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Parasite Killing in *Plasmodium vivax* Malaria by Nitric Oxide: Implication of Aspartic Protease Inhibition

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Nitric oxide (NO) is known to possess antiparasitic activity towards *Plasmodium* species. Parasite proteases are currently considered to be promising targets for antimalarial chemotherapy. In the present study, we have studied the inhibitory effect of NO on the activity of plasmepsin in *Plasmodium vivax*, the pepsin-like aspartic protease which is believed to be involved in the cleavage during hemoglobin degradation in *Plasmodium falciparum*. NO donors (±) (*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3), *S*-nitrosoglutathione (GSNO), and sodium nitroprusside (SNP) were found to inhibit this plasmepsin activity in a dose-dependent manner in purified *P. vivax* aspartic protease enzyme extracts. This inhibitory effect may be attributable to the nitrosylation of the cysteine residue at the catalytic site. However, an inhibitor of aspartic protease activity, namely pepstatin, was also found to inhibit (IC_{50} 3 μM) the enzyme activity, which we have used as a positive control. Our results therefore provide novel insights into the pathophysiological mechanisms, and will be useful for designing strategies for selectively upregulating NO production in *P. vivax* infections for antimalarial chemotherapy and also biochemical adaptations of the malaria parasite for survival in the host erythrocytes with a better understanding of the protease substrate interactions.

Key words: aspartic protease, malaria, nitric oxide, plasmepsin, *Plasmodium vivax*.

Abbreviations: plasmepsin, *P. falciparum* and *P. vivax* pepsin–like aspartic protease activity; NO, nitric oxide; GSH, glutathione; GSNO, *S*-nitrosoglutathione; NOR-3, (+)-(*E*)-4-ethyl-2-[(*E*)-hydroxyiminol-5-nitro-3-hexenamide; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; DTT, dithiothretol.

Nitric oxide (NO) has been determined to possess antiparasitic activity (*1*). NO has been shown *in vitro* to be toxic for the erythrocytic stage of P. berghei (*2*). In this respect , cytokines and reactive nitrogen intermediates (RNI) play a central role in malaria immune responses and pathogenesis, and cell-mediated killing of the malaria parasites (*3*). NO might also play an important role as an effector molecule in the development of resistance in mice (*4*, *5*). Excess release of mediators, namely tumor necrosis factor, interleukin 1 and NO, is believed to be correlated with the pathogenesis of severe malaria, however, their association is not yet well established. An inverse relationship has been observed between malaria severity and NO synthase type–2 induced NO production (*6*). More recently, in our studies we have shown that cytokines induce the production of NO in monocytes and may contribute to the pathology of host cells (*7*). However, the exact role of NO in the blood stages of malaria is unknown and the inhibition kinetics/mechanism of NO in the killing of malaria parasites is still a matter of debate .

During the erythrocytic stages of their life cycle, malaria parasites degrade hemoglobin as a major source of amino acids for protein synthesis. This process includes the transport of hemoglobin from the erythrocyte cytoplasm to the parasite acidic digestive vacuole, the precipitation of heme and the protease-catalysed hydrolysis of hemoglobin into small peptides. Finally, the exopeptidase activity of the malaria parasite converts the small peptides into individual amino acids for its growth and maturation (*8*, *9*). The malaria parasite thus produces a large number of proteases. These proteases are essential for the parasite's survival since they play important roles in processes such as host cell invasion, nutrition and growth, processing of precursor proteins, *etc*. (*10*). Specific protease inhibitors interfere with the protease functions and also affect normal parasite growth *in vitro* (*11*). These proteases are thus promising targets for antimalarial chemotherapy as inhibition of the *P. falciparum* proteases blocked parasite development *in vitro* (*12*) and also inhibition of the *P. vinckei* proteases cured murine malaria infection *in vivo* (*11*). Not much work has been done on the proteases of *P. vivax*.

Aspartic proteases perform a number of functions in a wide range of organisms, *e.g*. invasion, replication, antigen processing, hormone generation and cancer in humans (*13*). In Plasmodium species, aspartic proteases are suggested to be involved in the release of merozoites, the invasion of erythrocytes, and the digestion of haemoglobin (*14*). Aspartic protease inhibitors are also under study as potential candidate antimalarials in *P. falciparum* (*15*). Effective antimalarial protease inhibitors should ideally inhibit parasite proteases but not analogous host proteases. In this regard, studies on aspartic proteases inhibitors have demonstrated specificity

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Table 1. **Percentages of total recovered glutamate dehydrogenase and catalase in isolated *P. vivax* parasites.**

Enzyme	% of recovered parasites [parasite/(parasite + red cell supernatant) × 100]
Protein	5.62 ± 1.60
Glutamate dehydrogenase	78.30 ± 10.82
Catalase	0.97 ± 0.32

Values are mean ± SD.

between inhibition of plasmepsins I and II (*15*, *16*) and between these enzymes and cathepsin D (*14*). Cysteine protease inhibitors including NO (*17*) have not been rigorously evaluated as to specificity, but it is encouraging that a number of amino acids predicted to surround the active site of filliping are conserved in homologoues from nine other plasmodia species, but are not conserved in the host cysteine protease (*18*).

In the present study, we examined the abilities of NO donors and NO producers to inhibit in a dose-dependent manner the plasmepsin activity in purified *P. vivax* extracts. Our results provide new insights into the regulation of NO production in *P. vivax* malaria and also the mechanism of killing of malaria parasites via inhibition of protease activities, and may help us design novel strategies for selectively upregulating NO production for the inhibition of *P. vivax* malaria.

MATERIALS AND METHODS

The following reagents, GSH, leupeptin, pepstatin, NOR-3, SIN-1, SNP and DTT, were purchased from Sigma Chemical Co. (St. Louis, MO). NO-deprived NOR-3 (NOR-3*) was obtained by incubating a NOR-3 solution at alkaline pH and 25°C for 72 h (*19*). GNSO was prepared (*20*) by mixing equimolar concentrations of an aqueous solution of $NaNO_2$ and a freshly prepared GSH solution in 2.5 $\times 10^{-1}$ M HCl and 1.0×10^{-4} M EDTA (pH 1.5). The resulting mixture was incubated at 25°C for 5 min and then neutralized with NaOH. The GNSO solution was stored at 20°C. All the other products were obtained from BDH Chemicals. All chemicals used were of analytical grade.

Preparation of Samples—Blood samples were obtained from patients (with informed consent) from different geographical regions of India. Then they were screened microscopically for the presence of malaria (*P. vivax* + *ve*) parasites. Age- and sex-matched control samples were obtained from the same areas. Blood samples from *P. vivax*–infected patients (0.05–0.5 % parasitaemia) of either sex were collected in heparinized tubes. Blood was centrifuged at 1,500 × *g* for 10 min to separate the plasma and buffy coat, or the pellet was diluted with PBS and overlaid on Ficoll-paque for the removal of WBC, followed by centrifugation for 10 min in the cold at 1,500 × *g*.

Separation of Parasitized Red Cells on a Percoll Density Gradient—Percoll was adjusted to iso-osmolarity with 1.5 M PBS, pH 7.2 (9 parts Percoll: 1 part PBS). Different concentrations, 30, 45, 50 and 65%, were prepared. The cell suspension was overlaid on 30% Percoll, followed by centrifugation at 1,500 × *g* for 15 min in the cold. The top layer was then aspirated off and washed twice with complete medium to obtain a 10% suspension. This suspension was overlaid on a 45, 50 and 65% Percoll gradient. The top and bottom layers were collected and checked for purity by microscopic examination (*21*). Parasitized red cells were washed three times with normal saline and then lysed with 5 mM phosphate buffer, pH 8.0. Thereafter the lysed suspension was washed and centrifuged at 1,500 × *g* for 30 min. The uppermost layer of the haemolysate was separated for enzymatic estimation. An erythrocyte lysate from normal patients was prepared like those of parasitized cells and served as a control. Isolated parasites were kept at –70°C for further biochemical analysis. The purity of the parasites was established as described previously (*22*). The parasites were isolated with little contamination by host red cell cytoplasmic materials.

The hemoglobin concentration was determined by means of the cyanomethemoglobin method (*23*). Glutamate dehydrogenase was measured by the method of Walter *et al*. (*24*). Most of the glutamate dehydrogenase activity, which is considered to be a marker of isolated parasites, was found to be concentrated in the isolated parasites. However, the activity of catalase was found to amount to 17% of that present in the host cell cytoplasm (Table 1). Protein was estimated by the method of Lowry *et al*. (*25*), using BSA as a standard.

Characterization of the Protease Activity—Gelatin PAGE: Polyacrylamide (12.5%) was copolymerized with 0.1% gelatin (300 Bloom). After SDS-PAGE, the gels were washed in 2.5% Triton X-100 detergent (two 30 min washes) to remove SDS. They were then incubated in glycine buffer (0.1 M glycine, 0.002 M cad, pH 7.0) at 37°C for 72 h to allow digestion of the gelatin by the proteinases before staining of the gel with Commassie blue. Aspartic protease activity (40 kDa) was electroeluted out of the gel and reconstituted in the sample buffer for further analysis.

Aspartic acid proteinase purification: Aspartic proteinase activity isolated from the extract of *P. vivax* parasites by electroelution was purified further by HPLC on a Biogel HT hydroxyapatite column using a Shimadzu 10A HPLC apparatus by size exclusion (*26*). The column was eluted with a sodium phosphate gradient, 0.01–0.35 M, at pH 6.8. The flow rate was 0.8 ml/min, and 0.8 ml fractions were collected and assayed for aspartic protease activity. A 50 μl aliquot of the HPLC peak was subjected to electrophoresis on a 12% SDS-PAGE gel under reducing conditions. The gel was developed with Coomassie blue. The resultant peak specific activity on purification by HPLC was 252-fold purified as to the starting material, the yield being 8%. This proteinase activity migrated as a single band corresponding to M_r 40,000 on SDS-PAGE and also on HPLC gel filtration chromatography (Fig. 1). Definitive assignment will however, require purification to homogeneity and also N-terminal

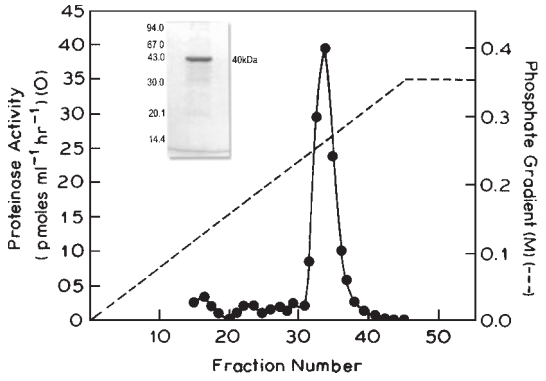

Fig. 1. **HPLC purification of aspartic protease activity on a Biogel HPHT column using a Shimadzu 10A apparatus.** The column was eluted with a sodium phosphate gradient, 0.01–0.35 M, at pH 6.8. The flow rate was 0.8ml/min, and 0.8 ml fractions were collected and assayed for aspartic protease activity as described under "MATERIALS AND METHODS." Inset: SDS-PAGE analysis. A 50 μl aliquot of the HPLC peak was subjected to electrophoresis on a 12 % SDS PAGE gel under reducing conditions. The gel was developed with Coomassie blue.

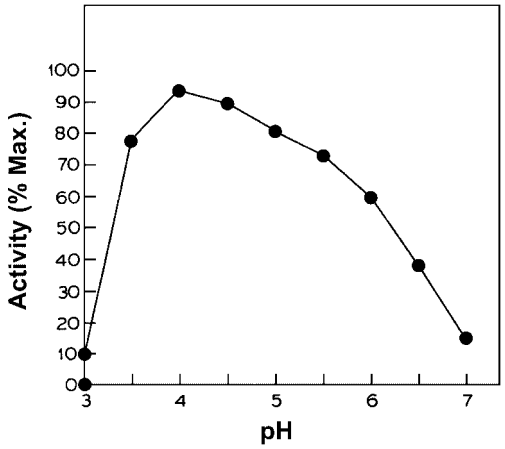

Fig. 2. **pH profile of the aspartic protease activity.**

sequencing to demonstrate that the protein shows homology with other aspartic proteinases.

Inhibitor studies: Aspartic proteinase inhibitor pepstatin (100 μg/ml) was incubated with samples for 60 min before the assay and before the addition of electrophoresis sample buffer. The inhibitor was also added to the glycine buffer in which the gels were incubated after electrophoresis. The inhibition of proteinase activity was examined by comparison with a control.

Protease assay: The protease activity of *P. vivax* parasites was measured by means of the casein/gelatin hydrolysis assay with a Quanti Cleave protease assay kit according to the instructions of the manufacturer (Pierce Co., Rockford, III). All these assays were performed on microtiter plates. Briefly, 100 μg of succinylated casein/gelatin in a 100 μl volume in 40 mM Tris HCl buffer, pH 7.4 (at a concentration of 1 mg/ml) was added to the wells in the left half of the plate, an equal (100 μl) amount of the buffer being added to the right half. A different amount of the sample (dissolved in 40 mM Tris-HCl buffer, pH 7.4) was added to each well. Equal amounts of samples were added to the blank buffer wells to subtract the background contributed by proteins present in the sample. The wells with buffer and substrate (succinylated casein/gelatin) served as a control. The samples were incubated at 37°C for 60 min and then 50 μl of (0.03%) trinitrobenzene sulfonic acid (TNBS) was added to each well, followed by incubation for 20 min at room temperature. The color development was measured at 450 nm.

In order to examine the effects of reducing agents on the aspartic protease activity present in the extracts of *P. vivax*, the aspartic protease was incubated with DTT at pH 5.5 at 25°C. The incubation time ranged from 20 s to 30 min, and the DTT concentration from 1.0×10^{-6} M to 1.0×10^{-3} M. The inactive protease activity obtained on treatment of aspartic protease with 1.0×10^{-4} M NOR-3 was also incubated with excess DTT and NOR-3 for 30 min at pH 4.0. Extracted *P. vivax* aspartic protease was also incubated simultaneously with DTT and NOR-3. The aspartic protease activity was determined as previously described.

The inhibitory effects of NO donors on the aspartic protease activity were determined by incubation of the extracted *P. vivax* protease activity with a reaction buffer of sodium acetate 0.1 M, pH 4.0, and an appropriate concentration of NOR-3, NOR-3*, GNSO, SIN-1, SNP, or pepstatin (ranging between 5.0×10^{-7} M and 1.0×10^{-3} M) for 30 min at 25°C. The protease activity was then determined as described above.

In Vitro Studies—Since it is not possible to culture *P. vivax in vitro* we examined the effects of these NO donors on the killing of *P. falciparum* parasites in culture. *P. falciparum* cultures were maintained in human O + ve erythrocytes at the hematocrit of 5% in a standard culture system, as previously described. The RPMI 1640 medium contained L-Asp, L-Glu, and L-Tyr. The assay used to measure parasite growth was performed as described elsewhere (*21*). Nitrate, nitrite, GNSO, SNP, *etc*. were tested *in vitro* studies. Parasite death was confirmed by examining smears made from these assay mixtures and IC_{50} values were calculated.

RESULTS AND DISCUSSION

Aspartic proteases are found in all eukaryotic organisms, where they are secreted and targeted to acidic organelles, and play important roles in protein degradation and protein processing (*9*). They usually exhibit an acidic pH optimum and are normally expressed as zymogens. The roles of aspartic proteases in the hemoglobin degradation process *in vivo* have not been fully examined, however, the mature naturally occurring enzymes from the malaria parasite have been reported to exhibit different globin cleavage specificities (*15*), and an inhibitor apparently specific for plasmepsins is capable of inhibiting parasite growth (*16*).

The present data indicate that aspartic protease (plasmepsin) activity purified from *P. vivax* extracts exhibited a pH profile for the degradation of haemoglobin over the pH range of 3–7 (Fig. 2). The activity was found to rapidly decrease above pH 4.5. The aspartic protease activity was inhibited by NO donors and reactive nitrogen intermediates, namely GNSO, NOR-3, SIN-1, and SNP (Figs. 3,

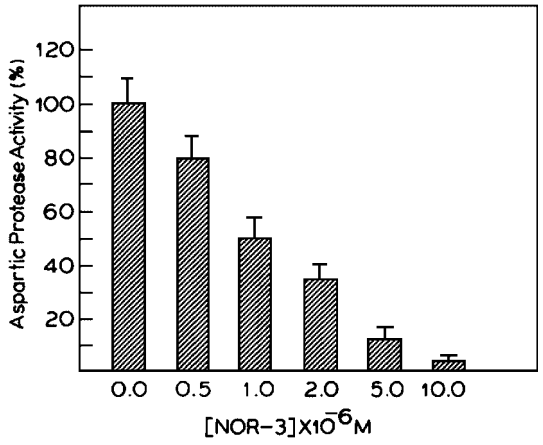

Fig. 3. **Effect of NO donor NOR-3 on the activity of *P. vivax* aspartic protease.** Dose-dependent inhibitory effect of NOR-3 on the enzyme activity. Each bar represents the mean + SEM for at least four experiments. All data were obtained at pH 4.0. For further details, see the text.

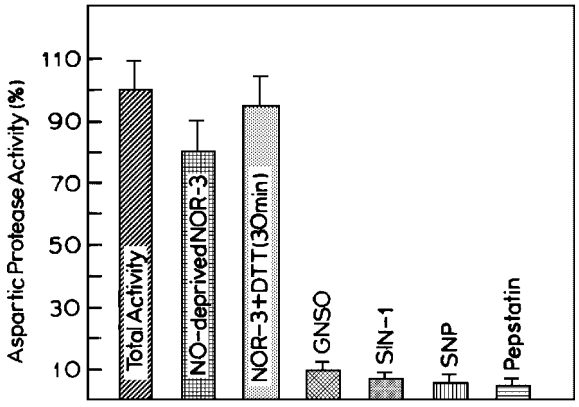

Fig. 4. **Effect of NO on the catalytic activity of *P. vivax* aspartic protease.** NO-deprived NOR-3 (NOR-3*; 1.0×10^{-5} M) does not affect the aspartic protease activity. The addition of DTT (1.0×10^{-3} M) to the inactive enzyme restores its activity. GNSO, SNP and SIN-1 block the aspartic protease activity. As a control, pepstatin, a typical aspartic protease inhibitor, supressess the aspartic protease activity. Each bar represents the mean + SEM for at least four experiments.

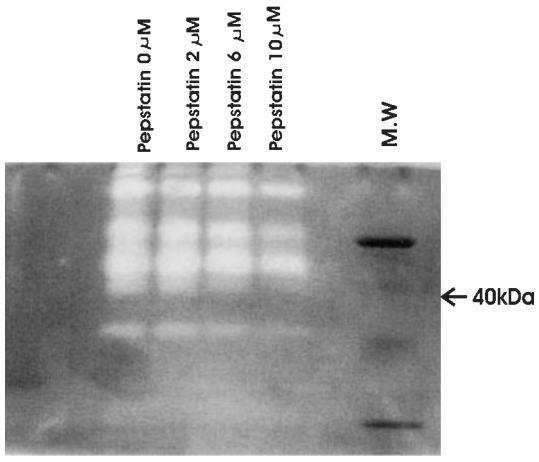

Fig. 5. **Zymogram (gelatin gel PAGE) showing the aspartic protease activity (40 kDa), and its inhibition by different concentrations of pepstatin (2, 6 and 10 μM).**

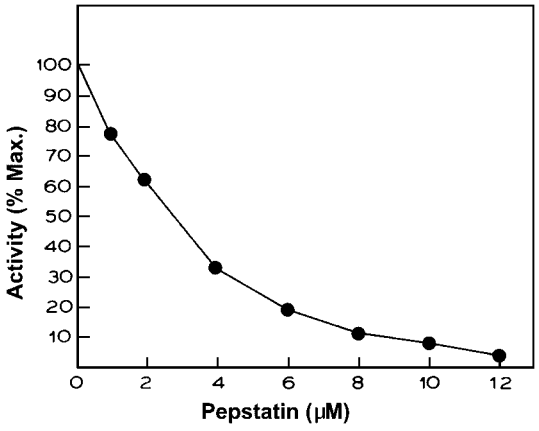

Fig. 6. **Inhibition of the protease activity by pepstatin.**

and 4). The purified *P. vivax* aspartic protease activity was found to be unaffected when the enzyme was incubated with NOR-3*(NO-deprived NOR-3). NOR-3 inhibited the aspartic protease activity in a dose-dependent manner (Fig. 3). When the NOR-3-treated inactive aspartic protease was incubated with DTT the activity was found to be restored (Fig. 4). Similarly, the activity was found to be unaffected when the enzyme was incubated simulataneously with NOR-3 and DTT, which shows that NOR-3 and DTT together prevent protease inhibition. Pepstatin, an inhibitor of aspartic protease activity, induces complete suppression of the aspartic protease activity which we have taken this as a positive control (Figs. 5 and 6), and it showed an IC_{50} value of 3.0 μM.

The generation of NO as a direct result of circulating cytokines may mediate the host pathology seen in malaria infections and in the production of RNI (*7*). The antiparasitic effects of the cytokines observed in different murine malaria infections may be mediated via the production of RNI (*27*), and may be due to the inhibition of protease activity. An effector molecules increase the production of RNI via different pathways, as inferred from *in vitro* studies (*28*). Thus, RNI production may be important for understanding the pathophysiology of *P. vivax* infections (*29*).

The catalytic activity of proteolytic enzymes is modulated by NO through binding to metal centers and also by chemical modification of the reactive residues (*30*). NO-mediated S-nitrosylation of the catalytic cysteine residues of cysteine proteases, including papain (*19*), caspases, cathepsins (*31*), and cysteine protease type-3, blocks the enzyme activity (*31*). The aspartyl protease activity of HIV-1 has been found to be inhibited via NO-mediated S- nitrosylation of the cysteine regulatory residues (*32*, *33*).

In our efforts to establish the role of NO compounds in malaria we have conducted experiments *in vitro* with *P.falciparum* in culture (Table 2). We have observed that the most potent compounds tested were *S*-nitrosoglutathione and S-nitrosocompounds, the reaction mixture for which requires 1,000 times less material on a molar basis than for either nitrate or nitrite. The parasiticidal effect

Table 2. **Killing of *P. falciparum in vitro* by reactive nitrogen intermediates.**

Reactive nitrogen intermediates (RNI)[a]	IC_{50} (μM)[b]	n[c]
$NaNO_3$	36,270 ± 7,240	5
$NaNO_2$	10,450 ± 3,490	5
Sodium nitroprusside	> 25,000	3
ON-S-Glutathione	41.80 ± 18.45	4
ON-S-Cysteine	38.20 ± 10.75	4

[a]Nitric oxide–releasing compounds: ON-S-, nitrosothiol group. [b]Mean ± SEM. [c]No. of experiments.

of these compounds is primarily because of their nitrosothiol contents (*34*), and they were found to be a thousand times more active (50% growth inhibitory concentration, approx. 40 μM) than nitrite. Once the nitrogen oxides have diffused into erythrocytes, nitrosothiol groups are formated on proteins, or more toxic chemical species such as peroxynitrites (*35*) or hydroxyl radicals, which could lead to inactivation of enzymes and thus changes in protein functions are produced. The constant generation of nitric oxide may be required for it to be parasiticidal because it reacts to crosslinked sulfhydral groups, therefore we are now conducting experiments to purify and express the nitric oxide synthase in order to test this hypothesis.

The present data thus indicate that the plasmepsins of *P. vivax* are inactivated by NO donors and also NO, probably through S-nitrosylation, this representing a novel approach for the inhibition of *P. vivax* infections. GSNO and nitroso-L-cysteine are known to kill *P. falciparum in vitro* (*3*), probably through the inhibition of a cysteine protease (falcipain). The concentrations of GNSO, NOR-3, SIN-1 and SNP used in the present study are consistent with the concentrations of GNSO and nitroso-L-cysteine used to kill malaria parasites (4.0×10^{-5} M). Since both cysteine proteases (*19*) and aspartic proteases (present study) are inhibited by NO donors and NO, it is postulated that the NO-releasing drugs which are used for the treatment of coronary artery diseases with limited toxicity appear to be potent useful tools in the therapeutic treatment of malaria.

NO has been shown to exhibit direct microbiocidal activity by interacting with enzymes, sulphydryl groups or superoxides (*1*, *36*). Moreover, it has also been shown that NO regulates IL2 and IFN-γ production in Th-1 cells, and might be important in the regulation of switching between Th-1 and Th-2 type immune responses (*37*). This can facilitate the design of strategies for upregulating NO-mediated S-nitrosylation of proteases in *P. vivax* infections, and for *P. falciparum* antimalarial chemotherapy through blocking of essential metabolic pathways for hemoglobin degradation by the parasite. The demonstration of the involvement of proteases and NO inhibitors of this activity could constitute a new approach for the treatment of malaria.

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