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THIOPURINE INDUCED DISTURBANCE OF DNA METHYLATION IN HUMAN MALIGNANT CELLS

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INTRODUCTION

In general, tumor cells have an accelerated metabolism as compared to their normal counterparts in accordance with their higher cell growth and proliferation rate (1–6). Overall anabolic routes are enhanced in tumor cells, whereas catabolic routes are less active. This imbalance increases with the malignant state of the tumor cells (1, 2, 7, 8) and can be used as a tool for cancer therapy. No wonder that drugs, which resemble naturally occurring cellular metabolites, are used in target-directed anticancer therapy.

Antimetabolites of purine and pyrimidine are of great interest because they may incorporate into DNA and RNA of tumor cells and as such may even inhibit the synthesis of these polynucleotides. The thiopurine antimetabolites 6-mercaptopurine (6MP) and 6-thioguanine (6TG) form the cornerstone of regimens in treatment of malignancies (9–12). The metabolic action of 6MP and 6TG are closely related (Fig. 1). 6MP as well as 6TG are initially converted by hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) to 6-thio-IMP (t-IMP) and 6-thio-GMP (t-GMP), respectively. Further conversion of t-IMP to t-GMP via 6-thio-XMP is catalyzed by two enzymes, IMP dehydrogenase (IMPDH, EC 1.2.1.14) and GMP synthetase (GMPS, EC 6.3.4.1) respectively. Subsequently, t-GMP is converted by consecutive steps to 6-thio-GTP (t-GTP) and 6-thio-deoxy-GTP (t-dGTP), which are incorporated into RNA and DNA, respectively.

At present it is generally accepted that incorporation of t-dGTP into DNA is the most important reason for cytotoxicity of 6MP and 6TG, inducing DNA damage, such as single strand breaks, DNA-protein cross-links, interstrand cross-links and sister chromatid exchanges (13–18).

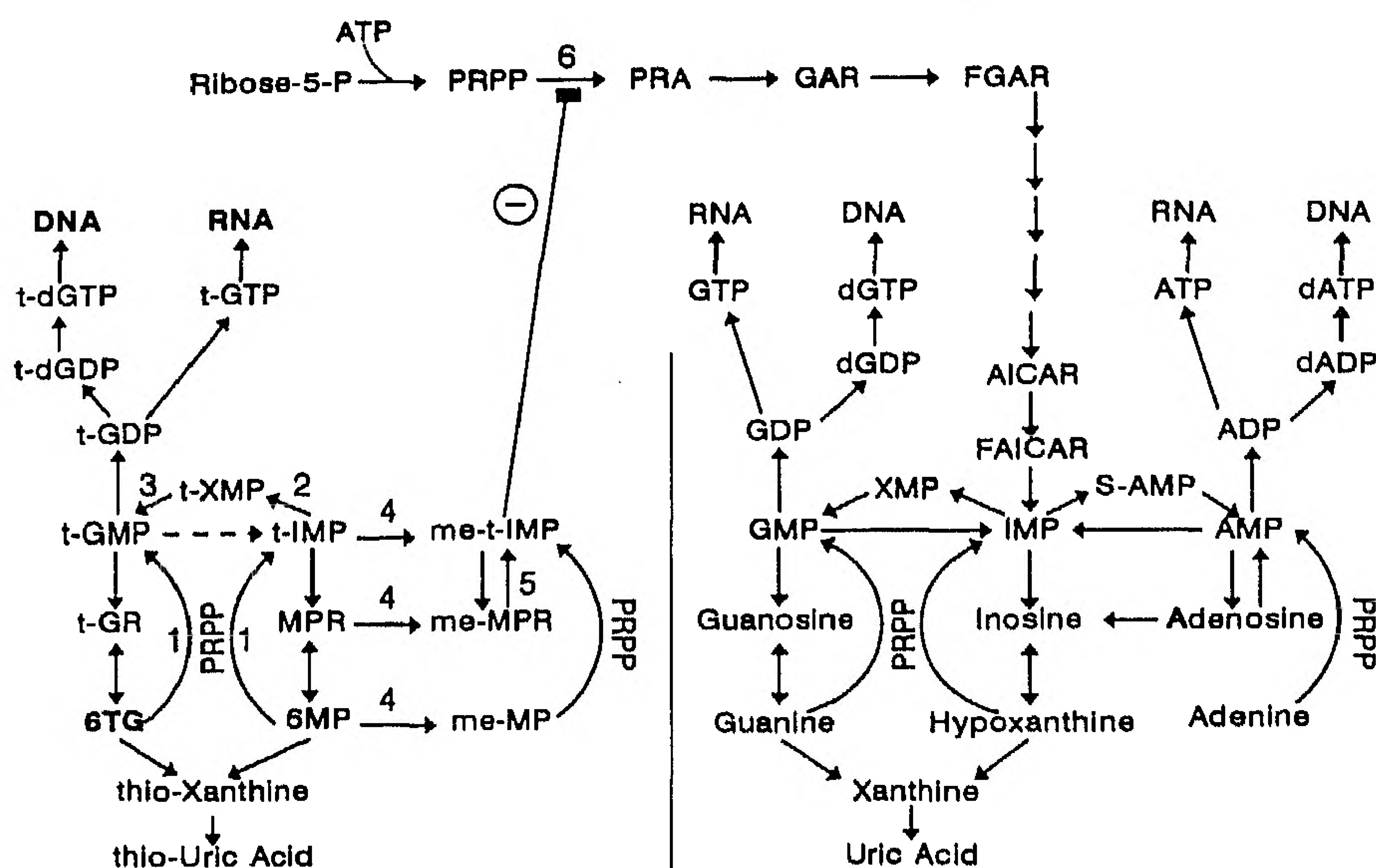


FIG. 1. 6-Thiopurine metabolism (left) and purine metabolism (right). 1. hypoxanthine-guanine phosphoribosyltransferase (HGPRT); 2. IMP dehydrogenase (IMPDH); 3. GMP synthetase (GMP synthetase); 4. thiopurine methyltransferase (TPMT); 5. adenosine kinase (AK); 6. PRPP amidotransferase.

However, 6MP seems to provoke cytotoxicity in a more complex manner than 6TG.

Metabolites from 6MP can also be methylated to methylmercaptapurines (Fig. 1). The thiopurine methylation is catalyzed by thiopurine methyltransferase (TPMT, EC 2.1.1.67) and is *S*-adenosyl-L-methionine dependent (19–21).

The importance of the methylation route for 6MP cytotoxicity has not yet been fully elucidated. Methyl-thio-IMP (me-t-IMP), the predominant metabolite formed by methylation of 6MP, is a strong inhibitor of PRPP amidotransferase (EC 2.4.2.14) the second enzyme of *de novo* purine synthesis (19, 22, 23). PRPP amidotransferase catalyzes the conversion of PRPP to PRA. This inhibition by me-t-IMP can induce cytotoxicity by several mechanisms.

Inhibition of PRPP amidotransferase results in accumulation of PRPP. Since PRPP is a co-substrate for intracellular conversion of 6MP to t-IMP, increase of PRPP induces an increased anabolism of 6MP and as such 6MP cytotoxicity is stimulated (23, 24).

A depletion of endogenous nucleotides is observed due to decreased *de novo* purine synthesis by me-t-IMP (23, 25). Tumor cells have a high need

of purine metabolites for cell growth and cell proliferation and these cells are highly dependent on *de novo* purine synthesis (PDNS). So, depletion of purine nucleotides results in decreased RNA and DNA synthesis (23).

Increase of PRPP, which results from inhibition of PRPP amidotransferase, induces an enhanced biosynthesis of pyrimidines, because this pathway is regulated by PRPP (23, 26). Increase of pyrimidine nucleotides in combination with decrease of purine nucleotides may lead to unbalanced cell growth and this may also contribute to cell death of tumor cells.

The effects of me-t-IMP could be confirmed, in experiments with methylmercaptapurine riboside (me-MPR) (26, 27). This cytostatic drug is an analogue of the purine nucleoside adenosine (Fig. 1). Earlier studies (28) demonstrated that me-MPR is effective in experimental tumor cells resistant to 6MP. Probably this is due to the fact that development of cellular resistance to 6MP is commonly associated with loss of HGPRT activity. Under these conditions the resistant cells are more dependent on the *de novo* purine synthesis. Me-MPR is converted into me-t-IMP by adenosine kinase (AK, EC 2.7.1.20). As discussed, me-t-IMP inhibits *de novo* purine synthesis and this may be the reason why cells resistant to 6MP are more sensitive to me-MPR.

Our recent *in vitro* studies confirm the important role of me-t-IMP formation and we have provided evidence that me-t-IMP may provoke cytotoxicity in tumor cells by a more complex mechanism (23, 27, 29, 30). The studies were performed in Molt F4 cells, a T-cell acute lymphoblastic leukemia cell line. The first indications for the importance of me-t-IMP for 6MP cytotoxicity is the observed synergism between 2 μM 6MP and 0.5 μM mycophenolic acid (MPA) on cell viability and cell growth (31). Since IMPDH is involved in the conversion of t-IMP to t-GMP, inhibition of this enzyme by MPA will decrease the formation of 6TG nucleotides. Ultimately, inhibition of the formation of 6TG nucleotides will result in diminished incorporation of 6TG nucleotides into DNA and RNA. Indeed, during the first 24 hr after MPA and 6MP, less t-GMP was measured as compared to 6MP alone. Furthermore, much higher me-t-IMP concentrations were detected after combination of 0.5 μM MPA (27). The observation that both cytotoxicity and Me-t-IMP concentration are increased after combination of MPA and 6MP treatment indicates that me-t-IMP formation is indeed of importance for cytotoxicity of 6MP in lymphoblastic cells with an active *de novo* purine synthesis. The inhibition of *de novo* purine synthesis is studied with increasing concentrations of me-MPR, and 0.1 μM me-MPR already resulted in maximal inhibition of *de novo* purine synthesis and increase of PRPP. The me-t-IMP concentration which induces a maximal PRPP concentration is approximately 500 pmol/ 10^6 cells in this cell line (27).

The inhibition of *de novo* purine synthesis by me-t-IMP is examined in

more detail by simultaneous prevention of purine nucleotide depletion by addition of aminocarboxamide ribonucleoside (AICAR). AICAR is incorporated distal to the site of inhibition of *de novo* purine synthesis by me-t-IMP. Addition of 50 μM AICAR indeed results in prevention of depletion especially of adenine nucleotides and subsequent decrease of cytotoxicity by 6MP and me-t-IMP (29). Similar effects are observed in combinations of intermediates of purine salvage adenosine, adenine, and inosine with 6MP or me-MPR, i.e., depletion of adenine nucleotides is prevented and less cytotoxicity is observed (30). So, these data clearly present evidence that me-t-IMP contributes to 6MP cytotoxicity in malignant cells with an active *de novo* purine synthesis.

MATERIALS AND METHODS

Materials. [^{14}C -CH $_3$]-methionine was purchased from Amersham (UK). 6MP, me-MPR, SAM and SAH were obtained from Sigma (St. Louis, MO, USA).

Procedures. Cell culture. The studies were performed with Molt F4 cells. Cells were grown in RPMI 1640 DM medium (Gibco, Netherlands), containing 10% non-dialyzed fetal calf serum (Gibco, Netherlands), 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 2 mM sodium pyruvate (BDH Chemicals Ltd, UK) and gentamicin/streptomycin, thrice a week. The cell cultures were maintained in a humidified 5% CO $_2$ atmosphere at 37°C. The absence of mycoplasma contamination and the presence of marker antigens were tested regularly.

Incubation procedure. Experiments were started with logarithmically growing cells at an initial cell number of 0.3×10^6 cells/ml, before addition of the drugs. 6MP and Me-MPR were added as a single dose in a small VOL (1/100v/v) and the cells were incubated for 24 hr with either of these drugs. After incubation, the cells were harvested and counted with a Coulter counter. Cell viability was determined by means of trypan blue exclusion. Cell growth was determined by counting the cells and correcting cell number for cell viability.

DNA methylation. After incubation with the drug, 500 μl pulse medium (0.198 mM [^{14}C -CH $_3$]-methionine (50.4 mCi/mmol) in RPMI 1640 DM) was added to the cell pellet and the cells were incubated for another 2 hr at 37°C. After pronase treatment, DNA was isolated by phenol/chloroform extraction and dissolved in 300 μl 100 mM Tris + 1 mM EDTA buffer, pH 7.5. From this solution, 100 μl was counted by liquid scintillation.

Determination of SAM concentrations. SAM concentrations were determined by HPLC in 10^7 viable cells according to the method described by Molloy *et al.* (32).

RESULTS AND DISCUSSION

Effects of 6-Mercaptopurine on the Transmethylation Route

Recently we postulated a new mechanism by which formation of me-t-IMP from 6MP may disturb various cellular processes, thereby inducing cytotoxicity (30). Treatment of Molt F4 cells, a human malignant lymphoblastic cell line, with 6MP results in a depletion of SAM and an increase of methionine and *S*-adenosyl-L-homocysteine (SAH), leading to a concentration dependent decrease of the intracellular SAM/SAH ratio (Fig. 2).

The depletion of SAM can be ascribed to two mechanisms. First, SAM acts as co-substrate for the methylation of 6MP metabolites into methylthiopurines (Fig. 3), so SAM may become depleted during the methylation of thiopurines. Second, as a result of Me-t-IMP formation, *de novo* purine synthesis is inhibited and ATP becomes depleted. Formation of SAM from methionine (Fig. 3) is ATP dependent, so conversion of methionine to SAM will be hampered under these conditions.

To discriminate between these two effects, experiments were performed with me-MPR. Overall the effects of me-MPR on the SAM, SAH and methionine pools closely resemble the effects of 6MP, i.e., SAM is depleted and SAH is increased (30). Me-MPR is already methylated, so this means that the conversion of me-MPR to me-t-IMP is a process which is independent of SAM. On the other hand, conversion of me-MPR to me-t-IMP by AK consumes ATP, since ATP is the co-substrate of this conversion. Moreover, me-MPR competitively inhibits the conversion of adenosine to AMP. So, me-MPR indirectly inhibits re-synthesis of ATP via salvage as well as *de novo* purine synthesis. Both inhibitions lead to depletion of ATP. These findings indicate that the effects of 6MP and me-MPR on SAM levels are most likely caused by reduced synthesis of SAM, due to decreased ATP levels. However, it cannot be excluded that, during treatment with 6MP, methyl transfer may also contribute to decreased SAM.

Enzymatic assays were performed to exclude direct inhibition of methionine adenosyltransferase (MAT, EC 2.5.1.6) or *S*-adenosylhomocysteine hydrolase (SAHH, EC 3.3.1.1) by metabolites of 6MP. Enzyme activities of MAT and SAH were measured in presence of 6MP, and me-MPR, t-IMP, me-t-IMP, respectively. None of these antimetabolites show any inhibition of MAT or SAHH (Table 1).

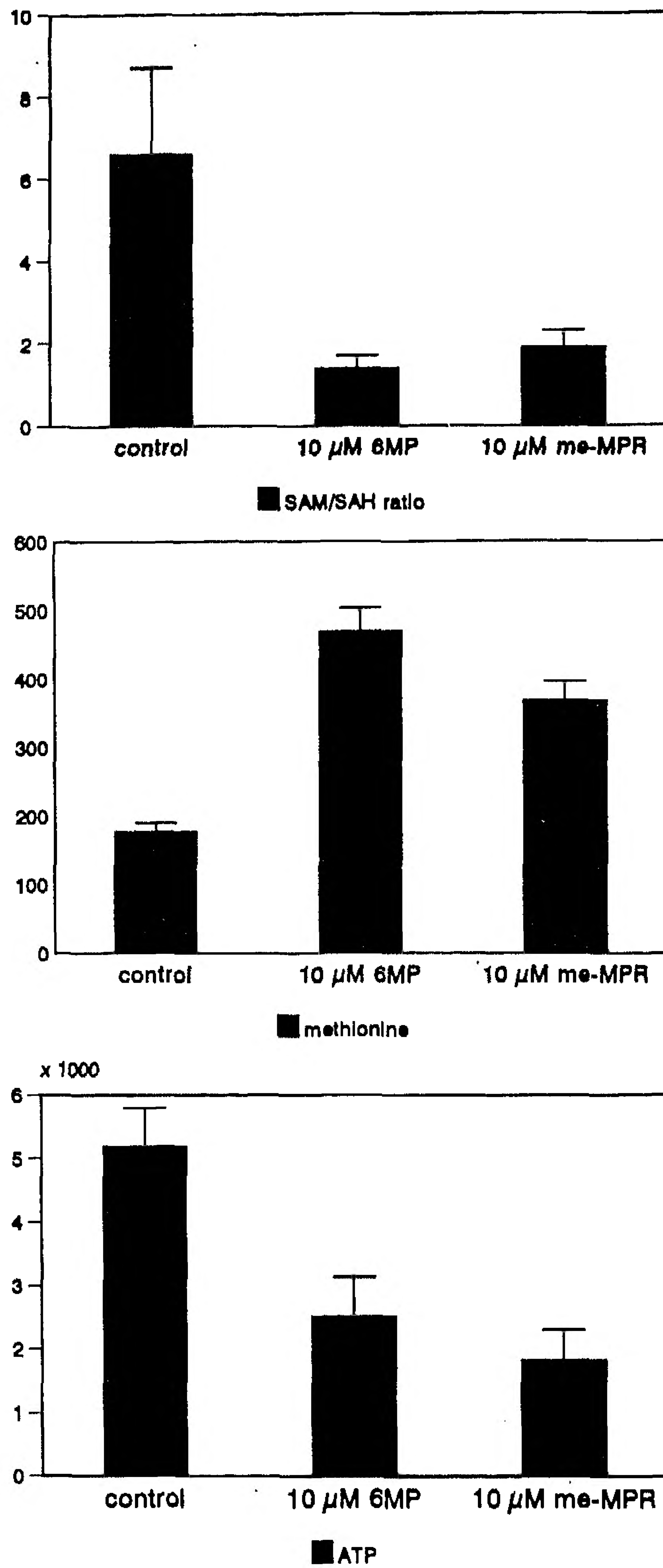


FIG. 2. Effects of 10 μM 6MP and 10 μM me-MPR, respectively, on the SAM/SAH ratio, the methionine concentration and the ATP concentration after 24 hr treatment of Molt F4 cells. Methionine and ATP concentrations are expressed as pmol/10⁶ viable cells; means and range (between brackets) of 4 experiments.

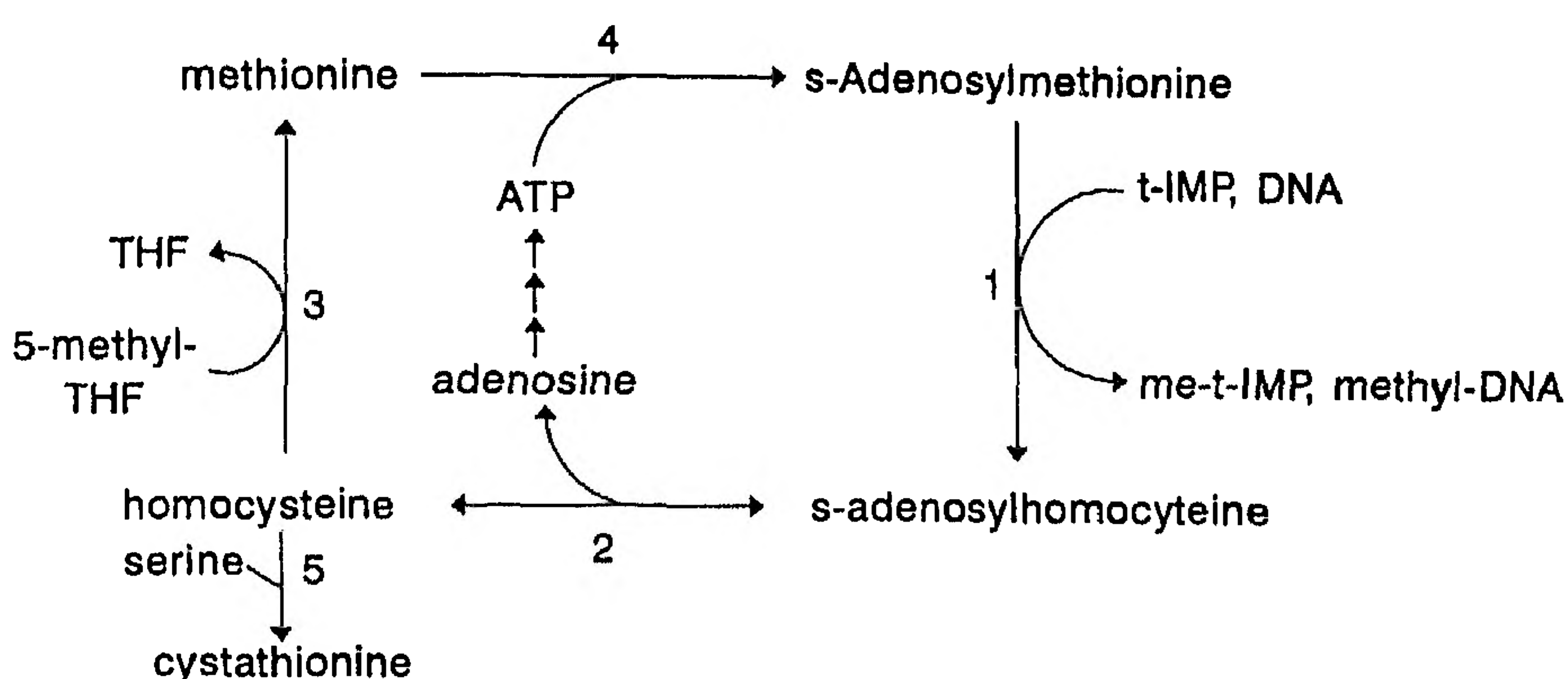


FIG. 3. Transmethylation pathway. 1. SAM-dependent methyltransferases (e.g. TPMT); 2. S-adenosyl-L-homocysteine hydrolase (SAHH); 3. methionine synthase; 4. methionine adenosyltransferase (MAT); 5. cystathionine synthase.

TABLE 1. MAT AND SAHH ACTIVITIES, MEASURED IN THE PRESENCE OF 6MP AND ITS METABOLITES

	MAT activity ($n=5$) (mean \pm SD)	SAHH activity ($n=6$) (mean \pm SD)
Without drug	2.85 \pm 0.52	4.01 \pm 0.35
10 μ M 6MP	2.87 \pm 0.22	3.97 \pm 0.35
10 μ M me-MPR	3.37 \pm 0.36	3.97 \pm 0.36
10 μ M t-IMP	2.84 \pm 0.38	4.65 \pm 0.07
10 μ M me-t-IMP	2.92 \pm 0.30	4.06 \pm 0.54

Enzyme activities are $\text{nmol} \times 10^{-6} \text{ cells} \times \text{hr}^{-1}$ and are measured in quadruplicate
(n = number of experiments, SD = standard deviation).

The observation that 6MP and me-t-IMP cause a decrease of the SAM/SAH ratio may help unravel the complexity of 6MP cytotoxicity. SAM is a methyl-donor for a great number of methyltransferase reactions, and is involved in methylation of e.g., DNA and RNA, proteins and phospholipids (33, 34).

Experiments have been performed to study whether depletion of SAM by 6MP or me-t-IMP results in disturbance of DNA methylation in human malignant cells. Logarithmically growing Molt F4 cells were incubated for 24 hr with either 2 μ M 6MP, 10 μ M 6MP, 0.5 μ M me-MPR or 2 μ M me-MPR. Untreated cells were used for reference. The 24 hr incubation was followed by a 2 hr 'pulse' with [^{14}C -CH $_3$]-methionine. DNA was isolated and quantified and radioactivity was counted.

TABLE 2. EFFECTS OF 6MP AND ME-MPR ON THE SAM CONCENTRATION AND DNA METHYLATION

	SAM (nmol/10 ⁷ viable cells)	[¹⁴ C-CH ₃]-DNA incorporation (% of control)
	(mean ± SD)	(mean ± SD)
Control (without drug)	5.1 ± 0.2	100
2 μM 6MP	2.8 ± 0.4	24 ± 1
10 μM 6MP	1.5 ± 0.8	18 ± 5
0.5 μM me-MPR	1.7 ± 0.4	22 ± 12
2 μM me-MPR	1.7 ± 0.3	19 ± 12

Mean ± standard deviation (SD) are from 3 experiments in quadruplicate.

DNA methylation is decreased significantly by 6MP and me-MPR as compared with untreated cells (Table 2).

The observation that 6MP and me-MPR cause DNA hypomethylation as a consequence of depletion of SAM may have a great impact on cytotoxicity and tumor progression. In human DNA, cytosine residues are methylated in specific sequences, by which two cytosine residues are sequenced by two guanine residues. The methylation status of these so-called CpG sites is involved in the multilevel genetic control system regulating differentiation processes and gene expression (35, 36). The extent to which DNA methylation contributes to the differentiation of hematopoietic cells is not yet fully understood.

In human malignant cells, regional areas of hypermethylation (CpG islands) co-exist with a more widespread hypomethylation status (36, 37). This is in contrast with normal cells, where most of the widely dispersed CpG sites in bulk chromatin and in the body of genes are methylated, while the CpG islands are normally not methylated. Furthermore, malignant cells have an increased cellular capacity for methylation (36).

From recent reports (35, 38, 39) it appears that global DNA hypomethylation may be a nonspecific change in tumors and potentially of less functional importance than the more regional hypermethylated CpG islands. There is a growing evidence that abnormal CpG island methylation is an important factor in the altered gene expression observed in human tumors. Abnormal CpG island methylation on autosomal chromosomes occurs at a surprisingly high frequency in immortalized rodent and human cells (40).

Recently abnormal DNA methylation patterns have been associated with hematopoietic malignancies. It has been reported (41) that the calcitonin gene is hypermethylated in human lymphoblastic and myeloid leukemia. Other reports (42, 43) show that the same gene is abnormally methylated in accelerated or blast crises phase of chronic myeloid leukemia. In a majority

of patients with acute myeloid leukemia (95%) and acute lymphoblastic leukemia (90%), DNA from tumor cells shows hypermethylation at the 5' end of the calcitonin gene (42).

Chemopreventive Effects of SAM

Several reports indicate that SAM and its precursor, methionine, may prevent the development of liver and mammary gland carcinomas or sarcomas induced in rats or mice by various carcinogens (44, 45). The mechanisms underlying the chemoprotective effect of SAM are far from being completely elucidated.

Recent data have excluded the dependency of this effect upon production of 5-methylthioadenosine, a catabolite of SAM which inhibits growth (46). The chemoprotective effect of SAM has been tentatively attributed to the methylation and inhibition of expression of growth-related genes, such as *c-myc*, *c-Ha-ras* and *c-Ki-ras*. These genes are highly hypomethylated and overexpressed in persistent nodules and hepatocellular carcinomas, in the absence of SAM (47). However, there is no definitive proof that the methylation of regulatory sequences of gene promoter is responsible for inhibition of expression of growth-related genes and nodule growth in SAM-treated rats. Nevertheless, the role of DNA methylation in SAM chemopreventive effect is proven by the finding that 5-azacytidine, a known inhibitor of DNA methyltransferases (48), largely overcomes the inhibitory effects of SAM on the growth of preneoplastic tissue (49). As mentioned, 6MP and me-MPR can similarly induce depletion of SAM levels and therefore may reverse the protective effects of SAM against carcinogenesis. Possible promoting effects of 6MP on carcinogenesis have been studied by Matsushima *et al.* (50) recently in rats. 6MP was given as a dietary supplement (50 ppm) for 35 weeks, subsequent to wide-spectrum initiation with *N*-ethyl-*N*-nitrosourea. Various tumors were observed in carcinogen-initiated groups. No significant influence of 6MP on their development, including the occurrence of leukemia, was apparent when comparing the 6MP/*N*-ethyl-*N*-nitrosourea group with the *N*-ethyl-*N*-nitrosourea group. The incidences of some proliferative lesions in the lung, intestine and kidney, however, in the 6MP/*N*-ethyl-*N*-nitrosourea group were slightly higher, although not significant. To confirm the negative result of promoting effects of 6MP, further studies have to be performed based on dose-dependent examinations using several 6MP-dose levels and other initiators.

Since 6MP therapy may also induce hypomethylation of DNA *in vivo*, it would be worthwhile to focus in future studies upon consequences for gene expression and tumor progression during 6MP treatment of patients with acute lymphoid leukemia. This may mark the effectiveness of 6MP

therapy against tumor progression. These approaches have not yet been reported.

SUMMARY

The studies described indicate that me-t-IMP formation is an important pathway, contributing to cytotoxicity in Molt F4 cells, which exhibit a highly active *de novo* purine synthesis. On three levels cytotoxicity is induced during methylation of thiopurines.

1. Purine synthesis *de novo* is inhibited during formation of me-t-IMP. Inhibition of PDNS results in depletion of purine nucleotides, with subsequently diminishing DNA and RNA synthesis.

2. The increased PRPP levels, induced by me-T-IMP, induce increased pyrimidine biosynthesis and cause an imbalance in purine nucleotides. This imbalance may lead to inhibition of cell growth and after prolonged exposure, to cell death.

3. The observed depletion of SAM and the decrease of the SAM/SAH ratio may be an additional mechanism by which 6MP and me-MPR exert their effects on cell growth and cell viability. Changes in SAM/SAH ratio may directly influence methylation reactions.

The significant decrease of DNA methylation by 6MP and me-t-IMP may influence gene regulation and tumor progression. Administration of SAM leads to chemoprevention of rat liver carcinogenesis, indicating a role of DNA methylation in tumor progression.

Besides the effects on methylation of DNA, a decrease of SAM/SAH ratio may also affect other processes, such as methylation of RNA, proteins and phospholipids, thereby disturbing their functionality.

In conclusion, decrease of the SAM/SAH ratio resulting from treatment with 6MP and me-MPR may exert many effects in these cells. This may open a new field of research, possibly contributing to a deeper understanding of the complex mechanisms by which 6MP provokes cytotoxicity.

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