

Investigating the Antimalarial Action of 1,2,4-Trioxolanes with Fluorescent Chemical Probes

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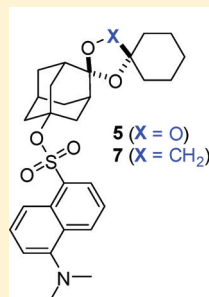
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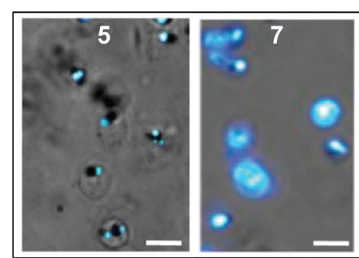
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Supporting Information

ABSTRACT: The 1,2,4-trioxolanes are a new class of synthetic peroxidic antimalarials currently in human clinical trials. The well-known reactivity of the 1,2,4-trioxolane ring toward inorganic ferrous iron and ferrous iron heme is proposed to play a role in the antimalarial action of this class of compounds. We have designed structurally relevant fluorescent chemical probes to study the subcellular localization of 1,2,4-trioxolanes in cultured *Plasmodium falciparum* parasites. Microscopy experiments revealed that a probe fluorescently labeled on the adamantane ring accumulated specifically in digestive vacuole-associated neutral lipid bodies within the parasite while an isosteric, but nonperoxidic, congener did not. Probes fluorescently labeled on the cyclohexane ring showed no distinct localization pattern. In their subcellular localization and peroxidative effects, 1,2,4-trioxolane probes behave much like artemisinin-based probes studied previously. Our results are consistent with a role for adamantane-derived carbon-centered radicals in the antimalarial action of 1,2,4-trioxolanes, as hypothesized previously on the basis of chemical reactivity studies.



Peroxide-dependent localization of trioxolane **5** to neutral lipid bodies in *Plasmodium falciparum* parasites

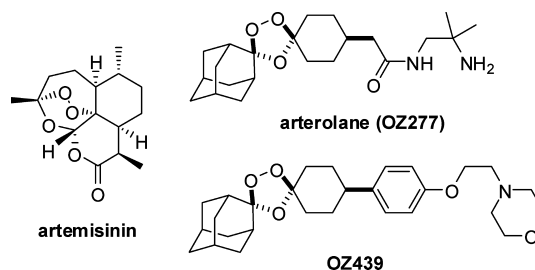


INTRODUCTION

The World Health Organization has recommended artemisinin combination therapy (ACT) as the preferred first-line treatment for uncomplicated *Plasmodium falciparum* malaria.¹ Artemisinin and its semisynthetic analogues are derived ultimately from the plant source, *Artemisia annua*. The need for affordable and reliably available antimalarial therapies has spurred research into alternate methods for their manufacture² and also the search for wholly synthetic molecules that confer an analogous antimalarial activity. An important advance with regard to the latter has been the appearance of various synthetic peroxides,³ most notably the investigational agent arterolane (OZ277),⁴ which contains a sterically hindered 1,2,4-trioxolane ring (Chart 1). Arterolane is currently in phase III clinical trials in combination with piperazine, whereas a next-generation trioxolane (OZ439) with improved in vivo properties is in early stage clinical trials.⁵

The antimalarial action of the artemisinins, 1,2,4-trioxolanes, and other synthetic peroxides is clearly associated with the presence of a peroxide bond, although the precise molecular mechanism of activity remains a topic of active research and healthy debate. Largely undisputed in the field is the

Chart 1

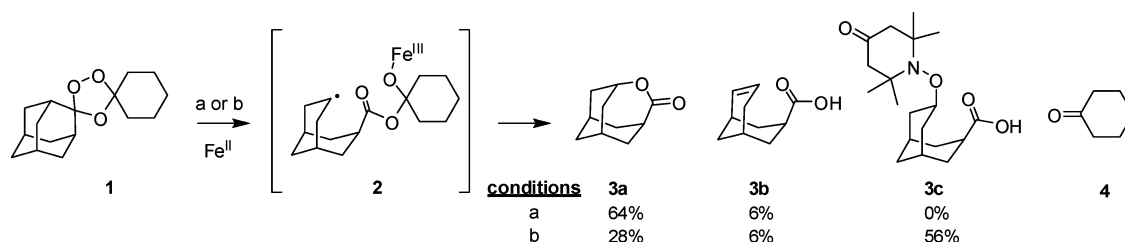


importance of ferrous iron (putatively inorganic Fe^{II}, free heme, or ferrous hemoglobin heme) in the activity of antimalarial peroxides, and the likely involvement of primary and/or secondary carbon-centered radical metabolites.^{3,6,7} For example, isosteric but nonperoxidic analogues of artemisinin and arterolane are generally at least 1000-fold less potent against *P. falciparum* parasites^{8,9} and the nonperoxidic isosteres do not antagonize the activity of their peroxidic congeners.¹⁰

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Scheme 1. Likely Mechanism and Observed Products^{7,10} in the Reaction of 1,2,4-Trioxolane 1 with Inorganic Ferrous Iron^a

^aReaction conditions: (a) 1.0 equiv ferrous bromide, tetrahydrofuran; (b) 1.5 equiv ferrous acetate, water–acetonitrile (1:1), 2.0 equiv 4-(oxo)-TEMPO.

Also favoring an iron-dependent activation hypothesis are findings that artemisinin and arterolane are potently active primarily against hemoglobin digesting parasites⁸ while iron chelating reagents like desferrioxamine (DFO) antagonize their action against *P. falciparum*.^{9,11}

The *P. falciparum* sarco/endoplasmic reticulum Ca²⁺ ATPase (PfATP6/SERCA) has been proposed as a specific target of the artemisinins.⁹ However, recent studies have questioned this hypothesis. The Ca²⁺-ATPase activity of purified PfATP6 was insensitive to inhibition by artemisinin and derivatives but sensitive to known SERCA inhibitors.¹² Morphological studies in *P. falciparum* have shown that initial cellular damage by artemisinin and experimental trioxanes is directed toward the digestive vacuole (DV) as opposed to the endoplasmic reticulum at relevant drug concentrations.¹³ Arterolane binds PfATP6 about 5000-fold more weakly ($K_i = 7900$ nM) than its antimalarial potency, making this an unlikely target of trioxolanes.¹¹

Other investigators have advanced a role for heme–artemisinin and heme–trioxolane conjugates in the antimalarial action of these agents. Supporting this view are findings that artemisinins alkylate human ferrous hemoglobin in vitro¹⁴ and that heme–artemisinin conjugates could be isolated from artemisinin-treated mice infected with *P. vinckei* but not from uninfected mice given an equivalent dose of drug.¹⁵ Attempts to correlate antimalarial activity with in vitro reactivity toward heme have been complicated by the fact that artemisinins are generally less efficient than trioxolanes at alkylating heme in vitro^{16,17} and are similarly poor at intercepting radical spin traps such as (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO).^{10,17} Thus it is problematic to interpret such correlation data across structural classes of peroxides. However, within a congeneric series of 1,2,4-trioxolane analogues, antimalarial activity was indeed well correlated with the extent of heme alkylation in vitro.¹⁶ Finally, a recent finding¹⁸ that artemisinin action requires the presence of hemoglobin degradation products is also consistent with a role for heme in the mode of action of artemisinins.

Heme conjugates have been proposed to confer parasite toxicity by interference with hemozoin formation¹⁹ or by disruption of DV morphology.¹³ One of us (Cooper) recently demonstrated with fluorescently labeled probes the peroxide-dependent localization of artemisinin species (or their reaction products) within DV-associated neutral lipid bodies (NLB).²⁰ This localization was correlated with oxidative damage to cellular lipids as determined in situ using an oxidation sensitive BODIPY lipid probe and also via analysis of lipids extracted from treated parasites. Recent experimental work suggests that neutral lipids can serve as a catalyst for hemozoin formation within the DV²¹ and that 1,2,4-trioxolanes (and also

tetraoxanes) mediate phospholipid peroxidation in vitro upon exposure to ferrous iron sources.²² Hence, current evidence indicates that both free ferrous heme and neutral lipids are present in the DV, producing a chemical environment favorable for the homolytic cleavage of peroxide bonds. We now describe the design and synthesis of a new series of fluorescent chemical probes intended to reveal the intracellular reactivity and subcellular localization of 1,2,4-trioxolane antimalarials and their reaction products.

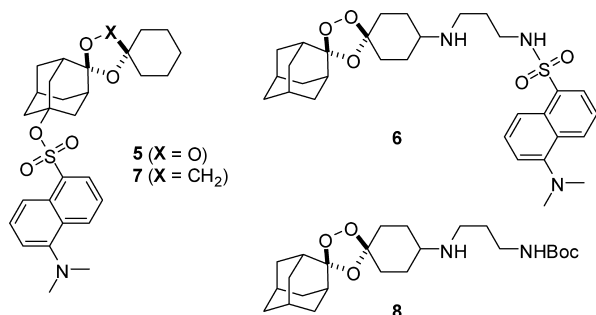
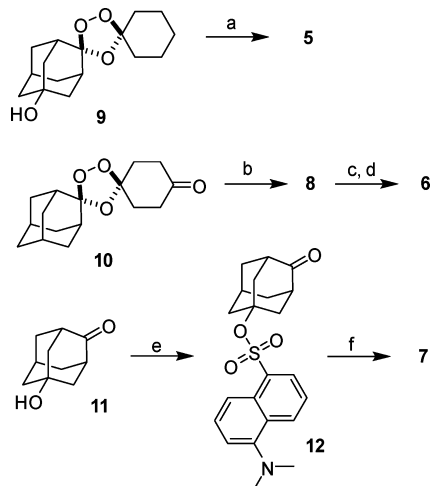
RESULTS AND DISCUSSION

Structurally relevant fluorescent probes were designed to explore the intracellular reactivity of 1,2,4-trioxolanes and more specifically to determine if such reactivity was consistent with trioxolane fragmentation mechanisms proposed previously⁷ on the basis of in vitro chemical reactivity studies. In the canonical mechanism (Scheme 1), reduction of the peroxide bond with Fe^{II} occurs regioselectively at the less hindered oxygen atom, presumably via the equatorial-peroxide conformer which best exposes the peroxide σ^* antibonding orbital for reaction. An initial oxygen-centered radical quickly collapses to a secondary carbon-centered radical 2, which reacts further to afford products of radical self-quenching (3a and 3b) and cyclohexanone (4). When the reaction is carried out in the presence of a radical spin trap reagent such as 4-(oxo)-TEMPO, high yields of the adamantane-derived TEMPO conjugate 3c are obtained.¹⁰ It is the adamantane-derived radical species 2 that is proposed to confer antimalarial effects in parasites, putatively via reaction with heme¹⁶ and/or by mediating the peroxidation of phospholipid.^{20,22}

If the chemical processes described above occur also in the parasite, one predicts that adamantane- and cyclohexane-derived reaction products should have different subcellular localization patterns. In recent studies employing cyclohexane-linked fluorescent probes, only diffuse labeling of the parasite cytosol or DV was observed.^{11,23} These studies did not address the possibility that such probes might well fragment within the parasite to produce fluorescently labeled cyclohexanone products without any predicted target or role in antimalarial action. To reveal the fates of both halves of 1,2,4-trioxolanes, we designed the probes 5 and 6, in which a dansyl fluorophore is attached via the adamantane or cyclohexane ring, respectively (Chart 2). The nonperoxidic dioxolane probe 7 was prepared to test the peroxide-dependence of antimalarial activity and subcellular localization, while the nonfluorescent trioxolane 8 was used in competition experiments and in experiments with the BODIPY lipid probe.

Compounds 5–8 were synthesized using established synthetic methods (Scheme 2).²⁴ No attempt was made to isolate the final compounds as single stereoisomers, a decision

Chart 2

Scheme 2. Synthesis of Chemical Probes 5–8^a

^aConditions: (a) dansyl chloride, DMAP, pyridine, 29%; (b) *N*-Boc-1,3-propanediamine, NaBH(OAc)₃, dichloroethane, 95%; (c) 4N HCl, dioxane; (d) dansyl chloride, triethylamine, dichloromethane, 8% over two steps; (e) dansyl chloride, DMAP, pyridine, 14%; (f) 1-(hydroxymethyl)cyclohexanol, camphor sulfonic acid, dichloromethane, 72%.

that was largely pragmatic but is justified in light of previous findings that stereoisomeric 1,2,4-trioxanes possess similar antimalarial activities.²⁵ The antimalarial activity of compounds 5–8, artemisinin, and arterolane⁴ were determined using a standard 72 h microplate assay with SYBR Green I detection to determine the half-maximal inhibitory concentration (IC₅₀) of each compound against *P. falciparum* parasites (Table 1).^{26,27}

Table 1. Growth Inhibitory Activities of Artemisinin, Arterolane, and Compounds 5–8 against Intraerythrocytic Ring Stage *P. falciparum* Parasites

compd	IC ₅₀ ± SEM (nM)	<i>P. falciparum</i> strain
artemisinin	8.0 ± 0.1	FCB
arterolane	2.5 ± 0.3 ^a	K1
5	17.9 ± 0.9	FCB
6	14.6 ± 1.1	FCB
7	24990 ± 1363	FCB
8	12.6 ± 0.4	FCB

^aIC₅₀ value from ref 4.

Importantly, the fluorogenic probes 5 and 6 retain a low nanomolar effect on parasites, as does compound 8. As expected, the nonperoxidic dioxolane probe 7 was at least 1000-fold less potent than 5 against parasites. Thus, the introduction

of the dansyl group does not prevent permeability across erythrocyte and parasite membranes; compounds 5–8 appear to be valid probes for studies of trioxolane action.

Next we examined fluorescence localization of compounds 5–8 in living intraerythrocytic *P. falciparum* parasites. Similar to previous findings^{11,23} with other cyclohexane-linked probes, the trioxolane probe 6 demonstrated a cytoplasmic accumulation pattern within mature parasites, with no distinct subcellular localization (Figure 1A). In contrast, we found that application of an equimolar concentration (500 nM) of the adamantyl-linked trioxolane 5 resulted in a punctate localization within small spherical structures closely associated with the parasite DV (Figure 1B). This precise compartmentalization pattern has been demonstrated previously by our laboratory to be evidence of fluorescent trioxane metabolite accumulation within parasite NLB.²⁰ NLB are small droplets consisting of neutral lipids hypothesized to promote hemozoin crystallization following hemoglobin digestion and free heme release within the parasite DV.^{28,29} In contrast to our observations with trioxolane 5, we found that the isosteric but nonperoxidic dioxolane probe 7 failed to exhibit NLB localization and instead distributed broadly throughout the parasite cytosol and membranous structures (Figure 1C). As noted in previous *in vitro* studies, the adamantane ring is the site of carbon radical formation (Scheme 1) as well as the site of covalent reaction with heme *in vitro*.¹⁶ Therefore, the specific localization to NLB observed with trioxolane 5 but not trioxolane 6 strongly suggests that NLB accumulation is associated with adamantane-derived products, possibly including heme conjugates. Our finding that dioxolane 7 accumulation patterns did not mirror those of trioxolane 5, indicates that localization of fluorescent metabolites within NLB is endoperoxide-dependent and therefore almost certainly involves reaction with heme or other ferrous iron sources in the parasite. The very different localization patterns observed for analogues 5–7 also establishes that NLB accumulation of 5 is not an artifact of the dansyl moiety itself.

Specific and endoperoxide-dependent trioxolane localization within NLB was clearly demonstrated by competition experiments between nonfluorescent trioxolane 8 and trioxolane 5. We found that pretreatment with trioxolane 8 significantly ($n = 3$; $p = 0.009$) reduced the number of detectable fluorescent NLB by $66.8 \pm 10.2\%$ within mature parasites labeled by trioxolane 5 (Figure 2B). In contrast, pretreatment with methanol vehicle had no effect on trioxolane 5 distribution, as evidenced by the expected exclusive pattern of fluorescent accumulation within NLB (Figure 2A). The ability of the nonfluorescent trioxolane 8 to competitively block NLB accumulation by fluorescent trioxolane 5 supports a common mechanism of action for these two trioxolane analogues and further suggests a *specific reaction partner* (plausibly free heme) that is depleted by pretreatment with 8 in this experiment.

Earlier research into the possible mechanism of trioxane (artemisinin) action provides evidence that artemisinin is capable of mediating the peroxidation of a parasite membrane-incorporated peroxy radical sensor (BODIPY 581/591 C₁₁) following a relatively short incubation period (~5 h), thereby suggesting that oxidation reactions initiated directly by the endoperoxide or by redox-active heme–artemisinin conjugates may ultimately lead to parasite death.²⁰ To examine the ability of trioxolanes to induce peroxidation reactions in the parasite, we incubated parasite cultures at each major asexual stage for 2.5 h with 250 nM of trioxolane 8, artemisinin, or the

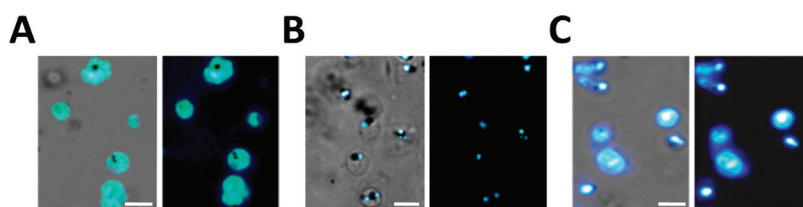


Figure 1. Accumulation of adamantyl-tagged trioxolane derivatives within NLB of mature *P. falciparum* parasites is endoperoxide-dependent. Parasites were incubated with 500 nM of (A) trioxolane 6, (B) trioxolane 5, or (C) inactive trioxolane 7. Fluorescence from trioxolane 6, containing the cyclohexane dansyl fluorophore, distributes within the parasite cytoplasm uniformly (A), while the adamantyl-tagged trioxolane 5 signal is seen only within spherical NLB (B). In contrast to trioxolane 5, the inactive trioxolane derivative 7 distributes indiscriminately in the parasite, suggesting specific NLB localization requires endoperoxide activation. Images show merged fluorescence with phase contrast view (left) and fluorescence view only (right). Scale bars represent 5 μ m.

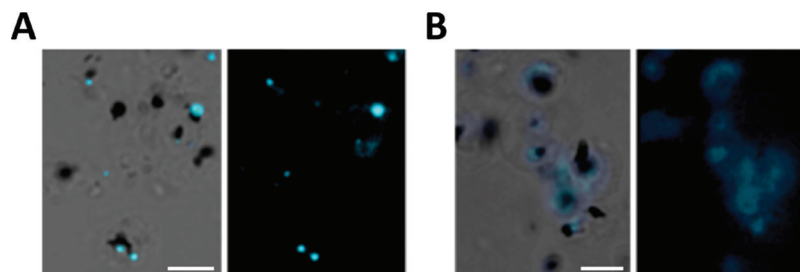


Figure 2. Preincubation with unlabeled trioxolane 8 decreases trioxolane 5 accumulation within NLB of late-stage parasites. Cultures were exposed for 10 min to either (A) 0.01% MeOH (drug vehicle) or (B) 500 nM trioxolane 8, followed by a 10 min exposure to 500 nM trioxolane 5. (A) Cultures preincubated with MeOH show intense labeling of NLB upon exposure to trioxolane 5 compared to (B) parasites preincubated with trioxolane 8, demonstrating competition between the two compounds. Images show phase contrast merged with fluorescence view (left) and fluorescence view only (right). Scale bars represent 5 μ m.

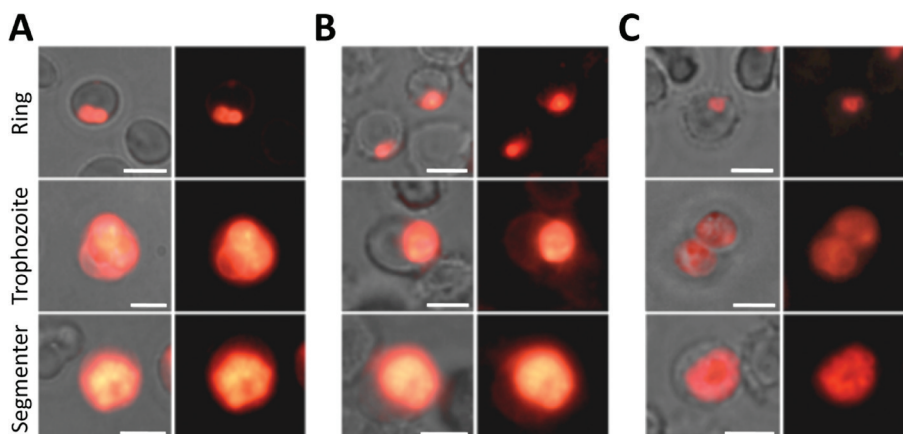


Figure 3. Trioxolane exposure induces peroxidation of parasite membrane-incorporated BODIPY 581/591 C_{11} radical sensor probe, similar to the trioxane artemisinin. Cultures were treated for 2.5 h with 250 nM (A) trioxolane 8, (B) artemisinin, or (C) the nonperoxidic artemisinin analogue dDHA, prior to a 0.5 h exposure to BODIPY probe. Rows represent asexual parasite stages. Trioxolane exposure induces probe peroxidation (indicated by the yellow emission shift) in all asexual stages, similar to the artemisinin control group, while the inactive derivative fails to induce probe peroxidation. Left columns show merged fluorescence with phase contrast view, while right columns show fluorescence only. Scale bars represent 5 μ m.

nonperoxidic artemisinin derivative deoxydihydroartemisinin (dDHA), followed by 0.5 h incubation with BODIPY 581/591 C_{11} (Figure 3). dDHA is roughly 100-fold less potent than typical artemisinin derivatives.²⁰ The BODIPY probe is a useful peroxy radical oxidation sensor as radical cleavage of the butadienyl group results in a loss of red spectral emission.³⁰ We found that exposure to trioxolane 8 induced peroxidation of membrane-incorporated probe in all asexual stages (Figure 3A), similar to the artemisinin control group (Figure 3B), as evidenced by a shift from red to yellow/green fluorescence. Late-stage parasites exhibited a greater degree of peroxidation than ring stages. In contrast, the dDHA derivative failed to

induce peroxidation at any asexual stage, confirming that peroxidation was endoperoxide-dependent (Figure 3C).

CONCLUSIONS

Initial studies with 5–8 suggest that these compounds are useful probes for dissecting the reactivity and localization of 1,2,4-trioxolanes in live *P. falciparum* parasites. Our initial studies provide evidence that the trioxolane ring does fragment within parasites and that fragmentation is accompanied by the accumulation in NLB of reaction products derived from the adamantane moiety. These results are thus consistent with the fragmentation mechanism proposed previously on the basis of

in vitro chemical reactivity studies and indirectly support the proposed involvement of covalent heme adducts, which may become localized in NLB due to the presence of free heme in DV lipids. Our findings further suggest a common mechanism of action for trioxolane and trioxane antimalarials involving peroxidative damage to the parasite mediated either by the endoperoxide species itself and/or by covalent adducts derived from them.

EXPERIMENTAL SECTION

In Vitro Cultures of *Plasmodium falciparum* Parasites. *P. falciparum* strains FCB and 7G8 (ATCC repository; MRA-926) were cultured in either human A⁺ or AB⁺ erythrocytes at 37 °C using RPMI 1640 media supplemented with 0.25% NaHCO₃, 25 mM HEPES, 0.37 mM hypoxanthine, 0.01 mg/mL gentamicin, and either 0.5% Albumax I (Invitrogen) or 10% human serum, under an atmosphere of 90% nitrogen, 5% oxygen, and 5% carbon dioxide.

Susceptibility Testing. Test compounds were prepared as 10 mM stock solutions in DMSO or MeOH and stored at −30 °C. The in vitro susceptibility of *P. falciparum* to test compounds was measured in a 72 h microplate growth inhibition assay using SYBR Green I (Molecular Probes) detection as described.^{26,27} Parasites were introduced into the assay only when a majority of the parasites (typically ≥80%) were at ring stage. Mean half-maximal inhibitory concentrations (IC₅₀ values) were derived by plotting percent growth inhibition against log compound concentration and fitting the response data to a variable slope sigmoidal curve-fit function using Prism 5.0d (GraphPad, San Diego, CA). IC₅₀ values represent means and standard error from at least three independent assays.

Fluorescence Microscopy and Image Analysis. For live cell imaging, culture samples were washed twice with PBS and mounted under coverslips with Slowfade antifade reagent (Invitrogen) in PBS. Samples were viewed with 100× oil-immersion on either an Olympus BX61 or an AX70 Olympus PROVIS U-CMAD3 epifluorescence microscope equipped with a 100 W mercury bulb using U-MWU or U-MWB filter sets. Micrographic images were recorded using the Olympus MicroSuite (TM)-B3SV or Olympus DP70 camera system and compiled using the Olympus DP Manager software and Adobe Photoshop CS v8.0. Mixed-stage *P. falciparum* cultures were exposed to 500 nM concentrations of trioxolane **5**, **7**, or **8** for 10 min in RPMI at 37 °C. Parasites were viewed directly or were first released from erythrocytes using 0.2% saponin.

Competition Experiments. Mid- to late-trophozoite-stage cultures were exposed to 500 nM trioxolane **8** or drug-vehicle (0.01% MeOH) in prewarmed complete media and incubated at 37 °C. After 10 min, the media was removed and replaced with fresh, prewarmed complete media containing 500 nM of trioxolane **5**. Following a 10 min incubation period, parasites were washed, released with 0.2% saponin, and processed for microscopy. The total number of NLB positive for detectable trioxolane **5** fluorescence from each pretreatment group was tabulated from ~10 random fields of view in three independent experiments (approximately 300 total parasites per treatment group) and divided by the total number of parasites counted. Results were reported as the average percent decrease in fluorescently labeled NLB among the trioxolane **8** pretreatment groups compared to the MeOH pretreatment control group. Statistical significance of pretreatment effects on the average accumulation of trioxolane **5** signal between the two pretreatment groups was evaluated using the student's *t* test (*p* < 0.05).

Lipid Peroxidation Studies Using BODIPY 581/591 C₁₁. BODIPY 581/591 C₁₁ peroxidation-sensitive probe (Molecular Probes) was reconstituted to 40 μM in DMSO and stored under N₂. At each asexual stage, cultures were exposed to 250 nM trioxolane **8**, artemisinin, dHA,²⁰ or vehicle control (0.01% MeOH) for 2.5 h at 37 °C. Cultures were then treated with 40 nM BODIPY 581/591 C₁₁ and incubated for an additional 30 min prior to imaging. Ring and segmenter-stage parasites occurred simultaneously in culture and were not saponin-released due to difficulty in pelleting freed ring-stage parasites, whereas trophozoite-stage parasites were released from erythrocytes using 0.2% saponin prior to visualization.

Synthesis: General Methods. ¹H NMR spectra were recorded on a Varian INOVA-400 400 MHz spectrometer. Chemical shifts are reported in δ units (ppm) relative to TMS as an internal standard. Coupling constants (*J*) are reported in hertz (Hz). Synthetic intermediates 1-(hydroxymethyl)cyclohexanol, **9**, and **10** were prepared according to literature methods as indicated below. All other reagents and solvents were purchased from Aldrich Chemical or Acros Organics and used as received. Column chromatography was carried out using a Biotage SP1 flash chromatography system and silica gel cartridges from Biotage. Analytical TLC plates from EM Science (Silica Gel 60 F254) were employed for TLC analyses.

Purity of Final Compounds. All compounds studied in parasites (**5**–**8**) were judged to be of 95% or higher purity based on analytical LC/MS analysis. LC/MS analyses were performed on a Waters Micromass ZQ/Waters 2795 separation module/Waters 2996 photodiode array detector system controlled by MassLynx 4.0 software. Separations were carried out on an XTerra MS C₁₈ 5 μm 4.6 mm × 50 mm column at ambient temperature using a mobile phase of water–acetonitrile containing 0.05% trifluoroacetic acid. Gradient elution was employed wherein the acetonitrile–water ratio was increased linearly from 5 to 95% acetonitrile over 2.5 min, then maintained at 95% acetonitrile for 1.5 min, and then decreased to 5% acetonitrile over 0.5 min and maintained at 5% acetonitrile for 0.5 min. Compound purity was determined by integrating peak areas of the liquid chromatogram, monitored at 254 nm.

Synthesis of Dispiro[adamantane-2,2'-[1,3,5]trioxolane-4',1'-cyclohexane]-5-yl 5-(Dimethylamino)naphthalene-1-sulfonate (5**).** To a solution of alcohol **9**³¹ (0.15 g, 0.54 mmol) and 4-(dimethylamino)pyridine (0.065 g, 0.54 mmol) in anhydrous pyridine (3 mL) at 0 °C was added dropwise a solution of dansyl chloride (0.29 g, 1.07 mmol) in dichloromethane (0.2 mL). The reaction was warmed slowly to room temperature and stirred overnight, at which time an additional 5 equiv of dansyl chloride were added and the reaction left to stir for an additional 72 h. The reaction mixture was then concentrated in vacuo, and ethyl acetate and water were added. The aqueous layer was separated and extracted with EtOAc (3 × 5 mL). The organic layers were combined and washed with water and brine and then dried (MgSO₄), filtered, and concentrated. The crude residue was purified by automated silica gel flash chromatography (7% EtOAc/hexanes) to afford the trioxolane **5** as a mixture of stereoisomers (0.079 g, 0.15 mmol, 29%): IR 2938, 2864, 2787, 1574, 1451, 1354, 1173, 1115, 1092, 1071 cm^{−1}. ¹H NMR (400 MHz, CDCl₃) δ 8.56 (dd, *J* = 8.4, 3.6 Hz, 1H), 8.22–8.31 (m, 2H), 7.49–7.60 (m, 2H), 7.19 (dd, *J* = 7.6, 3.2 Hz, 1H), 2.89 (s, 6H), 2.26–2.50 (m, 2H), 2.03–2.21 (m, 7H), 1.79–1.91 (m, 2H), 1.50–1.70 (m, 10H), 1.30–1.45 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 151.8, 135.8, 135.7, 130.9, 130.0, 129.9, 128.7, 128.4, 128.4, 123.3, 123.3, 119.9, 119.9, 115.5, 115.4, 109.8, 109.7, 109.1, 109.0, 90.2, 89.5, 45.6, 42.2, 42.0, 39.9, 39.7, 39.0, 38.7, 34.8, 34.6, 33.0, 33.0, 30.0, 29.6, 25.0, 23.9. LRMS calculated for C₂₈H₃₅NO₆ 513.65, found (*M* + *H*⁺) 514.7.

Synthesis of 5-(Dimethylamino)-N-[3-((dispiro[adamantane-2,2'-[1,3,5]trioxolane-4',1'-cyclohexane]-4"-yl)amino)propyl]naphthalene-1-sulfonamide (6**).** The amine **8** (0.30 g, 0.69 mmol) was dissolved in a 4N solution of HCl in dioxane (3.6 mL). The reaction mixture was stirred for 30 min and then concentrated in vacuo. A quarter of the crude product was dissolved in dichloromethane (0.5 mL) and treated with dansyl chloride (0.047 g, 0.17 mmol) and triethylamine (0.06 mL, 0.4 mmol). The reaction mixture was stirred overnight protected from light. The reaction mixture was then loaded directly onto a silica gel column and eluted with 2–5% MeOH/CH₂Cl₂ to provide the trioxolane **6** as a mixture of stereoisomers (8.1 mg, 0.014 mmol, 8% over 2 steps). ¹H NMR (400 MHz, CDCl₃) δ 8.49–8.55 (m, 1H), 8.20–8.33 (m, 2H), 7.47–7.59 (m, 2H), 7.15–7.21 (m, 1H), 2.97–3.04 (m, 2H), 2.89 (s, 6H), 2.57–2.64 (m, 2H), 2.35–2.45 (m, 1H), 1.48–2.02 (m, 22H), 1.25–1.34 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 152.1, 135.0, 130.2, 130.0, 129.8, 129.7, 128.4, 123.3, 119.1, 115.3, 111.6, 108.3, 55.1, 46.1, 45.6, 43.8, 36.9, 36.5, 35.0, 34.9, 32.9, 30.0, 28.0, 27.0, 26.6. LRMS calculated for C₃₁H₄₃N₃O₅S 569.29, found (*M* + *H*⁺) 570.25.

Synthesis of Dispiro[adamantane-2,2'-[1,3]dioxolane-4',1''-cyclohexane]-5-yl 5-(Dimethylamino)naphthalene-1-sulfonate (7). To a mixture of ketone **12** (0.050 g, 0.125 mmol) and 1-(hydroxymethyl)-cyclohexanol³² (0.021 mg, 0.16 mmol) in dichloromethane (1.5 mL) was added camphor sulfonic acid (0.035 g, 0.15 mmol). The reaction mixture was stirred 48 h at room temperature and then was washed sequentially with a saturated solution of NaHCO₃, water, and brine, and dried (MgSO₄), filtered, and concentrated. The crude residue was purified by automated silica gel chromatography (10% EtOAc in hexanes) to provide **7** as a mixture of stereoisomers (0.046 g, 0.09 mmol, 72%): IR 2933, 2860, 2788, 1589, 1576, 1478, 1453, 1387, 1354, 1342, 1203, 1193, 1173, 1123, 1100, 1062, 1045, 1024 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) δ 8.53–8.56 (m, 1H), 8.23–8.33 (m, 2H), 7.50–7.60 (m, 2H), 7.17–7.20 (m, 1H), 3.67–3.68 (m, 2H), 2.89 (s, 6H), 2.41–2.48 (m, 2H), 1.82–2.14 (m, 9H), 1.32–1.66 (m, 12H). ¹³C NMR (100 MHz, CDCl₃) δ 151.8, 151.7, 163.0, 130.8, 130.8, 130.1, 130.0, 129.9, 128.37, 128.7, 128.3, 128.3, 123.3, 120.1, 115.4, 109.3, 91.3, 91.0, 81.0, 80.9, 74.1, 73.6, 45.6, 42.6, 42.4, 40.8, 40.7, 40.0, 39.9, 37.0, 36.9, 33.0, 33.0, 30.2, 29.9, 25.5, 23.8, 23.7. LRMS calculated for C₂₉H₃₇NO₅ S 511.24, found (M + H⁺) 512.5.

Synthesis of tert-Butyl N-[3-((Dispiro[adamantane-2,2'-[1,3,5]trioxolane-4',1''-cyclohexane]-4''-yl)amino)propyl]carbamate (8). To a solution of ketone **10**²⁴ (0.35 g, 1.26 mmol) and commercially available N-Boc-1,3-propanediamine (0.26 g, 1.5 mmol) in dichloroethane (3 mL) was added sodium triacetoxyborohydride (0.40 g, 1.89 mmol). The reaction mixture was stirred for 1 h at ambient temperature and then diluted with dichloromethane, washed with saturated aqueous NaHCO₃ and brine, and dried (Na₂SO₄), filtered, and concentrated. The crude residue was purified by automated silica gel flash chromatography (5% MeOH/CH₂Cl₂) to provide **8** as a mixture of stereoisomers (0.55 g, 1.26 mmol, >95%). ¹H NMR (400 MHz, CDCl₃) δ 5.13–5.17 (m, 0.14H), 3.22–3.23 (m, 2H), 2.70–2.73 (m, 2H), 2.59 (m, 1H), 1.44–2.03 (m, 24H), 1.44 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 156.4, 111.8, 111.5, 108.5, 108.4, 79.2, 55.2, 54.9, 44.9, 44.8, 39.1, 36.9, 36.5, 36.4, 35.0, 34.9, 32.6, 32.2, 30.0, 29.8, 29.4, 28.5, 27.0, 26.6. LRMS calculated for C₂₄H₄₀N₂O₅ 436.29, found (M + H⁺) 437.25.

Synthesis of 4-Oxadadamantan-1-yl 5-(Dimethylamino)naphthalene-1-sulfonate (12). To a solution of 5-hydroxy-2-adamantanone (**11**) (0.15 g, 0.90 mmol) and a catalytic amount of 4-(dimethylamino)pyridine in anhydrous pyridine (1.5 mL) at 0 °C was added dropwise a solution of dansyl chloride (0.49 g, 1.81 mmol) in dichloromethane (0.2 mL). The reaction mixture was warmed to room temperature slowly and stirred overnight, at which time an additional 2 equiv of dansyl chloride were added and the reaction mixture stirred for another 18 h. The reaction mixture was concentrated in vacuo, and dichloromethane and water were added. The organic layer was separated and washed with water and brine and then dried (Na₂SO₄), filtered, and concentrated. The crude residue was purified by automated silica gel chromatography (20% EtOAc in hexanes) to provide 52 mg of the desired product **12** (0.13 mmol, 14%): IR 2936, 2863, 2788, 1725, 1575, 1455, 1397, 1350, 1337, 1234, 1203, 1172, 1153, 1060, 1031 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) δ 8.58 (d, J = 8.8 Hz, 1H), 8.25 (d, J = 7.6 Hz, 2H), 7.51–7.60 (m, 2H), 7.20 (d, J = 7.2 Hz, 1H), 2.89 (s, 6H), 2.61 (br s, 2H), 2.36–2.41 (m, 7H), 1.87–1.98 (m, 4H). ¹³C NMR (100 MHz, CDCl₃) δ 214.2, 152.0, 135.4, 131.3, 130.0, 129.9, 128.9, 128.6, 123.3, 119.6, 115.6, 87.8, 47.4, 45.6, 42.5, 41.8, 37.8, 30.6. LRMS calculated for C₂₂H₂₅NO₄ 399.50, found (M + H⁺) 400.5.

■ ASSOCIATED CONTENT

● Supporting Information

Copies of ¹H NMR spectra for analogues **5**–**8**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS USED

ACT, artemisinin combination therapy; DFO, desferrioxamine; PfATP6, *Plasmodium falciparum* sarco/endoplasmic reticulum Ca²⁺ ATP-ase; SERCA, sarco/endoplasmic reticulum Ca²⁺ ATP-ase; DV, digestive vacuole; TEMPO, (2,2,6,6-tetramethylpiperidin-1-yl)oxyl; NLB, neutral lipid bodies; dDHA, deoxydihydroartemisinin

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