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The spermidine synthase of the malaria parasite *Plasmodium falciparum*: Molecular and biochemical characterisation of the polyamine synthesis enzyme☆

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Abstract

The gene encoding spermidine synthase was cloned from the human malaria parasite *Plasmodium falciparum*. Northern and Western blot analyses revealed a stage specific expression during the erythrocytic schizogony with the maximal amount of transcript and protein in mature trophozoites. Immunofluorescence assays (IFAs) suggest a cytoplasmatic localisation of the spermidine synthase in *P. falciparum*. The spermidine synthase polypeptide of 321 amino acids has a molecular mass of 36.6 kDa and contains an N-terminal extension of unknown function that, similarly, is also found in certain plants but not in animal or bacterial orthologues. Omitting the first 29 amino acids, a truncated form of *P. falciparum* spermidine synthase has been recombinantly expressed in *Escherichia coli*. The enzyme catalyses the transfer of an aminopropyl group from decarboxylated *S*-adenosylmethionine (dcAdoMet) onto putrescine with K_m values of 35 and 52 μM, respectively. In contrast to mammalian spermidine synthases, spermidine can replace to some extent putrescine as the aminopropyl acceptor. Hence, *P. falciparum* spermidine synthase has the capacity to catalyse the formation of spermine that is found in small amounts in the erythrocytic stages of the parasite. Among the spermidine synthase inhibitors tested against *P. falciparum* spermidine synthase, *trans*-4-methylcyclohexylamine (4MCHA) was found to be most potent with a K_i value of 0.18 μM. In contrast to the situation in mammals, where inhibition of spermidine synthase has no or only little effect on cell proliferation, 4MCHA was an efficient inhibitor of *P. falciparum* cell growth in vitro with an IC_{50} of 35 μM, indicating that *P. falciparum* spermidine synthase represents a putative drug target.

Keywords: Polyamines; Putrescine; Spermidine; Spermine; *trans*-4-Methylcyclohexylamine

1. Introduction

Malaria represents an enormous global health problem with 300–500 million infected people annually and approximately 40% of the world's population living in areas of infection risk. The most virulent form of malaria that is caused by the protist *Plasmodium falciparum* accounts for at least 1.5 million deaths per year [1]. The lack of a vaccine and the spreading resistance against conventional anti-malarials urgently requires the identification and validation of new drug targets in the metabolism of the parasite.

The naturally occurring polyamines spermidine and spermine are involved in numerous cellular processes and were

Abbreviations: AdoMetDC; *S*-adenosylmethionine decarboxylase; APA; 1-aminooxy-3-aminopropane; APE; 5-amino-1-pentene; dcAdoMet; decarboxylated *S*-adenosylmethionine; EBSS; Earle's balanced salt solution; ODC; ornithine decarboxylase; 4MCHA; *trans*-4-methylcyclohexylamine; MTA; 5′-methylthioadenosine; PBS; phosphate-buffered saline; PP5; protein phosphatase 5

☆ *Note:* The protein sequence data reported in this paper are available in the EMBL, GenBank™ and DDJB under the accession number CAB71155.

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found to be essential for cell proliferation and differentiation of prokaryotes and eukaryotes [2,3]. Since inhibition of polyamine synthesis correlates with block of cell growth, the synthesis enzymes represent attractive targets for therapeutic intervention in cancer as well as in parasite infection treatment [4–6]. In the polyamine synthesis pathway, spermidine is formed by the transfer of an aminopropyl group from decarboxylated *S*-adenosylmethionine (dcAdoMet) to a terminal amino group of putrescine. This reaction that also produces 5′-methylthioadenosine (MTA) is catalysed by spermidine synthase. The precursors of spermidine synthesis, putrescine and dcAdoMet, are provided by the two key enzymes of the pathway, ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC). In some eukaryotes, a second aminopropyltransferase, spermine synthase, is responsible for the formation of spermine from spermidine, in a catalytic reaction equivalent to spermidine synthesis [7].

Compared to the situation in mammals, the polyamine synthesis of *P. falciparum* exhibits some peculiarities. The two key enzymes ODC and AdoMetDC are encoded by a common gene leading to a unique bifunctional protein with an N-terminal ODC and a C-terminal AdoMetDC joint by a hinge region [8]. Moreover, biochemical analyses of the bifunctional *P. falciparum* ODC/AdoMetDC revealed features distinct from the monofunctional mammalian counterparts, including feedback inhibition of ODC by putrescine at physiological levels, the lack of AdoMetDC stimulation by putrescine and a long half-life of the protein [9].

Regarding the polyamine metabolism of *Plasmodium*, previous studies mainly focused on ODC and AdoMetDC and their inhibitors, which affect the cell cycle of the parasite and as a consequence show anti-malarial activity at least in cultured parasites [10–15]. Up to now, relatively little attention has been turned to *P. falciparum* spermidine synthesis. However, dicyclohexylamine, a known inhibitor of spermidine synthase, was reported to deplete spermidine pools in *P. falciparum*-infected erythrocytes and block parasite development [16].

Here, we report on the cloning, recombinant expression and biochemical characterisation of *P. falciparum* spermidine synthase. In accordance with the data on the bifunctional ODC/AdoMetDC [8], the transcript of *P. falciparum* spermidine synthase exhibits a stage specificity during the erythrocytic cycle of the parasite, peaking at the old trophozoite stage. The biochemical characterisation reveals that, in contrast to its mammalian counterparts that have a high specificity for putrescine [17], the plasmodial enzyme to a lower degree also accepts spermidine as a substrate and, hence, has the capacity to form spermine. *trans*-4-Methylcyclohexylamine (4MCHA) is introduced as an effective inhibitor of *P. falciparum* spermidine synthase exhibiting plasmodicidal activity.

2. Material and methods

2.1. Chemicals and reagents

4MCHA, 5-amino-1-pentene (APE) hydrochloride and (*S*,*R*)-dcAdoMet were generous gifts from Keijiro Samejima (Josai University, Saitama, Japan). 1-Aminooxy-3-aminopropane (APA) was kindly provided by Alex R. Khomutov (Russian Academy of Sciences, Moscow, Russia). MTA, dicyclohexylamine and cyclohexylamine were purchased from Sigma-Aldrich. [1,4-^{14}C] Putrescine dihydrochloride (107 mCi/mmol) and [^{14}C] spermidine trihydrochloride (112 mCi/mmol) were from Amersham Biosciences. Spermidine and putrescine were acquired from Fluka.

2.2. Culture of P. falciparum

P. falciparum 3D7 parasites were maintained in continuous culture according to Trager and Jensen [18]. Parasites were grown in human erythrocytes (blood group A+) in RPMI 1640 medium (Life Technologies) supplemented with 25 mM HEPES, 20 mM sodium bicarbonate, 0.5% albumax and 40 μg/ml gentamicin at 5% hematocrit in 150 cm^2 flasks at 37 °C with a gaseous phase of 90% N_2, 5% O_2 and 5% CO_2. The percentage of infected erythrocytes and the development of the parasites were determined by light microscopy of Giemsa-stained thin smears. Synchronisation of the culture was carried out by incubation of the cells in two volumes of 0.3 M L-alanine, 10 mM HEPES, pH 7.4 for 5 min at 37 °C [19].

For saponin lysis, *P. falciparum*-infected erythrocytes at a hematocrit of 25% were incubated for 10 min in ice-cold Earle's balanced salt solution (EBSS) containing 0.15% (w/v) saponin before the addition of four volumes of EBSS. The reaction mixture was centrifuged at 1500 × *g* for 5 min at 4 °C and the resulting pellet was washed twice in ice-cold EBSS.

2.3. In vitro assay in P. falciparum culture

Spermidine synthase inhibitors were tested for their plasmodicidal activity using a [^{3}H] hypoxanthine incorporation assay [20]. Parasitized erythrocytes (1.5% haemotocrit at 1% parasitemia) were distributed in 96-well plates (Filtermat A 1450-421, Wallac). APE, cyclohexylamine, dicyclohexylamine, 2-mercaptoethylamine and 4MCHA were used in concentrations ranging from 0.01 to 1.00 mM. After incubation for 24 h, 100 nCi of [^{3}H] hypoxanthine (Amersham Biosciences) was added to each well. Cultures were incubated for a further 24 h, before the contents of each well were collected on standard filter microplates and washed using a cell harvester (Inotech). Parasite growth was assessed by measuring the radioactivity incorporated by the parasites using a multi-detector liquid scintillation and luminescence counter (Wallac). The 50% inhibitory concentrations (IC_{50})