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Antiplasmodial Activity of Targeted Zinc(II)-Dipicolylamine Complexes

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Abstract

This study measured the antiplasmodial activity of nine zinc-dipicolylamine (ZnDPA) complexes against three strains of *Plasmodium falciparum*, the causative parasite of malaria. Growth inhibition assays showed significant activity against all tested strains, with 50% inhibitory concentrations between 5 and 600 nM and almost no toxic effect against host cells including healthy red blood cells. Fluorescence microscopy studies with a green-fluorescent ZnDPA probe showed selective targeting of infected red blood cells. The results suggest that ZnDPA coordination complexes are promising antiplasmodial agents with potential for targeted malaria treatment.

Graphical abstract

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc??>.

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Keywords

malaria; antiparasmodial drugs; zinc dipicolylamine; fluorescence microscopy

1. Introduction

Malaria is the greatest parasitic killer in the world, especially in the developing regions of Asia, Africa, and South America.[1] In 2015, nearly half the world population – approximately 3.2 billion people – was at risk of contracting malaria. The disease is transmitted by female *Anopheles* mosquitos. The mosquito bite introduces parasites into a host's blood, which travel to the liver for maturation and reproduction. *Plasmodium Falciparum* (*P. falciparum*) is the predominate species of malaria parasite, responsible for 85% of world cases.[2] Almost all existing anti-*Plasmodial* drugs, particularly 4-aminoquinoline based drugs such as chloroquine, have been rendered ineffective due to chemoresistance.[3, 4] Artemisinin and related endoperoxide derivatives display the most rapid action of current drugs against *P. Falciparum* infections but the appearance of artemisinin-resistant parasites in Southeast Asia threaten its efficacy.[5] Additionally, low bioavailability and poor pharmacokinetic properties diminish their therapeutic potential.[6, 7] Discovering new and inexpensive treatments with novel modes of action is a top priority for improving health outcomes and controlling disease spread in endemic areas.

A promising new chemotherapeutic approach is to actively target drugs to red blood cells (RBCs) infected with *P. Falciparum* parasites (iRBCs). One notable example is immunoliposomes which have been extensively explored for drug delivery applications.[8, 9] However, immuno-based strategies are limited due to antigenic variation among parasite subspecies.[10] A universal biomarker among iRBCs is the presence of an anionic outer membrane. The exterior of healthy blood cells have an electrostatic charge that is close to neutral, with zwitterionic lipids predominating on the membrane surface. However, once cells experience oxidative stress or undergo cell death, anionic phosphatidylserine (PS) becomes exposed on the external cell surface. PS is a membrane phospholipid that is exclusively localized to the inner leaflet of the lipid bilayer and promotes cell adherence with immune scavenger cells when externalized. In iRBCs, programmed erythrocyte death (eryptosis) occurs as a result of parasite growth and reproduction which scrambles membrane lipids and exposes PS on the outer membrane.[11] Exposed PS stimulates adherence to CD36 and thrombospondin receptors on vascular endothelial cells. Additionally, exposure of parasite-derived knob proteins, which act as virulence factors and mediate cytoadherence of iRBCs to endothelial cells, also contribute negative charge to the membrane surface.[12]

The feasibility of using cationic molecules to target anionic iRBC is supported by reports that quaternary ammonium salts, cationic 2-phenylbenzofurans, and dicationic 3,5-diphenylisoxazoles have *in vitro* and *in vivo* efficacy.[13–15] Many positively charged amphipathic and cysteine-rich peptides are highly toxic towards isolated sporozoites *in vitro* as well as early sporogonic stages in the mosquito midgut.[16] Also, a wide variety of transition metal chelators and organometallic complexes have been broadly screened for

antimalarial activity. The most commonly explored are iron chelating agents with the aim of sequestering iron from parasites which require it for reproduction. Iron-chelating drugs inhibit parasite reproduction and have been used in conjunction with traditional antimalarial drugs.[17] Ferrocene-containing compounds are particularly active against clinical isolates of both *P. Falciparum* and *P. vivax*. [18, 19] However, these agents may also reduce iron availability to the patient thus imparting anemic side effects. Chloroquine complexed with gold, platinum, and palladium displayed better antiplasmodial activity than chloroquine alone.[20] Auranofin, a gold-based antiarthritic drug, strongly inhibited *P. Falciparum* growth *in vitro*. [21] Manganese complexes of cross-bridged tetraazamacrocyclic ligands are highly potent against *P. Falciparum* strains resistant to chloroquine.[22] A number of zinc complexes with ligands such as quinine and arginine have displayed unique activity against iRBCs.[23, 24] One complex in particular, zinc-desferrioxamine, was found to be more permeative into iRBCs than the desferrioxamine ligand alone as the former exchanges its bound zinc for ferric ions which enhanced uptake.[25]

Some of us have shown that fluorescent and radiolabeled zinc-dipicolylamine (ZnDPA) complexes can selectively target anionic cell surfaces in cell culture and in small animal models of disease, and used these probes for *in vivo* imaging of dead and dying mammalian cells (which expose PS) and bacterial infection (which expose anionic phosphatidylglycerol and cardiolipin).[26] Recently, we discovered that ZnDPA complexes selectively target and kill the trypanosomatid parasite *Leishmania major* (*L. major*), a causative agent of cutaneous leishmaniasis.[27] The surface of *Leishmania* parasites is negatively charged due to high fractions of anionic lipids and a thick coating of the anionic polysaccharide lipophosphoglycan. *In vivo* treatment efficacy studies showed that a ZnDPA treatment regimen significantly reduced parasite burden with minimal side effects to the local host tissue. These promising results have motivated us to determine if ZnDPA coordination complexes are active against other classes of parasites.

This study had two objectives. The first was to determine if a fluorescent ZnDPA probe was able to selectively target iRBCs and the second was to measure antiplasmodial activity in cell culture. A library of eight ZnDPA complexes was synthesized and screened for inhibitory activity against three different *P. Falciparum* species that have different levels of sensitivity towards the standard antimalarial chemotherapeutic agent, chloroquine. *Plasmodium* inhibition activity was assessed using fluorescent- and tritium-based assays, and the ZnDPA complexes were concurrently screened for toxicity against models of host tissue.

2. Results and Discussion

2.1 Synthesis

Shown in Scheme 1 are the ZnDPA complexes that were used in this study. The structures of complexes **1** – **5** vary in the amount of lipophilicity which was expected to influence membrane affinity. Complexes **6** and **7** were included because a previous study had shown that they have high affinities for anionic membranes.[28] The dimeric complex **8** allowed assessment of the effect gained by doubling the number of ZnDPA in one molecule. The fluorescent probe **mSeek** and control **BODIPY Dye** were prepared during previous studies.

[26] The ligands **apo-1** – **apo-5** were prepared by conducting Mannich reactions with the appropriate phenol derivative having different substituents at the 4-position (Scheme 2).[29] Dimeric ligand **apo-8** was made by conducting a copper-catalyzed azide-alkyne cycloaddition (CuAAC) using **apo-5**. Mixing these water insoluble ligands with the appropriate stoichiometry of $\text{Zn}(\text{NO}_3)_2$ in methanol produced the corresponding water soluble ZnDPA complexes in quantitative yield. All compounds were tested as aqueous solutions except for **3**, **6** and **7** which were dissolved in 10 % DMSO/water.

2.2 Fluorescence Microscopy

The size of *Plasmodium* parasites depends on the developmental stage but they are approximately five times smaller than the 6–8 μM diameter of a healthy RBC. ZnDPA targeting of iRBC was explored using the green fluorescent probe **mSeek** which was shown previously to stain bacteria and *Leishmania* parasites.[27, 30] Samples of red blood cells (10^4 cells per mL) infected with *P. Falciparum* W2 were treated with Hoechst nuclear stain which selectively localizes to iRBCs through binding parasite DNA. The same samples were co-treated with **mSeek** (5 μM) in HEPES buffer. After a 15 min incubation period, each sample was rinsed twice with fresh buffer and then subjected to fluorescence microscopy. As shown in Figure 1A, the fraction of iRBCs is low but in each case the **mSeek** fluorescence co-localizes with the Hoechst staining of *Plasmodium* parasites. The requirement for a ZnDPA targeting ligand to selectively bind infected cells was tested by performing the same staining procedure on separate samples with a control **BODIPY Dye**. Micrographs of infected blood cells stained with the control dye displayed nonspecific staining of both infected and uninfected blood cells, with strong localization to the blood cell membrane (Figure 1A, **bottom**).

To better visualize the location of **mSeek** staining, high magnification fluorescence microscopy of infected samples was performed using propidium iodide to identify iRBCs. Propidium iodide is a red-fluorescent DNA stain that is analogous to Hoechst but less susceptible to imaging artifacts due to background RBC autofluorescence. As expected, **mSeek** selectively localized to infected cells with strong accumulation at the cellular membrane (Figure 1B). The probe also appeared to enter the cell cytoplasm and label components of the intracellular *Plasmodium* parasite. This selective affinity is likely due to the electrostatic attraction of cationic ZnDPA for exposed anionic PS on the iRBC surface. These results suggest that the ZnDPA targeting ligand can selectively identify iRBCs which may be useful for identifying or studying malaria infection in blood samples.

2.3 Antiplasmodial Activity and Host Cell Toxicity

The antiplasmodial activities of **mSeek** and ZnDPA complexes **1** – **8** were evaluated against three *P. Falciparum* strains. Two strains are considered chloroquine-sensitive (*P. Falciparum* HB3 and *P. Falciparum* 3D7) and one strain is chloroquine-resistant (*P. Falciparum* W2). Parasite growth inhibition was evaluated using two different assays.¹ The inhibition of W2 and HB3 were evaluated using an incorporation assay with the tritiated nucleotide

¹ Authors from the University of Notre Dame tested inhibition of the W2 and HB3 strains using the hypoxanthine incorporation assay. Authors from Tulane University measured inhibition of their 3D7 strain using the SYBR green assay.

hypoxanthine ($[^3\text{H}]$ Hypoxanthine). Briefly, separate cultures of *P. Falciparum* were treated with a ZnDPA concentration for 48 hr followed by the addition of $[^3\text{H}]$ Hypoxanthine for 20 hr, which is incorporated into newly replicated DNA as a quantitative measure of viable parasites for calculated growth-concentration curves (IC_{50}). The inhibition of 3D7 was evaluated using SYBR® green instead of $[^3\text{H}]$ Hypoxanthine; this dye selectively binds to the minor groove of double stranded DNA which activates a green fluorescent signal upon blue excitation. The complexes were active against all strains, with IC_{50} values between ~600 and 5 nM (Figures S19–21, Table 1). The probe **mSeek** was the most inhibitory to parasite growth, displaying an IC_{50} of 15 nM against the *P. Falciparum* W2 strain and 5 nM against the HB3 strain. Among the unlabeled complexes, dimeric complex **8** displayed potent activity with IC_{50} values between 23 and 142 nM. This is potentially due to an enhanced affinity for the negatively charged iRBCs. Multimeric ZnDPA complexes have been previously shown to have higher affinity towards the anionic surfaces of dead and dying cells. [31] Nearly all the complexes were more inhibitory than chloroquine against the chloroquine-resistant W2 strain. The complexes were concurrently evaluated for host cell activity. Mammalian toxicity was assessed using a CellTiter-Blue® assay, which measures the ability of living cells to convert the redox dye resazurin into an orange-fluorescent product (resorufin) (Figure S22). Hemolytic activity was measured using the absorbance of extracellular hemoglobin released from lysed blood cells (Figure S23). Most complexes did not display toxicity at concentrations $\geq 100 \mu\text{M}$ (Table 1). Complexes **1**, **2** and **mSeek** were moderately toxic with IC_{50} values between 35 and 80 μM . Complex **3** was the most toxic ($\text{IC}_{50} = 17 \pm 2 \mu\text{M}$). The complexes displayed minimal hemolytic activity at concentrations $\geq 100 \mu\text{M}$ with the exception of complex **3** ($\text{HC}_{50} = 42 \pm 3 \mu\text{M}$).

To observe parasite status following treatment, iRBCs were treated with **mSeek** (100 nM) for 24 hours then stained with Giemsa followed by brightfield microscopy. Micrographs show the parasites are affected by the **mSeek** treatment. The schizont stages show abnormal morphology. (Figure 2, **top**). In contrast, micrographs of iRBCs treated with control **BODIPY Dye** displayed relatively healthy parasites in much greater quantity (Figure 2, **bottom**) (see Figures S24 and S25 for expanded views of multiple micrographs). At this point in time, the antiplasmodial mechanism of ZnDPA complexes remains unknown, but a few relevant points are worth highlighting. The precursor ligands are water insoluble organic compounds with very high affinities for transition metal cations such as Zn(II), Cu(II) and Fe(II/III). Conversion of the ligands to ZnDPA complexes induces water solubility and ability to selectively target the anionic membranes of iRBCs. But in-situ metal cation exchange is a possibility and the antiplasmodial activity may be due to alterations of metal cation concentrations within the blood cell cytosol. Zinc acquisition and trafficking are essential for parasite replication and a recent study demonstrated that decreasing zinc levels inhibited *Plasmodium* development.[32] Alternatively, ZnDPA binding may be destabilizing the parasite membrane or anionic DNA scaffold causing parasite death. The anionic iRBC membrane may also be targeted and disrupted which could inhibit the extracellular exit of parasites from host cells.

The World Health Organization recommends treating malaria with artemisinin combination therapy (ACT), consisting of artemisinin and another drug. Malaria strains resistant to ACT

have already emerged in Southeast Asia, demonstrating the need for new drugs with novel modes of action.[1] ZnDPA complexes may be useful as chemotherapeutic leads that target the anionic membrane of infected cells, a ubiquitous biomarker of iRBCs, as demonstrated by fluorescence microscopy (Figure 1). Moreover, ZnDPA-drug conjugates could selectively deliver chemotherapy to infected cells while reducing off-target accumulation. A ZnDPA-vancomycin derivative was recently used to treat antibiotic-resistant bacteria infections in mice without the development of chemoresistance.[33] In addition, ZnDPA-camptothecin conjugates have been reported to selectively target PS-exposing tumors in mice.[34]

3. Conclusion

The green-fluorescent ZnDPA probe, **mSeek**, selectively targets red blood cells infected with *P. falciparum*. *In vitro* activity assays indicate that ZnDPA complexes have significant and selective activity against *P. falciparum*, with little mammalian cell toxicity or red blood cell hemolytic activity. Further studies are warranted to determine the mechanism of action and *in vivo* performance.

4. Experimental

4.1 Synthesis and Characterization

All starting materials were reagent grade purchased from commercial sources and used without further purification. NMR spectra were recorded in CDCl₃ with chloroform (7.26 ¹H, 77.0 ¹³C) as an internal reference, using 400 MHz, 500 MHz Bruker or 600 MHz Varian spectrometers. Data are reported in the next order: chemical shift (ppm) multiplicities (s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet)), coupling constants, *J* (Hz) and integration. Reactions were monitored by TLC analysis conducted in silica-gel on TLC-Al foils. The fluorescent probe **mSeek**, control **BODIPY Dye, 6**, and **7** were prepared during previous studies.[28, 30]

General procedure for ligand synthesis—The appropriate 4-substituted phenol and formaldehyde were added to a solution of di-(2-picoly)amine (DPA) in EtOH/H₂O (12 mL/5 mL) and the mixture kept at reflux temperature for 48 hours. The solvent was removed and residue partitioned between CH₂Cl₂ and H₂O. The aqueous layer was extracted with CH₂Cl₂ three times and then dried over MgSO₄. The crude material was purified by silica gel chromatography (CH₂Cl₂/MeOH/NH₄OH; 94/5/1) to yield the desired product.

Apo-1: yellow oil in 72 % yield (682.0 mg, 1.190 mmol). ¹H NMR (400 MHz, CDCl₃) δ 10.77 (s, 1H), 8.52 (dd, *J* = 3.2, 1.5 Hz, 4H), 7.74 – 7.57 (m, 4H), 7.50 (d, *J* = 7.9 Hz, 4H), 7.20 – 7.05 (m, 4H), 7.01 (s, *J* = 5.5 Hz, 2H), 3.88 (s, 8H), 3.80 (s, *J* = 7.6 Hz, 4H), 2.50 (t, *J* = 7.6 Hz, 2H), 1.59 – 1.46 (m, 2H), 1.31 (td, *J* = 14.6, 7.3 Hz, 2H), 0.89 (td, *J* = 7.2, 1.0 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 159.4, 153.8, 148.9, 136.4, 132.5, 129.0, 123.6, 122.9, 121.9, 77.3, 59.8, 54.9, 34.7, 33.9, 22.3, 14.0. HRMS-ESI [*M* + *H*⁺] *m/z* 573.3336: calcd for C₃₆H₄₁N₆O 573.3337

Apo-2: pale yellow crystals in 63 % yield (600 mg, 1.05 mmol). ¹H NMR (400 MHz, CDCl₃) δ 8.54 – 8.49 (m, 4H), 7.59 (td, *J* = 7.6, 1.8 Hz, 4H), 7.50 (d, *J* = 7.8 Hz, 4H), 7.19 (s, *J* = 9.7 Hz, 2H), 7.11 (ddd, *J* = 7.3, 4.9, 1.1 Hz, 4H), 3.88 (s, 8H), 3.81

(s, 4H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 159.3, 153.5, 148.8, 140.9, 136.4, 125.9, 123.2, 122.9, 121.9, 59.9, 55.2, 33.9, 31.6. HRMS-ESI $[\text{M} + \text{H}^+]$ m/z 573.3336: calcd for $\text{C}_{36}\text{H}_{41}\text{N}_6\text{O}$ 573.3337.

Apo-3: yellow oil in 90 % yield (687.0 mg, 1.09 mmol). ^1H NMR (400 MHz, CDCl_3) δ 8.49 (d, $J = 4.2$ Hz, 4H), 7.56 (tt, $J = 7.8, 3.9$ Hz, 4H), 7.47 (d, $J = 7.8$ Hz, 4H), 7.08 (dd, $J = 6.6, 5.6$ Hz, 4H), 6.98 (s, 1H), 3.85 (s, $J = 8.7$ Hz, 8H), 3.77 (s, $J = 8.7$ Hz, 4H), 2.46 (dd, $J = 17.8, 10.0$ Hz, 2H), 1.51 (dd, $J = 14.4, 7.0$ Hz, 2H), 1.23 (d, $J = 14.2$ Hz, 10H), 0.83 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 159.4, 153.7, 148.9, 136.4, 132.6, 129.0, 123.7, 122.9, 121.9, 77.3, 59.8, 54.9, 35.1, 31.9, 31.7, 29.5, 29.3, 29.2, 22.6, 14.1. HRMS-ESI $[\text{M} + \text{H}^+]$ m/z 629.3962: calcd for $\text{C}_{40}\text{H}_{49}\text{N}_6\text{O}$ 629.3963.

Apo-4: yellow pale solid in 65 % yield (660 mg, 1.18 mmol). ^1H NMR (500 MHz, CDCl_3) δ 8.41 (d, $J = 4.2$ Hz, 4H), 7.49 (td, $J = 7.7, 1.7$ Hz, 4H), 7.38 (d, $J = 7.8$ Hz, 4H), 7.03 (dd, $J = 6.7, 5.3$ Hz, 6H), 6.98 (s, 2H), 3.86 (s, 8H), 3.79 – 3.68 (m, 6H), 2.68 (t, $J = 6.5$ Hz, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 158.1, 154.3, 148.6, 136.6, 130.3, 128.7, 123.1, 122.9, 122.1, 77.4, 63.2, 59.4, 55.0, 38.3. HRMS-ESI $[\text{M} + \text{H}^+]$ m/z 561.2963: calcd for $\text{C}_{34}\text{H}_{37}\text{N}_6\text{O}_2$ 561.2963.

Apo-5: yellow solid 45 % yield (419.0 mg.). ^1H NMR (400 MHz, CDCl_3) δ 8.48 (d, $J = 4.2$ Hz, 4H), 7.55 (td, $J = 7.7, 1.7$ Hz, 4H), 7.44 (d, $J = 7.8$ Hz, 4H), 7.07 (dd, $J = 6.8, 5.5$ Hz, 4H), 7.02 (s, 2H), 3.85 (s, 8H), 3.77 (s, 4H), 3.74 (s, 1H), 3.38 (t, $J = 7.2$ Hz, 2H), 2.75 (t, $J = 7.2$ Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 159.1, 154.8, 148.9, 136.5, 129.5, 127.6, 124.2, 123.0, 122.0, 100.5, 63.6, 59.8, 54.8, 52.8, 34.6. HRMS-ESI $[\text{M} + \text{H}^+]$ m/z 586.3037: calcd for $\text{C}_{34}\text{H}_{36}\text{N}_9\text{O}$ 586.3038.

Apo-8: **Apo-5** (126.0 mg, 0.215 mmol) and 4,7,10,13-tetraoxahexadeca-1,15-diyne (23.2 mg, 0.102 mmol) were dissolved in CHCl_3 (3 mL). Then, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (103.0 mg, 0.645 mmol) and sodium *L*-ascorbate (128.0 mg, 0.645 mmol) were mixed in a separated vial and added to the reaction mixture. The mixture was stirred overnight. The crude was washed with saturated aqueous solution of Na_4EDTA and saturated aqueous NaCl . The organic layer was dried over magnesium sulfate and concentrated under reduced pressure. The crude was purified by chromatography to yield the product in 38 % yield (27.8 mg, 0.02 mmol). ^1H NMR (500 MHz, MeOD) δ 8.38 (d, $J = 4.9$ Hz, 4H), 7.90 (s, 2H), 7.77 – 7.69 (m, 12H), 7.65 (d, $J = 7.8$ Hz, 4H), 7.39 (t, $J = 7.8$ Hz, 4H), 7.26 – 7.19 (m, 4H), 6.99 (s, 2H), 6.85 (d, $J = 7.3$ Hz, 4H), 6.77 (s, 4H), 6.41 (d, $J = 8.2$ Hz, 4H), 3.97 (t, $J = 6.0$ Hz, 4H), 3.76 – 3.67 (m, 12H), 3.54 (d, $J = 16.0$ Hz, 16H), 1.81 (ddd, $J = 19.6, 14.1, 6.8$ Hz, 8H). ^{13}C NMR (126 MHz, MeOD) δ 169.8, 160.9, 160.4, 158.2, 149.3, 141.6, 139.7, 138.6, 135.2, 133.3, 124.5, 124.0, 123.7, 122.4, 114.9, 112.7, 108.4, 79.5, 68.2, 60.8, 60.8, 59.7, 38.5, 27.7, 26.2. HRMS-ESI $[\text{M} + \text{H}^+]$ m/z 1397.7207: calcd for $\text{C}_{80}\text{H}_{89}\text{N}_{18}\text{O}_6$ 1397.7207

Zinc Complexation—Stock solutions of bis(di-2-picoyl)amine ligands and $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ were prepared in methanol (each 25 mM) and appropriate aliquots were mixed such that the $[\text{Zn}^{2+}]:[\text{DPA}]$ molar ratio was 1:1. The solutions were stirred for 1 hr before the solvent was removed and the residue held under vacuum for 1 hr. Each residue was

dissolved in water or 10 % DMSO/water in the case of **3**, **6** and **7** to give a stock solution of each ZnDPA complex.

4.2 Hypoxanthine Incorporation Growth Inhibition Assay

P. Falciparum strains W2 (Indochina) and HB3 (Honduras) were maintained in O-positive human red blood cells (BioChemed, Winchester, VA) and RPMI 1640 medium (Gibco) supplemented with 0.5% Albumax II (Gibco), 0.25% sodium bicarbonate (Corning), and 0.01 mg/mL gentamicin (Gibco) under an atmosphere of 90% N₂, 5% O₂, and 5% CO₂. Cultures underwent at least two life cycles prior to initiation of assays to ensure that normal growth was established. Dose-response curves were generated using a hypoxanthine incorporation assay. [35] Briefly, parasite cultures for assays were maintained as asynchronous cultures and required to reach a parasitemia of no less than 1%, with 70% of parasites in the early ring stage. Sample parasitemia and hematocrit were then adjusted to 0.2% and 2%, respectively, and the samples were added to test plates containing ZnDPA complexes. The appropriate control solution (water or 10% DMSO/water) were added to control wells. Parasites were exposed to drug dilutions for 48 h. [3H]hypoxanthine (PerkinElmer) was added to the plates and incubated for an additional 20 h before freezing at -80 °C for ~24 h. Plate contents were harvested and counted on a Trilux beta counter. The dose-response curves show means for three independent biological replicates.

4.3 SYBR® Green and Giemsa Staining

The *P. Falciparum* 3D7 (Netherland/Africa)[36] and W2 (Indochina) strains were maintained in continuous culture in RPMI-1640 medium (Gibco Grand Island, NY, USA) supplemented with 10% heat-inactivated non-immune AB human serum, 26 mg/L of hypoxanthine (Sigma-Aldrich St. Louis, MO, USA) and 10 mg/L of Gentamicin (Gibco). Uninfected O-positive erythrocytes (Interstate Blood Bank, Memphis, TN) in a 50% suspension were added every two days to maintain the hematocrit at 5%. Cultures were incubated at 37 °C in 5% O₂ and 5% CO₂ atmosphere with daily change of culture medium. Final dilutions of chloroquine (Sigma-Aldrich, St Louis, MO) were prepared in water. 96-well flat-bottom plates were coated with 10 µL of 11 different dilutions of each compound in duplicate in the following range: CQ (3.12 – 3,200 nM); ZnDPA complexes (4.88 – 5000 nM).

A parasite suspension of each strain with parasitemia at 0.5% (ring form higher than 80% of the total stage form) and hematocrit 1.5% was added (190 µL per well) to the microplates pre-dosed with ZnDPA and then incubated at 37 °C in an atmosphere of 5% O₂ and 5% CO₂ for 72 hr. For control parasites wells, appropriate 10 µL aliquots of water or 10% DMSO/water were added. After incubation, the plates were freeze-thawed at -80 °C and growth inhibition was determined using a SYBR® Green I-based method as previously described. [37, 38] Briefly, 100 µL of freeze-thaw homogenate were transferred to 96-well black plates. 100 µL of SYBR® Green lysis buffer I (2x SYBR® Green I, Thermo Fisher Scientific), Tris-HCl (100mM, pH 7.5), EDTA (10 mM), Saponin (0.016% w/v), and Triton X-100 (1.6% v/v) were added to each well. The plates were incubated in darkness at room temperature for 1 hr. The fluorescence was measured using a fluorescence plate reader (Synergy HT, Biotek Instruments, Inc., Winooski, VT, USA) at 485 nm wavelength using Gen 5 2.01 software. The 50 % inhibitory concentrations (IC₅₀) were calculated using the online program

ICEstimator 1.2 (<http://www.antimalarial-icestimator.net/>) [39] IC₅₀ values were acceptable only if the ratio of fluorescence intensity between the parasite at concentration 0 versus FU at maximum concentration, was equal or higher than 2.

Morphological assessment of 3D7 parasites after treatment with **mSeek** or control **BODIPY dye** was performed using a Giemsa staining procedure. A 200 μ L suspension of parasites at a parasitemia of 1% and a hematocrit of 1.5% was exposed at 100 nM of **mSeek** or **BODIPY dye** for a 24 hr period. After exposure, the parasite samples were stained with Giemsa using a 10% w/v aqueous solution for 15 minutes. Micrographs were acquired using an Olympus BX41 microscope with a 100 $\times$ objective and QIClic CCD Camera (QImaging).

4.4 Fluorescence Microscopy

Samples of *P. falciparum* W2 infected RBCs were aliquoted into 1.5-mL microcentrifuge tubes, followed by centrifugation (500 rpm, 5 min). The infected RBCs were resuspended in 1 mL of sterile Phosphate Buffered Saline (PBS, 10 mM PO₄³⁻, 145 mM NaCl, 2.6 mM KCl, pH 7.4) and treated for 15 min with ZnDPA probe **mSeek** or a control **BODIPY dye** (Abs max: 499 nm; Em max: 520 nm) (5 μ M). The samples were also stained with either Hoechst 33342 nuclear stain (Hoechst) or propidium iodide (PI). Hoechst-stained samples were co-treated with **mSeek** or control **BODIPY dye** using a 20 mM stock solution (Thermo Fischer Scientific) to reach a final concentration of 1 μ M in the sample for 15 minutes. Samples stained with PI were permeabilized with a PBS solution containing 0.01% saponin prior to incubation with PI (Sigma-Aldrich, 5 μ M) for 30 minutes. The samples were washed twice with fresh buffer to reduce background fluorescence, dispersed into fresh solution, and then aliquoted onto glass slides. Micrographs were acquired using a Nikon Eclipse TE2000-U epifluorescence microscope with a 10 $\times$ or 60 $\times$ objective and a Photometrics Cascade 512B CCD. Fluorescence images were captured using UV (excitation, 340/80 nm; emission, 435/85 nm), GFP (450/90, 500/50), and Cy5 (620/60, 700/75) filter sets.

4.5 Mammalian Cell Culture and Toxicity

Chinese hamster ovary cells (CHO-K1) were purchased from the American Type Culture Collection (CCL-61). Cells were grown to a confluence of 85% in F-12K media supplemented with 10% FBS and 1% streptavidin L-glutamate at 37°C and 5% CO₂. 10⁵ cells/mL were seeded in a 96-well plate and incubated in the presence of increasing concentrations of ZnDPA complex for 72 h at 37 °C, along with the appropriate solvent controls. Afterward, 20 μ L of CellTiter-Blue reagent was added to 100 μ L of the cell culture, followed by a 4-h incubation period at 37°C, and the fluorescence was measured (excitation, 555 nm; emission, 580 nm) using a Typhoon FLA 9500 laser scanner (GE Healthcare) and analyzed with ImageQuant TL software (GE Healthcare). The output fluorescence values were background subtracted from wells containing medium alone and normalized to wells containing untreated cells. Normalized fluorescence values were plotted against the ZnDPA complex concentration using GraphPad Prism 5 software.

4.6 Red Blood Cell (RBC) Lysis

RBC lysis was measured using a literature procedure.[40] Briefly, O-positive human red blood cells (BioChemed, Winchester, VA) were centrifuged at $500 \times g$ for 5 min, then the plasma was aspirated and discarded. The RBCs were washed two times with a 150 mM NaCl solution followed by replacement with PBS at pH 7.4. Aliquots (100 μ L) of RBCs suspended in PBS were added to 96 well plates. Increasing concentration of each ZnDPA complex was added to the wells in triplicate. For positive control wells, 10 μ L of 20% Triton X-100 were added. For negative control wells, 10 μ L of 10% DMSO/water were added. The plates were incubated at 37 °C and continuously rocked for three hours. After incubation, the plates were centrifuged for 5 min at $500 \times g$ to pellet intact erythrocytes. Using a multichannel pipet, 100 μ L of supernatant from each well were carefully transferred into clear, flat-bottomed 96-well plates. The supernatant absorbance ($\lambda = 450$ nm) was measured using a SpectraMax Plus 384 Microplate Reader (Molecular Devices). Each sample was background subtracted from negative control wells and normalized to positive control wells which represent 100% hemolysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

[A] Representative low magnification (10 $\times$) micrographs of red blood cells infected with *P. falciparum* W2 stained with Hoechst and either **mSeek** (*top*) or control **BODIPY Dye** (*bottom*) (5 μ M) for 10 minutes prior to blue and green fluorescence imaging. The yellow arrows indicate the location of iRBCs as determined from Hoechst staining. Scale bar = 20 μ m. [B] High magnification (60 $\times$) widefield micrographs of red blood cells infected with *P. falciparum* W2 stained with propidium iodide and **mSeek** (5 μ M) for 10 minutes prior to red and green fluorescence imaging. Scale bar = 15 μ m [PRINT FIGURE IN COLOR]

Figure 2. Brightfield microscopy of *P. falciparum* 3D7 infected red blood cells treated with **mSeek** (*top*) or control **BODIPY Dye** (*bottom*) (100 nM) for 24 hours followed by Giemsa staining. Red arrows indicate iRBCs. Scale bar = 15 μm [PRINT FIGURE IN COLOR]

Scheme 1.

Structures of compounds used in this study. All ZnDPA complexes were prepared and tested as nitrate salts.

Scheme 2.
Synthesis of precursor ligands.

Table 1

Inhibitory (IC₅₀) and hemolytic activity (HC₅₀) against mammalian cells, healthy red blood cells (RBCs), and RBCs infected with *P. falciparum* W2, HB3 and 3D7 strains. The values represent means ± standard error for 3 independent experiments.

		<i>P. falciparum</i>				
ZnDPA Complex		CHO-K1 IC ₅₀ (μM)	RBC HC ₅₀ (μM)	W2 IC ₅₀ (nM)	HB3 IC ₅₀ (nM)	3D7 IC ₅₀ (nM)
mSeek		44 ± 4	>100	15 ± 3	5 ± 2	n.m.*
BODIPY Dye		88 ± 4	>100	>100	>100	n.m.*
1		38 ± 1	>100	394 ± 49	135 ± 10	251 ± 11
2		78 ± 2	>100	221 ± 75	103 ± 8	196 ± 21
3		17 ± 2	42 ± 3	527 ± 88	195 ± 39	436 ± 23
4		>100	>100	347 ± 73	290 ± 28	567 ± 23
5		>100	95 ± 4	217 ± 64	540 ± 91	205 ± 91
6		>100	>100	198 ± 31	89 ± 34	424 ± 26
7		>100	>100	161 ± 52	50 ± 12	360 ± 30
8		>100	>100	79 ± 14	23 ± 3	142 ± 34
Chloroquine		n.m.*	n.m.*	488 ± 69	96 ± 8	52 ± 12

*
n.m. = not measured