

# In Vitro Susceptibility of *Leishmania infantum* to Artemisinin Derivatives and Selected Trioxolanes

Sofia Cortes,<sup>a,b</sup> Andreia Albuquerque,<sup>a</sup> Lília I. L. Cabral,<sup>c,d</sup> Liliana Lopes,<sup>a,e</sup> Lenea Campino,<sup>a,d,e</sup> Maria L. S. Cristiano<sup>c,d</sup>

Global Health and Tropical Medicine (GHTM), Unidade de Ensino e Investigação de Parasitologia Médica, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), Lisbon, Portugal<sup>a</sup>; Centro de Malária e Outras Doenças Tropicais, IHMT, UNL, Lisbon, Portugal<sup>b</sup>; Centro de Ciências do Mar (CCMAR), Universidade do Algarve (UALg), Campus de Gambelas, Faro, Portugal<sup>c</sup>; Departamento de Química e Farmácia, UALg, Campus de Gambelas, Faro, Portugal<sup>d</sup>; Departamento Ciências Biomédicas e Medicina, UALg, Campus de Gambelas, Faro, Portugal<sup>e</sup>

**Leishmaniasis is among the world's most neglected diseases. Currently available drugs for treatment present drawbacks, urging the need for more effective, safer, and cheaper drugs. A small library of artemisinin-derived trioxanes and synthetic trioxolanes was tested against promastigote and intramacrophage amastigote forms of *Leishmania infantum*. The trioxolanes LC50 and LC95 presented the best activity and safety profiles, showing potential for further studies in the context of leishmanial therapy. Our results indicate that the compounds tested exhibit peroxide-dependent activity.**

Leishmaniasis are a group of opportunistic and emerging diseases, representing the ninth largest health burden among individuals (1, 2). In the Mediterranean basin, infections by *Leishmania infantum* represent an important health problem, causing extensive canine and also human leishmaniasis mainly associated with immunosuppressed status, such as HIV coinfection (3). To date, the sole effective vaccination available in the Mediterranean region is for dogs (CaniLeish, marketed in 2011), and diagnostic tools show low sensitivity, due to deficient vector control measures, placing chemotherapy as the sole effective solution. However, currently available chemotherapeutic formulations (e.g., pentavalent antimonials, paromomycin, liposomal amphotericin B, and miltefosine) are expensive, show toxicity to the host, and have declining efficacy in some geographic regions, mostly due to increasing selection of resistance (4). WHO advocates an urgent need for new, efficient, safe, and affordable drugs for the treatment of leishmaniasis (1).

Artemisinin and derivatives (ARTs) and synthetic peroxides have demonstrated efficacy against protozoan parasites, such as *Plasmodium* (5–8) and *Perkinsus* species (9). ARTs exhibit antiparasitic activity against *Plasmodium falciparum* at nanomolar concentrations and are widely used for the treatment of malaria, as part of the artemisinin combination chemotherapy protocols recommended by the WHO (10). Similar antiparasitic activity was achieved by selected synthetic trioxolanes (8, 11) and tetraoxanes (12). These peroxides have been considered alternatives to artemisinin-derived antimalarials, and some have gone to clinical trials for use as malaria chemotherapy (12, 13). They are easily synthesized, and the preparation of a library of compounds for structure-activity relationship (SAR) studies and lead optimization is thus facilitated. The antiparasitic activity of peroxides against other human protozoans, namely, *Leishmania* spp., has scarcely been explored. Chollet et al. (14) reported on the activity of fluoroartemisinins against promastigote forms of *Leishmania donovani* (at micromolar concentrations) but observed no activity against corresponding intramacrophage amastigote forms. We now describe results of the susceptibility testing of *L. infantum* life stage forms (promastigote and amastigote) with respect to selected semisynthetic and synthetic peroxides and their cytotoxic-

ity. We demonstrate that the peroxide chemotype has potential as a tool for leishmaniasis chemotherapy in mammalian hosts.

An *L. infantum* strain (MHOM/PT/88/IMT151) from the Instituto de Higiene e Medicina Tropical (IHMT) cryobank, isolated from a visceral leishmaniasis human case without previous treatment, was selected. *Leishmania* parasites used for the *in vitro* experiments had <10 passages in culture to guarantee the virulence of the strains (15) and were used when the stationary phase of growth was reached (day 5 to 6), corresponding to the highest parasite density and percentage of infective promastigote (metacyclic) forms observed. Parasites were cultivated at 24°C ± 1°C in RPMI 1640 medium with L-glutamine (Sigma) supplemented with 10% fetal calf serum (FCS) (Gibco), penicillin (10,000 IU/ml) (Sigma), and streptomycin (10 mg/ml) (Sigma) (= complete RPMI).

Dihydroartemisinin (DHA), artesunate (ATS), deoxygenated dihydroartemisinin (deoxy-DHA), deoxygenated artesunate (deoxy-ATS), and synthetic trioxolanes (LC95, LC67, and LC50) (Table 1) were prepared according to procedures described in the literature (9, 13) and were tested for antileishmanial activity. Deoxy-DHA and deoxy-ATS were used for proof of concept regarding the involvement of the peroxide bridge as a pharmacophore. Full experimental details regarding the synthesis and chemical characterization of the compounds are included in the supple-

Received 5 February 2015 Returned for modification 5 March 2015

Accepted 18 May 2015

Accepted manuscript posted online 26 May 2015

Citation Cortes S, Albuquerque A, Cabral LIL, Lopes L, Campino L, Cristiano MLS. 2015. *In vitro* susceptibility of *Leishmania infantum* to artemisinin derivatives and selected trioxolanes. *Antimicrob Agents Chemother* 59:5032–5035. doi:10.1128/AAC.00298-15.

Address correspondence to Sofia Cortes, scortes@ihmt.unl.pt, or Maria L. S. Cristiano, mcristi@ualg.pt.

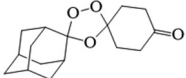
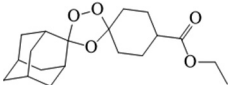
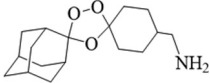
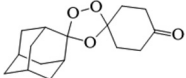
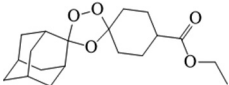
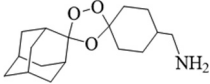
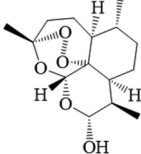
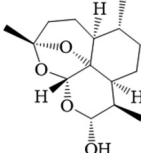
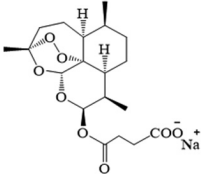
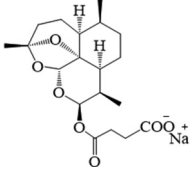
S.C., A.A., and L.I.L.C. contributed equally to this work.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00298-15>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00298-15

TABLE 1 Compounds and range of concentrations used in studies of cytotoxicity and *in vitro* antiparasitic activity on promastigote and intracellular amastigote forms of *L. infantum*

Compound	Structure	Concn range ( $\mu\text{M}$ ) in:		
		Cytotoxicity assay	Promastigote assay	Intracellular amastigote assay
Pentamidine		52.73–210.91	0.42–13.50	15.82–506.18
Miltefosine		38.35–153.35	19.17–613.39	23.00–736.07
Amphotericin B		1,082.25–2,164.50	0.27–10.82	10.15–324.68
LC50		3,592.73–7,185.46	0.90–35.93	11.68–1,077.82
LC67		2,972.47–5,944.95	14.86–445.87	27.87–3,566.97
LC95		3,408.32–6,816.63	1.70–20.45	1.07–409.00
DHA		228.74–439.89	27.49–3,519.14	27.49–219.95
Deoxy-DHA		1,197.00–3,356.08	29.13–3,728.98	29.13–932.24
ATS		169.19–325.37	2.03–260.29	20.34–650.74
Deoxy-ATS		871.86–2,444.46	21.2–2,716.06	21.22–679.02

mental material. Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma), and working solutions presented  $\leq 1\%$  DMSO. All subsequent dilutions were freshly made with RPMI 1640. Amphotericin B (Sigma), miltefosine (Sigma), and pentamidine (Sigma) were used as controls.

For the promastigote susceptibility tests, parasites were plated in 96-well flat-bottomed microtiter plates for 48 h, at a final parasite density of  $2.5 \times 10^6$  parasites/ml in complete RPMI, in the presence of the studied compounds (six replicates of six different concentrations) and with the control drugs (Table 1). A 3-(4,

5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay was used to assess parasite viability in order to estimate the 50% inhibitory concentration ( $\text{IC}_{50}$ ) (16, 17). Optical density was measured spectrophotometrically at  $\lambda$  of 595 nm, as described by Denizot and Lang (16). Three independent experiments were conducted.

*In vitro* intracellular amastigote susceptibility assays were performed by using the human acute monocytic leukemia cell line THP-1 (ATCC TIB-202) maintained in complete RPMI at  $37^\circ\text{C} \pm 1^\circ\text{C}$  and 5%  $\text{CO}_2$ . After 24 h of phorbol myristate acetate (PMA)

**TABLE 2** IC<sub>50</sub>, SI, and CC<sub>50</sub> of pentamidine, miltefosine, amphotericin B, synthetic trioxolanes, and artemisinin-derived trioxanes against the intracellular amastigote and promastigote forms of *L. infantum* and THP1 cell line<sup>a</sup>

Compound	Susceptibility of <i>L. infantum</i> parasites in form of:							
	Intracellular amastigotes			Promastigotes			Cytotoxicity (μM) in THP1	
	IC <sub>50</sub> (μM)	95% CI (μM) <sup>b</sup>	SI	IC <sub>50</sub> (μM)	95% CI (μM)	SI	CC <sub>50</sub>	95% CI
Pentamidine	18.96	ND	4.96	1.60	1.55–1.64	58.83	94.13	76.47–111.79
Miltefosine	61.41	ND	0.64	67.55	65.18–69.93	0.58	39.34	33.21–45.47
Amphotericin B	8.37	8.10–8.64	>57.05	1.15	0.92–1.37	>944.51	>1,082	ND
LC50	79.76	36.24–123.28	>90.08	9.35	5.71–12.99	>768.44	>7,185	ND
LC67	1,202.81	69.94–2,335.69	3.43	145.98	144.15–147.81	28.24	4,121.83	3,851.51–4,392.15
LC95	107.87	98.39–117.36	54.82	3.51	3.36–3.66	1684.74	5,913.43	5,516.96–6,309.90
DHA	104.06	103.58–104.54	2.41	123.06	96.25–149.88	2.04	251.24	172.68–329.80
Deoxy-DHA	457.36	410.22–504.50	2.91	1,249.33	1,082.02–1,416.64	1.07	1,332.18	1,262.38–1,401.98
ATS	122.52	33.75–211.29	2.43	40.68	35.79–45.56	7.32	297.78	286.49–309.06
Deoxy-ATS	80.27	46.87–113.68	11.95	747.73	636.80–858.66	1.28	959.31	947.07–971.56

<sup>a</sup> SI, selectivity index, defined as the ratio between the value of CC<sub>50</sub> against THP1 cells and the value of IC<sub>50</sub> against *Leishmania* parasites.

<sup>b</sup> 95% CI, 95% confidence interval; ND, not determined.

(25 ng/ml; Sigma) differentiation of  $1 \times 10^5$  cells/ml in sterile 16-chamber Lab-Tek culture slides (Nunc), the cells were infected with  $1 \times 10^6$  cells/ml promastigotes in a 10:1 parasite-to-host cell proportion (18) for 24 h. Treatment (two replicates of six different concentrations) was performed with an additional 48 h of incubation, as previously described (Table 1). Slides were fixed and stained, and the intensity of infection was evaluated by counting the number of parasites per 100 macrophages (19) in treated and nontreated cells. Two independent experiments were performed to determine the IC<sub>50</sub> of each compound.

The monocyte THP1 cell line was used in order to estimate the 50% cytotoxic concentration (CC<sub>50</sub>) of the peroxides in host mammalian cells. A total of  $1 \times 10^5$  cells/ml were plated in 96-well tissue culture plates, and after 24 h of differentiation, compounds (four replicates of three different concentrations) (Table 1) were added for an exposure of 48 h. After 24 h at  $37^\circ\text{C} \pm 1^\circ\text{C}$  and 5% CO<sub>2</sub>, 25 μl/well of resazurin (250 μg/ml; Sigma) was added, with an additional 24 h of incubation. Cell viability was evaluated by measuring fluorescence using a Triad multimode detector (Dynex Technologies) at excitation/emission wavelengths of 535/595 nm. Each compound was tested in three independent assays.

Drug activity was expressed as the percentage of the viability of the parasites compared to that of the untreated controls. IC<sub>50</sub> and CC<sub>50</sub> were calculated by using nonlinear regression analysis with variable slope in GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). The selectivity index (SI) was determined based on the ratio of CC<sub>50</sub>/IC<sub>50</sub>, as described elsewhere (20).

Our results showed that *L. infantum* parasites were susceptible to all peroxides scrutinized, at inhibitory concentrations (IC<sub>50</sub>) ranging between 3.51 μM and 1.25 mM for promastigote forms and between 79.76 μM and 1.20 mM for intramacrophage amastigote forms (Table 2). The IC<sub>50</sub>s were compared with those obtained for the control drugs. Artemisinin derivatives (DHA and ATS) showed lower antileishmanial activity than that of the control drugs in both forms of the parasite. On the other hand, the observed activities for synthetic trioxolanes LC50 and LC95 against promastigote (9.35 and 3.51 μM, respectively) and intracellular amastigote (79.76 and 107.87 μM, respectively) forms were close to those of the control drugs. Moreover, when tested for cytotoxicity, the trioxolanes LC50 and LC95 showed comparable or higher SI values than those of the control drugs (Table 2).

The results obtained demonstrate the potential of peroxides as scaffolds for leishmaniasis chemotherapy. Synthetic trioxolanes appear to be especially promising, considering their high antiparasitic activity, SI values, and the easy access to this chemotype by chemical synthesis. From the three trioxolanes tested, LC95 and LC50 presented the best activity and safety profiles. These compounds showed high antipromastigote activity and low host cell toxicity compared to those of the control drugs. The range of activity and safety data obtained for trioxolanes clearly show that the chemical nature of substituents linked to the endoperoxide core has an impact on performance. We believe that SAR studies on an extended library of trioxolanes will provide optimized leads.

Deoxygenated compounds (deoxy-DHA and deoxy-ATS), when tested in promastigotes, have shown higher IC<sub>50</sub> and lower SI values than those of their peroxide precursors, pointing to the importance of the peroxide linkage in activity and implicating the peroxide group as a putative pharmacophore, in keeping with previous results obtained for *Plasmodium* species (21). Surprisingly, when tested in amastigotes, the opposite happens. An explanation for this unexpected behavior requires a deep investigation into the macrophage-amastigote system and into the bioaccumulation and mode of action for this class of compounds. The cellular membrane of the host and the parasitophorous vacuole may act as barriers to the direct interaction with the parasite. Additionally, artemisinin derivatives might act by an alternative mode of action, inhibiting specific parasite enzymes in a peroxide-independent manner.

This work brings new perspectives to the field of leishmaniasis chemotherapy, demonstrating that peroxides may be considered antileishmanial agents. This search is quite relevant, as leishmaniasis is, among other tropical diseases, in the lead in the discovery for novel drugs, since several factors, such as high cost, poor compliance, drug resistance, low efficacy, and poor safety (15), limit the utility of the existing drugs in resource-poor settings. Synthetic peroxides were considered to circumvent known liabilities of ARTs, namely, poor bioavailability, short plasma half-life, relatively high cost due to low yield of extraction from *Artemisia annua*, and, although residual, some neurotoxicity (22). Extensive studies centered on the pharmacokinetic and toxicological properties of a wide and chemically diverse range of trioxolanes have demonstrated that compared to the parent artemisinin deriva-

tives, the plasma half-life of trioxolanes is longer, and the toxicity (including neurotoxicity) is lower (6, 8, 23, 24).

In conclusion, this study shows that synthetic trioxolanes have potential as antiproliferative agents for the *L. infantum* promastigote stage, with LC95 and LC50 as potential candidates for the treatment of *Leishmania* infection. There is evidence for peroxide-dependent toxicity, and *in vitro* results indicate that substituents linked to the trioxolane affect activity. Thus, a structure-based adjustment of chemical, biopharmaceutical, and treatment properties may be achieved and is facilitated by the ease of synthesizing analogues with chemical diversity. Although further studies are required for a deeper evaluation of the effectiveness of these compounds, using other *L. infantum* strains and also other *Leishmania* species, followed by *in vivo* studies (murine model) and safety profile studies (e.g., evaluation of genotoxicity), the data gathered and described here bring new molecules into consideration for use in *Leishmania* chemotherapy.

## ACKNOWLEDGMENTS

This work was supported by the FCT (Portuguese Science and Technology Foundation) and FEDER through projects PTDC/CVT/112371/2009, PEst-C/MAR/LA0015/2013, and PEst-C/MAR/LA0015/2014 and grant SFRH/BPD/44450/2008 (to S. Cortes).

L. I. L. Cabral thanks CCMAR/CIMAR for a fellowship.

## REFERENCES

- WHO. 2012. Accelerating work to overcome the global impact of neglected tropical diseases: a roadmap for implementation. WHO/HTM/NTD/2012. World Health Organization, Geneva, Switzerland. [http://www.who.int/neglected\\_diseases/NTD\\_RoadMap\\_2012\\_Fullversion.pdf](http://www.who.int/neglected_diseases/NTD_RoadMap_2012_Fullversion.pdf).
- Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, WHO Leishmaniasis Control Team. 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7:e35671. <http://dx.doi.org/10.1371/journal.pone.0035671>.
- Dujardin JC, Campino L, Cañavate C, Dedet JP, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M. 2008. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. *Emerg Infect Dis* 14:1013–1018. <http://dx.doi.org/10.3201/eid1407.071589>.
- Croft SL, Olliaro P. 2011. Leishmaniasis chemotherapy—challenges and opportunities. *Clin Microbiol Infect* 17:1478–1483. <http://dx.doi.org/10.1111/j.1469-0691.2011.03630.x>.
- Copple IM, Mercer AE, Firman J, Donegan G, Wong M, Chadwick J, Bringela AD, Cristiano MLS, van de Water B, Ward SA, O'Neill PM, Park BK. 2012. Examination of the cytotoxic and embryotoxic potential and underlying mechanisms, of next-generation synthetic trioxolane and tetraoxane antimalarials. *Mol Med* 18:1045–1055. <http://dx.doi.org/10.2119/molmed.2012.00154>.
- Dong Y, Chollet J, Matile H, Charman SA, Chiu FCK, Charman WN, Scorneaux B, Urwyler H, Santo Tomas J, Scheurer C, Snyder C, Dorn A, Wang X, Karle JM, Tang Y, Wittlin S, Brun R, Vennerstrom JL. 2005. Spiro and dispiro-1,2,4-trioxolanes as antimalarial peroxides: charting a workable structure-activity relationship using simple prototypes. *J Med Chem* 48:4953–4961.
- Gibbons P, Verissimo E, Araujo NC, Barton V, Nixon GL, Amewu RK, Chadwick J, Stocks PA, Biagini GA, Srivastava A, Rosenthal PJ, Gut J, Guedes RC, Moreira R, Sharma R, Berry N, Cristiano MLS, Shone AE, Ward SA, O'Neill PM. 2010. Endoperoxide carbonyl falcipain 2/3 inhibitor hybrids: toward combination chemotherapy of malaria through a single chemical entity. *J Med Chem* 53:8202–8206. <http://dx.doi.org/10.1021/jm1009567>.
- Tang Y, Wittlin S, Charman SA, Chollet J, Chiu FCK, Morizzi J, Johnson LM, Tomas JS, Scheurer C, Snyder C, Zhou L, Dong Y, Charman WN, Matile H, Urwyler H, Dorn A, Vennerstrom JL. 2010. The comparative antimalarial properties of weak base and neutral synthetic ozonides. *Bioorg Med Chem Lett* 20:563–566. <http://dx.doi.org/10.1016/j.bmcl.2009.11.088>.
- Araújo NC, Afonso R, Bringela A, Cancela ML, Cristiano MLS, Leite RB. 2013. Peroxides with antiplasmodial activity inhibit proliferation of *Perkinsus olseni*, the causative agent of perkinsosis in bivalves. 2013. *Parasitol Int* 62:575–582. <http://dx.doi.org/10.1016/j.parint.2013.06.010>.
- WHO. 2010. Guidelines for the treatment of malaria, 2nd ed. World Health Organization, Geneva, Switzerland. [http://whqlibdoc.who.int/publications/2010/9789241547925\\_eng.pdf](http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf).
- Vennerstrom JL, Dong YX, Chollet J, Matile H. November 2002. Spiro and dispiro 1,2,4-trioxolane antimalarials. U.S. patent 6,486,199B1.
- Marti F, Chadwick J, Amewu RK, Burrell-Saward H, Srivastava A, Ward SA, Sharma R, Berry N, O'Neill PM. 2011. Second generation analogues of RKA182: synthetic tetraoxanes with outstanding *in vitro* and *in vivo* antimalarial activities. *MedChemComm* 2:661–665. <http://dx.doi.org/10.1039/c1md00102g>.
- Fügi MA, Wittlin S, Dong Y, Vennerstrom JL. 2010. Probing the anti-malarial mechanism of artemisinin and OZ277 (arterolane) with nonperoxidic isosteres and nitroxyl radicals. *Antimicrob Agents Chemother* 54:1042–1046. <http://dx.doi.org/10.1128/AAC.01305-09>.
- Chollet C, Crousse B, Bories C, Bonnet-Delpon D, Loiseau PM. 2008. *In vitro* antileishmanial activity of fluoro-artemisinin derivatives against *Leishmania donovani*. *Biomed Pharmacother* 62:462–465. <http://dx.doi.org/10.1016/j.biopha.2008.04.003>.
- Moreira D, Santarem N, Loureiro I, Tavares J, Silva AM, Amorim AM, Ouaisi A, Cordeiro-da-Silva A, Silvestre R. 2012. Impact of continuous axenic cultivation in *Leishmania infantum* virulence. *PLoS Negl Trop Dis* 6:e1469. <http://dx.doi.org/10.1371/journal.pntd.0001469>.
- Denizot F, Lang R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 89:271–277. [http://dx.doi.org/10.1016/0022-1759\(86\)90368-6](http://dx.doi.org/10.1016/0022-1759(86)90368-6).
- Dutta A, Bandyopadhyay S, Mandala C, Chatterjee M. 2005. Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis. *Parasitol Int* 54:119–122. <http://dx.doi.org/10.1016/j.parint.2005.01.001>.
- Jain SK, Sahu R, Walker LA, Tekwani BL. 2012. A parasite rescue and transformation assay for antileishmanial screening against intracellular *Leishmania donovani* amastigotes in THP1 human acute monocytic leukemia cell line. *J Vis Exp* 70:4054.
- Maia C, Rolão N, Nunes M, Gonçalves L, Campino L. 2007. Infectivity of five different types of macrophages by *Leishmania infantum*. *Acta Trop* 103:150–155. <http://dx.doi.org/10.1016/j.actatropica.2007.06.001>.
- Weninger B, Robledo S, Arango GJ, Deharo E, Aragón R, Muñoz V, Callapa J, Lobstein A, Anton R. 2001. Antiprotozoal activities of Colombian plants. *J Ethnopharmacol* 78:193–200. [http://dx.doi.org/10.1016/S0378-8741\(01\)00346-4](http://dx.doi.org/10.1016/S0378-8741(01)00346-4).
- Stocks PA, Bray PG, Barton VE, Al-Helal M, Jones M, Araújo NC, Gibbons P, Ward SA, Hughes RH, Biagini GA, Davies J, Amewu R, O'Neill PM. 2007. A common “free iron” dependent mechanism of activation among diverse antimalarial endoperoxide structures. *Angew Chem Int Ed* 119:1–7. <http://dx.doi.org/10.1002/ange.200690172>.
- Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu CKF, Chollet J, Dong YX, Dorn A, Hunziker D, Matile H, McIntosh K, Padmanilayam M, Tomas JS, Scheurer C, Scorneaux B, Tang Y, Urwyler H, Wittlin S, Charman WN. 2004. Identification of an antimalarial synthetic trioxolane drug development candidate. *Nature* 430:900–904. <http://dx.doi.org/10.1038/nature02779>.
- Tang Y, Dong YX, Wittlin S, Charman SA, Chollet J, Chiu CKF, Charman WN, Matile H, Urwyler H, Dorn A, Bajpai S, Wang X, Padmanilayam M, Karle JM, Brun R, Vennerstrom JL. 2007. Weak base dispiro-1,2,4-trioxolanes: potent antimalarial ozonides. *Bioorg Med Chem Lett* 17:1260–1265.
- Keiser J, Kirchhofer C, Haschke M, Huwyler J, Dong YX, Vennerstrom JL, Vanhoff K, Kaminsky R, Malikides N. 2010. Efficacy, safety and pharmacokinetics of 1,2,4-trioxolane OZ78 against an experimental infection with *Fasciola hepatica* in sheep. *Vet Parasitol* 173:228–235. <http://dx.doi.org/10.1016/j.vetpar.2010.06.031>.