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Increased Concentrations of Methylated 6-Mercaptopurine Metabolites and 6-Thioguanine Nucleotides in Human Leukemic Cells *In Vitro* by Methotrexate

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ABSTRACT. The effect of methotrexate (MTX) on 6-mercaptopurine (6-MP) metabolism was studied in four human leukemic cell lines *in vitro*. CCRF-CEM, WI-L2, TBJ, and HL-60 all expressed thiopurine methyltransferase (TPMT) activity. The cells were grown in horse serum-supplemented RPMI 1640 medium to which was added 4 μM of 6-MP or 4 μM of 6-MP and 20 nM of MTX. The presence of MTX resulted in a 2.1-, 1.7-, 2.4- and 8-fold increase in the concentrations of methylmercaptopurine ribonucleotides (MMPRP) in CEM, WI-L2, TBJ, and HL-60 cells, respectively ($P < 0.0008$). The concentrations of 6-thioguanine nucleotides (6 TGN) increased 1.9-, 1.4-, 2.4- and 1.9-fold in the same cell lines ($P < 0.02$). The four cell lines differed with respect to the effect of MTX on the consumption of 6-MP from the medium; CEM consumed more 6-MP and WI-L2 less 6-MP from media containing MTX than from media containing 6-MP only ($P = 0.005$ and 0.02, respectively). MTX did not affect the consumption of 6-MP by TBJ cells ($P = 0.17$). Media in which HL-60 cells had been grown did not contain detectable amounts of 6-MP at the end of the experiment. The simultaneous increase in methylated 6-MP metabolites and 6-TGN represents a possible explanation for the synergism of MTX and 6-MP; however, the clinical importance of increased MMPRP remains to be elucidated. BIOCHEM PHARMACOL **55**;10:1641–1646, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. drug interaction; antimetabolites; acute lymphoblastic leukemia

The combination of 6-MP† and MTX constitutes the cornerstone of maintenance treatment of children's ALL. In most treatment protocols, daily peroral 6-MP is combined with weekly parenteral MTX. The increased effect of the combination is based on empirical data from clinical studies, whereas the biochemical interactions taking place are only partly known.

The thiopurine drug 6-MP is converted to active metabolites *in vivo*. The main steps in the metabolism of 6-MP are shown in Fig. 1. The key intermediate TIMP is either oxidized and aminated to 6-TGN or methylated to MMPRP, often referred to as methylthioinosine monophosphate, MTIMP. The latter reaction is catalyzed by TPMT (E.C.2.1.1.67), which also methylates 6-MP directly to form MMP. *In vitro* methylation of 6-MP to MMP is the basis of estimation of TPMT activity [1]. TPMT activity is genetically regulated and may vary more than 100-fold between individuals [2]. XO (E.C.1.2.3.2) catalyzes the oxidation of 6-MP to TUA.

The main mechanism of action of 6-MP is incorporation of 6-TGN into DNA and RNA, producing nonfunctional nucleotides and nucleic acids. Furthermore, it has been shown *in vitro* that MMPRP inhibits PRPP amidotransferase, the first enzyme in PDNS [3], resulting in an accumulation of PRPP. PRPP is the donor of the ribose phosphate moiety in nucleotide synthesis. PDNS inhibition depletes cellular ATP and decreases the conversion of AdoMet into methionine, thus altering the methylation state of the cells [4].

MTX is a folate antagonist that is metabolized to 7-hydroxy-MTX and MTX-polyglutamates *in vivo*. MTX itself and its metabolites inhibit DHFR, the enzyme that reduces inactive dihydrofolate to active tetrahydrofolate [5]. Furthermore, purine and pyrimidine synthesis are perturbed by MTX and its metabolites through the inhibition of two PDNS enzymes GAR and AICAR formyltransferases [6, 7]

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† *Abbreviations:* AdoMet, S-adenosylmethionine; AICAR, aminoimidazole-carboxamide ribonucleotide; ALL, acute lymphoblastic leukemia; DHFR, dihydrofolate reductase; GAR, glycinamide ribonucleotide; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; 6-MP, 6-mercaptopurine; MMP, methylmercaptopurine; MMPR, methylmercaptopurine riboside; MMPRP, methylmercaptopurine monophosphate; MTIMP, methylthioinosine monophosphate; MTGR, methylthioguanine riboside; MTGRP, methylthioguanine ribonucleotide; MTX, methotrexate; PDNS, purine *de novo* synthesis; PRPP, phosphoribosyl pyrophosphate; RBC, red blood cell; TIMP, thioinosine monophosphate; 6-TG, 6-thioguanine; 6-TGN, 6-thioguanine nucleotides; TPMT, thiopurine methyltransferase; TS, thymidylate synthetase; TUA, thioric acid; XO, xanthine oxidase.

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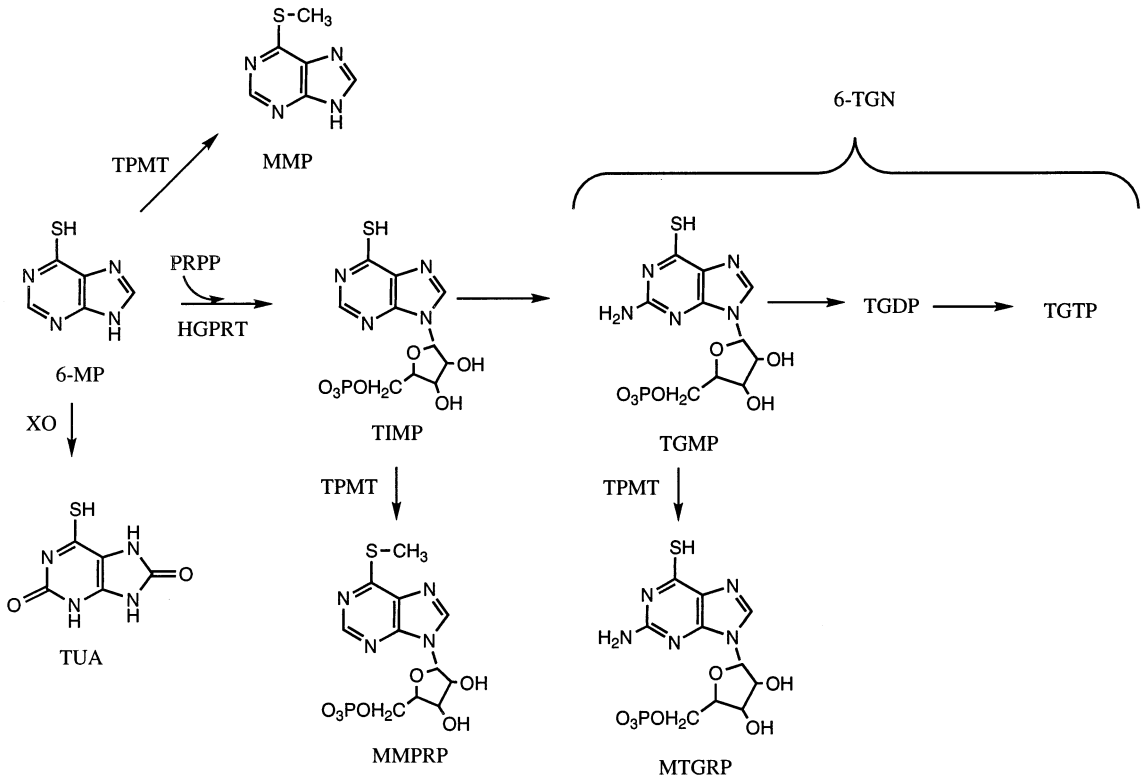

FIG. 1. The metabolism of 6-MP, 6-mercaptopurine; MMP, methylmercaptopurine; TUA, thiouric acid; TIMP, thioinosine monophosphate; MMPRP, methylmercaptopurine monophosphate; MTGRP, methylthioguanine ribonucleotide; TGMP, 6-thioguanine monophosphate; TGDP, 6-thioguanine diphosphate; TGTP, 6-thioguanine triphosphate; 6-TGN, 6-thioguanine nucleotides; TPMT, thiopurine methyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XO, xanthine oxidase.

and TS [5]. The purine degrading enzyme XO is also inhibited by MTX [8].

Recently, we observed increased concentrations of MMPRP in RBCs from rats given 6-MP plus MTX compared to rats given 6-MP alone. MTX had no effect on 6-TGN concentrations [9]. The present study was performed in order to elucidate the effect of MTX on 6-MP metabolism in leukemic cell lines of lymphocytic and myelocytic origin.

MATERIALS AND METHODS

Chemicals

Methotrexate® infusion concentrate 100 mg/mL was obtained from Lederle (American Cyanamid Company Lederle Laboratories Division, Wayne, NY) and calf intestine alkaline phosphatase was obtained from Promega. All other reagents were obtained from Sigma.

Cells

The lymphoblastoid cell lines CCRF-CEM (T-lymphoblasts), WI-L2 (B-lymphoblasts), TBJ (B- and T-cell hybrids) and HL-60 (promyelocytes) were gifts from Dr. M. S. Hershfield, Duke University Medical Center. Cells were grown in RPMI-1640 medium supplemented with 10% horse serum in an atmosphere of 5% CO_2 at 37° and counted in a Bürker chamber after trypan-blue exclusion.

Experimental

Ten mL of medium containing 1×10^5 cells/mL were added to culture flasks in replicates of 20. Twenty-four hours later, 200 μL of medium was added to 2 flasks (controls), 100 μL of medium and 100 μL 400 μM of 6-MP to 9 flasks, and 100 μL 400 μM of 6-MP and 100 μL 2 μM of MTX to 9 flasks (final drug concentrations: 4 μM of 6-MP, 20 nM of MTX). The cultures were maintained at 37° with 5% CO_2 for the next 24 hr. Eight mL of the cell suspension in each flask were centrifuged for 5 min at 160 *g* and the cell pellet was washed and resuspended in 1 mL of PBS. The cells were lysed by freezing/thawing three times and the membranes were removed by centrifugation for 5 min at 14,000 *g*. The lysates were stored at −80° until analysis. To obtain growth curves, 2.5 mL of medium containing 1×10^5 cells/mL were added to wells of a 24-well culture plate. Twenty-four hours later, drugs were added to the same final concentrations as above (four replicates of each drug, the combination and controls). The cells were counted every 24 hr for 3 days.

Analysis of Intracellular Methylated Metabolites

Methylated metabolites were detected as MMPR as described previously [10] after hydrolysis of the cell lysate with calf intestine alkaline phosphatase. To 85 μL of cell lysate was added 10 μL 0.5 M of Tris-HCI (pH 7.8) and 5 μL of calf intestine alkaline phosphatase 0.1 U/μL, and the mixture was incubated at 37° for 2 hr. The enzymatic hydrolysis was stopped by addition of 10 μL 1.8 M of perchloric acid. After centrifugation (5 min, 14,000 *g*, 4°), 70-μL aliqots of the supernatant were analyzed by HPLC. The mobile phase consisted of 0.18 M of KH_2PO_4, pH 6.85, with 24% methanol and was delivered at a rate of 1 mL/min. MMPR eluted at *ca*. 8 min and was detected at 290 nm (UV). Integration of the data was done by the Waters Millennium 2.1 software. The values are given as pmol MMPR per 10^6 cells.

Analysis of Intracellular 6-TGN

6-TGN were detected as 6-TG as described by Krynetski *et al*. [11]. A mixture of 85 μL of cell lysate and 85 μL 1.8 M of perchloric acid was heated at 100° for 1 hr, cooled and centrifuged as described above. Aliquots of 70 μL were analyzed by HPLC with a mobile phase consisting of 30 mM of $NH_4H_2PO_4$, pH 3.7, with 5% methanol. 6-TG eluted at *ca*. 2.3 min and was detected at 340 nm. The values are given as pmol 6-TG per 10^6 cells.

Analysis of 6-MP in the Medium

Five hundred μL of medium were added to 10 μL 1.8 M of perchloric acid and centrifuged for 5 min at 14,000 *g*, 4°. Aliqots of 50 μL were analyzed by HPLC with the same mobile phase as for the analysis of 6-TGN. 6-MP eluted at *ca*. 3.5 min and was detected at 320 nm.

Analysis of TPMT Activity

TPMT activity was measured radiochemically according to a modification [12] of the method of Weinshilboum [1]. The principle of the method is the conversion of 6-MP to radioactively labelled MMP with [^{14}C]-methyl-AdoMet as the methyl donor. One unit corresponds to the formation of 1 nmol MMP per hour per mL of packed RBCs. TPMT activity was measured in three lysates of each of the four cell lines prior to the experiment to ensure that the cells expressed the enzyme necessary to methylate 6-MP.

HPLC

The HPLC system consisted of a Waters™ 717 Autosampler, 600E Pump and System Controller and a 996 Photodiode Array Detector, equipped with an RCM C-18 column protected by a C-18 guard column.

Standard curves for MMPR or TG were prepared by adding known amounts of the chemicals to control lysates of each cell line. The standard curve for 6-MP was prepared in control medium.

Statistics

The Mann-Whitney U test (StatView II, Abacus Concepts, Berkeley, CA) was used to compare the levels of 6-MP in media and 6-MP metabolites in cells grown with 6-MP plus MTX or with 6-MP alone.

RESULTS

The growth of each of the cell lines was more suppressed by the combination of 4 μM of 6-MP and 20 nM of MTX than by either drug alone (Fig. 2).

All cell lines expressed TPMT activity. The activity (U/10^6 cells) was 0.04 in CEM, 0.10 in WI-L2, 0.22 in TBJ, and 0.17 in HL-60 cells.

Lysates from cells grown with MTX and 6-MP in the medium had higher intracellular concentrations of MMPRP than lysates from cells grown with 6-MP alone. MMPRP increased 2.1-fold in CEM, 1.7-fold in WI-L2, 2.4-fold in TBJ, and 8.0-fold in HL-60 cells in the presence of MTX (Fig. 3). The increase was statistically significant in all cell lines ($P \leq 0.0008$). MMPR was not detectable in lysates prior to hydrolysis, indicating that MMPR measured after hydrolysis was derived from the corresponding nucleotides.

6-TGN levels were higher in lysates from cells grown with MTX in the medium compared to cells grown with 6-MP alone. 6-TGN increased 1.9-fold in CEM, 1.4-fold in WI-L2, 2.4-fold in TBJ, and 1.9-fold in HL-60 cells in the presence of MTX (Fig. 4). The increase was statistically significant in all cell lines ($P \leq 0.02$).

CEM cells consumed more 6-MP from the medium in the presence of MTX than without (remaining 6-MP concentration 0.225 ± 0.06 (SD) μM vs 0.397 ± 0.12 (SD) μM, $P = 0.005$). In contrast, WI-L2 consumed less 6-MP from the medium when MTX was present (remaining 6-MP concentration 0.313 ± 0.11 (SD) μM vs 0.186 ± 0.06 (SD) μM, respectively, $P = 0.02$). In the case of TBJ, the amount of 6-MP consumed from the medium was not affected by MTX (remaining 6-MP concentration 0.280 ± 0.08 (SD) μM with the combination vs 0.344 ± 0.09 (SD) μM with 6-MP alone, $P = 0.17$). Media in which HL-60 had been grown did not contain measurable amounts of 6-MP at the end of the experiment.

DISCUSSION

This study confirms that MTX increases cellular concentrations of methylated 6-MP metabolites as previously shown in rats *in vivo* [9]. A novel observation is the MTX-mediated increase in cellular 6-TGN in leukemic cells *in vitro*.

Several mechanisms underlying the clinical synergism of MTX and thiopurines have been suggested. MTX increases

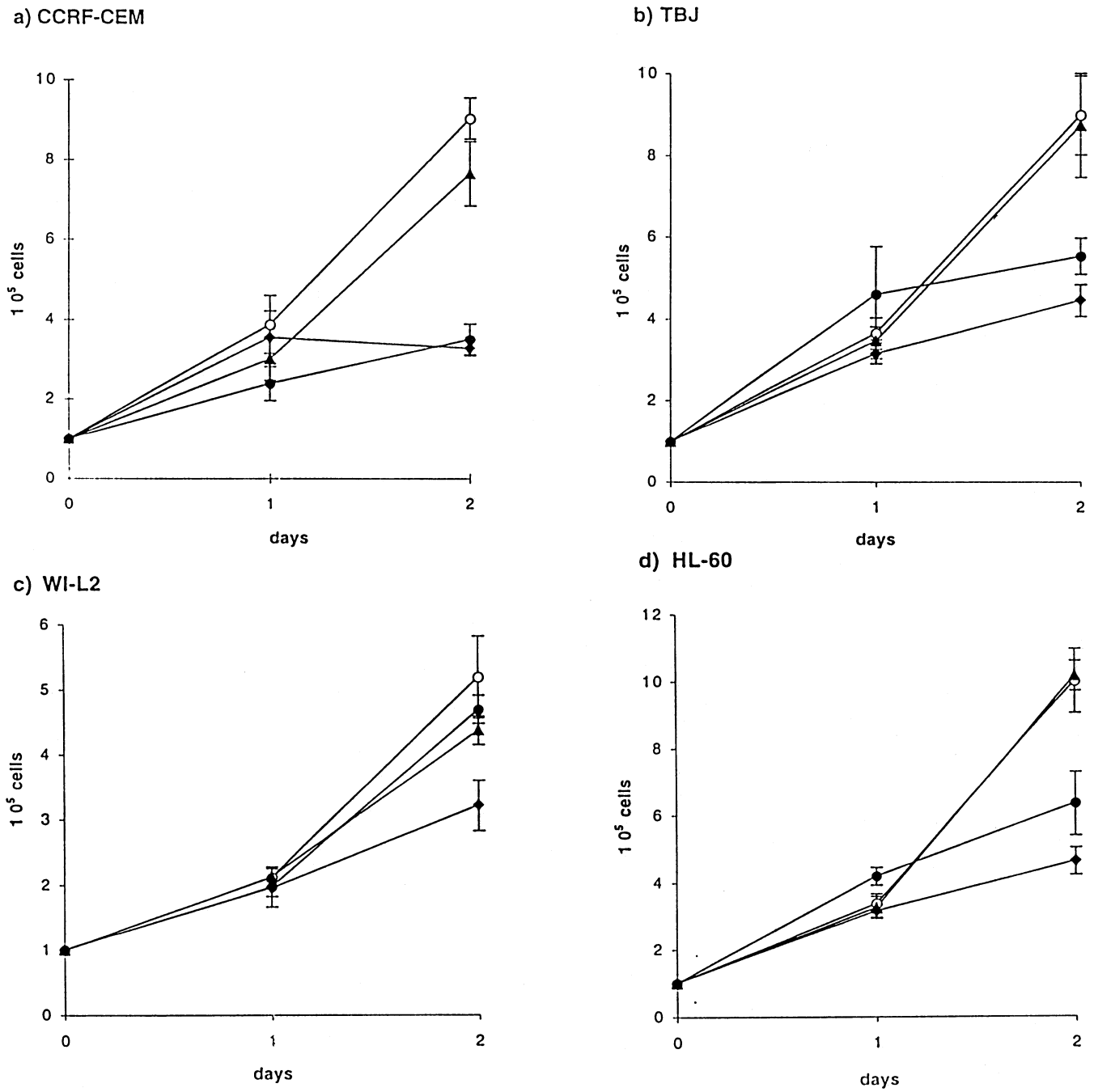

FIG. 2. Curves showing the number of cells (mean ± SD) when a) CCRF-CEM; b) TBJ; c) WI-L2; and d) HL-60 cells were grown in control medium (○) or in medium to which were added 4 μM of 6-MP (▲), 20 nM of methotrexate (MTX) (●), or both drugs (◆). Drugs were added at day 1.

the bioavailability of orally administered 6-MP, probably by inhibiting presystemic XO [13, 14]. Inhibition of XO in the cells, leaving more 6-MP for HGPRT and/or TMPT, might contribute to the increase in MMPRP and 6-TGN.

As shown in Fig. 1, TIMP is the common precursor for MMPRP and 6-TGN. Provided that the levels of cosubstrates and catalyzing enzymes are not rate-limiting, increased concentrations of *both* metabolites are a natural consequence of increased TIMP. MTX has been shown to increase the concentration of TIMP in murine WEHI-3b cells [15]. RBC 6-TGN concentration in rats *in vivo* was not affected by MTX [9], which may reflect that the *in vivo* model is far more complex pharmacokinetically.

The growth of the CEM cells was only weakly affected by 6-MP, whereas the presence of MTX in the medium reduced growth to the same degree as the presence of both drugs. Still, more 6-MP was consumed from the medium when MTX was present, which may be reflected in the higher final concentrations of MMPRP and 6-TGN. In the case of WI-L2, 6-MP gave the most pronounced reduction

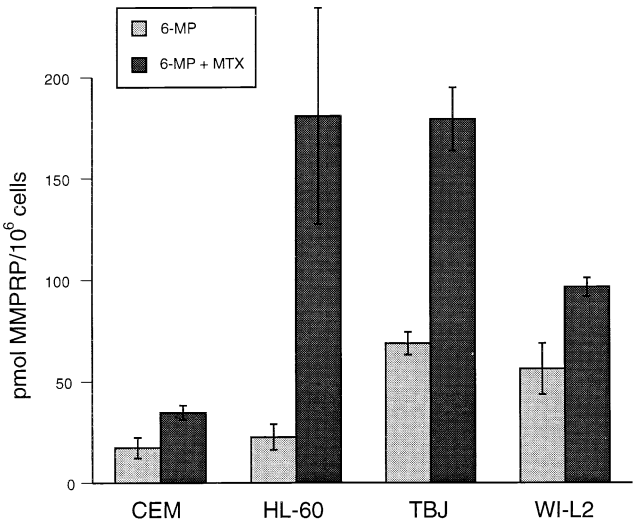

FIG. 3. Concentrations (mean ± SEM) of MMPRP in cells grown with 4 μM of 6-MP or 4 μM of 6-MP + 20 nM of MTX in the medium for 24 hr.

in cell number, whereas the presence of MTX resulted in higher final concentrations of 6-MP in the medium *and* higher concentrations of intracellular metabolites. This may indicate that the concentration of other 6-MP metabolites than those measured here are reduced by MTX in these cells. However, the interpretation of these data is complicated by the inability to calculate molar intracellular concentrations of metabolites, due to the unknown volume of the cells. The curves further indicate a synergistic effect of 6-MP and MTX, because the number of cells in the presence of both drugs seems to be more reduced than in the presence of either drug alone.

The simultaneous increase in 6-TGN and MMPRP provides a new mechanism for the interaction between 6-MP and MTX, and suggests a possible new explanation for the synergism between these drugs. Studies in patients indicate that the concentration of 6-TGN is associated with the clinical effect of 6-MP [16], whereas the association between methylated 6-MP metabolites and the clinical effect of the drug remains to be elucidated.

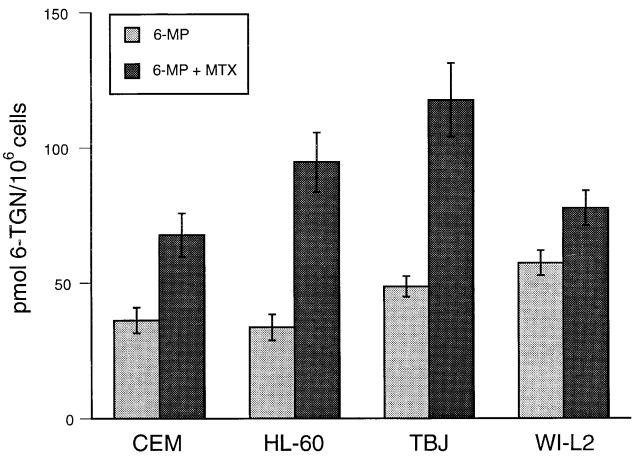

FIG. 4. Concentrations (mean ± SEM) of 6-TGN in cells grown with 4 μM of 6-MP or 4 μM of 6-MP + 20 nM of MTX in the medium for 24 hr.

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