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ORIGINAL ARTICLE

Down-regulation of hypusine biosynthesis in plasmodium by inhibition of *S*-adenosyl-methionine-decarboxylase

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Abstract An important issue facing global health today is the need for new, effective and affordable drugs against malaria, particularly in resource-poor countries. Moreover, the currently available antimalarials are limited by factors ranging from parasite resistance to safety, compliance, cost and the current lack of innovations in medicinal chemistry. Depletion of polyamines in the intraerythrocytic phase of *P. falciparum* is a promising strategy for the development of new antimalarials since intracellular levels of putrescine, spermidine and spermine are increased during cell proliferation. *S*-adenosyl-methionine-decarboxylase (AdoMETDC) is a key enzyme in the biosynthesis of spermidine. The AdoMETDC inhibitor CGP 48664A, known as SAM486A, inhibited the separately expressed plasmodial AdoMETDC domain with a Km_i of 3 μM resulting in depletion of spermidine. Spermidine is an important precursor in the biosynthesis of hypusine. This prompted us to investigate a downstream effect on hypusine biosynthesis after inhibition of AdoMETDC. Extracts from *P. falciparum* in vitro cultures that were treated with 10 μM SAM 486A showed suppression of eukaryotic initiation factor 5A (eIF-5A) in comparison to the untreated control in two-dimensional gel electrophoresis. Depletion of eIF-5A was also observed in Western blot analysis with crude protein extracts from the parasite after treatment with 10 μM SAM486A. A determination of the intracellular polyamine levels revealed an approximately 27% reduction of spemidine and a 75% decrease of spermine while putrescine levels increased to 36%. These data suggest that inhibition of AdoMetDc provides a novel strategy for eIF-5A suppression and the design of new antimalarials.

Keywords AdoMETDC · EIF-5A · Malaria · SAM486A

Abbreviations

AdoMetDC	*S*-adenosylmethionine decarboxylase
EIF-5A	Eukaryotic initiation factor 5A
ODC	Ornithine decarboxylase
DHS	Deoxyhypusine synthase
DOHH	Deoxyhypusine hydroxylase
SAM486A	4-amidinoindan-1-one-2′-amidinohydrazone
kDa	Kilodalton
p.i.	Post infection
RBC	Red blood cells
Put	Putescine
Spd	Spermidine
Spm	Spermine

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Introduction

During proliferation in the intraerythrocytic phase of the human host, the malaria parasite undergoes intense cell multiplication with an absolute requirement for the major

polyamines putrescine, spermidine and spermine. Depletion of polyamines leads to inhibition of cell proliferation.

The polyamine biosynthetic pathway in *P. falciparum* consists of two regulatory key enzymes, i.e. ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC). While ODC catalyzes the decarboxylation of ornithine to putrescine, AdoMetDC regenerates decarboxylated *S*-adenosylmethionine which is the aminopropyl moiety donor for the biosynthesis of spermidine (Haider et al. 2005). In *P. falciparum*, spermidine synthase can also catalyze the reaction of spermidine to spermine (Haider et al. 2005). This result strongly supported previous findings that no separate spermine synthase locus has been isolated from the parasite. ODC and AdoMetDC from *P. falciparum* have very peculiar features i.e. both activities are on the same open reading frame but acting independently (Müller et al. 2000). The bifunctional character of ODC provides a rational basis for drug design since there is only a monofunctional homolog present in the human host cell.

Classical inhibitors of AdoMetDC are derivatives of bis(guanyl)hydrazones which have been reported to have potent effects on parasites and tumor cells (Brun et al. 1996; Regenass et al. 1994). The drug CGP 48664A, also known as SAM 486A, potently blocked mammalian cell growth (Regenass et al. 1992). It has already been tested in phase I and phase II trials for its activities against solid tumors and relapsed or refractory non-Hodgkin's lymphoma (Pless et al. 2004; Seiler 2003). SAM 486A turned out to be an effective inhibitor of the separately expressed AdoMetDC from *P. falciparum* with a determined K_i of 3 μM (Das Gupta et al. 2006). Determination of intracellular polyamines showed that bis(guanyl)hydrazones resulted in depletion of spermidine (Das Gupta et al. 2006) which is an important precursor in the biosynthesis of hypusine, a unique, polyamine-derived amino acid present in eukaryotic initiation factor 5A (eIF-5A).

Hypusine is formed in a posttranslational modification (Wolff et al 2007) which involves two sequential enzymatic steps catalyzed by deoxyhypusine synthase (DHS) (Liao et al. 1998) [EC 1.14.9929] and deoxyhypusine hydroxylase (DOHH) [EC 1.14.9929] (Park et al. 2006). While DHS catalyzes the transfer of the aminobutyl moiety to a specific lysine residue in the eIF-5A precursor protein, DOHH completes hypusine biosynthesis through hydroxylation and thereby activates eIF-5A.

It has recently been shown that depletion of the intracellular spermidine level by the AdoMetDC inhibitor SAM486A 4-amidinoindan-1-one-2′-amidinohydrazone, also known as CGP 48664A, abolishes eIF-5A activity which is required for Rev trans-activation of HIV-1 (Schäfer et al. 2006). SAM486A suppressed the formation of progeny viruses by blocking Rev activity (Hauber et al. 2005; Hart et al. 2002; Andrus et al. 1998) and HIV-replication without affecting the metabolism of the host. Suppression of eIF-5A was attributed to spermidine depletion and thus prevention of deoxyhypusine formation in eIF-5A. The substrate spermidine is required for catalysis of DHS in the first step of hypusine modification. Hitherto, the function of this posttranslational modification remains unknown in the malaria parasite.

Hauber et al. (2005) demonstrated that suppression of the hypusine pathway is possible by inhibition of regulatory key enzymes i.e. SAMDC at the early steps of the polyamine pathway. In the present study we investigated the inhibitory effect of the AdoMetDC inhibitor SAM468A on eIF-5A formation in *P. falciparum* strain NF54 and its potency for antimalarial therapy. Our data show that the drug inhibits hypusinylation of eIF-5A by spermidine depletion in the parasite and protects *P. berghei* infected C57Bl/6 mice from cerebral malaria.

Materials and methods

Expression and purification of *P. vivax* eIF-5A on nickel-nitrilotriacetic acid spin columns under native conditions

Escherichia coli cell cultures harboring the *Plasmodium* eIF-5A expression plasmid (Kaiser et al. 2001) were grown in 200 ml Luria Bertani (LB) medium with the appropriate antibiotic (ampicillin 50 μg) for 15.5 h overnight at 37°C until an OD_{600} of 1.5–1.6 was reached. One hour after the addition of 50 ml LB medium, cells were induced with 0.4 mM IPTG. From the beginning of induction, samples of 1 ml were taken at intervals of 1 h and analyzed on 10% SDS polyacrylamide gels to control expression. *E. coli* BL21 (DE3) pLysS cells (Invitrogene, Germany) expressing either eIF-5A or truncated DHS from a 200 ml LB medium culture were thawed for 15 min and dissolved in 4 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8.0). Lysozyme was added at a concentration of 1 mg/ml and cells were incubated on ice for 30 min and sonicated for 10 s each, with a pause of 5 s in between. The lysate was centrifuged at 10,000 rpm for 20–30 min at 4°C. As much as 600 μl of the lysate was centrifuged for 2 min at 2,000 rpm on a nickel-NTA spin column, which was pre-equilibrated with lysis buffer. The Ni-NTA spin column was washed twice with 600 μl wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol, pH 8.0) for 2 min at 2,000 rpm. The protein was eluted with 200 μl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol, pH 8.0) for 2 min at 2,000 rpm. Different eluate fractions from native purification on

nickel-nitrilotriacetic acid spin columns were pooled and applied on to SDS protein gels for Western blot analysis.

Isolation of proteins from *P. falciparum* strain NF54

Isolation of proteins from parasite infected red blood cells (RBCs) was performed after lysis with saponin (Kaiser et al. 2001) as described previously. Saponin-lysed parasites were resuspended in 200 µl CelLytic B (Sigma Aldrich) and lysed at 37°C with constant shaking. For further homogenization, 50 mg glass beads were added and samples were shaken twice for 20 s each time and centrifuged at 15,000*g* at 4°C for 20 min. Proteins in the supernatant of the cleared lysate were precipitated with 4 VT of methanol and centrifuged at 13,000*g* at 4°C for 20 min according to a protocol of Wessel and Flügge (1983). Extraction with 200 µl chloroform with subsequent centrifugation for 1 min at 13,000*g* was performed. The interphase was re-extracted with 600 µl methanol, vortexed, centrifuged for 2 min at 13,000*g* and then pellet dried and applied for further analysis.

Western blot analysis

Western blot analysis was performed according to the "QIAexpress Detection and Assay Handbook" with minor changes. Proteins were blotted after SDS PAGE on a nitrocellulose membrane in semi-dry transfer buffer (25 mM Tris base, 15 mM glycine, 10% methanol, pH 5.3) in the Fastblot B34 (Whatman Biometra) assembly with transfer conditions of 5 mA/cm^2 for 30 min. After protein blotting the nitrocellulose membrane was stained for 2 min with Ponceau S stain and washed with water and TBS buffer (10 mM Tris–Cl, 150 mM NaCl, pH 7.5) for 10 min at RT (room temperature) before antibody detection. The blot was incubated overnight at 4°C in the appropriate diluted primary antibody solution and washed with TBS buffer for 10 min. The membrane was incubated with the secondary antibody; a goat anti-rabbit IgG biotin conjugate, which was diluted 1:5,000 fold in blocking buffer for 60 min at room temperature. The blot was subsequently incubated in strepavidin-alkaline phosphatase solution (Sigma; 1:10,000) for 30 min and washed 4 times in TBST (TBS plus Tween 20) buffer. Detection was performed in a nitroblue tetrazolium (NBT) and 5-brom-4-chlor-3-indoxylphosphate (BCIP) solution.

EIF-5A antiserum

The eIF-5A antiserum was obtained from 200 µg purified eIF-5A from *P. vivax* in a classical 3-month protocol by Eurogentec, Belgium. The specificity and validity of the antibody was checked by Western blot analysis with purified eIF-5A protein from *P. vivax*.

2D-gel electrophoresis

The pellet of saponin-lysed parasites from a 30 ml in vitro culture of *P. falciparum* strain NF54 was homogenized in a 240 µl 10 mM Tris–HCl, pH 8.0, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM Pefabloc SC and 0.1% (w/v) SDS. As much as 50 mg of glass beads was added and the sample was shaken 2 times 20 s and centrifuged at 15,000*g* at 4°C for 20 min. As much as 60 µl of the crude extract from the parasite was added to the rehydration buffer containing 2.5 ml of 8 M urea, 4% (w/v) CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 2% (v/v) IPG buffer (3–10), 25 mM DTT, and a trace of bromophenol blue and centrifuged for 10 min at 4,000 rpm. As much as 25 µg of the sample was loaded on an IPGphor strip holder (3–11) (Amersham) which was overlaid with 1.4 ml cover fluid. The strips were placed into an EttanIGphor (Amersham) with the following programme: step 1 500 V/h, step 2 1,000 V/h, and step 3 8,000 V/hour.

After isoelectric focusing the strip was incubated in equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) with bromophenol blue for 15 min. Subsequently the strip was incubated for 15 min at room temperature in equilibration buffer with 0.75 g iodoacetamide. The second dimension was run on a 9–16% T-gradient (total percentage of acrylamide and bisacrylamide) polyacrylamide gel. The gel was run at 200 V for 50 min. Staining of the gel was performed with Coomassie blue for 1 h while destaining was performed 3 times in destaining solution.

Determination of intracellular polyamines

Intracellular polyamines were determined according to a protocol by Redmond and Tseng (1978). A 10 ml in vitro culture of *P. falciparum* strain NF54 which was treated with 10 µM CGP was extracted with 2.5 ml 0.3 m perchloric acid and incubated on ice for 20 min under vigorous shaking. After centrifugation at 12,500*g* the supernatant was transferred to a glass micro reaction vessel and alkalized with 2 ml of 2 M NaOH solution. The derivatization was performed with 5 µl benzoyl chloride under shaking for 20 min at room temperature. Polyamine benzoyl derivatives were extracted with 2 ml chloroform and washed once with H_2O. The chloroform layer was evaporated under vacuum and the residue was dissolved in 100 µl methanol. Analysis of the polyamine derivatives was performed by the reverse-phase HPLC DAD system (Agilent 1100 series; Column: LiChrocart® 125-4 HPLC-Cartridge; Packaging material: LiChrospher® 60 RP-select B (5 µm).

Reverse phase chromatography

Analyses of the benzoylated polyamines were performed using the analytical HPLC system Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary solvent pump, a solvent degasser, an automatic sampler, a column thermostat, a diode array detector (DAD) and a data station with the ChemStation Software, Rev. A.09.03. For chromatographic separations, a LiChroCART® cartridge with a LiChrospher® 60 RP-select B analytical column, 125 mm in length and 4 mm inner diameter, with a particle size of 5 µm, and a guard column containing the same material, as well as isocratic conditions with a mobile phase of 55% (v/v) methanol and 45% (v/v) water were used. The flow rate of the mobile phase through the column was 1 cm^3 min^{-1} at 25°C. Samples of 10 µl were injected automatically. The UV spectra were recorded at a wavelength range from 190 to 400 nm with steps of 2 nm. The integrated chromatogram was acquired at 230 nm. The identifications of the benzoylated polyamines were confirmed by UV spectra of the reference polyamines. The quantification was done using 1,7-diaminoheptane as the internal standard.

Animals

The animal studies were performed according to the guidelines of the German animal rights (license number: 50.203. 2-BN). C57BL/6 mice were obtained from Harlan and infected intravenously with 1×10^5 parasitized erythrocytes from a homolog donor mouse, which had been infected with frozen polyclonal stocks of *P. berghei* ANKA (PbA) strain C57BL/6. PbA-infected susceptible mice develop overt clinical signs between day 6 (D6) and 9 (D9) and die within 48 h of that onset. In infected mice, where CM does not develop by D10, the parasitemia reaches high levels resulting in anemia and death 7 to 10 days later. Five to eight mice were used in each study group. Parasitemia was monitored daily by thin blood smears of tail blood stained with Giemsa. Treatment of mice was performed with SAM 486A dissolved in DMSO 40 mg/kg intra-peritoneally for the respective time points (Specht et al. 2008).

Results

In vivo protection against cerebral malaria of mice infected with *P. berghei*

Recent results from Das Gupta et al. (2006) demonstrated that SAM486A inhibited a *P. falciparum* in vitro culture with an IC_{50} value of 8.8 µM. The drug was used in concentrations from 2 to 20 µM and dissolved in DMSO. Since it was reported that SAM 486A also known as CGP48664A was less efficient in *P. falciparum* in vitro cultures (Das Gupta et al. 2006) compared to its potency in mammalian cells, we investigated whether the compound would protect C57/BL6 mice against cerebral malaria. Protection against cerebral malaria was achieved by treating the mice intra-peritoneally in a dose of 40 mg/kg body weight from day 1 to day 6 postinfection (Fig. 1a). Surprisingly, the survival rate of the infected mice was prolonged to 20 days p.i. in comparison to the untreated control mice that died 8 days p.i.

Next we investigated the effect of SAM486A on the parasitemia of the infected C57/BL6 mice between day 4 p.i. and day 19 p.i. Parasitemia was significantly reduced below 10% between 8 and 10 days p.i. and increased constantly after 10 days p.i. until day 14 to 50% and peaked on day 16 to approximately 70%. However, the significant increase in parasitemia did not change the prolonged survival rate of the mice (Fig. 1b).

Western blot analysis of protein extracts from *P. falciparum* strain NF54 in vitro cultures after treatment with 10 µM SAM486A

In a first set of experiments we investigated whether a polyclonal antibody directed against the eIF-5A protein from *Plasmodium* was able to detect the protein in crude extracts obtained from an in vitro culture of *P. falciparum* strain NF54. EIF-5A is present in its precursor and modified form. The acetylated form was characterized in human cells but its occurrence has not been described for *Plasmodium* (Klier et al. 1995). The anti-eIF-5A antibody recognizes either modified or unmodified eIF-5A protein. Western blot analysis (Fig. 2a) was performed with 1 µg of purified N-terminal histidine-tagged eIF-5A protein after nickel-chelate affinity chromatography (lane 1) as a control and 60 µg of crude, precipitated protein extract from an in vitro culture of *P. falciparum* strain NF54 (lane 2). As shown in Fig. 2a the anti-eIFA antibody detected the protein with an estimated size of 19 kDa in crude, precipitated protein extracts from the *P. falciparum* in vitro culture with a smaller intensity compared to the purified histidine tagged eIF-5A protein which had a molecular weight of approximately 20 kDa. The anti-eIF-5A antibody was applied in a concentration of 1:2,000.

Next we tested the inhibitory effect of the drug in a concentration of 10 µM on eIF-5A suppression in crude, enriched protein extracts from an in vitro culture of *P. falciparum* (lane 1) in comparison to the untreated control extract (lane 2) (Fig. 2b). Treatment with SAM 486A led to complete suppression of eIF-5A (Fig. 2b, lane 1) after 24 h while the untreated control exhibited

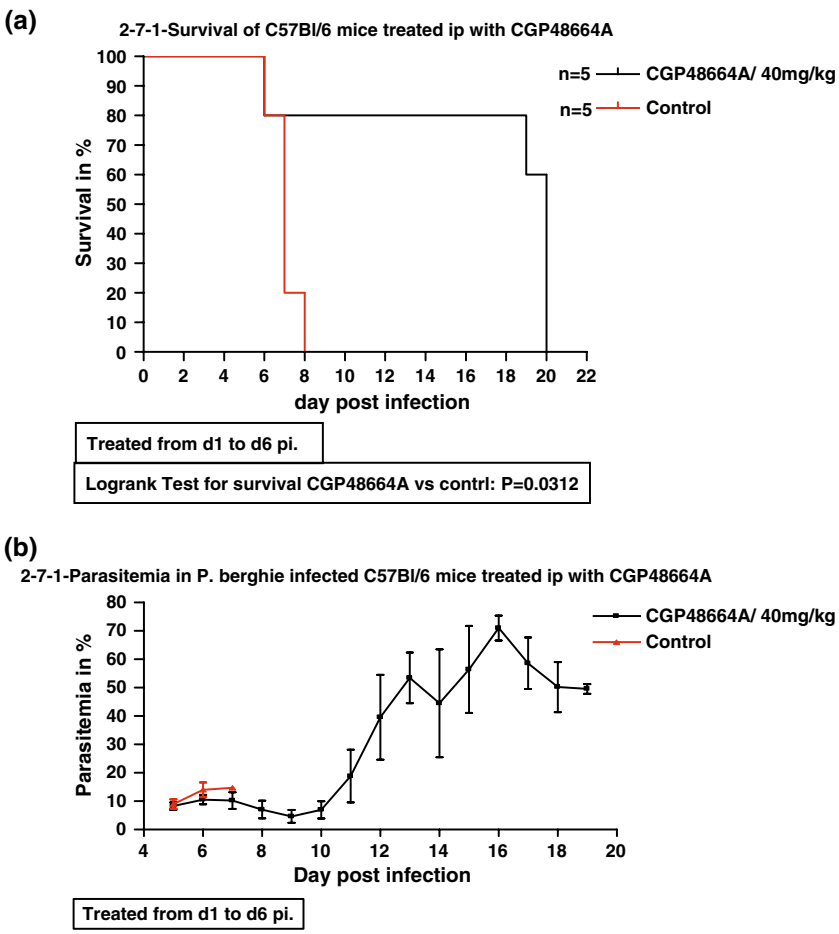

Fig. 1 **a** Survival rate of *P. berghei* ANKA-strain infected C57Bl/6 mice p.i. after treatment with SAM486A in a dose of 40 mg/kg body weight between day 1 (D1) and day 6 (D6) p.i. **b** Parasitemia *P. berghei* ANKA-strain infected C57Bl/6 mice p.i. after treatment with SAM486A in a dose of 40 mg/kg body weight between day 1 (D1) and day 6 (D6) p.i. Parasitemia was monitored from day 5 (D5) to day 19 (D19)

a band of 19 kDa (Fig. 2b, lane 2). In this experiment the primary antibody was diluted 1:2,000 fold.

Prevention of hypusine formation eIF-5A after treatment with 10 µM SAM486A

To investigate prevention of hypusine formation in in vitro cultures from *P. falciparum* a two-dimensional gel electrophoresis was performed with equal amounts of protein extracts from *Plasmodium* either in the absence (Fig. 3a) or presence of 10 µM SAM486A (Fig. 3b). In the first dimension isoelectric focusing was applied on an immobilized linear pH gradient (IPG) between 3 and 10 which was followed by SDS-PAGE separation in the second dimension. EIF-5A from *Plasmodium* was identified by its molecular weight of 19 kDa and its theoretical pI of 5.21 (Fig. 3a). Moreover, a protein landmarker (Serva) was applied to identify the eIF-5A spots. The two-dimensional gel electrophoresis showed the absence of eIF-5A after treatment with the *S*-adenosylmethionine decarboxylase inhibitor (Fig. 3b). These results were supported by immunoprecipitation of the identified protein spots by an anti-eIF-5A serum which could only weakly detect the eIF-5A protein after SAM486A treatment in comparison to the untreated control (Fig. 4).

Determination of intracellular polyamine concentrations after treatment with SAM486A

To determine the effect of SAM486A on the levels of the major polyamines i.e. putrescine, spermidine and spermine, intracellular levels of polyamines from the parasite were analyzed after benzoylation. The corresponding benzoyl derivatives were identified by reverse phase HPLC. 1,7-diaminoheptane was used as an internal standard for quantification. In correlation with their alkyl chains putrescine had a retention time of 3.79 min, spermidine of 7.25 min and spermine of 14.45 min (Fig. 5). The untreated control samples contained spermidine (0.781 nmol/10 µl injection volume from 10^{10} infected RBCs) and spermine (0.292 nmol/10 µl injection volume from 10^{10} infected RBCs) and to a less extent putrescine (0.083 nmol/10 µl injection volume from 10^{10} infected RBCs). After inhibition with SAM486A spermine (0.103 nmol/10 µl injection

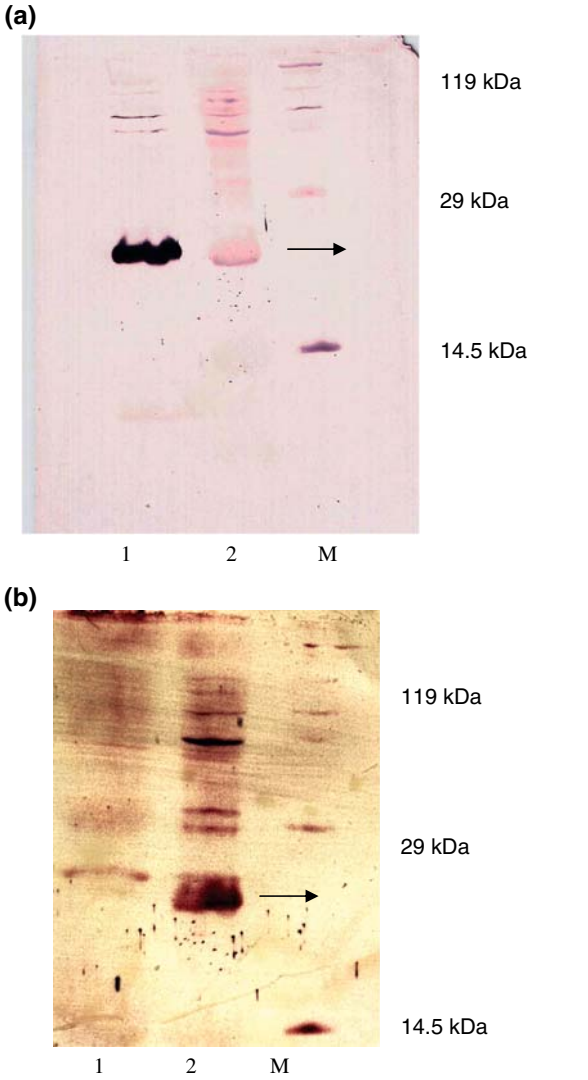

Fig. 2 Western blot analysis of crude protein extracts from *P. falciparum* strain NF54 **a** in the absence of SAM486A; 1 µg purified eIF-5A (*lane 1*); 60 µg purified protein extract (*lane 2*); Roti-mark protein marker (*lane 3*); **b** in the presence of 10 µM SAM486A; 60 µg protein of SAM486A treated protein extract (*lane 1*); 60 µg untreated protein extract (*lane 2*)

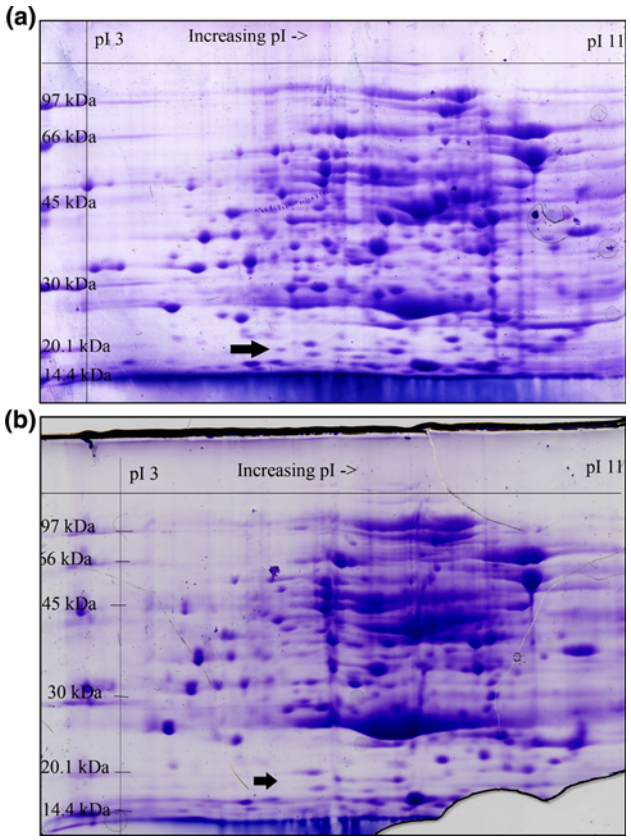

Fig. 3 Two-dimensional gel electrophoresis of total protein extracts prepared from *P. falciparum* in vitro cultures **a** untreated; **b** treated with 10 µM SAM486A for 48 h. Isoelectric focusing was performed in the first dimension on an immobilized linear pH gradient from pH 3 to 7.0 which was followed by SDS-PAGE separation in the second dimension. Molecular mass standards indicated in kilodalton are given on the *left*

volume from 10^{10} infected RBCs) and spermidine levels (0.572 nmol/10 µl injection volume from 10^{10} infected RBCs) decreased to 50% and to 25%, respectively, while putrescine levels increased 4-fold (0.230 nmol/10 µl injection volume from 10^{10} infected RBCs).

Discussion

In the present study we demonstrated that inhibition of AdoMetDC, which is a key regulatory enzyme of the polyamine pathway providing decarboxylated *S*-adenosylmethionine (dc-AdoMet) for the synthesis of spermidine and spermine, is an alternative strategy to suppress modification of eIF-5A. Spermidine is an important substrate of DHS, the enzyme which catalyzes the first step in the biosynthesis of hypusine modification in eIF-5A. In *Plasmodium* eIF-5A is involved in cell proliferation (Kaiser et al. 2003). A low-molecular weight drug like the AdoMetDC inhibitor SAM486A suppresses hypusine formation by spermidine depletion. SAM486A protected C57/BL6 infected mice against cerebral malaria without drug-induced cellular toxicity and prolonged the survival time in comparison to the untreated control to 75% when administered in a concentration of 40 mg/kg body weight 1 to 6 days p.i. These results are accompanied by a significant decrease in parasitemia below a level of 10% between days 8 and 10 p. i. after treatment. However, after day 10 (D10) p.i. parasitemia increased without affecting the survival rate of the infected C57/BL6 mice. The observed increase in parasitemia might either result from low levels of spermidine which is reduced to approximately 27% (Ramya et al. 2006) and the resulting low levels of

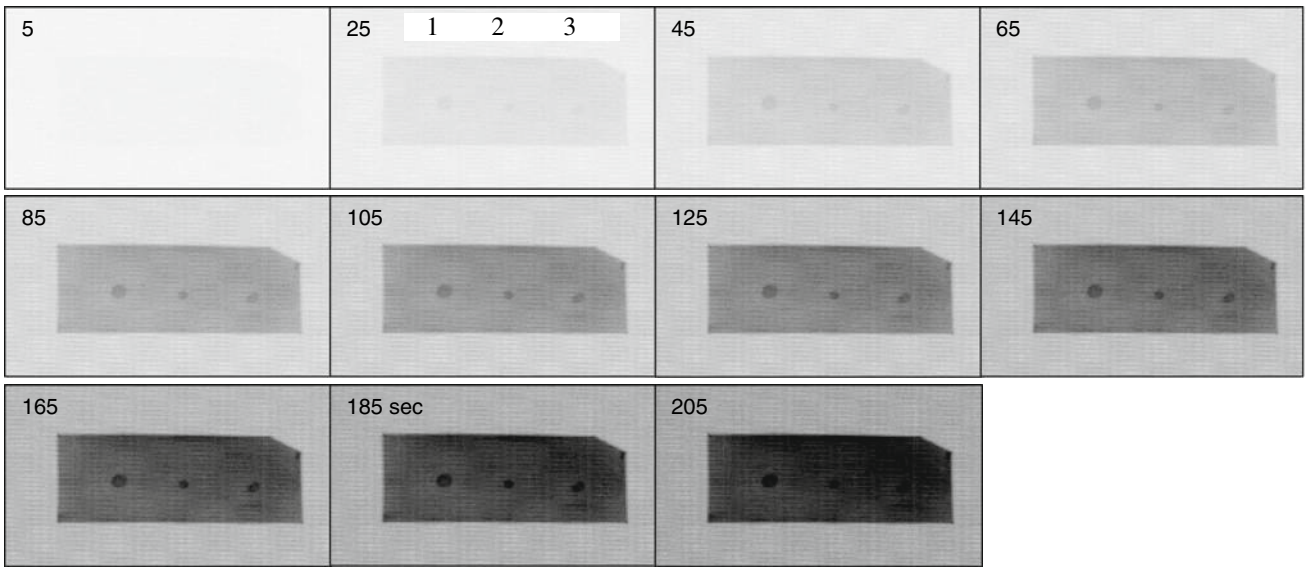

Fig. 4 Dot blot of different eIF-5A concentrations treated with 10 μM SAM486A obtained after immunoprecipitation and two-dimensional gel electrophoresis incubated with Rb-anti- eIF5A serum from *P. vivax* (1st Ab; 1:500) and Monkey-anti-Rb IgG HRP conjugated (2nd Ab; 1:1000) antibody. Detection was performed with the ECL chemiluminescent detection system (GE-Healthcare). *Lane 1* EIF-5A extracted and immunoprecipitated after two-dimensional gel electrophoresis; *lane 2* EIF-5A 1:10 diluted; *lane 3* EIF-5A 1:1 diluted. In the *left* corner the light emission times are given

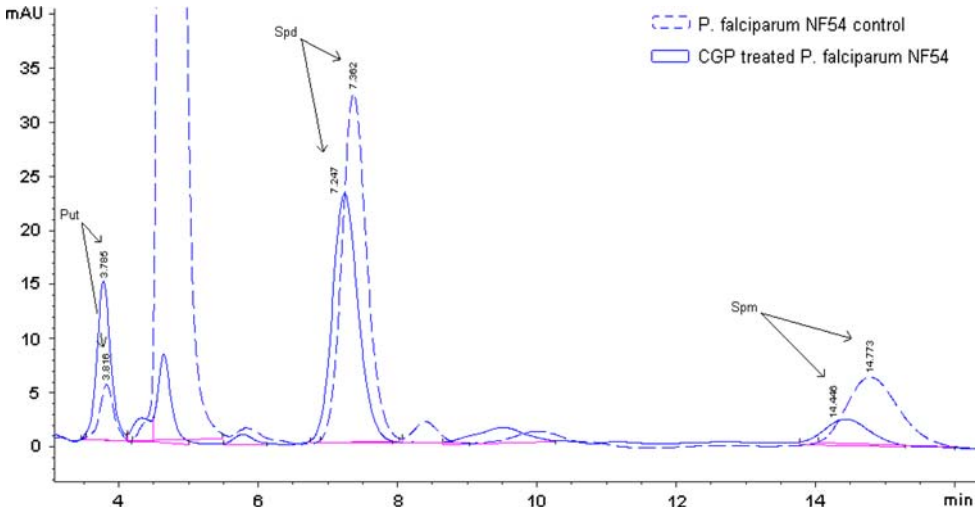

Fig. 5 Overlay of the two chromatograms from *P. falciparum* strain NF54 in the presence (**a** blue lane) and absence (**b** dotted blue lane) of 10 μM SAM486A. Differences in putrescine (Put), spermidine (Spd) and spermine (Spm) are shown

modified eIF-5A. Thus it seems likely that a combinatory drug regimen between an AdoMetDC inhibitor and an inhibitor of the hypusine pathway is necessary to efficiently eradicate the parasite.

The determination of intracellular polyamine levels shows that spermidine is the major polyamine present in *Plasmodium* which is in agreement with previously obtained results (Kaiser et al. 2001) suggesting its role of interaction with nucleic acids (Müller et al. 2001) and as a substrate donor in hypusinylation (Park 2006). Spermine has an intracellular concentration of 0.292 nmol/10 μl injection volume of 10^{10} infected RBCs which surpasses recently obtained results (Das Gupta et al. 2005). After treatment with SAM486A spermidine and spermine pools were decreased while the putrescine pool increased. This mode of action resembles AdoMetDC inhibitor MDL 73811 (Wright et al. 1991) which shows a 3-fold increase of putrescine and a 67% decrease of spermidine when administered in a dose of 5 μM. An investigation of a dose-dependent effect for SAM486A will be necessary to compare its efficacy in comparison to MDL 73811.

Western blot experiments performed with an anti-eIF-5A serum showed that protein extracts obtained from *Plasmodium* after treatment with 10 μM SAM468A did not detect eIF-5A protein (Fig. 3). This result suggests that the drug might change the half-life ($t_{1/2}$) of the eIF-5A protein.

However, eIF-5A either in its activated or unmodified form is known to be a very stable protein with $t_{1/2}$ more than 7 days (Nishimura et al. 2005). Mouse mammary carcinoma FM3 cells treated with alpha-difluoromethylornithine (DFMO) showed a decrease of activated eIF-5A when levels of spermidine began to fall beyond 8 ng/mg protein after 12 h of treatment (Nishimura et al. 2005). However with respect to the Western blot experiment at least the eIF-5A precursor protein had to be recognized by the antibody independent of the spermidine level. Further, experiments with protein extracts from *Plasmodium* labeled with [^{14}-C]-spermidine are necessary to elucidate the drug's mechanism. Consistent with this view that SAM486A might decrease $t_{1/2}$ of eIF-5A are the results obtained by two-dimensional gel electrophoresis (Fig. 4). However, the immunoprecipitated eIF-5A spot resulted in a faint band after drug treatment which could either be the unmodified or active form of eIF-5A since the anti-eIF-5A antibody detects both forms. To clarify this a two-dimensional gel electrophoresis with protein extracts labeled with [^{14}-C]-spermidine should be performed to differentiate between the different modified forms of eIF-5A by immunoprecipitation.

A determination of the half-life of eIF-5A with cycloheximide, an inhibitor of protein synthesis (Gregio et al. 2009) is required to delineate the drug's mode of action since eIF-5A is involved in translation initiation (Zanelli and Valentini 2007).

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References

Andrus L, Szabo P, Grady RW, Hanauske AR, Huima-Byron T, Slowinska B, Zagulska S, Hanauske-Abel HM (1998) Antiretroviral effects of deoxyhypusyl hydroxylase inhibitors: a hypusine-dependent host cell mechanism for relication of human immunodeficiency virus type 1 (HIV-1). Biochem Pharmacol 55:1807–1818

Brun R, Buhler Y, Sandmeier U, Kaminsky R, Bacchi CJ, Rattendi D, Lane S, Croft SL, Snowdon D, Yardley V, Caravatti G, Frei J, Stanek J, Mett J (1996) In vitro trypanocidal activities of new *S*-adenosylmethionine decarboxylase inhibitors. Antimicrob Agents Chemother 40:1442–1447

Das Gupta R, Krause-Ihle T, Bergmann B, Müller IB, Khomutov AR, Müller S, Walter RD, Lüersen K (2005) 3-Aminooxy-1-aminopropane and derivatives have an antiproliferative effect on cultured *Plasmodium falciparum* by decreasing intracellular polyamine concentrations. Antimicrob Agents Chemother 49:2857–2864

Gregio AP, Cano VP, Avaca JS, Valentini SR, Zanelli CF (2009) EIF5A has a function in the elongation step of translation in yeast. Biochem Biophys Res Commun 380:785–790

Haider N, Eschbach ML, Dias Sde S, Gilberger TW, Walter RD, Lüersen K (2005) The spermidine synthase of the malaria parasite *Plasmodium falciparum*: molecular and biochemical analysis of the polyamine synthesis enzyme. Mol Biochem Parasitol 142:224–236

Hart RA, Billaud JN, Choi SJ, Phillips TR (2002) Effects of 1, 8-diaminooctane on the FIV Rev regulatory system. Virology 304:97–104

Hauber I, Bevec D, Heukeshoven J, Krätzer F, Horn F, Choidas A, Harrer T, Hauber J (2005) Identification of cellular deoxyhypusine synthase as a novel target for antiretroviral therapy. J Clin Investig 115:76–85

Kaiser A, Gottwald A, Wiersch C, Lindenthal B, Maier W, Seitz HM (2001) Effect of drugs inhibiting spermidine biosynthesis and metabolism on the in vitro development of *Plasmodium falciparum*. Parasitol Res 87:963–972

Kaiser A, Gottwald A, Maier W, Seitz HM (2003) Targeting enzymes involved in spermidine metabolism of parasitic protozoa-a possible new strategy for antiparasitic treatment. Parasitol Res 91:508–516

Klier H, Csonga R, Joäo HC, Eckerskorn C, Auer M, Lottspeich F, Eder J (1995) Isolation and structural characterization of different isoforms of the hypusine-containing protein eIF-5A from Hela cells. Biochem 34:14693–14702

Liao DI, Wolff EC, Park MH, Davies DR (1998) Crystal structure of the NAD complex of the human deoxyhypusine synthase: an enzyme with a ball and chain mechanism for blocking the active site. Structure 6:23–32

Müller SA, Da'dara K, Lüersen C, Wrenger R, Das Gupta R, Madhubala R, Walter RD (2000) In the human malaria parasite *Plasmodium falciparum*, polyamines are synthesized by a bifunctional ornithine decarboxylase, *S*-adenosylmethionine decarboxylase. J Biol Chem 275:8097–8102

Müller S, Coombs GH, Walter RD (2001) Targeting polyamines of parasitic protozoa in chemotherapy. Trends Parasitol 17:242–249

Nishimura K, Murozumi K, Shirahata A, Park MH, Kashiwagi K, Igarashi K (2005) Independent roles of eIF5A and polyamines in cell proliferation. Biochem J 385:779–785

Park MH (2006) The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). J Biochem 139:161–169

Park JH, Aravind L, Wolff EC, Kaevel J, Kim YS, Park MH (2006) Molecular cloning, expression and structural prediction of deoxyhypusine hydroxylase: a HEAT-repeat-containing metalloenzyme. Proc Natl Acad Sci USA 103:51–56

Pless M, Belhadj K, Menssen HD, Kern W, Coiffier B, Wolf J, Herrmann R, Thiel E, Bootle D, Sklenar I, Muller C, Choi L, Porter C, Capdeville R (2004) Clinical efficacy, tolerability, and safety of SAM486A, a novel polyamine biosynthesis inhibitor, in patients with relapsed or refractory non-Hodgkin's lymphoma: results from a phase II multicenter study. Clin Cancer Res 10:1299–1305

Ramya TNC, Surolia N, Surolia A (2006) Polyamine synthesis and salvage pathways in the malaria parasite *Plasmodium falciparum*. Biochem Biophys Res Commun 348:579–584

Redmond JW, Tseng A (1978) High-pressure liquid chromatographic determination of putrescine, cadaverine, spermidine and spermine. J Chomatogr 170:479–481

Regenass U, Caravatti G, Mett H, Stanek J, Schneider P, Müller M, Matter A, Vertino P, Porter CW (1992) New *S*-adenosylmethionine decarboxylase inhibitors with potent antitumor activity. Cancer Res 52:4712–4718

Regenass U, Mett H, Stanek J, Mueller M, Kramer D, Porter CW (1994) CGP 48664, a new *S*-adenosylmethionine decarboxylase inhibitor with broad spectrum antiproliferative and antitumor activity. Cancer Res 54:3210–3217

Schäfer B, Hauber I, Bunk A, Heukeshoven J, Düsedau A, Bevec D, Hauber J (2006) Inhibition of multidrug-resistant HIV-1 by

interference with cellular S-adenosylmethionine decarboxylase activity. J Infect Dis 15:740–750

Seiler N (2003) Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. Curr Drug Targets 4:537–564

Specht S, Sarite SR, Hauber I, Hauber J, Görbig UF, Meier C, Bevec D, Hoerauf A, Kaiser A (2008) The guanylhydrazone CNI-1493: an inhibitor with dual activity against malaria-inhibition of host cell pro-inflammatory cytokine release and parasitic deoxyhypusine synthase. Parasitol Res 102:1177–1184

Wessel D, Flügge UI (1983) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138:141–143

Wolff EC, Kang KR, Kim YS, Park MH (2007) Posttranslational synthesis of hypusine: evolutionary progression and specificity of the hypusine modification. Amino Acids 33:341–350

Wright PS, Byers TL, Cross-Doersen DE, McCann PP, Bitonti AJ (1991) Irreversible inhibition of *S*-adenosylmethionine decarboxylase in *Plasmodium falciparum*-infected erythrocytes: growth inhibition in vitro. Biochem Pharmacol 41:1713–1718

Zanelli CF, Valentini SR (2007) Is there a role for eIF5A in translation? Amino Acids 33:351–358