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ORIGINAL ARTICLE

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Metabolism of 6-mercaptopurine in the erythrocytes, liver, and kidney of rats during multiple-dose regimens

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Abstract *Purpose*: To describe the metabolism of 6-mercaptopurine (6-MP) in erythrocytes and tissues of rats after repeated administration of 6-MP at two dose levels and to provide evidence that in vivo modulation of 6-MP anabolism can be obtained by simultaneous treatment with ribavirin or hydroxyurea, two inhibitors of enzymes involved in the bioactivation of 6-MP to the active 6-thioguanine nucleotides (6-TGN). *Methods*: Rats were treated i.p. with 6-MP at 12.5 and 25 mg/kg daily for 12 days and erythrocyte, liver, and kidney levels of 6-mercaptopurine nucleotides (6-MPN) and 6-TGN were investigated during the accumulation phase and for 50 days after the end of treatment. In combination studies, ribavirin at 75 and 100 mg/kg per day (for 6-MP, 25 and 12.5 mg/kg per day) or hydroxyurea at 200 mg/kg per day were given i.p. for 12 days. The measurements of thionucleotide levels in rat samples were performed by high-pressure liquid chromatography (HPLC). *Results*: The maximal concentration (C_{max}) and the area under the concentration versus time curve (AUC) of 6-MPN and 6-TGN in erythrocytes and tissues increased significantly after the administration of 6-MP at 25 mg/kg per day as compared with 12.5 mg/kg per day. In particular, the C_{max} and AUC of 6-TGN in erythrocytes of rats treated with 6-MP at 25 mg/kg per day were approximately 5-fold higher than the 6-TGN values observed following treatment at 12.5 mg/kg per day. Moreover, 6-TGN levels in erythrocytes were significantly higher than those of 6-MPN (910.9 ± 53.1 and 286.8 ± 23.4 pmol/8×10^8 cells for 6-TGN and 6-MPN, respectively, $P < 0.05$) after treatment with 6-MP at 25 mg/kg per day. The administration of ribavirin, an inhibitor of inosine monophosphate dehydrogenase, in association with 6-MP increased the amount of 6-MPN detected in erythrocytes and tissues while reducing 6-TGN levels in samples. The production and accumulation of 6-MPN and 6-TGN were increased in erythrocytes and tissues by hydroxyurea, an inhibitor of ribonucleotide reductase. Finally, a significant correlation between thionucleotide concentrations and erythrocyte counts was observed. *Conclusion*: The overall results demonstrate that 6-MP is actively metabolized in rats and that its biotransformation can be modulated by agents acting on enzymes of the purine metabolism, resulting in significant changes in erythrocyte and tissue levels of 6-MPN and 6-TGN. These findings provide evidence that the rat is a suitable model for investigation of the metabolism of 6-MP and its possible pharmacologic modulation.

Key words 6-MP · 6-TG · Rat tissues · Pharmacokinetics · Ribavirin · Hydroxyurea

Abbreviations *6-MP* 6-Mercaptopurine · *6-TG* 6-Thioguanine · *6-MPN* 6-Mercaptopurine nucleotide(s) · *6-TGN* 6-Thioguanine nucleotide(s) · *ALL* Acute lymphoblastic leukemia · *MTX* Methotrexate · *PBS* Phosphate-buffered saline · *DTT* Dithiothreitol · *HPLC* High-pressure liquid chromatography · C_{max} Maximal concentration · $t_{1/2}$ Terminal half-life · *AUC* Area under the concentration versus time curve · *PRPP* phosphoribosyl pyrophosphate · *IMPDH* Inosine monophosphate dehydrogenase

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Introduction

The purine analog 6-mercaptopurine (6-MP) is a chemotherapeutic antimetabolite that has been used in the treatment of childhood acute lymphoblastic leukemia (ALL) since the 1950s. Despite 40 years of clinical use, its metabolism and mechanism of action remain incompletely understood [17].

6-MP undergoes extensive intracellular metabolism resulting in the formation of several thionucleotides (Fig. 1). Among them, 6-thiodeoxyguanosine triphosphate appears to be the most active cytotoxic metabolite, and its incorporation into DNA is considered as the primary cytotoxic mechanism of 6-MP (Fig. 1) [2, 29]. Moreover, the role of methylated 6-TGN and 6-MPN in the overall antiproliferative effect of 6-MP is under investigation [28].

In the maintenance treatment of patients with ALL the concentrations of active intracellular metabolites of 6-MP in residual lymphoblasts within the bone marrow cannot be measured [12]. On the contrary, erythrocytes offer a valuable means of investigating the intracellular bioactivation of 6-MP, since they lack a nucleus and accumulate 6-MPN and 6-TGN, which reach steady-state levels after a few weeks that remain stable from day to day if the dose of 6-MP remains unchanged [3].

In leukemic children treated with 6-MP, increasing erythrocyte concentrations of 6-TGN result in enhanced cytotoxicity, which is a critical factor for long-term disease-free survival [18]. Relapse rate and myelosuppression in ALL patients show poor correlation with the 6-MP dose; on the contrary, the erythrocyte concentrations of 6-TGN appear to be better related than 6-MP plasma levels to the child's ability to form active cytotoxic metabolites [18, 24]. Indeed, modulation of 6-MP intracellular anabolism has been recognized as a critical factor for improvement of the cytotoxicity of the drug [1].

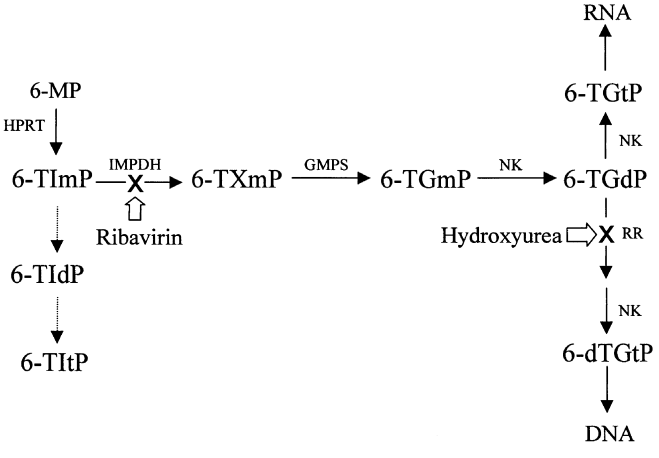

Fig. 1 Metabolic pathways of 6-mercaptopurine and sites of action of ribavirin and hydroxyurea. Thiopurine nucleotides: *6-MP* 6-mercaptopurine, *6-TImP* 6-thioinosine monophosphate, *6-TIdP* 6-thioinosine diphosphate, *6-TItP* 6-thioinosine triphosphate, *6-TXmP* 6-thioxanthosine monophosphate, *6-TGmP* 6-thioguanosine monophosphate, *6-TGdP* 6-thioguanosine diphosphate, *6-TGtP* 6-thioguanosine triphosphate, *6-dTGtP* 6-deoxythioguanosine triphosphate. Enzymes: *HPRT* hypoxanthine-guanine phosphoribosyl transferase, *IMPDH* inosine monophosphate dehydrogenase, *GMPS* guanine monophosphate synthetase, *NK* nucleotide kinase, RR ribonucleotide reductase

To date, the metabolism of 6-MP in erythrocytes and tissues of animals treated with 6-MP and its modulation by substances acting on 6-MP activation have not been investigated in detail. A report describes the effect of methotrexate (MTX) on 6-MP metabolism in rat erythrocytes [7], whereas the plasma concentration and tissue distribution of thiopurines have been studied in mice after a single dose of azathioprine, a prodrug of 6-MP [11].

The aim of the present work was to describe the metabolism of 6-MP in the erythrocytes and tissues of rats after repeated administration of 6-MP at two dose levels and to provide evidence that in vivo modulation of 6-MP anabolism can be obtained by simultaneous treatment with ribavirin or hydroxyurea, two inhibitors of enzymes involved in the bioactivation of 6-MP to the active 6-TGN triphosphates.

Materials and methods

Drugs and chemicals

6-MP, 6-TG, ribavirin, hydroxyurea, phosphate-buffered saline (PBS, pH 7.4), dithiothreitol (DTT), and phenyl mercury acetate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Methanol, toluene, amyl alcohol, and triethylamine were of HPLC grade as obtained from BDH (Poole, UK). Other chemicals not listed in this section were of analytical grade.

Thiopurine stock solutions for chromatography were obtained by dissolution of 1 mg of 6-MP and 6-TG in 1 ml of 0.1 *M* NaOH, and solutions were further diluted to a final concentration of 100 µg/ml with 0.1 *M* HCl. 6-MP for animal treatment was prepared extemporaneously each day by dissolution of the purine analog in 0.1 *M* NaOH and by its dilution with sterile 0.9% NaCl up to a final concentration of 2.5 or 5 mg/ml; the pH was adjusted to 8 with 0.1 *M* HCl. For combination studies, ribavirin and hydroxyurea solutions were made by dissolution of drugs in sterile 0.9% NaCl at 60 and 120 mg/ml, respectively.

Animals and treatments

Adult female Wistar rats with a mean body weight of 200 g (Nossan, Milano, Italy) were used. Animals were housed in cages (five rats/cage) for at least 1 week after their delivery to the laboratory and were maintained on a 12-h lighting cycle at an environmental temperature of 22–24 °C, with food and water being provided ad libitum. Care and handling of animals were undertaken in accordance with the provisions of European Economic Community Council Directive 86-609.

Rats were given 6-MP at two dose levels, i.e., 12.5 of 25 mg/kg per day, for 12 days by 1-ml i.p. injections. Additional animals were treated with 6-MP following the same schedule in combination with i.p. hydroxyurea at 200 mg/kg per day. Moreover, i.p. injections of ribavarin at 75 or 100 mg/kg per day for 12 days were given to rats treated with 6-MP at 25 or 12.5 mg/kg per day, respectively. A 25% lower ribavirin dose was given to animals treated with 6-MP at 25 mg/kg per day because of the occurrence of general toxicity as

shown in preliminary studies, whereas the addition of hydroxyurea at 200 mg/kg per day to the 6-MP treatment was well tolerated. Ribavirin and hydroxyurea were used on the basis of their potential biomodulating activity on thiopurine metabolism (Fig. 1). Indeed, ribavirin is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH) [21], whereas hydroxyurea exerts an effective suppression of ribonucleotide reductase [22, 33] in vitro and in vivo, and their doses were selected on the basis of previous reports [21, 22].

Three animals per time point were anesthetized with 1 g/kg urethane at 2, 4, 8, and 12 days after the beginning of treatment and at 4, 8, 12, 20, 28, 38, and 50 days after the end of 6-MP administration. In combination studies, five rats treated with 6-MP plus ribavirin or hydroxyurea were anesthetized and killed after 12 days of treatment. Samples of erythrocytes, liver, and kidney were collected as reported below for evaluation of the accumulation as well as the elimination of thiopurines.

Sample preparation

Blood samples were obtained from the carotid artery and collected in lithium heparin-coated tubes and the erythrocytes in each sample were counted by hemocytometry. Whole blood (0.5 ml) was transferred into 10-ml tubes, and erythrocytes were washed three times with 3 ml of PBS and then centrifuged to remove residual 6-MP contained in plasma. Finally, the pellets were resuspended with 0.5 ml of PBS and stored frozen at −20 °C until HPLC measurement of intracellular 6-MPN and 6-TGN.

The livers and kidneys were removed and an average of 500 mg of tissue was used for HPLC analysis; tissues were disrupted by a Potter homogenizer with a tight-fitting ground glass pestle in 0.5 ml of PBS and were stored frozen as described for erythrocytes. The thiopurine content of erythrocyte and tissue samples was analyzed within 2 weeks of their storage at −20 °C.

HPLC analysis of 6-MPN and 6-TGN

Thiopurines were extracted from erythrocytes and tissues and then analyzed by reversed-phase HPLC following the method described by Lennard and Singleton [14]. In brief, 0.3 ml of 20 m*M* DTT was added to thawed samples to protect the SH group from oxidation. Next, 0.5 ml of 1.5 *M* H_2SO_4 was added to samples, which were then shaken for 15 s and heated at 100 °C for 1 h in a Dri-Block (Techne, Cambridge, UK). This step hydrolyzes the thionucleotides of 6-MP and 6-TG and releases the free bases that can be extracted by phenyl mercury adduct formation. After the samples had cooled to room temperature, 5 *M* NaOH (0.5 ml) was added to each tube, immediately followed by 8 ml of toluene containing 170 m*M* amyl alcohol and 5.2 m*M* phenyl mercury acetate. Samples were shaken gently for 15 min and centrifuged at 1000 *g* for 10 min. Toluene (7 ml) was transferred into another tube, and 200 µl of 0.1 *M* HCl was added. After vortex mixing three times for 20 s each, samples were again centrifuged at 3000 rpm for 10 min, the toluene layer was discarded, and 50 µl of the acidic phase was injected into the analytical column.

The HPLC system consisted of a Waters LC Module I Plus (Waters, Milford, Mass.) equipped with a UV detector and a Waters Resolve Spherical C_{18} 5-µm column (3.9 × 300 mm; Waters, Milford, Mass.). The analytical column was protected by a Waters Resolve Spherical C_{18} precolumn (5-µm particle size). Thiopurines were eluted at 0.6 ml/min with a mobile phase consisting of methanol-H_2O (10:90, v/v) containing 100 m*M* triethylamine and 1 m*M* DTT and the eluate was adjusted to pH 3.2 with H_3PO_4. Working standards for 6-MP and 6-TG (5, 20, 40, 200, 1000, and 3000 pmol/8×10^8 erythrocytes or pmol/g of tissue) were prepared by the addition of thiopurine stock solutions to erythrocyte, liver, and kidney samples obtained from untreated rats. UV detection was accomplished at 342 nm, with the limit of sensitivity and reproducibility being 5 pmol/8×10^8 erythrocytes or pmol/g of tissue. Peak heights were measured and 6-MPN and 6-TGN concentrations were quantitated by comparison of the average peak response of the sample with that of the standards. During method reproduction and validation, quality-control samples were periodically used to monitor assay performance. Samples were not analyzed in duplicate unless misleading results were observed (e.g., interfering peaks). With regard to the assay precision, the average intraday and interday coefficients of variation were 0.8% (range 0.5–2%) and 6% (range 4–15%), respectively.

Pharmacokinetic and statistical analysis

The maximal concentration (C_{max}, in pmol/8×10^8 erythrocytes or pmol/g of tissue), terminal half-life ($t_{1/2}$, in days), and area under the concentration versus time curve ($AUC_{0\rightarrow62}$, in days × pmol/8×10^8 erythrocytes or days × pmol/g of tissue) of 6-MPN and 6-TGN were calculated in erythrocyte and tissue samples using the MW-Pharm software (MediWare, Gröningen, The Netherlands) following standard procedures [6]. The AUC from time 0 to the last sampling time point (62nd day) was computed for erythrocyte and tissue concentration data by addition of the AUC values of the accumulation phase with those of the elimination phase, both of which had been calculated using the trapezoidal method. Concentration-time curves were obtained by plotting of the average data points measured in separate experiments, and mean curves were than used to calculate the $t_{1/2}$ and AUC. Curves describing the elimination phase of thiopurines from erythrocytes, liver, and kidney were generated according to a biexponential equation using a weighting factor of unity.

All data are presented as mean values ± SE of *n* observations. For documentation of the possible correlation between the nucleotide level and treatment-related bone marrow damage in rats, linear regression analysis between thiopurine concentrations and erythrocyte counts (10^9 cells/ml of blood) measured at the corresponding time points was performed using the least-squares method (r = coefficient of correlation) [34]. Statistical comparison among the C_{max} of 6-MPN and 6-TGN in erythrocytes and tissues from rats treated with 6-MP alone or in combination with ribavirin and hydroxyurea was performed using Student's *t*-test for unpaired data. 6-MPN and 6-TGN C_{max} values in combination treatments were expressed as percentages of change as compared with 6-MP alone. For statistical analysis an a priori level of significance of $P < 0.05$ was set.

Results

The erythrocyte and tissue concentration-time data recorded at 6-MP doses of 12.5 and 25 mg/kg per day are reported in Figs. 2–4, and the resulting pharmacokinetic parameters, including the percentage of variation in the C_{max} of thiopurine nucleotides after the combination treatments with ribavirin or hydroxyurea, are shown in Tables 1 and 2.

After repeated injections of 6-MP at 12.5 mg/kg per day for 12 days the C_{max} values of 6-TGN and 6-MPN were 166.4 ± 23.9 and 130.3 ± 16.4 pmol/8×10^8 erythrocytes, respectively (n = 3, Fig. 2). The terminal half-lives of 6-TGN and 6-MPN in erythrocytes were 8.9 and 6.4 days, respectively, whereas the average $AUC_{0\rightarrow62}$ values were 2933 and 1993 days × pmol/8×10^8 erythrocytes, respectively (Table 1). The analysis of thiopurines in tissue samples after treatment with 6-MP at 12.5 mg/kg per day demonstrated that the highest tissue concentrations were reached by 6-MPN, with C_{max} values being 460.5 ± 12.1 pmol/g of tissue in the liver and 303.4 ± 16.3 pmol/g of tissue in the kidney

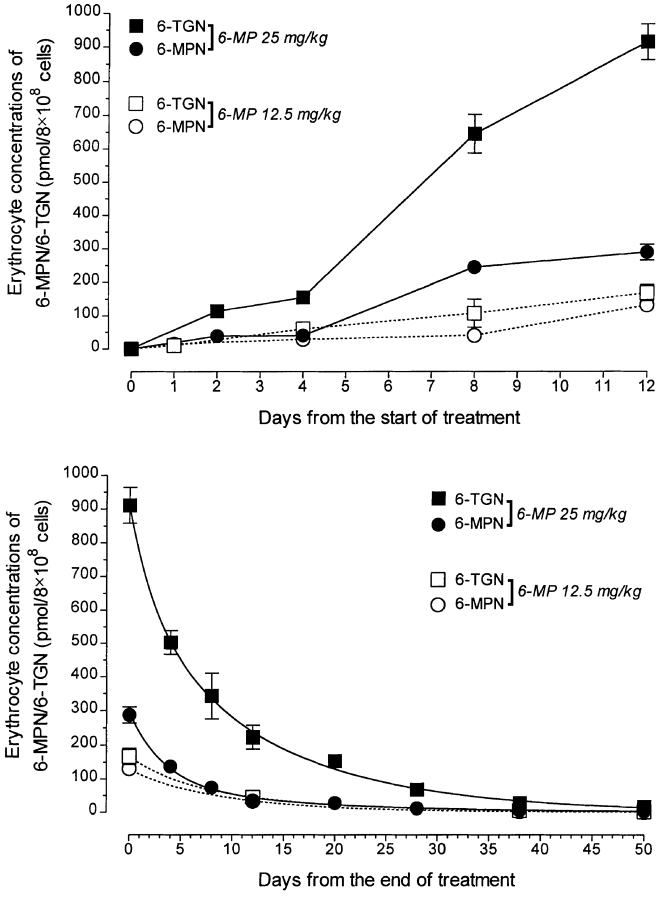

Fig. 2 Pharmacokinetic profiles of 6-MPN and 6-TGN in rat erythrocytes as obtained during a 12-day i.p. treatment with 6-MP at 12.5 and 25 mg/kg per day (*top*) and for 50 days following the end of treatment (*bottom*). Each point represents the mean value for three samples from different animals

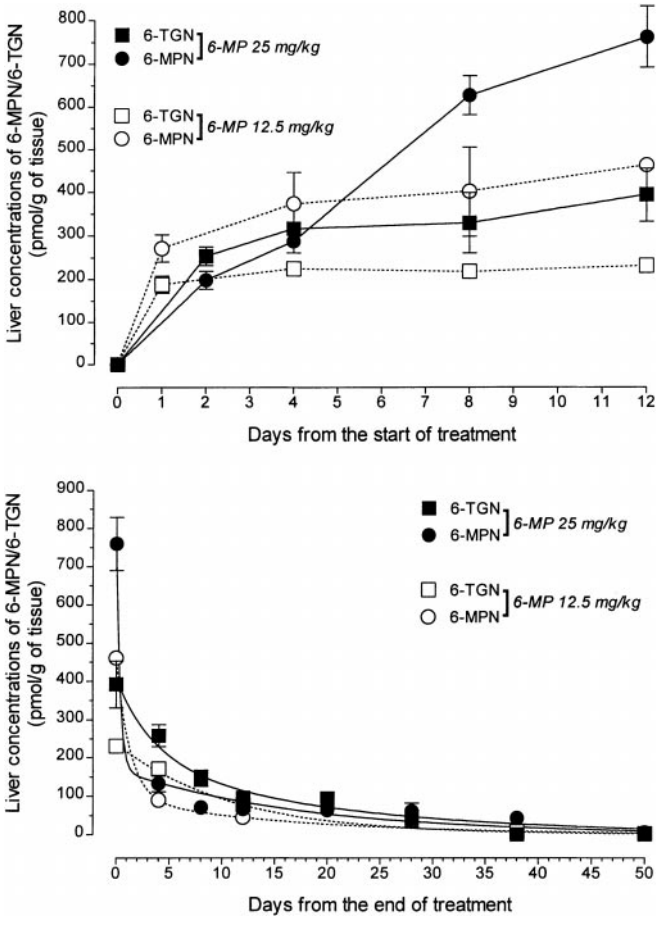

Fig. 3 Pharmacokinetic profiles of 6-MPN and 6-TGN in the liver of rats as obtained during a 12-day i.p. treatment with 6-MP at 12.5 and 25 mg/kg per day (*top*) and for 50 days following the end of treatment (*bottom*). Each point represents the mean value for three samples from different animals

Table 1 Pharmacokinetic parameters of 6-MPN and 6-TGN as determined in erythrocytes and tissues after i.p. administration of 6-MP at 12.5 mg/kg per day to rats and the percentage of variation in the thiopurine C_{max} after treatment with ribavirin at 100 mg/kg per day or hydroxyurea at 200 mg/kg per day

	Erythrocyte		Liver		Kidney	
	6-MPN	6-TGN	6-MPN	6-TGN	6-MPN	6-TGN
C_{max}[a]	130.3 ± 16.4	166.4 ± 23.9	460.5 ± 12.1[e]	230.4 ± 3.4	303.4 ± 16.3[e]	140.7 ± 4.9
+ Ribavirin[b]	+49.3 ± 5.2%[f]	− 69.1 ± 2.3%[f]	35.2 ± 4.9%[f]	−41.1 ± 5.2%[f]	+19.2 ± 2.4%	−51.4 ± 6.2%[f]
+ Hydroxyurea[b]	+52.3 ± 6.4%[f]	+52.1 ± 7.1%[f]	+68.7 ± 7.8%[f]	+10.8 ± 1.2%	+31.3 ± 3.7%[f]	+15.3 ± 1.8%
$t_{1/2}$[c]	6.4	8.9	11.2	7.4	6.8	9.4
$AUC_{0\rightarrow62}$[d]	1993	2933	6782	5492	3909	3359

[a] In pmol/8 × 10^8 erythrocytes or pmol/g or tissue
[b] Percentage of variation in the thiopurine C_{max} in samples from rats receiving additional treatment with ribavirin or hydroxyurea
[c] In days
[d] In days × pmol/8 × 10^8 erythrocytes or days × pmol/g of tissue
[e] $P < 0.05$ as compared with 6-TGN in the same tissue
[f] $P < 0.05$ as compared with the C_{max} of thiopurines in samples from rats treated with 6-MP alone

($n = 3$, $P < 0.05$ as compared with 6-TGN; Fig. 3, 4, Table 1). The $t_{1/2}$ values of thiopurines in tissues were 6.8 and 11.2 days for 6-MPN in the kidney and liver, respectively (Table 1). Following the administration of 6-MP at 12.5 mg/kg per day, maximal tissue exposure to thiopurines occurred in the liver, where average AUC values of 6782 and 5492 days × pmol/g of tissue were observed for 6-MPN and 6-TGN, respectively (Table 1).

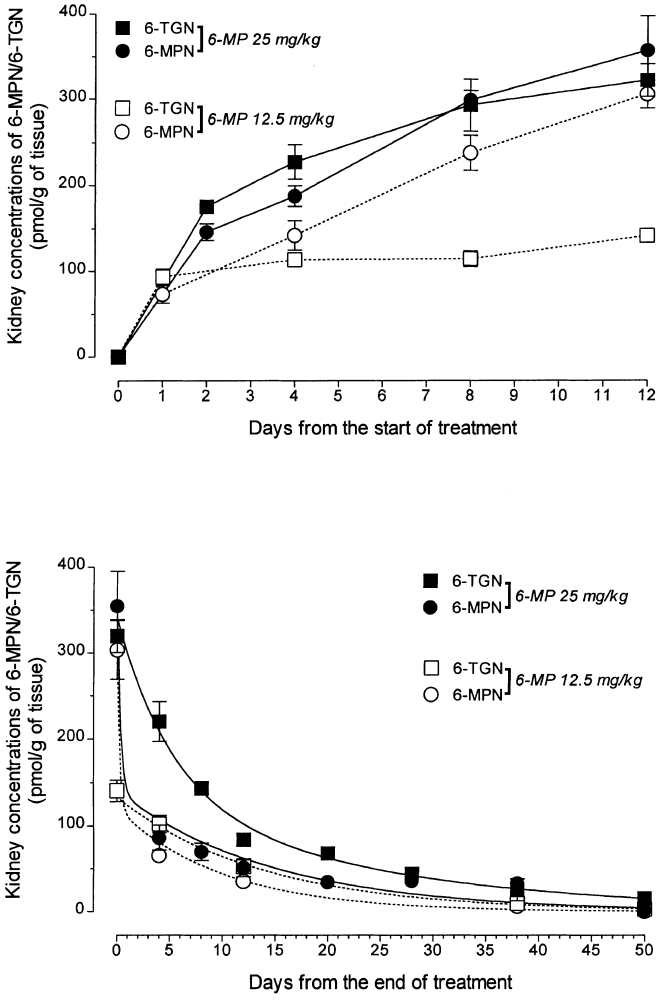

Fig. 4 Pharmacokinetic profiles of 6-MPN and 6-TGN in the kidney of rats as obtained during a 12-day i.p. treatment with 6-MP at 12.5 and 25 mg/kg per day (*top*) and for 50 days following the end of treatment (*bottom*). Each point represents the mean value for three samples from different animals

The combination of 6-MP at 12.5 mg/kg per day with ribavirin at 100 mg/kg per day significantly decreased C_{max} levels of 6-TGN in erythrocytes and tissues with respect to 6-MP alone. On the contrary, the C_{max} of 6-MPN significantly increased in erythrocyte and liver samples (Table 1). When animals were treated with 6-MP at 12.5 mg/kg per day plus hydroxyurea at 200 mg/kg per day the C_{max} of thiopurine nucleotides significantly increased in the liver (by 68.7 ±7.8%) and erythrocytes (by 52.3 ± 6.4%) with respect to the treatment with 6-MP alone (Table 1); however, the percentage of variation in 6-TGN observed in the liver and kidney did not reach the level of significance following combination treatment with hydroxyurea (Table 1).

At the 25-mg/kg dose level, 6-TGN concentrated in erythrocytes, with 6-MPN and 6-TGN C_{max} values being 286.8 ± 23.4 and 910.9 ± 53.1 pmol/8×10^8 erythrocytes, respectively ($n = 3$, $P < 0.05$ as compared with 6-MPN; Fig. 2, Table 2), and $t_{1/2}$ values being 9.3 (6-TGN) and 10.4 days (6-MPN; Table 2). The thiopurine AUC achieved in erythrocytes reflected the behavior observed for the C_{max} of 6-TGN and 6-MPN in erythrocytes; the 6-TGN AUC was 3-fold higher than that of 6-MPN (Table 2). In agreement with the findings obtained in rats treated with 6-MP at 12.5 mg/kg per day, the highest tissue levels of thiopurines were detected in the liver, with 6-MPN and 6-TGN C_{max} values being 759.1 ± 69.7 and 391.9 ± 61.0 pmol/g of tissue, respectively ($n = 3$, $P < 0.05$ for 6-MPN as compared with 6-TGN; Figs. 3, 4). The elimination of 6-TGN and 6-MPN from tissues revealed that in rats treated with 6-MP at 25 mg/kg per day the half-lives of 6-MPN and 6-TGN were higher than those observed in rats receiving 6-MP at the lower dose level, ranging from 10.1 to 15.6 days for 6-MPN and 6-TGN in the kidney, respectively (Table 2). Furthermore, a significant increase in C_{max} values was observed in a comparison of 6-MPN and

Table 2 Pharmacokinetic parameters of 6-MPN and 6-TGN as determined in erythrocytes and tissues after i.p. administration of 6-MP at 25 mg/kg per day to rats and the percentage of variation in the thiopurine C_{max} after treatment with ribavirin at 75 mg/kg per day or hydroxyurea at 200 mg/kg per day

	Erythrocyte		Liver		Kidney	
	6-MPN	6-TGN	6-MPN	6-TGN	6-MPN	6-TGN
C_{max}[a]	286.8 ± 23.4[f]	910.9 ± 53.1[e,f]	759.1 ± 69.7[e,f]	391.9 ± 61	354.7 ± 40.5	319.6 ± 19.2[d]
+Ribavirin[b]	+132.2 ± 12.4%[g]	−58.1 ± 6.4%[g]	+41.9 ± 4.7%[g]	−55.2 ± 6.1%[g]	+25.3 ± 3.4%[g]	−20.4 ± 2.9%[g]
+Hydroxyurea[b]	+24.6 ± 3.5%[g]	+18.3 ± 2.1%	+61.5 ± 6.9%[g]	+35.1 ± 4.2%[g]	+20 ± 2.8%[g]	+20.1 ± 3.1%[g]
$t_{1/2}$[c]	10.4	9.3	12.1	13.4	10.1	15.6
$AUC_{0\rightarrow62}$[d]	3778	13835	9547	7414	5354	6827

[a] In pmol/8×10^8 erythrocytes or pmol/g of tissue
[b] Percentage of variation in the thiopurine C_{max} in samples from rats receiving additional treatment with ribavirin or hydroxyurea
[c] In days
[d] In days × pmol/8×10^8 erythrocytes or days × pmol/g of tissue
[e] $P < 0.05$ as compared with 6-MPN or 6-TGN in the same tissue
[f] $P < 0.05$ as compared with the C_{max} of the corresponding thiopurine in the same tissue following treatment with 6-MP at 12.5 mg/kg per day
[g] $P < 0.05$ as compared with the C_{max} of thiopurines in samples from rats treated with 6-MP alone

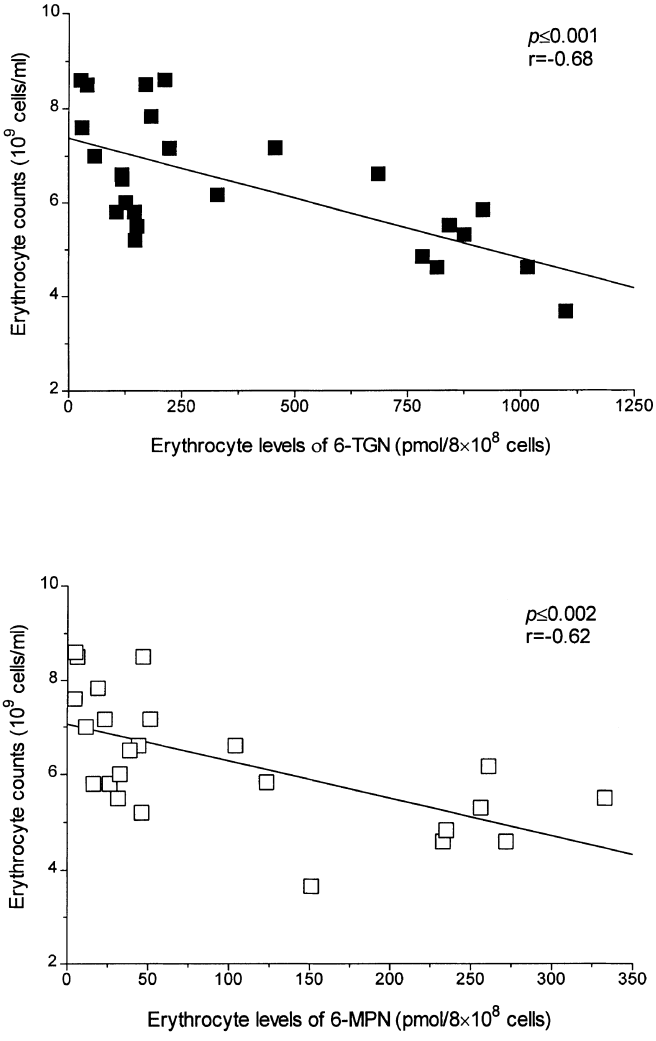

Fig. 5 Linear regression analysis between 6-MPN and 6-TGN concentrations detected in rat erythrocytes and erythrocyte counts measured at the corresponding time points in rats treated i.p. with 6-MP at 12.5 and 25 mg/kg per day

6-TGN in erythrocytes and of 6-MPN in the liver and 6-TGN in the kidney of rats treated at 25 versus 12.5 mg/kg per day (Tables 1, 2). According to the results obtained in combination treatments with ribavirin and 6-MP at 12.5 mg/kg per day, 6-TGN peak levels decreased significantly in erythrocytes and tissue samples of rats treated with 6-MP at 25 mg/kg per day plus ribavirin at 75 mg/kg per day with respect to the C_{max} values obtained after administration of 6-MP alone, with maximal inhibition being observed in erythrocytes ($-58.1 \pm 6.4\%$, Table 2). In addition, ribavirin treatment produced a significant increase in 6-MPN C_{max} in erythrocytes and tissues (Table 2). Moreover, a statistically significant increase in the C_{max} values of thiopurine nucleotides was observed when animals were treated with 6-MP at 25 mg/kg per day plus hydroxyurea at 200 mg/kg per day, the exception being 6-TGN C_{max} values achieved in erythrocytes (Table 2).

The linear regression analysis of 6-TGN and 6-MPN concentrations in erythrocytes and erythrocyte counts in rat whole blood, measured at the corresponding time points, showed a significant correlation between thiopurine concentrations and circulating erythrocytes ($P < 0.001$ for 6-TGN and $P < 0.002$ for 6-MPN), with the coefficient of correlation being -0.68 and -0.62 for 6-TGN and 6-MPN, respectively (Fig. 5).

Discussion

6-MP is important in the successful management of childhood ALL and is universally used as a major component of remission maintenance treatment [31]. In recent years, much attention has been devoted to the pharmacology of 6-MP [3, 20], since the concentrations of cytotoxic metabolites in erythrocytes are related to the likelihood of relapse and myelosuppression [18, 24]. At standard doses of 6-MP there is a large interindividual variation in the formation of 6-TGN [12] that depends on differences in the metabolic behavior of patients [5], and for these reasons the modulation of 6-MP metabolism by pharmacologic agents may improve its therapeutic activity [1]. Animal models of drug disposition have been used to investigate the poor systemic bioavailability and pharmacokinetics of thiopurines in studies involving the administration of 6-MP to monkeys and azathioprine mice [10, 11, 19, 23, 25] as well as the modulation of 6-MP metabolism by methotrexate in rats [7].

Although there is a large body of clinical work on the pharmacokinetics of thiopurines, their metabolism in animal models and the effects of combination treatments with agents that modulate the activity of enzymes of the purine metabolic pathway have not been addressed in detail. For these reasons the present study evaluated 6-MP biotransformation in erythrocytes and tissues of rats treated with multiple doses of 6-MP at 12.5 and 25 mg/kg per day alone or in combination with ribavirin or hydroxyurea. The i.p. route was chosen to maximize the absorption of 6-MP and because oral administration by gastric gavage is suboptimal for repeated treatment. The doses of 6-MP used in the present work are similar to those applied in previous studies in rats with oral and parenteral 6-MP at 10 mg/kg [10] and with 6-MP given subcutaneously at 20 mg/kg [7] and in mice treated at 50 mg/kg with a single dose of azathioprine, a prodrug of 6-MP [11]. High single doses of 6-MP (50–200 mg/kg i.p.) have been evaluated for their effect on the immune system [27]. Moreover, the present schedule was found to be well tolerated by animals, and it can be suggested for chronic treatment in future experimental studies.

The results indicate that after its administration by the i.p. route, 6-MP is well absorbed in rats and is converted into thiopurine nucleotides in red blood, hepatic, and renal cells, with 6-MPN and 6-TGN reaching

detectable levels at 24 h after the first dose. The cellular uptake of 6-MP and intracellular metabolism of its derivatives is rapid [11], and, unlike 6-MP, the polar ribonucleotide monophosphates with the thiopurine as the base cannot cross the cell membranes freely because of the presence of a charged phosphate moiety [3]. Ribonucleotide monophosphates are retained intracellularly, where they are converted to those with 6-TG as the base (Fig. 1) [3]. The present findings show that rats can metabolize 6-MP and, as based on this evidence, they have an active purine metabolism pathway similar to that of humans, whereby 6-TGN is produced after 6-MP dosing by the enzymes of the ribonucleotide interconversion pathway within cells [12]. It is noteworthy that phosphoribosyl pyrophosphate (PRPP), the cofactor required in thionucleotide synthesis, has been measured in various rat organs, including regenerating liver and developing kidneys [9, 26]. Furthermore, the increased rate of de novo purine biosynthesis observed in rat liver during regeneration as well as following burn injury is mediated by the elevated cellular levels of PRPP [4, 9]. A substantial similarity between humans and the rat model has been observed in the case of inosine monophosphate dehydrogenase, the key enzyme in the conversion of thioinosine monophosphate into 6-TGN, since the kinetic properties are similar to those of the enzyme extracted from rat hepatoma 3924A and from human myelocytic leukemic cells [30].

Kurowsky and Iven [11] observed the production of 6-MPN and 6-TGN in tissues of mice treated with azathioprine; in erythrocytes, 6-MPN and its hydroxylated metabolite were detectable, whereas 6-TGN was lacking. The absence of 6-TGN in erythrocytes indicates that the mouse is an unsatisfactory model for the study of thiopurine metabolism in vivo. In monkeys treated with i.v. infusions of [^{14}C]-6-MP the autoradiograms revealed high levels of radioactivity in the liver, bile, and intestine, indicating an extensive hepatic metabolism of 6-MP [25]. The present findings provide the first detailed description of in vivo metabolism of 6-MP in the tissues and erythrocytes of rats treated with 6-MP and of its modulation by agents active on enzymes of purine metabolism.

The kinetic behavior of thiopurine nucleotides in rat erythrocytes during the loading phase indicated that 6-MPN and 6-TGN did not reach steady-state concentrations after the 12-day treatment, and thionucleotide levels continued to increase after 20 days of 6-MP administration at 25 mg/kg per day (data not shown). In most patients, 6-TGN reaches high erythrocyte concentrations within 2 weeks of oral administration of 6-MP, but others show a slower increase in 6-TGN levels, with equilibrium concentrations being reached only after 28 days [13]. The progressive accumulation of thiopurines in rat erythrocytes, with respect to humans, might reflect a more active metabolism of 6-MP, its reduced body elimination, or both. Moreover, the terminal half-lives recorded for thiopurines in erythrocytes of rats are similar to the $t_{1/2}$ values noted for 6-TGN in patients after the withdrawal of therapy [16]; this indicates a long-term retention of thiopurine metabolites within erythrocytes that is measured in days rather than in hours [15].

The significant relationship observed between 6-MPN and 6-TGN and erythrocyte counts in the present study suggests that red cell metabolites reflect the amount of cytotoxic nucleotides within the bone marrow stem cells, with the numbers of circulating erythrocytes declining as the levels of 6-MPN and 6-TGN increase. In leukemic patients treated with 6-MP, Lennard et al. [15] have evidenced a direct relationship between 6-TGN levels in erythrocytes and bone marrow toxicity, suggesting that erythrocyte 6-TGN levels indicate the actual exposure of patients and could be useful for adjustment of the dose of 6-MP and improvement of the outcome of the maintenance treatment [18].

With regard to the distribution of thiopurine nucleotides in tissues, the results of this study indicate that hepatic and renal tissues of the rat actively metabolize 6-MP to its derivatives, according to the results obtained in mice [11]. It has previously been demonstrated that in the male rat kidney, thiopurine methyltransferase activity is higher than in female rats, indicating a gender-dependent metabolism of 6-MP in rats [8, 32]. Although thiopurine production in renal tissue reveals no significant difference between 6-MPN and 6-TGN concentrations, 6-MPN accumulates in the liver to a significantly greater extent than does 6-TGN. The C_{max} and average AUC values noted for 6-MPN in the liver could be dependent on the distribution of the drug from the peritoneal cavity to the hepatic tissue and on a preferential activation to 6-MPN at the intracellular level. Finally, although our data concerning the drug modulation of 6-MP metabolism must be considered as preliminary results to be further evaluated in future studies, the significant changes in 6-MPN and 6-TGN C_{max} values observed in the erythrocytes and tissues of animals receiving combination treatments with ribavirin and hydroxyurea indicate that the rat model behaves as expected toward drugs acting on enzymes of thiopurine metabolism. In particular, ribavirin proved to be active in preventing further biotransformation of 6-MPN to 6-TGN through the inhibition of IMPDH associated with accumulation of 6-MPN and reduction of 6-TGN levels. An increase in the metabolism of 6-MP to its nucleotide derivatives was obtained by activation of the purine salvage pathway via combination treatment with hydroxyurea, an agent known to inhibit the enzyme ribonucleotide reductase.

The findings of this study may be relevant to the clinical setting and could provide evidence that the rat is a suitable model for the investigation of novel approaches to the modulation of thiopurine biotransformation to improve the metabolism of thiopurines to cytotoxic compounds, particularly in patients with deficient activation of 6-MP to cytotoxic 6-TGN.

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