ABSTRACT

The accumulation, metabolism, and retention of mercaptopurine (MP) was studied in four human neoplastic cell lines (three acute leukemia lines Molt-4, CCRF-CEM, and HL-60; and one Burkitt's lymphoma line, Wilson), each of which was sensitive to MP. Two cell lines resistant to MP (Wilson and CCRF-CEM) were also studied. The cell lines were incubated for 3 h in 10 μM [14C]MP and then placed in drug-free media for an additional 3 h. Cell samples were obtained at regular intervals, and the intracellular MP metabolites were measured in the acid-soluble fractions by anion-exchange high-pressure liquid chromatography. MP accumulated progressively within cells during the 3-h drug exposure period and declined rapidly when the cells were placed in drug-free media. Over 80% of the intracellular MP was present in the form of three nucleotide metabolites, MP ribose monophosphate, thioxanthosine monophosphate, and thioguanosine monophosphate. MP ribose monophosphate was found in greatest amount, accounting for 59–85% of the intracellular metabolite pool. Thioxanthosine monophosphate thioguanosine monophosphate were detected in lesser amounts. Study of leukemic cells obtained from patients demonstrated a similar pattern of MP accumulation, metabolism, and retention, although the overall amounts of the various metabolites formed were less. In contrast, there was essentially no MP nucleotide metabolite formation in the two MP-resistant cell lines. A more complete understanding of the cellular pharmacokinetics of MP in human neoplastic cells is likely to lead to a more rational use of the drug in the clinical setting.

INTRODUCTION

MP,2 an analogue of hypoxanthine, was synthesized and introduced into clinical use over 30 years ago (1, 2). It has a well-established clinical role in the treatment of acute lymphoblastic leukemia (3). Although the plasma pharmacokinetics of MP in humans has been the subject of recent investigations (4, 5), the cellular pharmacokinetics of MP in human neoplastic cell lines and cells obtained directly from patients has not been carefully studied. The importance of examining the cellular disposition of MP lies in the fact that mercaptopurine itself is merely a prodrug. In order for it to have cytotoxic activity, it must be converted to a nucleotide, TIMP. This intracellular reaction is catalyzed by HPRT (EC 2.4.2.8) and requires PRPP (6–8). TIMP is known inhibitor of several enzymes in the de novo purine synthetic pathway (9). In addition, thioguanosine monophosphate and deoxythioguanosine monophosphate have been identified in RNA and DNA isolated from murine lymphoma cells treated with mercaptopurine, suggesting that TIMP can be further metabolized to thioguanine nucleotides (Chart 1) (10).

The present study was conducted in an effort to determine the extent and the time course of formation and retention of intracellular MP metabolites in human neoplastic cells, and to investigate possible relationships to the activities of HPRT, PRPP, and the major intracellular MP degradative enzyme, alkaline phosphatase (EC 3.1.3.1) (11).

MATERIALS AND METHODS

Materials. [14C]MP (specific activity, 30 mCi/mmol), labeled in the 8-position, was purchased from Research Products International, Mt. Prospect, IL. [14C]hypoxanthine (specific activity, 49 mCi/mmol) and [14C]orotic acid (specific activity, 52.2 mCi/mmol) were obtained from New England Nuclear, Boston, MA. MP, TX, ATP, GTP, TIMP, PRPP, alkaline phosphatase, p-nitrophenylphosphate, venom phosphodiesterase, and orotatephosphoribosyl-transferase-3'orthidyl-5-monophosphate were purchased from Sigma Chemical Co., St. Louis, MO. MP ribose triphosphate was purchased from P.L. Biochemicals. TMGMP and TU were generously provided by Drs. G. B. Elion and R. Miller, Burroughs Wellcome Co., Research Triangle Park, NC.

Cell Lines and Cells. All cell lines were of human origin and were maintained in 10% FBS and RPMI 1640 and passaged twice weekly. All cell lines were screened for Mycoplasma infection every 6–8 weeks. CCRF-CEM and MOLT-4 are acute lymphoblastic leukemia lines and HL-60 is an acute promyelocytic leukemia cell line. These 3 cell lines were obtained from American Type Culture Collection, Rockville, MD. The Wilson cell line is a Burkitt's lymphoma line and was kindly provided by D. Ian Magrath, Pediatric Branch, National Cancer Institute. The cell lines resistant to MP, CCRF-CEM and Wilson were selected by serial passage of the respective parental, drug-sensitive lines in progressively increasing concentrations of mercaptopurine. These cell lines were able to grow in 2 mm MP.

Cytotoxicity Studies. To determine the sensitivity of the various cell lines to MP, cells were seeded at an initial density of 1 x 10^6/ml in 10% FBS and RPMI 1640. The media were then adjusted to varying MP concentrations, ranging from 10 nM to 1 mM, and the cells were cultivated for 72 h at 37°C in a humidified atmosphere of 5% CO2. Cells were then counted with a Model ZBI Coulter Counter. The cytotoxicity of MP was determined by calculating the IC50 (drug concentration required to inhibit cell growth by 50% compared to control).

Leukemic Cells from Patients. Leukemic cells were obtained from 10 patients (7 at initial diagnosis and 3 at bone marrow relapse) with acute lymphoblastic leukemia being treated in the Pediatric Branch, National Cancer Institute. These cells were separated from other cellular elements by Ficoll-Hypaque gradients and were stored in 10% dimethyl sulfoxide at −190°C. Cell viability exceeded 90%, by trypan blue dye exclusion, at the time that the studies were performed.
Cellular Pharmacokinetics. During pharmacokinetic studies, all cell lines and cells were incubated in 10% dialyzed PBS and RPMI 1640 at a concentration of 1 x 10^6 cells/ml. Cells were incubated in 10 μM [³²P]-MP for 3 h and then washed in iced phosphate-buffered saline and placed in drug-free media for an additional 3 h. Cell aliquots of 1 x 10^6 cells were removed at the following times for analysis: 0.5, 1, and 3 h during the period of drug incubation, and then at 1 and 3 h after placement of the cells in drug-free media.

Sample Preparation and HPLC Nucleotide Assay. MP and its nucleotide metabolites were extracted from the cell aliquots with 100 µl of 10% trichloroacetic acid that contained 2 mM dithiothreitol, and precipitated proteins were removed by centrifugation. Samples were then diluted with 200 µl of distilled water, neutralized to pH 4-5 with 10 µl NaOH, and stored at -190°C until the time of analysis (usually 4-7 days).

Selective extraction of the thiol-containing nucleotide metabolites was done to confirm that ATP and GTP were metabolites of MP. This method was conducted as described elsewhere (13), using a mercurocellulose resin.

The method used for separating MP intracellular metabolites was based on the nucleotide separation assay described previously by Hartwick and Brown (14), and McKeag and Brown (15). A Waters HPLC system was used that consisted of a WISP Model 710B automatic sample injector, 2 Model 6000A solvent delivery systems, a Model 440 absorbance detector, a system controller and data module, and a Whatman Partisil-10 SAX anion exchange column. The low-concentration eluent was 0.01 M KH₂PO₄ and 0.01 M KCl (pH 4.0), and the high-concentration eluent was 0.25 M KH₂PO₄ and 0.50 M KCl (pH 5.0). All eluents were filtered and degassed prior to use. Following application of a 100-µl sample to the column, a No. 8 convex gradient was developed going from 100% low-concentration eluent to 100% high-concentration eluent over 30 min at a flow rate of 2 ml/min. After completion of the gradient, the column was eluted for an additional 25 min with 100% high-concentration eluent, the next sample was injected. The absorbance detector, a system controller and data module, and a Whatman Partisil-10 SAX anion exchange column. The low-concentration eluent was 0.01 M KH₂PO₄ and 0.01 M KCl (pH 4.0), and the high-concentration eluent was 0.25 M KH₂PO₄ and 0.50 M KCl (pH 5.0). All eluents were filtered and degassed prior to use. Following application of a 100-µl sample to the column, a No. 8 convex gradient was developed going from 100% low-concentration eluent to 100% high-concentration eluent over 30 min at a flow rate of 2 ml/min. After completion of the gradient, the column was eluted for an additional 25 min with 100% high-concentration eluent, the next sample was injected. The absorbance of the supernatant was injected onto the HPLC system.

Additional confirmation of the identity of the nucleotide metabolites of MP was obtained by collecting the TIMP, TGMP, ATP, and GTP peaks and then lyophilizing them. The samples were then incubated at room temperature for 2 h in 0.1% diethanolamine containing 7 µl of alkaline phosphatase (type VII-S) and 1.5 mg of phosphodiesterase. The samples were then boiled for 2 min, centrifuged (400 x g for 10 min), and the supernatant was injected onto the HPLC system.

The HPLC assay, modified from one previously reported (17), consisted of a 5-µm reverse-phase column with a 2-module (Waters Associates). The initial eluent was 50 mM ammonium acetate, pH 6.1, with a flow rate of 1 ml/min. The second eluent was 70% acetonitrile-30% water which was introduced into the mobile phase using a linear gradient, increasing from 0 to 28% second eluent over a period of 35 min (0.8%/min). The retention times of guanosine, TGR, MP, adenosine, and methyl-mercaptopurine riboside were 20.3, 22.7, 23.4, 25.3, and 40.5 min, respectively. The effluent that eluted with the authentic standards for MP, methylmercaptopurine riboside, TGR, adenosine, and guanosine and was collected and radioactivity was determined as described above.

HPRT Assay. The HPRT assay measured the conversion of hypoxanthine to inosine monophosphate (18, 19). Cells were washed 3 times in phosphate-buffered saline, resuspended in 1 ml of distilled water, and then lysed by 4 cycles of freezing-thawing. The supernatant resulting from a 30-min spin at 20,000 x g was subsequently heated for 10 min at 60°C and then assayed for HPRT. The assay incubate consisted of 5 mM MgCl₂, 1 mM PRPP, 0.1 mM glucose buffer, pH 10, 0.15 mM [³²P]-hypoxanthine, and 20 µl of cell supernatant in a total volume of 100 µl. The incubation was conducted for 15 min and terminated by placing the samples on ice and adding 1.25 µmol EDTA in 5 µl. Aliquots of 25 µl were then spotted on Whatman DE-81 filter discs. The samples were allowed to dry and then were washed 3 times with 1 mM ammonium bicarbonate, once with distilled water, and then once with methanol. After being allowed to dry, the discs were counted in a liquid scintillation counter in order to quantitate the amount of nucleotide formed.

RESULTS

Cell Line Characteristics. The Molt-4, CCRF-CEM, HL-60, and Wilson cell lines had doubling times in 10% FBS and RPMI 1640 of 24, 19, 30, and 19 h, respectively. Growth curves in the presence of varying concentrations of MP for the Molt-4, CCRF-CEM, HL-60, and Wilson lines revealed IC₅₀'s of 6.5, 2.4, 4.0, and 1.2 µM, respectively. These results indicate that all 4 cell lines were sensitive to MP. The CCRF-CEM and the Wilson lines had doubling times of 32 and 24 h, respectively, and were able to grow in 2 mM MP.

Biochemical Factors Influencing MP Cytotoxicity. HPRT, alkaline phosphatase, and PRPP activity were measured in all 6 cell lines. The results are shown in Table 1. HPRT, the enzyme that converts MP to its active metabolite, TIMP, was present to a similar extent in the 4 wild-type cell lines. In addition, these cell
lines were found to have similar levels of PRPP and alkaline phosphatase activity. In the CCRF-CEM and Wilson cell lines, however, HPRT activity was markedly reduced. There was no increase in alkaline phosphatase activity or decrease in PRPP levels in the 2 resistant cell lines.

**MP Pharmacokinetics in Human Neoplastic Cell Lines and Patients' Cells.** Following the 3-h incubation in 10 μM [14C]MP, 7 radiolabeled compounds, which accounted for 85–90% of radioactivity injected onto the HPLC system, were identified in the acid-soluble extracts of the cell lines. These were mercaptopurine, thioxanthine, and thiouric acid (these latter 2 metabolites could not be separated under the present assay conditions), TIMP, TXMP, TGMP, ATP, and GTP. A sample HPLC radiochromatogram demonstrating these compounds in a cell extract is shown in Chart 2. TIMP, TXMP, and TGMP, the active nucleotide metabolites of MP, accounted for 80–85% of the intracellular radioactivity during the entire time course of the experiment. The formation and retention of these 3 active metabolites in each of the cell lines studied is shown in Chart 3. TIMP was the predominant compound observed, accounting for 59–85% of the active MP nucleotide metabolites measured within cells during the period of drug incubation. However, within 1 h of being placed in drug-free media, TIMP accounted for 30–62% of the active MP nucleotides found within the cell lines. After 3 h in drug-free media, TIMP comprised only 34–47% of the MP nucleotide metabolite pool. During the period in drug-free media TIMP accounted for less and TXMP and TGMP accounted for more of the intracellular metabolite pool. TXMP was the MP nucleotide metabolite found in second highest amount, with peak levels ranging from 0.30–0.65 nmol/10^7 cells. TGMP was the MP nucleotide metabolite found in the lowest amount. TIMP, TXMP, and TGMP were formed rapidly, all being detectable at the earliest time point measured (30 min). During the period in which the cells were in drug-free media, TXMP and TGMP accounted for an increasing fraction of the MP nucleotide metabolite pool, as more TIMP underwent conversion to these 2 compounds.

A small amount of the intracellular radioactivity, 5–10%, was detected under the ATP and GTP peaks. Approximately 80–90% of this fraction coeluted with ATP and 10–20% with GTP. Additional confirmation of the identity of these 2 compounds was obtained by extracting the cell aliquots using a mercarial cellulose resin (13) that binds only thiolated compounds. When this was done, the peaks of radioactivity that eluted with authentic ATP and GTP disappeared. In addition, when the radiolabeled ATP and GTP were collected and enzymatically digested to the nucleoside level and chromatographed, all the radioactivity migrated with the authentic standards for adenosine and guanosine, not with MPR or TGR. This suggested that MP or TIMP, in human leukemia and lymphoma cell lines, undergoes desulfuration to hypoxanthine or inosine monophosphate, which is subsequently metabolized to ATP and GTP.

Mercaptopurine, thioxanthine, and thiouric acid comprised the remaining fractions of intracellular radioactivity. These 3 compounds accounted for 3–12% of the intracellular MP equivalents, with one-half being due to MP and one-half to TU and TX.

The nucleoside di- and triphosphate metabolites of MP, TX, and TG were not detected in any of the cell lines studied. Methyl-TIMP eluted too close to TIMP to be accurately quantitated by anion-exchange HPLC. However, when the TIMP formed was collected and enzymatically digested to the nucleoside level, greater than 95% of the radioactivity migrated with reference MPR and less than 5% with reference MMPR.

In contrast to the above results, there was very little TIMP, TGMP, or TXMP detected in the 2 resistant cell lines (Chart 3). The total amount of the MP nucleotide metabolite pool (TIMP, TGMP, and TXMP) was less than 0.01 nmol/10^7 cells. MP, TU, and TX accounted for a larger fraction of the intracellular radioactivity (25–35%) than was noted in the wild-type cell lines, with the remainder of the radioactivity due to small amounts of TIMP, TGMP, and TXMP.

The pharmacokinetics of MP was also studied in vitro in cells obtained from 10 patients with acute lymphoblastic leukemia. The time course of the formation and retention of the active MP metabolites, TIMP, TXMP, and TGMP are shown in Chart 4.

![Chart 2](chart2.png)  
Chart 2. Molt-4 cell line was incubated with 10 μM [14C]MP for 3 h and 1 x 10^7 cells were then extracted, neutralized, and 100 μl were injected onto the HPLC column. One-min fractions were collected and assayed for [14C]. Peaks, retention times of reference MP metabolites. Tu/Tx, TU/TX.

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HPRT (nmol/min/mg protein)</th>
<th>Alkaline phosphatase (nmol/min/mg protein)</th>
<th>PRPP (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molt-4</td>
<td>3.3 ± 0.2*</td>
<td>2.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>6.2 ± 2.0</td>
<td>2.7 ± 1.0</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>HL-60</td>
<td>2.9 ± 0.5</td>
<td>3.4 ± 0.8</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Wilson</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 1.0</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>0.06 ± 0.01</td>
<td>1.8 ± 0.7</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>Wilson</td>
<td>0.03 ± 0.02</td>
<td>3.2 ± 0.7</td>
<td>2.7 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± SD.

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**Table 2**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PRPP (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molt-4</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>HL-60</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Wilson</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

Mean ± SD.
Chart 3. Time course of formation and retention of TMP, TXMP, and TGMP in 3 human acute leukemia cell lines (CCRF-CEM, Molt-4, and HL-60) and one human Burkitt's lymphoma cell line (Wilson), all sensitive to MP and 2 MP-resistant cell lines, CCRF-CEM$^R$ and Wilson$^R$. Cells were incubated in 10$\mu$L MP for 3 h and then washed and placed in drug-free media for an additional 3 h. Samples were obtained at 0.5, 1, and 3 h, and then at 1 and 3 h after being placed in drug-free media and analyzed as described in "Materials and Methods."
MP cellular pharmacokinetics

Chart 4. Time course of formation and retention of TIMP, TXMP, and TGMP in cells obtained from 10 patients with acute lymphoblastic leukemia. Cells were incubated in 10 μM MP for 3 h, and then washed and placed in drug-free media for an additional 3 h. Samples were obtained at 0.5, 1, and 3 h, and then at 1 and 3 h after being placed in drug-free media. Columns, mean; bars, SD.

Overall pattern of MP metabolism was similar to that seen in the sensitive cell lines; however, the amounts of TIMP, TXMP, and TGMP were approximately 10-fold less. There was no apparent difference in the pattern of nucleotide metabolite accumulation and retention in cells obtained from newly diagnosed patients compared with those obtained at relapse; the overall number of patients, however, was small. As in the cell lines, the predominant MP nucleotide seen was TIMP, which comprised 71% of the MP nucleotide pool. TXMP accounted for the remaining 29% of this fraction. There was very little TGMP identified in any of the cell samples analyzed. Once placed in drug-free media, TIMP declined rapidly with only 10% remaining after 1 h. At this time point, TXMP was the predominant MP nucleotide metabolite accounting for two-thirds of this intracellular pool. As in the cell lines, the remainder of the intracellular radioactivity was due to MP, TU and TX, ATP and GTP. Neither methylated MP nucleotides nor nucleoside di- or triphosphate metabolites of MP were identified in any of the cell samples studied.

Discussion

Despite its long history of clinical use, little is known about the intracellular time course of MP accumulation, metabolism, and retention. A more complete understanding of this aspect of the pharmacology of the drug is relevant because it is the cellular metabolites of MP, that is, TIMP, TGMP, and TXMP, along with their higher phosphate derivatives, that are responsible for the antineoplastic effect of the drug. With this intended goal, the cellular pharmacokinetics of MP in cell lines derived from patients with acute lymphoblastic leukemia and Burkitt’s lymphoma were examined. The results indicate that MP accumulates in cells primarily in the form of 3 nucleotide metabolites, the monophosphate derivatives of theophylline, theophanolosine, and thioguanosine. The present study demonstrates that in human neoplastic cell lines, MP nucleotide metabolites are formed rapidly and are easily detectable at the earliest time point (30 min) measured. This contrasts with previously published data in a murine lymphoma cell line which suggested that incubations of 8-24 h were required before substantial amounts of MP nucleotides could be seen (23). Although TIMP was the predominant nucleotide metabolite identified, TXMP and, to a lesser extent, TGMP, were also readily identified. Of interest, in a recent report, TIMP was identified as a major metabolite of MP when whole blood was incubated in vitro with 10 μM MP. However, when erythrocytes obtained from patients on p.o. MP therapy were assayed, TGMP was the major metabolite identified (24). Another metabolic pathway identified in the present study was that of apparent desulfuration of MP or TIMP, with the subsequent detection of 14C under the ATP and GTP peaks. This observation is consistent with previous reports of the ability of neoplastic cells in vitro to desulfurate MP and TIMP (9, 23, 25, 26). Although quantitatively minor, it does demonstrate another means by which MP can be detoxified, in addition to the better known catabolic route mediated by xanthine oxidase. It is possible that methyl-TIMP and a larger amount of TGMP, as well as their nucleoside di- and triphosphate metabolites, would have been detected in these studies if longer drug incubations (up to 24 h) had been conducted. Short drug incubations were chosen for these experiments because they more closely resemble the clinical situation, where an exposure of 2-4 h is achieved following p.o. MP administration to patients with acute lymphoblastic leukemia receiving maintenance chemotherapy (5). It was apparent that HPRT and PRPP were present in all 4 cell lines, and all these lines demonstrated the ability to form the 3 major MP nucleotide metabolites.

The importance of the ability of the neoplastic cell to form TIMP, TGMP, and TXMP is demonstrated by the nearly complete inability of the 2 resistant cell lines to form these nucleotide metabolites. In the cell lines sensitive to MP, however, there was no correlation between the IC50 and the amount of TGMP formed during a 3-h incubation in MP. These results show that, in general terms, there is a relationship between sensitivity to mercaptopurine and the extent to which a cell can form the nucleotide metabolites of MP. The formation of these metabolites probably does not represent the final event in the mechanism of MP cytotoxicity, as prior studies have demonstrated the importance of ultimate MP conversion to TG nucleotides, and their subsequent incorporation into DNA and RNA (10). However, the formation of TIMP, TXMP, and TGMP does appear to be a necessary first step in MP activation. Although MP resistance in the above cell lines, as well as in most cell lines selected for in vitro MP resistance, was the result of decreased HPRT activity, caution should be used in extrapolating this finding to the clinical
situation. In other studies, only a minority of leukemic patients judged to be clinically resistant to MP had low HPRT activity (27, 28). This suggests that other factors may be involved in the development of clinical thiopurine resistance.

Pharmacokinetic studies in leukemic cells obtained directly from patients and incubated in vitro with [14C]MP revealed a similar pattern of MP nucleotide formation, as well as a rapid decline in the amounts of these compounds once the cells were placed in drug-free media. However, the amounts of TIMP, TXMP, and TGMP measured were only 10% of those measured in human leukemia cell lines. Although a paucity of patients' cells precluded measurement of HPRT or PRPP, this difference may be due to the much lower amounts of PRPP reported to be present in human leukemic cells compared to leukemia cell lines (29). In support of this, the PRPP levels measured in the MP-sensitive cell lines in this report were substantially higher than those measured in leukemic cells obtained directly from patients, as previously reported (30). Further studies to explore the biochemical differences within patients are in progress.

In order to confirm and extend these in vitro observations, the cellular pharmacokinetics of MP is currently being characterized in vivo in neoplastic cells obtained from patients on i.v. MP therapy. Detailed information regarding the intracellular pharmacokinetics of mercaptopurine provides a greater understanding of the relationship between drug administration schedule and drug action at the cellular level, which hopefully will lead to more rational use of this drug.

REFERENCES

Cellular Pharmacokinetics of Mercaptopurine in Human Neoplastic Cells and Cell Lines


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