On the Paradoxically Concentration-dependent Metabolism of 6-Mercaptopurine in WEHI-3b Murine Leukemia Cells

Jan Liliemark, Birgitta Pettersson, Britt Engberg, Pierre Lafolie, Michèle Masquelier, and Curt Peterson

Department of Medicine, Karolinska Institute at Huddinge Hospital, S-141 86 Huddinge [J. L., B. E.], and Department of Clinical Pharmacology, Karolinska Hospital, S-104 01 Stockholm [B. P. L., M. M., C. P.], Sweden

ABSTRACT

The intracellular metabolism of 6-mercaptopurine (6-MP) was studied in a murine leukemia cell line, WEHI-3b. Cells were incubated 3 to 24 h with 10 nM to 50 μM 6-MP. Nucleotides were extracted with perchloric acid, and the 6-thiophosphate nucleotides were isolated on mercurial cellulose. The endogenous ribonucleotides in the perchloric acid extracts as well as 6-thiophosphate nucleotides were separated and quantified with anion exchange high-performance liquid chromatography. The concentration of 6-thioinosinate (6-TIMP) and 6-thioxanthinate (6-TXMP) increased with an increasing 6-MP dose. The concentration of 6-thioguanosine nucleotides (6-TGN) increased with 6-MP concentrations between 10 nM and 1 μM. However, further increase in 6-MP concentration led to a decrease in the formation of 6-TGN. At 50 μM 6-MP, the concentration of 6-thioguanosine 5'-triphosphate was reduced with 6-MP concentrations between 10 nM and 1 μM. The incorporation of 6-[35S]mercaptopurine into DNA was also slightly higher at 1 μM compared with 50 μM. The cytotoxic effect on clonogenic cells was one log greater at 1 μM 6-MP compared with 50 μM 6-MP.

The decrease of 6-TGN was accompanied not only by an increased 6-TIMP concentration but also by an inhibition of the purine de novo synthesis presumably by 6-TIMP.

INTRODUCTION

6-MP3 has been used in the treatment of acute leukemias since the 1950s. Today it is a cornerstone in the maintenance treatment of acute lymphoblastic leukemia. Despite 30 years of clinical use, the knowledge of its metabolism and mechanism of action is sparse. The importance of the intracellular conversion of 6-MP to phosphorylated metabolites has been emphasized (1). It has also been shown that the incorporation of 6-MP into DNA as 6-thioguanine is one but not the only prerequisite for its lethal action (2). In 1983 it was reported that there is a paradoxical relationship between the 6-MP concentration and the lethal effect on clonogenic cells in vitro (3). The cytotoxic effect was in fact lower with a higher concentration of 6-MP. Nucleotides were extracted with perchloric acid and the 6-thiopurine nucleotides were separated and quantified with anion exchange high-performance liquid chromatography; PBS, phosphate-buffered saline; 6-TGMP, 6-thioguanalylate; 6-TGDP, 6-thioguanosine 5'-diphosphate; 6-TGTP, 6-thioguanosine 5'-triphosphate.

The aim of the present investigation was to delineate the cellular metabolism of 6-MP in vitro as a tool to optimize the design of future clinical studies.

MATERIALS AND METHODS

Chemicals. 6-[14C]Mercaptopurine, 55 Ci/mol, was purchased from CEA, Siren, France, and elemental sulfur-35, 30 Ci/mol, from Amersham. 6-TIMP, 6-thioinosine 5'-diphosphate, 6-thioinosine 5'-triphosphate, 6-TXMP, and 6-TGN were synthesized by incubation of erythrocytes with 6-MP, 6-thioxanthine, and 6-thioguanine, respectively, as previously described (4). 6-[35S]Mercaptopurine was synthesized as described by Moravec (7). From the crude preparation, [35S]6-MP was separated from labeled sulfur by HPLC to 98% radiochemical purity. The fraction containing [35S]6-MP were collected, lyophilized, reconstituted in methanol, and stored at -70°C. Tiazofurin was generously provided by Dr. Ven Narayanan, National Cancer Institute, Bethesda, MD. All other chemicals were purchased from Sigma.

Cell Lines. WEHI-3b, a murine leukemia cell line with myelomonocytic features, was kindly provided by Dr. Malcolm A. S. Moore (Memorial Sloan-Kettering Cancer Center, New York, NY). Cells were regularly monitored for Mycoplasma infection. Cells were cultured in RPMI-1640 and supplemented with 10% fetal calf serum at 37°C and 5% CO2. Penicillin G (50 IU/ml) and streptomycin (50 μg/ml) were added to prevent bacterial and fungal infections. Cells were recultivated twice a week and found to grow exponentially between 5 x 104 and 1.5 x 106 cells/ml. All experiments were performed with 106 cells at a concentration of 105 cells/ml except where otherwise stated. All results are derived from duplicate experiments unless otherwise stated.

Colonies Formation in Soft Agar. Cells (104 to 105) were plated in 1 ml of McCoy's Medium A5 in 0.3% agar and 15% fetal calf serum. The feeder layer (1 ml) consisted of McCoy's Medium A5 in 0.5% agar. The number of colonies (>40 cells) was counted in replicate plates on Day 8. Plates with 20 to 500 colonies were accepted. The plating efficiency in the controls was approximately 40%.

Determination of the 6-MP Concentration in Culture Medium. One ml aliquots of culture medium were collected after centrifugation (550 x g, 5 min) before and after the incubations. 6-MP was determined with HPLC as previously described (8).

Determination of Endogenous Nucleotides and Intracellular 6-MP Metabolites. The method used was modified from that published by Lavi and Holcenberg (9). After incubation, cells were cooled in ice water and washed once with PBS. One aliquot was removed for cell counting. The rest was suspended in 500 μl of 6-MP-containing medium, pH 7.4, and the supernatant was collected after centrifugation. The pellet was washed with PCA (0.5 M) and the supernatants were pooled and neutralized with KOH/Na2HPO4, 1.2/0.5 M. Two mg of mercurial cellulose (10) and 200 pmol of 4-thiouridine 5'-monophosphate were added to the PCA extract, vortexed for 30 s, and centrifuged at 1000 x g, 5 min. The supernatant was stored at -20°C until the analysis of endogenous nucleotides. The pellet was washed 3 times with PBS. After addition of 300 μl of mercaptoethanol (20 mM) and centrifugation, the supernatant was collected, and 100 μl were injected on to the HPLC column with a CMA/240 autoinjector (Carnegie Medicine, Inc., Stockholm, Sweden). The nucleotides were separated on a Whatman Partisil-10 SAX anion exchange column. At a flow rate of 3 ml/min, a gradient was run between 100% KCl/KH2PO4, 0.01/0.005 M, pH 6, and 100% KCl/KH2PO4, 0.5/0.25 M, pH 5.5, over 20 min following Curve 2 on a Milton-Roy CM4000 solvent delivery system, followed by 10-min

Received 5/24/89; revised 9/25/89; accepted 10/3/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1Supported by grants from the Swedish Cancer Society, the Cancer Society in Stockholm, the Swedish Medical Research Council (Project No. 08640), the Swedish Medical Society, and the Swedish Child Cancer Foundation.2To whom requests for reprints should be addressed.3The abbreviations used are: 6-MP, 6-mercaptopurine; 6-TIMP, 6-thioinosine 5'-diphosphate; 6-TXMP, 6-thioxanthate; 6-TGN, 6-thioguanosine nucleotides; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; 6-TGMP, 6-thioguanalylate; 6-TGDP, 6-thioguanosine 5'-diphosphate; 6-TGTP, 6-thioguanosine 5'-triphosphate.
isocratic elution with 100% of the latter buffer. The metabolites were quantified by UV absorbance at 340 nm with a Milton-Roy SM 3100 absorbance detector. The peak areas were integrated with a Macintosh SE computer with the ChroMac 354 chromatography software connected with a DCU interface (Drew Scientific, Ltd., London, United Kingdom). The response factors were determined by comparing the integrated area and the dpm of the metabolites in triplicate samples when cells were incubated with [14C]6-MP.

The PCA extracts were used for the HPLC assay of endogenous nucleotides as previously described (11). The intracellular concentration of endogenous nucleotides and 6-MP metabolites was determined by dividing the amount of nucleotide with the cell number x mean volume as calculated on a Coulter mull ¡MAT (Coulter Electronics, Ltd., Luton, United Kingdom).

Determination of the Purine de Novo Synthesis. Leukemic cells were incubated for 14 h with 1 or 50 μM 6-MP. Two h before termination of incubation, 70 nmol of [14C]glycine, 4 μCi, were added to each 100-ml flask. The nucleotides were extracted from the leukemic cells, and the nucleotides were separated as described above. Fractions of the mobile phase, corresponding to the nucleotides, were collected, and the amount of radioactivity was determined by scintillation counting with a Packard Tri-carb 460C scintillation counter.

Isolation of DNA and Determination of Drug Incorporation. Cells were incubated with [14C]6-MP or [35S]6-MP at a concentration of 1 or 50 μM. After incubation, cells were washed with PBS, and the DNA was purified on a cesium chloride gradient (12). DNA concentrations were measured by spectrophotometry, and the radioactivity of incorporated 6-MP was counted.

RESULTS

The isolation of thiometabolites on mercurial cellulose provided a sample where no endogenous nucleotides were found. Baseline separation was achieved among all the 6-thiornucleoside metabolites. 6-Thioinosine 5'-di- and triphosphate could also be separated from the 6-TGN, 6-TIMP, and 6-TXMP. The 5'-di- and triphosphate 6-thioinosine were, however, not found in any sample. The intersample variation (SD) was less than 20%. The limits of detection for the metabolites were 3 to 15 pmol at 340 nm. Due to the purity of the samples after the mercurial cellulose workup step, the detection limit in terms of concentration was dependent on the amount of cells used. The degradation of 6-thiometabolites in 20 mM mercaptoethanol was <5% when stored at −20°C for 2 mo.

The cellular concentrations of 6-TIMP and 6-TGMP in WEHI-3b cells incubated with 1 or 50 μM 6-MP for 1 to 24 h are shown in Fig. 1. The concentration of the metabolites increased linearly with time to reach a maximum after 12 h. Thereafter, the concentrations declined. Therefore, overnight incubations were chosen for the subsequent experiments. The concentration of 6-MP in the medium was measured before and after incubation, and its decrease was found to be a limiting factor for the accumulation of metabolites when low incubation concentrations of 6-MP were used. At an incubation concentration of 1 μM, only 10 to 20% of 6-MP remained in the medium after a 14-h incubation. At 50 μM the corresponding figure was >50% (data not shown).

Cells were incubated with 1 μM 6-MP and thereafter put in ice water, washed 3 times with ice-cold PBS, and reincubated in drug-free medium at 37°C. Aliquots were taken after 15 and 30 min and 1, 2, and 4 h. The t½ for cellular 6-TGMP and 6-

Fig. 1. a, the cellular concentration of 6-TIMP during incubation with 1 (□) and 50 μM (■) 6-MP. Points, mean from duplicate experiments; bars, SD. b, the cellular concentration of 6-TGMP during incubation with 1 (□) and 50 μM (■) 6-MP. Points, mean from duplicate experiments; bars, SD.
TGTP was 2.1 and 0.7 h, respectively (Fig. 2). The $t_{1/2}$ of 6-TIMP could not be measured at 340 nm due to the low initial concentration at this incubation concentration. When the washout experiment was done with 50 μM 6-MP, 6-TGN continued to accumulate during the washout period, and 6-MP was found in the "drug-free medium" probably due to redistribution of drug from the cells (data not shown).

The decrease of the 6-MP concentration in the medium during the incubations and the short $t_{1/2}$ of intracellular metabolites have to be taken into account when studying Fig. 3 showing the concentrations of nucleotide 6-MP metabolites in the leukemic cells at various incubation concentrations. The concentration of 6-TIMP was <0.1 μM at incubation concentrations <0.1 μM 6-MP. There was a linear increase of 6-TIMP with an increasing 6-MP concentration up to 10 to 20 μM. The 6-TXMP also increased with 6-MP but to a lower concentration level. The 6-TGN could be detected already at an initial 6-MP concentration of 10 nM. The concentration of 6-TGN reached a maximum at initial 6-MP concentrations around 1 to 5 μM. At 50 μM 6-MP, the concentration of 6-TGN was around one fifth that at 1 μM. The three 6-TGN (6-TGMP, 6-TGDP, and 6-TGTP) showed essentially the same pattern with the 6-TGMP concentration being 5 to 10 times that of 6-TGDP and 6-TGTP. The relation of 6-TGTP to 6-TGDP showed a considerable variation with 6-TGTP concentrations generally being higher. Similar results were obtained with L-1210 or CCRF-CEM (a human lymphoblastic lymphoma cell line) cells (data not shown).

The purine de novo synthesis was strongly inhibited when cells were incubated with 50 μM 6-MP compared with that of the control or at 1 μM (Fig. 4). Consequently the ATP concentration fell to ~50% of control at 50 μM 6-MP. The decrease in the formation of 6-TGN, the increasing concentration of 6-TIMP, and the decrease in the ATP concentration are seen at the same 6-MP level. 6-TGMP is formed by transamination of 6-TXMP by the enzyme guanylate synthetase, and ATP serves as a cofactor. To test the hypothesis that the decrease in formation of 6-TGN at a high 6-MP concentration is due to a depletion of ATP due to an inhibition of the purine de novo synthesis presumably by 6-TIMP, we treated leukemic cells
simultaneously with 6-MP and antimycin A. Antimycin A blocks the respiratory chain between cytochrome b and cytochrome c and thus the formation of ATP by oxidative phosphorylation. Incubation with 1 µM 6-MP and 0.3 µM antimycin A decreased the ATP concentration in the leukemic cells to the level seen with 50 µM 6-MP alone (Fig. 5). The concentration of 6-TGN also decreases to the level seen with 50 µM 6-MP. Concomitantly, the concentration of 6-TIMP increased, but that of 6-TXMP decreased somewhat.

To elucidate the significance of the metabolism of 6-MP for its cytotoxicity, WEHI-3b cells (10⁵ cells/ml) were incubated with 1, 10, and 50 µM 6-MP during 24 and 48 h. The effect on clonogenic cell survival is shown in Fig. 6. Cell kill was one log greater at 1 µM compared with 50 µM 6-MP. Mycophenolic acid and tiazofurin are inhibitors of inosinate dehydrogenase, the enzyme converting 6-TIMP to 6-TXMP. When cells were incubated with 0.5 µM mycophenolic acid or 20 µM tiazofurin concomitantly with 1 µM 6-MP, the formation of 6-TXMP and 6-TGN diminished, and the cell kill was 1 to 2 logs lower in cells treated with mycophenolic acid or tiazofurin concomitantly with 6-MP compared with cells treated with 6-MP alone (data not shown).

Cells were incubated with 1 and 50 µM 6-MP of either [14C]- or [35S]6-MP, and the DNA was isolated as described in "Materials and Methods." At 1 µM, slightly less radioactivity was found in DNA compared with 50 µM 6-MP when [14C]6-MP was used. With [35S]6-MP, the opposite was found (Fig. 7). The total amount of radioactive drug or metabolites found in DNA was 5 times higher when [14C]6-MP was used.

**DISCUSSION**

It has previously been shown that the incorporation of 6-MP into DNA is related to the delayed cytotoxicity of the 6-MP (2). However, after digestion of DNA, only deoxythioguanosine but no deoxythioinosine is found. This indicates that the conversion of 6-TIMP to 6-TGN is compulsory for its lethal effect (2). This conclusion is supported by the decrease in toxicity of 6-MP when the cells were treated concomitantly with tiazofurin or mycophenolic acid inhibiting the conversion of 6-TIMP to 6-TXMP and 6-TGN. It is unclear why 6-MP is generally used for treatment of acute lymphoblastic leukemia and 6-thioguanine for treatment of acute myeloblastic leukemia. The data in this and a previous study (2) suggest that substitution of 6-thioguanine for 6-MP might be advantageous because it is metabolized directly to 6-TGN.

Because appropriate deoxyribonucleotide standards of thioguanine are not commercially available, we have not been able to separate ribo- and deoxyribonucleotides of 6-MP metabolites in this study. Unfortunately deoxythioguanosine is not resistant to periodate oxidation (data not shown) which would otherwise have been a useful method for separation of ribo- and deoxyribonucleotides (13). However, a comparison of the chromatographic retention times of standard ribonucleotides and the metabolites we find suggests that what we measure are the ribonucleotides. Thus, the metabolites discussed in this study

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**Fig. 5.** Top, cellular concentration of 6-MP metabolites in leukemic cells incubated with 1 or 50 µM 6-MP of either 6-MP and 0.3 µM antimycin A. Bottom, cellular concentration of ribonucleoside triphosphates in the same experiments. Columns, mean and the lower SD (shaded areas) from duplicate experiments.

**Fig. 6.** The survival of clonogenic cells (percentage of control) after incubation of leukemic cells with 1 (▲), 10 (▼), and 50 µM 6-MP (●). Points, mean from triplicate experiments; bars, SD.

**Fig. 7.** The cellular concentration of 6-TIMP (■), 6-TXMP (●), 6-TGMP (▲), 6-TGDP (○), and 6-TGTP (△) and the incorporation of radioactivity into DNA after incubation of leukemic cells with 1 or 50 µM [14C]-6-MP or [35S]6-MP (○).
are merely intermediates between 6-MP and the deoxyribonucleotides that can be incorporated into DNA. This has to be taken into account when interpreting the results of this study.

This study shows that, in vitro, 6-MP has a "self-limiting" type of metabolism; i.e., above a certain dose level, the concentration of its important metabolites, the 6-TGN, will decrease with increasing dose. It has previously been shown that 6-MP is a strong inhibitor of purine de novo synthesis (14). The low purine de novo synthesis with high 6-MP and high intracellular 6-TIMP concentrations and the decrease of 6-TGN formation following addition of antimycin A seen in this study suggest that ATP is important for the conversion of 6-TIMP to 6-TGMP. This conclusion is supported by ATP being a cofactor for guanylate synthetase. The decrease of the 6-TXMP concentration after addition of antimycin A probably reflects disturbances in the cellular utilization of NAD, which is a cofactor for inosinate dehydrogenase, the enzyme converting 6-TIMP to 6-TXMP. An alternative mechanism would be a direct inhibition of guanylate synthetase by 6-TIMP. Studies on the isolated enzyme to investigate this possibility are under way.

When the incorporation of 6-MP into DNA is studied, the site of the radioactive label is of great importance for the results. With the [14C] label in the purine ring structure, 5 times more radioactivity was found in DNA, and more "drug" was incorporated at 50 μM than at 1 μM 6-MP. This indicates that some of the 14C-labeled 6-MP is oxidized in position 6 to hypoxanthine and incorporated into DNA as adenine or guanine. When [35S]6-MP is used, such an artifact is ruled out. Thus, the lower incorporation of radioactivity into DNA seen at 50 μM, compared with 1 μM [35S]6-MP, represents a more accurate incorporation of metabolites of this drug. As it has been shown previously that 6-MP is incorporated into DNA as 6-thioguanine (2), this is also in line with the lower concentration of 6-TGN seen with 50 μM 6-MP in this study.

It has been shown by others and also confirmed in this study that the lethal effect on clonogenic cells is, within a certain concentration range, inversely related to the dose of 6-MP. The hypothesis presented to explain these paradoxical results was based on cell kinetic perturbations (3), but it is obvious from the results of the present study that the cellular metabolism of 6-MP might constitute an additional explanation.

In maintenance treatment of acute lymphoblastic leukemia, the standard dose of 6-MP is 75 mg/m² p.o. once daily. Although the interindividual variation of the plasma concentrations achieved with this dose is great, a peak concentration in the range of 1 μM is normal (8). In the present study the highest concentrations of 6-TGN were achieved with an initial incubation concentration of 1 to 5 μM, but as emphasized before, this corresponds to a concentration of 0.2 to 1.5 μM at the end of the incubation. Because of the short retention of the 6-MP nucleotides in the leukemic cells (Fig. 2), it is probable that the concentration of the metabolites relates more to the final 6-MP concentration. It is assumed that these results from murine leukemia cells in vitro can be extrapolated to human leukemia cells in vivo, it is obvious that raising the peak plasma concentration to >2 μM by increasing the dose in the clinical situation might not increase the antileukemic effect of the treatment. In contrast, shortening of the dose interval and increasing the trough concentration while little affecting the peak concentra-

ACKNOWLEDGMENTS

The valuable discussions of this work with Dr. Gertrude Elion, Dr. Bertil Fredholm, Dr. William Plunkett, and Dr. Peter Reichard are greatly appreciated. We are also thankful to Dr. A. Rheidin for the linguistic revision.

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