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Plasma concentrations and organ distribution of thiopurines after oral application of azathioprine in mice*

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Summary. The plasma concentrations and tissue distribution of thiopurines were studied in mice after oral administration of 50 mg/kg azathioprine (AZA) using HPLC analysis. Peak concentrations of AZA and three other thiopurine metabolites in plasma [thiouric acid (TUA) >6-mercaptopurine (6-MP) >AZA >8-hydroxy-AZA] were observed as early as 10 min after drug application, thus indicating fast absorption and extensive metabolism of AZA, and were followed by a rapid decline. The extraction of thiopurines from organs (intestinal mucosa, liver, kidney, testes, spleen, and bone marrow) and from red blood cells (RBCs) was preceded by an acid hydrolysis procedure resulting in the release of thiopurine bases from their corresponding ribonucleotides. 6-MP, 6-thioxanthine (6-TX), 6-thioguanine (6-TG), TUA, and 8-hydroxy-6-MP (8-OH-6-MP) were extracted from the organs, whereas only 6-MP and 8-OH-6-MP were found in the processed RBCs. Initially, high concentrations of TUA, the endpoint of metabolic AZA degradation, were detected in the intestinal mucosa and in the liver. This provides evidence for a first-pass metabolism of AZA in these two organs. The initial concentrations of 6-MP extracted from the organs were about 10-fold those found in plasma. This indicates rapid cellular uptake of 6-MP and an accumulation of 6-MP derivatives that can be explained by formation of the 6-MP ribonucleotide thioinosine monophosphate (TIMP). With the exception of plasma and RBCs, 6-TG, which may originate from intracellular 6-thioguanosine nucleotides (TGNs), was extracted from all organs examined in the study. From the sequence of appearance of 6-MP, 6-TX, and 6-TG extracted from spleen and bone marrow homogenates, it can be assumed that formation of TGN occurs via the nucleotide interconversion pathway TIMP → 6-thioxanthosine monophosphate → 6-thioguanosine monophosphate. The highest concentrations of 6-TG derivatives were found in the spleen and bone marrow. This correlates with the clinical and experimental observation that AZA cytotoxicity mainly affects bone-marrow stem cells and lymphocytes and supports the hypothesis (derived from in vitro experiments) that the incorporation of TGN into DNA is the cytotoxic mechanism of AZA and 6-MP.

Introduction

Azathioprine (AZA), a derivative of the cytostatically active antimetabolite 6-mercaptopurine (6-MP), was synthesized in the late 1950s. For >25 years AZA has been used extensively for immunosuppression in organ transplantation and in a variety of immunologically mediated diseases [6, 27]. Despite its long clinical use, the mode of action of AZA is not fully understood. This is predominantly due to the complex metabolism of AZA, which (mainly derived from numerous in vitro experiments) is shown in Fig. 1.

The proposed biologically active metabolites of AZA and 6-MP are the thiopurine ribonucleotides 6-thioinosine monophosphate (TIMP) and the 6-thioguanosine nucleotides (TGNs). Whereas, TIMP interferes in vitro with several enzymes of the purine de novo synthesis and the purine nucleotide interconversion pathway and is therefore thought to be responsible for the antiproliferative action of AZA and 6-MP [1], the incorporation of TGNs into DNA has been shown to be the cytotoxic mechanism of 6-MP in cell cultures [26]. However, as the organ distribution of thiopurine metabolites after application of AZA or 6-MP has thus far not been studied, it is not known exactly where or to what extent the thiopurine ribonucleotides, which have shown activity in vitro, are formed in vivo.

Recently, TGNs were found to be the major metabolites of AZA and 6-MP in human erythrocytes (RBCs) [12]. In subsequent studies, elevated TGN concentrations in RBCs

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Abbreviations: TGNs, 6-thioguanosine nucleotides, RBC, red blood cell; PBS, phosphate-buffered saline; DTT, dithiothreitol

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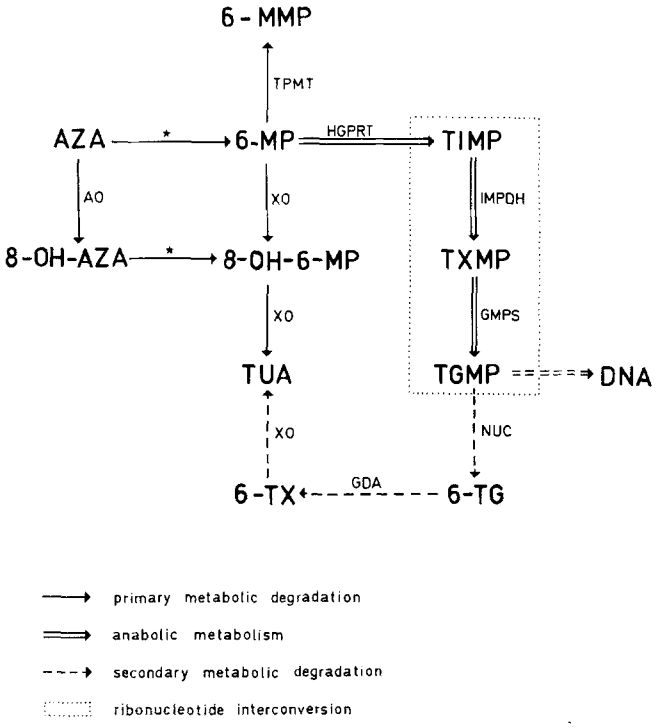

Fig. 1. Metabolic pathways of azathioprine (modified from [1, 27]). Thiopurine metabolites: *AZA*, azathioprine; *8-OH-AZA*, 8-hydroxy-azathioprine; *6-MP*, 6-mercaptopurine; *8-OH-6-MP*, 8-hydroxy-6-mercaptopurine; *6-TG*, 6-thioguanine; *6-TX*, 6-thioxanthine; *TUA*, thiouric acid; *6-MMP*, 6-methyl-mercaptopurine; *TIMP*, 6-thioinosine monophosphate; *TGMP*, 6-thioguanosine monophosphate; *TXMP*, 6-thioxanthosine monophosphate. Enzymes: *AO*, aldehyde oxidase; *XO*, xanthine oxidase; *TPMT*, thiopurine methyltransferase; *HGPRT*, hypoxanthine guanine phosphoribosyl transferase; *IMPDH*, inosine monophosphate dehydrogenase; *GMPS*, guanine monophosphate synthetase; *NUC*, nucleotidases; *GDA*, guanine deaminase; *, nonenzymatic, SH-dependent product

could be associated with myelosuppression [13, 14] and with the development of skin cancer in renal transplant recipients [15]. However, again the origin of TGNs in RBCs and their possible correlation with active AZA or 6-MP metabolites in other tissues remained unclear. As an approach to obtain a solution to these problems, we studied the pharmacokinetics and tissue distribution of thiopurines after a single oral dose of AZA in mice.

Materials and methods

Chemicals. AZA (sodium salt, Imurek ampules) was obtained from German Wellcome Pharmaceutical Co. (Burgwedel, FRG). 6-MP, 6-thioxanthine (6-TX), and 6-thioguanine (6-TG) were all purchased from Sigma Chemical Co. (Deisenhofen, FRG). 8-Hydroxy-azathioprine (8-OH-AZA), and 8-hydroxy-6-mercaptopurine (8-OH-6-MP) were kindly supplied by Burroughs Wellcome Co. (Research Triangle Park, N. C., USA). TUA was synthesized in our laboratory using a previously published method [17]. All other chemicals were of analytical reagent grade and were purchased from Merck Co. (Darmstadt, FRG).

Animal treatment and organ preparation. Male NMRI mice (35–40 g) were used throughout the study. For the oral application of 50 mg/kg AZA, a solution (5 mg/ml H_2O) of the AZA sodium salt was prepared. The mice (six animals per group) were killed by cardiac puncture while under CO_2 anesthesia at 10, 20, 40, 60, and 90 min and at 2, 4, 6, 8, 10, 12, 16, 20, and 24 h after drug administration. A 1.2- to 1.5 ml sample of blood was aspirated into a heparinized syringe and immediately put on ice, and plasma and blood cells were separated within minutes by centrifugation at 4° C. The plasma (0.6 ml) was stored at –20° C. The buffy coat of the blood cell layer was removed and the remaining RBCs were then washed twice with phosphate-buffered saline (PBS). After being washed, the RBCs were "packed" by centrifugation and stored at –20° C. The liver, kidneys, testes, and spleen were removed, weighed, and also stored at –20° C. The entire small intestine was removed and divided into segments, which were then opened, carefully rinsed with PBS, and shock-frozen in liquid nitrogen. The mucosa (250–300 mg) was scraped off with a scalpel and stored at –20° C. For the preparation of bone marrow samples, all four long bones of the hind legs were used. The ends of each bone were clipped and the marrow was ejected with chilled PBS using a small injection needle. After centrifugation, the supernatant was carefully removed by suction and the bone marrow pellets (35–40 mg/mouse) were stored at –20° C.

Assay of AZA, 8-OH-AZA, 6-MP, and TUA in plasma. For the separation of lipophilic AZA and 8-OH-AZA from the more hydrophilic metabolites 6-MP and TUA, 400 µl plasma was extracted with 3 ml ethyl acetate for 15 min. After centrifugation, the aqueous phase was processed as described below for determination A_1 and the organic phase as described for determinations B and C.

For determination A, 200 µl aqueous phase and 20 µl 1 *N* acetic acid were heated for 5 min (protein precipitation) and centrifuged; 100 µl supernatant was injected onto the HPLC system. By this step, all TUA and the major portion of 6-MP (80%–85%) present in the plasma sample could be quantified. The ethyl acetate phase was evaporated under a gentle stream of air and the residue was redissolved in 400 µl ice-cold 0.1 *N* NaOH. For determination B, one half (200 µl) of this redissolved sample was immediately neutralized with 20 µl 1 *N* HCl. A 100-µl aliquot was injected onto the HPLC system. This step covered the portion of 6-MP (15%–20%) that was extracted into the organic phase. To obtain the total 6-MP concentration originally present in the native plasma sample, the results derived from determinations A and B had to be added. For determination C, the other half (200 µl) of the redissolved sample was kept at 95° C for 10 min. In this way AZA and 8-OH-AZA were completely hydrolized, releasing 6-MP and 8-OH-6-MP, respectively. After heating, the sample was neutralized with 20 µl 1 *N* HCl. A 100-µl aliquot was injected onto the HPLC system. The 8-OH-6-MP concentration detected by this procedure represented the equimolar amount of all 8-OH-AZA that was present in the native plasma sample. The 6-MP concentration measured by determination C included all 6-MP released from AZA by hydrolysis and the portion of 6-MP that was extracted from the plasma into the ethyl acetate phase. To obtain only the amount of 6-MP derived from AZA, the 6-MP concentration measured using determination B had to be subtracted from that detected by determination C.

Calibration samples were run in parallel following the addition of the respective thiopurines to the plasma of untreated animals. The lower limits of detection for AZA, 6-MP, 8-OH-AZA, and TUA were 66, 66, 60, and 54 nmol/l plasma, respectively.

Assay of thiopurine metabolites in tissue samples. This assay is a modification of methods that have been reported elsewhere for the quantification of thiopurines in plasma and RBCs [11, 12, 19]. It consists of an acid hydrolysis step (release of thiopurines from their corresponding ribonucleotides), a derivatization procedure (formation of lipophilic thiopurine adducts), the extraction of these adducts into an organic solvent, and the back-extraction of the thiopurines into an aqueous phase (release of the thiopurines from the lipophilic adducts formed by derivatization). Due to the acid hydrolysis procedure, the thiopurines measured using this assay may partly originate from ribonucleotide forms: TIMP, TXMP, and TGMP are assayed as 6-MP, 6-TX, and 6-TG, respectively (see Discussion). Since a free SH-group is required for the derivatization procedure, 6-methyl-mercaptopurine (6-MMP, see Fig. 1), another important metabolite of AZA and 6-MP in humans [16], is not quantified by this assay.

After thawing, the solid organs (liver, kidney, testis, and spleen) were minced. To 1 part of tissue, 4 parts (w/v) of 1.25 *N* H_2SO_4 containing

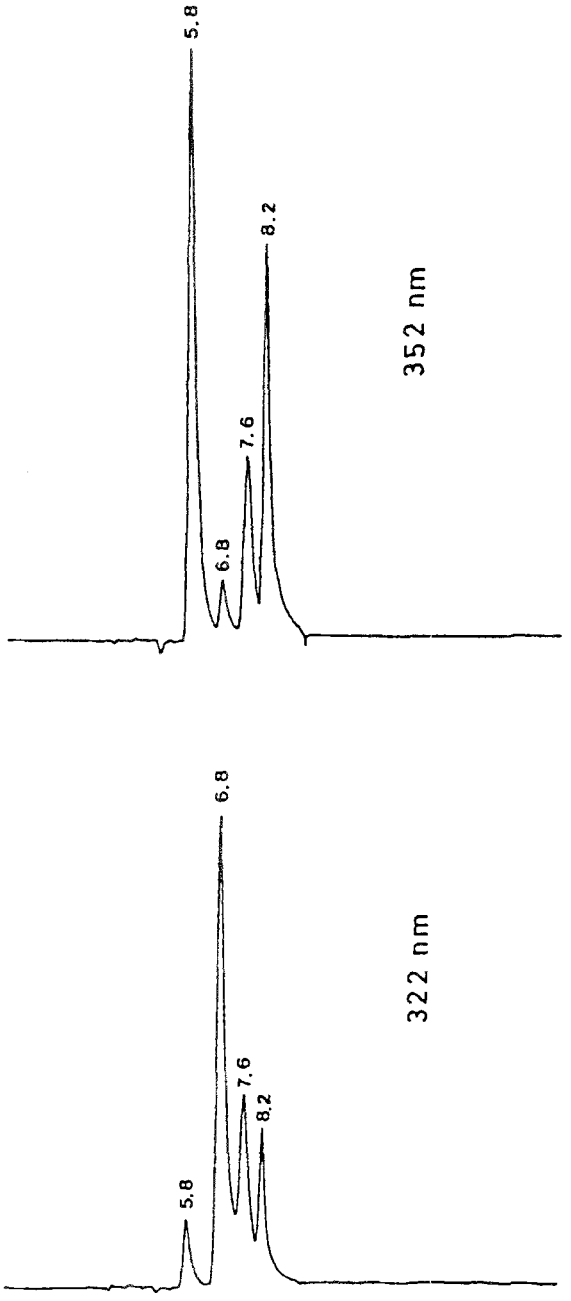

Fig. 2. HPLC chromatograms of a sample spiked with TUA (5.8 min), 6-MP (6.8 min), 6-TX (7.6 min), and 6-TG (8.2 min) as obtained at 322 nm and 352 nm

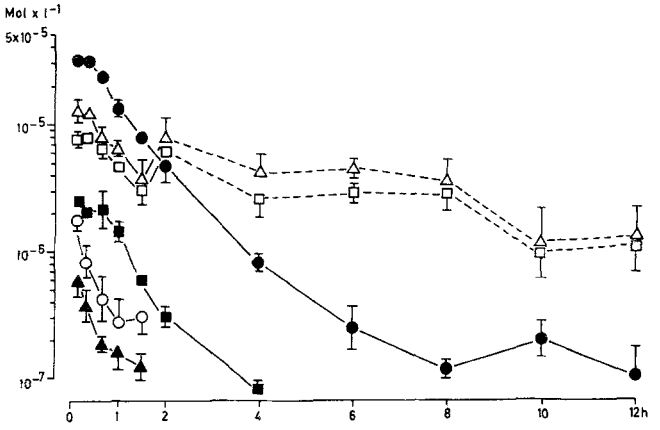

Fig. 3. Plasma concentrations (mol/l) of AZA (○), 8-OH-AZA (▲), 6-MP (■) and TUA (●) in mice after oral administration of 50 mg/kg AZA. Concentrations of 6-MP (□) and 8-OH-6-MP (△) as extracted from RBCs (mol/l "packed" RBCs) are included for comparison ($\bar{x}_{geo} \pm SD_S\bar{x}$, $n = 6$)

2.5 mM dithiothreitol (DTT) were added. The samples were homogenized for 30 s with an Ultraturrax (Ika-Werk, Staufen, FRG) at 20,000 rpm. The intestinal mucosa and the bone marrow pellets were suspended in 4 parts (w/v) of the H_2SO_4-DTT solution and then homogenized. Since a minimal weight of 200 mg tissue/sample was required, two spleens had to be combined for one sample, resulting in three samples per group. Furthermore, the bone marrow of all six mice per group was pooled. Next, 1 ml of the homogenates (representing 200 mg tissue) was transferred to Eppendorf tubes and kept at 95° C for 1 h. This resulted in a complete release of thiopurine bases from their corresponding ribonucleotides via acid hydrolysis [12].

After heating, the homogenates were centrifuged and 700 µl supernatant was transferred to glass tubes; 350 µl 5*N* NaOH and 2 ml 9 mM phenyl mercury acetate were added, resulting in the formation of lipophilic phenyl mercury-thiopurine derivatives [19]. The mixture was extracted for 20 min with 8 ml toluene containing 1.5% *n*-amyl alcohol (v/v) and centrifuged. A 7-ml aliquot of the toluene layer was shaken with 150 µl 0.1 *N* HCl for 20 min; in this way, the thiopurines were released from their phenyl mercury adducts into the aqueous phase [19]. After centrifugation, 120 µl acidic reextract was neutralized with 12 µl 1 *N* NaOH and 100-µl aliquot was injected onto the HPLC system.

No suitable internal standard could be found for the assay. Therefore, calibration curves (consisting of a minimum of six samples for each metabolite) were prepared daily following the addition of 6-MP, 6-TG, 6-TX, 8-OH-6-MP, and TUA to tissue homogenates of untreated animals. The curves (peak height vs concentration) for all metabolites were linear over the entire concentration range examined in this study. The recovery rates (which differed slightly, depending on the organs used) for 6-MP, 6-TG, 6-TX, 8-OH-6-MP, and TUA were approx. 75%, 80%, 70%, 65%, and 25%, respectively; the lower limits of detection were 50, 60, 60, 60, and 110 nmol/kg tissue (wet wt.), respectively.

Assay of thiopurine metabolites in RBCs. This assay is in principle the same as that described above. After thawing, the "packed" RBCs were hemolyzed by the addition of a 100 µl aliquot to 1.2 ml 3.3 mM DTT in glass tubes. Thereafter, 700 µl 3 *N* H_2SO_4 was added, and the samples were kept at 95° C for 1 h. After heating, 700 µl 5 *N* NaOH and 2 ml 9 mM phenyl mercury acetate were added. Using this mixture, the extraction of the formed phenyl mercury-thiopurine derivatives into toluene (8 ml) and the back-extraction of the thiopurines into 0.1 *N* HCl (150 µl) were performed as described above. For calibration, samples were prepared by the addition of thiopurine metabolites to RBCs of untreated mice. The recovery rates for 6-MP, 6-TG, 6-TX, and 8-OH-6-MP were 65%, 60%, 60%, and 55%, respectively. In agreement with a previous report [11], we could not extract TUA from RBCs by the phenyl mercury derivatization procedure. The lower limits of detection for 6-MP and 8-OH-6-MP were 130 and 180 nmol/l "packed" RBCs, respectively.

Chromatography. TUA, 8-OH-6-MP, 6-MP, 6-TX, and 6-TG (as derived from the processed plasma and tissue samples) were separated by HPLC using a cation-exchange column (Whatman Partisil SCX, 10 µm, 250 × 4.6 mm). To separate the thiopurines with short retention times (TUA and 8-OH-6-MP) from the void fraction, the cation-exchange column was preceded by a reverse-phase column (Shandon ODS Hypersil, 5 µm, 60 × 4.6 mm). The eluent, a 200 mM ammonium formate buffer (pH 3.2) containing 0.1% mercaptoethanol (v/v), was pumped at a flow rate of 1 ml/min. UV detection at 322 and 352 nm was carried out using two variable-wavelength monitors (SP4; Gynkotek, Germering, FRG) in series. According to their maximal UV absorption (indicated in parentheses), 8-OH-6-MP (330 nm) and 6-MP (320 nm) were monitored at 322 nm, whereas TUA (355 nm), 6-TX (340 nm), and 6-TG (342 nm) were monitored at 352 nm. The retention times for TUA, 8-OH-6-MP, 6-MP, 6-TX, and 6-TG were 5.8, 5.8, 6.8, 7.6, and 8.2 min, respectively. If TUA and 8-OH-6-MP had to be determined in the same sample, the

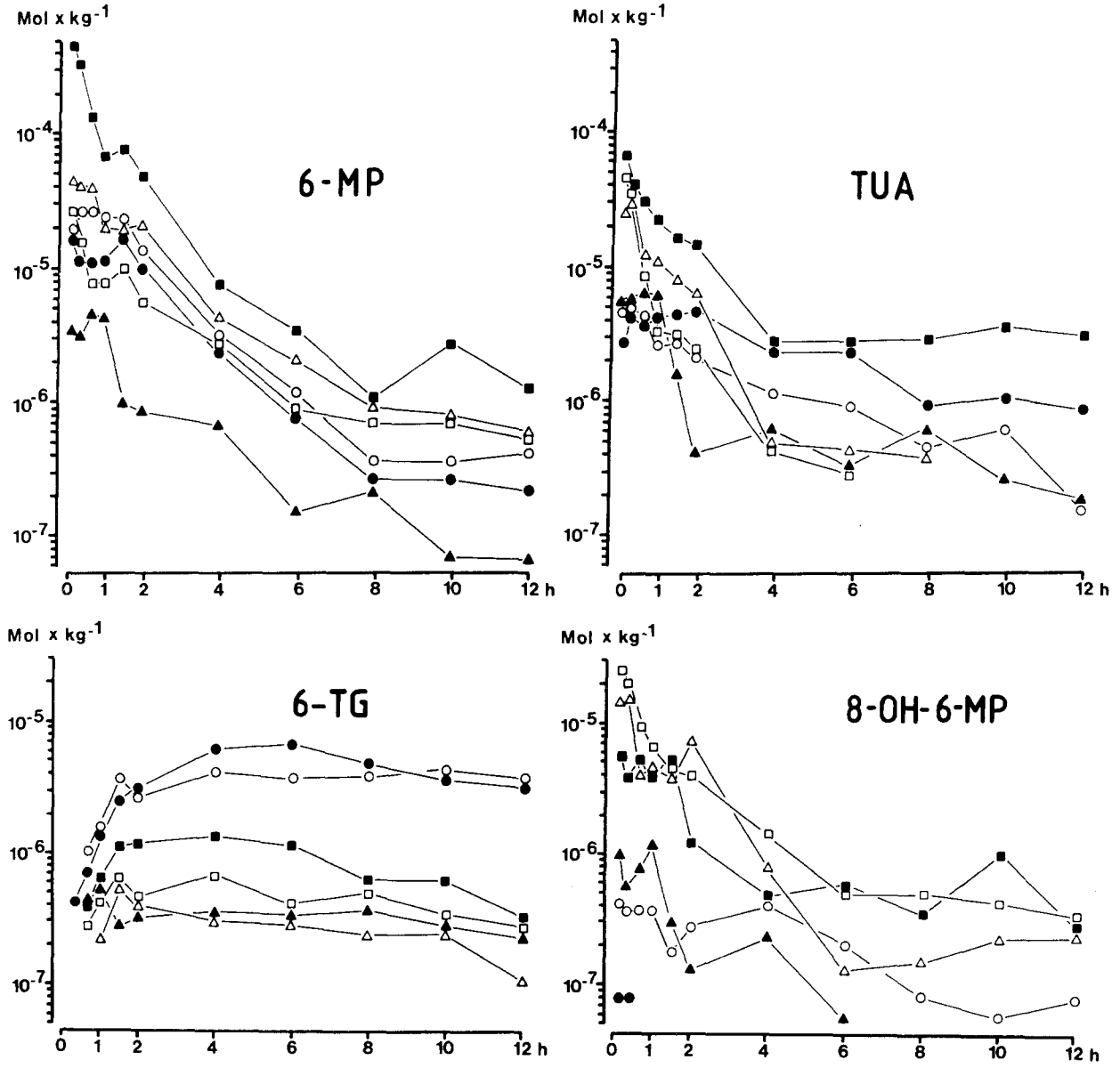

Fig. 4. Concentrations (mol/kg tissue, wet weight) of 6-MP (*upper left*), 6-TG (*lower left*), TUA (*upper right*), and 8-OH-6-MP (*lower right*) as extracted from the organs of mice after oral administration of 50 mg/kg AZA. Shown are the geometric means in bone marrow (●), spleen (○), intestinal mucosa (■), liver (□), testes (▲), and kidney (△)

simultaneous two wavelength registration (with the constant ratios of the peak heights at 322–352 nm for pure TUA and 8-OH-6-MP being known), made it possible to calculate the individual contributions of the two compounds. Figure 2 shows the chromatograms of a sample spiked with TUA, 6-MP, 6-TX, and 6-TG as obtained at 322 and 352 nm.

Results

Pharmacokinetics of thiopurines in plasma

The concentration-time profiles for AZA, 8-OH-AZA, 6-MP, and TUA are shown in Fig. 3. Peak concentrations of all thiopurines were detected at as early as 10 min after the application of AZA (first sample). Due to a rapid decline, AZA and 8-OH-AZA reached the detection limit after 90 min and 6-MP, after 4 h. TUA concentrations were 10-fold those of 6-MP and were detectable for up to 12 h after AZA dosing. At subsequent times (16, 20, and 24 h), no thiopurines could be detected in the plasma samples.

Thiopurine metabolites in RBCs

Figure 3 also shows the concentration-time curves for 6-MP and 8-OH-6-MP as extracted from RBCs after the acid hydrolysis procedure. Peak concentrations of both thiopurines were determined in the 10-min samples. The initial concentration of 6-MP in RBCs was 3-fold that in plasma and did not decline substantially within the first 8 h. The 8-OH-6-MP concentrations paralleled those of 6-MP. For both metabolites a gradual decrease was observed after 8 h. At up to 24 h after the administration of AZA, no 6-TG or 6-TX was detected in RBCs.

Thiopurine metabolites in organs

The concentration-time curves for 6-MP, 6-TG, TUA, and 8-OH-6-MP as extracted from tissue homogenates after the acid hydrolysis procedure are shown in Fig. 4. 6-MP peak concentrations either were determined in the 10-min samples (intestinal mucosa, kidney, liver, bone marrow) or were reached within 40 min after the application of AZA (spleen, testes). The initial 6-MP concentrations in most organs were 6- to 16-fold those in plasma. In testes, the maximal 6-MP level lay in the range of the peak 6-MP plasma concentration. In the intestinal mucosa, the 6-MP concentration exceeded that in the other organs by 1 order of magnitude. In all organs, initially high 6-MP concentrations declined over a period of 8 h and persisted at relatively low levels for the next 4 h.

6-TG could be extracted from all organs examined in this study except plasma and RBCs. In most organs, 6-TG appeared at 40 min after the application of AZA (bone marrow, 20 min; kidney, 60 min). The 6-TG concentrations rose during the first 2–4 h and subsequently remained constant for several hours. Small amounts of 6-TG were extracted from the liver, kidney, and testes, and higher concentrations were found in the intestinal mucosa. The concentrations of 6-TG extracted from bone marrow and spleen exceeded those in other tissues by 1 order of magnitude.

Considerable amounts of TUA and 8-OH-6-MP were extracted from most organs within 1–2 h after the administration of AZA; again, peak concentrations of both metabolites were often found in the 10-min samples. The high concentrations observed initially were followed by a decline over a period of approx. 8 h. Between 8 and 12 h, TUA and 8-OH-6-MP remained detectable at constant and low levels in most organs. As compared with plasma levels, the initial TUA concentrations in intestinal mucosa and liver were higher (2.1- and 1.5-fold, respectively).

The fifth thiopurine extracted from tissue homogenates after the acid hydrolysis procedure was 6-TX. Considerable amounts of this metabolite were found in the spleen and bone marrow. Low concentrations were detected in the intestinal mucosa and testes of some animals during the first 6 h (data not shown), but no 6-TX could be found in the liver or kidneys. Figure 5 shows the concentration-time curves for 6-TX and, for purposes of comparison, those for 6-MP and 6-TG extracted from the spleen (Fig. 5a) and bone marrow (Fig. 5b).

The initial concentrations of 6-MP in the spleen and bone marrow were about 10-fold those in plasma. During the first 90 min, 6-MP levels in both organs remained relatively stable and then declined with a half-life of about 70 min for up to 8 h after drug application. Low concentrations of 6-TX had been detected in the 10-min samples. In contrast to 6-MP, 6-TX concentrations increased during the first 90 min and then declined in parallel with 6-MP. As compared with 6-TX, 6-TG concentrations rose with some delay, exceeding the 6-MP and 6-TX levels after 4 h and reaching a peak in the 4- and 6-h samples. After 8 h, 6-TG levels declined with a half-life of approximately 3 h. At timepoints of >16 h, no thiopurines other than 6-TG were extracted from the spleen or bone marrow.

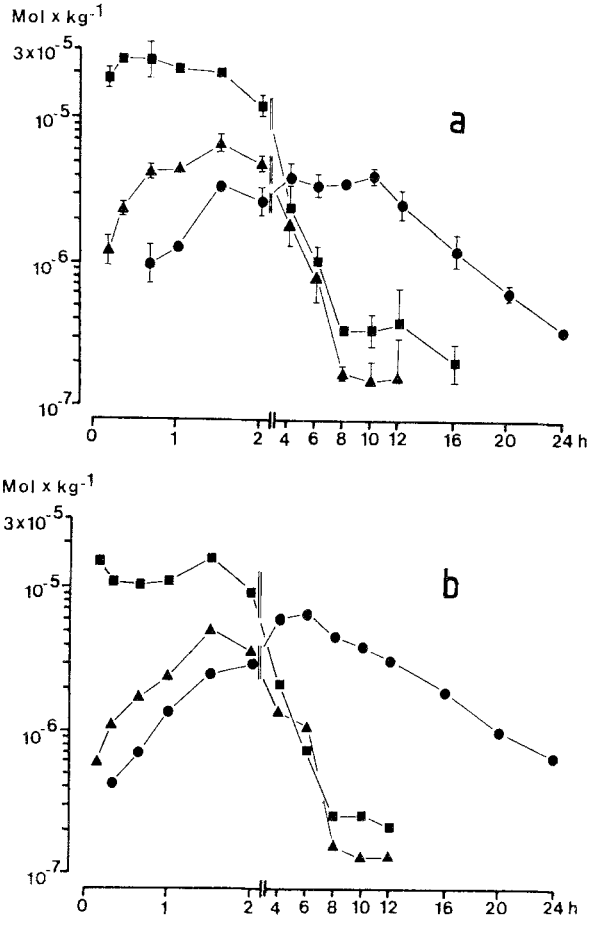

Fig. 5a, b. Concentrations (mol/kg tissue, wet weight) of 6-MP (■) 6-TX (▲), and 6-TG (●) as extracted from **a** the spleen and **b** bone marrow of mice after oral administration of AZA (50 mg/kg)

Discussion

Thiopurines in plasma

The pharmacokinetics of thiopurines in plasma after the administration of AZA has repeatedly been investigated in animals and man. These studies covered only a maximum of two metabolites, e.g., AZA and 6-MP [18, 20], 6-MP and TUA [4, 7], or 6-MP and 8-OH-6-MP [5]. We detected and quantified four thiopurines (AZA, 8-OH-AZA, 6-MP, and TUA) in the plasma of AZA-treated mice. Peak levels of all thiopurines were observed at as early as 10 min after oral drug application (Fig. 3). This indicates rapid absorption and extensive metabolism of the parent compound. AZA rapidly disappeared from plasma; this has also been reported after i.v. administration of AZA to rhesus monkeys and renal transplant recipients [5, 18, 20]. After oral administration of therapeutic doses, very little (if any) AZA is detectable in patient plasma [18, 20].

Concentrations of 8-OH-AZA, which is enzymatically generated from AZA by an aldehyde oxidase (Fig. 1), declined in parallel with AZA levels. 8-OH-AZA has previously been identified in the rhesus monkey and in man but was not quantified [5, 19].

AZA was rapidly metabolized to 6-MP, the concentrations of which were higher than those of the parent com-

pound. Like AZA, 6-MP soon disappeared from the plasma (Fig. 3). Similar observations have been reported in mice [24], the rhesus monkey [5] and man [18–20].

By far the highest plasma concentrations were found for TUA. These were 10- to 20-fold those of 6-MP (Fig. 3). Similar findings have been reported in patients after oral application of AZA [4, 7]. After i.p. injection of an almost equimolar dose of 6-MP in mice, Van Scoik et al. [28] observed TUA plasma concentrations in the same range as we did; however, these authors also reported a secondary increase in TUA concentration that started at 8 h after 6-MP administration and had not reached its maximum by the end of the 12-h observation period. This secondary TUA peak, which is also known from 6-MP-treated cell cultures [2], was explained by the metabolic degradation of intracellularly formed thiopurine nucleotides (Fig. 1). We could not detect any thiopurine metabolites in the plasma of AZA-treated mice at timepoints of >12 h after drug application. In agreement with our results, no secondary increase in TUA concentrations has been found in patients receiving oral AZA therapy [4].

Metabolic degradation, first-pass metabolism

TUA is the endpoint of the metabolic degradation of AZA (Fig. 1) and its main urinary metabolite [6]. In our study, TUA concentrations found in the intestinal mucosa and in the liver were higher than those in plasma (Fig. 4). This may indicate the preferential role of these two organs in AZA metabolism. The main enzyme involved in the formation of TUA is xanthine oxidase (XO). Unlike 6-MP, AZA itself is not a substrate of XO [6]. Thus, before oxidative degradation via XO can take place, the imidazole moiety of AZA must be cleaved from the purine structure. This step has been shown to occur nonenzymatically in the presence of SH-groups, e.g., glutathione [6]. From the observation that RBCs enable the conversion of AZA to 6-MP in vitro, it was concluded that RBCs play a major role in this step in vivo as well [6]. In contrast to this assumption is the previous finding that the glutathione depletion in rats following i.p. administration of AZA was more pronounced in the liver than in RBCs, which may indicate that the liver is the main site of 6-MP formation from AZA [10]. The extremely high 6-MP concentrations measured in the intestinal mucosa of AZA-treated mice (Fig. 4) indicate a substantial transformation of AZA to 6-MP in the gut wall.

It has been reported that the glutathione content in the intestinal mucosa of mice is as high as that in the liver [25]. This would enable the SH-dependent splitting of AZA into its imidazole moiety and 6-MP, a prerequisite for further oxidation of 6-MP to TUA by XO. The high TUA levels found in the intestinal mucosa are in good agreement with the high XO activity previously found in the small intestine of the mouse, which exceeded by several times even that detected in the liver [9]. Furthermore, it has been demonstrated in experiments using murine isolated jejunal loops that 6-MP is extensively metabolized to TUA during absorption [3]. In vivo, a substantial first-pass metabolism of 6-MP, which could be markedly inhibited by coadministration of the XO inhibitor allopurinol, has been shown in the rat and in man [22, 29]. For AZA, a first-pass metabolism after oral intake has thus far not been demonstrated. From our results we conclude that in addition to the liver, the intestinal wall plays an important role in the first-pass metabolism of AZA.

Anabolic metabolism

AZA-derived 6-MP can escape the primary metabolic degradation pathway if it enters intracellular ribonucleotide metabolism (Fig. 1). Due to the acid hydrolysis procedure used in the extraction process (see Materials and methods), the 6-MP concentrations measured in the processed organs of AZA-treated mice may originate not only from the thiopurine base 6-MP but also from its ribonucleotide TIMP. It has been shown in tumor cell cultures that the formation of TIMP from 6-MP proceeds rapidly [2]. The assumption that rapid intracellular formation of TIMP also took place in our in vivo study is supported by the following observations.

The initial concentrations of 6-MP extracted from most organs ranged 1 order of magnitude higher than the corresponding 6-MP plasma concentrations (Fig. 4). This indicates rapid cellular uptake of 6-MP and intracellular accumulation of its derivatives. Whereas 6-MP plasma levels declined rapidly (Fig. 3), the concentrations measured in some organs (e.g., spleen and bone marrow; Fig. 5) reached a plateau at up to 90 min after drug application. Unlike 6-MP, the polar ribonucleotide TIMP cannot cross cell membranes freely and is thus "captured" intracellularly. In a previous study [24], the 6-MP concentrations in the spleens of mice treated i.v. with AZA were only 50% of the corresponding 6-MP plasma levels, reached their maximum at as early as 5 min after drug administration, and lay below the detection limit after 30 min. The assay used in that study did not cover the 6-MP derivative TIMP. From these results we conclude that the high 6-MP concentrations that we detected in spleen homogenates after the acid hydrolysis procedure originated almost exclusively from TIMP.

We have extracted substantial amounts of 6-TG from the processed organs of AZA-treated mice (Fig. 4). The long persistence of these metabolites in the tissues and the absence of 6-TG in plasma again indicate the intracellular formation of 6-TG ribonucleotides (TGNs), which cannot cross cell membranes. In 6-MP-treated cell cultures, the intracellular formation of TGNs can be reduced by pretreatment with the IMPDH and GMPS inhibitor mycophenolic acid [26]. This suggests that TGNs are formed from TIMP by nucleotide interconversion, with 6-thioxanthosine monophosphate (TXMP) being an intermediate product (see Fig. 1).

This hypothesis is supported by our finding that in the processed spleen and bone marrow samples, the metabolite that first reached its peak concentration was 6-MP, followed by 6-TX and, finally, by 6-TG (Fig. 5). This sequence would be expected from the nucleotide interconversion pathway shown in Fig. 1 and indicates that the thiopurine bases extracted after the acid hydrolysis procedure originated from their corresponding ribonucleotides

TIMP, TXMP, and TGMP, respectively. A similar sequence of appearance of TIMP, TXMP, and TGMP has been observed in 6-MP-treated cell cultures [2], whereas the in vivo formation of TGN from AZA or 6-MP via the nucleotide interconversion pathway has not previously been demonstrated.

Figure 4 shows the large variability of the 6-TG concentrations extracted from the different organs, which could reflect the differing extent of TGN formation. The incorporation of TGN into DNA has been shown to be the cytotoxic mechanism of 6-MP in cell cultures [26]. More recently, the molecular mechanism of DNA damage resulting from TGN incorporation has been elucidated [8]. From clinical experience, bone marrow depression is known to be the main cytotoxic effect of AZA, which is also cytotoxic to lymphocytes, an effect that causes a substantial reduction in spleen weight in mice [23]. We found the highest concentrations of 6-TG derivatives in the bone marrow and spleens of AZA-treated mice (Fig. 4). This may indeed indicate that 6-TG derivatives are essentially involved in the development of AZA toxicity.

The incidence of cancer in renal transplant recipients is significantly higher than that in the control population. The question has repeatedly been raised as to whether this is due only to the suppression of "immunosurveillance" or whether the drugs used in the immunosuppression protocols may also have direct carcinogenic properties [21]. Our experimental results cannot provide an answer to this question, but the formation of TGN shown in the present study and the previous finding that TGN incorporation into DNA causes DNA damage [8] raise the possibility that AZA itself is a carcinogenic agent. In this context, it is interesting that elevated TGN levels in RBCs of patients on AZA or 6-MP therapy are associated not only with myelosuppression [13, 14] but also with the development of skin cancer [15].

TGNs have been found to be the major AZA metabolites in RBCs of patients on long-term therapy [12]. It has tentatively been concluded that the liver may play an important role in the ultimate appearance of TGNs in RBCs [12]. Our results do not support this hypothesis: the concentrations of 6-TG derivatives in the liver were rather low (Fig. 4), and we did not detect any TGNs in the RBCs of mice that had received a single dose of AZA. However, high concentrations of 6-TG derivatives were measured in the bone marrow samples (Fig. 5). We therefore assume that the TGNs found in the RBCs of patients on long-term AZA or 6-MP therapy are formed in bone marrow cells during erythropoiesis and persist in the mature RBCs.

In agreement with this assumption, a slow accumulation of TGNs in human RBCs after the onset of AZA or 6-MP therapy (steady-state conditions were achieved only after several weeks) and a long persistence of TGN levels in these cells (even after the discontinuation of AZA) have been observed [13, 14]. If the TGNs in RBCs of patients on long-term AZA therapy originated from the bone marrow, the TGN concentrations in the former cells could be a reflection of the cytostatically active nucleotides within the bone-marrow stem cells. Thus, the measurement of TGN concentrations in RBCs would be a reasonable approach to therapeutic drug monitoring during AZA or 6-MP therapy.

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