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Clinical and experimental pharmacokinetic interaction between 6-mercaptopurine and methotrexate

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Abstract Clinical and experimental pharmacokinetic interaction between 6-mercaptopurine (6-MP) and methotrexate (MTX) was investigated in patients as well as in rats and in HL-60 human leukemic cells. Ten children affected by acute lymphoblastic leukemia (ALL) in remission received daily doses of 6-MP given at 25 mg/m^2 and i.v. infusion of high-dose MTX at 2 or 5 g/m^2 once every other week. When 6-MP was given alone, the mean peak plasma concentration (C_{max}) and area under the curve (AUC) of 6-MP were 72.5 ng/ml and 225.3 h ng ml^{-1}. Concurrent treatment with MTX at 2 or 5 g/m^2 resulted in a mean increase of 108% and 121% in the C_{max} and of 69% and 93% in the AUC, respectively. In rats treated with an oral dose of 6-MP at 75 mg/m^2, MTX given i.p. at 5 g/m^2 produced mean increases of 110% and 230% in the C_{max} and AUC of 6-MP, respectively. In HL-60 human leukemic cells incubated with 6-MP at 250 ng/ml, the cumulative intracellular concentration of 6-thioguanine and 6-MP nucleotides was not significantly modified by treatment with 20 μg/ml of MTX. The present findings indicate that high-dose MTX enhances the bioavailability of 6-MP as evidenced by the observed increases in the plasma C_{max} and AUC of 6-MP in humans and animals.

Key words 6-Mercaptopurine · Pharmacokinetics · Methotrexate · Lymphoblastic leukemia · Rat

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Introduction

The purine antimetabolite 6-mercaptopurine (6-MP) and its related compound 6-thioguanine (6-TG) are prodrugs that undergo extensive intracellular metabolism resulting in the formation of several thionucleotides [15]. Among them, 6-thiodeoxyguanosine triphosphate appears to be the most active cytotoxic metabolite [15], which is eventually incorporated into the DNA [33]. Among the metabolites of 6-MP and 6-TG, the role of the methylated 6-TG and 6-MP nucleotides produced by the enzyme thiopurine methyltransferase in the overall cytotoxic effect of 6-MP is presently under investigation [30].

Since the early 1960s, administration of daily oral doses of 6-MP for a period of 2–3 years in combination with weekly methotrexate (MTX) treatment has been the backbone of maintenance regimens for childhood acute lymphoblastic leukemia (ALL) [5, 13]. Presently, 6-MP is considered one of the standard agents for the treatment of children with ALL in remission [25, 28]; however, despite a success rate of about 90% in inducing remission, 30%–40% of such patients have relapses afterward [25]. In these protocols, 6-MP is given in standardized doses that are titrated on the basis of routine peripheral white blood cell counts, an approach that frequently leads to misinterpretation and suboptimal treatment.

The availability of high-performance liquid-chromatography (HPLC) methods for the determination of plasma 6-MP concentrations has revealed low and highly variable drug levels following oral administration [12, 26, 31, 36]. Zimm et al. [36] found that an average of 16% of the dose was bioavailable and that the interindividual variability ranged from 5% to 37% after an oral dose of 75 mg/m^2, suggesting that subjects receiving similar amounts of 6-MP per unit of body size may be exposed systemically to different amounts of the drug. The low and variable bioavailability of 6-MP has been suggested as one of the possible causes of

relapse in children with leukemia receiving maintenance chemotherapy [9, 12, 26, 31, 36]. The importance of optimizing 6-MP therapy and achieving high and predictable systemic drug exposure, as defined by the area under the plasma concentration-time curve (AUC), has encouraged the use of i.v. 6-MP in patients with ALL [21].

The limited bioavailability is the result of variable absorption and presystemic metabolism of the drug by the intestinal and hepatic xanthine oxidases, which convert 6-MP to the inactive metabolite 6-thiouric acid. Pretreatment with the xanthine oxidase inhibitor allopurinol markedly increases the bioavailability of oral 6-MP [35]. Several preclinical and clinical studies have shown synergism between the antineoplastic activities of MTX and 6-MP [1, 8]; for this reason, MTX is used in combination with 6-MP during the maintenance phase of therapy for ALL [19].

To examine further the complex pharmacology of 6-MP, we investigated the pharmacokinetic interaction between 6-MP and MTX in humans and animals and in the HL-60 leukemic cell line in vitro.

Subjects and methods

Drugs and chemicals

Drugs for human use were MTX (Methotrexate; Cyanamid, Catania, Italy) and 6-MP (Purinethol; Wellcome, London, UK). Chemicals for experimental use were 6-MP, MTX, methanol, 1-heptanesulfonic acid, DL-dithiothreitol (DTT), ethyl acetate, diethyl ether, glacial acetic acid, phenylmercuric acetate (PMA) and toluene (Aldrich, Milano, Italy), RPMI-1640, penicillin, streptomycin, L-glutamine, and fetal calf serum (GIBCO-BRL, Paisley, UK). Chemicals not listed in this section were of analytical grade.

Pharmacokinetic study of 6-MP in patients

Ten children (six boys and four girls) aged between 4 and 16 years (median, 9 years) and affected by ALL were enrolled into the study. Eight of them were classified as being at low risk and two, as being at average risk on the basis of blast count, immunophenotype of leukemic cells, and hepatic and splenic dimensions. Children were treated with consolidation chemotherapy according to Italian Association of Pediatric Hematology and Oncology (AIEOP) treatment protocols 8801-02 and 9101. The duration of treatment was 8 weeks and the therapy consisted of daily oral doses of 6-MP (25 mg/m^2) and a 24-h i.v. infusion of high-dose MTX given at 5 g/m^2 (8801-02 protocol, seven patients) and at 2 g/m^2 (9101 protocol, three patients) on days 7, 21, 35, and 49; L-folinic acid was given i.v. after MTX. Intrathecal MTX, cytosine arabinoside, and prednisone were also given. The patients had normal renal and hepatic function tests and no clinical symptoms of malabsorption. The study was authorized by the local ethics committee and informed consent was obtained from the parents of each child.

Patients were admitted to the Pediatric Clinic of the University of Pisa, Italy. Each subject was studied on 2 consecutive days, first after an oral dose of 6-MP (25 mg/m^2) given at 6:00 p.m. and, on the next day, concurrently with a 24-h i.v. infusion of high-dose MTX (2 or 5 g/m^2) starting at 9:00 a.m. Patients were fasted for 1.5 h before and after receiving oral 6-MP; they were not allowed to take other medication and/or methylxantine-containing foods for at least 24 h before the study and until blood sampling was completed. Blood (1.5 ml) for the analysis of 6-MP plasma levels was obtained from the antecubital vein before and at 30 min as well as 1, 2, and 4 h after the dose of 6-MP and was then heparinized. Plasma was immediately separated by centrifugation at 400 *g* and stored at − 20°C until 6-MP analysis as described below.

Pharmacokinetic study of 6-MP in rats

Plasma levels of 6-MP were examined in Wistar female rats (Nossan, Milano, Italy) with a mean body weight of 250 g. Their care and handling were in accordance with the provisions of European Economic Community (EEC) Council Directive 86–609 as recognized and adopted by the Italian Government. The rats were randomly divided in 2 groups of 20 animals; the first group was treated with 6-MP at 75 mg/m^2 by gastric gavage, and the second group received 6-MP at the same dose plus i.p. MTX at 5 g/m^2. At various times after drug administration (15 and 30 min and, 1, 2, and 4 h), four animals from each group were anesthetized by diethyl ether and blood samples (1.5 ml) were drawn by cardiac puncture and collected in heparinized tubes. Plasma was immediately separated by centrifugation and stored at − 20°C until analysis; 6-MP was extracted and assayed as described below.

Biotransformation of 6-MP by HL-60 leukemic cells

The HL-60 human promyelocytic leukemia cell line, obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA), was maintained at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium, supplemented with 20% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 µg/ml), and L-glutamine (2 m*M*). Cells were seeded at 2×10^6 cells/well in flat-bottomed 24-well plates (Costar, Cambridge, Mass., USA) with 1.5 ml of culture medium. Cells were treated with 6-MP (250 ng/ml) alone or in combination with MTX (20 µg/ml). After 24 h of incubation, cells were collected by centrifugation; the cell pellets were resuspended in 1 ml of phosphate-buffered saline (pH 7.4) and intracellular concentrations of 6-MP and 6-TG nucleotides were measured as reported below.

Sample processing and analysis

6-MP was extracted from human and rat plasma as previously reported [22] with minor modifications. Briefly, to 250 µl of plasma we added 500 µl of toluene, 25 µl of 0.4 *N* NaOH, and 500 µl of ethyl acetate containing PMA at 0.3 mg/ml; samples were mixed for 15 s and centrifuged at 12,000 *g* for 2 min. The organic layer (800 µl) was transferred to a second tube, and 200 µl of 0.1 *N* HCl was added. The tubes were vortexed for 15 s and centrifuged at 12,000 *g* for 1 min. Finally, 180 µl of the acid phase was available for the HPLC analysis. The 6-MP concentration in human and rat plasma was measured by means of a reverse-phase HPLC method [34] using a Waters LC Module I liquid chromatograph (Waters, Milford, Mass., USA) equipped with a Model 116 variable-wavelength UV detector. The analytical column was a Dynamax 300-Å, 5-µm C_{18}, 250 × 4.6-mm column coupled with a Dynamax 5-µm C_{18} precolumn (Rainin, Woburn, Mass., USA). UV absorbance detection was accomplished at 322 nm, with the limit of detection being 5 ng/ml of 6-MP. The mobile phase consisted of methanol/water at 3:97 (v/v) containing 2 ml of glacial acetic acid/l, 1 n*M* DTT, and 1 mg of 1-heptanesulfonic acid/ml. Acid extracts (70 µl) were injected into the system and chromatographed at a mobile phase flow of 1 ml/min. Peak heights were measured and 6-MP concentrations were quantitated by comparing the peak responses

of the samples with those of the standards. Cellular concentrations of the nucleotides of 6-MP and 6-TG in HL-60 leukemic cells were measured exactly as previously reported [16] with the HPLC apparatus described above.

Pharmacokinetic and statistical analysis

Results are expressed as mean values ± SE of n observations. The peak plasma concentrations (C_{max}) and the time required to reach them (T_{max}) were obtained by visual inspection of the concentration-time curves. The AUC of 6-MP for the interval 0–4 h ($AUC_{0\to4}$) was calculated by the trapezoidal rule. Student's paired t-test was used to determine the statistical significance of differences in the C_{max} and AUC values found for 6-MP after its administration alone or in combination with MTX. Significance was assessed at the 5% level ($P < 0.05$).

Results

Pharmacokinetics study of 6-MP in patients

Following the administration of 6-MP at 25 mg/m^2 to ten patients, a mean plasma C_{max} value of 72.5 ng/ml was obtained 30 min after dosing (Fig. 1); the mean $AUC_{0\to4}$ value was 225.3 h ng ml^{-1}. The combined treatment with MTX resulted in a significant modification of the pharmacokinetic parameters with respect to those recorded for 6-MP alone. As shown in Table 1, the mean C_{max} values obtained for 6-MP after the administration of 2 or 5 g/m^2 MTX were 150.8 ($n = 3$) and 160.9 ng/ml ($n = 7$), respectively, corresponding to percentages of change of 108% and 121%, respectively ($P < 0.05$ as compared with 6-MP alone). Moreover, MTX administration increased the T_{max} value recorded for 6-MP to 1 h as compared with 30 min for 6-MP alone (Fig. 1). The mean $AUC_{0\to4}$ values obtained for 6-MP after the administration of MTX at 2 and at 5 g/m^2 were 380.6 ($n = 3$) and 435.1 h ng ml^{-1} ($n = 7$), respectively; these differences were statistically significant ($P < 0.05$) as compared with 6-MP administration alone (Table 1).

Table 1 Plasma pharmacokinetic parameters (mean values ± SE) of 6-MP given to patients alone or in combination with MTX at 2 ($n = 3$) and 5 g/m^2 ($n = 7$), respectively

Treatment	C_{max} (ng/ml)	T_{max} (min)	$AUC_{0\to4}$ (h ng ml^{-1})
6-MP	72.5 ± 11.9	30	225.3 ± 26.2
6-MP + MTX 2 g/m^2	150.8 ± 23.4*	60*	380.6 ± 48.2*
6-MP + MTX 5 g/m^2	160.9 ± 25.2*	60*	435.1 ± 55.6*

*$P < 0.05$ vs 6-MP alone

Pharmacokinetics study of 6-MP in rats

Figure 2 depicts the plasma concentration-time profile of 6-MP given at 75 mg/m^2 alone and after an i.p. dose of MTX at 5 g/m^2. The C_{max} value (158.1 ± 27.6 ng/ml) obtained following treatment with 6-MP alone was reached at 30 min after dosing, with the $AUC_{0\to4}$ value being 147.4 ± 24.3 h ng ml^{-1}. The administration of MTX determined a considerable and statistically significant increase in 6-MP C_{max} (328.4 ± 40.2 ng/ml; increase, 110%) and $AUC_{0\to4}$ (484.8 ± 63.4 h ng ml^{-1};

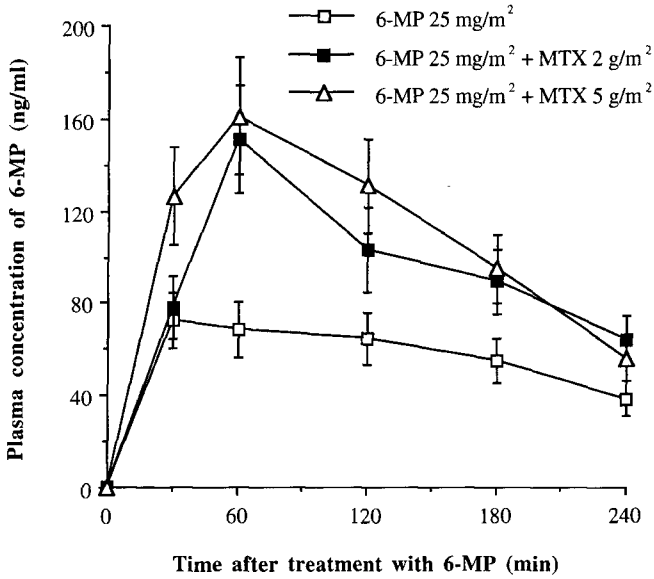

Fig. 1 Plasma concentration-time curves of 6-MP in patients ($n = 10$) after an oral dose of 25 mg/m^2 given alone or concurrently with MTX at 2 ($n = 3$) or 5 g/m^2 ($n = 7$). Results are expressed as mean values (*points*) ± SE (*vertical bars*)

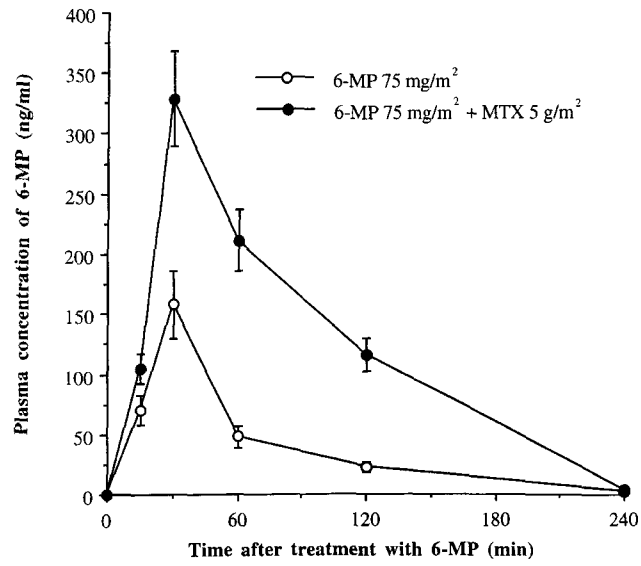

Fig. 2 Plasma concentration-time curves of 6-MP in rats ($n = 4$ per time point) after an oral dose of 75 mg/m^2 given alone or concurrently with i.v. MTX at 5 g/m^2. Results are expressed as mean values (*points*) ± SE (*vertical bars*)

increase, 230%), whereas the T_{max} value remained unmodified (30 min).

Biotransformation of 6-MP by HL-60 leukemic cells

The measurement of the cellular concentration of the active metabolites of 6-MP after treatment of HL-60 cells for 24 h with 6-MP at 250 ng/ml revealed that cells actively metabolized the drug to 6-TG (9.4 ± 1.2 ng/10^6 cells) and 6-MP nucleotides (9.2 ± 1.8 ng/10^6 cells). Combined treatment with MTX at 20 μg/ml produced a marked change in the nucleotide levels (6-TG, 3.8 ± 0.7 ng/10^6 cells; 6-MP, 16.3 ± 2.1 ng/10^6 cells), although their cumulative concentration did not change significantly.

Discussion

In recent years, much attention has been devoted to the combination of anticancer agents to increase the therapeutic effect of drugs in the treatment of children with ALL. MTX is frequently used in combination with 6-MP in the maintenance chemotherapy of standard-risk ALL, since data from studies in patients have revealed an increased efficacy of this drug combination [8]. Recently, high-dose MTX and 6-MP have been widely employed after the achievement of a complete remission so as to consolidate the latter and to prevent central nervous system involvement due to the ability of high-dose MTX to cross the blood-brain barrier [27, 32]. The present results obtained in patients confirm the usefulness of both drugs; high-dose MTX enhanced the bioavailability of 6-MP as evidenced by the statistically significant increase in both the C_{max} and AUC of 6-MP. The present increase in 6-MP plasma concentrations measured in patients can be explained by the assumption that MTX is an inhibitor of xanthine oxidase [19], the enzyme responsible for the catabolism of 6-MP to the inactive metabolite 6-thiouric acid [4]. Moreover, the increment obtained with coadministration of 2 g/m^2 MTX was slightly different from that obtained with 2.5-fold higher dose of MTX, indicating that a substantial increase in the bioavailability of 6-MP could be reached with a lower dose of MTX. Balis et al. [1] observed a mean 26% change in the C_{max} and 31% increase in the AUC of 6-MP after concurrent administration of MTX in patients receiving maintenance chemotherapy. It is noteworthy that the findings of the present work differ considerably from those described in the aforementioned report, since both the C_{max} and the AUC obtained after an oral dose of 6-MP at 25 mg/m^2, as presented in this study, are very similar to those obtained by Balis et al. [1] after p.o. dosing of 6-MP at 75 mg/m^2. Furthermore, the increase in the 6-MP C_{max} and AUC values reported in the present study after treatment with MTX at 2 and 5 g/m^2 is proportionally much lower than that obtained by Balis et al. [1] after the administration of MTX at 20 mg/m^2. These findings might indicate that the inhibition of xanthine oxidase is maximal at low doses of MTX.

The biochemical basis for this synergic drug combination was proposed by Bokkerink et al. [2] and Cadman et al. [6]. These investigators noted that MTX increased the intracellular concentrations of phosphoribosylpyrophosphate (PRPP) by virtue of its inhibitory effect on de novo purine synthesis. PRPP is a cofactor required together with 6-MP to form the 6-MP nucleotides, and intracellular PRPP levels could potentially regulate the formation of the cytotoxic metabolites [13]. A schedule-dependent synergy between MTX and 6-MP has been reported in malignant human T-lymphoblasts. The increased availability of PRPP after MTX pretreatment enhances the metabolite formation from 6-MP; this synergism may occur in malignant lymphoblasts, which produce active de novo purine synthesis [2].

Our analysis of the plasma pharmacokinetics of 6-MP revealed a wide interpatient variation; similar findings were observed after both i.v. and oral administration of 6-MP in leukemic children [35] or of azathioprine, a 6-MP derivative, in renal transplant patients [20]. In the present study the degree of intersubject variability of 6-MP plasma concentrations was not altered by MTX coadministration, as also observed by Balis et al. [1]. A low 6-MP AUC value is considered one possible cause of the relapses of ALL [12, 25], and interindividual kinetic variability may also contribute to the therapeutic failure or myelotoxicity of 6-MP. The importance of optimizing 6-MP therapy and achieving high and predictable systemic drug exposure, as defined by the AUC, has encouraged the use of i.v. 6-MP in patients with ALL [21]. Furthermore, it has been shown that improved absorption of 6-MP can be achieved by oral administration of 6-MP riboside, which is more soluble than 6-MP [24].

Although the antileukemic and cytotoxic effects of 6-MP have been related to the generation of intracellular nucleotides derived from 6-MP rather than to plasma 6-MP concentrations [14, 17, 18], several reports have suggested that a pharmacokinetic assessment of oral 6-MP in children with acute leukemia may be useful for a better characterization of the correlation between dose and therapeutic outcome [9, 10, 31]. It is noteworthy that the most appropriate end point for monitoring 6-MP therapy remains under evaluation. In addition, new fields of investigation are offered by the role of the cellular production of methylated 6-TG and 6-MP nucleotides by the enzyme thiopurine methyltransferase in the overall cytotoxic effect of 6-MP [30] as well as by the development of therapeutic modalities to overcome the resistance to 6-MP that is dependent on the lack of the cellular enzyme hypoxanthine-guanine phosphoribosyltransferase [23].

The present pharmacokinetic parameters of 6-MP in rats revealed that 6-MP given alone was rapidly absorbed into the systemic circulation and soon disappeared from plasma, as has also been observed in mice [29] and rhesus monkeys [7]. Due to this rapid decline, 6-MP plasma levels decreased to the limit of detection 4 h after dosing: identical kinetic behavior can be observed in mice after oral administration of 50 mg/kg azathioprine [11]. Moreover, high concentrations of 6-thiouric acid, the end point of metabolic azathioprine and 6-MP degradation, were detected in the intestinal mucosa and in the liver of mice, providing evidence for a first-pass metabolism in these organs; a similar metabolic pathway has been observed in humans [11].

In the present study the administration of MTX at the same dose (5 g/m^2) to children and animals determined a different modification in the pharmacokinetics of 6-MP; the increase in the 6-MP AUC observed in patients was lower than that seen in rats. These results are probably due to a higher extent of 6-MP metabolism by hepatic and intestinal xanthine oxidases in rats.

A synergistic effect of the 6-MP/MTX combination was also demonstrated in vitro by Bokkerink et al. [3], who studied the cell proliferation and the biochemical pharmacology of simultaneous and sequential combination treatment with MTX and 6-MP in MOLT-4 malignant T-lymphoblasts. Our in vitro experiments in HL-60 human leukemic cells showed that MTX markedly affected the ratio of the 6-MP and 6-TG nucleotides inside the cells, although their cumulative concentration did not change significantly. This effect might be explained on the basis of the effect of MTX on the enzymes of the nucleotide interconversion pathway [13].

In conclusion, the present clinical and experimental studies concerning the pharmacokinetic interaction between 6-MP and MTX demonstrate that high-dose MTX administration produces a significant increase in the bioavailability of 6-MP and that MTX does not affect the cumulative amount of metabolites of 6-MP occurring in leukemic cells in vitro. These findings provide further support for the usefulness of this combined treatment in consolidation and maintenance therapy of ALL.

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