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INHIBITION OF HUMAN LEUKOCYTE PYRIMIDINE DEOXYNUCLEOSIDE SYNTHESIS BY ALLOPURINOL AND 6-MERCAPTOPURINE

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Abstract—Allopurinol and 6-mercaptopurine, two clinically useful purine antagonists, are inhibitors of deoxythymidine phosphorylase and pyrimidine deoxyribosyltransferase from human leukocytes. The inhibition of these enzyme activities is only in the direction of nucleoside synthesis and is not competitive with either substrate (thymine or deoxyribose-1-phosphate). 6-Mercaptopurine, but not allopurinol, also inhibits uridine phosphorylase.

DURING an investigation of the enzymatic mechanisms for pyrimidine deoxynucleoside synthesis in homogenates of human leukocytes, we observed that some naturally occurring purine bases markedly inhibited synthesis of deoxythymidine and deoxyuridine. It was therefore of interest to investigate the effect of purine analogs, especially those in wide clinical use, on these reactions. As shown in this report, allopurinol [4-hydroxypyrazolo (3,4-d) pyrimidine] (allo) and 6-mercaptopurine (6-MP)*, whose mechanisms of action had not been shown previously to be involved directly with pyrimidine metabolism, are potent inhibitors of deoxythymidine and deoxyuridine synthesis *in vitro*.

Allopurinol inhibits xanthine oxidase *in vitro* and *in vivo*,[1] thereby preventing xanthine production from hypoxanthine and uric acid formation from xanthine. It is now employed for the prevention of hyperuricemia and uric acid nephropathy both in gouty individuals and in patients with neoplastic diseases, particularly in those with leukemia or lymphoma when it is anticipated that anti-tumor therapy will result in a large increase in nucleic acid breakdown.[2–5]

6-MP inhibits tumor growth in animals and is used extensively in the treatment of childhood acute leukemia. The principal inhibitory effects of 6-MP are through its ribonucleotides, which act on several enzymatic reactions involved in purine biosynthesis, although other inhibitory properties have been described.[6–8]

One pathway for the synthesis of deoxythymidine in human leukocytes is mediated by deoxythymidine phosphorylase (EC 2:4:2:4).[9] This enzyme catalyzes the reversible reaction: thymine + deoxyribose-1-phosphate ⇋ deoxythymidine + Pi.[10, 11] It has recently been shown that the deoxythymidine phosphorylase and pyrimidine deoxyribosyltransferase activities are associated with the same protein,[12] the latter catalyzing the following reaction: thymine + deoxynucleoside ⇋ deoxythymidine + free base.

* Abbreviations used: 6-MP, 6-mercaptopurine.

MATERIALS AND METHODS

Allopurinol and deoxyribose-l-phosphate were obtained from Calbiochem, Los Angeles, Calif.; other purine bases and all pyrimidine bases and nucleosides were obtained from Sigma Chemical Co., St. Louis, Mo. ^{14}C-labeled bases and nucleosides were obtained from New England Nuclear Corp., Boston, Mass.

Leukocytes were obtained by differential centrifugation of whole blood from normal individuals (60–80 per cent granulocytes). Crude extracts were prepared by homogenizing 5×10^8 leukocytes in 5 ml of 0·1 M Tris-HCl buffer, pH 7·3. A 30,000 *g* supernatant fraction of the crude homogenate was used as the enzyme source. The final reaction mixture for assaying deoxythymidine synthesis contained in a total volume of 0·5 ml: 1 μmole ^{14}C-thymine (0·5 μc/μmole); 25 μmole sodium succinate buffer, pH 7·2; 25 μmole Tris-HCl buffer, pH 7·3; and 5 μmole deoxyribose-l-phosphate. Incubations were for 30 min at 37°. Reactions were stopped by boiling for 5 min. An aliquot was applied to Whatman No. 1 paper with deoxythymidine and thymine as carriers. The labeled compounds were separated by descending paper chromatography in a system consisting of the upper phase of a mixture of ethyl acetate:water:formic acid (12:7:1).[13] Thymine and deoxythymidine were located under u.v. light. The spots were cut

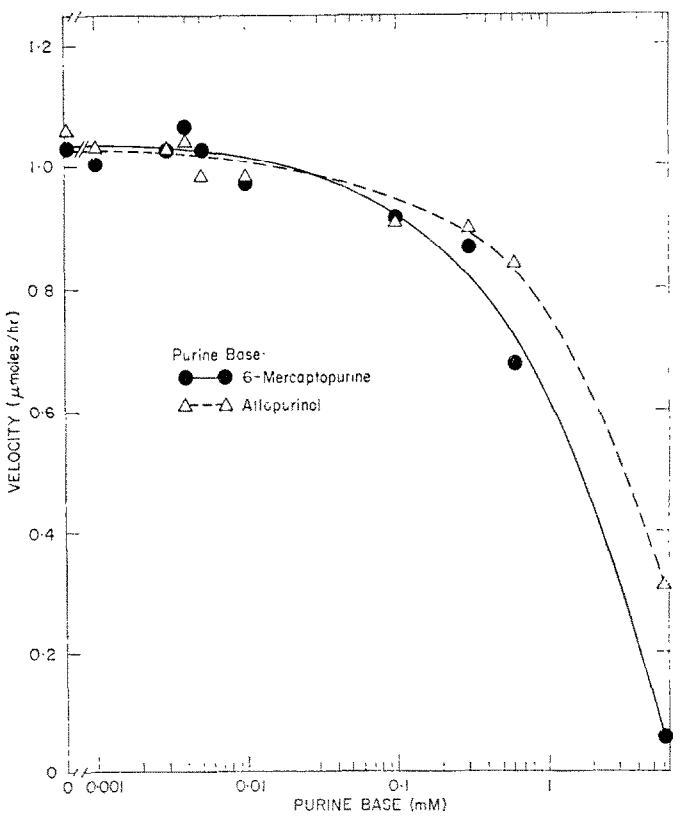

FIG. 1. The effect of increasing concentrations of allopurinol and 6-mercaptopurine on deoxythymidine synthesis catalyzed by deoxythymidine phosphorylase. The experimental conditions are described in Methods. The reaction mixture contained 0·75 mg protein, 2 mM thymine and a saturating concentration of deoxyribose-l-phosphate (10 mM).

into strips and counted in toluene phosphor at a ^{14}C efficiency of 60 per cent in a liquid scintillation spectrometer. There was no significant catabolism of thymine or deoxyribose-1-phosphate under the conditions of assay for deoxythymidine synthesis, since essentially complete recoveries were obtained after a 30-min incubation of either substrate incubated alone with the crude cell extract. Furthermore, recent kinetic studies have shown that the K_m values for both substrates are the same with the crude leukocyte extract and with a 250-fold purified enzyme (manuscript in preparation). Enzymatic activity is expressed as μmoles of product formed per hr/ml of reaction mixture. Protein was determined by the method of Lowry *et al.*[14]

RESULTS AND DISCUSSION

Both allopurinol and 6-MP inhibit deoxythymidine synthesis from thymine and deoxyribose-1-phosphate (Fig. 1). A comparison of the enzymatic activities of leukemic leukocytes (chronic myelogenous leukemia with 50 per cent myelocytes) with normal leukocytes (75 per cent granulocytes) did not reveal any significant differences in the magnitude of inhibition of deoxythymidine synthesis by allopurinol or 6-MP.

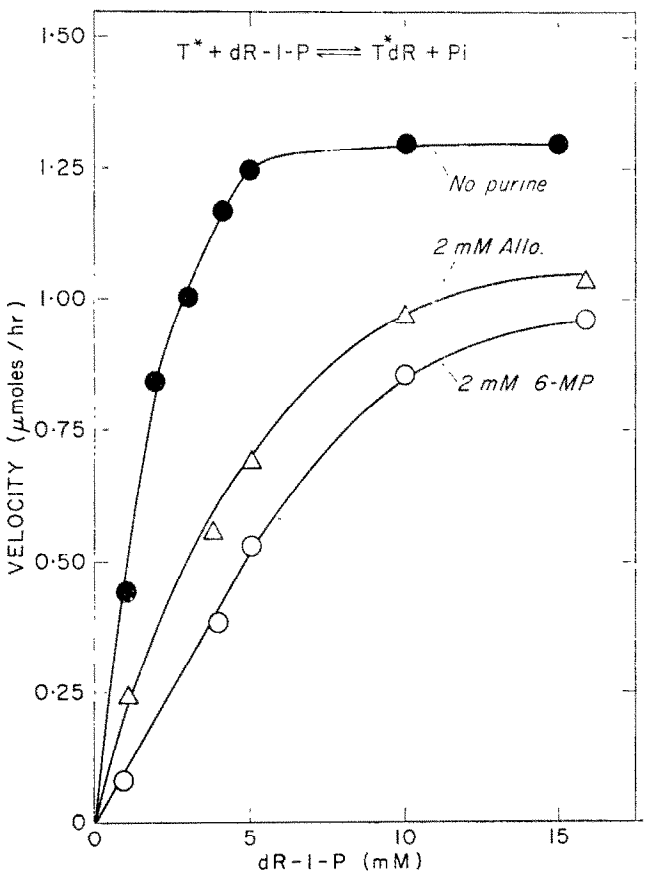

FIG. 2. Plot of the velocity of deoxythymidine synthesis vs. deoxyribose-1-phosphate concentration in the presence of 6-mercaptopurine and allopurinol (Allo.). The experimental conditions are described in Methods. The reaction mixture contained 0·72 mg protein, 2mM thymine and the indicated concentrations of deoxyribose-1-phosphate (dR-1-P).

As shown in Fig. 2, there is inhibition of deoxythymidine synthesis at all deoxyribose-l-phosphate concentrations. Inhibition at low concentrations of deoxyribose-l-phosphate is partly due to depletion of deoxyribose-l-phosphate by a reaction with the purine base catalyzed by purine nucleoside phosphorylase. However, it is to be noted that inhibition is still observed at saturating concentrations of deoxyribose-l-phosphate, so that even if a stoichiometric amount of the pentose were removed by reaction with purine, concentrations of deoxyribose-l-phosphate for optimal rates would still be present (Fig. 2).

The inhibition was also analyzed in the presence of various concentrations of thymine as shown in Fig. 3. Inhibition by 6-MP and allopurinol is observed at all

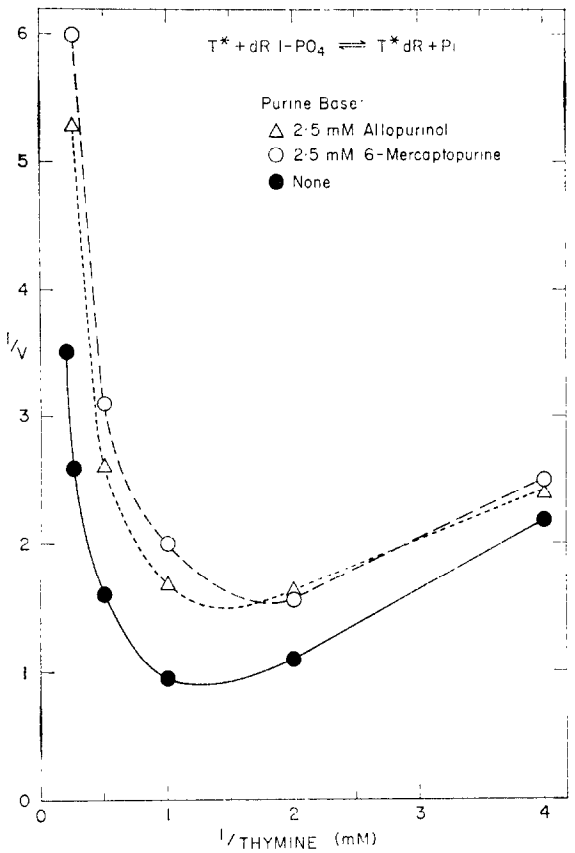

FIG. 3. Double reciprocal plot of the velocity of deoxythymidine synthesis vs. the thymine concentration in the presence of 6-mercaptopurine and allopurinol. The experimental conditions are described in Methods. The reaction mixture contained 0·65 mg protein, a saturating concentration of deoxyribose-l-phosphate (10 mM) and the indicated concentrations of thymine.

thymine concentrations. The calculation of a thymine K_m or a K_i for purines is complicated by substrate inhibition by thymine. A description of thymine substrate inhibition has been reported elsewhere.[9] It is apparent, however, that at high concentrations of thymine the 6-MP and allopurinol curves do not meet with the control curve. In the case of a purely competitive inhibitor complicated by the presence of

substrate inhibition, the curves join as one line at high substrate concentrations, as illustrated and discussed by Webb.[15] Furthermore, if the data are plotted by the method shown in Fig. 4, competitive inhibitors in the presence of inhibition by one of the substrates will yield curves that converge at inhibitory substrate concentrations.[15] However, the curves presented in Fig. 4 diverge at high thymine concentrations, which is characteristic of the noncompetitive or mixed type of inhibition.

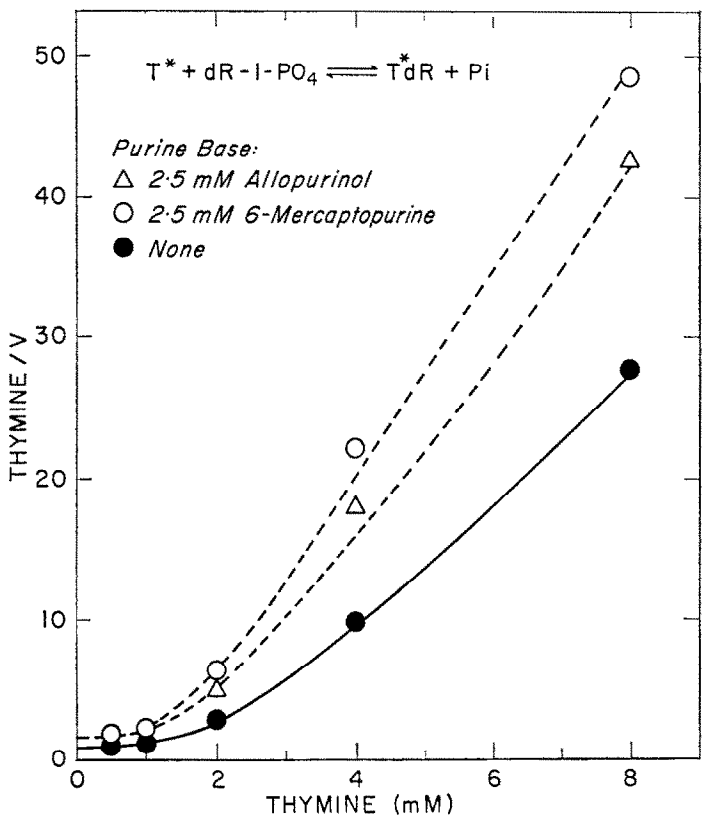

FIG. 4. Inhibition of deoxythymidine synthesis by 6-mercaptopurine and allopurinol over varying concentrations of thymine plotted as thymine/V vs. thymine concentration to illustrate that the inhibition by purines is not due to competition with thymine at the catalytic site. Data obtained from Fig. 3.

Allopurinol and 6-MP also inhibit deoxythymidine synthesis catalyzed by pyrimidine deoxyribosyltransferase (Table 1). However, the degree of inhibition is much less than the inhibition of deoxythymidine synthesis catalyzed by deoxythymidine phosphorylase (Fig. 1). At a thymine concentration of 2 mM, the apparent K_m for deoxyuridine is approximately 15 mM.[9] At deoxyuridine concentrations greater than 30 mM, there is no inhibitory effect by either allopurinol or 6-MP (data not shown). In contrast to their inhibitory effect on deoxythymidine synthesis, the analogs have no effect on the cleavage of deoxythymidine (Table 1).

TABLE 1. EFFECT OF ALLOPURINOL AND 6-MERCAPTOPURINE ON DEOXYTHYMIDINE SYNTHESIS AND CLEAVAGE*

Enzymatic reaction measured	Purine added†	Velocity‡ (μmole/hr)
Synthesis of deoxythymidine by transferase	none	0·62
	6-MP (2·5 mM)	0·40
	Allo (2·5 mM)	0·37
Cleavage of deoxythymidine by phosphorylase	none	0·68
	6-MP (2·5 mM)	0·69
	Allo (2·5 mM)	0·74

* Synthesis of deoxythymidine catalyzed by pyrimidine deoxyribosyltransferase was measured under the same conditions as described in Methods, except that the reaction mixture contained 2·5 μmole deoxyuridine instead of deoxyribose-l-phosphate and 50 μmole sodium arsenate was added to prevent formation of deoxyribose-l-phosphate.[19] The cleavage of deoxythymidine was followed by measuring the production of ^{14}C-thymine from ^{14}C-deoxythymidine (0·1 μc/μmole) in the presence of 50 μmole sodium phosphate or sodium arsenate, pH 5·9. The conditions were otherwise the same as described in Methods for deoxythymidine synthesis. The reaction mixture contained 0·52 mg protein.

† Abbreviations: 6-MP, 6-mercaptopurine; Allo, allopurinol.

‡ All values are the mean results from at least two experiments.

Synthesis of deoxyuridine from uracil and deoxyribose-l-phosphate, also catalyzed by deoxythymidine phosphorylase,[10, 11, 16] was inhibited by both 6-MP and allopurinol; however, uridine synthesis from uracil and ribose-l-phosphate, catalyzed by uridine phosphorylase, was inhibited only by 6-MP (Table 2).

TABLE 2. EFFECT OF ALLOPURINOL AND 6-MERCAPTOPURINE ON DEOXYURIDINE AND URIDINE SYNTHESIS*

Reaction measured	Purine added†	Velocity (μmoles/hr)
Deoxyuridine synthesis	none	1·52
	6-MP (0·5 mM)	0·60
	6-MP (2·5 mM)	0·35
	Allo (0·5 mM)	0·67
	Allo (2·5 mM)	0·68
Uridine synthesis	none	0·43
	6-MP (2·5 mM)	0·26
	Allo (2·5 mM)	0·47

* The incubation mixture is described in Methods, except that it contained 2·5 μmole ^{14}C-uracil (0·2 μc/μmole) instead of thymine, and for uridine synthesis it contained 7·5 μmole of ribose-l-phosphate. The reaction mixture contained 0·72 mg protein.

† Abbreviations: 6-MP, 6-mercaptopurine; Allo, allopurinol.

The metabolic functions of the protein containing deoxythymidine phosphorylase and pyrimidine deoxyribosyltransferase activities have not been clarified. The demonstration that the enzymatic activities of this protein can be subjected to substrate inhibition,[9, 17] product inhibition[18] and inhibition by purine bases suggests that it may

play an essential role in the intracellular metabolism of pyrimidine deoxynucleosides. Moreover, pyrimidine deoxynucleosides synthesized by this protein may constitute a precursor pool for thymine deoxynucleotide synthesis. It is possible, therefore, that the inhibition of pyrimidine deoxynucleoside synthesis by 6-MP and allopurinol decreases the level of pyrimidine precursors for DNA synthesis.

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