Inhibition by 6-Mercaptopurine of Purine Phosphoribosyltransferases from Ehrlich Ascites-Tumour Cells that are Resistant to this Drug

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1. A strain of Ehrlich ascites-tumour cells that showed little inhibition of growth in the presence of 6-mercaptopurine accumulated less than 5% as much 6-thioinosine 5′-phosphate *in vivo*, in the presence of 6-mercaptopurine, as did the sensitive strain from which it was derived. 2. Specific activities of the phosphoribosyltransferases that convert adenine, guanine, hypoxanthine and 6-mercaptopurine into AMP, GMP, IMP and 6-thioinosine 5′-phosphate were similar in extracts of the resistant and the sensitive cells. 3. As found previously with sensitive cells, 6-mercaptopurine is a competitive inhibitor of guanine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase from the resistant cells and does not inhibit the adenine phosphoribosyltransferase from these cells. Michaelis constants and inhibitor constants of the purine phosphoribosyltransferases from resistant cells did not differ significantly from those measured with the corresponding enzymes from sensitive cells. 4. Resistance to 6-mercaptopurine in this case is probably not due to qualitative or quantitative changes in these transferases.

6-Mercaptopurine is a powerful competitive inhibitor of the conversion of guanine into GMP and of hypoxanthine into IMP, but does not inhibit the conversion of adenine into AMP, in the presence of 5-phosphoribosyl pyrophosphate and the corresponding purine phosphoribosyltransferases in extracts of Ehrlich ascites-tumour cells (Atkinson & Murray, 1965). The convenient assay, with radioactive purines, that was developed in this investigation has now been used to measure the Michaelis constants and specific activities for conversion of adenine, guanine, hypoxanthine and 6-mercaptopurine into their nucleotides with extracts of Ehrlich ascites-tumour cells that are resistant to the action of 6-mercaptopurine. The results show that 6-mercaptopurine is a competitive inhibitor of formation of GMP from guanine and of IMP from hypoxanthine but does not inhibit formation of AMP from adenine in extracts of resistant cells. Michaelis constants and inhibitor constants measured with extracts of resistant cells do not differ significantly from those found with sensitive cells, and the specific activities of the purine phosphoribosyltransferases from the two types of cells are similar.

A resistant strain of Ehrlich ascites-tumour cells has been shown (Paterson, 1960; Paterson & Hori, 1962) to be much less effective than a sensitive strain in converting 6-mercaptopurine into 6-thioinosine 5′-phosphate. Paterson (1962) found that extracts of resistant cells contained 6-mercaptopurine phosphoribosyltransferase and suggested that decreased synthesis of 6-thioinosine 5′-phosphate was due to loss of the transport mechanism by which 6-mercaptopurine enters the cells. Our results support Paterson's (1962) findings and also show that resistance is not due to decreased sensitivity of guanine or hypoxanthine phosphoribosyltransferases to the inhibitory activity of 6-mercaptopurine.

EXPERIMENTAL

Substrates and inhibitors. Samples of radioactive and non-radioactive purines were those described by Atkinson & Murray (1965). 6-Mercapto[8-^{14}C]purine, from California Corp. for Biochemical Research, contained no contaminants that could be detected by spectrophotometry or by radioautography after chromatography in the propanol–aq. NH_3 system (R_F 0·54) and in the butanol–propionic acid system (R_F 0·48) described by Atkinson & Murray (1965).

Development of a strain of Ehrlich ascites-tumour cells resistant to the action of 6-mercaptopurine. After 20 successive inoculations in the presence of 6-mercaptopurine, as described by Paterson (1960), a strain of ascites-tumour cells that resisted the action of the antimetabolite was obtained. Growth of these resistant cells during 7 days in animals injected daily with 38 mg. of 6-mercaptopurine/kg. body weight was 84±4% (in 20 animals) of the growth in control animals injected with 0·9% NaCl (for conditions of the test,

see Paterson, 1960). In the same conditions, growth of sensitive cells was 11±2% (in 20 animals) of that in controls. Extracts for assays of purine-phosphoribosyltransferase activity were prepared as described by Atkinson & Murray (1965).

Tests for conversion of 6-mercaptopurine into 6-thioinosine 5′-phosphate in vivo *with sensitive and resistant cells*

Analysis by electrophoresis. Groups of three mice (body wt. about 25 g.) were inoculated with about 2×10^7 sensitive or resistant cells and after 7 days the mice were injected intraperitoneally with 5 μmoles of 6-mercaptopurine in 0·9% NaCl. The injection was repeated twice at 24 hr. intervals, and 1 hr. after the last injection the ascitic fluid was collected and centrifuged for 5 min. at 2000 *g*. After measurement of the volume of the packed cells (4–5 ml.) they were washed twice with 10 ml. of cold 0·9% NaCl and suspended in 10 ml. of 5% (w/v) trichloroacetic acid at 1°. After extraction of the insoluble residue with cold 5% trichloroacetic acid (2 × 5 ml.) the combined extracts were extracted with ether (3 × 50 ml.) and the aqueous phase was evaporated at 25–30°/15 mm. The residue was dissolved in water (0·1 ml./ml. of packed cells used) and 0·05 ml. portions of the solution were subjected to electrophoresis in 0·05 M-citrate (adjusted to pH 4·8 with tris) on Whatman 3MM paper under carbon tetrachloride at 30 v/cm. for 2·5 hr. Areas corresponding to thio-IMP (which was added as an internal standard to replicate samples and migrated towards the anode directly ahead of AMP) were eluted with 10 ml. of water and extinctions were measured against blanks from corresponding areas of paper after adjustment to pH 5·5 with NaOH. Concentrations of thio-IMP* were calculated from the millimolar extinction coefficient of 24·1 at 321·5 mμ (Atkinson, Morton & Murray, 1963).

Analysis by anion-exchange chromatography. In one experiment with sensitive cells the final injection was of 6-mercapto[8-^{14}C]purine (5 μmoles; 1·6 μc). The nucleotide fraction, after extraction with ether, was applied to a column (14 cm. × 2 cm.^{2}) of DEAE-cellulose (HCO_3^- form). Water (200 ml.) was passed through the column, followed by linear gradients of NH_4HCO_3 (600 ml. from 0 to 50 mM and 1 l. from 50 to 200 mM) and then by 250 ml. of 0·4 M-NH_4HCO_3. Extinctions of each 7 ml. fraction were measured at 260 mμ and 320 mμ and the radioactivity of a 0·2 ml. sample from each fraction with $E_{260m\mu}$ or $E_{320m\mu}$ greater than 0·05 was measured by liquid-scintillation counting with a scintillator suitable for aqueous solutions (Herberg, 1960). Thio-[8-^{14}C]IMP was eluted between ADP and ATP by 140–165 mM-NH_4HCO_3 and after removal of volatile material at 25°/15 mm. was further purified by electrophoresis in the tris–citrate system described above. The spectroscopic, chromatographic and electrophoretic properties of the product were identical with those of synthetic thio-IMP (cf. Atkinson *et al.* 1963).

* Abbreviation: thio-IMP, 6-thioinosine 5′-phosphate.

Kinetic studies with purine phosphoribosyltransferases from resistant ascites-tumour cells

Conditions of assay with radioactive adenine, guanine and hypoxanthine in the presence and absence of non-radioactive 6-mercaptopurine, and procedures for calculation of kinetic parameters, were those used before (Atkinson & Murray, 1965). In assays with 6-mercapto[8-^{14}C]purine, material with a specific activity 0·47 μc/μmole was used in the assay system described before for [8-^{14}C]hypoxanthine, 6-mercaptopurine replacing hypoxanthine; final concentrations of 6·29, 12·6, 31·5 and 63·0 μM-6-mercaptopurine were used, and radioactivity in the nucleotide fraction was measured at 0, 1, 2 and 4 min. for each concentration. The Michaelis constant of 6-mercaptopurine with an extract of sensitive cells was also measured by this method (for results see Table 1). In assays with 6-mercapto[8-^{14}C]purine the disks of DEAE-cellulose were washed with 8 mM-NH_4HCO_3; 6-mercaptopurine is a stronger acid than hypoxanthine and is not completely removed by the 4 mM-NH_4HCO_3 used before.

To test for the validity of the assay with 6-mercapto-[8-^{14}C]purine, 95 mμmoles (56 mμc) of this purine were treated with extracts of sensitive and of resistant cells (6·0 and 6·6 mg. of protein respectively) in the conditions described before for [8-^{14}C]hypoxanthine. On chromatography of the products in the butanol–propionic acid system (Atkinson & Murray, 1965) the only radioactive compounds detected were thio-IMP (R_F 0·14) and unchanged 6-mercaptopurine.

Table 1. *Inhibition of purine phosphoribosyltransferases from Ehrlich ascites-tumour cells by 6-mercaptopurine*

Values in parentheses are the coefficients of variation of the means.

Substrate	K_m (μM)	V (mμmoles/min./mg. of protein)	Concn. of 6-mercaptopurine (μM)	K_p (μM)	V_p (mμmoles/min./mg. of protein)	K_i (μM)
Adenine	0·89 (0·046)	1·08 (0·01)	32	0·91 (0·10)	1·06 (0·03)	—
Hypoxanthine	7·85 (0·15)	0·87 (0·03)	32	37·3 (0·05)	0·78 (0·03)	8·49 (0·19)
Guanine	1·60 (0·13)	0·55 (0·02)	34	30·7 (0·07)	0·56 (0·05)	1·82 (0·16)
6-Mercaptopurine	8·56 (0·11)	0·34 (0·03)	—	—	—	—
6-Mercaptopurine*	7·55 (0·17)	0·38 (0·05)	—	—	—	—

* Measured with an extract from sensitive cells.

An extract of resistant cells was tested as described before for enzymes which could catalyse cleavage of AMP, GMP, IMP or thio-IMP in the conditions of the assay.

RESULTS AND DISCUSSION

Ehrlich ascites-tumour cells grown in mice that were injected with 6-mercaptopurine became more resistant to the inhibitory activity of this drug, as reported by Paterson (1960). The accumulation of thio-IMP by resistant cells in mice injected with 6-mercaptopurine was compared with that in sensitive cells *in vivo*. Paterson (1960) found that resistant cells accumulated only traces of thio-IMP in these conditions, whereas sensitive cells accumulated up to 0·42 μmole of thio-IMP/ml. of packed cells. The resistant cells used in the kinetic studies reported here accumulated about 0·01 μmole of thio-IMP/ml. of packed cells and the sensitive cells accumulated up to 0·27 μmole of the nucleotide/ml. of packed cells. It is not known if the synthesis of thio-IMP was due to a small proportion of sensitive cells in the resistant population. If this were the case the proportion of sensitive cells could have been no more than 5%, and the corresponding contamination of the transferase preparation used in kinetic studies would not have caused serious errors in measurement of kinetic parameters.

As with the extract from sensitive cells, there was no evidence that the extract from resistant cells contained enzymes that would interfere to a significant extent with the assays of purine phosphoribosyltransferases. When the standard assay systems with radioactive adenine, guanine, hypoxanthine and 6-mercaptopurine were allowed to react with extract from resistant cells for 15 min. the extent of conversion of these purines into their nucleotides was 95, 93, 96 and 98% respectively. When 5·2 μM-[8-^{14}C]IMP was treated with the extract of resistant cells in the presence of 5-phosphoribosyl pyrophosphate and non-radioactive hypoxanthine in the conditions of the assay (cf. Atkinson & Murray, 1965) 6–7% of the nucleotide was converted into hypoxanthine and 0·1–0·2% of inosine was detected. In a corresponding experiment with 6 μM-[8-^{14}C]AMP 1% of the nucleotide was converted into adenine and 0·4% was converted into IMP. In the conditions of the assay, but without 5-phosphoribosyl pyrophosphate, the change in the extinction at 255 mμ was less than 0·002/10 min. in a 1 cm. cell with 25 μM-guanine or with 23 μM-GMP, nor was there a detectable change in the extinction at 320 mμ in corresponding experiments with 24 μM-6-mercaptopurine or with 19 μM-thio-IMP. From these results there is no evidence that resistance to 6-mercaptopurine is due to more rapid destruction of thio-IMP in the cells. However, insoluble components of the homogenate of broken cells were not examined in this respect.

The specific activities of adenine, guanine, hypoxanthine and 6-mercaptopurine phosphoribosyltransferases (1·08, 0·55, 0·87 and 0·34 mμmoles/min./mg. of protein respectively; see Table 1) were 64, 45, 130 and 89% of values found with corresponding extracts from sensitive cells (cf. Table 1 and Atkinson & Murray, 1965). Paterson (1962) reported no significant difference in the last of these activities with extracts from sensitive or from resistant cells.

With the extract from resistant cells, extrapolated maximum specific activities of guanine phosphoribosyltransferase in the presence or in the absence of 34 μM-6-mercaptopurine (0·56 $\pm$ 0·03 and 0·55 $\pm$ 0·01 mμmole/min./mg. of protein respectively) did not differ significantly, nor did the corresponding specific activities of hypoxanthine phosphoribosyltransferase (0·78 $\pm$ 0·02 and 0·87 $\pm$ 0·03 mμmole/min./mg. of protein) in the presence or absence of 32 μM-6-mercaptopurine. However, the apparent values of the Michaelis constants were increased from 1·60 $\pm$ 0·2 μM-guanine to 30·7 $\pm$ 2·1 μM-guanine and from 7·85 $\pm$ 1·2 μM-hypoxanthine to 37·3 $\pm$ 1·9 μM-hypoxanthine in the presence of 34 and 32 μM-6-mercaptopurine respectively. 6-Mercaptopurine is therefore a competitive inhibitor of guanine and hypoxanthine phosphoribosyltransferases from resistant Ehrlich ascites-tumour cells. The inhibitor constants (1·8 $\pm$ 0·3 and 8·5 $\pm$ 1·6 μM-6-mercaptopurine respectively) are similar to the corresponding values found with the extract of sensitive cells (3·4 and 8·3 μM-6-mercaptopurine; Atkinson & Murray, 1965). The Michaelis constants found with the extract of resistant cells are similar to those (2·8 μM-guanine and 11·0 μM-hypoxanthine) found previously (Atkinson & Murray, 1965) with extracts of sensitive cells.

The extrapolated maximum specific activities of adenine phosphoribosyltransferase in the extract from resistant cells and the Michaelis constants for adenine (1·06 $\pm$ 0·03 and 1·08 $\pm$ 0·01 mμmoles/min./mg. of protein; 0·91 $\pm$ 0·10 μM and 0·89 $\pm$ 0·04 μM-adenine; cf. Table 1) were not significantly different in the presence of 32 μM-6-mercaptopurine or in the absence of this purine. The corresponding enzyme in extracts of sensitive cells was also not inhibited by 6-mercaptopurine (25 μM) and had the same Michaelis constant (0·89 μM-adenine) as that reported here.

These kinetic studies provide no evidence of major qualitative or quantitative differences between the purine phosphoribosyltransferases of Ehrlich ascites-tumour cells that are resistant or sensitive to 6-mercaptopurine and indicate that the mechanism of resistance is not related to the properties of these enzymes.

The results reported above support Paterson's

(1962) suggestion that a possible mechanism of the change in development of resistance is the exclusion of 6-mercaptopurine from the corresponding phosphoribosyltransferase inside the cell. It is not known at present if this exclusion is through a change in permeability to 6-mercaptopurine or to a change in an enzymic process involved in the transfer of the drug into the cells. The results are consistent with the suggestion (Atkinson & Murray, 1965) that inhibition by 6-mercaptopurine of biosynthesis of IMP and GMP from hypoxanthine and guanine is a potential site of action of the drug.

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