549-MTAP<sup>-</sup> cells may have resulted from dilution of the nucleotide pool with endogenous nucleotides derived from the salvage pathway. Free TGNs increased with 6-TG dose in the A549-MTAP<sup>+</sup> cells but the lack of a dose-dependent increase in free TGNs in A549-MTAP<sup>-</sup> cells implies that free TGN levels had already reached saturation at 4 μM 6-TG in the A549-MTAP<sup>-</sup> cells. This interpretation of free TGN changes in terms of alterations in the contributions of DNPS and the salvage pathway to endogenous nucleotide pools is speculative and likely to be an over-simplification. Since the pathways of nucleotide synthesis and regulation are complex, and will also vary in relation to cell type and proliferative status of the cells, to understand the system fully we need robust computerized models on which to base experimental design and interpretation. The cell lines and thiopurine drugs investigated here in which the salvage pathway and level of DNPS can be manipulated experimentally will provide test-beds to develop and validate such an approach.

Deletion of the chromosome 9p21 region containing the MTAP gene, the CDNK2B locus encoding p15<sup>INK4b</sup> and the CDNK2A locus encoding the two CDK inhibitors, p16<sup>INK4a</sup> and p14<sup>ARF</sup>, has been characterized in cancer cell lines and primary tumors and is deleted in up to 58% and 17% of T- and B-lineage ALL, respectively (3). The consequences of loss of this locus and the effect on prognosis are controversial (33-35). If p16<sup>INK4a</sup> and p14<sup>ARF</sup> inactivation does have an adverse effect on prognosis this may mask or confound any clinical benefit for MTAP-deleted patients treated with 6-MP compared to patients without 9p deletions. Mirebeau et al. (34) considered the effect of deletion of the region, including MTAP and the CDNK2A...
loci, but did not find a difference in the 6-year event free survival rate or the incidence of CNS relapse in patients with MTAP inactivation compared to those without; however, the relationship between MTAP and TPMT status in the context of 6-MP dose has not been taken into account, and this, as we have shown, may be critical to clinical response.

In conclusion, this study shows that the absence of MTAP activity, in cells with normal levels of TPMT expression, increases the sensitivity of these cells to 6-MP. This suggests that thiopurines should be most effective in MTAP-deleted cells and may facilitate greater targeting to patients in whom the drug will be most effective. Many solid cancers have 9p-deletions (2, 36), which will include the MTAP locus, and since thiopurines have an excellent track record in leukaemia therapy they could also be an effective drug for 9p-deleted solid tumors lacking MTAP expression or in tumors with low MTAP activity in combination with MTAP inhibitors (37). In view of the increasing costs of drug treatment there is considerable interest in targeting existing drugs more effectively and in new therapeutic contexts. Clearly, 6-MP warrants consideration as a relatively tumor-specific therapeutic strategy for a wider range of tumour types than at present. Furthermore, the greater efficacy of 6-TG in a 9p-deleted, MMR-proficient cell line suggests that the individualization of therapy based on MTAP and MMR status may be a key factor in widening the therapeutic use of thiopurines. Nevertheless, these data should be confirmed in xenograft models before extending to the clinic as some of the drug concentrations used i.e. EC50 values of 6-MP, are not all achievable *in vivo*. Clearly, a consideration of pharmacogenetic markers is imperative for the improvement of cancer therapy with drugs already in clinical use; markers such as MTAP and TPMT in the context of thiopurines may increase efficacy by optimising drug dosing according to tumour genotype.

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References


34. Mirebeau D, Acquaviva C, Suciu S, et al. The prognostic significance of CDKN2A, CDKN2B and MTAP inactivation in B-lineage acute lymphoblastic leukemia of
childhood. Results of the EORTC studies 58881 and 58951. Haematologica 2006;91:881-5.


Figure 1
Simplified schematic diagram of the metabolism of the thiopurines in the context of
methylthioadenosine phosphorlyase (MTAP). 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine;
MMPR, methylmercaptopurine riboside; TPMT, thiopurine methyltransferase; TIMP,
thioloinosine 5'-monophosphate; TXMP, thioxanthine monophosphate; TGMP, thioguanosine
monophosphate; IMP, inosine monophosphate; GMP, guanine diphosphate GTP, guanine
triphosphate; AMP, adenosine monophosphate; MTA, methylthioadenosine; MeTIMP,
methylthioinsine monophosphate; MeTGMP, methylthioguanosine monophosphate.

Figure 2
The effect of MTAP on sensitivity of Jurkat-MTAP+ve, Jurkat-MTAP-ve, A549-MTAP+ve and
A549-MTAP-ve to etoposide and Methylmercaptopurine. Error bars represent the S.D. of the
means from three separate experiments. No difference in sensitivity to etoposide was observed
for Jurkat-MTAP+ve (■) and Jurkat-MTAP-ve (▲) cell lines (A; P < 0.05) or A549-MTAP (■)
and A549-MTAP-ve (▲) (B; P < 0.05). Jurkat-MTAP+ve cells (■) were more resistant than
Jurkat-MTAP-ve cells (▲) when treated with MMPR (C; P < 0.05), as were A549-MTAP+ve (■)
compared to A549-MTAP-ve (▲) cells (D; P < 0.05). The inserts on graphs A and B are
Western blots of Jurkat-MTAP+ve (1) and Jurkat-MTAP-ve (2) cells (A) and A549-MTAP+ve (1)
and A549-MTAP-ve (2) cells (B) demonstrating expression of MTAP in MTAP+ve cells and the
absence of MTAP expression in MTAP-ve cells.

Figure 3
The effect of MTAP on sensitivity of Jurkat-MTAP+ve, Jurkat-MTAP-ve (A), A549-MTAP+ve and
A549-MTAP-ve (B) cells (in the presence or absence of 1µM hypoxanthine) to 6-
mercaptopurine.
Error bars represent the S.D. of the means from three separate experiments. MTAP\textsuperscript{ve} cells with (Δ) or without 1\,\mu M hypoxanthine (▲) were significantly (two-way ANOVA) more sensitive to 6-MP than MTAP\textsuperscript{+ve} cells for both Jurkat and A549 paired cell lines (P<0.05 for both cell line pairs), with viability/growth partially rescued by hypoxanthine supplementation in MTAP\textsuperscript{ve} cells with (Δ) or without 1\,\mu M hypoxanthine (▲) (P<0.05 for both cell line pairs).

**Figure 4**

*The effect of MTAP on MeTIMP and free TGN levels in Jurkat-MTAP\textsuperscript{+ve}, Jurkat-MTAP\textsuperscript{ve}, A549-MTAP\textsuperscript{+ve} and A549-MTAP\textsuperscript{ve} cells after exposure to 6-mercaptopurine.* Error bars represent the S.D. of the means from three separate experiments. Significantly more MeTIMP was produced in the A549-MTAP\textsuperscript{ve} (□) compared to the A549-MTAP\textsuperscript{+ve} (■) cells (A; t\textsubscript{(4)} = -8.17, P=0.001 and in the Jurkat-MTAP\textsuperscript{ve} (□) compared to Jurkat-MTAP\textsuperscript{+ve} (■) cells (B; t\textsubscript{(10)} = -9.373, P<0.0001). There were significantly more free TGNs in the A549-MTAP\textsuperscript{ve} (□) compared to the A549-MTAP\textsuperscript{+ve} (■) cells with 200.0\,\mu M 6-MP (C; t\textsubscript{(2)} = -1.675, P<0.0001) and in the Jurkat-MTAP\textsuperscript{ve} (□) compared to the Jurkat-MTAP\textsuperscript{+ve} (■) with 65 \,\mu M 6-MP (C; t\textsubscript{(10)} = -24.926, P<0.0001).

**Figure 5**

*The effect of MTAP on sensitivity of Jurkat-MTAP\textsuperscript{+ve}, Jurkat-MTAP\textsuperscript{ve}, A549-MTAP\textsuperscript{+ve} and A549-MTAP\textsuperscript{ve} cells (in the presence or absence of 1\,\mu M hypoxanthine) to 6-thioguanine.* Error bars represent the S.D. of the means from three separate experiments. Jurkat-MTAP\textsuperscript{+ve} with (□) or without 1\,\mu M hypoxanthine (■) showed no difference compared to Jurkat-MTAP\textsuperscript{+ve} cells in sensitivity to 6-TG, (P>0.05, A). A549-MTAP\textsuperscript{+ve} with (□) or without 1\,\mu M hypoxanthine (■) cells were more resistant than A549-MTAP\textsuperscript{ve} with (Δ) or without 1\,\mu M hypoxanthine (▲) to 6-TG (B; P<0.05).
Figure 6

The effect of MTAP on free TGNs (A, C) and dG⁺ incorporated into DNA (B, D) in Jurkat-MTAP⁺ve and Jurkat-MTAP⁻ve cells (A, B), and A549-MTAP⁺ve and A549-MTAP⁻ve cells (C, D) after exposure to 6-thioguanine.

Error bars represent the S.D. of the means from at least three separate experiments. In B and D, the ordinate scale is dG⁺ incorporation into DNA normalised to thymidine as a dG⁺-independent measure of nucleotide recovery from DNA. There was a statistically significant difference in free TGNs at equitoxic doses, comparing Jurkat-MTAP⁺ve (■) 3.4µM 6-TG versus Jurkat-MTAP⁻ve 2µM 6-TG (□) (A; t (9) = -5.181, P<0.0001) and at an equimolar dose of 3.0µM 6-TG, comparing Jurkat-MTAP⁺ve (■) versus Jurkat-MTAP⁻ve (□) (A; t (10) = -5.749, P<0.0001). There was no statistical difference in DNA incorporated dG⁺ at equitoxic doses, comparing Jurkat-MTAP⁺ve (■) 3.4µM 6-TG versus Jurkat-MTAP⁻ve 2µM 6-TG (□) (B; t (4) = 1.178, P=0.304), or at an equimolar dose of 3.0µM 6-TG, comparing Jurkat-MTAP⁺ve (■) versus Jurkat- MTAP⁻ve (□) (B; t (4) = 1.178, P=0.304). In the A549 cells there was no statistically significant difference in free TGNs at equitoxic doses of 6-TG; comparing A549-MTAP⁺ve 170.0µM 6-TG (■) versus A549- MTAP⁻ve 4.0µM 6-TG (□) (C; t (4) = -0.854, P=0.441), or equimolar dose of 170 µM 6-TG comparing A549-MTAP⁺ve (■) versus A549- MTAP⁻ve (□) (C; t (4) = -0.941, P=0.400) though there was a difference in free TGNs at 4µM 6-TG comparing A549-MTAP⁺ve (■) versus A549-MTAP⁻ve (□) (C; t (4) = 5.233 P=0.006). There was no statistically significant difference in the amount of dG⁺ in the DNA comparing equitoxic doses of 6-TG in A549-MTAP⁺ve 170.0µM 6-TG (■) versus A549- MTAP⁻ve 4.0µM 6-TG (□) (D; t (4) = 2.511, P=0.066) or equimolar doses of 170µM 6-TG in A549-MTAP⁺ve (■) versus A549-MTAP⁻ve (□) (D; t (4) = 1.602, P= 0.184) or at 4µM A549-MTAP⁺ve (■) versus A549-MTAP⁻ve (□) (D; t (4) = -1.165, P=0.309).
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