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## Hypoxanthine-guanine phosphoribosyltransferase-independent toxicity of azathioprine in human lymphoblasts

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6-Mercaptopurine (6-MP\*) and its imidazole derivative, azathioprine (AZ), are clinically useful immunosuppressive agents. AZ in particular has gained widespread use and has been cited as "the most widely used cytotoxic immunosuppressive agent in clinical medicine" [1]. The biochemical and metabolic effects of these thiopurines have been reviewed [1-4]. However, the precise mechanism of their immunosuppressive activity is not clearly understood [1,2].

The cytotoxicity of 6-MP is dependent upon its phosphoribosylation to thioinosinic acid (TIMP) which is catalyzed by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8). Cell lines which are resistant to 6-MP have been shown to lack HPRT activity [5], and neither 6-MP nor AZ has activity in HPRT-deficient patients [6]. TIMP is an effective inhibitor of purine nucleotide biosynthesis and interconversion (see Fig. 1). It is a competitive inhibitor of adenylosuccinate synthetase, adenylosuccinate lyase, and IMP dehydrogenase, and it is a pseudo feedback inhibitor of glutamine-phosphoribosylpyrophosphate amidotransferase, the first enzyme unique to the *de novo* purine nucleotide biosynthetic pathway [3,4]. Other effects of TIMP include incorporation into nucleic acid, the inhibition of coenzyme formation and function, and the inhibition of protein synthesis [3,4].

Many of the metabolic effects of AZ are dependent upon its cleavage to 6-MP and the subsequent phosphoribosylation to TIMP. However, there has been increasing evidence which indicates that AZ has metabolic effects which may account for its distinct and more widespread clinical appli-

cability as an immunosuppressant as compared to 6-MP [1-4,7,8]. These effects might be attributable to biologically active metabolites unique to AZ or could possibly be due to the reaction of the methylnitroimidazole moiety with active metabolites or with potentially reactive groups on cellular proteins [2].

In this study, utilizing HPRT<sup>+</sup> and HPRT-deficient human lymphoblastoid cell lines, we have found evidence which suggests that the cytotoxicity of AZ in cell culture, unlike that of 6-MP, is, in part, independent of HPRT activity.

### Materials and methods

**Chemicals.** Azathioprine, 6-mercaptopurine, glutamine and the purine nucleosides and bases used were purchased from the Sigma Chemical Co. Heat-inactivated horse serum and RPMI 1640 medium were purchased from the Grand Island Biological Co. RPMI 1640 contains glutathione (1 mg/l) as one of its components.

**Cell lines.** Human lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 2 mM glutamine. The MGL-8 cell line (HPRT<sup>+</sup>) was derived from a normal individual and was a gift from J. Epstein, Johns Hopkins University. The GM-130 cell line, derived from a normal individual, and the GM-467 (HPRT-deficient, mutagenized) cell line were obtained from the Human Genetics Mutant Cell Repository. The HPRT-deficient cell line HD was derived from a patient presenting with the Lesch-Nyhan Syndrome by Epstein-Barr virus transformation by the method described previously [9]. The MOLT-4 cell line (HPRT<sup>+</sup>), originally derived from a patient presenting with the Lesch-Nyhan Syndrome by Epstein-Barr virus transformation by the method described previously [9]. The MOLT-4 cell line (HPRT<sup>+</sup>), originally derived from a patient with acute

\* Abbreviations: 6-MP, 6-mercaptopurine; AZ, azathioprine; TIMP, thioinosinic acid; and HPRT, hypoxanthine-guanine phosphoribosyltransferase.

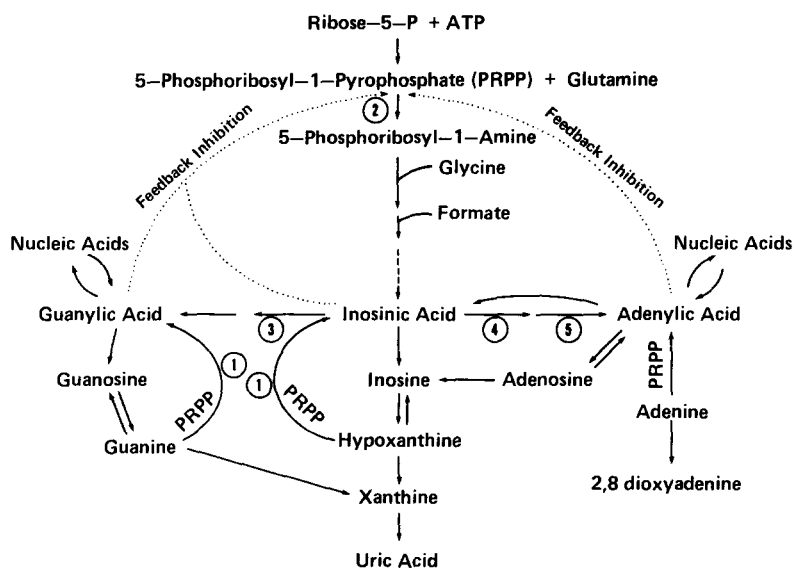


Fig. 1. Purine metabolic pathways. The phosphoribosylation of 6-MP to thioinosinic acid (TIMP) is catalyzed by (1) hypoxanthine-guanine phosphoribosyltransferase (HPRT). TIMP can potentially interfere with purine nucleotide biosynthesis by inhibiting the activities of (2) phosphoribosylpyrophosphate amidotransferase, (3) IMP dehydrogenase, (4) adenylosuccinate synthetase, and (5) adenylosuccinate lyase [2-4].

lymphoblastic leukemia, was obtained from HEM Research. The levels of HPRT enzyme activity in the MGL-8 and GM-130 cell lines were reported to be  $18.4 \pm 1.5$  and  $11.4 \pm 2.5$  mU/mg, respectively, while the levels of HPRT immunoreactive protein in these cell lines were reported as  $442 \pm 45$  and  $264 \pm 19$  ng HPRT/mg respectively [9]. Using similar methodology, the GM-467 and HD cell lines were found to have undetectable HPRT enzyme activity or HPRT immunoreactive protein (data not shown).

**Growth studies.** All lymphoblast growth studies were initiated at a cell density of approximately  $2.0 \times 10^5$  cells/ml. After 3 days of incubation at  $37^\circ$ , viable cells were counted using a hemacytometer, with trypan blue dye exclusion used as an index of viability. The toxic effects of AZ and 6-MP on the growth of HPRT<sup>+</sup> and HPRT-deficient human lymphoblasts were evaluated by determining the concentration of each drug at which there was a 50% reduction in the control cell density after 3 days of incubation ( $IC_{50}$ ).

#### Results and discussion

The effects of 6-MP and AZ on the growth of normal (HPRT<sup>+</sup>) and HPRT-deficient human lymphoblasts are summarized in Table 1 and in Fig. 2. The HPRT<sup>+</sup> lymphoblasts were sensitive to both 6-MP and AZ, with AZ appearing to be somewhat less toxic than 6-MP at equimolar doses, especially in the HPRT<sup>+</sup> T lymphoblasts. The average  $IC_{50}$  values of 6-MP and AZ for the HPRT<sup>+</sup> B cells were 1.8 and 3.8  $\mu$ M respectively. In the HPRT<sup>+</sup> T cell line, MOLT-4, the  $IC_{50}$  for 6-MP was 1.0  $\mu$ M and for AZ, 7.6  $\mu$ M. The lower toxicity of AZ as compared to 6-MP at equivalent doses is in accordance with previous reports, and has been postulated as an explanation for the reported clinical efficacy of AZ as compared to 6-MP [2]. As expected, based on the inability of the cells to convert 6-MP to thioinosinic acid, the growth of the HPRT-deficient B lymphoblast cultures was unaffected by 6-MP concentrations as high as 200  $\mu$ M, the highest concentration used in these experiments. However, AZ was found to be toxic to both the chemically mutagenized HPRT-deficient B cell line (GM-467) and the B lymphoblastoid cell line derived from a patient presenting with the Lesch-Nyhan

Syndrome (HD), with respective  $IC_{50}$  values of 30 and 32  $\mu$ M. These values are approximately 8-fold higher than the  $IC_{50}$  values for the HPRT<sup>+</sup> B lymphoblasts, indicating that there was some resistance to the toxic effects of AZ in the HPRT-deficient cells; however, these cell lines were resistant to 6-MP concentrations at least 100-fold greater than the  $IC_{50}$  of 6-MP for the HPRT<sup>+</sup> B lymphoblasts. The discrepancy between the resistance of the HPRT-deficient cells to 6-MP and AZ indicates that, unlike the toxicity of 6-MP, the toxicity of AZ at concentrations greater than 25  $\mu$ M was independent of HPRT activity.

Supplementation of cultures with either adenine or inosine protected HPRT<sup>+</sup> lymphoblasts from growth inhibition by 6-MP at concentrations at least as high as 180  $\mu$ M (Table 1 and Fig. 2A). Supplementation of cultures with adenine, deoxyadenosine, deoxyinosine, or hypoxanthine similarly protected these cells from growth inhibition by 6-MP (data not shown). However, supplementation of cultures with guanosine, deoxyguanosine, or xanthosine did not appear to affect the toxicity of 6-MP (data not shown). Both guanosine and deoxyguanosine can be converted to guanine in these cells. Because guanine and hypoxanthine are both substrates for HPRT, it is highly unlikely that the effect of the other purines was due solely to competition for HPRT by hypoxanthine accumulated as a metabolite of these purines. The principal mechanism by which the metabolites of 6-MP exert toxicity is through the inhibition of *de novo* purine biosynthesis and purine nucleotide interconversions [1-4]. The purines which protect the cells from the toxicity of 6-MP can be utilized by the purine salvage and nucleotide interconversion pathways in the HPRT<sup>+</sup> lymphoblasts to form both adenine and guanine nucleotides (Fig. 1). However, guanosine and deoxyguanosine can only be utilized to form guanine nucleotides as the ability to convert guanine nucleotides to adenine nucleotides is absent in mammalian cells [10]. It appears most likely that, by providing an alternative source for purine nucleotide synthesis, purine supplementation protects the cells from effects of the inhibition of the *de novo* purine biosynthetic pathway by TIMP and other metabolites of 6-MP.

The addition of 50  $\mu$ M inosine or adenine to cultures of

HPRT<sup>+</sup> B lymphoblasts incubated with 6-MP raised the  $IC_{50}$  for 6-MP in these cell lines by over 100-fold (from 1.8 to >180  $\mu$ M for 50  $\mu$ M inosine and to >180  $\mu$ M for 50  $\mu$ M adenine). Higher concentrations of inosine further protected the cells from growth inhibition by 6-MP (Fig. 2A). In contrast, when 50  $\mu$ M adenine or inosine was added to cultures of HPRT<sup>+</sup> B lymphoblasts incubated with AZ, the  $IC_{50}$  for AZ in these cells was raised approximately 10-fold

(Table 1 and Fig. 2B). Higher concentrations of either adenine or inosine did not further affect the toxicity of AZ. Interestingly, the  $IC_{50}$  values of AZ for the HPRT<sup>+</sup> cell lines supplemented with adenine or inosine are comparable to those observed in the HPRT-deficient cell lines. Neither adenine nor inosine had any marked effect on the growth inhibition of the HPRT-deficient lymphoblasts by AZ. Although inosine cannot be utilized as a purine source in HPRT-deficient cells since B lymphoblasts lack the ability to phosphorylate inosine, adenine can be utilized in these cells to form both adenine and guanine nucleotides. In addition we observed no effect on the toxicity of AZ from the addition of 50  $\mu$ M adenine to cultures of an adenine phosphoribosyltransferase (EC 2.4.2.7)-deficient human B lymphoblast cell line which cannot utilize adenine to form purine nucleotides. In contrast, 50  $\mu$ M inosine raised the  $IC_{50}$  of AZ in this cell line from 2.7 to 39  $\mu$ M. Again, this is comparable to the  $IC_{50}$  of AZ in the HPRT-deficient cell lines. In the HPRT<sup>+</sup> lymphoblasts, supplementing cultures with adenosine, deoxyadenosine, deoxyinosine or hypoxanthine had effects on the toxicity of AZ which were similar to those of adenine and inosine, and supplementing the cultures with guanosine, deoxyguanosine or xanthosine did not appear to affect AZ toxicity (data not shown). Again, only supplementation of cultures with purines which can be utilized to form both adenine and guanine nucleotides affects the toxicity of AZ. These results suggest that the addition of purine relieves only the HPRT-dependent toxicity of AZ, presumably by circumventing the effects of the inhibition of *de novo* purine biosynthesis and/or nucleotide interconversions. Because added purines do not protect cells from the HPRT-independent effects of AZ, these effects are probably not directly due to purine nucleotide depletion.

The data presented in this communication strongly suggest that the cytotoxicity of AZ, unlike that of 6-MP, is, in part, independent of HPRT activity. The HPRT-independent toxicity of AZ was not affected by purine supplementation and is therefore unlikely to be associated with the inhibition of *de novo* purine biosynthetic or interconversion pathways. The culture medium concentrations at which the HPRT-independent toxicity of AZ exceeds the reported therapeutic plasma range concentration (upper limit 7  $\mu$ M [2]) for AZ in man, and the relationship of our observations to clinical differences between AZ and 6-MP, remain unestablished. A preliminary report of our results was presented earlier [11].

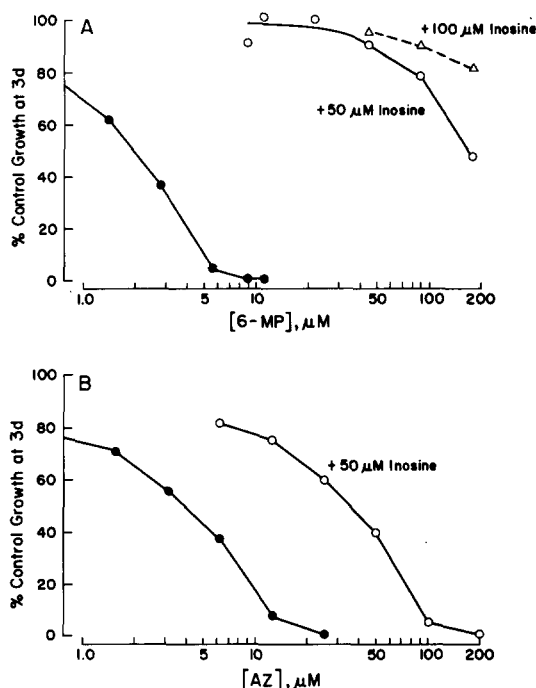


Fig. 2. Effect of inosine on the toxicity of (A) 6-MP and (B) AZ in normal (HPRT<sup>+</sup>) B lymphoblasts (MGL-8). The effect of each drug alone (●—●) is compared to the effect of the drug in the presence of 50  $\mu$ M inosine (○—○) and, for 6-MP, 100  $\mu$ M inosine (Δ—Δ). There was little difference between the effects of 100  $\mu$ M inosine and 50  $\mu$ M inosine on the toxicity of AZ (see Table 1).

Table 1. Effects of 6-MP and AZ on the growth of normal (HPRT<sup>+</sup>) and HPRT-deficient human lymphoblasts\*

Cell line	$IC_{50}$ ( $\mu$ M)									
	6-Mercaptopurine					Azathioprine				
	6-MP	+ Inosine		+ Adenine		AZ	+ Inosine		+ Adenine	
	50 $\mu$ M	100 $\mu$ M	50 $\mu$ M	100 $\mu$ M		50 $\mu$ M	100 $\mu$ M	50 $\mu$ M	100 $\mu$ M	
<b>B lymphoblasts</b>										
MGL-8	1.8	179	>180	>180	>180	3.7	35	42	54	38
GM-130	1.7	>180	>180	>180	>180	4.0	40	34	43	41
<b>HPRT-deficient B Lymphoblasts</b>										
GM-467	>200	—	—	—	—	30	29	21	34	36
HD	>200	—	—	—	—	32	31	26	46	33
<b>T Lymphoblasts</b>										
MOLT-4	1.0	175	>200	>200	>200	7.6	36	41	40	34

\* The toxic effects of AZ and 6-MP on normal (HPRT<sup>+</sup>) and HPRT-deficient human lymphoblasts were evaluated by determining the concentration of each drug which caused a 50% inhibition of the growth of the culture over a 3-day incubation ( $IC_{50}$ ) relative to control cultures (no drug added). The initial cell density in these experiments was approximately  $2 \times 10^5$  cells/ml. The growth of each culture was determined by subtracting the initial cell density from the cell density after a 3-day incubation at 37°. Each  $IC_{50}$  value represents one to eleven determinations.

To summarize, the toxic effects of AZ and 6-MP on normal (HPRT<sup>+</sup>) and HPRT-deficient human lymphoblasts were evaluated. HPRT-deficient B lymphoblasts were resistant to 6-MP at concentrations as high as 100-fold greater than for HPRT<sup>+</sup> lymphoblasts. In contrast, the IC<sub>50</sub> values for AZ in the HPRT-deficient lymphoblasts were less than 10-fold greater than for the HPRT<sup>+</sup> B cell lines. The data suggest that the growth-inhibitory effects of AZ, unlike those of 6-MP, are, in part, independent of phosphoribosylation by HPRT. We also found that supplementing the cells with a purine which could be utilized to form both adenine and guanine nucleotides prevented the HPRT-dependent inhibition of lymphoblast growth, presumably by alleviating the effects of the inhibition of *de novo* purine biosynthesis. However, added purines did not protect the cells from the HPRT-independent effects of AZ, which suggests that these effects are not due to purine nucleotide depletion.

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