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NOTES

Killing of *Plasmodium falciparum* In Vitro by Nitric Oxide Derivatives

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We have investigated the in vitro susceptibility of the human malaria parasite *Plasmodium falciparum* to killing by nitric oxide and related molecules. A saturated solution of nitric oxide did not inhibit parasite growth, but two oxidation products of nitric oxide (nitrite and nitrate ions) were toxic to the parasite in millimolar concentrations. Nitrosothiol derivatives of cysteine and glutathione were found to be about a thousand times more active (50% growth inhibitory concentration, approximately 40 μM) than nitrite.

Since the discovery that mammalian cells produce nitric oxide (15), considerable attention has been focused on its role in the cell-mediated killing of microbes. Recently, macrophages (48), neutrophils (36), and mast cells (44) have all been shown to be major producers of this molecule. Its induction by tumor necrosis factor (TNF) (30), which in some indirect way inhibits *Plasmodium* sp. (10), focused our attention on the possibility that this parasite is also sensitive to nitric oxide and related molecules. These molecules, including the nitric oxide radical ($NO\cdot$) and its oxidized forms, nitrite (NO_2^-) and nitrate (NO_3^-), are termed reactive nitrogen intermediates (RNI) and are known to have antibacterial and antiviral activities in vitro (25, 34, 47). More recently, the triggering of leukocytes has been shown to cause intracellular arginine to be converted by the enzyme nitric oxide synthase (EC 1.14.23) (6) to citrulline and nitric oxide (35, 51). This reaction can be competitively inhibited by a number of arginine analogs, in particular, N^G-methyl-L-arginine (11). Pathogens reported to be inhibited by cell-derived RNI include herpes simplex virus (46), *Cryptococcus neoformans* (16, 17), *Leishmania major* (20, 21), *Schistosoma mansoni* (27), and *Plasmodium* liver stages (20, 21, 41). A role for RNI parasite killing in vivo has also been demonstrated for *L. major* (33) and *Toxoplasma gondii* (1).

There is recent evidence that *S*-nitrosothiol derivatives, such as *S*-nitrosocysteine and *S*-nitrosoglutathione, which may be formed in mammalian tissues, are more vasoactive than nitric oxide itself (40). They have also been shown to be toxic to bacteria (25, 39). In examining the toxicity of RNI for *Plasmodium falciparum*, we have accordingly extended the work to include these *S*-nitrosothiol compounds.

S-Nitrosothiol derivatives of cysteine (Sigma Chemical Co., St. Louis, Mo.) and glutathione (Sigma) were prepared by reacting the parent thiols with sodium nitrite (BDH, Kilsyth, Victoria, Australia) under acidic conditions. In brief, 1 mmol of thiol compound was dissolved in 1 ml of distilled water at 4°C, and 10 μl of 10 M HCl (1 mmol) was added with shaking. Solid $NaNO_2$ (BDH) (1 mmol) was added to the acidified thiol, and the mixture was shaken for 30 s. The formation of *S*-nitrosothiol was indicated by a pink-red color. The solution was then immediately frozen in liquid nitrogen, freeze-dried, and stored in a dessicator at −20°C. To assay these compounds for nitrosothiol, 5 mg of the dried product was dissolved in 0.5 ml of distilled water, and the concentration of nitrite/nitrate was measured by a modification of the method of Green et al. (19), while the method of Saville (45) was modified to measure nitrosothiols.

Dilutions of $NaNO_2$ (BDH), $NaNO_3$ (BDH), or test compounds were made in distilled water in 96-well, flat-bottom plates (NUNC, Roskilde, Denmark) to a final volume of 50 μl. Twenty microliters of NH_4Cl-borate buffer was added to all wells requiring analysis for nitric oxide/nitrite or *S*-nitrosothiol, and 20 μl of catalyst (see below) was added to wells to be tested for nitrate. After standing for 5 min at room temperature, the nitrate test samples were transferred to new wells, leaving the catalyst behind.

Fifty microliters of Griess reagent [1% sulfanilamide plus 0.1% *N*-(1-napthyl)ethylenediamine dihydrochloride (Sigma) in 2 M H_2SO_4] (22) was then added to wells to be analyzed for nitric oxide/nitrite or nitrate, while wells for *S*-nitrosothiol measurement received 50 μl of modified Griess reagent (100 ml of Griess reagent plus 2 mg of $HgCl_2$ [BDH]). The Hg^{2+} ions react with *S*-nitrosothiol groups, liberating nitrite (45). The plate was read at 540 nm (test) (reference, 630 nm) and nitric oxide and nitrite concentrations were read directly from a nitrite standard curve. Nitrate concentrations were calculated as $[OD_{(\text{with catalyst})} - OD_{(\text{without catalyst})}]$, where OD is the optical density, and compared with a nitrate standard curve. The *S*-nitrosothiol contribution to the OD was calculated as $[OD_{(\text{modified Griess})} - OD_{(\text{Griess})}]$ and compared with a nitrite standard curve.

The catalyst was prepared by a modification of the method of Davison and Woof (12). Powdered zinc (BDH; 1 g) was suspended in 100 ml of distilled water by stirring. A saturated solution of cadmium acetate (BDH) was added dropwise to the zinc until the suspension cleared. The precipitate was washed extensively in distilled water, added to 100 ml of 5% $CuSO_4$ (BDH) for 30 s, and then again washed extensively in distilled water. Finally, the catalyst was stored in a 5% NH_4Cl–borate buffer (BDH) brought to pH 8.5 with

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TABLE 1. Nitrite, nitrate, and *S*-nitrosothiol composition of *S*-nitrosocysteine and *S*-nitrosoglutathione preparations

Prepn	% Composition (mean ± SEM)[a]		
	Nitrite	Nitrate	Nitrosothiol
S-Nitrosocysteine	3.68 ± 1.87	3.95 ± 2.49	92.38 ± 4.37
S-Nitrosoglutathione	6.47 ± 2.44	2.09 ± 0.86	91.44 ± 3.12

[a] Values are taken from at least three experiments.

$Na_2B_4O_7$ (BDH) as previously described (19) at 4°C. The catalyst was always used within 1 week of preparation. Catalyst that was black rather than a metallic copper color invariably gave the best results.

Analyses of the nitrosothiol compounds are shown in Table 1. As indicated, >90% of the RNI in each mixture was *S*-nitrosothiol and the remaining 10% consisted of nitrite and nitrate. These results also demonstrated that the *S*-nitroso group was stable in the presence of the catalyst, and we have found that the nitrosothiols completely withstood several freeze-thaw cycles in solution (data not shown).

From the data in Table 1, it was possible to prepare solutions containing a precise molarity of each of the nitrosothiol compounds. Along with $NaNO_2$, $NaNO_3$, and nitric oxide, these were screened against *P. falciparum*. Nitric oxide was produced by reaction between $NaNO_2$ and acidified $FeSO_4$ solutions as previously described (4) and bubbled through phosphate-buffered saline (PBS) that had been purged with nitrogen. The nitric oxide content was measured at room temperature by using Griess reagent and was compared with a nitrite standard. Nitric oxide solutions exceeding 2 mM, indicating that the purging with nitrogen had not completely removed the dissolved oxygen, were discarded (a saturated solution of nitric oxide at room temperature has a maximum concentration of 2.06 mM [2]).

P. falciparum FC27 (28) was maintained in human O-positive erythrocytes at a hematocrit of 5% in a standard culture system (52), except that the RPMI 1640 medium (38) contained (milligrams per liter) L-Asp (56.82), L-Glu (59.15), and L-Tyr (24.86); $Ca(NO_3)_2$ was omitted. The assay used to measure parasite growth was performed as described elsewhere (43), except that all samples (apart from nitric oxide, which was prepared in PBS) were prepared in complete medium immediately before testing and sterilized by 0.22-μm-pore-size filtration. The concentrations (mean ± the standard error of the mean) of these compounds required to inhibit parasite growth by 50% are shown in Table 2. Parasite death was confirmed by examining smears made from these assays.

It was not possible to obtain a 50% growth inhibitory concentration (IC_{50}) for nitric oxide because this value is greater than the concentration of a saturated solution (2 mM [2]). It is possible to dissolve more than the predicted amount of nitric oxide in water (or other solutions), but this occurs only when nitric oxide has reacted with some component in the solution, usually oxygen (42). When nitric oxide reacts with oxygen, nitrite and/or nitrate are produced, and these are toxic to *P. falciparum* (Table 2). Therefore, constant generation of nitric oxide may be required for it to be parasiticidal, so at present we are purifying the NO synthase enzyme in order to test this hypothesis. Even in this case, the active parasiticidal species may be other RNI generated from nitric oxide (42).

TABLE 2. Killing of *P. falciparum* in vitro by RNI

RNI[a]	IC_{50}, μM (mean ± SEM)	n[b]
$NaNO_3$	34,150 ± 7,690	5
$NaNO_2$	11,030 ± 3,360	7
NO·	>20,000	3
ON–*S*-cysteine	39.10 ± 18.46	4
ON–*S*-glutathione	41.86 ± 10.77	7

[a] NO, nitric oxide; ON–*S*-, nitrosothiol group.
[b] Number of experiments.

The most potent compounds tested were *S*-nitrosocysteine and *S*-nitrosoglutathione, the reaction mixtures of which require 1,000 times less material on a molar basis than did either nitrite or nitrate. The parasiticidal effect of the mixtures was evidently due specifically to their nitrosothiol content since, at their respective IC_{50}s, the nitrite and nitrate contents were approximately 100 nM and 4 μM, far below the respective IC_{50}s for these ions. Also, the observed parasiticidal effect could not be attributed to unreacted cysteine or glutathione, as these had no effect on parasite growth at micromolar concentrations; indeed, they occasionally enhanced growth (data not shown).

RNI may react in several ways to cause cell death. Once the oxides of nitrogen have diffused into erythrocytes, the formation of nitrosothiol groups on proteins could lead to the inactivation of enzymes or changes in protein function. These groups can react further to cross-link sulfhydryl groups (37), one effect of which would be to oxidize glutathione and so reduce the parasite oxidant defense capacity. This process may create more nitrosothiol groups rather than form nitrite or nitrate (37) and thus initiate a chain reaction. In an erythrocyte, one target for RNI attack is oxyhemoglobin. Nitrite reacts with this molecule to form either nitrosothiolhemoglobin (54) or methemoglobin (53), and one product of the latter reaction is hydrogen peroxide, itself toxic to malaria parasites (9, 14). This property of RNI to reduce metal ions means that other metal-centered proteins would also be affected (24, 31, 50). Besides these oxidation-reduction reactions, RNI can form toxic alkylating agents by reacting with secondary amines (26).

A pharmacokinetic explanation could account for nitrosothiols being more toxic on a molar basis to *P. falciparum* than either nitrite or nitrate. Thus, the parasites might actively take up these cysteine and glutathione analogs, thereby concentrating the toxic nitroso group intracellularly, where the above reactions can occur.

Cytokines are involved in the induction of RNI synthesis (13, 49), and therefore these molecules may play a role in malaria in vivo. There is now general agreement that levels of TNF are increased in the sera of human malaria patients (7, 18, 29, 32), and we have recent evidence for raised lymphotoxin levels in the same circumstances (8a). Upon injection into malaria-primed mice, both TNF and lymphotoxin generated levels of RNI in plasma similar to those shown here (Table 2) to be toxic to cultured *P. falciparum* (42a). This implies that the increase in RNI caused by TNF could contribute to the antimalarial effects of TNF in vivo (10) and that lymphotoxin could have a similar action. The antiparasite effect may be a direct result of RNI alone as discussed above or RNI in synergy with oxygen radicals known to be toxic to malaria parasites (9, 14). Alternatively, nitric oxide may go on to form chemical species that are even more toxic, such as peroxynitrite (5, 23) or hydroxyl radicals (3). Since a number of cell types produce NO· , particularly endothelial cells, this mechanism could explain how P/J

mice, whose macrophages have poor oxidative capacity, can kill *Plasmodium chabaudi* as effectively as can normal mice (8).

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