RESEARCH LETTER

Nicotinamide inhibits *Plasmodium falciparum* Sir2 activity *in vitro* and parasite growth

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Abstract

Plasmodium falciparum sirtuin, PfSir2, contains histone deacetylase (HDAC) activity that may be central to the regulation of virulence gene expression in the parasites. Although a few reports have been published recently regarding *in vitro* and *in vivo* function of PfSir2, expression of the endogenous protein (*c.* 30 kDa) has not been shown yet. Here we report the presence of PfSir2 in the parasite at the protein level by specific antibodies. HDAC activity of PfSir2 can be inhibited by nicotinamide, a product of sirtuin reaction. Surprisingly, we find that nicotinamide also delays parasite growth significantly in culture. These findings further our knowledge on PfSir2 and raise the possibility of using an inexpensive agent like nicotinamide as an antimalarial in combination with other antiparasitic drugs.

Introduction

Plasmodium falciparum 'silent information regulator 2' (PfSir2) is a member of class III histone deacetylases (HDACs) that has been shown recently essential for epigenetic regulation of virulence genes central to the malaria pathogenesis (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005; Merrick & Duraisingh, 2007). Disruption of PfSir2 leads to activation of silenced *var* genes by chromatin modification (Duraisingh *et al.*, 2005). Another study suggests that PfSir2 forms a heterochromatin structure with histone hypoacetylation at the telomeric and subtelomeric region. Activation of telomere-associated *var* gene results in removal of PfSir2 and acetylation of promoter region suggesting a direct role of PfSir2 in *var* gene regulation (Freitas-Junior *et al.*, 2005).

Like *Saccharomyces cerevisiae* Sir2 protein, recombinant PfSir2 shows NAD+-dependent HDAC activity. Additionally, PfSir2 is a unique deacetylase that can ADP-ribosylate histones and itself (Merrick & Duraisingh, 2007). The activity of PfSir2 can be inhibited by nicotinamide, one of the products of the deacetylation reaction.

Although, the above studies reflect fundamental role of PfSir2 in *var* gene silencing, the expression of endogenous PfSir2 (*c.* 30 kDa) has remained elusive. Anti-PfSir2 antibodies used by Freitas-Junior *et al.* (2005) detected a prominent band of *c.* 70 kDa, much higher than the expected molecular mass of PfSir2. Similarly, the recent study on PfSir2 highlighting its biochemical activities shows the expression of the protein using a His_6-tagged parasite line (Merrick & Duraisingh, 2007). These studies raise the issue whether PfSir2 is truly expressed in the parasites at the protein level or its expression level is really low limiting its detection by western blot experiments.

Both nicotinamide (NAm) and nicotinic acid (NAc) are intermediate products of NAD^+-dependent sirtuin activities of Sir2 (Bitterman *et al.*, 2002). Interestingly, nicotinamide is an inhibitor of *S. cerevisiae* Sir2 and human SirT1. Additionally, nicotinamide inhibits yeast transcriptional silencing, increases rRNA gene recombination and reduces

yeast life span significantly when used at the physiological concentration suggesting nicotinamide is a regulator of Sir2 enzymatic activity (Bitterman *et al.*, 2002).

Nicotinamide has been widely used against pellagra. It also shows healing effects against osteoarthritis and inflammation (Jonas *et al.*, 1996). The antimicrobial effect of nicotinamide is documented ranging from *Mycobacterium tuberculosis* to HIV (Murray, 2003). More recently, nicotinamide has been shown to contain antileishmanial activity *in vitro* (Sereno *et al.*, 2005). The above studies indicate that nicotinamide has the potential to be used against different diseases although the exact mechanism for disease prevention is not known yet.

We are interested in biochemical and functional analysis of this protein *in vitro* and *in vivo*. Using specific polyclonal antibodies against PfSir2, here we show the presence of a specific band (*c.* 30 kDa) in the parasite lysate following Western blot experiments corresponding to the estimated molecular mass of PfSir2. Recombinant PfSir2 shows NAD^+-dependent HDAC activity that can be inhibited by nicotinamide but not by NAc. These results confirm the recently published data on biochemical activities of PfSir2 (Merrick & Duraisingh, 2007). Finally, we show that nicotinamide delays parasite growth significantly when added directly to the culture. These findings reconfirm the presence of a Sir2-like protein in the parasites and raise the possibility of using nicotinamide as an antimalarial along with other drugs amidst widespread drug-resistance in malaria.

Materials and methods

Parasite culture and synchronization

Plasmodium falciparum strain 3D7 was cultured in human erythrocytes essentially following the method described elsewhere (Trager & Jensen, 1976). Synchronization of parasites was done by the 5% sorbitol method (Lambros & Vanderberg, 1979).

DNA manipulations

Plasmodium falciparum Sir2 ORF (PF13_0152) was amplified by PCR using *P. falciparum* (strain 3D7) genomic DNA as template and PfSir2Fw and PfSir2Rv primer sets. For protein purification, full length (822 bp) PfSir2 was cloned in expression vector $pGEX_{6p2}$ (Novagen) at the BamHI restriction site and sequenced subsequently.

PfSir2 BamHI Fw

5′-CGGGATCCATGGGTAATTTAATGATTTCCTTTTTG-3′

PfSir2 BamHI Rv

5′-CGGGATCCCTACATTATTTTCTTATTTTTTTCAC-3′

Protein purification and raising polyclonal antibodies

Escherichia coli strain BL21 Codon plus were transformed with $pGEX_{6p2}$-PfSir2 recombinant plasmid. One liter of Luria–Bertani (LB) was inoculated with overnight culture and incubated at 37 °C until $A_{600\,nm}$ reached 0.5–0.6. The culture was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside and kept at 22 °C for 8–10 h. The bacterial pellet was resuspended in 10 mL lysis buffer (1 × PBS, 10 mM dithiothreitol, 2 mM EDTA, 100 μM phenylmethanesulfonyl fluoride). The lysate was sonicated and incubated with 0.1% Triton X-100 at 4 °C for 30 min. Lysate was clarified by high-speed centrifugation. The soluble fraction was incubated with pre-equilibrated Glutathione Sepharose 4B beads (Amersham Biosciences) at 4 °C for 1 h. Beads were washed with lysis buffer containing 300 mM NaCl. Protein was eluted with elution buffer (50 mM Tris-Cl, pH 8.0, 10 mM dithiothreitol, 20 mM reduced glutathione, 0.1% NP-40, 10% Glycerol, 100 mM NaCl) at 4 °C for 1 h. Purified protein was dialyzed and stored at − 80 °C.

To raise polyclonal antibodies against PfSir2, purified protein was mixed with Freund's adjuvant (Sigma) and the mixture was used to immunize multiple mice as described elsewhere (Gupta *et al.*, 2006).

Immunodetection and immunolocalization

For immunodetection, parasite pellets containing ring, trophozoites and schizont stages obtained by saponin lysis were washed with 1 × PBS and then boiled with 1 × sodium dodecylsulfate (SDS) gel loading buffer and loaded on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Western blot analysis was done using affinity purified anti-PfSir2 antibodies (1 : 5000) using the standard protocol.

For *in vivo* localization studies, immunofluorescence analysis was performed in different asexual stages of the parasite essentially following the protocol described elsewhere (Mehra *et al.*, 2005). Affinity purified anti-PfSir2 and preimmune sera were used at the dilution of 1 : 2000 and secondary antibodies (Alexa Fluor 594; goat anti-mice IgG from Molecular Probes) were used at the dilution of 1 : 1000.

HDAC assay

HDAC assay was carried out using the Fluorimetric Drug discovery kit-AK-555 (Biomol) as per the manufacturer's instructions. Briefly, deacetylase reaction was carried out in a 50 μL reaction mixture containing 25 μM Fluor de Lys as fluorogenic acetylated peptide substrate, *c.* 1 μg recombinant PfSir2 protein, 50–500 μM NAD^+ and Sirtuin assay

buffer. Two millimolar nicotinamide or NAc was added in the reaction as inhibitors. Reaction mixture was incubated at 37 °C for 10 min followed by the addition of Fluor de Lys developer. Deacetylation-dependent fluorescent signal was allowed to develop for 1 h. Fluorescence was measured with a Fluoremetric plate reader Infinite M200 (Tecan) (excitation 360 nm, emission 460 nm, gain = 85). The assay was carried out in triplicate.

Nicotinamide inhibition assay

Synchronized late ring-stage parasite culture (1–2% parasitemia) was incubated for 80 h in the presence of three different concentrations of nicotinamide (5, 10 and 20 mM) or NAc (20 mM). Complete RPMI media supplemented with nicotinamide or NAc were changed after every 24 h. Samples were taken out from treated and untreated flasks at 8 h intervals and thin slides were made for Giemsa staining.

Results and discussion

PLASMODB.ORG shows the presence of two putative homologs of Sir2 family of proteins in *P. falciparum*. PF14_0489 codes for an ORF (*c.* 1304 amino acid residues) containing a putative HDAC domain. PF13_0152 codes for a smaller protein (*c.* 273 amino acid residues) that contains a conserved core domain present in *S. cerevisiae* Sir2 (Fig. 1a). The deletion of the smaller gene coding for the putative Sir2 homolog resulted in the activation of *var* genes that were originally silenced in the wild-type background (Duraisingh *et al.*, 2005). Another related study failed to detect the endogenous Sir2 protein corresponding to *c.* 30 kDa using two different sets of polyclonal antibodies (Freitas-Junior *et al.*, 2005). Although these studies collectively show the importance of PfSir2 in *var* gene regulation, the failure to detect the endogenous PfSir2 protein raises issues related to antibody specificity.

We first amplified the ORF of PfSir2 using *P. falciparum* genomic DNA and specific primers as shown in the 'Materials and methods', followed by cloning the amplified DNA product in $pGEX_{6p2}$ vector. The GST-PfSir2 fusion protein was expressed and purified from *E. coli* strain BL21 (codon plus). Initially, the protein was found completely in the inclusion bodies. However, expression of the protein at low temperature (22 °C) allowed us to purify GST-PfSir2 protein in soluble form as shown in Fig. 1b (left panel). The purified protein was injected into mice for immunization as discussed in the 'Materials and methods'. Polyclonal antibodies were further immuno-affinity purified against purified antigens and checked for specificity. These affinity-purified antibodies cross-reacted with GST-PfSir2 protein specifically because unrelated protein HpDnaB did not cross-react with these antibodies, as shown in Fig. 1b (middle panel). The right panel shows the presence of the proteins on the Coomassie-stained gel following transfer of the proteins on polyvinylidene difluoride membrane.

In order to investigate whether these antibodies can recognize the endogenous PfSir2 protein, we performed Western blot analysis using parasite lysate obtained from trophozoite stage parasites. An equivalent amount of lysate from uninfected RBC was also loaded as a negative control. Western blot analysis reveals the presence of a distinct band of *c.* 30 kDa molecular mass only in the lane containing lysate from the parasites. No cross-reacting band was found in the uninfected RBC lane (Fig. 1c, left panel). Additionally, preimmune sera fail to recognize any band under the same experimental conditions, suggesting the specificity of these antibodies (Fig. 1c, middle panel). These results clearly indicate that PfSir2 is indeed expressed in *P. falciparum*.

To investigate the localization of this protein in a stage-specific manner, we performed immunofluorescence experiments using anti-PfSir2 immuno-affinity purified antibodies against thin smears on glass slides prepared from synchronized parasites collected from ring, trophozoite and schizont stages. ALEXA Fluor 594 secondary antibodies were used for immunolocalization and the fluorescence was monitored using a Nikon fluorescence microscope. Immunolocalization data reveals that PfSir2 is mostly expressed during trophozoite and schizont stage parasites. These data match the expression profile of PfSir2 at the mRNA level as shown in PLASMODB.ORG (transcriptome data). During the trophozoite stage, PfSir2 shows typical polarized signal at the end of the nucleus (Fig. 1d). We also found discrete foci of PfSir2 around the nucleus during trophozoite stage (data not shown). The punctate staining pattern of PfSir2 was more visible and clear during the late schizont stage. Preimmune sera under the same experimental conditions failed to detect any signal. This pattern of polar and discrete foci is similar to the pattern found in *S. cerevisiae* (Gotta *et al.*, 1997).

We also performed Western blot analysis using anti-PfSir2 polyclonal antibodies against parasite lysate from ring, trophozoite and schizont stages. The expression of PfSir2 was greatly enhanced during trophozoite and schizont stages compared with the ring stage parasites (Fig. 1e, top panel). The Coomassie-stained gel following protein transfer on the membrane is shown as loading control (bottom panel). The same blot was also probed with conserved anti-human actin antisera that recognized a band (*c.* 45 kDa) corresponding to PfActin confirming the presence of proteins in all three lanes (middle panel).

In order to explore the HDAC activity of recombinant PfSir2, we performed a novel class III HDAC assay that produces fluorescent signal following deacetylation of acetylated substrate as described in the 'Materials and methods'. Incubation of GST-PfSir2 with acetylated substrate in the

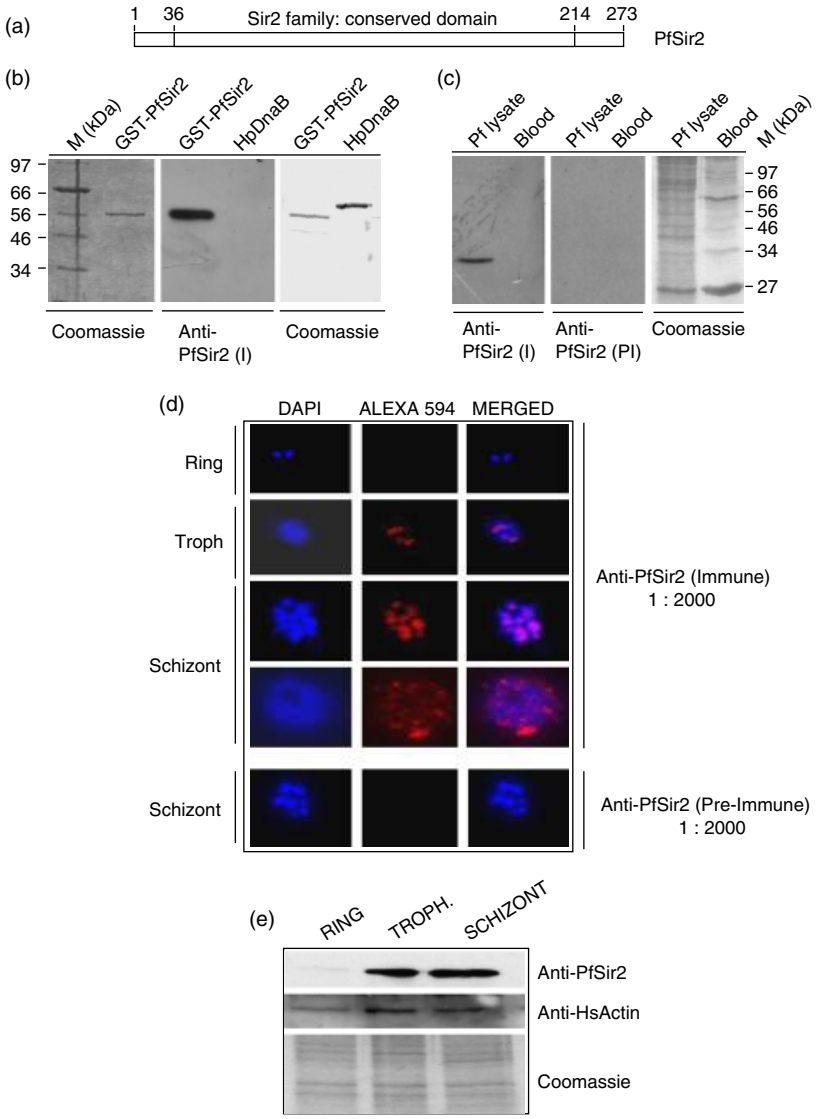

Fig. 1. (a) Schematic diagram of PfSir2. Conserved domain present in Sir2 family of proteins is indicated with amino acid co-ordinates. (b) Purification of GST-PfSir2 protein and specificity of anti-PfSir2 antibodies. The left panel shows the Coomassie stained gel showing the purified GST-PfSir2 protein as described in the 'Materials and methods'. The molecular mass marker is also shown. The middle panel shows the Western blot analysis of purified GST-PfSir2 protein and control HpDnaB protein using affinity purified anti-PfSir2 antibodies. These antibodies detected specifically PfSir2 only. The right panel shows the Coomassie stained gel following protein transfer to show the presence of both the proteins. (c) 3D7 parasites enriched in late trophozoite stages were boiled in the SDS-PAGE loading buffer followed by SDS-PAGE analysis of the lysate and western blot analysis using either preimmune or immune anti-PfSir2 sera. An equivalent amount of lysate from uninfected RBC was also loaded as control. A band of *c.* 30 kDa was detected only in the *Plasmodium* lysate containing lane and not in the RBC lysate containing lane (left panel) using immune sera (I). Preimmune sera (PI) did not cross-react with any band (middle panel) and the right panel shows the Coomassie stained gel following transfer to show the presence of proteins in each lane. (d) Immunofluorescence assay. Glass slides containing thin smears of *Plasmodium falciparum* infected erythrocytes from the different erythrocytic stages (as mentioned on the left of the different panels) were incubated with either preimmune or affinity purified PfSir2 antibodies (1 : 2000 dilution) followed by red ALEXA Fluor 594 fluor conjugated goat anti-mouse secondary antibodies. The slides were washed extensively with PBS buffer and scanned under a Nikon fluorescence microscope for the detection of fluorescence. The presence of PfSir2 was detected in trophozoite- and schizont-stage parasites whereas no signal was detected in the ring stage parasites. As a control, preimmune sera failed to detect any signal in the schizont-stage parasites. Diamidino-2-phenylindole indicates the nuclear staining in each case. (e) Western blot analysis. Equal amount of parasite lysate from different stages were loaded on SDS-PAGE followed by Western blot analysis using anti-PfSir2 antibodies (top panel). The middle panel shows the western blot analysis of the same blot using anti-human actin (HsActin) antibodies. The bottom panel shows the Coomassie-stained gel following protein transfer on the membrane (loading control).

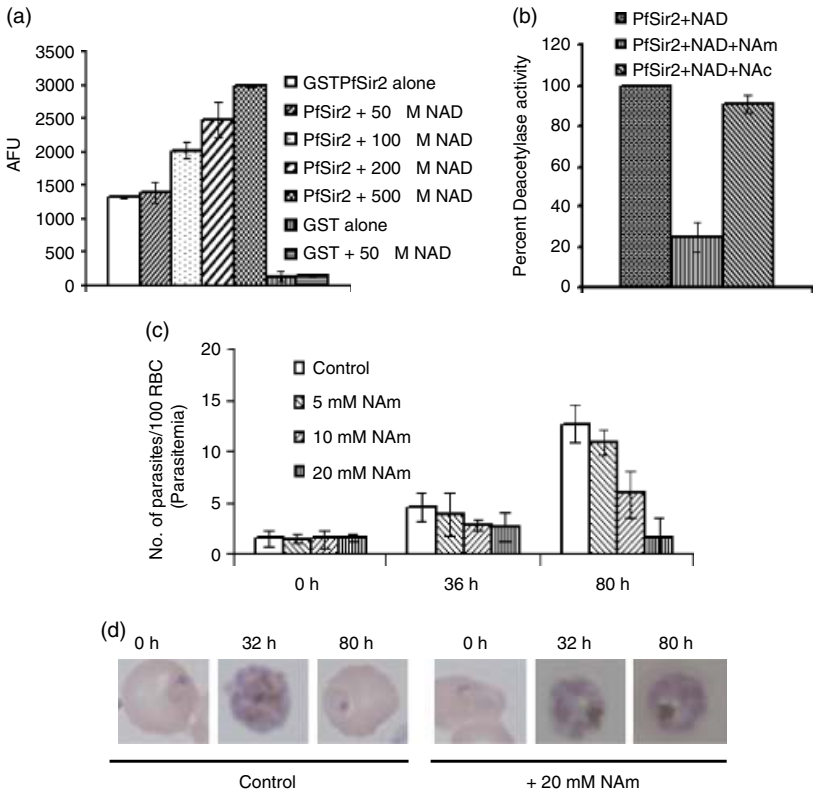

Fig. 2. (a) NAD^+-independent and -dependent HDAC activity of PfSir2. GST-PfSir2 protein (*c.* 1 μg) was incubated in the absence or the presence of different concentrations of NAD^+ (as indicated) and processed for HDAC activity as mentioned in 'Materials and methods'. Each reaction was performed in triplicate. The arbitrary fluorescence units obtained from each reaction were plotted accordingly. As a control, GST protein was also used in the absence and the presence of NAD^+. (b) NAD^+-dependent HDAC activity was performed in the absence or the presence of 2 mM nicotinamide and 2 mM NAc as indicated. The relative HDAC activity was plotted accordingly. (c) Effect of nicotinamide and NAc on parasitic growth. Synchronized ring-stage parasite culture was incubated in the absence or the presence of different concentrations of nicotinamide and NAc as indicated and parasitemia was calculated at different time points as indicated and plotted. (d) Giemsa staining of the thin smears of parasites at different time points following nicotinamide treatment along with untreated parasites to follow the parasite growth progression.

absence of NAD^+ gives a strong fluorescent signal compared with the GST alone signal (Fig. 2a). These results suggest that GST-PfSir2 contains intrinsic deacetylase activity. Interestingly, we find that the deacetylase activity of PfSir2 is also NAD^+ stimulated because an increasing amount of NAD^+ further increases the deacetylase activity. The control equimolar GST protein does not show any stimulation of deacetylase activity (Fig. 2a). Nicotinamide has been reported to inhibit both yeast Sir2 and human SIRT1 deacetylase activity (Bitterman *et al.*, 2002). A range of nicotinamide concentration was used initially for inhibition studies of NAD^+-dependent deacetylase activity of PfSir2 (data not shown). Surprisingly, we find that PfSir2 activity is drastically reduced at 2 mM concentration whereas NAc shows almost no effect on PfSir2 activity under the same experimental conditions, suggesting specific inhibition of PfSir2 using nicotinamide (Fig. 2b). This is consistent with the recently published report where maximum inhibition of recombinant PfSir2 is shown at higher concentration (*c.* 5–10 mM) (Merrick & Duraisingh, 2007).

As nicotinamide shows significant antileishmanial activity *in vitro* (Sereno *et al.*, 2005), we were interested to see the effect of nicotinamide in *P. falciparum* culture. Synchronized late ring stage parasite culture (*c.* 16–18 h) was treated with an increasing concentration of nicotinamide and incubated for 80 h continuously (with change of media every 24 h) and parasite growth was monitored at different time points. We find that 5 mM nicotinamide does not show much effect on parasite growth whereas 10 and 20 mM nicotinamide delay the parasitic growth significantly in a dose-dependent manner (Fig. 2c). The effect of nicotinamide on parasitemia is minimal during the first developmental cycle (*c.* 36 h) post-drug treatment whereas this effect becomes very prominent during the second developmental cycle (*c.* 80 h). The parasitemia of 20 mM nicotinamide treated culture (*c.* 2%) is reduced drastically after 80 h compared with the untreated culture (*c.* 13%) under the same experimental conditions. The remaining parasites in 20 mM nicotinamide-treated culture eventually stopped growing (data not shown). In contrast, NAc does not show similar growth inhibitory effect in *Plasmodium* culture under the same experimental conditions (data not shown). These results suggest that although nicotinamide and NAc are products of Sir2 dependent enzymatic pathways, nicotinamide shows unique inhibitory activity on parasite growth. The 50% inhibitory concentration of nicotinamide (IC_{50}) was found to be 9.9 ($\pm$ 1.25) mM. The dramatic effect of nicotinamide on parasitemia prompted us to examine the progression of the parasite growth at different time points by Giemsa staining of the blood smears. The average number of parasites at different developmental stages (ring, trophozoite and schizont) following drug treatment are shown in Table 1. These results

Table 1. Number of parasites at different stages (%) at different time points and parasitemia (%, post 80 h) following nicotinamide treatment

Time posttreatment (h)	0 h (*c.* 16–18 h postinvasion)			36 h (*c.* 52–54 h postinvasion)			80 h (*c.* 96–98 h postinvasion)			Parasitemia
Parasite stage (%)	Ring	Troph	Schizont	Ring	Troph	Schizont	Ring	Troph	Schizont	(post 80 h)
Control	83.3 (±3.84)	16.7 (±4.44)	0	94.7 (±3.2)	0	5.3 (±0.89)	89.4 (±1.89)	1.6 (±2.1)	9 (±1.48)	12.96 (±1.89)
5 mM nicotinamide	81.9 (±2.89)	18.1 (±2.09)	0	94.8 (±7.98)	0	5.2 (±4.67)	84.8 (±14.7)	1.8 (±1.5)	13.4 (±8.1)	11.1 (±1.32)
10 mM nicotinamide	80.4 (±1.9)	19.6 (±2.09)	0	77.14 (±11.56)	8.36 (±9.4)	14.5 (±8.7)	78.8 (±15.1)	7.4 (±3.18)	13.4 (±7.77)	6.06 (±2.4)
20 mM nicotinamide	84.8 (±3.4)	15.2 (±3.1)	0	58.85 (±17.2)	15.96 (±10.1)	25.19 (±4.87)	19.7 (±4.95)	15.4 (±8.9)	64.9 (±16.1)	1.85 (±1.9)

Values in parentheses show SD from three independent experiments.

reveal that the development of the parasites from ring to trophozoite and schizont is greatly affected in the presence of 20 mM nicotinamide compared with the control untreated parasites. This effect is more drastic during the second cycle of the development. The analysis of Giemsa-stained parasites following 20 mM nicotinamide treatment revealed two important features. Firstly, the average number of nuclei in the affected schizonts was smaller (*c.* 8–10) from nicotinamide treated parasites compared with untreated parasites (*c.* 16–32) (data not shown). Secondly, the nuclei were clumped together, suggesting difficulty in segregation of these nuclei during development in the nicotinamide-treated parasites (Fig. 2d). The combined effect of the smaller number of parasites and defect in maturation leading to the death of the parasites might explain the sharp decline in the parasitemia of nicotinamide-treated samples.

Nicotinamide causes delocalization of Sir2 in the yeast *S. cerevisiae* (Bitterman *et al.*, 2002). To find out whether treatment of nicotinamide in *P. falciparum* culture will result in redistribution of PfSir2, we performed immunofluorescence analysis using parasites from nicotinamide-treated culture, and compared the PfSir2 signal with untreated culture. Untreated parasites showed punctate staining of PfSir2 at the schizont stage whereas the majority of the affected schizonts from the 20 mM nicotinamide-treated culture showed diffused staining of PfSir2 (supplementary Fig. S1). These results indeed suggest that nicotinamide treatment causes redistribution of PfSir2 in the parasites.

Nicotinamide inhibits PfSir2 deacetylase activity at higher concentrations (mM level) than yeast Sir2 and human SIRT1 homolog (μM level) (Bitterman *et al.*, 2002). PfSir2 contains intrinsic deacetylase activity that can be stimulated in the presence of NAD^+. It is possible that the higher concentration of nicotinamide may be required for inhibition of NAD^+-independent activity of PfSir2. Alternatively, the lack of conservation between PfSir2 and yeast and human counterparts may be responsible for this difference.

What is the cellular target of nicotinamide? It has been proposed earlier that nicotinamide is a noncompetitive inhibitor of Sir2-like enzymes (Bitterman *et al.*, 2002). However, the deletion of PfSir2 does not show any effect on parasite growth. We can hypothesize that the other Sir-like protein present in *Plasmodium* (PF14_0489) will be essential for the survival of the parasite if these proteins are the targets for nicotinamide *in vivo*. Alternatively, some unidentified essential physiological functions could be the target for nicotinamide. These issues can be addressed using the PfSir2 knockout parasite line as reported earlier (Duraisingh *et al.*, 2005). The authenticity of the anti-PfSir2 antibodies can also be tested using this PfSir2 KO parasite line.

The reduced number of nuclei in the affected schizonts and their clumping nature suggest that nicotinamide may affect the segregation of nuclei during schizont stages. It has been suggested earlier that human SIRT2 protein might have a regulatory role in mitosis during the normal cell cycle (Inoue *et al.*, 2007). Because schizogony is comparable to mitosis in parasites, the role of PfSir2 in this process may get inhibited in the presence of nicotinamide.

Nicotinamide is very cheap, has low toxicity and can be introduced at a high dose (10 g day^{-1}). There are widespread incidences of chloroquine and other drug resistance in *P. falciparum* (Olliaro, 2001; Ridley, 2002). Although the IC_{50} of nicotinamide is very high (*c.* 10 mM), it might represent a useful anti-malarial combination drug.

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Authors' contribution

D.P., P.M. and S.S. contributed equally to this work.

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Supplementary material

The following material is available for this article online:

Fig. S1. Immunofluorescence assay. Nicotinamide causes redistribution of PfSir2 localization.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2008.01135.x (This link will take you to the article abstract).

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