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Original article

Exploring new inhibitors of *Plasmodium falciparum* purine nucleoside phosphorylase

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ABSTRACT

Plasmodium falciparum purine nucleoside phosphorylase (*Pf*PNP) has a central role in purine salvage and inhibitors of the enzyme have been shown to have antiplasmodial activity. The enzyme preferentially uses inosine as substrate ($K_m = 5$ μM, $k_{cat}/K_m = 7.4 \times 10^4$ M^{-1} s^{-1}), but can also use uridine, albeit less efficiently ($K_m = 85$ μM, $k_{cat}/K_m = 306$ M^{-1} s^{-1}). In an effort to identify new *Pf*PNP inhibitors, two series of compounds were prepared. Series **1** was based on known human uridine phosphorylase inhibitors whilst series **2** was uracil equivalents of purine-based PNP transition state inhibitors. These two series of compounds were assayed for inhibition of both *Pf*PNP activity and growth of *P. falciparum*. The transition state analogues were found to be moderate inhibitors of *Pf*PNP (most potent compound, $K_i = 6$ μM).

1. Introduction

It is estimated that malaria causes 300–500 million clinical cases and 2 million deaths annually [1]. *Plasmodium falciparum* is responsible for the majority of deaths due to malaria. Drug resistance is a major problem for treatment and there is a need for new drugs particularly those that have a new mode of action [2,3].

P. falciparum cannot synthesize purines *de novo*, and is entirely reliant on the salvage of extracellular purines. Therefore, the purine salvage pathway is a potential target for antimalarial chemotherapy [4]. An enzyme involved in this pathway is *P. falciparum* purine nucleoside phosphorylase (*Pf*PNP), which catalyzes the phosphorolysis of inosine to ribose-1-phosphate and hypoxanthine (Fig. 1). Uridine is also known to be a substrate for this *Pf*PNP, although the affinity ($K_m = 85$ μM, $k_{cat}/K_m = 306$ M^{-1} s^{-1}) is lower than for inosine ($K_m = 5$ μM, $k_{cat}/K_m = 7.4 \times 10^4$ M^{-1} s^{-1}) and the catalytic efficiency is 240-fold higher with the latter [5].

*Pf*PNP is essential for parasite survival. Thus targeted gene disruption in a *P. falciparum* strain in order to ablate PNP expression has resulted in parasites that exhibit growth defects at physiological concentrations of hypoxanthine and require exogenous purines for sustained growth [9]. Chemical validation of *Pf*PNP has also been performed and transition state analogues developed that inhibit hPNP and *Pf*PNP and kill *P. falciparum in vitro* [10].

Immucillin-H (**3**) (Fig. 2) is a PNP transition state analogue inhibitor, which inhibits hPNP very potently ($K_d = 56$ pM) and the *Plasmodium* enzyme *Pf*PNP ($K_i = 860$ pM) slightly less strongly [11]. Immucillin-H was designed using kinetic isotope measurements which allowed for the identification of the transition state [7,12]. The study indicated that catalysis proceeds via a classic S_N1 nucleophilic substitution reaction in which, at transition state, the ribose ring forms an oxocarbenium ion (Fig. 1) [8,13]. In these transition state inhibitors, oxygen is replaced by a basic nitrogen, which mimics the positive charge of the oxocarbenium ion, and the distance between the ribose and the base in Immucillin-H is 1.5 Å, which is very close to the distance between the ribose and the leaving base in the transition state [13]. The substrate specificity, catalytic site structure, and subunit structure of *Pf*PNP are distinct from hPNP [6]. Potentially this can be used to design inhibitors which are selective for *Pf*PNP over hPNP. However, most

Abbreviations: ADA, adenosine deaminase; BBB, 5-(*m*-benzyloxy)benzylbarbituric acid; BBBA, 1-[(2-hydroxyethoxy)methyl]-5-(*m*-benzyloxy)benzylbarbituric acid acyclonucleoside; NOESY, nuclear Overhauser effect spectroscopy; *Pf*PNP, *Plasmodium falciparum* purine nucleoside phosphorylase; MT-immH, 5′-methylthio-immucillin-H; UP, uridine phosphorylase.

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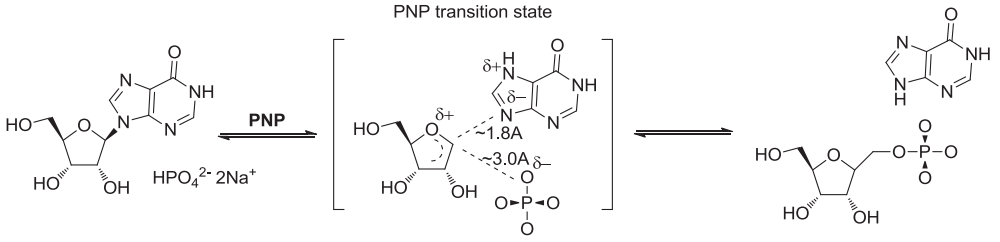

Fig. 1. Inosine phosphorylase catalyzed by purine nucleoside phosphorylase. The reaction is an S_{N1} type mechanism [6–8].

of the reported transition state inhibitors bind preferentially to hPNP rather than *Pf*PNP [5,10]. There are several exceptions to this: for example MT-immH (5′-methylthio-immucillin-H) gives the largest selectivity between *Pf*PNP and hPNP so far reported, a 100-fold difference between *Pf*PNP ($K_i = 2.7$ nM) and hPNP ($K_i = 303$ nM) [14]; although MT-immH still shows nanomolar potency against hPNP. It is proving challenging to obtain compounds that are highly selective towards *Pf*PNP. However, it should be noted that the antiparasitic activity of these PNP inhibitors may in part be due to simultaneous inhibition of hPNP, preventing generation of hypoxanthine by the erythrocytes [5,9,10].

In the study we report here, two series of compounds have been designed as potential inhibitors of *Pf*PNP. In particular, the fact that *Pf*PNP can use uridine as a substrate has been exploited.

First, human uridine phosphorylase (UP) has been considered as target for cancer therapy and many nucleoside analogues have been evaluated as inhibitors of the enzyme [15–17]. As *Pf*PNP shows significant activity with uridine and similarity to human uridine phosphorylase, a potent human uridine phosphorylase inhibitor 5-(*m*-benzyloxy)benzylbarbituric acid acyclonucleoside (BBBA **2**, $K_i = 1.1$ nM) [15] and its intermediate-5-(*m*-benzyloxy)benzylbarbituric acid (BBB **1**, $K_i = 2.3$ μM) [15] were made to test for activity against *Pf*PNP (Fig. 2). If active and selective, these could provide a starting point for a more focused discovery programme.

Second, different generations of purine-based transition state inhibitors of human and *Plasmodium* PNP have been reported [11,13,18]. Compound **4** (Fig. 2) belongs to a simplified series of purine-based PNP transition state inhibitors [19] and has a K_d of 5.5 nM for hPNP, although binding is weaker than that of immucillin-H **3** ($K_d = 6$ pM). In contrast, the substrate inosine has a K_d of 10,000 nM for hPNP [19]. We were interested to replace the purine base of the transition inhibitors with a uracil base to generate a new series of compounds and investigate if they show activity against *Pf*PNP. Therefore uracil containing derivatives of compound **5** were designed, prepared and tested for inhibition against *Pf*PNP. Compound **5** is the general structure for the proposed transition state inhibitor with the purine replaced by uracil. In our study, we prepared a series of inhibitors (**18**–**22** and **24**, **25**) which exhibit an alkyl chain length of two to four carbon atoms between these two nitrogen atoms.

2. Chemistry

2.1. Series *1*

The first series of compounds prepared were barbituric acid derived inhibitors of human uracil phosphorylase. 5-(*m*-Benzyloxy) benzylbarbituric acid (BBB, **1**) and 1-[(2-hydroxyethoxy)methyl]-5-(*m*-benzyloxy)benzylbarbituric acid (BBBA, **2**) were prepared as shown in Scheme 1. Barbituric acid (**6**) was coupled to benzaldehyde or *m*-benzyloxybenaldehyde to give the derivatives **7** and **8** respectively [20]. Products **7** and **8** were used crude as they contained more than 80% product and the removal of unreacted aldehydes proved problematic. The α, β-enone was reduced with sodium borohydride. Work was then carried out to introduce the side chain. Attempts to react compound **1** with 2-acetoxyethyl acetoxymethyl ether were unsuccessful [21]. However the reaction was successfully completed using (2-acetoxyethoxy)methyl bromide following a literature procedure [22]. (2-Acetoxyethoxy)methyl bromide **12** was prepared [23] by stirring acetyl bromide and 1,3-dioxolane together at room temperature, and then separating the required product by distillation at 113 °C. Compound **1** was first silylated using bis(trimethylsilyl)acetamide and then stirred with the (2-acetoxyethoxy)methyl bromide to give the required ester **13**. The ester **13** was deprotected to give the final product **2**.

2.2. Series *2*

The second series of inhibitors, compounds **18**–**22** and **24**, **25**, were prepared as shown in Scheme 2. Uracil was stirred with 1,2-dibromoethane, 1,3-dibromopropane or 1,4-dibromobutane [Br $(CH_2)_nBr$ ($n = 2$–4)] in Cs_2CO_3 in DMF at 40 °C for 2 days. Chromatographic purification was used to separate the final products. The dibromoalkanes could alkylate either N1 or N3 of the uracil. In order to investigate this, NOESY spectra were run on compounds **15**–**17** and **23**. Coupling could be observed for compounds **15**–**17** between the proton of N6 and the protons of alkyl chain (H1′ and H2′), but not for compound **23** (Fig. 3). Therefore, in compound **23**, alkylation had occurred on the undesired N3. Compounds **15**–**17** and **23** were then reacted with pyrrolidine or *l*-prolinol in ethanol at 150 °C under microwave conditions to give the final compounds **18**–**22**, **24** and **25**.

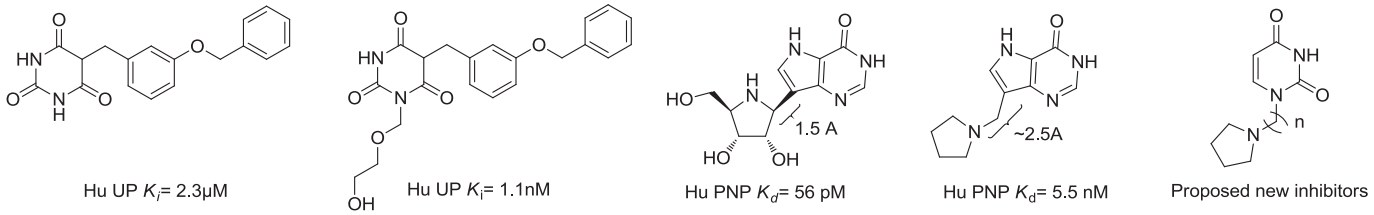

Fig. 2. Four selected known inhibitors and one proposed new inhibitor: (**1**) 5-(*m*-benzyloxy)benzylbarbituric acid, BBB; (**2**) 5-(*m*-benzyloxy)benzylbarbituric acid acyclonucleoside, BBBA; (**3**) Immucillin-H; (**4**) Immucillin-H analogue; (**5**) Proposed new inhibitors, based on compound **4**.