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Anti-Plasmodial Activity of Aroylhydrazones and Thiosemicarbazones Iron Chelators: Effect on Erythrocyte Membrane Integrity, Parasite Development and the Intracellular Labile Iron Pool

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Abstract

Iron chelators inhibit the growth of the malaria parasite, *Plasmodium falciparum*, in culture and in animal and human studies. We previously reported the anti-plasmodial activity of the chelators, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311), 2-hydroxy-1-naphthylaldehyde 4-methyl-3-thiosemicarbazone (N4mT), and 2-hydroxy-1-naphthylaldehyde 4-phenyl-3-thiosemicarbazone (N4pT). In fact, these ligands showed greater growth inhibition of chloroquine-sensitive (3D7) and chloroquine-resistant (7G8) strains of *P. falciparum* in culture compared to desferrioxamine (DFO). The present study examined the effects of 311, N4mT and N4pT on erythrocyte membrane integrity and asexual parasite development. While the characteristic biconcave disk shape of the erythrocytes was unaffected, the chelators caused very slight hemolysis at IC₅₀ values that inhibited parasite growth. The chelators 311, N4mT and N4pT affected all stages of the intra-erythrocytic development cycle (IDC) of *P. falciparum* in culture. However, while these ligands primarily affected the ring-stage, DFO inhibited primarily trophozoite and schizont-stages. Ring, trophozoite and schizont-stages of the IDC were inhibited by significantly lower concentrations of 311, N4mT, and N4pT (IC₅₀ = 4.45 ± 1.70, 10.30 ± 4.40,

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and $3.64 \pm 2.00 \mu\text{M}$, respectively) than DFO ($\text{IC}_{50} = 23.43 \pm 3.40 \mu\text{M}$). Complexation of 311, N4mT and N4pT with iron reduced their anti-plasmodial activity. Estimation of the intracellular labile iron pool (LIP) in erythrocytes showed that the chelation efficacy of 311, N4mT and N4pT corresponded to their anti-plasmodial activity, suggesting that the LIP may be a potential source of non-heme iron for parasite metabolism within the erythrocyte. This study has implications for malaria chemotherapy that specifically disrupts parasite iron utilization.

Keywords

Aroylhydrazone; Thiosemicarbazone; Iron Chelator; *Plasmodium falciparum*; Erythrocytes

1. Introduction

The initiation of human malaria begins with the female *Anopheles* mosquito injecting sporozoites into the blood circulation during a blood meal [1]. These sporozoites migrate to the liver, pass through Kupffer cells and then actively invade hepatocytes. Each invading sporozoite differentiates and divides mitotically into thousands of liver merozoites that, when released, invade erythrocytes, thereby beginning the asexual lifecycle of *Plasmodium falciparum* [1]. The merozoites then mature asexually during the parasite's intra-erythrocytic development cycle (IDC) through the ring, trophozoite, and schizont-stages [2]. The complete cycle spans approximately 48 h [1,2].

Maturation of the parasite to the schizont-stage entails: (i) proteolytic degradation of the host hemoglobin; (ii) detoxification of the liberated heme to prevent damage to both parasite and host cell; (iii) reduction of the intra-erythrocytic hemoglobin concentration and consequently intra-erythrocytic colloid-osmotic pressure; (iv) initiation of biochemical reactions involving proteases that destabilize the erythrocyte cytoskeleton; and (v) eventual host-cell lysis. These events lead to the release of 15–32 non-motile merozoites into the circulation, which are capable of invading new erythrocytes to begin a new IDC [2–5]. The simultaneous appearance of merozoites every 48 h causes the fever seen in patients with clinical malaria [6, 7]. The ever increasing mortality and morbidity rate of *P. falciparum* malaria due to drug-resistance underscores the urgent need to develop effective, less expensive drugs that allow for the exploration of new therapeutic strategies against this disease.

Intra-erythrocyte development and growth of *P. falciparum* is dependent on iron and is repressed by iron chelators, as demonstrated by the anti-malarial activity of the clinically-used ligand, desferrioxamine (DFO; Fig. 1A) [8–10]. This finding prompted research into the anti-malarial activity of the lipophilic aroylhydrazone class of iron chelators, such as pyridoxal isonicotinoyl hydrazone (PIH; Fig. 1A), 2-hydroxy-1-naphthaldehyde *m*-fluorobenzoyl hydrazone (HNFBH) and salicylaldehyde isonicotinoyl hydrazone (SIH; Fig. 1A) [11, 12]. Aroylhydrazone chelators have been shown to be orally effective, economical to synthesize and markedly membrane-permeant, thus overcoming the many clinical disadvantages of DFO [13].

We previously showed that 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311; Fig. 1A), 2-hydroxy-1-naphthylaldehyde 4-methyl-3-thiosemicarbazone (N4mT; Fig. 1A), and 2-hydroxy-1-naphthylaldehyde 4-phenyl-3-thiosemicarbazone (N4pT; Fig. 1A), are effective inhibitors of the *in vitro* growth of chloroquine-sensitive 3D7 and chloroquine-resistant 7G8 strains of *P. falciparum* [14]. The chelators, 311, N4mT and N4pT, are Schiff base compounds formed between hydrazides or thiosemicarbazides and an aldehyde [15]. In comparison to the hexadentate iron chelator, DFO, the aroylhydrazone, 311, and

thiosemicarbazones, N4mT and N4pT, are tridentate chelators that strongly bind iron and possess high iron-chelation and anti-proliferative efficacies *in vitro* [13, 15–18].

The efficacy of iron chelators at inhibiting *P. falciparum* development and growth indicates the important role of iron in its life cycle [8–12, 14]. Indeed, iron is required for the activity of a number of plasmodial proteins, including the rate-limiting enzyme, ribonucleotide reductase, which catalyzes the *de novo* synthesis of deoxyribonucleotides that are required for DNA synthesis in the parasite [19, 20].

Since malaria parasites are cultured in human erythrocytes, the effect of anti-malarial drugs on the growth and proliferation of various stages of the parasite during the IDC could be due to direct effects within the *P. falciparum* cell, and/or to indirect effects elicited by drug interactions within the host erythrocyte or at the erythrocyte membrane [21–23]. As invasion and survival of *P. falciparum* depends on the normal functioning of the erythrocyte membrane [22], changes in its properties are likely to interfere with the IDC of the parasite. Ziegler and colleagues [23, 24] have shown that a number of amphiphiles that cause formation of stomatocytes (*i.e.*, erythrocytes with an elongated area of central pallor), but not echinocytes (*i.e.*, spiculated erythrocytes), inhibit growth of *P. falciparum*. In other studies, it was demonstrated that licochalcone A, a potent membrane-active agent, is incorporated into the erythrocyte membrane, transforming normal discoid parasitized and non-parasitized erythrocytes into echinocytes [24]. The erythrocyte shape changes caused by licochalcone A correlated with its apparent half-maximal inhibitory concentration (IC₅₀) value in inhibiting growth of *P. falciparum*, although no data were reported on cellular hemolysis [24].

In the present study, we designed experiments to determine the effect of 311, N4mT and N4pT on uninfected human erythrocyte morphology and membrane integrity (estimated by hemolysis) by incubating erythrocytes at concentrations similar to those used in the *in vitro* inhibition of parasite growth. We also examined the effect of these chelators on specific stages of *P. falciparum* development and growth during the IDC. The mechanism by which the chelators inhibit parasite development and growth was assessed after their complexation with iron and also by analyzing the labile iron pool (LIP) in erythrocytes. These studies indicate that the ring, trophozoite and schizont-stages of the parasite IDC are inhibited by 311, N4pT, and N4mT, at lower concentrations than DFO. The chelators did not alter red cell shape, but they did cause very slight hemolysis. Furthermore, results obtained from iron complexation experiments, and the use of the iron-sensing fluorescent probe, calcein, indicate that the ligands readily permeate erythrocytes to chelate intracellular labile iron.

2. Materials and Methods

2.1. Chemicals

Synthesis and characterization of PIH, 311, N4mT and N4pT were performed using standard techniques [15,16]. The chelator, 2,2'-bipyridine (BIP, >99% pure; Fig. 1A), was purchased from Acros Organics, Morris Plains, NJ. Anti-calcein antibody, which quenches calcein fluorescence, was a gift from Professor Z.I Cabantchik, Department of Biological Sciences, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel.

Salicylaldehyde isonicotinoyl hydrazone (SIH) was a gift from Professor Prem Ponka (Lady Davis Institute for Medical Research, Montréal, Canada). DFO was purchased from Novartis Pharma AG, Basel, Switzerland and DMSO was from Fisher Scientific, Norcross, GA. Calcein-acetoxymethyl ester (calcein-AM) was obtained from Molecular Probes, Eugene, OR. Other reagents used include: RPMI-1640 (Mediatech Cellgro, Alexandria, VA), human A+ serum (pooled from different A+ serum from non-immune donors; Interstate Blood Bank Inc., Memphis, TN), 2,8-[³H]-hypoxanthine (Amersham, Pharmacia

Biotech, Little Chalfont, England), D-sorbitol and scintillation fluid (FluorSafe; Fisher Scientific, Norcross, GA).

2.2. Cell culture

The chloroquine-sensitive strain of *P. falciparum* (3D7) used in these studies was provided by O. Muratova and D. Keister (National Institutes of Health, Bethesda, MD). Parasites were maintained as continuously growing cultures in washed human erythrocytes (A+) using culture medium containing RPMI-1640 supplemented with 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 23 mM NaHCO₃, 10 mM D-glucose and 10% (v/v) heat-inactivated human serum (A+), in accordance with the methods of Trager & Jensen [25]. Cultures were maintained at 3–5% parasitemia and 2% hematocrit in an atmosphere containing 90% N₂, 5% CO₂ and 5% O₂. Synchronization to the ring-stage (90% rings) was achieved by using 5% D-sorbitol treatment [26]. The gross morphological characteristics of the parasites were assessed by light microscopic examination of thin blood smears stained with Giemsa. The degree of parasitemia was examined by counting a representative field of 1000 cells and determining the percentage of cells parasitized. For the determination of IC₅₀ values, parasites were cultured at 2% parasitemia and 2% hematocrit. When required for drug assays, synchronized ring-stage cultures were used after suspension in RPMI-1640 supplemented with 10% heat-inactivated human serum (A+) and buffered to pH 7.4 with 25 mM HEPES 23 mM NaHCO₃ and 10 mM D-glucose.

2.3. Preparation of iron chelators

Aroylhydrazone and thiosemicarbazone chelators were dissolved in DMSO as 10 mM stock solutions [18] and diluted in culture medium, so that the final concentration of DMSO was 0.5% (v/v). In contrast, DFO was prepared in culture medium alone due to its high solubility. Stock solutions were then filtered through a Swinnex Millipore filter (pore size 0.2 µm) for sterilization. We demonstrated in previous experiments that DMSO at this low concentration had no effect on parasite growth [14]. All drug solutions were prepared fresh before an experiment and used immediately.

2.4. Effects of 311, N4mT and N4pT on erythrocyte shape

Non-parasitized human erythrocytes were incubated with 311, N4mT, or N4pT at the same molar concentrations used for *P. falciparum* culture following the methods of Ziegler *et al.* [24]. Control wells contained non-parasitized erythrocytes without the chelators in culture medium. Non-parasitized erythrocytes in wells containing 0.5% DMSO in culture medium served as negative controls. Briefly, various concentrations of the test compound were added to 600 µL of non-parasitized washed human erythrocytes (blood group A) at 3% hematocrit in parasite growth medium (see above) in Eppendorf tubes, then 200 µL of each suspension were pipetted into Falcon 96-well microtiter plates. Each chelator concentration was tested in duplicate wells. Cells were incubated in a Thermo Forma Model 370 Steri-Cycle Incubator at 37°C in a humidified atmosphere of 90% N₂, 5% CO₂ and 5% O₂. After a 48 h incubation period, the plates were placed on a shaking table for 1 min and 100 µL of each sample were then transferred into Eppendorf tubes and treated with 50 µL of 3% glutaraldehyde in phosphate buffer (pH 7.4) for 1 h. The cells were washed three times with sterile phosphate-buffered saline (PBS, pH 7.4; Biofluids, Biosource International, Inc., Camarillo, CA). A fraction (20 µL) of this sample was spread on microscope slides, allowed to dry, treated with methanol and stained with Giemsa for light-microscopic examination of erythrocyte shape [24]. Representative views of the cells under the microscope were photographed using different light filters to generate clear images.

2.5. Assessment of potential chelator-induced erythrocyte hemolysis

Assays were conducted according to the method of Pradines and colleagues [27] with modifications. Human erythrocytes were washed three times and resuspended in RPMI-1640 medium (without phenol red) supplemented with 10% human serum and buffered to pH 7.4 with 25 mM HEPES and 25 mM NaHCO₃ to a hematocrit of 2–2.5%. The erythrocyte suspensions were aliquoted at 200 µL/well into Falcon 96-well microtiter plates in triplicate. Final concentrations of 311, N4pT, and N4mT ranged from 0.5–3.0 µM. Erythrocytes incubated in RPMI-1640 medium (without phenol red) and without any of the chelators, but containing 0.5% DMSO, served as a negative control. For comparison, erythrocytes were also incubated with DFO or PIH (10–60 µM). Standard hemoglobin solutions (Fisher Scientific, Norcross, GA) were diluted with RPMI-1640 medium (without phenol red) and used to prepare the standard curve for the hemolysis assay.

The optical density of the standard hemoglobin solutions and the supernatants of the cell suspensions were measured using an automatic plate reader (PerkinElmer Victor2TM MultiLabel Counter, PerkinElmer, Inc., Shelton, CT) at a wavelength of 415 nm. These data were expressed as a percentage of the mean corpuscular hemoglobin concentration (MCHC) obtained after complete cellular lysis. As 10% human serum (A+) was used in the standard culture media for parasite growth [12, 14, 25, 28], we also assessed the hemoglobin concentration in the autologous plasma. Therefore, aliquots of the autologous plasma of the donor blood were also diluted with RPMI-1640 medium (without phenol red) to either 10% or 50% plasma, or used without dilution (100%). The rationale for using these plasma concentrations was to spectrophotometrically compare their heme-containing proteins. This was required, since 10% plasma is the concentration used in the culture medium for parasite growth. Additionally, 50% plasma and 100% (*i.e.*, undiluted) plasma were also included to assess the effect of an increase in heme-containing proteins at higher % plasma concentrations. The optical density of the diluted and undiluted plasma (100%) was measured at 415 nm for hemoglobin (*i.e.*, the absorption maximum of the hemoglobin Soret band). The results of the diluted and whole autologous plasma without any of the chelators, but containing 0.5% DMSO, served as an additional negative control. These results were compared to the supernatant optical density of the RPMI-DMSO control and the supernatants of cell suspensions incubated with chelators.

2.6. Assessment of chelator effect on stage-specific parasite growth

To evaluate the effects of the chelators on specific stages of parasite growth, synchronized (90% rings) parasite cultures [26] were incubated with 311, N4mT, N4pT (at 2.5–15 µM) or DFO (10–60 µM) in duplicate wells in 24-well plates. Plates were transferred to a candle jar and incubated at 37°C for 48 h [25]. Control cultures without the chelators were incubated concurrently under the same conditions. Giemsa-stained slides were prepared from the parasite cultures from the different chelator concentrations at 0, 24 and 48 h after drug incubation and examined using a light microscope. The sensitivity of parasite development to the chelators was determined by counting the number of rings, trophozoites and schizonts per 10³ erythrocytes at the different time intervals. Representative views of the parasite IDC under the microscope were photographed using different light filters for clear images.

2.7. Effect of chelators and their Fe^{III} complexes on parasite growth

To determine the role of iron chelation in the anti-plasmodial effects observed, we assessed the growth of parasites cultured with chelators pre-complexed with iron. The Fe^{III} complexes of the chelators were prepared by adding FeCl₃ to the ligands in a 1:1 metal:ligand equimolar ratio for the hexadentate chelator (DFO), and a 2:1 metal:ligand ratio for the tridentate chelators (311, N4mT and N4pT), using previous methods [29]. The

solutions were then mixed thoroughly and incubated for 1 h at 37°C before being added to parasite cultures. Complexation of the chelators with Fe³⁺ ions was monitored spectrophotometrically between 200 nm and 800 nm [30]. The effect of the chelators and their Fe^{III} complexes on parasite growth was examined by adding various concentrations to 600 µL of ring-stage synchronized parasitized (3D7) red cells (2.0% parasitemia and 2.0% hematocrit) in a 24-well plate. In all these studies, DFO served as a positive control, while negative-control wells contained only the vehicle (0.5% DMSO). After a 24 h/37°C incubation of the parasitized erythrocytes with the chelators or their Fe^{III} complexes, [³H]-hypoxanthine was added to the wells (1 µCi/well) and incubated for a further 24 h (48 h culture). The parasites were then harvested to determine [³H]-hypoxanthine incorporation. The half-maximal inhibitory concentration (IC₅₀) values were determined by analyzing these data implementing non-linear regression analysis of log-dose-response curves using GraphPad Prism® Analysis software (GraphPad Prism® Software, Inc., San Diego, CA).

2.8. Intracellular iron chelation properties of 311, N4mT and N4pT

To evaluate the intracellular chelating activities of 311, N4mT, and N4pT in intact red cells, we used the iron-sensing fluorescent probe, calcein, as described by Epsztejn and colleagues [31] and Darbari and colleagues [32]. The ligands, BIP and SIH, were used as Fe^{II} and Fe^{II}/Fe^{III} control chelators, respectively. Erythrocyte suspensions (2 × 10⁹ cells/mL) were briefly incubated for 30 min/37°C in medium containing NaCl (125 mM), HEPES (20 mM; pH 7.35), D-glucose (5 mM) and calcein-AM (250 nM). Following this incubation, the cells were washed four times with HEPES-saline-glucose buffer to remove extracellular calcein-AM and the cell suspension was adjusted to 10⁷ cells/mL. One milliliter of the calcein-loaded erythrocytes (10⁷ cells/mL) was pipetted into a spectrofluorometer cuvette, followed by the addition of an aliquot of anti-calcein antibody (1:1000 final titer). The intracellular calcein fluorescence was then recorded on a PerkinElmer LS-50B Luminescence Spectrometer (PerkinElmer, Inc. Shelton, CT). The addition of anti-calcein antibody to the erythrocyte suspensions quenched any extra-cellular fluorescence in the suspension (which was less than 1% of the initial signal) and assured that all recorded fluorescence signals originated from within the erythrocytes [32].

The fluorescence of calcein is quenched by the binding of iron [31, 32], and in the current study, calcein fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 512 nm. Following a stable basal fluorescence signal (F₁), aliquots of 311, N4mT, N4pT, BIP or SIH (100 µM final) were added to the suspension, which caused an increase in the fluorescence signal (F₂). Considering this, the addition of the chelator presumably led to competitive binding of intracellular labile iron, subsequent release of calcein-bound iron, and an increase in fluorescence intensity. Therefore, the fractional increase in fluorescence (dF) reflects the labile iron concentration. This measurement together with the values of the dissociation constant (K_d) and the total intracellular calcein concentration [CA]_t, which were determined as described by Epsztejn and colleagues [31] and Darbari and colleagues [32], was used to calculate and compare the chelation of labile iron in erythrocytes. The erythrocyte labile iron concentration, [LIP], was calculated using the equation:

$$[LIP] = (dF \times [CA]_t) + [K_d \times dF \div (1 - dF)]$$

where dF represents the fractional increase in fluorescence intensity after addition of BIP, SIH, 311, N4mT, or N4pT to the erythrocyte suspensions, K_d is the dissociation constant of calcein and iron within human erythrocytes [28, 32], and [CA]_t is the total intracellular concentration of calcein [31,32].

2.9. Statistical analysis

Data are expressed as mean $\pm$ standard deviation. Data were compared against the respective control in experiments using the Mann-Whitney test which is appropriate for small sample sizes. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of 311, N4mT and N4pT on erythrocyte shape

Incubation of human erythrocytes in RPMI-1640 culture medium containing 0.5% DMSO (solvent control), or 2.5–15 μ M of 311, N4mT or N4pT had no apparent effect on erythrocyte shape, as shown by Giemsa-staining (Fig. 1B). Indeed, as determined by light microscopy, the cells used in this study demonstrated the characteristic biconcave, discoid shape of normal, healthy erythrocytes. The peripheral zone of such erythrocytes is thicker (resulting in a longer optical path length) and so appears darker, whereas the central zone is thinner (due to the biconcave shape) and consequently appears pale (Fig. 1B). The size of this zone of central pallor and the overall staining intensity of the erythrocyte is related to the content of hemoglobin. The control and the chelator-treated cells both showed the normal minor characteristic variations in shape and size seen in Giemsa-stained preparations (Fig. 1B) [33]. After 48 h of incubation with the chelators (2.5–15 μ M), the erythrocytes remained as biconcave disks (*i.e.*, discocytes), with the same typical features as control cells. Thus, under the present incubation conditions, the chelators did not affect erythrocyte morphology.

3.2. Effects of 311, N4mT and N4pT on erythrocyte membrane integrity

The hemolytic effect of 311, N4mT, N4pT, DFO, or PIH on erythrocytes was assessed by comparing the supernatant hemoglobin concentrations of the cells incubated in vehicle-containing control medium [RPMI 1640 (without phenol red), 0.5% DMSO and 10% human plasma] to the supernatant hemoglobin concentration of cells incubated with various chelator concentrations (Table 1). As shown in Table 1, the supernatant hemoglobin in the controls, expressed as a percentage of MCHC, was $0.0175 \pm 0.0005\%$. As 10% serum was used in the standard culture media for *P. falciparum*, and was used in the present hemolysis experiments, we also measured the hemoglobin concentration in the autologous plasma to determine the extent to which it contributed to the apparent degree of hemolysis. The concentrations of hemoglobin (determined spectrophotometrically at 415 nm) in 10% plasma, 50% plasma, and whole (100%) autologous plasma, expressed as percentage of MCHC, were $0.0158 \pm 0.0013\%$, $0.0564 \pm 0.0029\%$ and $0.0892 \pm 0.0039\%$, respectively. The level of hemoglobin measured in the 50% and 100% autologous plasmas was significantly higher ($p < 0.01$) than the levels detected in the supernatant of the RPMI-1640-DMSO control and 10% plasma (Table 1).

When erythrocytes were incubated in the RPMI-1640 control media containing increasing concentrations of the chelators reflecting those used to inhibit *P. falciparum* growth (see below), there was a very slight, but significant increase in hemolysis for all concentrations tested compared to the control (Table 1). The supernatant hemoglobin values from cells incubated with the chelators were significantly ($p < 0.05$) less than the apparent hemoglobin concentration in the 50% and 100% autologous plasmas. Importantly, all cell supernatant hemoglobin values (except for 20 μ M DFO; $p > 0.05$) were significantly greater ($p < 0.05$) than the apparent hemoglobin values of the 10% autologous plasma. Thus, the incubation of the erythrocytes with the chelators caused a minor, albeit significant, increase in hemolysis. It is also worth noting that the incubation of erythrocytes in control medium alone was sufficient to cause a small, but significant ($p < 0.05$) increase in hemolysis when compared to the 10% autologous plasma.

3.3. Effect of chelators on stage-specific parasite growth

As previously reported, the chelators, 311, N4mT and N4pT, are significantly more effective at inhibiting the growth of chloroquine-resistant (7G8) and chloroquine-sensitive (3D7) strains of *P. falciparum* *in vitro* than DFO [14]. The IC₅₀ values for these aroylhydrazone and thiosemicarbazone chelators were significantly ($p < 0.001$) lower than those determined for DFO (Table 2). The chelators, 311 and N4pT, were the most effective among these compounds, with mean IC₅₀ values more than 4- times lower than the IC₅₀ for DFO in the 7G8 and 3D7 strains of the parasite (IC₅₀ = 18.5 μ M and 23.5 μ M), respectively. Furthermore, the IC₅₀ values of 311 and N4pT were 11- to 14-times lower than the IC₅₀ of their parent compound, PIH (IC₅₀ = 50 μ M), in the 3D7 malaria strain [14].

The asexual IDC of *P. falciparum* involves specific molecular and biochemical mechanisms that result in the manifestation of the major morphological stages (detectable by Giemsa stains of thin blood films) throughout the IDC represented as ring-stage, trophozoite-stage, or schizont-stage parasites. In the present study, the effects of various concentrations of 311, N4mT, and N4pT (2.5–15 μ M) and DFO (10–60 μ M) on the developmental stages of the synchronized chloroquine-sensitive strain of *P. falciparum* (3D7) cultures were investigated by determining the numbers of rings, trophozoites and schizonts at 0, 24 and 48 h of incubation with each of the chelators. Each chelator caused inhibition of all developmental forms of the parasite (Table 3). However, markedly higher concentrations of DFO were required to inhibit the development of rings to trophozoites and schizonts compared with 311, N4mT and N4pT. Indeed, relative to DFO, these novel chelators significantly ($p < 0.01$) inhibited the developmental progression of the parasite at its ring-stage at concentrations as low as 2.5 μ M (Fig. 2A). Additionally, during a 48 h incubation, 311, N4pT and N4mT caused significantly greater stage-specific parasite growth inhibition than DFO (Table 3).

Figure 2B demonstrates the effect of the chelator N4pT (2 μ M) on the stage-specific growth of chloroquine-sensitive *P. falciparum* (3D7) cultured within human erythrocytes. Figures 2B*i*–2B*ii* show control (*i.e.*, pre-treatment, 0 h) Giemsa-stained erythrocytes harboring the ring (Fig. 2B*i*) and schizont (Fig. 2B*ii*) stages of chloroquine-sensitive *P. falciparum* (3D7). Notably, during the ring and schizont-stages of the IDC, the infected erythrocyte membrane surface showed the characteristic protruding cyto-adherent knobs or “blebs” (see arrows, Fig. 2B*i* & 2B*ii*) that are indicative of increased host cell permeability [34].

Figures 2B*iii*–2B*iv* show *P. falciparum* (3D7)-containing erythrocytes originally containing ring- and schizont-stage parasites that were incubated with vehicle-control medium (*i.e.*, culture medium + 0.5% DMSO) for 24 h (2B*iii*; ring-stage) or 48 h (2B*iv*; schizont-stage). As Giemsa specifically stains phosphate groups within DNA, the parasite stains markedly more intensely with Giemsa than the enucleated host cell. It should be noted that Maurer’s dots or clefts, which appear as pink-stained stippling or dots in the erythrocyte cytoplasm or attached to the plasma membrane, are also apparent (see arrows, Fig. 2B*v* and Fig. 2B*vi*). These are single-membrane organelles localized beneath the host plasma membrane. The parasite generates this membranous network connected to both the parasitophorous vacuoles and the erythrocyte plasma membrane for nutrient uptake, immune evasion, cell signaling, merozoite egress, phospholipid biosynthesis and possibly other biochemical pathways [34].

Notably, 24 h after incubation with 2 μ M of N4pT, all parasite stages ceased to develop (Fig. 2B*v*). Also, incubation of the parasite with 2 μ M of N4pT for 48 h revealed significant inhibition and no schizonts, compared to the vehicle-control medium, but showed putative non-viable parasites that did not develop further (Fig. 2B*vi*). Confirmation of parasite non-viability was achieved by washing the parasites that were previously incubated in 311, N4mT, or N4pT for 24 or 48 h experiments several times with culture medium, and then re-

culturing these parasites according to standard methods. Reexamination of Giemsa-stained slides prepared from these cultures showed no parasite growth even after more than 48 h (data not shown). This observation strongly suggests that the normal molecular and biochemical mechanisms required for *P. falciparum* growth during the IDC had been irreversibly repressed by the chelators.

It is also worth noting that after a 24 h or 48 h incubation with N4pT (2 μ M) the erythrocyte morphology was altered to pre-lytic swollen cells (*i.e.*, spherocytes; Figs. 2B v and 2B vi). The spherocyte morphology reflects the adaptation of the parasite to the host environment, whereby it proteolytically degrades the host cell hemoglobin and detoxifies the liberated heme to prevent damage to both parasite and host cell. Importantly, this process progressively reduces the intraerythrocytic hemoglobin concentration, and consequently reduces the colloid-osmotic pressure within the erythrocyte that leads to the entrance of water into the cell [35, 36, 37].

3.4. Effect of chelators and their Fe^{III} complexes on parasite growth

To examine the role of iron in the mechanism of parasite growth inhibition, the iron complexes of 311, N4pT and N4mT were prepared (which are saturated with iron, and thus, cannot deplete cellular iron) and added to synchronized ring-stage 3D7 parasites. Iron-free chelators incubated with synchronized ring-stage parasites served as a control. Figure 3A shows the IC₅₀ values of the free ligands and their Fe^{III} complexes. Complexation of DFO, 311, N4mT and N4pT with Fe^{III} significantly ($p < 0.01$ or $p < 0.05$) inhibited their anti-plasmodial activity in terms of parasite development and growth when compared to the ligand alone. However, the effect of Fe^{III} complexation varied with the individual chelators examined. For instance, the IC₅₀ of DFO and N4mT increased by 53.6% and 47.0%, respectively, whereas the IC₅₀ of 311 and N4pT increased by 102.1% and 191.8%, respectively. These results suggest that the inhibitory effect of the chelators on 3D7 parasite development and growth during the IDC is mediated, at least in part, through iron-binding.

3.5. Estimation of labile iron by calcein dequenching studies

To further verify that the chelator-mediated effect on parasite development and growth is due to host iron-binding, we employed the iron-sensing fluorescent probe, calcein [31, 32]. Erythrocyte cytosolic labile iron is thought to be in dynamic equilibrium between the Fe^{II} and Fe^{III} oxidation states. Therefore, in the calcein-based assay for the determination of intracellular labile iron, SIH (an Fe^{II}/Fe^{III} chelator) [38] served as a well characterized control. Further, the effect of SIH was compared to 311, N4mT and N4pT that are also ligands with affinity for Fe^{II}/Fe^{III} [15] whereas BIP served as a control for exclusive Fe^{II} chelation [39, 40]. Hence, we performed parallel measurements of the erythrocyte labile iron concentration in cell suspensions using the Fe^{II} or Fe^{III} specific chelators.

After a stable baseline fluorescence signal (F_1) was obtained, each of the following chelators, BIP, SIH, 311, N4mT, or N4pT (100 μ M final concentration), was added to erythrocyte suspensions. This caused an increase in the fluorescence signal (F_2) due to the competitive binding to intracellular labile iron and the subsequent release of iron bound to calcein. Hence, the resulting fractional increase in fluorescence (dF) was reflective of the labile iron concentration. The total intracellular calcein concentration [Ca_t] was calculated and compared upon the chelation of labile iron in the cells by BIP, SIH, 311, N4mT, and N4pT, as described in the *Materials and Methods*.

Using the calcein-BIP (Fe^{II}) chelator system, the labile Fe^{II} concentration estimated in three separate experiments was $1.55 \pm 0.23 \mu$ M. On the other hand, the average labile iron concentration estimated using calcein and the Fe^{II}/Fe^{III} chelators, SIH and 311, was $2.93 \pm$

0.44 and $3.07 \pm 0.46 \mu\text{M}$, respectively. In contrast, calcein-N4mT and calcein-N4pT chelator systems gave labile $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ estimates of $7.77 \pm 1.17 \mu\text{M}$ and $10.93 \pm 1.56 \mu\text{M}$, respectively. Thus, the measurement of erythrocyte labile Fe^{3+} using calcein-N4mT or calcein-N4pT chelator systems was 2.6- and 3.5-times greater than those of SIH and 311, respectively (Fig. 3B), and this difference was statistically significant ($p < 0.001$). The measured variables (dF, [calcein], and [LIP]) using the Fe^{II} chelator, BIP were also significantly ($p < 0.001$) lower than the values obtained with the $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ chelators, SIH, 311, N4mT and N4pT. The lower free calcein concentration in cells incubated with BIP probably reflects the balance between Fe^{II} and Fe^{III} in calcein-loaded cells. Considering this, in its mono-protonated form, calcein chelates Fe^{III} with a low reduction potential, large stability constant ($\log K_{111} = 33.9$) and high pFe^{III} value [41]. However, calcein also binds Fe^{II} [31] and the dequenching of calcein fluorescence obtained after the addition of a ferrous iron-specific chelator such as BIP provides a measure of labile Fe^{II} levels within cells. The latter process depends on BIP's ability to rapidly permeate the cell membrane and competitively bind Fe^{II} with high affinity [42, 43].

4. Discussion

We previously reported the inhibitory activity of aroylhydrazones and thiosemicarbazones Fe chelators, including 311, N4mT and N4pT, against the growth of chloroquine-resistant and chloroquine-sensitive *P. falciparum* during the IDC [14]. These compounds are tridentate ligands that strongly bind ferric iron (Fe^{3+}) and have lower affinity for ferrous iron (Fe^{II}) [13, 30]. In the present study, we demonstrated the effectiveness of the chelators, 311, N4pT, and N4mT, to inhibit the ring, trophozoite and schizont-stages of *P. falciparum* development at 3- to 4-times lower concentrations compared to DFO. Among the chelators, 311 and N4pT were most effective at inhibiting the trophozoite and schizont-stages of parasite growth.

In response to chemical treatments, erythrocytes may alter their shape [21, 44]. Hence, we also investigated the effects of 311, N4mT and N4pT on erythrocyte shape using non-parasitized erythrocytes treated with anti-plasmodial concentrations of the chelators. Echinocytes are induced by agents that cause red cell membrane evagination, whereas stomatocytes are induced by chemicals that cause invagination of the cell membrane [21, 44]. Some amphiphilic agents induce echinocytes or stomatocytes depending on the distribution and incorporation of the amphiphile into the outer or the inner membrane leaflet [21, 45, 46]. This leads to expansion of one leaflet relative to the other. Furthermore, some compounds have been shown to modify the shape of erythrocytes by becoming incorporated into the erythrocyte membrane, making the cells unsuitable as *P. falciparum* hosts. Such chemically-altered cells were shown to inhibit parasite growth not directly related to the drug effect, but *via* an indirect mechanism on the host cell [23, 24].

In the present studies, microscopic examination of Giemsa-stained erythrocytes incubated with the chelators, 311, N4mT, N4pT, showed characteristic, biconcave disks, with an area of central pallor. This suggests that, unlike other reported amphiphiles that cause erythrocytes to transform into stomatocytes [23] or echinocytes [24], 311, N4mT, N4pT do not cause such effects. In evaluating the effect of the chelators on erythrocyte integrity, we demonstrated that all three chelators caused a very slight, yet statistically significant, increase in hemolysis of uninfected erythrocytes after correction for the apparent hemoglobin levels in 10% autologous plasma.

As an appropriate approach to elucidate aspects of the mechanism of Fe chelator-mediated anti-plasmodial activity, we used the iron-sensing fluorescent probe, calcein, to determine the intra-erythrocytic iron chelation efficacy of host labile iron by BIP (Fe^{II}), SIH, 311,

N4mT, and N4pT ($\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$). With calcein and the $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ chelators (SIH, 311, N4mT, N4pT; [13, 30]), we found significant differences in the erythrocyte total cytosolic labile iron concentrations. As shown in Fig. 3B, the chelation efficacy is as follows: N4pT > N4mT > 311 > SIH > BIP. The greater efficacy of N4pT and N4mT relative to 311 and SIH is probably related to their higher lipophilicity [15]. Additionally, it is well known that aroylhydrazone chelators have a low affinity for Fe^{II} [13, 15, 30] and that strong Fe^{III} chelators such as PIH can “push” the redox equilibrium between Fe^{II} and Fe^{III} to the right in solutions containing oxygen [47]. Therefore, the estimates of cytosolic labile iron concentration using calcein in the presence of N4mT, N4pT, 311, and SIH can be regarded as a composite measure of both Fe^{II} and Fe^{III} pools, depending on the $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ redox equilibrium in the cell.

The lower free calcein concentration observed in the cells treated with BIP (an Fe^{II} chelator) probably reflects the shift of the Fe^{II} and Fe^{III} redox balance in calcein-loaded cells. Calcein chelates both Fe^{III} and Fe^{II} [31, 41] and when present in intracellular compartments, it will bind labile iron that is complexed to other endogenous chelators with relatively low affinities for iron ions, (e.g., citrate) [48] and/or putative iron-transporting chaperones (e.g., poly-r(C)-binding proteins 1–4) [49]. Hence, the level of fluorescence of free calcein indicates the variations in the availability of iron complexed to these low affinity ligands. The affinity of calcein for Fe^{III} is evident from the large stability constant ($\log K_{111}$) and the large pFe^{III} values reported by Thomas *et al.* [41]. The dequenching obtained after addition of BIP provides a measure of the size of the cytosolic labile Fe^{II} pool. The higher concentrations of calcein estimated in cells treated with N4mT and N4pT probably reflect the marked ability of these ligands to rapidly permeate the plasma membrane and mobilize intracellular iron, which has been previously shown using SK-N-MC neuroepithelioma cells in culture [15].

Erythrocytes contain up to 20 mM of ferrous iron (Fe^{II}) coordinately bound to the protoporphyrin moiety of heme, but the concentration of “free” cytosolic ferrous (Fe^{II}) iron in the erythrocytes is reportedly very low [28, 32]. This labile iron may constitute metabolically active and catalytically reactive iron and is thought to be complexed to endogenous ligands [50, 51]. Non-heme iron is essential for the asexual growth of the *P. falciparum* in mature erythrocytes. During intra-erythrocytic infection, *P. falciparum* generates large millimolar levels of free heme within its acidic food vacuole *via* hemoglobin digestion and limited *de novo* biosynthesis [52]. Although it has been proposed that some portion of heme iron may be liberated from hemoglobin and mobilized by the parasites for their utilization [52 – 54], there is no direct evidence for mobilization of host iron from heme for parasite metabolism. Notably, Goldberg and colleagues recently demonstrated that the parasite lacks the ubiquitous heme oxygenase (HO) pathway that enzymatically degrades some heme to biliverdin, or its downstream metabolite, bilirubin [55]. Thus, the parasite presumably must rely on an alternative mechanism for heme detoxification and iron acquisition during the IDC. Loyevsky and colleagues [28] demonstrated that labile iron pools are present within normal and *P. falciparum*-infected erythrocytes and that such iron pools are also a potential source of iron for the parasite. More recent studies have also indicated the existence of a chelatable pool of iron in normal erythrocytes that can be bound by lipophilic thiosemicarbazone ligands [56]. Our results demonstrate that the iron chelation efficacies of 311, N4mT and N4pT correspond to their anti-plasmodial activities, suggesting that the intracellular LIP may be a potential source of non-heme iron for the parasite within the mature erythrocyte.

Our data also suggest that the inhibition of the intra-erythrocytic development of the *P. falciparum* is probably *via* chelation of iron in the parasite and/or the host cell. Complexation of iron by chelators has been used in several different experimental models to

elucidate cellular mechanisms of action. To examine the effect of the complexation on the anti-plasmodial activity of each chelator, the chelators and their Fe^{III} complexes were added at various concentrations to *P. falciparum* cultures. Complexation of 311, N4mT and N4pT with Fe^{III} decreased their anti-plasmodial activity, strongly suggesting that Fe chelation is necessary for their efficacy.

In summary, we have demonstrated that 311, N4mT, and N4pT have significantly greater anti-plasmodial activities compared to DFO. We also showed that the ring, trophozoite and schizont-stages of *P. falciparum* are more vulnerable to 311, N4pT, and N4mT compared to DFO. Iron complexation experiments revealed that the inhibitory effect of the chelators on parasite growth is mediated, in part, through iron-binding and iron-deprivation. Direct estimation of the labile iron concentration shows that the chelation efficacies of 311, N4mT and N4pT correspond to their anti-plasmodial activities. These results suggest that the labile iron pool may be a potential source of non-heme iron for parasite metabolism within the mature erythrocyte. Furthermore, the promising *in vitro* data presented in this study should now be followed up by using *in vivo* models of *P. falciparum* malaria to confirm the therapeutic efficacy of the novel thiosemicarbazone chelators, N4pT and N4mT.

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Highlights

- Iron chelators inhibit the growth of the malaria parasite, *Plasmodium falciparum*, in culture and in animal and human studies.
- The novel iron chelators, N4mT and N4pT, did not affect erythrocyte shape, but caused very slight hemolysis at IC₅₀ values that inhibited parasite growth.
- The chelators 311, N4mT and N4pT affected all stages of the intra-erythrocytic development of *P. falciparum* development in culture.
- Complexation of 311, N4mT and N4pT with iron reduced their anti-plasmodial activities.
- The labile iron pool may be a potential source of non-heme iron for parasite metabolism within the erythrocyte.

Figure 1.

(A) Line drawings of the chemical structures of the iron chelators studied, including DFO, 311, N4mT, N4pT, PIH, SIH and BIP. (B) Giemsa-stained normal human erythrocytes viewed by light microscopy after a 48 h incubation with: (i) control medium; (ii) 0.5% DMSO; (iii) 311 (15 μ M); (iv) N4mT (15 μ M); or (v) N4pT (15 μ M). Magnification: 1000 x, oil immersion.

Figure 2.

(A) The inhibitory effects of DFO, 311, N4mT and N4pT on the ring-stage of chloroquine-sensitive (3D7) *P. falciparum*. Parasites were evaluated by examining Giemsa-stained microscope slides prepared from synchronized cultures at 24 h after incubation with the chelators to determine the sensitivities of different stages of parasite development. One thousand cells were counted for each compound. DFO served as the standard. The results are expressed as percent inhibition relative to that determined in the control cultures without chelators at the ring-stage of parasite development. Each data point is mean $\pm$ SD (3 experiments). ** $p < 0.01$ comparing the respective chelator concentration with the untreated control cultures.

(B) Effect of N4pT incubation on parasitized (ring- and schizont-stages) of chloroquine-sensitive *P. falciparum* (3D7). (i) Control ring-stage at 0 h (*i.e.*, pre-treatment); (ii) Control schizont-stage at 0 h (Note: arrows in (i) and (ii) indicate the presence of protruding cyto-adherent knobs or “blebs” indicative of increased host cell permeability [34]); (iii) Control ring-stage at 24 h after incubation with vehicle-control; (iv) Control schizont-stage at 48 h after incubation with vehicle-control. (v) *P. falciparum*-infected erythrocytes were incubated for 24 h with 2 μ M N4pT; Only dead parasites and

Maurer's dots or clefts (see arrows) are seen at ring-stage. (vi) *P. falciparum*-infected erythrocytes were incubated for 48 h with 2 μ M N4pT (Note: arrows in (v) and (vi) indicate non-viable parasites and Maurer's dots or clefts (see arrows) are seen at the schizont-stage. Non-viable parasites did not recover after re-culturing). Magnification: x1000, oil immersion.

Figure 3.

(A) Effect of DFO, 311, N4mT and N4pT and their iron complexes on *P. falciparum* growth in culture. The Fe^{III} complexes of the chelators were prepared by adding Fe (as FeCl₃) to the ligands in a 1:1 ligand:metal ratio for the hexadentate chelator (DFO), and a 2:1 ligand:metal ratio for the tridentate chelators (311, N4mT and N4pT), as described in the *Materials and Methods*. Inhibitory effects of the chelators with and without FeCl₃ were assessed. Parasite growth was assessed after 48 h in the presence of the compounds by using [³H]-hypoxanthine incorporation. The results are mean ± SD (6 experiments). ** $p < 0.01$ or * $p < 0.05$ in comparison to the free ligand.

(B) Intracellular iron chelation properties of 311, N4mT and N4pT compared to BIP and SIH. The fractional increase in fluorescence intensity (dF) was used together with the measured values of the dissociation constant (K_d) and the total intracellular calcein concentration [Ca_i], to calculate and compare the chelation properties of the chelators in the erythrocytes. Results are from typical experiments with the chelators and the numerical indices are expressed as means ± SD (3 experiments). Calculation of: [calcein] (μM); [LIP]

(μM); and dF are described in the *Materials and Methods*. *** $p < 0.001$ in comparison to the [LIP] measured with SIH, 311 or BIP.

Table 1
Comparative hemolytic effect of 311, N4mT, N4pT, DFO or PIH

Supernatant hemoglobin was measured at 415 nm and expressed as a percentage of the mean corpuscular hemoglobin concentration (MCHC). Heme-containing proteins in the autologous plasma (*i.e.*, 10% plasma, 50% plasma, and whole (100%) plasma) are shown for comparison to the supernatant hemoglobin concentrations in control and chelator-treated samples. Results are mean $\pm$ SD (3 experiments). The percent MCHC in the supernatants after incubation under the various conditions was compared to the control using the Mann-Whitney test

Sample	Concentration (μ M)	% MCHC	<i>p</i> value
Control	-	0.0175 $\pm$ 0.0005	-
10% Plasma	-	0.0158 $\pm$ 0.0013	<i>p</i> < 0.05
50% Plasma	-	0.0564 $\pm$ 0.0029	<i>p</i> < 0.01
100% Plasma	-	0.0892 $\pm$ 0.0039	<i>p</i> < 0.01
311	0.5	0.0193 $\pm$ 0.0005	<i>p</i> < 0.05
	1	0.0195 $\pm$ 0.0010	<i>p</i> < 0.05
	1.5	0.0200 $\pm$ 0.0006	<i>p</i> < 0.05
	2	0.0203 $\pm$ 0.0005	<i>p</i> < 0.05
	3	0.0197 $\pm$ 0.0005	<i>p</i> < 0.05
N4mT	0.5	0.0211 $\pm$ 0.0004	<i>p</i> < 0.05
	1	0.0208 $\pm$ 0.0007	<i>p</i> < 0.05
	1.5	0.0213 $\pm$ 0.0002	<i>p</i> < 0.05
	2	0.0208 $\pm$ 0.0007	<i>p</i> < 0.05
	3	0.0214 $\pm$ 0.0004	<i>p</i> < 0.05
N4pT	0.5	0.0215 $\pm$ 0.0006	<i>p</i> < 0.05
	1	0.0218 $\pm$ 0.0002	<i>p</i> < 0.05
	1.5	0.0222 $\pm$ 0.0004	<i>p</i> < 0.05
	2	0.0227 $\pm$ 0.0006	<i>p</i> < 0.05
	3	0.0225 $\pm$ 0.0006	<i>p</i> < 0.05
DFO	10	0.0193 $\pm$ 0.0005	<i>p</i> < 0.05
	20	0.0182 $\pm$ 0.0005	<i>p</i> > 0.05
	30	0.0186 $\pm$ 0.0008	<i>p</i> > 0.05
	45	0.0192 $\pm$ 0.0001	<i>p</i> < 0.05
	60	0.0182 $\pm$ 0.0002	<i>p</i> < 0.05
PIH	10	0.0195 $\pm$ 0.0004	<i>p</i> < 0.05
	15	0.0188 $\pm$ 0.0003	<i>p</i> < 0.05
	20	0.0190 $\pm$ 0.0006	<i>p</i> < 0.05
	25	0.0193 $\pm$ 0.0004	<i>p</i> < 0.05
	30	0.0192 $\pm$ 0.0008	<i>p</i> < 0.05

Table 2

Growth inhibition efficacy (IC_{50} ; μM) of chelators in cultures of chloroquine-sensitive (3D7) and chloroquine-resistant (7G8) *Plasmodium falciparum*. Results are mean $\pm$ SD ($n = 6$)

Chelator	3D7 <i>P. falciparum</i>	7G8 <i>P. falciparum</i>	Statistical Significance [*]
DFO	23.43 $\pm$ 3.40	18.54 $\pm$ 4.70	N/A
311	4.45 $\pm$ 1.70	4.46 $\pm$ 0.30	311 vs. DFO, $p = 0.001$
N4mT	10.30 $\pm$ 4.40	10.81 $\pm$ 2.00	N4mT vs. DFO, $p = 0.001$
N4pT	3.64 $\pm$ 2.00	2.68 $\pm$ 1.90	N4pT vs. DFO, $p = 0.001$

^{*} Significance for both 3D7 and 7G8 *P. falciparum*; N/A, not applicable

Table 3
Inhibition of *P. falciparum* stage development after 24 h and 48 h incubation with chelators

IDC	24 hour culture <i>P. falciparum</i> IDC stage				48 hour culture <i>P. falciparum</i> IDC stage			
	Rings	Trophozoites	Schizonts	% Inhibition	Rings	Trophozoites	Schizonts	% Inhibition
Control (%)	0	20 ± 2.0	20 ± 2.0	0	0	20 ± 2.0	20 ± 2.0	20 ± 2.0
Chelator	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition
DFO (30 µM)	80 ± 6.0	74 ± 7.0	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5	95 ± 4.8	100 ± 9.5
311 (7.5 µM)	99 ± 8.4	90 ± 8.6	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5
N4mT (7.5 µM)	80 ± 4.0	60 ± 5.7	80 ± 6.0	80 ± 6.0	80 ± 6.0	80 ± 6.0	80 ± 6.0	80 ± 6.0
N4pT (7.5 µM)	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5	100 ± 9.5

Parasites were synchronized at the ring-stage. Giemsa-stained slides of parasite culture were examined microscopically for ring-, trophozoite-, or schizont-stage parasites after a 24 and 48 h incubation at 37°C. The results are expressed as percent of observed inhibition relative to the control cells without chelators during-stages of parasite development. One thousand cells were counted for each compound. Each data point is the mean of triplicates. The range of results did not exceed 10% of the mean parasitemia in the ring-stage. IDC: intra-erythrocytic developmental cycle.