***Helicobacter pylori* Xanthine-Guanine-Hypoxanthine Phosphoribosyltransferase – a Putative Target for Drug Discovery against Gastrointestinal Tract Infections**

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ABSTRACT

Helicobacter pylori (*Hp*) is a human pathogen that lives in the gastric mucosa of approximately 50% of the world's population causing gastritis, peptic ulcers and gastric cancer. An increase in resistance to current drugs has sparked the search for new *Hp* drug targets and therapeutics. One target is the disruption of nucleic acid production which can be achieved by impeding the synthesis of the 6-oxopurine nucleoside monophosphates, precursors of DNA and RNA. These metabolites are synthesised by *Hp* xanthine-guanine-hypoxanthine phosphoribosyltransferase (XGHPRT). Here, nucleoside phosphonates have been evaluated which inhibit the activity of this enzyme with K_i values as low as 200 nM. Prodrugs of these compounds arrest the growth of *Hp* at a concentration of 50 μ M in cell based assays. The kinetic properties of *Hp*XGHPRT have been determined together with its X-ray crystal structure in the absence and presence of 9-[(*N*-3-phosphonopropyl)-aminomethyl-9-deazahypoxanthine, providing a basis for new antibiotic development.

INTRODUCTION

Gastritis, peptic ulcers, two forms of cancer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma have been shown to be associated with the presence of *Helicobacter pylori* (*Hp*) in the gastric mucosa. The finding that *Hp* is the etiological agent for these diseases was made in 1982 by two Australian scientists, Marshall and Warren,¹ who were subsequently awarded the Nobel Prize for Physiology and Medicine in 2005 for this discovery. *Hp* is found in the gastric mucosa of at least 50% of the world's population,² though this number is closer to 100% in some countries.³ Its presence is asymptomatic and carriers have no prior knowledge of when, or if, life-threatening symptoms could take hold.⁴⁻⁹ As well as causing the devastating symptoms of gastritis and peptic ulcers, *Hp* is the third leading cause of cancer and the most common cause of cancer due to infectious agents, ranking it above papillomavirus and hepatitis B.¹⁰

Together with other gram negative infections, there has been an alarming increase in multi-drug resistant strains (MDR) of *Hp* (where MDR is defined as resistance to $\geq$ three antibiotics of different classes) in recent years.^{4, 11} This resistance has been found to be as high as 15-20% and, in some locations, this percentage is higher.¹²⁻¹³ This led the WHO to include *Hp* in the list of the top twelve pathogens for urgent drug discovery and prompted the drive to find new drug targets and new antibiotics to combat the diseases caused by this organism.¹⁴⁻¹⁵

One possible target for innovative therapies to treat *Hp* infections is the metabolic production of nucleic acids. If this can be interrupted, or significantly slowed, the organism will have a diminished ability to survive. All organisms need to be able to synthesise their own DNA/RNA and this means that they must have a constant supply of precursor nucleoside monophosphates. There are two metabolic routes whereby an organism can obtain these compounds: (i) by the synthesis of the purine ring in a 10-step

energetically demanding metabolic pathway (*de novo*) or (ii) by the transport of purine bases or nucleosides from their host (salvage). *Hp* has adopted the second of the two strategies with the full complement of genes coding for the *de novo* pathway being redundant.^{16,17}

Mendz and colleagues undertook the first studies on the purine salvage pathway in *Hp*¹⁸ and established the role of *Hp*XGHPRT in the synthesis of its purine nucleotides. That *Hp* requires exogenous purine bases/nucleosides for growth has been documented previously.¹⁹ For microbes, the rate at which they generate AMP and GMP pools has also been found to be the limiting step in their growth²⁰ and, hence, the need for a fully functional salvage pathway. **Fig. 1** shows the enzymes available to the parasite needed to synthesize the nucleoside monophosphates, GMP and IMP, which are then incorporated directly into its DNA and RNA.

Fig. 1. The purine salvage pathway in *Hp*. The green oval indicates the cell membrane of *Hp*. A. GMP synthase; B. IMP dehydrogenase; C. Adenylosuccinate synthase; D. Adenylosuccinate lyase; E. GMP

reductase; F. Purine nucleoside phosphorylase (PNP); APRT: Adenine phosphoribosyltransferase; XGHPRT: Xanthine-guanine-hypoxanthine phosphoribosyltransferase. Adapted with permission from Liechti and Goldberg.¹⁹

A diagram for the cycling of bases, nucleobases and nucleotides within *Hp* is given in **Fig. 2**.

Fig. 2. The cycling of purine bases, nucleosides and nucleotides in *Hp*. The gene coding for the 5'/3'-nucleotidase is *surE*. Adapted with permission from Liechti and Goldberg.¹⁹ The red cross signifies that nucleoside monophosphates are unable to cross the cellular membrane into the bacteria.

A central enzyme for the supply of the purine nucleoside monophosphates is xanthine-guanine-hypoxanthine phosphoribosyltransferase (XGHPRT) (**Fig. 2**). Thus, it is a candidate for drug intervention of *Hp*. XGHPRT belongs to a family of enzymes known as the 6-oxopurine phosphoribosyltransferases (PRTases). The reaction catalysed by these enzymes is given in **Fig. 3**.

Fig. 3. The reaction catalysed by the 6-oxopurine PRTases. When R=NH₂, the base is guanine, when R=H, the base is hypoxanthine and when R=OH, the base is xanthine.

It has previously been demonstrated that inhibitors of *Plasmodium falciparum* (Pf)HGXPRT, *Plasmodium vivax* (Pv)HGPRT or *Mycobacterium tuberculosis* (Mt)HGPRT show activity as anti-malarial²¹⁻²⁵ and anti-tuberculosis agents.²⁶ One class of inhibitor is broadly referred to as the “nucleoside phosphonates” as they contain at least one carbon-phosphorus bond, making them stable to enzymatic or chemical hydrolysis. These compounds are designed to mimic either the substrates or products of the catalytic reaction or that of the transition-state. The nucleoside phosphonate inhibitors of the 6-oxopurine PRTases can be categorised into six groups: the acyclic nucleoside phosphonates (ANPs);²³ the aza-ANPs;²¹ the symmetric and asymmetric acyclic nucleoside bis-phosphonates (ANbPs);²⁷⁻²⁸ the acyclic immucillin phosphonates (AIPs);²⁴ the pyrrolidine nucleoside phosphonates (PNPs);²⁹ and the pyrrolidine nucleoside bis-phosphonates (PNBPs).²² The scaffolds for these inhibitors are shown in **Fig. 4**.

Fig. 4. The chemical templates of the 6-oxopurine PRTase phosphonate inhibitors. **(A)** The first acyclic nucleoside phosphonates (ANPs). X denotes the atoms connecting the two functional groups, the base and the phosphonate group, and can be either carbon or oxygen atoms. R₁ denotes where further chemical attachments have been made.²¹ **(B)** The aza-ANPs. Attachments at (R₂) allow a functional group to reach into a third cavity in the active site, the pyrophosphate binding pocket.²² **(C)** The acyclic immucillin

phosphonates (AIPs) containing a positively charged nitrogen in the linker mimicking the positively charge on the ribose oxygen which is predicted to occur at the transition state. Variable attachments have been made at R₃.²⁵ (D) The pyrrolidine nucleoside phosphonates (PNPs). The length of the linker (m) can vary.²⁶ (E) The pyrrolidine nucleoside bisphosphonates (PNBPs). A second phosphonate group is added to the carbon atom in the pyrrolidine ring. Rotation around the five-membered ring allows either of the phosphonate groups to point to the two different areas in the active site that can bind phosphate groups.²⁷

Crystal structures of different representatives from these five classes of nucleoside phosphonate inhibitors have been obtained in complex with the 6-oxopurine PRTases from homo sapiens, *Escherichia coli* (Ec) *Mycobacterium tuberculosis* (Mt), *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv). The K_i values for the inhibitors differ for each of these enzymes. Thus, the crystal structures show not only how they bind in the active site but provide the explanations for the range of binding constants and offer clues as to how the inhibitor can be tailor-made to fit more tightly in the active site of the 6-oxopurine PRTases from the individual organisms.

The original design of these inhibitors was based on the successful antiviral drugs including tenofovir, adefovir and cidofovir which also contain a nucleobase (either adenine or cytosine) linked by a series of atoms to a phosphonate group.³⁰⁻³¹ To improve the biological activity of those drugs, the negative charges were masked by hydrophobic attachments to increase membrane transport and, thus, their biological activity.³²⁻³³ Such compounds are prodrugs since the masking groups have to be hydrolysed by intracellular enzymes present in the target cell to release the effective therapeutic.³²⁻³³ This prodrug strategy was thus adopted to convert phosphonate inhibitors of *Plasmodium falciparum* (Pf) HGXPRT and *Mycobacterium tuberculosis* (Mt) HGPRT into compounds that arrest the growth of *Pf*²¹ and *Mt* in cell-based assays.²⁶

Based on previous studies, *Hp*XGHPRT is suggested as a target enzyme for the development of chemotherapeutics against *H. pylori* infections. Herein, recombinant *Hp*XGHPRT has been expressed and purified and its kinetic properties evaluated. Non-naturally occurring 6-oxopurines were also assayed to determine if they are substrates for the enzyme and if they possess anti *H. pylori* activity in cell assays. Such compounds have potential to be developed into new therapeutics. In addition, we present the first crystal of *Hp*XGHPRT which is used to explain substrate specificity and to show how phosphonates newly synthesised in our laboratories, inhibit this enzyme. We then demonstrate that prodrugs of these compounds arrest the growth of *Hp* in cell-based assays.

RESULTS and DISCUSSION

Catalytic properties of the target enzyme, *Hp*XGHPRT. Recombinant hexa-his tag *Hp*XGHPRT was expressed and purified to homogeneity. The kinetic constants of this enzyme for the naturally-occurring 6-oxopurine bases and 5-phospho- α -D-ribosyl-1-pyrophosphate (*PRib-PP*) were determined. These are compared with those for the human 6-oxopurine PRTase as well as three bacterial 6-oxopurine PRTases to assess its relative catalytic efficiency (**Table 1**).

Table 1. The kinetic constants for *Hp*XGHPRT, human HGPRT and three bacterial 6-oxopurine PRTases.

Enzyme	k_{cat} (s ⁻¹) (G)	k_{cat} (s ⁻¹) (Hx)	k_{cat} (s ⁻¹) (X)	
<i>Hp</i> XGHPRT	7.3±0.2	4.5±0.4	2.3±0.3	
<i>Hs</i> HGPRT ³⁴	8.2±0.6	5.2±0.4	NS ^b	
<i>Ec</i> XGPRT ³⁵	28.0±0.1	13.7±1.1	38.5±0.7	
<i>Ec</i> HGPRT ³⁶	10.2±0.7	59.0±3.5	0.008±0.0001	
<i>Mt</i> HGPRT ²⁶	0.62±0.02	0.55±0.01	NS ^b	
Enzyme	K_m (μM) (G)	K_m (μM) (Hx)	K_m (μM) (X)	K_m (μM) (<i>PRib-PP</i>)
<i>Hp</i> XGHPRT	3.1±0.2	8.4±0.5	20±2	27±4 ^a
<i>Hs</i> HGPRT ³⁴	1.9±0.4	3.4±1.0	NS	65±5 ^a
<i>Ec</i> XGPRT ³⁵	4.3±0.3	91±11	30.5±2.6	139±16 ^a
<i>Ec</i> HGPRT ³⁶	294±20	12.5±2.4	25±6.1	193±7 ^c
<i>Mt</i> HGPRT ²⁶	4.4±0.4	8.3±0.7	NS ^b	465±15 ^c
Enzyme	k_{cat}/K_m (μM ⁻¹ s ⁻¹) (G)	k_{cat}/K_m (μM ⁻¹ s ⁻¹) (Hx)	k_{cat}/K_m (μM ⁻¹ s ⁻¹) (X)	k_{cat}/K_m (μM ⁻¹ s ⁻¹) (<i>PRib-PP</i>)
<i>Hp</i> XGHPRT	2.3	0.5	0.10	0.3 ^a
<i>Hs</i> HGPRT ³⁴	4.3	1.5	NS ^b	0.13 ^a
<i>Ec</i> XGPRT ³⁵	6.5	0.15	1.3	0.2 ^a

<i>Ec</i> HPRT ³⁶	0.03	4.7	0.03	0.3 ^c
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^aGuanine as the substrate. ^bNS=Not a substrate. ^cHx as substrate.

In terms of its k_{cat} and K_m values for hypoxanthine, guanine and *PRib-PP*, *HpXGHPRT* is more akin to human HGPRT than to the three bacterial enzymes (**Table 1**). Where it differs from the human enzyme is that xanthine is not a substrate for the human enzyme but it is a good substrate for *HpXGHPRT* with a k_{cat} value only three times lower than for guanine and 1.7 lower for hypoxanthine (**Table 1**). The K_m value of 20 μM demonstrates that it also binds this substrate well, slightly better than *EcXGPRT* which has a K_m for xanthine of 30 μM (**Table 1**).

The K_m of *PRib-PP* for *HpXGHPRT* is also similar to the human enzyme but is five times lower than for *EcXGPRT*, seven times lower than for *EcHPRT* and seventeen times lower than for *MtHGPRT*. Thus, the *Hp* enzyme has a higher affinity for this substrate than *EcXGPRT*, *EcHPRT* or *MtHGPRT* (**Table 1**). The two *Ec* enzymes have higher k_{cat} values compared with *HpXGHPRT* for the purine bases. As denoted by their name, *EcXGPRT* prefers xanthine (19-fold higher) and guanine (4-fold higher) while *EcHPRT* prefers Hx (16-fold higher). In contrast, these purine bases have much higher K_m values for the *Ec* enzymes, resulting in little difference in catalytic efficiency between these three enzymes (**Table 1**). *MtHGPRT* has similar K_m values for guanine and Hx as *HpXGHPRT* but the major difference lies in their respective k_{cat} -values (approximately 10-fold lower for both bases for the *Mt* enzyme) making *MtHGPRT* the slowest of these five enzymes, but still highly active (**Table 1**). Thus, *HpXGHPRT* is an efficient enzyme, consistent with its central role in the synthesis of the nucleoside monophosphates *in vivo*. Furthermore, the recombinant enzyme is suitable for screening and testing of compounds that could be developed as drug leads.

The catalytic properties of this enzyme can be used to arrest the growth of *Hp* *in vitro*. The purine base analogs, 6-mercaptopurine and 6-thioguanine, have been shown to possess anti-cancer properties³⁷ and they also arrest the growth of *Plasmodium falciparum* *in vitro*.³⁴ The mechanism of action of these

two anti-cancer agents is via conversion to their toxic metabolites, the corresponding nucleoside monophosphates, by human HGPRT.³⁷ Thus, *Hp*XGHPRT could play a similar role in *H. pylori*.

6-Thioxanthine is not a substrate for human HGPRT and it functions as a non-toxic drug in human cells. This purine base requires the introduction of the *Ec gpt* gene into the cancer cells before cell proliferation can cease or be reduced.³⁸ It then exerts its ant-cancer activity by the action of the expressed *Ec*XGHPRT enzyme which catalyses the formation of the corresponding 6-thioxanthine nucleoside monophosphate. GMP synthase converts this nucleoside into the lethal 6-thioguanine monophosphate.³⁸ This is the principle used in suicide gene therapy whereby an innocuous compound can be transformed into an active drug.³⁸

The kinetic constants of 6-thioxanthine, 6-thioguanine and 6-mercaptopurine for *Hp*XGHPRT were determined with a view to ascertaining if they were substrates of *Hp*XGHPRT which would convert them into toxic metabolites (**Table 2**). All three have similar kinetic properties to those of the naturally occurring bases (**Table 1**). The substitution of the 6-oxo group by a 6-thio group has only a small effect on k_{cat} and K_m values for either guanine or xanthine (*cf* **Table 1**). The biggest difference is a five-fold increase in the K_m for 6-mercaptopurine when compared with hypoxanthine. The naturally-occurring purine bases, guanine, hypoxanthine and xanthine, have been shown to be transported into the *Hp* cells from its host cell (**Fig. 1**), so it is assumed that these three bases would be transported in a similar manner. **Table 2** shows that 6-thioguanine and 6-thioxanthine exhibit activity against *Hp* in cell culture. Thus, purine base analogs have potential as drug leads against *Hp*. 6-Thioxanthine should exhibit a favourable therapeutic index as it is not a substrate for the human enzyme and it is non-toxic to human cells.

Table 2. Kinetic constants of the 6-thio substituted substrates for *Hp*XGHPRT and their activity in *Hp* cell assays

Base	Structure	k_{cat} (s ⁻¹)	$K_{m(app)}$ (μM)	<i>Hp</i> activity
6-thioxanthine		1.1	32 ± 2	(-) ^a
6-thioguanine		8.8	3.3 ± 0.2	(+) ^b
6-mercaptopurine		2.4	41 ± 4	ND ^c

^a(-) No growth at 50 μM concentration of the base in the cell culture. ^b(+) Some inhibition of growth at 50 μM concentration of the base in the cell culture. ^cND = not determined.

Inhibitors of this enzyme and assessment of their prodrugs in arresting the growth of *Hp* in cell based assays. Eleven compounds were chosen from our nucleoside phosphonate inventory and their K_i values determined for *Hp*XGHPRT (**Table 3**). All are competitive inhibitors with respect to *PRib-PP*. Prodrugs of each of these inhibitors were then synthesised to assess their biological activity. The prodrug approach was adopted as the negative charges of the phosphonyl oxygens of these inhibitors severely reduces, or abolishes, their ability to traverse cell membranes.³²⁻³³ Four different masking groups were trialled to determine the prodrug of choice for *Hp*. The compound number and structure of each inhibitor together with its K_i value and cell based activity is given in **Table 3**.

Table 3. K_i values of *Hp*XGHPRT inhibitors and the biological activity of their prodrugs against *Hp* in cell based assays.

Structure of the inhibitor	K_i (μM)	Structure of the prodrug ^a	<i>Hp</i> Activity
I²²
0.2±0.05	I-A	(-) ^c
		I-B	(+++) ^c
II²⁶
0.2±0.03	II-A^b	(+) ^d
		III-A	(-) ^c

0.3 ± 0.04 **IV²⁷****IV-A****(+)^d** 0.4 ± 0.1 **V²¹****V-A** 1.1 ± 0.3 **VI²⁹****VI-C^b****(+)^d**

1.9 ± 0.4 **VI-D^b** **(-)^c****VII²⁸****VII-A** 2.1 ± 0.4 **(+)^d****VIII²¹****VIII-A** 3.4 ± 0.4 **(-)^c****IX²⁷****IX-A** 5 ± 1 **(+)^d**

X³⁹ **X-A**

5±1

(-)^c**XI²¹****XI-A**

32±4

(+++)^c

^a**A, B, C and D** denote the chemical structure of the masking groups. ^bSynthesis in methods, unpublished compound. ^c(-) Indicates that there is no growth at 50 μ M concentration of the compound in the cell culture. ^d(+) Indicates that there was some inhibition of growth at 50 μ M concentration of the compound in the cell culture. ^e(+++) Indicates that the addition of the inhibitor to the cell culture at 50 μ M concentration made no difference to the growth.

Compound **I** has one of the lowest K_i values for *Hp*XGHPRT in this series of compounds and one of its prodrugs, **IA**, inhibits *Hp* growth (**Table 3**). However, the second prodrug of this compound, **IB**, had no biological effect, even at 50 μ M, suggesting that a phenyl group is the preferred option compared with a methyl group for delivery and activation in *Hp*. Other instances where the substitution of alanine for

phenylalanine influences the biological effectiveness of the prodrug *in vitro* have also been reported.^{33,}

⁴⁰ It is not known if the differences in biological activity are due to increased transport or to more efficient hydrolysis of the masking group within the cell or whether it is a combination of both.

Compounds **II** and **III** have similar K_i values as **I** ($\sim 0.2 \mu\text{M}$) and their masking group is the same as **IA**. The prodrug of **IIA** is slightly more effective *in vitro* than **IIIA**. The only difference between **II** and **III** is the number of carbon atoms in the linker connecting the hydroxyl group to the nitrogen atom with **II** having one extra carbon. Though the effect on the anti-bacterial growth is not marked, this extra carbon in the linker effects either transport or hydrolysis in the *Hp* cells.

There is little difference in the K_i values between **IV** and **V** (0.4 and $1.1 \mu\text{M}$) and they have the same masking groups. Therefore, it is not unexpected that the prodrugs, **IV-A** and **V-A**, have a similar ability to prevent *Hp* growth *in vivo*. Compound **VI** has a slightly higher K_i value ($1.9 \mu\text{M}$) than **I-V** and two different prodrugs of this compound were synthesised (**VI-A** and **VI-D**). Surprisingly, the prodrug with masking group “**D**” has better *Hp* activity than **A**. This masking group was originally prepared for use as an anti-malarial drug formulation as it is a mimic of primaquine. However, it works well as a prodrug for *Hp*.

Compounds **VII-X** have K_i values between 2 and $5 \mu\text{M}$. With similar masking group all possess activity against *Hp* though **VIII-A** and **X-A** are slightly more effective. **X-A** has good biological activity and its parent compound is a good inhibitor. Its activity against the growth of *Hp in vitro* could be due to the fact that there is only one phosphonate group and, therefore, only two masking groups have to be hydrolysed as opposed to four. Furthermore, the attachment of the hydrophobic phenyl group could facilitate transport. This prodrug also possesses excellent IC_{50} values against *Pf* and *Mycobacterium*

tuberculosis in cell culture (1.5 and 1.1 μM) suggesting that this form of drug and prodrug as a good lead for chemotherapeutic development.

Compound **XI** is the guanine analog of **V** but it has a higher K_i value (*cf* 32 μM with 1.1 μM) (**Table 3**). The masking group in both of these prodrugs is the same, so the only difference is in the value of the inhibition constant. **V-A** has good biological activity *in vitro* but **XI-A** has no effect, suggesting that the parent compound has to be a good inhibitor of *Hp*XGPRT to be effective *in vitro*.

The cytotoxicity in human cells for a number of these compounds has been determined previously. These values range from 100 to >300 μM , higher concentrations than those needed to kill *Hp* in culture.^{21-22, 26-29, 39, 41} This good selectivity index is an indication that these compounds are forerunners for antibiotic development against *Hp*.

The subunit size and amino acid sequence of *Hp*XGHPRT. A bioinformatic study was performed to assess the similarity and differences in the amino acid sequences between *Hp*XGHPRT and the four 6-oxopurine PRTases in (**Fig. 5**).⁴²⁻⁴³ The subunit of *Hp*XGHPRT consists of 153 amino acids making it, along with *Ec*XGPRT (152 amino acids), one of the two smallest of these five enzymes (*Hs*HGPRT contains 218 residues, *Ec*HGPRT contains 178 residues and *Mt*HGPRT contains 202) (**Fig. 5**).

Fig. 5. Sequence alignment between *HpXGHPRT* and the 6-oxopurine PRTases from humans, *Ec* and *Mt*. The residues highlighted in cyan are identical and those in red are homologous. The "boxes" indicate the binding site of functional groups *i.e.* purine base, 5'-phosphate group of the nucleoside monophosphates or *PRib-PP*, the divalent metal ion and PP_i (Fig. 3). These locations are based on the known crystal structures of the human and bacterial enzymes in complex with substrates or products of the reaction or with inhibitors. *HpXGHPRT* (WP_00559479); (*EcXGPRT* (POA9M5); *HsHGPRT* (POO492); *EcHPRT* (POA9M2); *MtHGPRT* (POA5TO).⁴³

There is little amino acid sequence identity between *HpXGHPRT* and the other four enzymes with *HpXGHPRT* having 29% identity with *EcXGPRT*, 24% identity with human HGPRT, 36% with *EcHPRT* and 29% with *MtHGPRT*. Overall, there are only eight completely conserved amino acid

residues (G36, L43, S59, Y60, D92, G94, K121 and P136) (**Fig. 5**). Based on this sequence alignment, six of the eight conserved residues are in the expected binding site of the functional groups of the substrates/products of the reaction while L43 and P136 do not appear to have a significant role in either catalysis or substrate/product binding.

The biological unit of *Hp*XGHPRT in solution. On gel filtration in 0.1 M Tris-HCL, 0.01 M MgCl₂, pH 7.4 containing 1 mM DTT, *Hp*XGHPRT elutes as a fully active dimer with a molecular mass of 38.6±1.9 kDa. This compares with the calculated molecular mass of 36.9 kDa. It is, therefore, likely that the active biological unit is dimeric. Under the identical conditions, *Ec*XGPRT eluted from the same column with a mass of 69.3±2.8 kDa, consistent with the calculated mass of a tetramer (65.6 kDa). *Mt*HGPRT exhibited a mass of 91±3.9 kDa which is tetrameric (calculated mass of 92 kDa). *Ec*XGPRT has previously been found to elute as an active tetramer on Superose-12 gel filtration³⁵ while analytical studies³⁶ and gel filtration of *Ec*HGPRT confirm that, in solution, this enzyme is also a tetramer suggesting that this is its active biological unit. Active human HGPRT also exists as a tetramer in solution.⁴⁴ Thus, not only is *Hp*XGHPRT a catalytically efficient member of the 6-oxopurine PRTase family but it is the smallest of these enzymes in its molecular mass in solution.

The crystal structure of *Hp*XGHPRT. In order to explain how inhibitors bind to *Hp*XGHPRT and to provide a framework for rational design, the knowledge of the 3D structure of the enzyme is essential. Two crystal structures of *Hp*XGHPRT have, therefore, been obtained. One is in the absence of added ligand and the second in the presence of 9-[(*N*-3-phosphonopropyl)-aminomethyl]-9-deazahypoxanthine (compound **XII**).²⁴⁻²⁵ In both cases, the asymmetric unit (ASU) contains a dimer. Data collection, processing and refinement statistics are given in **Table 4**.

Table 4. Data collection, processing and refinement statistics for two crystal structures of *Hp*XGHPRT

	No inhibitor	(+) compound XII
<u>Crystal Data</u>		
X-ray wavelength	0.95365	1.3776
Unit cell length a, b, c (Å)	40.20, 51.54, 82.69	40.22, 51.49, 82.88
Unit cell angle α , β , γ (°)	90, 96.09, 90	90, 96.21, 90
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁
<u>Diffraction data</u>		
Temperature (K)	100	100
Resolution range (Å)	43.67-1.47 (1.49-1.47) ^a	43.78-1.96 (2.08-1.96) ^a
Observations	389,538 (16,723)	3,335,000 (473,183) ^b
Unique reflections	57,196 (2,566)	24,376 (2,370)
Completeness (%)	99.0 (89.2)	99.5 (96.7)
R_{merge}^c	0.080 (0.592)	0.088 (0.592)
R_{pim}^d	0.036 (0.264)	0.020 (0.101)
$\langle I \rangle / \langle \sigma(I) \rangle$	10.6 (2.0)	74.1 (15.8)
<u>Refinement statistics</u>		
Resolution limits (Å)	34.54-1.47 (1.52-1.47)	19.99-1.97 (2.04-1.97)
R_{work}	17.09	15.09
R_{free}	19.17	19.41
Clashscore ^e	3.12	4.38
RMSD bond lengths (Å)	0.015	0.013
RMSD bond angles (deg)	1.36	1.06

Subunits per asymmetric unit	2	2
Inhibitors	0	1
Sulfate ions	4	5
Water molecules	312	344

Ramachandran statistics

Favoured	99.32	99.30
Outliers	0	0

Amino acids with visible density

Subunit A	-1 to 63; 65 to 153	-1 to 59; 68 to 153
Subunit B	-3 to 57; 68 to 153	-3 to 58; 68 to 153

B-factors (Å²)

Subunit A	23.15	21.66
Subunit B	20.31	20.40
Sulfate	26.33	28.83
XII	Not present	48.65
Water molecules	35.91	35.95

^a The values in parentheses are for the outer resolution shell. ^b A high redundancy of data was obtained because we were anticipating the need for anomalous phasing of the data. However, the structure was ultimately solved by molecular replacement. There was strong electron density for the sidechains of all the amino acids including those that are negatively charged, indicating that no radiation damage occurred during the time of data collection. ^c $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$

^d $R_{p.i.m.} = \sum_{hkl} \left[\frac{1}{[N(hkl)-1]} \right]^{1/2} \sum_i |I_i(hkl) - (I(hkl))| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and is the average intensity obtained from multiple observations of symmetry related reflections. ^eClashscore is defined as the number of bad overlaps ≥ 0.4 Å per thousand atoms

The subunit 3D structure of *Hp*XGHPRT. The subunit structure of *Hp*XGHPRT in the absence of added ligand is shown in **Fig. 6**. The electron density map of this structure shows two tetrahedral shapes which we have ascribed to sulfate ions arising from the crystallization solution. This assignment was confirmed by an anomalous difference map showing a strong signal for sulfur in Chain B at the 5'-phosphate binding pocket. There is also a weaker signal in the density in the predicted PP_i binding site for sulfur but, as this is just above the background, it was less conclusive. The location of the eight completely conserved amino acids in the five 6-oxopurine PRTases in the subunit structure are also shown together with the two acidic amino acids predicted to be involved with binding a divalent metal ion.

Fig. 6. Crystal structure of subunit A of *HpXGHPRT* in the absence of inhibitor. The eight conserved residues across five 6-oxopurine PRTases are in green and the two bound sulfate ions are shown as spheres. Red indicates the location of the long flexible loop (S59-I71) which is visible in this subunit except for the main chain atoms of H64. Two highly conserved acidic amino acids, 88D and 89E in *HpXGHPRT* (DD in *EcXGPRT* and ED in the other three amino acid sequences), are in dark blue.

Comparison of the subunit structure of *HpXGHPRT* with the human and three other bacterial enzymes. The secondary structure of *Hp* consists of four α -helices and four β -sheets and has a core structure that is similar to four other 6-oxopurine PRTases (**Fig. 7**). The most significant structural differences between these five enzymes occurs at the C-terminus (residues 122-153 in *HpXGHPRT*). *HsHGPRT*, *EcHPRT* and *MtHGPRT*. All have two β -sheets at their C-terminal while, for *EcXGPRT*,

the C-terminus reaches into the active site of the adjoining subunit. *Hp*XGHPRT, in contrast, has an α -helix of eleven amino acids at the C-terminus.

Fig. 7. Comparison of the subunit structure of *Hp*XGHPRT with *Hs*HGPRT and three other bacterial 6-oxopurine PRTases. **(A)** *Hp*XGHPRT (153 amino acids); **(B)** human (*Hs*) HGPRT (218 amino acids; PDB code: 4RAD).²¹ Ligand is 9-[(*N*-phosphonoethyl-*N*-phosphonoethoxyethyl)-2-aminoethyl]-guanine; **(C)** *Ec*XGPRT (152 amino acids; PDB code: 1A97).⁴⁵ Ligand is GMP; **(D)** *Ec*HPRT (178

amino acids; PDB code: 1G9S.³⁶ Ligand is IMP); and (E) *Mt*HGPRT (202 amino acids); PDB code: 4RHU.²⁶ Ligand is 2-((2-(guanin-9-yl)ethyl)(2-((2-hydroxyethyl)(2-phosphonoethyl)amino)ethyl)phosphonic acid.

The quaternary crystal structure of *Hp*XGHPRT. The dimeric structure of *Hp*XGHPRT is shown in **Fig. 8**. The two sulfate ions are located where the phosphate groups of the two products of the reaction (**Fig. 3**) *i.e.* the 5'-phosphate group of XMP/GMP/IMP and pyrophosphate are bound.

Fig. 8. The dimeric crystal structure of *Hp*XGHPRT with two sulfate ions in each subunit. The red cartoon is the long flexible loop (Subunit A) which is predicted to move over the active site during catalysis.⁴⁶

The long flexible loop. S59-711 in the amino acid sequence of *Hp*XGHPRT corresponds to 105S-I117 in the human HGPRT amino acid sequence (**Fig. 5**). This region of the human HGPRT structure is called the “long flexible loop” which closes over the active site when the transition state analogs are bound.⁴⁶ This flexible loop is resolved and visible in one of the subunits (Subunit A) of the *Hp*XGHPRT structure

(**Fig. 8**), but is disordered in subunit B. Thus, this structure represents two slightly different states which are either prior to, or just after, the catalytic reaction.

The two phosphate binding sites. One of the sulfate ions binds in the predicted 5'-phosphate binding site and the bonding pattern that the sulfonyl oxygens make with main chain and side chain amino acids as well as water molecules is shown in **Fig. 9A**. The electron density for the sulfate group is unequivocal (**Fig. 9A**). Thus, the sulfonyl group is firmly anchored in place by a stretch of amino acids, 92-DSGNS-96. D92 and G94 are two of the completely conserved residues across these five 6-oxopurine PRTases (**Figs. 5 and 6**). The second sulfate ion is found in that area of the enzyme that is predicted to bind pyrophosphate and includes R34 and G35 (**Figs. 5 and 9B**). The “RG” motif is highly conserved across 6-oxopurine PRTases, though in the human and *Mt* enzymes, arginine is replaced by the similarly charged lysine. R34 helps to keep a stable dimeric structure by formation of a bond between the NH1 atom of its side chain and the carbonyl of L49 in the adjoining subunit. Once again, the electron density for the sulfate ion and nearby waters is unequivocal (**Fig. 9B**). **Fig. 9C** shows that water molecules bridge the two sulfate ions located in the active site. This is the only known structure where the 5'-phosphate binding site and a phosphate mimic in the pyrophosphate binding site are occupied.

Fig. 9. The sulfate binding sites in *HpXGHPRT* **A. (Left)** The interactions that the sulfonfyl oxygen atoms form in the predicted 5'-phosphate binding site of *HpXGHPRT*. **(Right)** The $2F_o - F_c$ electron density for the sulfate group and four water molecules (green spheres) is overlaid as blue mesh. These are contoured at a sigma level of 1.0. **B (Left)** The location of the second sulfate ion in *HpXGHPRT*.

The loop in subunit A (grey; M33-G350) joins an α -helix to a β -sheet. The hydrogen bonding pattern when the sulfate ion binds. Subunit A is blue and subunit B is pink. **(Right)** The $2F_o - F_c$ electron density map for the bound sulfate group and nearby waters is overlaid in blue mesh. **C.** The two sulfate ions in the PP_i binding site [I] to the sulfate ion in the 5'-phosphate binding site [II] and connected by a network of water molecules (green spheres).

Crystal structure of the *Hp*XGHPRT in complex with 9-[(N-3-phosphonopropyl)-aminomethyl]-9-deazahypoxanthine (compound XII). Diffracting crystals were obtained in the presence of phosphonopropyl)-aminomethyl]-9-deazahypoxanthine which is one of two compounds known as the acyclic immucillin phosphonates (AIPs).²⁴ These compounds are partial mimics of the transition state analogs and were designed and synthesized by Schramm and his colleagues.²⁴ They contain 9-deazahypoxanthine as the purine base as well as a positively charged nitrogen in the linker connecting this base to a phosphonate group (**Fig. 10A and 10B**). The replacement of the N^9 atom by a carbon atom decreases the pK_a of the N^7 atom in the purine ring from 8.5 to 2.3⁴⁶⁻⁴⁷ and, thus, this atom is protonated under physiological conditions. This protonation was designed so that a hydrogen bond could form between the carboxylate of the highly conserved aspartic acid residue (D92 in *Hp*XGHPRT; **Fig. 5**) and N^7 of the purine base, reflecting the prediction of the transition state. Therefore, substitution of the naturally-occurring purine base by the deaza base could assist in tighter binding of the inhibitor. 9-Deazaguanine is a competitive inhibitor with guanine for *Hp*XGHPRT having a K_i of $3.0 \pm 0.2 \mu M$ which is similar to the K_m for guanine ($3.1 \mu M$; **Table 1**). It is likely, therefore, that 9-deazahypoxanthine would to bind in the same location in the active site as the naturally-occurring base.

The positively charged nitrogen in the linker (**XII and XIII**) mimics that of the oxygen in the chemical structure of the transition-state during the catalytic reaction (**Fig. 10C**). Its primary role appears to be in positioning a pyrophosphate group in the active site.

Fig. 10. The chemical structure of two known acyclic immucillin phosphonates (AIPs) and the proposed transition state. (A) Compound **XII**. 9-[(N-3-Phosphonopropyl)-aminomethyl]-9-deazahypoxanthine.²⁴ (B) Compound **XIII**. 9-[(N-3-(4-Hydroxyphosphonobutyl))-aminomethyl]-9-deazahypoxanthine.²⁴ (C) The proposed transition state of the catalytic reaction.⁴⁶⁻⁴⁷

There is only one published crystal structure of a 6-oxopurine PRTase in complex with an AIP. This is *Plasmodium falciparum* (*Pf*) HGXPRT in complex with **XIII**.²⁴ This complex also contains one pyrophosphate molecule and one magnesium ion per subunit.

Compound **XII** is a competitive inhibitor of *Pf*HGXPRT and *Hs*HGPRT and is highly selective for the *Pf*enzyme.²⁴ The K_i values of **XII** for *Hs*HGPRT and *Pf*HGXPRT are 4940 nM and 11 nM, respectively. The K_i value of **XII** for *Hp*XGHPRT is 2400 ± 0.4 nM, a value closer to that of the human enzyme. In comparison, the K_i values of **XIII** for *Hs*HGPRT and *Pf*HGXPRT are much lower, being 385 and 0.65 nM, respectively, suggesting that the addition of the -CH₂OH group plays a significant role in determining the K_i value.

Fig. 11A shows the Connolly surface of **XII** and sulfate bound in the active site of *Hp*XGHPRT (subunit A). **XII** was not observed in subunit B and just two sulfate ions were found in this subunit. The specific interactions that **XII** forms when bound in the active site of *Hp*XGHPRT are shown in **Fig. 11B**.

Fig. 11. Crystal structure of *HpXGHPRT* in complex with **XII**. **(A)** Connolly surface of *HpXGHPRT* dimer in complex with **XII** and one sulfate ion with subunit B and two sulfate ions in subunit A. **(B)** **XII** bound in the active site of *HpXGHPRT* and the interactions that it forms with backbone and side chain atoms of amino acids and also water molecules. Green dashed lines show the hydrogen bond network; red dashed lines show the distances in Å between the ligand and the side chain atoms of two highly conserved amino acids, D92 and K121.

In this structure, the side chain atom of D92 has not moved to form a hydrogen bond with the N⁷ atom of the purine ring as occurs when compound **XIII** (together with pyrophosphate and a magnesium ion) or the transition state analog (together with pyrophosphate and two magnesium ions) bind to either human HGPRT or *Pf*HGXPRT (distance is 2.8 Å in both those cases). Further, the NZ atom of the invariant lysine residue, K121, is too far away to form a hydrogen bond with the 6-oxo group of 9-deazahypoxanthine.

The positively charged nitrogen in linker. **Fig. 12A** shows the Connolly surface of the *Pf*HGXPRT in complex with **XIII** and shows that the large flexible loop is firmly closed over the active site and the ligand is “buried” in the active site.²⁴ The *HpXGHPRT*.**XII** complex has been superimposed onto this

complex to compare their modes of binding in an attempt to provide a rationale for the range of inhibition constants. **Figs. 12B** and **12C** show the interactions that the two ligands make in the active sites of their respective enzymes.

Fig. 12. Interactions between two ANPs and *HpXGHPRT* and *PfHGXPRT*. **(A)** Connolly surface of the crystal structure of the *PfHGXPRT*.XIII complex (PDB code: 3OZG) with the structure of the *HpXGHPRT*.XII complex superimposed. The green sphere is a magnesium ion. **(B)** The interactions that the positively charged nitrogen atom of XII forms in the active site of *HpXGHPRT*. **(C)** The interactions that the positively charged nitrogen atom of XIII forms in the active site of *PfHGXPRT*. All distances shown are in Å. Water molecules are shown as red spheres in B and C.

In the *HpXGHPRT*.XII complex, the positively charged nitrogen is linked, via two bridging waters, to two of the oxygens of the sulfate group located in the pyrophosphate binding site. One of these water molecules is then linked to the OD1 atom of the conserved acidic residue, E89 (**Figs. 12A** and **B**). The location of this sulfate ion mimics that of the second phosphate group of pyrophosphate which is found in the active site of *PfHGXPRT*.XIII complex (**Figs. 12A** and **C**).²⁴ In the *HpXGHPRT*.XII structure, the second water molecule fulfils the role of one of the oxygen atoms of pyrophosphate group in the *PfHGXPRT*.XIII complex (*cf.* **Figs. 12B** and **C**).

Thus, the role of the positively charged nitrogen atom in both these structures is to contribute to holding the sulfate ion (or a phosphate ion which can substitute for sulfate) or PP_i (*Pf*HGXPRT). It also helps in positioning the side chain of the conserved acidic residue (*cf.* **Figs. 12B and C**). When the transition state analog, ImmHp, binds to *Pf*HGXPRT (PDB code: 1CJB)⁴⁷ together with pyrophosphate and two magnesium ions, a similar role is attributed to the positively charged atom as it is also linked to the phosphoryl oxygens of pyrophosphate.

Thus, the *Hp*XGHPRT.**XII** is a partial reflection of the transition state in catalysis of this enzyme. Attempts to crystallize with pyrophosphate included in the active site together with compound **XII** were unsuccessful. This is attributed to the fact that this binding site may have been already occupied by sulfate and pyrophosphate was unable to displace it.

Positioning of the purine ring. In Subunit B of the *Hp*XGHPRT structure in the absence or presence of ligand, the side chain of W138 forms a π -stacking arrangement with the side chain of a histidine residue (**Fig. 13**). This residue is two residues distal to the start of the authentic amino acid sequence (HYSY) and is the fifth histidine residue at the N-terminus in the added hexa-his tag. In subunit A, in the absence of the inhibitor, the same histidine residue has not formed this packing arrangement and is freely rotating. Hence, its side chain is not visible and has been modelled in as alanine (**Fig. 13**). The side chain of W138 is then not restricted and freely rotates (**Fig. 13**). The B-factor for this side chain is 62 Å² indicative of its flexibility. In contrast, the B-factor in chain A is 22 Å² as it is firmly clamped in its position.

In all the 6-oxopurine PRTases, the purine base of the substrate or inhibitor is also “locked” into position by a π -stacking arrangement with the side chain of an aromatic amino acid. In *Ec*XGPRT, this amino

acid residue is tryptophan while, in *HsHGPRT*,⁴⁶ *EcHGPRT*³⁶ and *MtHGPRT*,²⁶ it is phenylalanine (**Fig. 5**). The aromatic amino acid is located on a flexible loop where it is freely rotatable and it only moves over the active site when a purine base, either free as in the *PfHGXPRT*.Hx.PP_i.Mg²⁺ complex (PDB code: 3OZF)²⁴ or as the nucleoside monophosphate or a phosphonate inhibitor enters the active site of these enzymes.^{22, 48}

Fig. 13. The movement of the side chain of the N-terminal and the side chain of W138 in *HpXGHPRT*. **(Left)** In the absence of inhibitor. Subunit A is orange; Subunit B is gold. In Subunit B, there is no density for the side chain of His(-2) and this side chain is modelled as alanine. **(Right)** In the presence of **XII** in the active site. Subunit A is pink; Subunit B is silver. The (-2) signifies the number of amino acids distal to the start of the integral amino acid sequence (HYSY).

Xanthine is a substrate for *HpXGHPRT*. Xanthine binds to *HpXGHPRT* and *EcXGPRT* with similar K_m values, 20 and 30 μ M, respectively (**Table 1**). However, this purine base is not a substrate for human HGPRT. In the *EcXGPRT*.GMP complex, the exocyclic amino group forms two hydrogen bonds, one with a bridging water to the OD1 atoms of E136 and D140 and the second to the carbonyl of I135. When xanthine binds, the exocyclic oxygen atom forms a bond with the water molecule but it is not possible

to make a bond with the carbonyl of I135 so the position of the base shifts slightly to avoid repulsion. In the *Hp*XGHPRT.**XII** complex, there is only a proton in the 2-position of the purine ring. In this structure, the side chain of E144 points towards the active site. This makes the distance between C² and the OD oxygen of E144, 3.4 Å. Thus, there is room to accommodate an exocyclic oxo group in this area of the active site and accounts for the fact that xanthine is a good substrate for this enzyme.

The C α backbone of human HGPRT adopts a different configuration in this region (*cf.* **Figs. 14A and B**). The exocyclic amino group of, the inhibitor, compound **I** (**Table 3**) forms a hydrogen bond with the carbonyl of D193. Thus, if xanthine tried to bind to this enzyme, repulsion would exist between the exocyclic oxygen atom and the carbonyl oxygen of D193 with the result that this purine base cannot bind to the human enzyme.

Fig. 14. Comparison of the position of the purine base in the *Hp*XGHPRT, *Ec*XGPRT and *Hs*HGPRT complexes. (A) The interactions that the N¹ atom in the purine ring make when the exocyclic amino group at the C² position of the purine base make when **XII** or GMP are bound in the active site of *Hp*XGHPRT or *Ec*XGPRT (PDB code: 1A97), respectively.⁴⁵ Pink is *Hp*XGHPRT and blue is

*Ec*XGPRT. The blue sphere is the bridging water between the exocyclic amino group and the two amino acid side chains in the *Ec*XGPRT.GMP complex. **(B)** Human HGPRT in complex with the inhibitor, [3*R*,4*R*-4-guanin-9-yl-3-((*S*)-2-hydroxy-2-phosphonoethyl)oxy-1-*N*-(phosphonopropionyl)pyrrolidine (Compound **1**, **Table 3**. PDB code: 5HIA).²²

Binding site for a divalent metal ion. In the 6-oxopurine PRTases, there are two highly conserved acidic amino acid residues (**Fig. 5**). These are D88 and E89 in *Hp*XGHPRT (**Fig. 15**). They occupy a similar location in subunits A and B in both of the two *Hp*XGHPRT structures. These two corresponding amino acid residues in the 6-oxopurine PRTase structures have been found directly coordinated to magnesium in the absence of ligand³⁶ or in the presence of a phosphonate inhibitor.²² When the transition state analogues are bound, they are indirectly coordinated to a magnesium ion via a hydrogen bond to the iminoribitol hydroxyl groups.⁴⁶⁻⁴⁷ In contrast, there is no electron density found in the two high resolution structures of *Hp*XGHPRT to show the presence of metal ion within coordination distance of the side chains of these two residues and, instead, they are turned toward each other and, via water molecules, to the sulfonyl oxygens of the sulfate group located in the binding pocket of the β -phosphate pocket and the carbonyl of V82. This residue is conserved as either valine or leucine in this group of the 6-oxopurine PRTases (**Fig. 5**).

Fig. 15. The interactions that the side chains of D88 and E89 form at the active site of *Hp*XGHPRT (Subunit A, no inhibitor present). This sulfate ion is located in the pyrophosphate binding pocket.

The role of K121. K121 in *Hp*XGHPRT, is highly conserved across the 6-oxopurine PRTases (**Fig. 5**). It has been proposed that its role is to confer specificity for this class of enzyme by forming a hydrogen bond between the NZ atom and the exocyclic oxo group. Thus, the 6-oxopurine phosphoribosyltransferases are unable to use adenine as a substrate because repulsion would occur with the exocyclic amino group.

In the *Hp*XGHPRT complex, the NZ atom of K121, which is located on a flexible loop is 5.9 Å away from the 6-oxo atom of the purine ring and, consequently, unable to form a bond with the 6-oxo group (**Fig. 11**). Instead the 6-oxo exocyclic group forms a hydrogen bond with a bridging water molecule and, via this molecule, to the backbone carbonyl of the conserved proline residue, P136 (**Fig. 16**). The NZ atom of K121 forms a hydrogen bond with a water molecule which is then linked to the carbonyl backbone oxygen of A137. This network appears to be the basis for the 6-oxopurine specificity of the *Hp* enzyme. *Hp*XGHPRT retains its specificity though for the 6-oxo group and adenine is not a substrate. *Hp*XGHPRT (360 nM, final concentration) was added to the cuvette containing 0.1 M Tris-HCl, 0.01 M MgCl₂, pH 7.4, 114 µM adenine, 550 µM *PRib-PP* at 25°C and the reaction followed spectrophotometrically at 255 nm for 600 secs. The concentration of adenine is 16 times that of the K_m value for guanine, 25 times higher than for Hx and 50 times higher than for xanthine (**Table 1**) and the enzyme concentration is ten times than when guanine was used as substrate. However, there was no change in absorbance (<0.001) over this time period so the enzyme is unable to convert adenine to AMP. Thus, the method adopted by *Hp*XGHPRT to bind the 6-oxopurine base has not broadened its specificity for the purine base and it retains its specificity for an exocyclic 6-oxo group.

*Ec*XGPRT also appears to adopt a modified network of bonds and is maybe less reliant that the other 6-oxopurine PRTases on the ubiquitous lysine residue.⁴⁵ The distance between the 6-oxo group and the NZ atom is 3.6 Å when GMP is bound but this is 3.2 Å in the presence of the inhibitor, (*S*)-3-(guanin-9-yl)pyrrolidine-*N*-ylacetylphosphonic acid (**Fig. 14**). This bacterial enzyme may place greater reliance on the hydrogen bond with the amide of I135.⁴⁵ This residue is highly conserved (**Fig. 5**) and completely conserved between *Ec*XGPRT and *Hp*XGHPRT but it does not perform the same role in the *Hp* enzyme as it is located too far away from the 6-oxogroup. Thus, though the proline amino acid residue is completely conserved across this group of the 6-oxopurine PRTases, it is only in the *Hp*XGHPRT enzyme that it appears to have an important role to play in the enzyme function.

Fig. 16. Comparison of inhibitor binding to two 6-oxopurine PRTases. **(A)** Superimposition of *Hp*XGHPRT.XII complex, the *Ec*XGPRT.GMP complex (PDB code:1A97) and the *Ec*XGPRT.(*S*)-3-(guanin-9-yl)pyrrolidin-*N*-ylacetylphosphonic acid complex (PDB code: 4JIT).⁴⁵ XGPRT and GMP are in blue; (*S*)-3-(guanin-9-yl)pyrrolidin-*N*-ylacetylphosphonic acid is in green; *Hp*XGHPRT and XII are in pink. The purine ring and the five membered ring of the *Ec* complexes are located in identical locations. **(B)** The hydrogen bond network that K121 forms at the active site in the *Hp*XGHPRT.XII complex and the distances in angstroms between the NZ atom of this amino acid. The distance between the OD atom of D92 and the N⁷ atom of the purine ring is also shown.

Derivative of compound XII. A derivative of **XII** was synthesized with the aim of positioning a second phosphonate group in the pyrophosphate binding pocket. The chemical structure of this compound (**XIV**) is given in **Fig. 17**.

Fig. 17. The chemical structure of **XIV**, 9-[(*N,N*-(bis-3-phosphonopropyl))-aminomethyl]-9-deazahypoxanthine.⁴⁹

This molecule inhibited *Hp*XGHPRT with a K_i of 4 ± 0.7 μM . Thus, though this modification did not result in the hoped-for decrease in the inhibition constant, compound **XIV** does not inhibit human HGPRT, even at a concentration of 46 μM in the assay. Thus, like xanthine and its derivatives, it is highly selective for *Hp*XGHPRT. This result suggests possibilities for the design of inhibitors totally specific for the bacterial enzyme. Thus, like 6-thioxanthine, this compound can constitute a lead molecule for therapeutics.

Possible binding modes of compounds I and III to *Hp*XGHPRT. In an attempt to explain the lower K_i values of compounds **I** and **III** compared with compound **XII**, two known structural complexes were superimposed onto that of the *Hp*XGHPRT.**XIII** complex. These are the human HGPRT.**I** complex (PDB code: 5HIA)²² and the *Mt*HGPRT.**III** complex (PDB code: 4RHX)²⁶ (**Fig. 18**).

Fig. 18. Superimposition of the complexes of two known crystal structures onto that of the *HpXGHPRT.XII* complex. (A) Human HGPRT.I complex (PDB code: 5HIA: green) with the *HpXGHPRT.XII* complex. (B) *MtHGPRT.III* complex (PDB code: 4RHX) with the *HpXGHPRT.XII* complex. A putative location of the pyrophosphate was obtained by superimposition of the human HGPRT structure in complex with the transition state analog (immHP) and PP_i. (PDB code: 1BZY)⁴⁶.

Both **I** and **III** contain a second phosphonate group covalently attached to a nitrogen atom in the pyrrolidine ring (**Fig. 18A**) or in the acyclic linker (**Fig. 18B**) (**Table 3**). In the human HGPRT.I complex, this phosphonate group reaches further into the putative pyrophosphate binding site than that of the phosphonate group in the *MtHGPRT.III* complex. In both cases, the phosphonyl oxygens would be expected to form additional interactions in the active site compared with compound **XII**, thus contributing to the decrease in the K_i values (2.4 μM for **XII** *cf* 0.2 μM for **I** and 0.3 μM for **III**; **Table 3**). These superimpositions are possible binding modes to show these two compounds bind in the active site of *HpXGHPRT* but other variations are possible given the flexible nature of the active site of the enzyme and the compounds themselves. Nonetheless, the superposition provides a basis for proposing future chemical modifications to increase affinity.

CONCLUSIONS

*Hp*XGHPRT is one of the smallest and most compact of the 6-oxopurine PRTases with a subunit mass of 17,327 Da and existing, in solution, as an active dimer. It is catalytically efficient, using all three naturally-occurring purine bases with k_{cat}/K_m values of 2.3, 0.5 and 0.1 $\mu\text{M}^{-1} \text{s}^{-1}$ for guanine, hypoxanthine and xanthine, respectively. These kinetic properties suggest that the nucleoside phosphonates, a class of inhibitors of other 6-oxopurine PRTases, may also inhibit *Hp*XGHPRT activity. All twelve such compounds investigated here were found to inhibit this enzyme with the lowest K_i value being 200 nM. Prodrugs, designed to increase membrane transport of these inhibitors, arrested the growth of *Hp* in cell-based assays. The catalytic properties of *Hp*XGHPRT can also be used to convert foreign substrates into toxic metabolites which we show to be effective *in vitro* against *Hp* growth. Thus, the enzymatic properties of *Hp*XGHPRT can be used in two ways to prevent the growth of *Hp*. The inhibitors and the substrate analog have low to negligible toxicity in human cells. The first crystal structures of *Hp*XGHPRT have been obtained. These structures provide clues for chemical modifications to the inhibitors to increase their potency. The innovative therapeutics described here are specific for *Hp*XGHPRT as they based on the chemical structure of its substrates/products. As such, it is unlikely that they could potently bind to other proteins. The enzymological properties of *Hp*XGHPRT combined with its 3D structure thus provides a platform for the design of new chemotherapeutics aimed at the treatment of *Hp* infections.

EXPERIMENTAL SECTION

Expression and Purification of *Hp*XGHPRT. Hexa-his tag *Hp*XGHPRT was expressed and purified to homogeneity as for human hexa-his tag HGPRT.²¹ The purified enzyme was stored at -80°C in 0.1 M Tris-HCl, 0.1 M MgCl₂, pH 7.4, containing 1 mM DTT and 400 μM PRib-PP. The concentration of protein was calculated using an $A^{1\%}_{1 \text{ cm}}$ of 1.38 $\text{M}^{-1} \text{cm}^{-1}$ and a subunit molecular mass of 18 481.1 Da.

Determination of kinetic constants. The kinetic constants, k_{cat} and K_m , were measured using a continuous spectrophotometric assay in 0.1 M Tris-HCl, 0.01 M MgCl₂, pH 7.4, 25°C. The reaction was initiated by the addition of an aliquot of enzyme to the cuvette containing the assay buffer and the two substrates, the purine base and *PRib-PP*. The reaction was followed for 60 s over an absorbance range of 0-0.05 AU. The reaction for each base was followed at these wavelengths: guanine (257.5 nm), hypoxanthine (245 nm); xanthine (255 nm); 6-mercaptopurine (300 nm); 6-thioguanine (355 nm); 6-thioxanthine (355 nm); and adenine (255 nm). The $\Delta\epsilon$ values for the different bases are: guanine (5816.5 M⁻¹ cm⁻¹); hypoxanthine (2439 M⁻¹ cm⁻¹); xanthine (4685 M⁻¹ cm⁻¹); 6-mercaptopurine (3284 M⁻¹ cm⁻¹); 6-thioguanine (4310 M⁻¹ cm⁻¹); 6-thioxanthine (3740 M⁻¹ cm⁻¹); and adenine (3300 M⁻¹ cm⁻¹).³⁴ The K_m values for the bases were measured under saturating conditions of *PRib-PP* (580 μ M) and the K_m values for *PRib-PP* under saturating conditions of guanine, 64 μ M. All experiments were performed as technical replicates in triplicate. The kinetic constants were calculated using GraphPad Prism.

Determination of K_i values. The two substrates and the inhibitor were incubated in the assay buffer, 0.1 M Tris-HCl, 0.01 M MgCl₂, pH 7.4, at 25°C, and the reaction initiated by the addition of an aliquot of enzyme. $K_{m(app)}$ was first calculated at different concentrations of inhibitor using the equation, $v_o = V_{max} \cdot [S]_o / (K_{m(app)} + [S]_o)$. The concentration of inhibitor was then chosen so that the $K_{m(app)}$ was at least five times the concentration of the variable substrate. The K_i values were determined at a constant concentration of guanine (64 μ M) and a range of concentrations of *PRib-PP* (10-1200 μ M). The range of inhibitor concentrations varied from 1-140 μ M, depending on the degree of inhibition by that particular inhibitor. Initial velocity (v_o) was determined at substrate saturation concentration. All experiments were performed as technical replicates in triplicate. For compound **XII**, the K_i was calculated using the Morrison equation⁵⁰ as for the inhibition of the human and *Pf* 6-oxopurine PRTases by this compound.²⁴

Gel filtration. Gel filtration was performed using Size-Exclusion Chromatography coupled to Multi-Angle Light Scattering (SEC-MALS) and with a G-75 Sephadex column equilibrated in 0.1 M Tris-HCl, 0.01M MgCl₂, pH 7.4 containing 1 mM DTT.

Crystallization and Structure Determination. The concentration of enzyme used to obtain highly diffracting crystals was 0.22 mM in terms of subunits (*i.e.* 4 mg ml⁻¹). To obtain crystals for the *Hp*XGHPRT.**XII** complex, the enzyme was incubated with or without **XII** (5.75 mM) on ice for 5 minutes prior to setting-up the crystal trays. The crystallization conditions were found by using the crystallization screening plates prepared by the Mosquito® Protein Crystallization Robot with 100 nl of the screening condition and 100 nl of protein. The crystallization screen was JCSG Plus™ from Molecular Dimensions. The crystallization conditions are 0.1 M Bis-Tris-propane, pH 5.5, 0.2 M (NH₄)₂SO₄, 25 % PEG3350. *Hp*XGHPRT crystals, in the presence and absence of **XII**, were obtained using the hanging drop method with 2 µl of enzyme and 2 µl of well solution. The trays were incubated at 18° C and crystals became visible after 14 days. The cryoprotectant was 10% glycerol in the well solution to which was added compound **XII** to give a final concentration of 1 mM. Crystals were cryocooled in liquid nitrogen for transport to the Australian Synchrotron where they were robotically placed in a cryostream (100 K). X-ray data were collected remotely at MX2 beamline.⁵¹ All data sets were processed with XDS.⁵²

The structure of the *Hp*HGXPRT.**XII** complex was determined by molecular replacement with Auto-Rickshaw⁵³⁻⁵⁴ with MoRDa⁵⁵ identifying and using PDB accession code, 5BQP, *Sulfolobus solfataricus* HGXPRT as a template model. The template model indicated about 27% sequence identity against the target molecule. The resulting model was subjected to rigid-body, positional and B-factor refinement in CNS⁵⁶ and continued to refine with REFMAC5⁵⁷ resulting in R/R_{free} to 45.6%/49.5%. At this stage, density modification was carried out with the model phase in PIRATE⁵⁸ and the improved phases were

used for model building in ARP/wARP.⁵⁹ The initial model contained 246 out of total 306 residues and of which 226 residues were correctly fitted in the electron density and refinement of the model resulted in R/Rfree to 27.4%/31.4%. Subsequent refinement and manual model building were performed with PHENIX 1.8⁶⁰ and COOT 0.8⁶¹, respectively. The liganded structure was solved by MR using the program PHASER⁶² and the unliganded structure of *Hp*XGHPRT as a starting model within PHENIX 1.8⁶⁰ and refinement and model building were carried out as described above. The data collection, processing, and refinement statistics for all of the datasets are presented in **Table 4**.

***Hp in vitro* assays.** Stock solutions of the prodrug (50 mM) were prepared in dimethyl sulfoxide. These solutions were then diluted further to give a final concentration of 1 mM in dimethyl sulfoxide. Aqueous stock solutions of the purine bases (approx. 2 mM) were also diluted to give a final concentration of 1 mM.

Helicobacter pylori strain 26695 was cultivated under microaerobic conditions (6% O₂, 6% CO₂ and 88% N₂) at 37 °C in brain heart infusion broth (BHI) supplemented with 10% (v/v) Foetal Calf Serum and 0.2% (v/v) Skirrow's supplement.⁶³ Cell suspensions were diluted with the BHI media to give a cell density of 10⁶ bacteria/ml.

Horse blood agar (950 µL) containing 8% (v/v) horse blood and 0.2% (v/v) Skirrow's supplement⁶⁴, was added into each well. 50 µL of the compounds or 50 µL of DMSO was added into each well. DMSO alone was added to determine that any decrease in cell growth was not due to DMSO itself. The final concentration of DMSO was 5%. To each well, 10 µL of the cell suspension was then added. The plates were then incubated at 37°C for three days in microaerobic condition (6% O₂, 6% CO₂ and 88% N₂). Cell growth was then observed.

Bioinformatic Analysis. The protein sequences for *HpXGHPRT*, *EcXGPRT*, *HsHGPRT*, *EcHPRT* and *MtHGPRT* were obtained from UniProtKB.⁴³ ClustalW2 was used to perform the sequence alignments of the enzyme.⁴²

Synthesis of the inhibitors and their prodrugs.

Except for the three prodrugs, **II-A**, **VI-C** and **VI-D** (synthesis described in the paragraphs below), the inhibitors and their prodrugs (listed below) were synthesized as described previously. They were all stored at 5°C, under which conditions they are stable for > 10 years. These compounds are >95% pure as assessed by LC/MS. [3*R*,4*R*]-4-Guanin-9-yl-3-((*S*)-2-hydroxy-2-phosphonoethyl)oxy-1-*N*-(phosphonopropionyl)pyrrolidine (**I**, **Table 3²²**); tetra-(L-phenylalanine ethyl ester) prodrug of [3*R*,4*R*]-4-guanin-9-yl-3-((*S*)-2-hydroxy-2-phosphonoethyl)oxy-1-*N*-(phosphonopropionyl)pyrrolidine (**I-A**, **Table 3²²**); tetra-(L-alanine ethyl ester) prodrug of [3*R*,4*R*]-4-guanin-9-yl-3-((*S*)-2-hydroxy-2-phosphonoethyl)oxy-1-*N*-(phosphonopropionyl)pyrrolidine (**I-B**, **Table 3²²**); (2-((2-(guanin-9-yl)ethyl)(2-((2-hydroxypropyl)(2-phosphonoethyl)amino)ethyl)amino)ethyl)phosphonic acid (**II**, **Table 3²⁶**); (2-((2-(guanin-9-yl)ethyl)(2-((2-hydroxyethyl)(2-phosphonoethyl)amino)ethyl)amino)ethyl)phosphonic acid (**III**, **Table 3²⁶**); tetra-(L-phenylalanine ethyl ester) prodrug of (2-((2-(guanin-9-yl)ethyl)(2-((2-hydroxyethyl)(2-phosphonoethyl)amino)ethyl)amino)ethyl)phosphonic acid (**III-A**, **Table 3²⁶**); sodium salt of [3-(guanine-9-yl)-2-((2-phosphonoethoxy)methyl)propoxy]methyl phosphonic acid (**IV**, **Table 3²⁷**); tetra-(L-phenylalanine ethyl ester) prodrug of [3-(guanine-9-yl)-2-((2-phosphonoethoxy)methyl)propoxy]methyl -phosphonic acid (**IV-A**, **Table 3²⁷**); 9-[(*N*-phosphonoethyl-*N*-phosphonoethoxyethyl)-2-aminoethyl]hypoxanthine (**V**, **Table 3²¹**); tetra-(L-phenylalanine ethyl ester) prodrug of 9-[(*N*-phosphonoethyl-*N*-phosphonoethoxyethyl)-2-aminoethyl]hypoxanthine (**V-A**, **Table 3²¹**); diisopropyl (*S*)-(2-(guanin-9-yl)pyrrolidin-1-yl)-2-oxoethyl)phosphonate (**VI**, **Table 3²⁹**); sodium salt of [(2-(guanine-9-yl)propane-1,3-diyl)bis(oxy)]bis(ethane-2,1-diyl) diphosphonic acid (**VII**, **Table 3²⁸**); tetra-(L-phenylalanine ethyl

ester) prodrug of [(2-(guanine-9-yl)propane-1,3-diyl)bis(oxy)]bis(ethane-2,1-diyl)}diphosphonic acid (**VII-A**, **Table 3**²⁸); [(2-[(guanin-yl)methyl]opropane-1,3-diyl)bis(oxy)]bis(methylene) diphosphonic acid (**VIII**, **Table 3**²¹); Tetra-(L-phenylalanine ethyl ester) prodrug of [(2-[(guanin-9-yl)methyl]propane-1,3-diyl)bis(oxy)]bis(methylene) diphosphonic acid (**V-III A**, **Table 3**²¹); sodium salt of [3-(8-bromoguanine-9-yl)-2-((2-phosphonoethoxy)methyl)propoxy]methyl phosphonic acid (**IX**, **Table 3**²⁷); tetra-(L-phenylalanine ethyl ester) prodrug of [3-(8-bromoguanine-9-yl)-2-((2-phosphonoethoxy)methyl)propoxy]methyl phosphonic acid (**IX-A**, **Table 3**²⁷); (2-((2-(benzyloxy)ethyl)(2-(guanin-9-yl)ethyl)amino)ethyl)phosphonate (**X**, **Table 3**³⁹); bis-(L-phenylalanine isopropyl ester) prodrug of (2-((2-(benzyloxy)ethyl)(2-(guanin-9-yl)ethyl)amino)ethyl)phosphonate (**X-A**, **Table 3**³⁹); 9-[(*N*-phosphonoethyl-*N*-phosphonoethoxyethyl)-2-aminoethyl]guanine (**XI**, **Table 3**²¹); tetra-(L-phenylalanine ethyl ester) prodrug of 9-[(*N*-phosphonoethyl-*N*-phosphonoethoxyethyl)-2-aminoethyl]guanine (**XI-A**, **Table 3**²¹); 9-[(*N*-3-phosphonopropyl)-aminomethyl]-9-deazahypoxanthine (**XII**, **Fig. 10**²⁴); 9-[(*N,N*-(bis-3-phosphonopropyl))-aminomethyl]-9-deazahypoxanthine (**XIV**, **Fig. 17**⁴⁹) were prepared previously as described previously (**Table 3**).

Synthesis of tetra-(L-phenylalanine ethyl ester) prodrug of (2-((2-(guanin-9-yl)ethyl)(2-((2-hydroxypropyl)(2-phosphonoethyl)amino)ethyl)amino)ethyl)phosphonic acid (II-A). A mixture of diethyl (2-((2-(guanin-9-yl)ethyl)(2-((2-hydroxypropyl)(2-phosphonoethyl)amino)ethyl)amino)ethyl)phosphonate²⁹, (0.5 mmol), dry pyridine (8 mL) and BrSiMe₃ (1 mL) was stirred overnight at room temperature under argon. After evaporation and co-distillation with pyridine (2 x 5 mL) under argon atmosphere, the residue was dissolved in dry pyridine (10 mL) and ethyl (L)-phenylalanine hydrochloride (1.75 g, 7.1 mmol) and triethylamine (3.1 mL) were added. The mixture was heated to 70 °C under argon atmosphere and then a solution of aldrithiol (2.31 g, 10.5 mmol) and triphenylphosphine (2.75 g, 10.5 mmol) in dry pyridine (8 mL) was added. The reaction mixture was heated at 70 °C for 3 days, the solvent was evaporated, and the residue co-evaporated with toluene

(2 x 10 mL) and purified by column chromatography on silica gel (gradient CHCl₃-MeOH). The crude product further purified by preparative HPLC. The phosphoramidate prodrug was obtained as foam, yield 34%. ¹H NMR (DMSO-d₆): 10.31 (s, 1H, NH); 7.60 (s, 1H, H-8); 7.24 (m, 20H, Ar); 6.21 (s, 2H, NH₂); 5.91 (s, 1H, OH); 4.23 (m, 4H, NH); 4.04 (m, 14H, Et, CHNH and H-1'); 3.48 (m, 2H, H-11'); 2.95 (m, 10H, CH₂Ph and CH₂), 2.76 (m, 4H) and 2.61 (m, 8H, CH₂-N); 1.63 (m, 4H, CH₂-P); 1.13 (m, 12H, Et). ¹³C NMR (DMSO-d₆): 172.53 m, 4 C (CO); 156.45 (C-6); 153.27 (C-2); 150.91 (C-4); 136.96 (C-8); 136.90 (m, 4C), 128.92 (m, 8C), 127.72 (m, 8C) and 126.08 (m, 4C, Ar); 116.57 (C-5); 60.00 (m, 4C, Et); 58.82 (C-OH); 53.88 (m, 4C, NHCH); 53.39, 52.27, 50.75, 50.36 (2C), 50.24 (C-N); 47.23; 43.71 (C-1'); 26.43 (d, 2C, JP, C = 111.5, C-P); 13.47 (m, 4C, Et). MS (ESI): m/z = 1212 [M + H]⁺. This compound is >95% pure as assessed by LC/MS.

Synthesis of Bis-(2-pivaloylthioethyl) diamide prodrug of diisopropyl (S)-(2-(guanine-9-yl)pyrrolidin-1-yl)-2-oxoethyl)phosphonate (VI-C). 2-Chloro-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide (0.6 g, 3.25 mmol) was added to the mixture of triethylammonium salt of (S)-3-(guanine-9-yl)-pyrrolidin-N-ylacetylphosphonic acid (synthesized according published procedure²²) (0.29 g, 0.65 mmol), trioctylamine (0.45 g, 1.3 mmol) and 2-pivaloylthioethanol (0.32 g, 1.96 mmol) in pyridine (10 mL). The reaction was stirred overnight under argon atmosphere at rt. The reaction mixture was concentrated in vacuo, dissolved in chloroform (20 mL) and washed with a saturated solution of NaHCO₃ (20 mL) and 1% citric acid (20 mL). The organic phase was dried over Na₂SO₄ and the desired product was obtained by column chromatography on silica gel using a linear gradient of ethanol in chloroform in 70% yield (0.29 g, 0.46 mmol) in the form of a white amorphous solid. The product was lyophilized from dioxane. According to NMR an ~ 1:1 mixture of A and B rotamers was obtained.

¹H NMR (499.8 MHz, CD₃OD, 25 °C): 1.209, 1.211, 1.226, 1.231 (4 × s, 4 × 9H, (CH₃)₃C-A,B); 2.48 (m, 1H, H-4'b-B); 2.51-2.59 (m, 3H, H-4'a-B, H-4'-A); 3.14-3.20 (m, 8H, SCH₂CH₂O-A,B); 3.21-3.28

(m, 4H, CH₂P-A,B); 3.63 (bm, 1H, H-5'b-B); 3.76 (bm, 1H, H-5'a-B); 3.85 (bm, 1H, H-5'b-A); 3.92 (bdd, 1H, $J_{\text{gem}} = 12.7$, $J_{2'b,3'} = 5.0$, H-2'b-A); 3.98 (bm, 1H, H-2'a-A); 4.00 (bm, 1H, H-5'a-A); 4.09 (dd, 1H, $J_{\text{gem}} = 10.8$, $J_{2'b,3'} = 6.0$, H-2'b-B); 4.15-4.23 (m, 9H, H-2'a-B, SCH₂CH₂O-A,B); 5.05 (m, 1H, H-3'-A); 5.11 (m, 1H, H-3'-B); 7.78, 7.83 (2 × s, 2 × 1H, H-8-A,B).

¹³C NMR (125.7 MHz, CD₃OD, 25 °C): 27.66, 27.68 ((CH₃)₃C-A,B); 29.67, 29.70 (d, $J_{\text{C,P}} = 6.5$, SCH₂CH₂O-A,B); 30.61 (CH₂-4'-B); 32.29 (CH₂-4'-A); 34.70 (d, $J_{\text{C,P}} = 133.0$, CH₂P-A,B); 45.78 (CH₂-5'-B); 47.18 (CH₂-5'-A); 47.51 ((CH₃)₃C-A,B); 51.62 (CH₂-2'-A); 52.62 (CH₂-2'-B); 54.47 (CH-3'-A); 55.51 (CH-3'-B); 66.40, 66.48, 66.51 (d, $J_{\text{C,P}} = 6.4$, SCH₂CH₂O-A,B); 117.95, 117.98 (C-5-A,B); 137.69, 137.88 (CH-8-A,B); 153.08 (C-4-A,B); 155.14 (C-2-A,B); 159.37 (C-6-A,B); 165.41, 165.55 (d, $J_{\text{C,P}} = 5.7$, NCO-A,B); 207.05 (SCO-A,B). ³¹P ¹H NMR (202.3 MHz, CD₃OD, 25 °C): 23.42, 23.49. HRMS (ESI-) for C₂₅H₃₉N₆O₇PNa (M+Na)⁺ : calcd 653.19515, found 653.19519. This compound is >95% pure as assessed by LC/MS.

Synthesis of N⁴-(6-methoxyquinolin-8-yl)pentane-1,4-diamine (primaquine) diamide prodrug of diisopropyl (S)-(2-(guanin-9-yl)pyrrolidin-1-yl)-2-oxoethyl)phosphonate (VI-D).

Bromotrimethylsilane (0.3 ml, 2.2 mmol) was added to the solution of diisopropyl (S)-(2-(guanin-9-yl)pyrrolidin-1-yl)-2-oxoethyl)phosphonate (synthesized according to a published procedure²²) (0.19 g, 0.44 mmol) in pyridine (5 ml) at rt under argon atmosphere and the reaction mixture was stirred overnight. This reaction mixture was concentrated in vacuo at exclusion of moisture and dissolved in the same solvent (5 ml). Primaquine free base (0.57 g, 2.2 g), triphenylphosphine (0.92 g, 3.52 mmol), DIPEA (0.3 ml, 1.76 mmol), and aldrithiol (0.77 g, 3.52 mmol) were added to the solution and reaction mixture was stirred under argon atmosphere at rt for two days. This reaction mixture was concentrated in vacuo and the desired product was obtained by column chromatography on silica gel using a linear gradient of methanol in chloroform followed by preparative reversed phase HPLC using a linear gradient of methanol in water in 80% yield (mg, mmol). ~ 1:1 mixture of amidic rotamers (each of them is a

mixture of 4 diastereoisomers) = 8 isomers. **¹H NMR** (500.0 MHz, CD₃OD): 1.19 – 1.29 (m, 48H, CH₃CH(NH)CH₂CH₂CH₂N); 1.48 – 1.72 (m, 64H, CH₃CH(NH)CH₂CH₂CH₂N); 2.24 – 2.43 (m, 16H, H-4'); 2.79 – 2.94 (m, 48H, CH₂P, CH₃CH(NH)CH₂CH₂CH₂N); 3.47 – 3.54 (m, 4H, H-5'); 3.54 – 3.72 (m, 24H, H-5', CH₃CH(NH)CH₂CH₂CH₂N); 3.73 – 3.82 (m, 8H, H-2',5'); 3.820, 3.826, 3.827, 3.831, 3.835, 3.837, 3.845 (7 × s, 48H, CH₃O); 3.86 – 3.95, 4.05 – 4.13 (2 m, 12H, H-2'); 4.88 – 4.95 (m, 8H, H-3'. overlapped with water signal); 6.24 – 6.31 (m, 16H, H-7-quin); 6.36 – 6.44 (m, 16H, H-5-quin); 7.27 – 7.35 (m, 16H, H-3-quin); 7.69, 7.743, 7.749, 7.752, 7.760 (5 × s, 8H, H-8); 7.93 – 8.02 (m, 16H, H-4-quin); 8.42 – 8.49 (m, 16H, H-2-quin).

¹³C NMR (125.7 MHz, CD₃OD): 20.83, 20.84 (CH₃CH(NH)CH₂CH₂CH₂N); 29.63, 29.67 (CH₃CH(NH)CH₂CH₂CH₂N); 30.56, 32.12 (CH₂-4'); 34.72, 34.74, 34.76, 34.89 (CH₃CH(NH)CH₂CH₂CH₂N); 37.23, 37.71 (2 × d, *J*_{C,P} = 100.3, CH₂P); 40.91 – 41.29 (m, CH₃CH(NH)CH₂CH₂CH₂N); 45.49, 47.10, 47.12 (CH₂-5'); 48.46 (CH₃CH(NH)CH₂CH₂CH₂N); 51.39, 51.42, 52.34, 52.36, 52.39, 52.41 (CH₂-2'); 54.24, 55.29 (CH-3'); 55.63 (CH₃O); 92.89, 92.95 (CH-5-quin); 98.29, 98.31, 98.36 (CH-7-quin); 117.92, 117.95 (C-5); 122.92, 122.94 (CH-3-quin); 131.55, 131.56, 131.57 (C-4a-quin); 136.29, 136.30, 136.31, 136.33 (CH-4-quin); 136.43, 136.44, 136.45 (C-8a-quin); 137.59, 137.70, 137.72, 137.75, 137.77 (CH-8); 145.295, 145.300, 145.317, 145.325 (CH-2-quin); 146.14, 146.16 (C-8-quin); 152.988, 152.994, 153.037, 153.044 (C-4); 155.10 (C-2); 159.38, 159.39 (C-6); 160.95, 160.96, 160.97, 160.98 (C-6-quin); 167.65 – 167.86 (m, COCH₂P). **³¹P ¹H NMR** (202.4 MHz, CD₃OD): 26.07; 26.12; 26.13; 26.15; 26.21; 26.22; 26.25. **IR** *v*_{max} (CHCl₃) 3360 (w, br), 2928 (m), 2860 (m, w), 2834 (sh, w), 1693 (s, ws), 1654 (sh, m), 1640 (sh, s), 1632 (sh, s), 1618 (vs), 1577 (s), 1521 (vs), 1444 (sh, m), 1389 (s), 1374 (sh), 1255 (m), 1238 (m), 1198 (s), 1166 (s), 1121 (m), 1091 (m, w), 1052 (m, w), 1032 (w), 888 (w), 821 (m), 847 (m), 641 (w). **HR-ESI** C₄₁H₅₄O₅N₁₂P (M+H)⁺ calcd 825.40723, found 825.40794. This compound is >95% pure as assessed by LC/MS.

ASSOCIATED CONTENT

Molecular *formula strings* (CSV)

PDB ID Codes

The atomic coordinates and structure factors of *Hp*XGHPRT in the presence (7KL6) and absence (7KL7) of 9-[(*N*-3-phosphonopropyl)-aminomethyl]-9-deazahypoxanthine (compound **XII**) have been deposited in the Protein Data Bank. Authors will release the atomic coordinates upon article publication.

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The manuscript was written through contributions from all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Acknowledgement

Initial crystallographic conditions were determined using the University of Queensland Remote-Operation Crystallization and X-ray Diffraction facility (UQROCX). X-ray measurements were performed at the Australian Synchrotron, ANSTO. This research was undertaken in part using the MX2 beamline at the Australian Synchrotron and made use of the Australian Cancer Research Foundation (ACRF) detector. The work was funded by the National Health and Medical Research Foundation, grant number 1147368 and by the Czech Science Foundation (Grant No. 19-07707S) to D.H. Research at the

Hudson Institute was funded by the NHMRC (Senior Research Fellowship APP1079904) and the Victorian Government's Operational Infrastructure Support Program.

Abbreviations Used

HGXPRT, hypoxanthine-guanine-xanthine phosphoribosyltransferase; PRTase, phosphoribosyltransferase; *PRib-PP*, 5-phospho- α -D-ribosyl-1-pyrophosphate; *Hp*, *Helicobacter pylori*; *Ec*, *Escherichia coli*; *Mt*, *Mycobacterium tuberculosis*; *Hs*, *Homo sapiens*; *Pf*, *Plasmodium falciparum*; ANP, acyclic nucleoside phosphonate; PP_i, pyrophosphate; AIP, acyclic immucillin phosphonate.

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