Increased Sensitivity to Thiopurines in Methylthioadenosine Phosphorylase–Deleted Cancers

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Abstract

The thiopurines, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), are used in the treatment of leukemia. Incorporation of deoxythioguanosine nucleotides (dG) into the DNA of thiopurine-treated cells causes cell death, but there is also evidence that thiopurine metabolites, particularly the 6-MP metabolite methylthioguanosine monophosphate (MeTGMP), inhibit de novo purine synthesis (DNPS). The toxicity of DNPS inhibitors is influenced by methylthioadenosine phosphorylase (MTAP), a gene frequently deleted in cancers. Because the growth of MTAP-deleted tumor 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 leukemic cell line, Jurkat, transfected with MTAP cDNA under the control of a tetracycline-inducible promoter, and a lung cancer cell line (A549-MTAP+) transfected to express MTAP constitutively (A549-MTAP-). Sensitivity to 6-MP or methyl mercaptopurine riboside, which is converted intracellularly to MeTIMP, was markedly higher in both cell lines under MTAP+ conditions. Measurement of thiopurine metabolites support the hypothesis that DNPS inhibition is a major cause of cell death with 6-MP, whereas dG 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.

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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 catalyzing the phosphorylization 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 p15INK4B gene, which is located on chromosome 9p21, 100 kb telomeric to the p16INK4A gene. MTAP is expressed ubiquitously in hematopoietic cells (1) but deletion of the MTAP gene is frequent in several cancer types (2), including hematologic 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; ref. 6) to target MTAP-deleted tumor 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; ref. 8).

The thiopurines 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) have been used extensively for treatment of leukemia. 6-MP is used in the current UKALL2003 trial (9). These drugs are metabolized extensively to cause their cytotoxic effect reviewed by Fotoohi and colleagues (ref. 10; Fig. 1). The main mechanism of cytotoxicity was initially thought to be via incorporation of deoxythioguanosine nucleotides (dG) into DNA, and in this context cytotoxicity varies in relation to MMR status (11, 12). Studies within the past decade have revealed additional mechanisms of cytotoxicity, particularly inhibition of DNPS (13, 14), interference of the rac1-vav–signaling pathway (15) and alteration of the methylation status of DNA (16). Although 6-MP via methylthioinosine monophosphate (MeTIMP), and to a lesser extent 6-TG via methylthioguanosine monophosphate (MeTGMP), inhibit DNPS, the influence of MTAP on the cytotoxic action of the thiopurines is unknown. This could be an
issue of considerable importance in leukemia because 9p abnormalities occur in 14% to 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; ref. 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 MeTIMP and MeTGMP, respectively (14). This study was undertaken to test the hypothesis that MTAP activity differentially affects sensitivity to 6-MP and 6-TG using 2 different cancer cell types as a model, a T-cell leukemia and a lung cancer cell line, both with TPMT activity in the normal range.

Materials and Methods

Cell culture

The MTAP-deleted T-cell leukemic line, Jurkat-MTAP<sup>−</sup> (Clontech), 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<sup>−</sup> parental cells were maintained in RPMI containing 10% dialyzed fetal calf serum (dFCS; Cambrex) and 100 μg/mL Geneticin (Invitrogen). Jurkat-MTAP<sup>+</sup> transfected cells were maintained in the same medium supplemented with 200 μg/mL of Geneticin and were checked regularly for mycoplasma contamination.

Cellular drug metabolite levels, MeTIMP, free thioguanine nucleotides (TGN) and dGs 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 3 separate occasions. Cells were washed twice in PBS and 5 × 10<sup>6</sup> cells pelleted and stored at −80°C before analysis.

MTAP activity assay

For MTAP activity assays, viable PBS-washed cells (5 × 10<sup>6</sup>) were snap frozen in liquid nitrogen and pellets stored at −80°C. Just before analysis, cells were thawed on ice and resuspended in 200 μL of 50 mmol/L K<sub>2</sub>PO<sub>4</sub> (pH 7.4), 0.1 mmol/L dithiothreitol, sonicated on ice for 3 seconds, 3 times, at an amplitude of 12 μm, and centrifuged at 20,800 × g for 30 minutes at 4°C. Protein concentration was measured on an aliquot of the supernatant using the Pierce BCA kit (Thermo Scientific), as described according to the manufacturer’s instructions. Measurement of MTAP activity (23) was based on a method by Savarese and colleagues (7).

TPMT activity assay

TPMT activity in Jurkat-MTAP<sup>−</sup> and Jurkat-MTAP<sup>+</sup> cells was measured as described (23) using an adaptation of the method originally published by Weinshilboum and
colleagues (24). TPMT activity was measured after induction with doxycycline for 96 hours in the presence of drug control vehicle and in the A549 cell lines 96 hours after seeding in the presence of drug control vehicle, that is, 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 3 separate occasions.

**Immunoblotting**

To confirm the expression of MTAP in the transfected A549-MTAP⁺ cells, 5 × 10⁶ cells were lysed in 100 µL of 2 × Laemmlı buffer [8% SDS, 40% glycerol, 20% 2-mercaptetoethanol, 0.04% bromophenol blue, and 0.25 mol/L Tris HC1 (pH 6.8)], heated for 7 minutes at 95°C and centrifuged at 20,800 × g at 4°C for 10 minutes. Protein concentration of the cell lysate was quantified using the Pierce kit. Ten micrograms of lysate was separated on 4% to 12% Tris/Glycine gels (Invitrogen) and blotted onto polyvinylidene difluoride (Bio-Rad). Membranes were blocked for 1 hour in blocking buffer [100 mmol/L Tris (pH 7.5), 0.9% NaCl, 0.05% Tween 20 containing 5% nonfat dried milk] and probed with either 1/4,000 dilution of mouse polyclonal MTAP antibody (Salmidix Inc.) in blocking buffer overnight at 4°C or with 1/80,000 dilution of mouse monoclonal α-tubulin antibody (Sigma-Aldrich) for 1 hour, as a loading control, and detected using HRP-conjugated goat anti-mouse secondary antibody. Visualization was achieved with 1/2,000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Dako) for 45 minutes at room temperature followed by enhanced chemiluminescence using ECL Plus [GE Healthcare (Amersham) Ltd.], according to the manufacturer’s instructions, and exposure to Kodak Medical X-ray film (GRI Medical Products Inc.).

**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 µmol/L or 16 µmol/L 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 × 10⁵ per 100 µL (in the presence of 2 µg/mL of doxycycline to induce expression of MTAP) and A549 cells at 1.5 × 10⁵ per 100 µL in a 96-well plate. After 24 hours, 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) according to the manufacturer’s protocol. Cells were treated with 6-MP, 6-TG (with and without 1 µmol/L 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). dG⁻ incorporation into DNA was measured as described previously (14, 23, 26). Briefly, DNA was extracted from 5 × 10⁶ cells resuspended in 200 µL of PBS using a commercially available spin column method according to the manufacturer’s instructions (QIAamp DNA Mini Kit; Qiagen). Purified DNA was eluted with 200 µL of 10 mmol/L Tris-HCl, 0.1 mmol/L EDTA (pH 9.0) and stored at −20°C before analysis (23). All metabolite assays were done in triplicate on 3 separate occasions.

**Statistical analysis**

Each experiment was repeated independently on at least 3 occasions. Drug sensitivity assays were analyzed using GraphPad PRISM software, in which sigmoidal dose-response curves (variable slope) were fitted to all data; drug concentrations were logged and the log EC₅₀ measured from the curve automatically. Other comparisons for treatments on MTAP-expressing and nonexpressing cell lines were carried out using 1- and 2-way ANOVA, as appropriate, with contrasts to compare treatment categories, using Systat version 15 (SPSS Inc.). 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⁺) and antisense (A549-MTAP⁻) MTAP cDNA have been characterized previously (22); the expression of MTAP at the protein level in the A549-MTAP⁺ and A549-MTAP⁻ 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 characterized. MTAP activity in the transfected Jurkat cells was measured after 24, 48, 72, and 96 hours in the presence and absence of 2 µg/mL of doxycycline. Doxycycline increased MTAP activity from 0.83 to 1.87 U/mg protein/minute between 24 and 96 hours 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/minute. 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⁻ cells. MTAP-transfected cells treated with doxycycline are referred to as Jurkat-MTAP⁺ cells. Because thiopurine metabolism and the extent of DNPS are 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+ and Jurkat-MTAP− was 3.03 ± 0.33 and 2.50 ± 0.23 mmol/L/μg protein/hour, respectively, and 1.94 ± 0.09 and 1.62 ± 0.21 mmol/L/μg protein/hour for the A549-MTAP− and A549-MTAP+ cells, respectively. The difference between cell lines and the effect of MTAP status independently of cell type was statistically significant (2-way ANOVA on log-transformed data, cell type: $F_{1,8} = 62.8, P < 0.001$; MTAP status: $F_{1,8} = 11.5, P = 0.01$; 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. MMRP 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− and Jurkat-MTAP+ cells ($P > 0.05$; Fig. 2A) or between the A549-MTAP− and A549-MTAP+ cells ($P > 0.05$; Fig. 2B) in sensitivity to etoposide. Conversely, in the case of MMRP there was a highly significant reduction in viability of Jurkat-MTAP− compared with Jurkat-MTAP+ cells ($P < 0.05$; Fig. 2C) and also with the A549-MTAP− compared with the A549-MTAP+ 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 (IMP). Jurkat-MTAP− cells were more resistant to 6-MP than Jurkat-MTAP+ 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+ 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, gray lines), producing an increase in 6-MP EC$_{50}$ from 64.3 μmol/L to 123.0 μmol/L.
Differences in levels of free TGNs could contribute to differences in cell viability between MTAP-expressing and nonexpressing cells after 6-MP treatment; at equimolar doses of 6-MP, there were significantly higher levels of free TGNs in the MTAP− cells compared with paired MTAP+ cells for Jurkat (65 μmol/L 6-MP, P < 0.0001; Fig. 4B) and A549 cells (200 μmol/L 6-MP, P ≤ 0.0001; Fig. 4B).

Drug sensitivity to 6-TG in relation to MTAP expression

Jurkat-MTAP+ and Jurkat-MTAP− 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− cells were considerably more sensitive to 6-TG than A549-MTAP+ cells. Hypoxanthine supplementation had no effect on the sensitivity of A549-MTAP− cells to 6-TG (P > 0.05; Fig. 5B), in contrast to the A549-MTAP− cells, in which the addition of hypoxanthine resulted in a significant decrease in sensitivity (P > 0.05; Fig. 5B) from an EC50 of 2.56 to 52.11 μmol/L.

To assess the contribution of TGN to drug toxicity, free TGNs were measured at equimolar (3 μmol/L) and equitoxic (3.4 μmol/L and 2 μmol/L for MTAP+ and MTAP−, 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 F1,20 = 56.7, P < 0.001) from 3.7 to 2.7 pmol/10^6 cells, but no effect of drug dose (dose or dose×MTAP interaction, F1,20 < 0.6, P > 0.4; Fig. 6A). However, there were no differences between Jurkat-MTAP− and Jurkat-MTAP+ exposed to either equitoxic or equimolar 6-TG treatments in the levels of dG incorporated into DNA (1-way ANOVA, F1,2 = 0.6, P = 0.62; Fig. 6C).

In the A549 cells, free TGNs were measured at equitoxic doses (170 μmol/L and 4 μmol/L for MTAP+ and MTAP−, respectively) and at doses of 170 μmol/L for MTAP− and 4 μmol/L for MTAP+ to allow equimolar comparisons (Fig. 6B). There was no significant difference in free TGN levels between MTAP+ and MTAP− A549 cells after treatment with equitoxic concentrations of 6-TG (1-way ANOVA, contrasts tγ = −0.04, P > 0.9). Maximal levels of free TGNs seems to be obtained with 4 μmol/L 6-TG, because these were not increased with 170 μmol/L 6-TG (contrasts tγ = −1.1, P = 0.323). However, TGN levels were much lower in the MTAP− cells than the MTAP+ cells with 4 μmol/L 6-TG (1-way ANOVA, contrasts tγ = 3.9, P = 0.006; Fig. 6B). As observed with the Jurkat cells, there was no difference between A549-MTAP− and A549-MTAP+ cells at either equitoxic or equimolar doses of 6-TG in the incorporation of dG into DNA (1-way ANOVA contrasts |t| < 2.4, P ≥ 0.128; Fig. 6D).
Higher levels of dG\textsuperscript{2} incorporation into DNA were obtained with the Jurkat cells treated with 2.0 to 3.4 µmol/L 6-TG (Fig. 6), compared with A549 cells treated with up to 170 µmol/L 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. The idea 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\textsuperscript{+/C0} cells than Jurkat-MTAP\textsuperscript{+/C0} cells perhaps due to greater inhibition of DNPS as a result of higher MeTIMP levels in the A549-MTAP\textsuperscript{+/C0} 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 IMP dehydrogenase activity in Jurkat.
Although the Jurkat cells (regardless of MTAP status) main mechanism of 6-TG toxicity in these cells.

MTAP status, thiopurine incorporation is likely to be the rated into Jurkat DNA did not differ in response to (B; (76x354) or without 1 mol/L hypoxanthine) to 6-TG. Error bars represent the SD of the mean values from 3 separate experiments. Jurkat-MTAP & A549-MTAP &/C0 mol/L hypoxanthine (<sup>m</sup>) cells were more resistant than A549-MTAP &/C0 cells (in the presence or absence of 1 μmol/L hypoxanthine) to 6-TG. Error bars represent the SD of the mean values from 3 separate experiments. Jurkat-MTAP + with (∆) or without 1 μmol/L hypoxanthine (■) showed no difference compared with Jurkat-MTAP + cells in sensitivity to 6-TG (A; P > 0.05). A549-MTAP + with (∆) or without 1 μmol/L hypoxanthine (■) cells were more resistant than A549-MTAP + with (∆) or without 1 μmol/L hypoxanthine (■) to 6-TG (B; P < 0.05). CV, control vehicle.

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<sup>-</sup> 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). Because the levels of dG<sup>+</sup> 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<sup>-</sup> A549 cells had similar sensitivity to 6-TG, Jurkat cells had greater levels of dG<sup>+</sup> 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 dG<sup>+</sup> incorporation in response to thiopurines (31). As there was no dose-dependent effect of 6-TG on dG<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> and MTAP<sup>-</sup> A549 cells used for this study, the A549-MTAP<sup>+</sup> 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 μmol/L 6-TG in the A549-MTAP<sup>+</sup> cells compared with 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 μmol/L 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 oversimplification. Because 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 CDKN2B locus encoding p15<sup>INK4B</sup> and the CDKN2A locus encoding the 2 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 acute lymphoblastic leukemia, respectively (3). The consequences of loss of this.
The effect of MTAP on free TGNs (A, C) and dG\(^\text{5}\) incorporated into DNA (B, D) in Jurkat-MTAP\(^+\) and Jurkat-MTAP\(^-\) cells (A, B) and A549-MTAP\(^+\) and A549-MTAP\(^-\) cells (C, D) after exposure to 6-TG. Error bars represent the SD of the mean values from at least 3 separate experiments. B and D, the ordinate scale is dG\(^\text{5}\) integrated into DNA normalized to thymidine as a dG\(^\text{5}\)-independent measure of nucleotide recovery from DNA. There was a statistically significant difference in free TGNs at equitoxic doses, comparing Jurkat-MTAP\(^+\) (■) 3.4 μmol/L 6-TG versus Jurkat-MTAP\(^-\) 2 μmol/L 6-TG (□; A: t\(_{250}\) = 5.181, P < 0.0001) and at an equimolar dose of 3.0 μmol/L 6-TG, comparing Jurkat-MTAP\(^+\) (■) versus Jurkat-MTAP\(^-\) (□; A: t\(_{250}\) = 5.749, P < 0.0001). There was no statistical difference in DNA incorporated dG\(^\text{5}\) at equitoxic doses, comparing Jurkat-MTAP\(^+\) (■) 3.4 μmol/L 6-TG versus Jurkat-MTAP\(^-\) 2 μmol/L 6-TG (□; B: t\(_{250}\) = 1.178, P = 0.304) or at an equimolar dose of 3.0 μmol/L 6-TG, comparing Jurkat-MTAP\(^+\) (■) versus Jurkat-MTAP\(^-\) (□; B: t\(_{250}\) = 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\(^+\) 170.0 μmol/L 6-TG (■) versus A549-MTAP\(^-\) 4.0 μmol/L 6-TG (□; C: t\(_{250}\) = −0.854, P = 0.441) or equimolar dose of 170 μmol/L 6-TG comparing A549-MTAP\(^+\) (■) versus A549-MTAP\(^-\) (□; C: t\(_{250}\) = 5.233 P = 0.006). There was no statistically significant difference in the amount of dG\(^\text{5}\) in the DNA comparing equitoxic doses of 6-TG in A549-MTAP\(^+\) 170.0 μmol/L 6-TG (■) versus A549-MTAP\(^-\) 4.0 μmol/L 6-TG (□; D; t\(_{250}\) = 2.511, P = 0.066) or equimolar doses of 170 μmol/L 6-TG in A549-MTAP\(^+\) (■) versus A549-MTAP\(^-\) (□; D; t\(_{250}\) = −1.165, P = 0.309).

locus and the effect on prognosis are controversial (33–35). If p16\(^\text{NK4A}\) and p14\(^\text{ARF}\) 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 with patients without 9p deletions. Mirebeau and colleagues (34) considered the effect of deletion of the region, including MTAP and the CDKN2A loci, but did not find a difference in the 6-year event free survival rate or the incidence of central nervous system relapse in patients with MTAP inactivation compared with 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 because thiopurines have an excellent track record in leukemia 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 tumor types than at present.

Figure 6. The effect of MTAP on free TGNs (A, C) and dG\(^\text{5}\) incorporated into DNA (B, D) in Jurkat-MTAP\(^+\) and Jurkat-MTAP\(^-\) cells (A, B) and A549-MTAP\(^+\) and A549-MTAP\(^-\) cells (C, D) after exposure to 6-TG. Error bars represent the SD of the mean values from at least 3 separate experiments. B and D, the ordinate scale is dG\(^\text{5}\) integrated into DNA normalized to thymidine as a dG\(^\text{5}\)-independent measure of nucleotide recovery from DNA. There was a statistically significant difference in free TGNs at equitoxic doses, comparing Jurkat-MTAP\(^+\) (■) 3.4 μmol/L 6-TG versus Jurkat-MTAP\(^-\) 2 μmol/L 6-TG (□; A: t\(_{250}\) = 5.181, P < 0.0001) and at an equimolar dose of 3.0 μmol/L 6-TG, comparing Jurkat-MTAP\(^+\) (■) versus Jurkat-MTAP\(^-\) (□; A: t\(_{250}\) = 5.749, P < 0.0001). There was no statistical difference in DNA incorporated dG\(^\text{5}\) at equitoxic doses, comparing Jurkat-MTAP\(^+\) (■) 3.4 μmol/L 6-TG versus Jurkat-MTAP\(^-\) 2 μmol/L 6-TG (□; B: t\(_{250}\) = 1.178, P = 0.304) or at an equimolar dose of 3.0 μmol/L 6-TG, comparing Jurkat-MTAP\(^+\) (■) versus Jurkat-MTAP\(^-\) (□; B: t\(_{250}\) = 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\(^+\) 170.0 μmol/L 6-TG (■) versus A549-MTAP\(^-\) 4.0 μmol/L 6-TG (□; C: t\(_{250}\) = −0.854, P = 0.441) or equimolar dose of 170 μmol/L 6-TG comparing A549-MTAP\(^+\) (■) versus A549-MTAP\(^-\) (□; C: t\(_{250}\) = 5.233 P = 0.006). There was no statistically significant difference in the amount of dG\(^\text{5}\) in the DNA comparing equitoxic doses of 6-TG in A549-MTAP\(^+\) 170.0 μmol/L 6-TG (■) versus A549-MTAP\(^-\) 4.0 μmol/L 6-TG (□; D; t\(_{250}\) = 2.511, P = 0.066) or equimolar doses of 170 μmol/L 6-TG in A549-MTAP\(^+\) (■) versus A549-MTAP\(^-\) (□; D; t\(_{250}\) = −1.165, P = 0.309).
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, that is, 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 optimizing drug dosing according to tumor genotype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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