The adenosine deaminases of Plasmodium vivax and Plasmodium falciparum exhibit surprising differences in ligand specificity

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Abstract

Plasmodium vivax and P. falciparum cause malaria, so proteins essential for their survival in vivo are potential anti-malarial drug targets. Adenosine deaminases (ADA) catalyze the irreversible conversion of adenosine into inosine, and play a critical role in the purine salvage pathways of Plasmodia and their mammalian hosts. Currently, the number of selective inhibitors of Plasmodium ADAs is limited. One potent and widely used inhibitor of the human ADA (hADA), erythro-9-(2-hydroxy-3-nonly)adenine (EHNA), is a very weak inhibitor \( (K_i = 120\mu M) \) of \( P. falciparum \) ADA (pfADA). EHNA-like compounds are thus excluded from consideration as potential inhibitors of \( P. vivax \) ADA in general. However, EHNA activity in \( P. vivax \) ADA (pvADA) has not been reported. Here we applied computational molecular modeling to identify the mechanisms of the ligand recognition unique for \( P. vivax \) and \( P. falciparum \) ADA. Based on the computational studies, we performed molecular biology experiments to show that EHNA is at least 60-fold more potent against pvADA \( (K_i = 1.9\mu M) \) than against pfADA. The D172A pvADA mutant is bound even more tightly \( (K_i = 0.9\mu M) \). These results improve our understanding of the mechanisms of ADA ligand recognition and species-selectivity, and facilitate the rational design of novel EHNA-based ADA inhibitors as anti-malarial drugs. To demonstrate a practical application of our findings we have computationally predicted a novel potential inhibitor of pvADA selective versus the human ADA.

Keywords

computational modeling; drug design; ligand recognition; selective inhibition; site-directed mutagenesis; adenosine deaminase

INTRODUCTION

The World Health Organization (WHO) reported 243 million cases of malaria around the world in 2008; about 1 million of them were fatal. Four distinct Plasmodium species cause human malaria: \( P. falciparum \), \( P. vivax \), \( P. malariae \), and \( P. ovale \). Among them, \( P. vivax \)
and *P. falciparum* are the most common. *P. vivax* is more widely distributed, but *P. falciparum* is the deadliest. Recently, Das et al. highlighted interesting differences in whole genome sequences of *P. vivax* and *P. falciparum*. *P. vivax* contains 23% less A+T nucleotide content than *P. falciparum*. 82% *P. vivax* and *P. falciparum* genes are conserved, while 18% genes are species specific. These genomic dissimilarities suggest that proteins of these two parasites can differ significantly in structure, and thus, in their molecular mechanisms of ligand recognition.

All four Plasmodium species express adenosine deaminase (ADA), which catalyzes the deamination of adenosine (1, Scheme 1) to form inosine. This reaction is part of the essential purine salvage pathway. ADA inhibition is fatal to the parasite, so ADA is a promising target for malaria treatment. The rational design of Plasmodium-specific ADA inhibitors, however, remains a challenge. In contrast, many potent hADA inhibitors have been proposed. Deoxycoformycin, a transition-state analogue (also known as pentostatin, 2), is among the best known and most potent inhibitors of hADA and pADA. The structure of its complex with the pvADA was recently solved. Erythro-9-(2-hydroxy-3-nonly)adenine (EHNA, 3) is another well known and widely used inhibitor of hADA. It is, however, almost inactive against pfADA. Most assume that it lacks activity against the other Plasmodium ADAs, but this assumption has not to our knowledge been tested experimentally. Here we describe computational and biological studies of ligand recognition by pvADA and pfADA.

**EXPERIMENTAL PROCEDURES**

*Sequence alignment and homology modeling:* the sequences of pvADA and pfADA (UNIPROT ID: A5KE01 and Q8IJA9, respectively) were aligned with ClustalX, using the BLOSUM Protein Weight Matrix (Scheme 2). Modeller 9.7 (number of models to generate = 25, library_schedule = 10000, md_level = refine.very_slow) was used to construct a homology model of pfADA based on the crystal structure of pvADA (PDB ID: 2PGR). Water molecule #687, which appeared in both pvADA-adenosine and pvADA-pentostatin crystal structures, zinc cation, and the pentostatin ligand were included in our model. The most accurate model was selected based on the values of DOPE, GA341, and normalized DOPE scoring functions calculated for each model. The geometry of amino acid residues was also analyzed with the Ramachandran plot generated with the Maestro program of the Schrödinger Suite. The further refinement of both, pvADA crystal structure and pfADA model, was performed using the Protein Preparation Wizard implemented in the Schrödinger Suite.

Inhibitors were docked to protein structures using the Glide program of the Schrödinger Suite. The pfADA model was superimposed upon the pvADA crystal structure complexed with adenosine (compound 1 in Scheme 1). Thus, 1 was prepositioned inside the pfADA binding site. For both proteins, the protein grid generation was performed for the box with the center in the centroid of 1. The box size was determined automatically. The value of 1.0 and 0.8 were used for receptor and ligand van der Waals scaling, respectively. The extra precision (XP) mode of Glide was used with its default values for all parameters. All ligands were prepared using LigPrep program of the Schrödinger Suite. The protein-ligand complexes obtained after molecular docking were optimized with the Powell-Reeves conjugate gradient energy minimization method using the maximum of 2000 interactions and the value of threshold of 0.05. The same procedure was utilized for molecular docking of compound 4 to pvADA and hADA (PDB ID: 3IAR) crystal structures.

*Protein cloning and site-directed mutagenesis:* the 1116 bp pvADA cDNA was synthesized (GeneArt, Regensburg, Germany), and inserted into the pET28a(+) expression vector (Novagen, Madison, WI) between its NdeI and Xhol sites. The pvADA D172A mutant was
constructed by QuikChange site-directed mutagenesis\textsuperscript{17}. The PCR reaction was performed with the Phusion DNA polymerase, and a pair of primers (mutated codons are underlined):

\begin{align*}
5'\text{-}cctgatgtgcattggtgctaccggtcatgaagcag-3' \text{ and} \\
5'\text{-}ctgcttcatgaccggtagcaccaatgcacatcagg-3' .
\end{align*}

The custom synthesis of oligonucleotides was ordered from the Integrated DNA Technologies Inc. (Coralville, IA, USA). The mutation was confirmed by the sequence analysis performed by Macrogen (Rockville, MD, USA)

**Protein expression and purification**

*Escherichia coli* BL21(DE3) Gold cells (Strategene, La Jolla, CA) were transformed with pvADA-pET28a(+) encoding the wild-type and D172A mutant proteins. The transformed cells were propagated to mid-log phase (A600 nm of 0.6) at 37 °C in 200 mL LB medium supplemented with 50 ug/mL kanamycin, and 1 mM zinc sulfate. Gene expression was induced for 20 h with 1 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG). The cells were harvested by centrifugation, resuspended in 20 mL of the Ni\textsuperscript{2+}-binding buffer [10 mM imidazole, 50 mM potassium phosphate, 300 mM sodium chloride, pH 7.5], and lysed in two passages through a French Press pressure cell. The cell debris was removed by centrifugation (14,000g, 1 h).

The supernatant was filtered through a 20 micron filter, and passed twice through the 5 mL HiTrap Ni-NTA column (GE Healthcare, Uppsala, Sweden). To exclude cross-contamination during purification two different HiTrap Ni-NTA columns were used for pvADA wild-type and D172A mutant. The column-bound protein was washed with 60 mL of buffer [20 mM imidazole, 50 mM potassium phosphate, 300 mM sodium chloride, pH 7.5]. The proteins were eluted with 15 mL of the elution buffer [500 mM imidazole, 50 mM potassium phosphate, 300 mM sodium chloride, pH 7.5]. The pvADA WT and D172A mutant were concentrated with the 30K concentration units (Milipore, Billerica, MA, USA) to final volumes of 500 uL and subjected to the size-exclusion chromatography with the ÄKTA purifier using Superdex 200 16/60 column (GE Healthcare) and an appropriate buffer [50 mM potassium phosphate pH 7.5, 300 mM sodium chloride]. The 800 uL fractions were collected and analyzed with the silver-stained SDS-PAGE gel electrophoresis. Then, most pure fractions were combined and concentrated with the 30K Millipore concentration units. The final concentrations of his-tagged wild-type pvADA (2.8 mg/mL) and D172A mutant (3.2 mg/mL) were measured with the Bradford method using BSA as a standard.

**Enzymatic assay**

Adenosine and EHNA were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Santa-Cruz Biotechnology Inc. (Santa Cruz, CA, USA), respectively. Enzymatic activities were analyzed on Shimadzu UV-1601 spectrophotometer by measuring the rate of ADA-dependent decrease of adenosine (absorbance at 265 nm at 30 °C in quartz cuvettes with a path length of 1 cm). Assays were performed in 50 mM potassium phosphate buffer pH 7.5. To calculate the $K_M$ value, pvADA WT activities were measured over six different concentrations of adenosine (from 0.5 to $2.5 \times K_M$). Six concentrations of EHNA (from 0 to 50 uM), and 100 uM adenosine were used to calculate the binding constants of EHNA at pvADA WT and D172A mutant. In all measurements 11.8 nM (0.5 ug/mL) enzyme was used. Assays were repeated at least three times.
RESULTS AND DISCUSSION

Molecular modeling of pvADA and pfADA

EHNA is a very weak inhibitor of pfADA, but its reactivity with pvADA has not been reported. The crystal structure of pvADA was recently solved, but that of pfADA has not. It seemed likely that the two enzymes were quite similar in the configuration of their ligand binding sites, so we constructed a homology model of pfADA based upon the crystal structure of pvADA. Although, the amino acid sequences of pvADA and pfADA share 72% identity, superimposition of the three-dimensional structures of these two enzymes revealed that several amino acid residues in the binding pockets differ. In particular, Val51, Leu97, Thr174, Ile179, Ala204 of pfADA were found in positions occupied by Leu47, Ile93, Ile170, His175, Gly200 of pvADA, respectively (Scheme 2, Fig. 1). All these residues are located within 3Å from the atoms of 1 in the pvADA crystal structure, and are thus directly involved in ligand recognition.

Computational molecular docking was utilized to investigate the inhibitor binding modes of pvADA and pfADA. Initially, to confirm the reliability of the docking procedure, 2 was extracted from and re-docked to the crystal structure of pvADA. As expected, the re-docked structure was identical to its experimentally determined position in the pvADA crystal structure (RMSD = 0.2 Å). Then, 2 was docked to the binding pocket of pfADA. The binding mode of 2 with pfADA obtained after molecular docking was very similar to the one observed in pvADA crystal structure (RMSD_\text{HEAVY-ATOM} = 0.7 Å). The 3′-OH group of 2 formed a hydrogen bond with the carboxylic group of Asp172. The OH-group at position six was H-bonded to His257, and an amino group at 1-position of pentostatin formed a hydrogen bond with Glu233. Also, the N3 nitrogen atom was hydrogen bonded to the NH-group of Gly205 of pfADA.

The binding modes of 2 with pvADA and pfADA differed most in the coordination of the 5′-OH group; the corresponding oxygen atoms differed in position by 1.65 Å. In the pvADA crystal structure the hydroxyl group of 2 formed H-bonds with the carboxylic group of Asp46 and the imidazole ring of His44, and was located more than 3 Å of hydrophobic Ile170. In contrast, the pfADA structure shows Thr174 in the position of pvADA Ile170. The 5′-OH group of 2 was located between the imidazole ring of His48 and the OH-group of Thr174, forming hydrogen bonds with both residues. It did not, however, form a H-bond with Asp46 of pfADA. The structural differences between the pvADA and pfADA complexes with pentostatin correspond to a 2-fold difference in the energy scoring function calculated for the 2/pfADA (Glide_\text{emodel} = −64) and 2/pvADA (Glide_\text{emodel} = −106) complexes. This calculation is also in agreement with the binding constants reported for coformycin in pfADA ($K_I = 14 \text{ nM}$) and pvADA ($K_I = 7.4 \text{ nM}$).\(^8\) These findings indicate that modest differences in binding site composition can produce observable effects on ligand binding modes, even for a transition-state analogue inhibitor such as 2.

The molecular docking results of 2 with pvADA and pfADA were in good agreement with the crystal structure of pvADA-2 complex, so we used the same procedure to study the binding mode of 3 with these proteins. Although 3 is a very weak inhibitor of pfADA, it still can bind to the ligand-binding pocket with the $K_I$ value of 120μM.\(^10\) In both the pfADA and pvADA complexes, the adenine ring of 3 occupied the same position as the corresponding ring of 2 (Fig. 2). In contrast, the nonan-2-ol fragment of 3 was oriented differently in those two models. In the pfADA-2 complex, the hydrophobic heptyl chain of the nonan-2-ol fragment was found in unfavorable position between hydrophilic functional groups of His48, Asp50, Ser133, Thr174, and Asp176. The hydroxyl group of the nonan-2-ol moiety was oriented toward the water molecule, and hydrophobic Val93 and Ala96. Furthermore, the position of this ligand OH-group did not correspond to any of the functional groups of 2.
The nonan-2-ol fragment assumed the opposite orientation in the pvADA-3 model. In particular, the long hydrophobic heptyl chain of the nonan-2-ol fragment appeared in proximity to hydrophobic amino acid residues, namely Phe88, Val89, Ala92, Ile93, and Phe132. The significant difference in the orientations of the nonan-2-ol fragment of 3 in two similar binding sites should not be surprising. For example, the crystal structures of pvADA bound to 2 or its methylthio-analogue (MT-coformycin) indicated that the ribose fragment, which is analogous to the nonan-2-ol fragment of 3, assumed a completely different orientation in the same binding pocket.

The methyl group of the nonan-2-ol chain of 2 was found in unfavorable proximity (3.2 Å) to the carboxylic oxygen atom of Asp172. In contrast, the hydroxyl group of the nonan-2-ol fragment was located near His44, and formed a hydrogen bond with its imidazole ring. The same hydrogen bond was observed for the 5′-OH group of 2 in the crystal structure of pvADA. The superimposition of 2 and 3 inside the binding center of pvADA demonstrated that the OH-group of the nonan-2-ol moiety of 3 was located between the oxygen atoms of 5′-OH group and the ribose ring of 2.

These observations also agree with the values of the energy scoring functions obtained for the models. The Glide XP scoring function calculated for 2 and 3 docked to pvADA differed by 18%; in pfADA models the difference of 31% was observed (the values of the Glide XP scoring function calculated for 2 were defined to be 100%). Thus, our qualitative and quantitative molecular docking analysis revealed differences in the binding mode of 3 at pvADA and pfADA. It also suggested that configuration of pvADA binding site is more favorable for 3 than the ligand binding center of pfADA, and that more potent inhibition of pvADA by 3 can be expected.

**Kinetic Assay**

Recently, the important role of Asp172 pvADA ligand recognition was reported by Larson et al.\(^9\) Our molecular modeling analysis suggested that the carboxylic oxygen atom of Asp172 unfavorably interacted with the hydrophobic methyl group of 3. To further understand the functional role of Asp172 in the ADA structure we performed site-directed mutagenesis, heterologous expression and in vitro kinetic characterization. The wild-type and D172A mutant forms of pvADA were expressed in BL21(DE3) Gold cells (as described in the Methods section), and purified with the Ni\(^{2+}\)-affinity chromatography and the size-exclusion chromatography on a Superdex 200 column. The purity of the protein was confirmed by SDS-PAGE gel electrophoresis (Fig. 3). The \(K_M\) and \(k_{cat}\) values of the wild-type and D172A pvADA variants in reactions with EHNA have been previously reported. For this reason, we did not perform a precise steady-state kinetics experiments here.

We measured the \(K_M\) value of WT pvADA in reactions with adenosine to confirm that our techniques were consistent with the published work. Our value \((K_M = 71 \pm 16 \text{ uM})\) was congruent with that reported Ho et al. \((K_M = 60 \pm 6 \text{ uM})\)\(^8\). We also compared the adenosine deamination activities of the WT and D172A pvADA enzymes by monitoring the changes in absorbance at 260nm during the reaction of 100uM adenosine with 11.8 nM enzyme. In agreement with the published data,\(^8\) the D172A pvADA mutant was 40% less active than the wild type. Thus, these experiments confirmed the consistency of our data with the previously reported results, and encouraged us to use both proteins in further enzyme inhibition assays.

We observed an inhibitory effect of 3 in a concentration range from 0.1 to 50uM on the ADA deamination activity in the reactions of pvADA WT and D172A mutant with 100uM adenosine. In contrast to the previous data reported for \(P. falciparum\) ADA, significant inhibition (more than 20%) was observed for pvADA WT with low (0.5 uM) concentrations of 3. Moreover, only 10% of the enzyme activity remained in the presence of 50 uM 3. The
inhibition of the pvADA D172A mutant was even more potent. In particular, the addition of 3 at 0.5 uM concentration decreased the mutant activity by 31%, and at 50 uM concentration pvADA activity was not detectable. The calculated $K_I$ values (Fig. 3) show that 3 is a relatively potent inhibitor of pvADA WT ($K_I = 1.9$ uM) compared with the pfADA WT ($K_I = 120$ uM), and is even more potent inhibitor of pvADA D172A mutant ($K_I = 0.9$ uM).

Interestingly, Asp172 is conserved among all plasmodium ADAs, but the human ADA has Met at that position. The D172A mutation in pvADA abrogates binding to methylthioformycin or methylthioadenosine.

### Computational design of novel selective inhibitor of pvADA

We designed a putative selective inhibitor of pvADA inhibitor to illustrate the practical applications of our findings. We modified 3 by substituting the methyl group of the nonan-2-ol chain with an ethylamine fragment. The resulting compound 4 (Scheme 1) was docked to the pvADA and hADA crystal structures. The results of molecular docking revealed that 4 fits pvADA binding site well with the same binding mode as 3 itself (Fig. 4). In addition, the newly introduced amino group of 4 formed new H-bonds with Asp172 and Ser129 side chains. In contrast, this compound was unable to fit the binding site of hADA (no reasonable binding mode was detected with the molecular docking). We predict that 4 will bind pvADA with greater affinity than EHNA, 3, and exhibit much less reactivity against hADA. We intend to validate this prediction in future studies.

In summary, our studies have indicated significant differences in structural organization and molecular mechanisms of ligand recognition of plasmodium adenosine deaminases. We observed a 60-fold difference in EHNA binding affinity of pvADA and pfADA. We expect that other EHNA-based compounds, such as 4, could provide higher affinity and selectivity for Plasmodium ADA versus its human homolog. Previous workers showed that Asp172 enables binding interactions with adenosine derivatives, including a transition-state analogue pentostatin. Our data reveals the critical role of Asp172 in recognition of inhibitors, which are structurally distinct from adenosine due to the absence of the ribose ring. We therefore speculate this residue would similarly play a decisive role in a selective binding of any substrate or inhibitor to adenosine deaminase of plasmodium parasites. The divergent amino acid residues located in this position in the plasmodium (Asp) and human (Met) homologues provide an opportunity for rational design of novel anti-malarial drugs.

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### References


Highlights

1. We have constructed homology model of Plasmodium (P) falciparum adenosine deaminase (ADA)
2. We identified differences in the inhibitor binding mode at the P. vivax and P. falciparum ADAs with the computational molecular docking
3. We have validated the results of the modeling studies experimentally.
4. We have identified amino acid residues critical for species-selective ADA ligand recognition
5. We have computationally designed novel potential inhibitor of P. vivax ADA selective versus the human ADA.
FIGURE 1.
The superimposition of the ligand binding sites of the crystal structure of pvADA complexed with pentostatin (protein atoms are colored in orange, pentostatin carbon atoms – in green) and a homology model of pfADA with the docked pentostatin (protein atoms are colored in grey, pentostatin carbon atoms – in yellow). The amino acid residues of pvADA and pfADA are labeled in black and magenta, respectively. The H-bonds are indicated by green lines.
FIGURE 2.
The putative binding mode of EHNA (2) with the molecular model of pfADA (a) and the crystal structure of pvADA (b). In the model of pfADA, the hydrophobic chain of 2 was unfavorably oriented toward hydrophilic residues, and the ligand hydroxyl group was observed in proximity to hydrophobic Val93 and Ala96. In contrast, this hydroxyl group of 2 formed a hydrogen bond with His44 of pvADA (H-bonds are indicated by green lines), and the hydrophobic chain of the ligand was located near hydrophobic amino acid residues. The methyl group of 2 was located at a distance of 3.2 Å from the carboxylic oxygen atoms of Asp172 (indicated by a red line).
FIGURE 3.
The purity of pvADA WT and D172 mutant obtained after immobilized metal affinity chromatography and gel filtration was confirmed by SDS-PAGE (a). Inhibitory curves (b) were obtained for the pvADA WT and D172A mutant in reactions with 100uM adenosine and EHNA at concentrations of 0.1 to 50 uM. Assays were performed in 50 mM potassium phosphate buffer pH 7.5. Six concentrations of EHNA (from 0 to 50 uM), and 100 uM adenosine were used to measure the binding constants of EHNA at pvADA WT and D172A mutant. In all measurements 11.8 nM enzyme (0.5 ug per cuvette) was used. All assays were repeated at least three times.
FIGURE 4.
A hypothetical inhibitor of pvADA, 4, proposed here, was computationally docked into pvADA crystal structure. The general binding mode of 4 is identical to the binding mode of its parent compound 3. In addition new H-bonds were formed between the amino group of 4 and Asp172 and Ser129 side chains.
SCHEME 1.
The ligands of adenosine deaminase: a native substrate, adenosine (1), transition-state analogue inhibitor, pentostatin (2), a potent inhibitor of hADA, EHNA (3), a novel potential inhibitor of pvADA (4).
SCHEME 2.
The amino acid sequence alignment of pvADA and pfADA was used to construct the homology model of pfADA. The overall sequence identity is 72%. The residues located in the binding site and which are not identical in these two proteins are shown in bold.