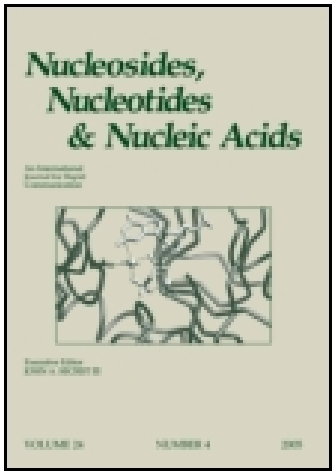

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L-NUCLEOSIDE ANALOGUES AS POTENTIAL ANTIMALARIALS THAT SELECTIVELY TARGET *PLASMODIUM FALCIPARUM* ADENOSINE DEAMINASE

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ABSTRACT: The L-stereoisomer analogues of D-coformycin selectively inhibited *P. falciparum* adenosine deaminase (ADA) in the picomolar range (L-isocoformycin, K_i 7 pM; L-coformycin, K_i 250 pM). While the L-nucleoside analogues, L-adenosine, 2,6-diamino-9-(L-ribofuranosyl)purine and 4-amino-1-(L-ribofuranosyl)pyrazolo[3,4-*d*]-pyrimidine were selectively deaminated by *P. falciparum* ADA, L-thioinosine and L-thioguanosine were not. This is the first example of 'non-physiological' L-nucleosides that serve as either substrates or inhibitors of malarial ADA and are not utilised by mammalian ADA.

Introduction

Malaria, mostly caused by *Plasmodium falciparum*, devastates the world's tropical zone. The World Health Organisation indicates renewed activity of this endemic disease, with 300 to 500 million cases per year, 2.3 billion persons at risk, and 1.5 to 2.7 million deaths per year.[1] Vaccination is considered to be an approach that will complement other strategies for prevention and control of the disease in the future, and in the last 10 years studies have been aimed at the development of a malaria vaccine. However, while several epitopes of limited polymorphism have been reported to generate a degree of protective immunity no completely effective vaccine for malaria has been developed.[2] Effective chemotherapy of malarial infections is also faltering because of multidrug resistance in *P. falciparum*. Reports of resistance to the 'mainstay of malaria chemotherapy', the quinoline-containing antimalarial drugs (chloroquine, quinine and

mefloquine) are increasing.[3,4] However, artesunate is being explored as an antimalarial to treat multidrug-resistant infections.[5] Hence, the continued development of novel antimalarials is essential to supplement the current limited treatment options for multidrug-resistant *P. falciparum* malaria.

The design of novel antimicrobials often exploit metabolic differences observed between the pathogen and its host. *P. falciparum* is incapable of *de novo* purine biosynthesis and therefore relies on salvaging preformed purine nucleosides which are essential for replication.[6] The intraerythrocytic parasite induces specific transport sites in the erythrocyte membrane, the properties of which are unique to the parasite-infected erythrocyte.[7] This site selectively transports preformed purine nucleosides.[8,9] Unlike normal erythrocytes, the induced nucleoside transport sites are not stereoselective, and low concentrations of L-nucleoside analogues are selectively transported into infected erythrocytes but not normal erythrocytes.[10] Thus, a unique opportunity exists for exploiting these changes in transport phenomena as a strategy for gaining selective access to the malaria parasite.

The design of substrates or inhibitors of parasite metabolic pathways which (a) gain selective entry to parasitised cells, and (b) target only malarial enzymatic pathways, would be a powerful combination in the design of a parasite specific agent. A major enzymatic difference between uninfected erythrocytes and malaria infected erythrocytes is the induction of adenosine deaminase (ADA), which catalyses the deamination of adenosine to inosine in the purine salvage pathway. ADA in *P. falciparum* infected erythrocytes is upregulated some 1000 fold over that of uninfected erythrocytes.[11] Previous researchers have exploited this observation by using the potent inhibitor of ADA, 2'-deoxy-D-coformycin, to successfully eliminate *Plasmodium knowlesi* infection in rhesus monkeys.[12] Preliminary data from Roth *et al.*,[13] suggest that 2'-deoxy-D-coformycin does not confer toxicity to cultures of *P. falciparum*. 2'-Deoxy-D-coformycin is a ubiquitous inhibitor of ADAs[14] which are distributed in most human tissues and cell lines including erythrocytes and lymphocytes and it has been shown that ADA deficiency causes severe combined immunodeficiency disease (SCID) in humans.[15,16,17] Therefore, the design of an antimalarial targeting ADA as a mode of activation or as an inhibitor, would need to be strictly specific for *P. falciparum* ADA because of this broad distribution of ADA in most human tissues.

Here, we show that two L-nucleoside structural analogues of 2'-deoxy-D-coformycin, namely L-isocoformycin and L-coformycin, are specific inhibitors of *P. falciparum* ADA and have no effect on host ADA. We also show that *P. falciparum* ADA is able to accept a broad range of L-nucleoside analogues including 2,6-diamino-9-(L-ribofuranosyl)purine and 4-amino-1-(L-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine, as

substrates. These analogues are not substrates for host erythrocyte ADA. These data provide the first evidence that L-nucleoside stereoisomers of physiological inhibitors and substrates have the potential to exploit parasite metabolism and therefore may be used in the development of novel parasite-specific antimalarials.

Results and Discussion

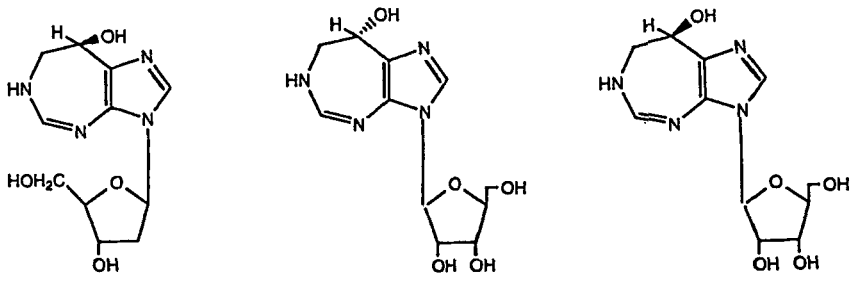
In preparations where infected red blood cells were lysed and used as a source of ADA, we found that the erythrocyte haemolysate interfered with both the indolephenol[18] and spectrophotometric[19] assays for ADA. Therefore we prepared 'free' *P. falciparum* trophozoites using saponin to lyse the erythrocyte membrane and a silicone oil clean-up step to remove erythrocyte haemolysate. The free parasite lysate was then used as the source of ADA when assaying for the malarial enzyme. Removal of the haemolysate effectively removed erythrocyte ADA, thus ensuring that the ADA being assayed was solely attributable to the parasite.

The ADA activity in *P. falciparum* using both assay methods gave a specific activity averaging ~15 U g haemoglobin^{-1}. This is comparable previous levels published for ADA (13 U g haemoglobin^{-1}) in another *P. falciparum* clone.[13] We observed a lower K_m value for adenosine (7 μM) compared to previous studies (50 μM).[20] It should be noted however that Daddona *et al.*[20] did not prepare ADA from free parasites; in that study whole infected red blood cell lysate was used as the source of ADA which may explain differences in K_m values, since such a lysate would contain erythrocyte ADA as a contaminant.

2'-Deoxy-D-coformycin (**1**) is commonly used as a specific inhibitor of all ADAs[14] and in this study it potently inhibited *P. falciparum* ADA (Fig. 1), and erythrocyte and purified bovine ADA, both of which were totally inhibited at 2 μM . *P. falciparum* ADA exhibited a K_i for 2'-deoxy-D-coformycin of 11.5 pM. This is comparable to that of erythrocyte ADA which had a K_i for 2'-deoxy-D-coformycin of 15 pM.[19] Previous reports have shown *P. falciparum* to have higher K_i values for 2'-deoxy-D-coformycin (320 pM).[20] However for the erythrocyte ADA higher K_i values for 2'-deoxy-D-coformycin values were also reported (420 pM).

We then investigated a series of novel L-nucleosides as inhibitors of ADA using the indolephenol assay.[18] L-coformycin (**2**) is the stereoisomer of D-coformycin and L-isocoformycin (**3**) is a structural analogue of L-coformycin.

P. falciparum ADA exhibited a K_i for L-coformycin of 250 pM and 7 pM for L-isocoformycin (Fig. 1). Thus a minor modification to a single hydroxyl group on the purine ring of L-coformycin enhanced the efficacy of the inhibitor some 35 fold. Like 2'-deoxy-D-coformycin, L-coformycin and L-isocoformycin were competitive inhibitors of



1 2 3

P. falciparum ADA (Fig. 1). 2'-Deoxy-D-coformycin, L-coformycin, and L-isocoformycin were also examined for their inhibitory effect on mammalian ADA. HPLC was used to monitor the deamination of adenosine to inosine. Retention times of standard nucleosides were determined with adenosine, inosine and hypoxanthine having retention times of 9.1, 6,9 and 5,6 min respectively. D- and L-adenosine were found to co-elute using the conditions described (data not shown). Under standard assay conditions D-adenosine, when added to erythrocyte lysates, was deaminated to inosine by ADA. Inosine was then converted to hypoxanthine by resident purine nucleoside phosphorylase. After a 20 min incubation neither adenosine nor inosine were observed, with all adenosine being metabolised to hypoxanthine which was the only peak observed (Fig. 2 A). However for the erythrocyte ADA, 2'-deoxy-D-coformycin completely inhibited the enzyme with D-adenosine being the only peak observed (r.t. 9.1 min) (Fig. 2 D). Purified bovine ADA was also completely blocked by 2'-deoxy-D-coformycin (data not shown). With L-coformycin and L-isocoformycin (both at 200 M), no inhibition of erythrocyte ADA was observed, with all added D-adenosine being metabolised to hypoxanthine after 20 min (Fig. 2B and 2C).

The above results suggest that, unlike mammalian ADAs, the *P. falciparum* ADA does not demonstrate stereospecificity toward either D- or L-nucleoside inhibitors. Furthermore, the results show that L-isocoformycin and L-coformycin are able to discriminate between host and parasite ADAs which is a desired characteristic of most chemotherapeutic agents. We then investigated a range of L-nucleoside analogues {L-adenosine, 2,6-diamino-9-(L-ribofuranosyl)purine and 4-amino-1-(L-ribofuranosyl)-pyraz-olo[3,4-*d*]pyrimidine} for their effectiveness as substrates for both *P. falciparum* and mammalian ADA. No formation of inosine nor hypoxanthine was detected when erythrocyte lysate was incubated with L-adenosine instead of D-adenosine, with L-adenosine being the only peak observed (Fig. 3E). Like L-adenosine, 2,6-diamino-9-(L-ribofuranosyl)purine and 4-amino-1-(L-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine did not serve as substrates for either erythrocyte ADA nor purified bovine ADA (data not

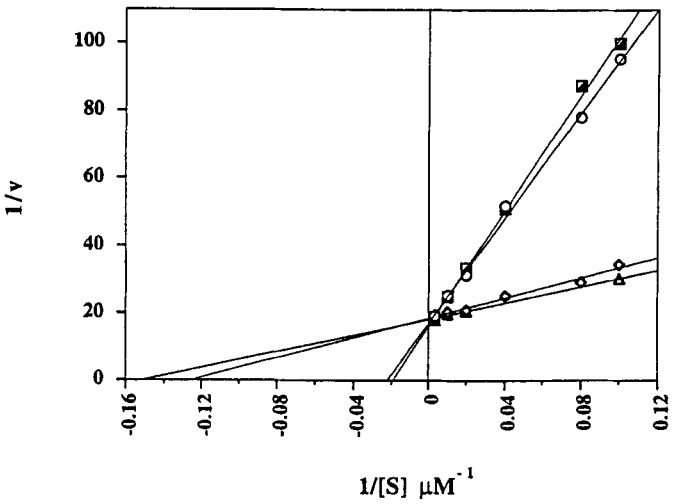


Figure 1. Lineweaver-Burke plot[30] of *P. falciparum* adenosine deaminase in the presence of 2'-deoxy-D-coformycin, L-isocoformycin and L-coformycin. *P. falciparum* adenosine deaminase (~0.01 units) was pre-incubated with each respective inhibitor (50 pM) for 15 min prior to the addition of adenosine ([S], concentrations indicated) to initiate the reaction. Assays were conducted in 50 mM potassium phosphate buffer, pH 7.5 at room temperature. v: ΔAbs_{630nm} min^{-1} □, 2'-deoxy-D-coformycin; ○, L-isocoformycin; ◇, L-coformycin; Δ, control assay without added inhibitors.

shown). However, when assayed with *P. falciparum* lysates, L-adenosine was deaminated to L-inosine which was the major peak observed at ~ 7 min (Fig. 3B). L-inosine apparently does not serve as substrate for malarial purine nucleoside phosphorylase since hypoxanthine was not detected as a metabolic product. The deamination of L-adenosine to L-inosine was completely inhibited by both 2'-deoxy-D-coformycin and L-isocoformycin (data for L-isocoformycin is shown in Fig. 3C), thus confirming that the enzyme responsible was *P. falciparum* ADA.

The other L-nucleoside substrates tested were 2,6-diamino-9-(L-ribofuranosyl)-purine and 4-amino-1-(L-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (data for the former nucleoside are shown in Fig. 4.). From neat injections, 2,6-diamino-9-(L-ribofuranosyl)-purine and L-guanosine have retention times of 7.5 min and 7.3 min respectively. In assays using free parasite lysates and 2,6-diamino-9-(L-ribofuranosyl)-purine without added inhibitor the major product observed was L-guanosine (~7.3 min; peak 2) with a minor amount of unmetabolised compound at ~7.5 min (Peak 1) (Fig. 4C). This was confirmed by comparing the spectra of each peak with that of the known compound (see Fig. 4D, 4E, 4F and 4G). The deamination of 2,6-diamino-9-(L-ribofuranosyl)purine to

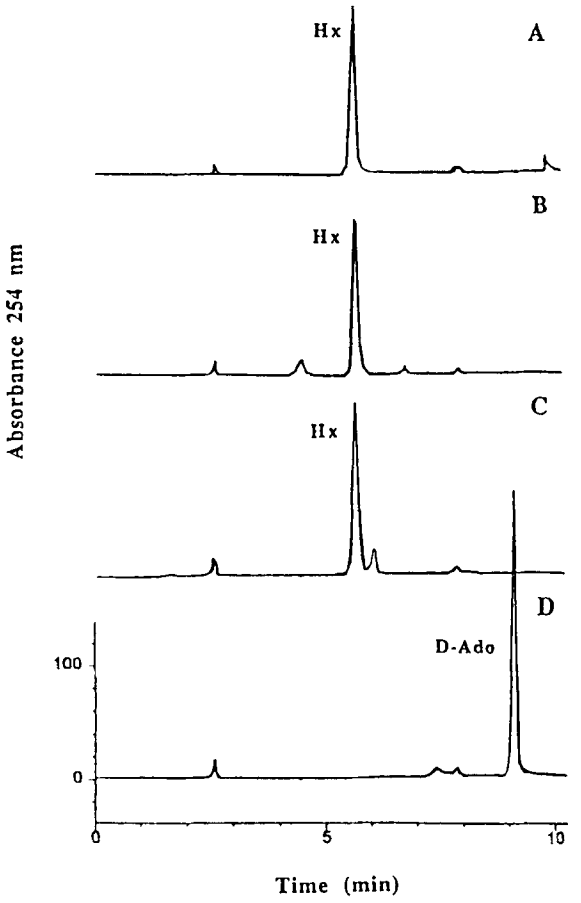


Figure 2. HPLC chromatographs comparing L-coformycin, L-isocoformycin and 2'-deoxy-D-coformycin as specific inhibitors of erythrocyte ADA. A. Standard ADA assay containing D-adenosine (500 M) and erythrocyte lysate. B. Standard assay where lysate was preincubated for 15 min with L-coformycin (200 M). C. Standard assay only lysate was preincubated for 15 min with L-isocoformycin (200 M). D. Standard assay only lysate was preincubated for 15 min with 2'-deoxy-D-coformycin (2 M). Hx, hypoxanthine; D-Ado, D-adenosine.

guanosine was blocked by both L-isocoformycin (Fig. 4B) and 2'-deoxy-D-coformycin thereby confirming that *P. falciparum* ADA was causing the deamination of each compound. Similarly, the deamination of 4-amino-1-(L-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine was also blocked by both L-isocoformycin (Fig. 4B) and 2'-deoxy-D-coformycin (data not shown). The other L-nucleoside analogues tested in this study were L-thioinosine, L-thioguanosine and 4-thio-1-(L-ribofuranosyl)pyrazolo[3,4-

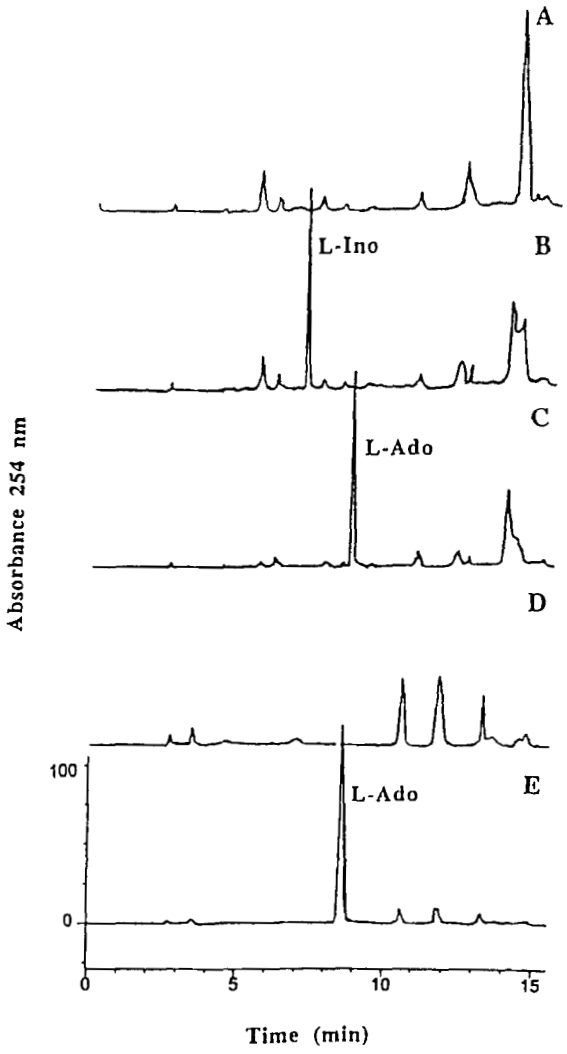


Figure 3. HPLC chromatographs comparing L-adenosine (500 M) as substrate for ADA from *P. falciparum* and normal erythrocytes. A. Free parasite lysate control. B, Free parasite lysate incubated with L-adenosine. C. Free parasite lysate preincubated L-iso-coformycin (2 M) for 15 min followed by incubation with L-Adenosine. D. Erythrocyte lysate control. E. Erythrocyte lysate incubated with L-adenosine.

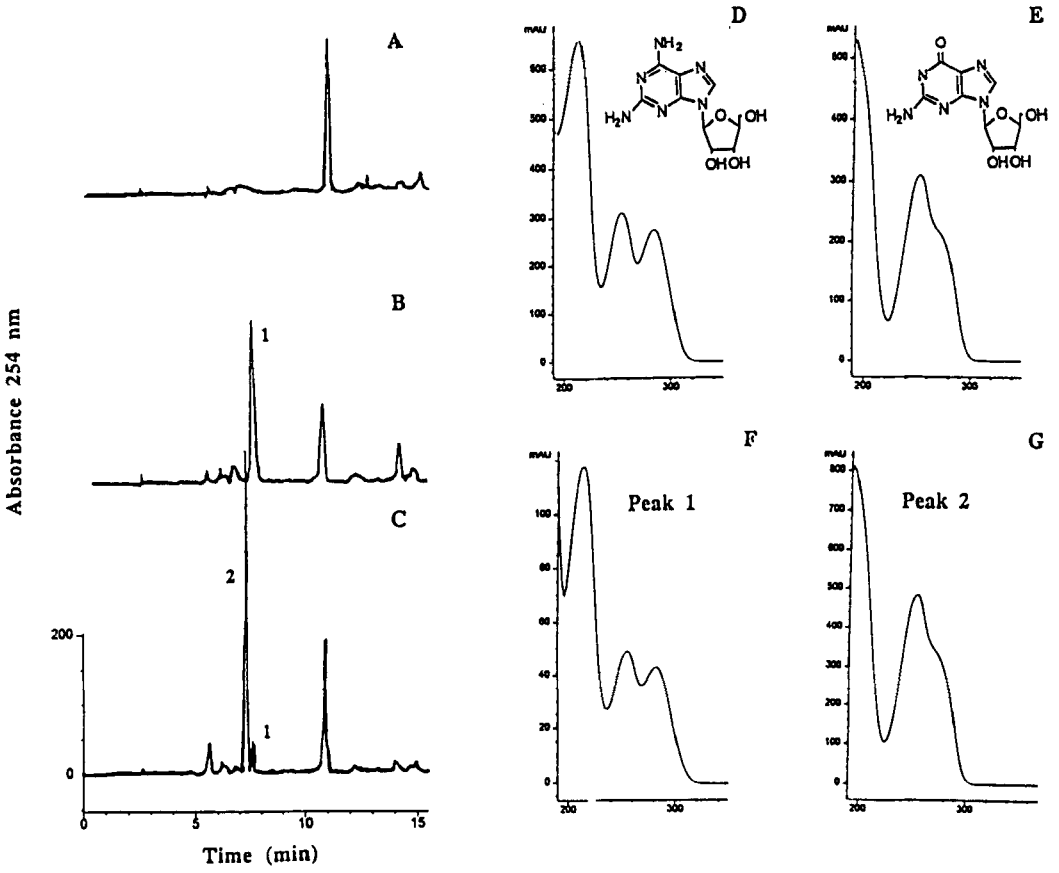


Figure 4. HPLC chromatographs following the deamination of 2,6-diamino-9-(L-ribofuranosyl)purine by *P falciparum* ADA and identification of metabolic products using wavelength scans of individual peaks. A. Free parasite control. B. Free parasite lysate preincubated L-isocoformycin (2 M) for 15 min followed by incubation with of 2,6-diamino-9-(L-ribofuranosyl)purine (500 M). C. Free parasite lysate incubated with of 2,6-diamino-9-(L-ribofuranosyl)purine. D and E show chemical of structures of 2,6-diamino-9-(L-ribofuranosyl)purine and L-guanosine respectively and their corresponding UV spectra. F. UV spectrum of Peak 1 in chromatograph C (labelled 1). The UV spectrum of peak 1 in chromatograph C matched the UV spectrum of peak 1 in chromatograph B. G. UV spectrum of peak 2 in chromatograph C (labelled 2).

d]pyrimidine however, they were not utilised as substrates (data not shown). These data show that *P. falciparum* ADA can accept a broad range of both D- and L- nucleosides as substrates whilst in contrast, mammalian ADAs are unable to utilise L-nucleoside analogues as shown in mouse tissue.[21] The data presented here emphasise the potential of designing novel L-nucleosides [22] for the selective delivery of toxic L-nucleoside

compounds to *Plasmodium* infected erythrocytes that target selective parasite enzymes such as the ADA.

Experimental

Materials - Deionised water from a MilliQ (Millipore) water treatment system was used throughout and all chemicals were of analytical grade or better. 2'-Deoxy-D-coformycin, L-coformycin, L-isocoformycin, 2,6-diamino-9-(L-ribofuranosyl)purine, 4-amino-1-(L-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine, L-thioinosine and L-thioguanosine were prepared by the Chu *et al*, details to be published shortly. Saponin was from Sigma and HPLC grade Tetrabutylammoniumphosphate was from Fluka.

P. falciparum *culture* - Uninfected whole blood containing type O^+ erythrocytes was collected from normal healthy volunteers, provided by the New South Wales Red Cross Blood Transfusion Service. Whole blood was washed with an equal volume of phosphate buffered saline (PBS) followed by centrifugation at 3000g for 5 min and the supernatant (white cell layer) removed by aspiration. This cycle was repeated a further 3 times to ensure adequate removal of white cells. Erythrocytes were then resuspended in complete RPMI-1640 medium (RPMI medium supplemented with 25 mM Hepes-KOH, pH 7.2, 25 mM $NaHCO_3$, 50 μg ml^{-1} gentamycin and 20% v/v human type O serum).

P. falciparum isolate FCQ-27 was maintained in culture using the methods of Trager and Jensen.[23] Cultures contained 1% suspensions of parasitised human type O erythrocytes in complete RPMI-1640 and were maintained at a haematocrit of 2% under an atmosphere of 3% O_2:7% CO_2:90% N_2 in a modular chamber (Flow) at 37°C. Infected erythrocytes were periodically synchronised using sterile D-sorbitol.[24] On the day of experimentation, synchronous cultures of high parasitaemias (10 %) were harvested and concentrated using Percoll gradients.[25] Infected erythrocytes at the trophozoite stage were diluted to a 25% haematocrit in PBS and 12 ml of this solution was layered over 30 ml of 70% Percoll of density 1.084-1.089 g ml^{-1}. The Percoll columns were centrifuged at 1000 g for 20 min. Approximately 90% of the cells harvested at the PBS/Percoll interface were found to be *Plasmodium* infected erythrocytes at the trophozoite life stage and were washed 3 times in PBS and used immediately for experimentation. For cell counting, cultures samples were thinly smeared on slides and methanol fixed followed by consecutive staining in Field stains A and Fields stain B.[25] Cells were counted microscopically to determine the percentage of parasitised cells.

L-nucleoside analogues as substrates and inhibitors of malarial and mammalian ADA - ADA activity was assayed in normal and malaria-infected human erythrocytes.

Purified ADA from bovine spleen (Sigma) was used as a control. All subsequent steps were performed at 4°C unless stated otherwise.

Preparation of malarial and erythrocyte lysates for assays of ADA–P. falciparum infected erythrocytes purified by Percoll, as described above, were subjected to saponin lysis[26] to release trophozoites from infected erythrocytes. Briefly, PBS was added to a packed cell volume of Percoll harvested infected erythrocytes to a final haematocrit of 25%. Saponin (0.5% stock in PBS) was added to yield a final concentration of 0.05% and the solution allowed incubated at room temperature for 10 min. The lysate was then centrifuged on a benchtop microfuge for 20 sec. 'Free' parasites were removed from haemolysate by centrifuging through silicone oil (16,000 *g*, 15 s).[10] The free parasite preparation was then examined for intact erythrocytes microscopically using the staining technique described above. Another saponin lysis cycle was performed if intact erythrocytes were detected to ensure complete lysis and removal of all erythrocytes. Free parasites were collected, washed in PBS and lysed by diluting with an equal volume of deionised H_2O followed by three cycles of freeze-thawing in liquid nitrogen. The lysate was then adjusted to 50 mM potassium phosphate buffer pH 7.5 with a 1M stock of the same buffer and centrifuged at 100,000*g*. The supernatant was removed and was used immediately as the source of malarial ADA for all assays.

Erythrocyte ADA was prepared from washed erythrocytes used for culture as described above. Packed erythrocytes were washed in PBS to remove complete RPMI-1640 medium and lysed by diluting with an equal volume of deionised water followed by freeze thawing (3 cycles). The lysate was then brought to 50 mM potassium phosphate, pH 7.5 as above and then centrifuged on a benchtop microfuge (16, 000*g*, 30 min^{-1}). The supernatant was then used as a source of erythrocyte ADA.

Pure bovine spleen ADA was diluted to 0.1 U ml^{-1} in 50 mM potassium phosphate buffer pH 7.5 immediately prior to assay. Haemoglobin content was determined using the method of Rodkey *et al.*[27] Protein concentration was determined using the method of Bradford.[28] ADA was assayed using 3 methods with the final method employing HPLC. All assays were conducted at room temperature. The first method used was that of Agarwal *et al.*[19] Briefly, assays contained 50 mM potassium phosphate buffer, pH 7.6 and 50 μM D-adenosine. Enough lysate was added to give a rate of $\Delta Abs_{265\ nm} = 0.08$ to initiate the assay. Activity was monitored using a Beckman™ DU 7500 spectrophotometer by measuring the change in absorbance at 265 nm due to adenosine deamination. The effect of inhibitors on ADA using this technique was determined by pre-incubating each inhibitor (2 M) with lysate for 15 min at RT prior to the addition of adenosine. Rates of adenosine deamination was determined using $\varepsilon_{265} = 8.1$ M cm^{-1} adenosine. One unit of activity was defined as the amount of enzyme which provided 1 mol of product min^{-1}. Specific activity was expressed as U g of haemoglobin^{-1}.

The second assay for ADA employed the method of O'Donovan [18] which monitors the production ammonia using the indolephenol reaction. The method was modified to enable the assay to be carried out in 96-well plates. Assays were set up according to the method of Agarwal *et al.*[19], only instead of monitoring the reaction spectrophotometrically, aliquots (50 µl) of reaction mixture were removed at 30 sec intervals over a time course of 3 minutes. Each aliquot was immediately aspirated into 100 l of solution A (50g l^{-1} phenol and 0.25 g l^{-1} sodium nitroprusside dissolved in deionised water) which ceased ADA activity. After the time course 100 µl of solution B (24 g l^{-1} sodium hydroxide and 2.1 g l^{-1} sodium hypochlorite dissolved in deionised water) was added. The 96-well plate was then analysed for absorbance at 630 nm using a Molecular Devices¶ (Spectra Max 340) Plate Reader. A linear rate curve was generated comparing absorbance with time to enable the determination of a rate of NH_3^+ produced min^{-1}. One unit of ADA activity was defined as the amount of enzyme required to produce 1 µmol NH_3^+ min^{-1}. Standard curves comparing NH_3^+ concentration and absorbance were generated between the ranges of 0–5 nmol NH_4Cl. For determination of K_m, assays were set up containing 20–300 M substrate. For determination of K_i, each reaction was pre-incubated with each respective inhibitor (50 pmol) for 15 min prior to addition of adenosine at the respective concentrations used for the determination of K_m for adenosine.

Analysis of L-nucleoside metabolism by HPLC. L-nucleoside metabolism was analysed using by HPLC using the method of Toguzov *et al.*[29] Briefly, cell lysates (40 l) (both free parasite and erythrocyte) at 10 mg ml^{-1} protein were incubated with L-nucleoside substrate (300 M) at 37C for 20–40 min, and the reaction terminated by the addition of 3 M perchloric acid. For inhibition studies inhibitors at concentrations noted in the text were pre-incubated with cells lysate for 15 min at 37C. Substrate was then added to and the reaction proceeded as described above. Following neutralisation with 1.3 M K_2CO_3, (10 µl), samples (25 µl) were separated on an adsorboshpere nucleoside-nucleotide column (Activon) using a Hewelett Packard™ HP1100 HPLC and monitored using diode array detection, the monitoring wavelength being 254 nm. The buffer system employed was 10 mM potassium phosphate, 2 mM tetrabutylammonium phosphate and a linear pH (5.25–7.25) and acetonitrile (3û20%) gradient over 10 min. Isocratic HPLC was performed from 10–40 min.

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