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INHIBITION OF HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE *

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Abstract—The affinities of eighteen purines or purine analogs for human erythrocytic hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8; HGPRTase) were compared to assess the feasibility of obtaining active inhibitors of the enzyme. Three compounds appeared to inhibit the utilization of hypoxanthine by L5178Y cells *in vitro* due to inhibition of the enzyme rather than depletion of the intracellular 5-phosphoribosyl-1-pyrophosphate pool. The three competitive inhibitors and their affinity constants (K_i) using 6-mercaptopurine as substrate were: 6-mercapto-9-(tetrahydro-2-furyl)-purine, 37 μM; 2,6-bis-(hydroxyamino)-9-β-D-ribofuranosyl-purine, 12 μM; and 6-iodo-9-(tetrahydro-2-furyl)-purine, 108 μM. The K_m for 6-mercaptopurine was 9 μM. Thus, the enzyme tolerates bulky substitution at N^9. 6-Mercapto-9-(tetrahydro-2-furyl)-purine also potentiated the chemotherapeutic effect of azaserine, an inhibitor of *de novo* purine biosynthesis, in L5178Y ascites tumor-bearing mice. Four 2-substituted, oxazolo-[5, 4-*d*]-pyrimidine-7-ones and 2-methylthiazolo-[5, 4-*d*]-pyrimidine-7-one had K_i values in the range of 84–173 μM. Consequently, isosteric substitution at N^9 may also be a fruitful and logical course to pursue in the design and synthesis of more potent inhibitors of this important enzyme.

Purine salvage enzymes are of special significance in the purine metabolism of cells [1] and organisms [2–4] in which purine biosynthesis *de novo* is deficient or inhibited. HGPRTase† may play a major role in purine salvage in humans since circulating levels of adenosine and adenine are low (10^{-7} M or less [5]), and the ubiquitous adenosine deaminase and purine nucleoside phosphorylase provide a continual supply of hypoxanthine. HGPRTase is important for the cytotoxic effects of purine analogs [6], and absence of HGPRTase is associated with the neurologic disorder, the Lesch–Nyhan syndrome [7].

Inhibitors of HGPRTase are needed to: (1) evaluate the role of this enzyme in maintaining steady-state pools of purines in various tissues and tumors; (2) potentially treat schistosomiasis and malaria, diseases in which the parasites lack the *de novo* purine biosynthetic pathway [2–4]; (3) potentiate the actions of other agents which inhibit *de novo* biosynthesis, effects of which may be "bypassed" due to HGPRTase; and (4) possibly mimic in animals the genetic disease which occurs in man as a result of HGPRTase deletion, i.e. the Lesch–Nyhan Disease.

Various compounds have been found to inhibit HGPRTase, including diazoacetylglycinamide [8], 6-azauridine [9], GMP and its periodate oxidation products [10], oxyphenbutazone and flufenamic acid [11], naptho-[2, 3-*d*]-imidazole-4,9-dione and its 1-β-D-ribofuranosyl derivative [12], and other purines and purine analogs [13–15]. However, the relative affinities (K_i values) of these inhibitors have not usually been determined, or the effects against the isolated enzyme have not been corroborated in intact cells, or vice versa.

This report describes the affinities of some inhibitors of HGPRTase in an attempt to define further the structural requirements for inhibition. A preliminary abstract of this work has appeared [16] in which several benzimidazole-like compounds were reported to inhibit the enzyme from human red blood cells. Subsequently, we have found the apparent inhibition by benzimidazoles to be artifactual and due to slight decreases in pH produced by addition of the putative inhibitors.

MATERIALS AND METHODS

Materials. [8-^{14}C]Adenine (43.95 mCi/m-mole) and [8-^{14}C]hypoxanthine (42.4 mCi/m-mole) were obtained from New England Nuclear, Boston, MA. 2,7-Disubstituted oxazolo-pyrimidines and the thiazolopyrimidine were synthesized as reported previously [17]. 6-Iodo-9-(tetrahydro-2-furyl)-purine (NSC No. 45152), 6-mercapto-9-(tetrahydro-2-furyl)-purine (NSC No. 45153), 7-deazanebularine (NSC No. 107519), 4-amino-7-(3-deoxy-β-D-erythro-pentofuranosyl)-pyrrolo-[2, 3-*d*]-pyrimidine (NSC No. 124154), 2,6-bis(hydroxyamino)-9-β-D-ribofuranosylpurine (NSC No. 130263), and inosine dialdehyde (NSC No. 118994) were obtained from the

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† Abbreviations used are: PRPP, 5-phosphoribosyl-1-pyrophosphate; 6-MP, 6-mercaptopurine; 6-MPTF, 9-(tetrahydro-2-furyl)-6-purinethiol; HGPRTase, IMP-GMP pyrophosphate phosphoribosyltransferase (EC 2.4.2.8); PNPase, purine-nucleoside: orthophosphate ribosyltransferase (EC 2.4.2.1); and APRTase, AMP: pyrophosphate phosphoribosyltransferase (EC 2.4.2.7).

Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Xanthine oxidase was purchased from ICN Pharmaceuticals, Inc., Cleveland, OH. Whatman microgranular DE 52 anion exchanger with exchanger capacity of 1 mEq/g dry weight was obtained from Whatman, Inc., Clifton, NJ. All other chemicals were of the highest purity available through commercial sources. PEI-cellulose plastic sheets (Baker flex) were obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ. Spectrophotometric analyses were performed with a Gilford model 250 spectrophotometer equipped with a Gilford model 6050 recorder and Haake model FE constant temperature circulator monitored at 37°. Radioactivity was determined in Scintanalyzed toluene (Fisher Scientific Co., Houston, TX) containing Spectrofluor (Amersham/Searle, Arlington Heights, IL) using a Searle Isocap/300 model 6872 liquid scintillation counter.

Enzyme preparation. A method modified from that of Kong and Parks [18], and Olsen and Milman [19] was used to obtain a partially purified preparation containing HGPRTase, APRTase and PNPase from human red blood cells. Human red blood cells (250 ml) were washed twice with equal volumes of 0.9% NaCl. The cells were lysed by diluting four times with distilled water and repeated freezing at −20°. Cellular debris was removed by centrifugation at 16,000 *g* for 10 min at 4°. DEAE cellulose (65 g), previously equilibrated at 0.01 M potassium phosphate, pH 7.4, was used to remove hemoglobin. The adsorbed protein was eluted by stirring twice with 800 ml portions of 0.2 M KCl in 0.01 M potassium phosphate, pH 7.4, followed by two washes with 400 ml. The washes were combined and brought to 40% saturation with solid ammonium sulfate. The precipitate thus obtained was discarded and the solution was brought to 70% saturation with ammonium sulfate. The precipitate was dissolved in a solution containing 0.01 M potassium phosphate, pH 7.5, 0.2 M KCl, and 0.005 M dithiothreitol. This enzyme preparation had a HGPRTase specific activity of 0.13 μ molar units/mg of protein with 6-MP as substrate.

Enzyme assays. HGPRTase was assayed in 1.0-ml reaction mixtures containing 10 mM Tris–HCl buffer, pH 7.5; 2 mM $MgSO_4$; 1 mM PRPP; and concentrations of 6-MP from 10 to 40 μM. The reaction was started by adding the enzyme preparation. The rate of formation of 6-mercaptopurine ribonucleoside 5′-phosphate was measured by the increase in absorbance at 320 nm [18]. Inhibitor constants (K_i) were calculated using a weighted, least squares regression analysis [20] with a Hewlett–Packard 9825-A desk top calculator.

A radiochemical method was also used to measure the HGPRTase and APRTase activity as follows: [8-^{14}C]hypoxanthine and [8-^{14}C]adenine (11.75 and 11.2 μM respectively) were used as substrates in the above reaction mixtures instead of 6-MP. The reaction was started by adding enzyme, and the mixture was incubated in a Dubnoff metabolic shaking incubator at 37°. The reaction was stopped by adding 50 μl of 4.2 M perchloric acid. After centrifugation, the acid-soluble extract was neutralized by adding 50 μl of 4.2 M KOH. The supernatant fraction (25 μl) was used for thin-layer chromatography. The reaction mixtures were applied to prewashed PEI-cellulose plates as 1 cm streaks from the bottom. The plates were developed with methanol–water (1:1) overnight. Under these conditions, nucleotides remain at the origin, whereas nucleosides and bases move with the solvent. PEI-cellulose plates were prewashed in 4 M sodium formate buffer, pH 3.4, for 5 hr and in methanol–water (1:1) overnight. Spots containing nucleotides were marked under u.v. light, cut and counted. Reaction mixtures were also applied as 2 × 2 cm spots which were counted similarly to obtain total counts in the reaction mixture. In some experiments, the thin-layer chromatographic method of Crabtree and Henderson [21] was used to isolate IMP and AMP to confirm their identity.

Xanthine oxidase was analyzed by monitoring the increase in absorbance at 293 nm due to the formation of uric acid in 1 ml reaction mixtures containing 100 μM hypoxanthine; 100 μM Tris–HCl buffer, pH 7.5; and 0.0015 μM units of the enzyme. PNPase was analyzed using the method of Kim *et al.* [22]. The reaction mixtures contained the following components in 1 ml final volume: inosine, 50 μM; potassium phosphate buffer, pH 7.5, 100 μM; xanthine oxidase, 0.015 μM units; and a limiting amount of PNPase. PNPase was added after incubating the reaction mixture for 1 min at room temperature, and the increase in absorbance at 293 nm due to the formation of uric acid was measured.

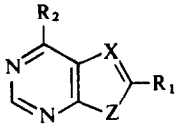
Whole cell experiments. L5178Y cells were obtained from BD_2F1 mice bearing the ascites tumor. Red blood cells were removed by washing with isotonic NH_4Cl [23]. The cells (2×10^7) were incubated in 1 ml of Fischer's medium (Grand Island Biologicals, NY) at 37° for 15 min. [8-^{14}C]Hypoxanthine (25 μM) and the desired inhibitor (100 μM) were added and the incubation was allowed to proceed for another 30 min. The incubation was stopped by centrifugation, the cells were extracted with perchloric acid as above, and the radioactivity was measured. PRPP levels in L5178Y ascites cells were determined using the method of Henderson and Khoo [24].

RESULTS

Isosteric substitution at N^9 of hypoxanthine and guanine with O, S or C should yield inhibitors of HGPRTase, since the isosteres would not have a displaceable hydrogen atom, but may retain affinity for the enzyme. The isosteres which were available for testing, i.e. four substituted oxazolopyrimidines and a thiazolopyrimidine, demonstrated weak affinity for HGPRTase (Table 1). The 2-phenyloxazolo-[5, 4-*d*]-pyrimidine-7-one was the most active. The two pyrrolopyrimidines available for testing were isosteric replacements of N^7 and were inactive.

As reported by others (for example in Refs. 13, 15 and 18), the substrates hypoxanthine, guanine, 6-MP and 6-thioguanine have affinities (K_m or K_i) for HGPRTase in the range of 1–10 μM (Table 2). Of the purine analogs tested, 2,6-bis-(hydroxyamino)-9-β-D-ribofuranosyl-purine was the most active ($K_i \sim 12\,\mu$M), and the inhibition was competitive with 6-MP. 6-MPTF was also found to be a competitive inhibitor of HGPRTase with a K_i of 37 μM. Both 6-MPTF and the bis-(hydroxyamino) compound were also competitive with respect to the other substrate,

Table 1. Inhibition constants of purine isosteres for human erythrocytic HGPRTase*



Purine isostere substituent

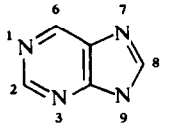
Inhibitor	X	Z	R_1	R_2	K_i (μM)	Type of inhibition
2-Methyloxazolo-[5, 4-*d*]-pyrimidine-7-one	N	O	CH_3	OH	163	Competitive
2-Ethyloxazolo-[5, 4-*d*]-pyrimidine-7-one	N	O	C_2H_5	OH	165	Competitive
2-Butyloxazolo-[5, 4-*d*]-pyrimidine-7-one	N	O	C_4H_9	OH	173	Competitive
2-Phenyloxazolo-[5, 4-*d*]-pyrimidine-7-one	N	O	C_6H_5	OH	84	Competitive
2-Methylthiazolo-[5, 4-*d*]-pyrimidine-7-one	N	S	CH_3	OH	136	Competitive
7-Deazanebularine	CH	*N*-ribose	H	NH_2		Inactive
4-Amino-7-(3-deoxy-β-erythro pentofuranosyl)-pyrrolo [2, 3-*d*]-pyrimidine	CH	*N*-deoxy ribose	H	NH_2		Inactive

* The conversion of 6-MP to 6-ThioIMP was measured by the increase in absorbance at 320 nm upon addition of the enzyme to reaction mixtures at 37° containing in 1 ml: substrate 10, 20, 30, and 40 nmoles; PRPP, 1μmole; $MgSO_4$, 2μmoles; Tris–HCl, 100μmoles; pH 7.5. Results shown are averages of duplicate determinations using at least two levels of inhibitor. The K_m for 6-MP was 8.9 $\pm$ 0.7μM ($\pm$ S.E., n = 20). Inactive, < 30 per cent inhibition at 100μM.

PRPP [illustrated for the bis-(hydroxyamino) compound in Fig. 1]. 6-MPTF did not inhibit APRTase, PNPase or xanthine oxidase, whereas the bis-(hydroxyamino) compound was active against APRTase and xanthine oxidase (data not shown). 6-Iodo-9(tetrahydro-2-furyl)-purine was active against HGPRTase ($K_i \sim 100\mu$M; Table 2), but it also inhibited xanthine oxidase (data not shown).

Hypoxanthine utilization by L5178Y cells *in vitro* was reduced significantly (Table 3) by the three most active nucleoside analogs, i.e. 6-MPTF, 2,6-bis-(hydroxyamino)-9-β-D-ribofuranosyl-purine, and 6-iodo-9-(tetrahydro-2-furyl)-purine. The reduction in hypoxanthine utilization produced by these agents was not due to a decrease inPRPP pool size. On the other hand, xylosyladenine and 7-deazanebularine probably prevent hypoxanthine utilization by depletion of PRPP (Table 3), since these compounds were inactive against isolated HGPRTase (Table 2). Although the oxazolopyrimidines and the thiazolopyrimidine were weakly active against isolated HGPRTase (Table 1), they were inactive as inhibitors of hypoxanthine utilization by L5178Y cells in experiments such as those shown in Table 3.

Table 2. Inhibition constants of purines for human erythrocytic HGPRTase*



Purine substituent

Inhibitor	2	6	9	K_i (μM)	Type of inhibition
Purine	H	H	H		Inactive
Hypoxanthine	H	OH	H	7	Competitive
Guanine	NH_2	OH	H	5	Competitive
Adenine	H	NH_2	H		Inactive
2,6-Diaminopurine	NH_2	NH_2	H		Inactive
2,6-Bis-(hydroxamino)-9-β-D-ribofuranosyl-purine	NHOH	NHOH	Ribose	12	Competitive
6-Iodo-9-(tetrahydro-2-furyl)-purine	H	I	Tetrahydrofuran	108	Competitive
6-Mercapto-9-(tetrahydro-2-furyl)-purine	H	SH	Tetrahydrofuran	37	Competitive
Inosine dialdehyde	H	OH	Diglycolicaldehyde		Inactive
Xylosyladenine	H	NH_2	Xylose		Inactive

* Conditions similar to those described in Table 1 were used.

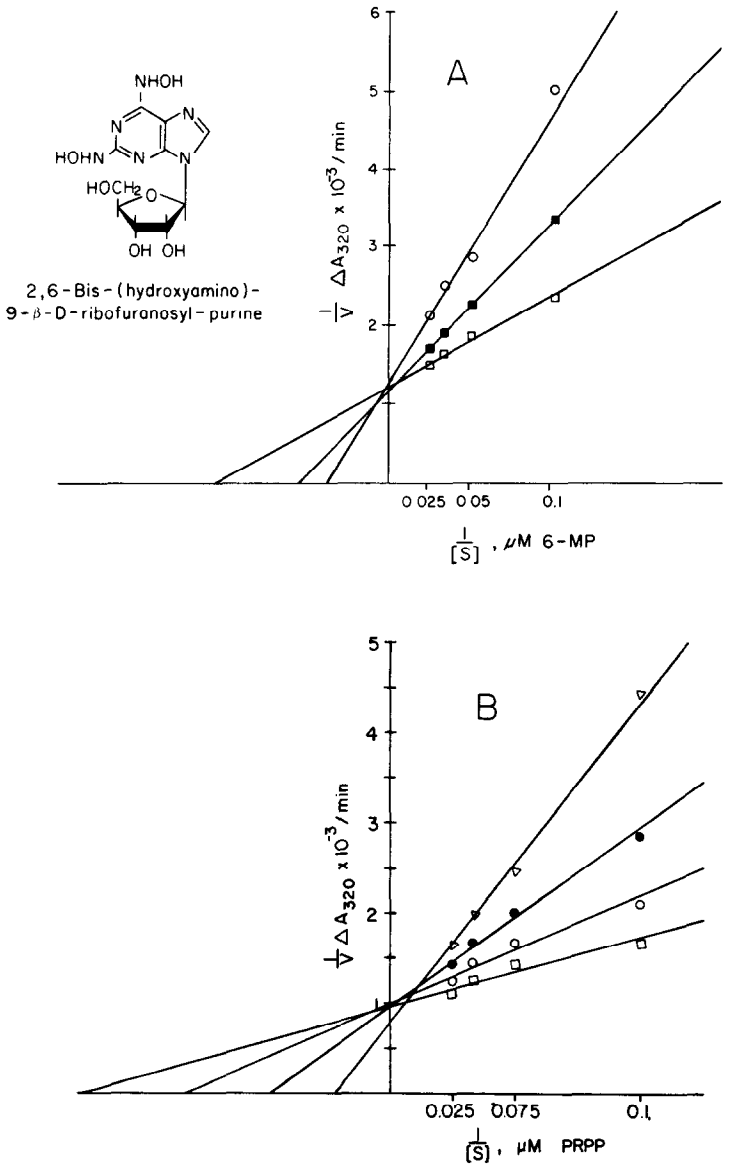


Fig. 1. Double-reciprocal plots of the inhibition on HGPRTase by 2,6-bis-(hydroxyamino)-9-β-D-ribofuranosyl-purine for the two substrates, 6-MP (A) and PRPP (B). 6-MP (A) (10–40 μM) was incubated at 37° with at least two levels of inhibitor, 1 mM PRPP and 2 mM $MgSO_4$ in 100 mM Tris–HCl, pH 7.5. The change in absorbance at 320 nm was measured as described in Materials and Methods. PRPP (B) (25–100 μM) was similarly incubated with 25 μM 6-MP. Key: —□— no inhibitor, —■— 10 μM, —○— 20 μM, —●— 40 μM, and —△— 60 μM 2,6-bis-(hydroxyamino)-9-β-D-ribofuranosyl-purine.

Table 3. Effects of purine analogs on the uptake of [8-^{14}C] hypoxanthine and PRPP levels of L5178Y cells

Inhibitor	Per cent of total radioactivity in cells *	PRPP† (nmoles/10^9 cells)
	30 ± 2	100 ± 5
7-Deazanebularine	22 ± 3	40 ± 4
Xylosyladenine	ND‡	20 ± 4
2,6-Bis-(hydroxyamino)-9-β-D-ribofuranosyl-purine	15 ± 3	100 ± 3
6-Iodo-9-(tetrahydro-2-furyl)-purine	20 ± 2	101 ± 5
6-Mercapto-9-(tetrahydro-2-furyl)-purine	18 ± 3	100 ± 2

* L5178Y cells were preincubated for 15 min at 37° in Fischer's medium. [8-^{14}C]Hypoxanthine was then added to a final concentration of 25 μM. Inhibitors (100 μM) were added at the same time as hypoxanthine. After 30 min, the reaction was stopped by centrifugation. Then radioactivity in the neutralized perchloric acid extracts of the cells was determined by liquid scintillation spectrometry. Average values ± range for two experiments are given.

† Ascites cells were incubated in Fischer's medium containing 1 mg/ml of glucose with and without 100μ M inhibitor at 37° for 30 min. The cells were lysed and the PRPP released was measured by reacting with [8-^{14}C]adenine (200μ M), as described in Materials and Methods. Results are average values ± range of duplicate analysis.

‡ Not determined.

6-MPTF has been shown to have chemotherapeutic activity [25], and it is especially effective when administered in combination with azaserine (Table 4). The life span of mice bearing the L5178Y ascites tumor is prolonged significantly by the combination chemotherapy, whereas either agent alone is without marked activity against this tumor, using the dosing schedule shown in Table 4.

DISCUSSION

Of the purine analogs tested for inhibition of HGPRTase, 6-MPTF was the most specific since it was inactive against APRTase, PNPase or xanthine oxidase. 2,6-Bis-(hydroxyamino)-9-β-D-ribofuranosyl-purine and 6-iodo-9-(tetrahydro-2-furyl)-purine were also active against HGPRTase; however, these compounds also inhibited APRTase and/or xanthine oxidase. Of 104 purine and pyrimidine derivatives tested for inhibition of HGPRTase by Lau and Henderson, twelve active agents were identified [14], including the three mentioned above. Nine of the twelve were purine nucleoside derivatives or analogs, of which eight were 9-substituted compounds. The phenylhydrazone derivatives of the diglycoaldehydes of adenosine or guanosine were also active. Clearly, HGPRTase from human erythrocytes tolerates extremely bulky and diverse substitution at N^9. This tolerance may relate to the nature of the transition-state intermediate. Since inversion occurs in the reaction, displacement of the pyrophosphoryl moiety may proceed via attack of the unshared pair of electrons of N^9 on the partially positive C_1 atom in PRPP [26, 27]. A tendency toward 6-membered ring formation may be envisioned due to hydrogen bond formation between the N^9 hydrogen atom and an oxygen in the pyrophosphate moiety of PRPP. Transition-state analogs may be potent, specific inhibitors [28]; therefore, agents which inhibit HGPRTase may result from simulation of the 6-membered ring in the postulated transition-state intermediate.

Table 4. Effects of azaserine and 6-MPTF on the survival time of mice bearing L5178Y ascites tumor*

Treatment	Median life span (days ± range)
Control	13 ± 1
Azaserine (2 mg/kg)	15 ± 1
6-MPTF (50 mg/kg)	17 ± 2
Combination	23 ± 2

* Drugs were administered i.p. one time daily for 7 days beginning one day after implantation of 10^5 cells; n = 5 animals per group.

6-MPTF and its 6-iodo-analog are active against some animal tumors [25, 29]. Whether these 9-substituted compounds are active as such or following conversion to the base has not been determined. The 1-(tetrahydrofuryl) derivative of 5-fluorouracil appears to require conversion to the base *in vivo* for antitumor effectiveness [30]. Similar to the combination of 6-mercaptopurine and inhibitors of *de novo* purine biosynthesis [31], 6-MPTF potentiates the effect of azaserine in the L5178Y tumor-bearing mouse (Table 4). This potentiation may indicate conversion of 6-MPTF to 6-MP *in vivo*, prevention of "escape" from the *de novo* blockade by inhibition of HGPRTase, or some other mechanism. Interestingly, 6-MPTF has a chemotherapeutic index superior to that of 6-MP [25]. Taken together, these observations suggest that 6-MPTF should be subjected to additional studies concerning its metabolism and modes of action *in vivo*.

Previous workers have reported inhibition of HGPRTase by high levels (0.1 to 1 mM) of diverse non-purine compounds, such as 6-azauridine [9], diazoacetylglycine amide [8], oxyphenbutazone and flufenamic acid [11], and naphtho[2, 3-*d*]imidazole-4,9-

dione and its 1-β-D-ribofuranosyl derivative [12]. The periodate-oxidized analogs of GMP or IMP are potent, irreversible inhibitors of HGPRTase [10]; however, whether these phosphorylated compounds are active in whole cells has not been reported. Using intact Ehrlich ascites tumor cells, Smith *et al.* [32] reported apparent inhibition of HGPRTase by 36 purine-like compounds using radioactive hypoxanthine accumulation, a method which would not differentiate inhibition of the enzyme directly from inhibition of PRPP availability [33, 34]. Krenitsky *et al.* [13] reported K_i values for a number of purines, and only those purines which served as substrates had K_i values similar to the K_m for hypoxanthine.

Although the number of compounds employed is limited, it appears from this work and that of others [13–15] that substitution at the 6-position of the purine ring with electron withdrawing groups such as iodo, mercapto or hydroxyamino increases the affinity for HGPRTase. 6-Amino substitution generally eliminates activity (a notable exception is the phenylhydrazone of the diglycoaldehyde of adenosine [14]). Since the enzyme reaction occurs at N^9, the so-called "business end" of the molecule, inhibitors should result with isosteric substitution at this position, i.e. O, S or C. Encouraging this approach, the substituted oxazolopyrimidines and the thiazolopyrimidine available for testing did show weak affinity for HGPRTase (Table 1).

The observation (Table 3) that some agents are capable of preventing hypoxanthine utilization in intact cells without lowering PRPP pools, coupled with the knowledge that they inhibit isolated HGPRTase, indicates that inhibition of HGPRTase is possible in whole cells. Furthermore, the tolerance of the enzyme toward bulky and diverse substitution at N^9 suggests that highly active, specific inhibitors may be obtainable.

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