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EFFECT OF ALLOPURINOL AND OXIPURINOL ON PYRIMIDINE SYNTHESIS IN CULTURED HUMAN FIBROBLASTS

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Abstract—Oxipurinol (4,6-dihydroxypyrazolo (3,4-*d*) pyrimidine) and to a lesser extent allopurinol (4-hydroxypyrazolo (3,4-*d*) pyrimidine) inhibit pyrimidine biosynthesis *de novo* in cultured human fibroblasts. This inhibitory effect can be localized to one of the two steps involved in the conversion of orotic acid to uridine 5′-monophosphate (UMP). It is unrelated to the inhibitory effect of allopurinol or oxipurinol on xanthine oxidase; and it is not dependent on the presence of hypoxanthine-guanine phosphoribosyltransferase.

We have examined the effects of allopurinol and oxipurinol as well as their major known metabolites on the substrate, phosphoribosylpyrophosphate (PP-ribose-P) and the enzymes, orotate phosphoribosyltransferase and orotidylic decarboxylase, essential for the conversion or orotic acid to UMP. Allopurinol at a concentration of 1×10^{-3} M depletes intracellular PP-ribose-P although oxipurinol has no effect. Allopurinol, oxipurinol, allopurinol ribonucleoside, and allopurinol ribonucleotide have no inhibitory effect on orotate phosphoribosyltransferase. Allopurinol ribonucleotide but not the other compounds tested is a potent inhibitor of orotidylic decarboxylase. Although these observations provide several possible mechanisms to account for the modest inhibitory effect of allopurinol on pyrimidine biosynthesis, they fail to account for the even more striking inhibition produced by oxipurinol in cell culture.

ALLOPURINOL (4-hydroxypyrazolo (3,4-*d*) pyrimidine), an analogue of hypoxanthine, interferes with the metabolism of purines in several ways. Both allopurinol and its major metabolic product oxipurinol (4,6-dihydroxypyrazolo (3,4-*d*) pyrimidine) are potent inhibitors of xanthine oxidase, the enzyme which catalyzes the final two steps of uric acid synthesis.[1–4] Allopurinol also inhibits purine biosynthesis *de novo* by a mechanism that is dependent on the presence of hypoxanthine-guanine phosphoribosyl transferase.[5,6]

In addition to these well-known effects on purine metabolism, recent studies suggest that allopurinol and oxipurinol may also interfere with pyrimidine biosynthesis. The administration of either of these drugs to man is followed by a prompt and striking increase in the urinary excretion of orotidine and orotic acid.[7,8] The increased excretion of these two pyrimidines is qualitatively and quantitatively similar to that observed during the administration of a similar dose of the pyrimidine antimetabolite, azauridine.[9,10] By analogy with the suspected site of inhibition of this compound, the effect of allopurinol and oxipurinol has been attributed to inhibition of orotidylic

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decarboxylase, the enzyme which catalyzes the irreversible decarboxylation of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP)[7,8] The finding that allopurinol ribonucleotide and xanthosine 5′-monophosphate (XMP) are potent inhibitors of this enzyme has provided indirect support for this hypothesis.[8]

In the present study, we have demonstrated that allopurinol and oxipurinol inhibit pyrimidine biosynthesis *de novo* in cultured human cells. This inhibitory effect in cell culture is not dependent on the presence of xanthine oxidase or hypoxanthine-guanine phosphoribosyltransferase and, therefore, cannot be attributed to the formation of allopurinol ribonucleotide or XMP. Although it is possible to localize the site of inhibition to the steps involved in the conversion of orotic acid to UMP the exact mechanism remains to be defined.

MATERIALS AND METHODS

Materials

Guanine-8-^{14}C (7·9 and 31·6 mc/mmole), adenine-8-^{14}C (5·5 and 27·2 mc/mmole), orotic acid 6-^{14}C (43·5 mc/mmole), orotidine 5′-monophosphate-7-^{14}C (21 mc/m mole) and uridine-6-^{3}H (10·4 c/mmole) were obtained from New England Nuclear Corporation. Phorphoribosylpyrophosphate (PP-ribose-P) and orotidine 5′-monophosphate (OMP) were obtained from Calbiochem. Thymidine triphosphate (TTP) was purchased from Sigma Chemical Company. Allopurinol, oxipurinol, allopurinol ribonucleoside, and allopurinol ribonucleotide were the generously provided gift of Dr. Gertrude B. Elion, Burroughs Wellcome and Company, Research Triangle Park, N.Y. All other reagents were the highest quality commercially available.

Cell-culture technique

Fibroblasts derived from punch biopsies of human skin were cultured in a routine manner as described previously.[6]

Orotic acid and uridine incorporation

The incorporation of orotic acid ^{14}C and uridine ^{3}H into nucleic acids was assessed in human fibroblasts growing in monolayer culture essentially as described previously for purine bases.[6] Uridine ^{3}H was diluted with cold uridine to a final specific activity of 104 mc/mM. Orotic acid and uridine were added to monolayer cultures at a final concentration of 1·2 μM; and the cells were cultured at 37° in a 5% CO_2–95% air environment. After 3–5 days, the cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS) pH 7·4, and nucleic acids were precipitated with cold trichloroacetic acid at a final concentration of 5%. The acid insoluble material was collected on a 0·45 μ Millipore filter which was then washed, dried, and counted in a Packard Tricarb liquid scintillation spectrometer at an efficiency of 63 per cent for ^{14}C and 1·3 per cent for ^{3}H. The results were essentially the same whether orotic acid and uridine were added together to the same cell culture or each added separately to different subcultures of the same cell strain.

Enzyme assays

Fibroblasts were harvested by rapid trypsinization, thoroughly washed with PBS, suspended in 0·01 M tris buffer pH 7·40 at a concentration of 60 mg/ml based on wet

weight and lysed by rapid freezing and thawing. The extract was then dialyzed against 0·01 M tris buffer pH 7·40 for 2 hr. Cell extracts were always prepared on the day the studies were performed; and all cells were grown to confluence before harvesting.

Hypoxanthine-guanine phosphoribosyltransferase was assayed in cultured fibroblasts by a radiochemical assay described previously.[11]

Orotidylic decarboxylase was assayed in fibroblast extracts essentially as described for human erythrocyte lysates by collecting the $^{14}CO_2$ evolved after incubation of carboxyl-labeled OMP ^{14}C with extract containing 30 to 150 μg fibroblast protein.[8,12]

Orotate phosphoribosyltransferase was assayed in erythrocyte lysates by a radiochemical method utilizing azauridine 5′monophosphate (azaUMP) to inhibit the further metabolism of orotidine 5′monophosphate. Erythrocytes were washed, lysed by freezing and thawing, and dialyzed for 2 hr against 0·01 M tris buffer pH 7·40. The reaction mixture contained 5·5 μmoles tris pH 7·40, 0·5 μmoles $MgCl_2$, 0·1 μmoles PRPP, 4 nmoles orotic acid-6-^{14}C, 0·1 μmoles azaUMP, and 1·0–1·2 mg erythrocyte protein in a final volume of 100 μl. After incubation for 1 hr at 37°, the reaction was stopped by the addition of 2 μmoles EDTA. Twenty μl of the reaction mixture was applied to No. 1 Whatman paper with 50–100 nmoles of orotidine, orotidine 5′-monophosphate (OMP), and uridine 5′-monophosphate (UMP) as carriers. Reaction products were separated by descending chromatography (Butyl alcohol–acetic acid–H_2O, 50: 25: 25; R_F: orotic acid, 39; orotidine, 30; UMP, 23; OMP, 12). The area of paper containing OMP was located by inspection of the paper under ultraviolet light and cut out and counted in a Packard Tricarb liquid scintillation spectrometer at 63 per cent efficiency. The formation of OMP exhibited a linear relationship to protein concentration and incubation time. Less than 7 per cent of the OMP formed under these conditions was converted to UMP or orotidine.

Intracellular PRPP content

PRPP content was assayed in human fibroblasts as described previously.[13,14]

Protein was estimated by the method of Lowry *et al.*[15]

RESULTS

The mean incorporation of orotic acid and uridine into nucleic acids in one to three experiments in three to six different cell strains is illustrated in Table 1. Human fibroblasts in monolayer are capable of synthesizing pyrimidine ribonucleotides by both the *de novo* and "salvage" pathways. In addition, the presence or absence of hypoxanthine-guanine phosphoribosyltransferase has no apparent effect on the incorporation of orotic acid into nucleic acids.[14] The data illustrated in Table 1 represent the control values for the studies illustrated in Figs. 1 and 2.

The effect of allopurinol and oxipurinol on the incorporation of orotic acid into nucleic acids is illustrated in Fig. 1. The data plotted represent the mean $\pm$ S.E. in nine experiments in five different cell strains with normal hypoxanthine-guanine phosphoribosyltransferase and the mean of two experiments in one cell strain markedly deficient in this enzyme. Oxipurinol produced significant inhibition in the normal cells at all concentrations studied ($P < 0{\cdot}01$); allopurinol had a significant inhibitory effect only at a concentration of 1 mM ($P < 0{\cdot}001$). A similar inhibitory effect of oxipurinol and allopurinol was observed in the mutant cell strain markedly deficient

TABLE 1. INCORPORATION OF OROTIC ACID AND URIDINE INTO COLD ACID-INSOLUBLE MATERIAL IN CULTURED HUMAN FIBROBLASTS WITH NORMAL AND DEFICIENT HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY

Cell strain	Passage	Hypoxanthine-guanine phosphoribosyltransferase activity (nmoles/mg prot./hr)	Orotic acid*-6-^{14}C (nmoles/mg prot.)	Uridine*-6-^{3}H (nmoles/mg prot.)
Normal hypoxanthine-guanine phosphoribosyltransferase				
167	4–5	114	18	291
169	4–5	141	20	366
170	4–5	153	15	385
163	5	131	32	—
154	7	—	19	—
Hypoxanthine-guanine phosphoribosyltransferase deficiency				
123	12–17	0·1–2·9	27	—

* Mean of one to three different experiments in each cell strain.

in hypoxanthine-guanine phosphoribosyltransferase. As indicated in Fig. 2, allopurinol and oxipurinol had no inhibitory effect on the incorporation of uridine into nucleic acids in four experiments in three different cell strains with normal hypoxanthine-guanine phosphoribosyltransferase activity. In fact, at the highest concentrations studied, both compounds produced a modest increase in the incorporation of uridine into nucleic acids. Neither allopurinol or oxipurinol at concentrations of 1 and 0·3 mM respectively had a demonstrable effect on protein synthesis or cell growth.

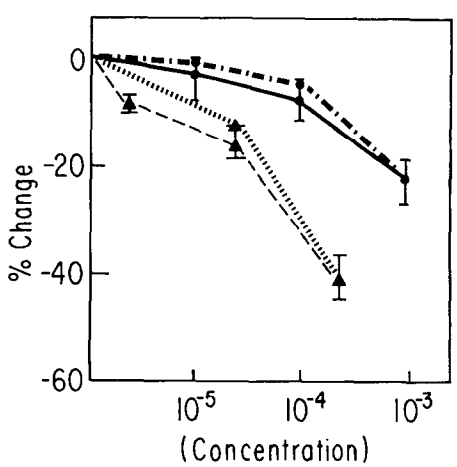

FIG. 1. Effect of allopurinol and oxipurinol on the incorporation of orotic acid-^{14}C into nucleic acids. Values represent mean ± S.E. expressed as per cent change from control values (Table 1): ——, allopurinol, normal hypoxanthine-guanine phosphoribosyltransferase activity; – · –, allopurinol, hypoxanthine-guanine phosphoribosyltransferase deficiency; — —, oxipurinol, normal hypoxanthine-guanine phosphoribosyltransferase activity; – – – –, oxipurinol, hypoxanthine-guanine phosphoribosyltransferase deficiency.

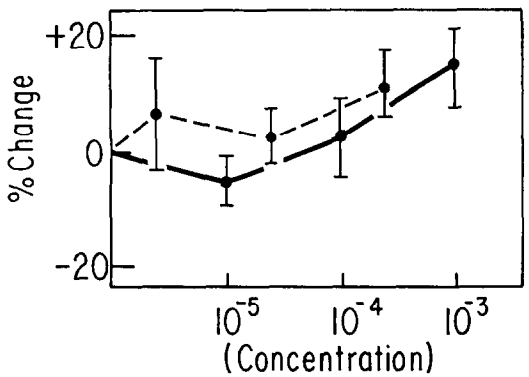

FIG. 2. Effect of allopurinol and oxipurinol on the incorporation of uridine-^{3}H into nucleic acids. The values represent mean ± S.E. expressed as per cent change from control values (Table 1): ——, allopurinol; – – –, oxipurinol.

The effects of oxipurinol and allopurinol on intracellular PP-ribose-P content in 1 cell strain with normal hypoxanthine-guanine phosphoribosyltransferase activity is indicated in Table 2. Although allopurinol at a concentration of 1·0 mM led to a modest depletion of PP-ribose-P, oxipurinol at this concentration had no detectable effect. Similar results have been observed in two additional experiments. This depletion of PRPP by allopurinol in cell culture appears to be at least partially dependent on the presence of hypoxanthine-guanine phosphoribosyltransferase since 1 mM allopurinol produced only a 15 per cent decrease in PRPP content in three experiments in two cell strains genetically deficient in this enzyme.

TABLE 2. EFFECT OF ALLOPURINOL AND OXIPURINOL ON INTRACELLULAR PP-RIBOSE-P CONTENT IN HUMAN FIBROBLASTS WITH NORMAL HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY

		PP-ribose-P	
Compound	Concentration (mM)	(nmoles/g prot.)	(Per cent of control)
None	—	4767	—
Allopurinol	0·1	4467	94
	1·0	2991	63
Oxipurinol	0·1	4917	103
	1·0	4764	100

As indicated in Table 3, allopurinol and its major known metabolic products including oxipurinol, allopurinol ribonucleoside, and allopurinol ribonucleotide as well as several intermediates of uric acid synthesis including hypoxanthine and xanthine at a concentration of 1 mM had no effect on the activity of orotate phosphoribosyltransferase obtained from human erythrocytes.

Previous studies have shown that allopurinol ribonucleotide and XMP are potent inhibitors of orotidylic decarboxylase from human erythrocytes.[8] As indicated in Table 3, these compounds are also potent inhibitors of this enzyme derived from cultured human fibroblasts. None of the other compounds tested inhibited this enzyme obtained from either fibroblasts or erythrocytes.

TABLE 3. EFFECT OF ALLOPURINOL AND ITS MAJOR KNOWN METABOLITES AND PRECURSORS OF URIC ACID SYNTHESIS ON HUMAN OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDYLIC DECARBOXYLASE ACTIVITY *in vitro*

Compound	Concentration (mM)	Orotate phosphoribosyltransferase activity* (hemolysate) (per cent of control)	Orotidylic decarboxylase activity† (fibroblast extract) (per cent of control)
None	—	100	100
Allopurinol	1	96	97
Oxipurinol	1	102	82
Allopurinol ribonucleoside	1	87	97
Allopurinol ribonucleotide	1	98	2
Hypoxanthine	1	88	92
Xanthine	1	100	77
XMP	1	—	2

Control activity.
* Orotate phosphoribosyltransferase—0·65 nmoles/mg prot./hr.
† Orotidylic decarboxylase—14·8 nmoles/mg prot./hr.

DISCUSSION

In the present study, we have demonstrated that oxipurinol, and to a lesser extent, allopurinol, inhibit the incorporation of orotic acid into nucleic acids in human cell culture. Thus, as suggested from observations *in vivo*, these purine analogs inhibit pyrimidine biosynthesis. The additional finding that allopurinol and oxipurinol have no inhibitory effect on the incorporation of uridine into nucleic acids provides strong evidence that the observed inhibition of pyrimidine biosynthesis involves one of the steps involved in the conversion of orotic acid to uridine 5′-monophosphate (UMP). Since human fibroblasts lack xanthine oxidase, this inhibitory effect of oxipurinol and allopurinol on pyrimidine synthesis in cell culture cannot be a result of xanthine oxidase inhibition. The minimal inhibitory concentration of oxipurinol in cell culture is less than the concentration of this compound found in plasma following the administration to man of either allopurinol or oxipurinol.

The two reactions involved in the synthesis of UMP from orotic acid include (1) the conversion of orotic acid to orotidine 5′-monophosphate (OMP) in the presence of PP-ribose-P which is catalyzed by orotate phosphoribosyltransferase and (2) the irreversible decarboxylation of OMP to UMP which is catalyzed by orotidylic decarboxylase. The inhibitory effect of oxipurinol and allopurinol does not appear to be due to interference with the conversion of orotic acid to orotidine 5′-monophosphate. Although allopurinol reduces the intracellular concentration of PP-ribose-P, an essential substrate for this reaction, oxipurinol has no effect. In addition, allopurinol,

oxipurinol, allopurinol ribonucleoside, and allopurinol ribonucleotide have no demonstrable inhibitory effect on orotate phosphoribosyltransferase, the enzyme catalyzing this reaction.

Inhibition of orotidylic decarboxylase has previously been proposed as a possible mechanism to account for the postulated inhibitory effect of allopurinol and oxipurinol on pyrimidine biosynthesis *in vivo*.[7,8] The observation that allopurinol ribonucleotide and XMP are potent inhibitors of the human enzyme derived from erythrocytes or fibroblasts provides a potential mechanism to account for this inhibition.[8] However, several findings in the present study indicate that the inhibition of pyrimidine biosynthesis observed in cell culture cannot be attributed to either allopurinol ribonucleotide or XMP. The inhibitory effect of allopurinol or oxipurinol on pyrimidine biosynthesis in cell culture is not modified by the absence of hypoxanthine-guanine phosphoribosyltransferase which is the enzyme necessary for catalyzing the conversion of allopurinol to allopurinol ribonucleotide[16,17] and xanthine to XMP.[18] In addition, oxipurinol, a more potent inhibitor of pyrimidine synthesis than allopurinol, cannot be converted to allopurinol ribonucleotide and, since human fibroblasts lack xanthine oxidase, oxipurinol should not lead to accumulation of xanthine or, therefore, to an increased synthesis of XMP.

Although the present studies in cell culture illustrate that oxipurinol and allopurinol are capable of inhibiting pyrimidine biosynthesis *de novo* at some portion in the pathway responsible for the conversion of orotic acid to UMP, they fail to elucidate the exact mechanism responsible for this inhibition. It seems most likely that orotate phosphoribosyltransferase or orotidylic decarboxylase is inhibited by a metabolite of allopurinol or oxipurinol which has not been examined in the present study. The only known metabolites of either drug which we have not been able to study are two ribonucleoside derivatives of oxipurinol; one which is found in small quantities in the urine after the oral administration of oxipurinol and another which is not found *in vivo* but which can be formed enzymatically.[19] Although we have been unable to detect the formation of these ribonucleoside derivatives from radioactive oxipurinol in extracts of human fibroblasts, we cannot exclude the possibility that either of these metabolites are responsible for the changes observed. The possibility must also remain that the alteration in pyrimidine metabolism is due to another metabolite of these drugs which has yet to be identified.

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