

# Development of a Bacterial Screen for Novel Hypoxanthine-Guanine Phosphoribosyltransferase Substrates

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## Abstract

The lack of *de novo* purine biosynthesis in many parasitic protozoans makes the enzymes in the salvage of purines attractive chemotherapeutic targets. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is a key enzyme for purine salvage and bacterial complementation screens for HGPRT inhibitors are known. The low  $K_M$ s for purine bases makes purine analogs unattractive as competitive inhibitors for this enzyme. Despite the availability of many crystal structures of HGPRTs, it is only recently that selective inhibitors of the enzyme have been developed. Therefore, novel purine analogs which act as substrates for the HGPRT reaction and thereby inhibit downstream enzymes or get incorporated into the nucleotide pool are an attractive alternative for drug design. We have used a combination of two *E. coli* strains S $\phi$ 606 (*ara*,  $\Delta$ *pro-gpt-lac*, *thi*, *hpt*) and S $\phi$ 609 (*ara*,  $\Delta$ *pro-gpt-lac*, *thi*, *hpt*, *pup*, *purH,J*, *strA*) to identify inhibitors and substrates of HGPRT. *E. coli* S $\phi$ 609 is deficient in both *de novo* synthesis as well as salvage enzymes of purine nucleotide synthesis, while *E. coli* S $\phi$ 606 is deficient in salvage enzymes only. Hence, expression of functional HGPRTs in *E. coli* S $\phi$ 606 grown in minimal medium makes it susceptible to HGPRT substrates, which inhibit downstream processes. Growth of *E. coli* S $\phi$ 609 in minimal medium can be made conditional for the expression of a functional HGPRT and this growth would be susceptible to both HGPRT substrate analogs and inhibitors. A substance that strictly acts as an inhibitor will affect growth of transformed *E. coli* S $\phi$ 609 only. For this purpose, we compared the human and *P. falciparum* enzymes with known HGPRT substrate analogs. Our data with 6-mercaptopurine, 6-thioguanine and allopurinol show that these compounds act by being substrates for HGPRT. Our results with allopurinol suggest that it is a better substrate for *P. falciparum* HGXPRT than the human enzyme. Therefore, species-specific substrates can be

tested out successfully in *E. coli* S $\phi$ 606. The formation of products from substrates like allopurinol lacking a labile proton at N7 raises the possibility that the deprotonation of substrates might occur at N9 rather than at N7 or a purine anion might be the true substrate for the reaction.

## Introduction

The rapid emergence of drug resistance in the human malaria parasite, *P. falciparum*, calls for an urgent need to develop effective antimalarials (Nabarro and Taylor, 1998). This requires the identification of metabolic differences between the parasite and the host. The reliance of the parasite on the salvage of hypoxanthine present in the erythrocyte of the host, coupled with the absence of *de novo* purine biosynthesis, has made the purine salvage enzyme HGPRT a target of rational drug design for more than a decade (Craig and Eakin, 1997; Craig and Eakin, 2000). It has been shown that depletion of hypoxanthine from parasite cultures leads to the death of the organism indicating that IMP formation from this purine, catalyzed by HGPRT, is not by-passed by an alternate reaction (Berman *et al.*, 1991). Compared to human HGPRT, the *P. falciparum* enzyme has additional substrate specificity for xanthine apart from hypoxanthine and guanine (Queen *et al.*, 1988). However, the crystal structure of the parasite enzyme exhibits no major difference on comparison with the human HGPRT structure (Shi *et al.*, 1999). Based on the proposed oxocarbenium ion transition state of the reaction, inhibitors have been designed which, although active at nanomolar concentrations, failed to inhibit the parasite enzyme specifically (Li *et al.*, 1999). The additional specificity of the malarial enzyme for xanthine, which was supposed to aid in the design of species specific inhibitors, has so far not been successfully utilized.

The recombinant human and *P. falciparum* enzymes complement bacterial HGXPRT deficiency (Eakin *et al.*, 1995). A bacterial complementation system using *E. coli* strain S $\phi$ 609 for screening inhibitors of HGPRT from parasites has been in use for sometime. This screen has been adapted to liquid culture by Eakin and coworkers and a few inhibitors having slightly better selectivity for the parasite HGPRTs were identified (Canyuk *et al.*, 1998). They have also proposed that some of the compounds tried could indeed be acting as substrate analogs. However, no attempt was made to confirm this. Allopurinol, allopurinol riboside and tubercidin are all prodrugs which get incorporated into the nucleotide pools of different parasitic protozoa, resulting in parasite death (Nelson *et al.*, 1979; Hassan and Coombs 1988). The identification of novel substrates that undergo phosphoribosylation by HGPRT,

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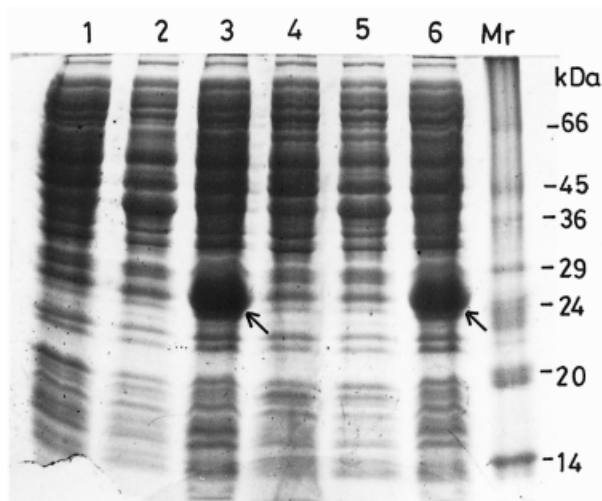


Figure 1. SDS-PAGE profile of expression constructs in *E. coli* Sφ606 and Sφ609. Lane 1: pTrc99A / Sφ606, Lane 2: pHu1 / Sφ606. Lane 3: pPf1 / Sφ606, Lane 4: pTrc99A / Sφ609, Lane 5: pHu1 / Sφ609. Lane 6: pPf1 / Sφ609, Mr: Marker. Arrow indicates hyperexpressed recombinant *P. falciparum* HGXPRT.

which leads to inhibition of subsequent enzymes or incorporation into the nucleic acid pool resulting in parasite death, may provide yet another approach to exploit the HGPRTs of parasitic protozoans. In a recent study it has been shown that allopurinol has an additive effect to quinine in the treatment of acute falciparum malaria (Sarma *et al.*, 1998).

In this paper we report a screening method using the *E. coli* strain Sφ606 (Jochimsen *et al.*, 1975) which lacks only the purine salvage enzymes. This strain can grow in a minimal medium without purine bases and can be used as a convenient way to identify and differentiate novel HGPRT substrates from inhibitors. We have used the well characterized substrate analogs of HGPRT namely, 6-mercaptopurine, 6-thioguanine and allopurinol for this purpose. The same analogs were also tested in *E. coli* Sφ609 (Jochimsen *et al.*, 1975) for comparison. Presence of hypoxanthine/guanine/xanthine in the screen dramatically reduces the extent to which the purine analogs are phosphoribosylated. The protection afforded by hypoxanthine to the phosphoribosylation of the analogs is the highest confirming that this purine has the highest affinity for the enzyme, followed by guanine and xanthine. The results reported here demonstrate the utility of *E. coli* Sφ606 for screening prodrugs acting through HGPRT.

Table 1. Specific activities (nmol/min/mg) in crude lysates of *E. coli* Sφ606 and Sφ609 transformed with human and *P. falciparum* HG(X)PRT constructs.

Construct / strain	Hypoxanthine	Guanine	Xanthine
pHu1 / Sφ606	953.8	2030.7	ND
pHu1 / Sφ609	256.9	724.4	ND
pPf1 / Sφ606	43.8	3.5	77.0
pPf1 / Sφ609	23.7	7.5	33.9

ND: Not detected

## Results

Expression and specific activities of the two HG(X)PRTs in *E. coli* Sφ606 and Sφ609 are shown in Figure 1 and Table 1. High levels of protein expression could not be achieved in the case of human HGPRT but high levels of activity in crude lysates indicated that active protein was expressed, albeit in low quantities. Although *P. falciparum* HGXPRT was expressed to high levels, activity in crude lysates was low. Further, the *P. falciparum* HGXPRT lysates had to be stored immediately after sonication in 10mM DTT to retain significant enzyme activity. This activation by DTT is also reported for the enzyme isolated from the parasite (Queen *et al.*, 1988). No detectable H/G/X PRT activity was found in lysates of *E. coli* Sφ606 and Sφ609 transformed with the vector alone.

*E. coli* strain Sφ606 transformed with human (pHu1) or *P. falciparum* HG(X)PRT (pPf1) expression plasmids or the vector alone grew in minimal medium without added purine bases. The addition of purine bases did not significantly increase their growth (Figure 2). *E. coli* Sφ609 cells transformed with pHu1 expressing human HGPRT grew in hypoxanthine and guanine, while cells transformed with pPf1 expressing *P. falciparum* HGXPRT grew on all three bases hypoxanthine, guanine and xanthine reflecting their purine base specificities. We used the human and *P. falciparum* HG(X)PRTs for standardizing this screen with compounds known to act through HGPRT. We used high concentrations of purine bases and analogs so that the intracellular concentrations of these did not become limiting at any point of growth. Many control experiments were done and strain reversions were not detected. We used an initial inoculum of  $\sim 10^6$  cells/ml in each experiment and  $A_{600}$  was recorded after 15 hours of growth at 37°C.  $A_{600}$  was recorded against water in the reference cuvette and the small values obtained for controls were due to slight precipitation of salts and nongrowing cells. No increase in growth could be seen in controls (Sφ609 transformed with pTrc99A) even after 48 hours, ruling out the occurrence of revertants which could arise from mutation in the *hpt* loci of this *E. coli* strain. In Sφ606 and Sφ609 the *hpt* loci has been inactivated by only UV irradiation unlike the *gpt* loci which has been deleted.

*E. coli* Sφ609 transformed with the vector alone failed to grow in minimal medium (Figure 3). As seen clearly from Figure 2, the expression of human and *P. falciparum* HG(X)PRT in *E. coli* Sφ606 made them susceptible to the HGPRT analogs 6-mercaptopurine, 6-thioguanine and allopurinol, indicating that these compounds are incorporated into the metabolic pool of *E. coli* and hence causing cell death. The addition of purine bases to cultures of *E. coli* Sφ606 transformed with expression plasmids, and containing the analogs, reversed the growth inhibitory effect of these compounds. Similar results were obtained with *E. coli* Sφ609 transformed with pHu1 and pPf1 (Figure 3). 6-mercaptopurine was the best substrate analog for human and *P. falciparum* HG(X)PRT enzymes. Allopurinol was found to be a better substrate for the *P. falciparum* enzyme. The reduced inhibition of growth by 6-thioguanine of *E. coli* Sφ606 transformed with pPf1 compared to Sφ609 (similarly transformed) indicates that this compound is a poor substrate for the *P. falciparum* HGXPRT but a good

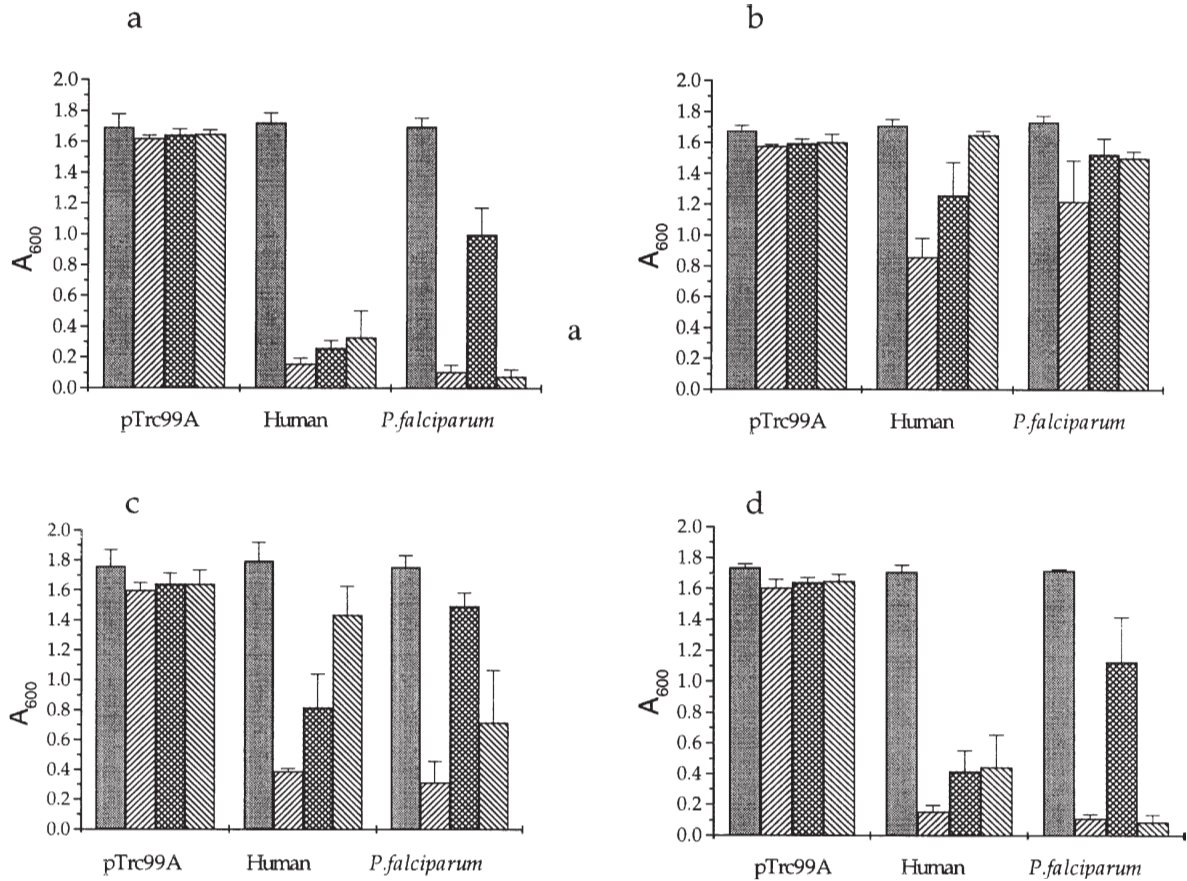


Figure 2. Growth, in minimal medium, of *E. coli* Sφ606 transformed with the indicated constructs. Control cells were grown without oxypurine analog (■) and the test samples contained 2.5mM 6-mercaptopurine (▨), 6-thioguanine (▩) or allopurinol (▧). Growth medium was supplemented with a) no purine base, b) 500μM hypoxanthine, c) 500μM guanine and d) 500μM xanthine.

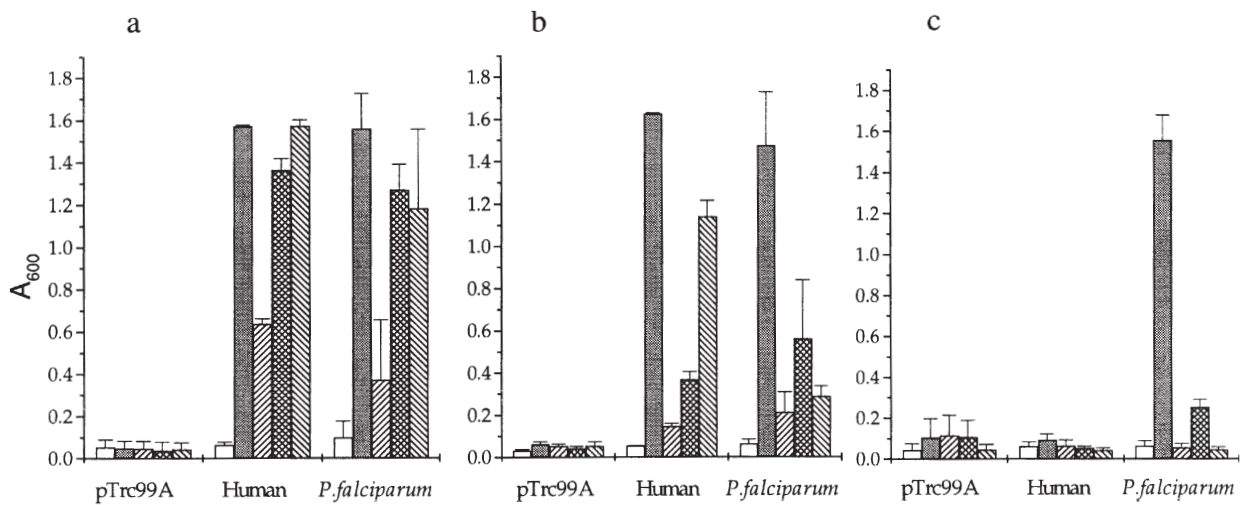


Figure 3. Growth, in minimal medium, of *E. coli* Sφ609 transformed with the indicated constructs. Control cells were grown without oxypurine base (□) and the test samples contained a) 500μM hypoxanthine, b) 500μM guanine and c) 500μM xanthine in the presence of no oxypurine analog (■), 2.5mM 6-mercaptopurine (▨), 6-thioguanine (▩) or allopurinol (▧).

Table 2. Percentage inhibition of *E. coli* S $\phi$ 606 growth<sup>§</sup>.

Construct / strain	No purine base			Hypoxanthine <sup>**</sup>			Guanine <sup>**</sup>			Xanthine <sup>**</sup>		
	6-MP*	6-TG*	AL*	6-MP*	6-TG*	AL*	6-MP*	6-TG*	AL*	6-MP*	6-TG*	AL*
pTrc / S $\phi$ 606	7.5	5.6	5.7	6.0	4.8	4.2	9.1	6.3	6.3	7.5	5.2	5.2
pHu1 / S $\phi$ 606	91.5	85.8	81.8	49.4	26.5	2.9	78.8	54.7	20.1	91.2	76.0	74.3
pPf1 / S $\phi$ 606	94.1	41.8	95.9	29.5	12.1	11.8	82.3	14.9	59.4	93.6	34.9	94.8

<sup>§</sup>*E. coli* S $\phi$ 606 transformed with the indicated constructs was grown in minimal medium containing the purine analogs; 6-MP – 6-mercaptopurine, 6-TG – 6-thioguanine, AL – allopurinol.

<sup>\*\*</sup>500 $\mu$ M purine base,

\*2.5mM analog

inhibitor (Tables 2 and 3). For both *E. coli* S $\phi$ 606 and S $\phi$ 609 transformed with pHu1 or pPf1, hypoxanthine afforded the highest protection to the bacteria from action of the prodrugs, followed by guanine and xanthine. The other analogs tested were 1-methyl xanthine, 3-methyl xanthine, 6-O-methyl guanine and 6-chloroguanine. 6-chloroguanine was cytotoxic as it killed *E. coli* S $\phi$ 606 cells transformed with the vector alone (data not shown). Other compounds tested did not inhibit *E. coli* S $\phi$ 606 growth to any significant extent although 6-O-methyl guanine showed marginal inhibition of growth of S $\phi$ 606 transformed with pPf1 (data not shown).

## Discussion

Chemotherapy for parasitic infections in principle relies on exploiting metabolic differences between the parasite and the host. Although the obligatory nature of purine salvage in parasites is known, its exploitation for chemotherapy has seen limited success. Recently, inhibitors which act on parasite HGPRT at submicromolar range have been designed and one of them does inhibit *T. foetus* growth in culture (Aronov *et al.*, 2000). Inhibitors which mimic the proposed oxocarbenium ion transition state are slow binding and their bioavailability has not been reported. These inhibitors require that the active site be occupied by Mg<sup>2+</sup>-pyrophosphate to enable their binding (Li *et al.*, 1999). However, this may not be possible *in vivo* where the enzyme is in the process of actively phosphoribosylating purine bases. Analogs, which can be phosphoribosylated, do not have this limitation and could potentially yield molecules with therapeutic value. Testing of a large number of substrate analogs, generated by synthetic chemistry, on parasites in culture can be quite laborious. Such parasite growth inhibition assays do not provide any indication of the mode of action of the drug. Hence, a bacterial assay providing a rapid screen for potential inhibitors/ prodrugs

is useful.

In the case of parasitic HG(X)PRTs, a bacterial screen for inhibitors already exists (Eakin *et al.*, 1995; Canyuk *et al.*, 1998). Compounds like allopurinol and tubercidin are incorporated into the nucleotide pools of the parasite, thereby causing their death (Hassan and Coombs, 1988). We are not aware of any bacterial screen where potential HGXPRT substrates and inhibitors can be differentiated conveniently in a completely defined medium. The potential use of *E. coli* S $\phi$ 606 for this purpose has been mentioned in the literature but not demonstrated (Ullman and Carter, 1995). We have used the human and *P. falciparum* HG(X)PRTs for standardizing this screen with compounds known to act through HGPRT. Our results (Figure 2a) show that substrate analogs of recombinant HG(X)PRT continue to inhibit cell growth even after 15 hours of incubation indicating that end point measurements are reliable. Such an end point measurement may not readily distinguish very weak from inactive purine analogs. Growth curve analysis would aid in identifying such weak substrate analogs. Plasmid loss during growth of *E. coli* S $\phi$ 606 transformed with pHu1 or pPf1, arising from depletion of ampicillin by secreted  $\beta$ -lactamase did not occur under assay conditions. Our results clearly show that molecules, which are efficient substrate analogs continue to keep cell growth checked at the time (15 hours) of cell density measurement.

Transition state analogs of HGPRT, which are potent inhibitors of the enzyme, being charged may not be cell permeable. But novel purine base analogs, which are uncharged, can permeate parasite membranes and subsequently get metabolized to toxic products, alleviating the problem of drug delivery. The uptake of inhibitors into *E. coli* and resultant cell death gives a good estimate of the bioavailability and general toxicity of a particular compound. Allopurinol, an analog of hypoxanthine is relatively nontoxic in mammals, but is metabolized by *Leishmania donovani*, resulting in its death (Looker *et al.*,

Table 3. Percentage inhibition of *E. coli* S $\phi$ 609<sup>§</sup>.

Construct / Strain	Hypoxanthine <sup>**</sup>			Guanine <sup>**</sup>			Xanthine <sup>**</sup>		
	6-MP*	6-TG*	AL*	6-MP*	6-TG*	AL*	6-MP*	6-TG*	AL*
pHu1 / S $\phi$ 609	59.6	13.4	0.0	91.4	77.8	29.6	-	-	-
pPf1 / S $\phi$ 609	76.1	18.7	23.9	85.7	62.6	81.0	96.4	83.9	97.4

<sup>§</sup>*E. coli* S $\phi$ 609 transformed with the indicated constructs was grown in minimal medium containing the purine analogs; 6-MP – 6-mercaptopurine, 6-TG – 6-thioguanine, AL – allopurinol.

<sup>\*\*</sup>500 $\mu$ M purine base

\*2.5mM analog

1986). Purified *P. falciparum* HGXPRT phosphoribosylates allopurinol very well compared to the HGPRT from the human host (Keough *et al.*, 1999). Allopurinol differs from hypoxanthine in having a carbon at position 7 instead of a nitrogen. The reaction mechanism detailed in the literature proposes a base assisted deprotonation at N7 mediated by a conserved aspartic acid residue in HGPRT (Xu and Grubmeyer, 1998). Mutating this aspartic acid results in a poorly active enzyme but does not eliminate activity (Xu and Grubmeyer, 1998). The carbon 7 proton of allopurinol cannot be easily abstracted but allopurinol is still a substrate for the malarial and Leishmanial HGPRTs. This raises an important question as to the site of proton removal, which might be at N9. In fact it has been speculated that the substrate could be a purine anion (Xu and Grubmeyer, 1998). The appreciable phosphoribosylation of allopurinol by the *P. falciparum* enzyme however, does not result in significant inhibition of *in vitro* parasite growth (Queen *et al.*, 1990). This lack of antiparasitic activity could arise from the inhibition of xanthine oxidase by allopurinol thereby, leading to an increase in the availability of hypoxanthine for the parasite (Berman *et al.*, 1991). However, in a recent study carried out by Sarma *et al.* (1999) allopurinol has been shown to have an additive effect in clearing parasitemia when administered along with quinine. The data on 6-thioguanine with *E. coli* S $\phi$ 606 and S $\phi$ 609 transformed with pPf1 indicate that this compound is phosphoribosylated poorly by *P. falciparum* HGXPRT, but serves as a good inhibitor when guanine and xanthine are the substrates. However, the greater phosphoribosylation of 6-thioguanine by the human enzyme hints at subtle differences in the active sites of human and *P. falciparum* enzymes, which can potentially be exploited for drug design. The relaxed substrate specificity of the parasitic HGXPRTs can be exploited to make specific inhibitors of downstream enzymes. The prior knowledge of multiple substrates utilized by parasite and host HGPRT using the screen described in the paper will therefore prove useful in limiting the number of compounds to be tested on the parasite. Situations are also known where the incorporation of nucleotide analogs into the nucleic acid pools did not have any detrimental affect on parasite growth (Dovey *et al.*, 1985). Therefore, caution should be exercised while using the screen, because a novel HGXPRT substrate inhibiting *E. coli* growth need not also inhibit parasite growth.

## Experimental Procedures

### Materials

Purine bases and analogs were procured from Sigma chemical company, USA. Chemicals for minimal medium were from Sigma chemical company, USA and Ranbaxy laboratories, India. *E. coli* bacterial strains S $\phi$ 609 (*ara*,  $\Delta$ *pro-gpt-lac*, *thi*, *hpt*, *pup*, *purH,J*, *strA*) and S $\phi$ 606 (*ara*,  $\Delta$ *pro-gpt-lac*, *thi*, *hpt*) were gifts from Dr. Per Nygaard. Human and *P. falciparum* HG(X)PRT expression clones in the vector pTrc99A were constructed in the laboratory (pHu1 and pPf1 respectively).

### Expression and Activity Measurements

Levels of expression of recombinant HG(X)PRTs were analyzed by SDS-PAGE. For specific activity measurements, transformed cells were induced as reported by Subbayya *et al.* (2000), and lysed in a buffer containing 50mM Tris-HCl pH 7.5, 2mM MgCl<sub>2</sub>, 10% glycerol, 0.1mM PMSF and 1mM DTT by sonication. DTT was added to a final concentration of 10mM for the *P. falciparum* HGXPRT lysates. Enzyme assays were carried out using conditions reported earlier (Subbayya *et al.*, 2000). Protein estimation was done by the Bradford method with BSA as the standard (Bradford., 1976).

### HGXPRT Functional Complementation and Screening

*E. coli* strain S $\phi$ 609 transformed with the expression plasmids pHu1 or pPf1 complemented HG(X)PRT deficiency. However, *E. coli* S $\phi$ 606 is capable of growth in the absence of recombinant HGPRT in minimal medium. Both bacterial strains transformed with vector alone were used as controls. Transformed *E. coli* S $\phi$ 609 cells were grown overnight in LB medium with 100 $\mu$ g/ml of ampicillin and 25 $\mu$ g/ml streptomycin. *E. coli* S $\phi$ 606 cells transformed with pHu1 and pPf1 were grown similarly, but with ampicillin only. These cells were washed 3 times with 1X M9 salt solution and resuspended in 1X M9 salt solution. A 1% inoculum of these cells was added to 2 ml minimal medium in 15 ml test tubes containing 1X M9 salts, 1mM MgSO<sub>4</sub>, 0.1mM CaCl<sub>2</sub>, 1mM thiamine hydrochloride, 1mM proline, 0.2% glucose, 0.3mM IPTG, 25 $\mu$ g/ml streptomycin, 100 $\mu$ g/ml ampicillin and 0.5mM hypoxanthine /guanine /xanthine. The cells were allowed to grow for 15 hours at 37°C and A<sub>600</sub> was recorded. All analogs were tested at a single concentration of 2.5mM. The varying solubilities of purine bases and analogs were circumvented by dissolving all of them in 0.4N NaOH. The final concentration of NaOH in the growth media is 1.3mM. At this concentration the pH of the media is not altered. A total of seven analogs were tested using these combined screens. Experiments with 6-mercaptopurine, 6-thioguanine and allopurinol were done in triplicates while others compounds (1-methyl xanthine, 3-methyl xanthine, 6-O-methyl guanine and 6-chloroguanine) were tested in duplicates as they were not available in sufficient amounts.

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