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► **To cite this version:**

Yan Lin, Yih Ching Ong, Sarah Keller, Rafika Bouchene, Eric Manoury, et al.. Synthesis, Characterization and Antiparasitic Activity of Organometallic Derivatives of the Anthelmintic Drug Albendazole. Dalton Transactions, 2020, 49 (20), pp.6616-6626. 10.1039/d0dt01107j . hal-02555378

HAL Id: hal-02555378

<https://hal.science/hal-02555378>

Submitted on 27 Apr 2020

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Synthesis, Characterization and Antiparasitic Activity of Organometallic Derivatives of the Anthelmintic Drug Albendazole

Yan Lin,^a Yih Ching Ong,^a Sarah Keller,^a Johannes Karges,^a Rafika Bouchene,^{b,c} Eric Manoury,^b Olivier Blacque,^d Joachim Müller,^e Nicoleta Anghel,^e Andrew Hemphill,^e Cécile Häberli^{g,h}, Aya C. Taki,^f Robin B. Gasser,^f Kevin Cariou,^{a,*} Jennifer Keiser,^{g,h,*} Gilles Gasser^{a,*}

a) Chimie ParisTech, PSL University, CNRS, Institute of Chemistry for Life and Health Sciences, Laboratory for Inorganic Chemical Biology, 75005 Paris, France.

b) CNRS, LCC (Laboratoire de Chimie de Coordination), Université de Toulouse, UPS, INPT, 205 Route de Narbonne, BP 44099, 31077 Toulouse, France.

c) Département Sciences de la Matière, Faculté des Sciences Exactes, Université Larbi Ben M'hidi, BP 358, Route de Constantine, 04000 Oum El Bouaghi, Algeria.

d) Department of Chemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland.

e) Institute of Parasitology, University of Bern, Länggass-Strasse 122, 3012 Bern, Switzerland.

f) Department of Veterinary Biosciences, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia.

g) Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Socinstr. 57, Basel, Switzerland.

h) University of Basel, Basel Switzerland.

Abstract

Helminthiases, a group of neglected tropical diseases, affect more than one billion people mainly in tropical and subtropical regions. Moreover, major intestinal protozoa have a significant impact on global public health. Albendazole (**ABZ**) is a broad-spectrum anthelmintic recommended by the World Health Organisation (WHO). However, drug resistance is emerging due to its widespread use. In order to tackle this problem, taking into account the spectacular results obtained with the organometallic derivatization of the antimalarial drug chloroquine, we have prepared, in this study, a series of new ferrocenyl and ruthenocenyl derivatives of the organic drug **ABZ** and assessed their activity against different helminths and protozoans, namely *Trichuris muris*, *Heligmosomoides polygyrus*, *Schistosoma mansoni*, *Giardia lamblia*, *Haemonchus contortus* and *Toxoplasma gondii*. The ferrocene-containing **ABZ** analogue **2d** exhibited over 70% activity against *T. muris* adults *in vitro* at 200 μ M and no toxicity to mammalian cells ($IC_{50} > 100 \mu$ M). *H. polygyrus* adults were not affected by any of the derivatives tested. Against *T. gondii*, the ferrocene-containing **ABZ** analogues **1a** and **2d** showed better *in vitro* activity than **ABZ** and low toxicity to the host cells. The activity of the analogous ruthenocenyl compound **2b** against *S. mansoni* and *T. gondii* *in vitro* might be attributed to its toxicity towards the host cells rather than a specific antiparasitic activity. These results demonstrate that the derivatives show a species specific *in vitro* activity and the choice of the organometallic moieties attached to the organic drug is playing a very important role. Two of our organometallic compounds, namely **1b** and **2d**, were tested in *T. muris* infected mice. At a 400 mg/kg dose, the compounds showed moderate worm burden reductions but low worm expulsion rates. Overall, this work, which is one of the first studies reporting the potential of organometallic compounds on a very broad range of parasitic helminths and protozoan, is a clear confirmation of the potential of organometallic complexes against parasites of medical and veterinary importance.

KEYWORDS: *anthelmintics; bioorganometallic chemistry; ferrocene; medicinal inorganic chemistry; medicinal organometallic chemistry.*

INTRODUCTION

Neglected tropical disease (NTDs) affect the poorest populations without adequate water and sanitation in tropical and subtropical regions. More than one billion people, which amounts to one-sixth of the world's population, are affected by NTDs within 149 countries.^{1,2} Even though the morbidity exceeds the mortality, more than half a million deaths per year are caused by NTDs.³ Among NTDs, worm infection, or helminthiasis, has been the target of global elimination programs.⁴ Because of the large number of individuals affected, a resolution was passed by the World Health Organisation (WHO) in 2001 to control several helminth infections through preventive chemotherapy (PC), employing anthelmintics on populations at risk to prevent morbidity.^{5,6} Considering the rapid re-infection following treatment and the possible development of resistance in species such as *Trichuris* or *Schistosoma*, there is an urgent need for research on novel and structurally different classes of potential anthelmintic drugs. Despite the urgency to develop new anthelmintics, the budget invested in this field of research is limited. The poorest population affected by NTDs is often living on less than US\$ 2 per day. Therefore, most of them cannot afford expensive medicines.⁶ Moreover, drugs in PC programs are donated in large scale treatment programs, which have mitigated effect.⁷ Concurrently, there is little motivation for the pharmaceutical industry to develop new drugs that might not provide return on huge research and development investment, making the discovery of new anthelmintics an even greater challenge.

Albendazole (**ABZ**, Figure 1) is an anthelmintic that was recommended by the WHO to treat soil-transmitted helminth infections.⁸ **ABZ** shows a broad-spectrum of activity in domestic animals and was subsequently licensed for human use in 1982.⁹ Because of its effectiveness, safety and low price, **ABZ** is one of the main drugs used in PC programs.¹⁰ The excellent safety record of **ABZ** is related to its mechanism of action, which involves selective binding to β -tubulin and disruption of microtubule polymerization.¹¹ However, to the best of our knowledge, the exact manner how **ABZ** binds β -

tubulin is unclear.¹⁰ Nevertheless, there are several drawbacks associated with its use, such as poor drug absorption and the lack of water solubility.¹² Importantly, its widespread use has resulted in reduced susceptibility of helminths of veterinary importance.¹³ Taking these effects into account is at the heart of the One Health initiative,¹⁴ as the development of drugs for human use can imply consequences for veterinary uses and vice-versa.

With this in mind, our research groups have started a program to derivatize **ABZ** with various organometallic moieties in order to evaluate their activity on various human and animal parasites, including, but not limited to, helminths. Organometallic compounds, which are defined as metal complexes containing at least one direct, covalent metal-carbon bond, have recently^{15,16,17} found numerous applications in medicinal chemistry,^{18,19,20,21} including against helminths^{22,23,24} and NTDs like schistosomiasis.^{3,25,26,27} For instance, Brocard, Biot and co-workers synthesized ferroquine (FQ, Figure 1), a ferrocenyl derivative of chloroquine (ACQ, Figure 1), which is currently undergoing phase II trials against malaria.^{28,29} It was demonstrated that ferroquine has an activity against both chloroquine-susceptible and chloroquine resistant *P. falciparum*. Ferrocene is a small, rigid, lipophilic molecule that can easily cross cellular membranes.³⁰ These characteristics, along with its reversible electrochemical behavior, make ferrocenyl derivatives interesting candidates for biological applications and further drug design. For ferroquine or ferrocifen (Fc-TAM, Figure 1) – an anticancer drug analogue of tamoxifen (TAM, Figure 1) developed by the Jaouen group³¹ – the addition of a ferrocenyl moiety into a known organic drug has allowed a remarkable increase in activity compared to the parent organic compounds. Inspired by these successes, we herein report our findings concerning the antiparasitic activity of five ferrocenyl and two ruthenocenyl derivatives of **ABZ** with the aim to bypass drug-resistance pathways and/or to increase the potency and therapeutic scope of **ABZ**. The ruthenocenyl derivatives were synthesized because ferrocene and

ruthenocene are isoelectronic and have a similar geometry and steric demand, yet they exhibit completely different electrochemical behavior and redox properties.²⁰

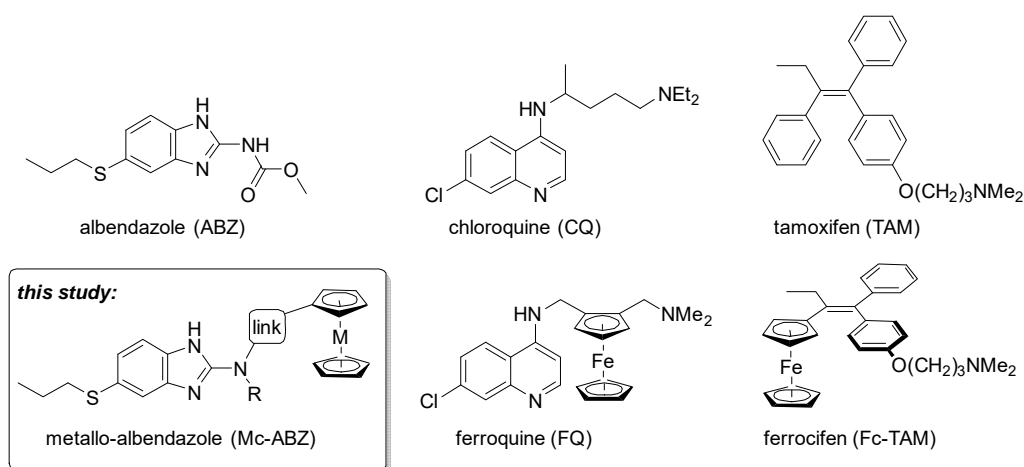


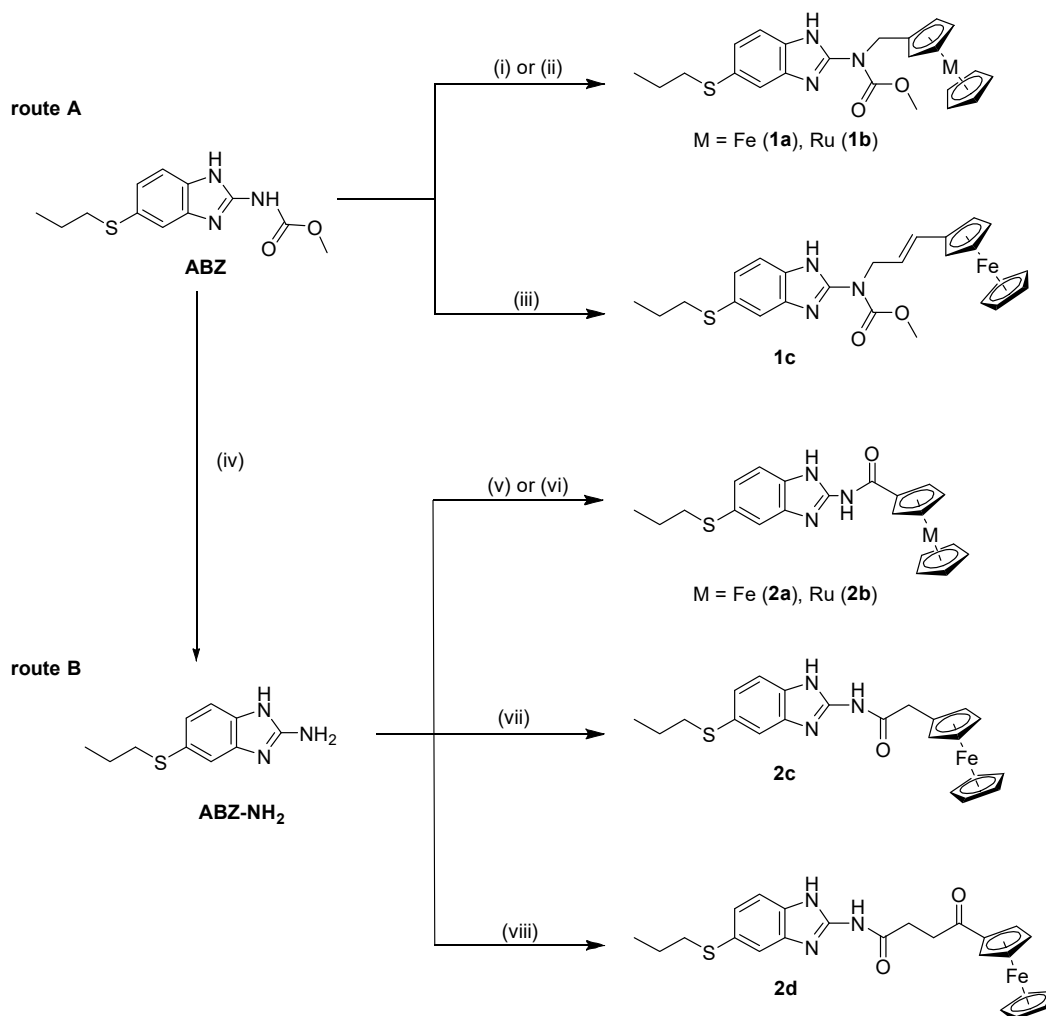
Figure 1. Structures of albendazole, chloroquine and tamoxifen and their metallocene derivatives.

RESULTS AND DISCUSSION

Synthesis and characterization

In this study, two strategies were used to synthesize **ABZ** analogues incorporating a metallocene moiety (**Scheme 1**). In the first strategy, (route A) the *N*-methylcarbamate group was left untouched and the metallocenyl fragment added on the nitrogen. In the second (route B), the carbamate function was replaced by an acyl metallocene group (with various linkers). Compounds **1a**, **1b** and **1c** were prepared by direct treatment of **ABZ** with (ferrocenylmethyl)-trimethylammonium iodide, (ruthenocenylmethyl)-trimethylammonium iodide and (2-ferrocenylvinyl)methyl-trimethylammonium iodide, respectively, with K_2CO_3 in acetonitrile using a similar synthetic method employed by Spiccia and co-workers.³³ Route B started by the diethylenetriamine-mediated cleavage of the unactivated carbamate in **ABZ** to give **ABZ-NH₂** in good yield.³⁴ Compound **ABZ-NH₂** was then converted into **2a-d** by treatment with the ferrocene- (**2a,c,d**) or ruthenocene- (**2b**) containing carboxylic acid derivatives *via* a TBTU mediated amide coupling reaction. Comparing the activities of ruthenocenyl analogues (**1b**, **2b**) with their ferrocene counterparts (**1a**, **2a**) should allow detecting whether the iron-mediated redox properties are responsible for the anthelmintic activity.

All newly synthesized compounds were unambiguously characterized by ^1H , ^{13}C NMR spectroscopy, IR spectroscopy and ESI mass spectrometry. The purity of individual compounds was confirmed by microanalysis. Details on synthesis and characterization of the complexes can be found in the Supporting Information.



Scheme 1. Synthesis of the metallocene analogues of albendazole. Reagents and conditions: (i) (ferrocenylmethyl)-trimethylammonium iodide, K_2CO_3 , dry MeCN, 90°C ; (ii) trimethylammoniomethylruthenocene iodide, K_2CO_3 , dry MeCN, 90°C ; (iii) (2-ferrocenylvinyl) methyl-trimethylammonium iodide, K_2CO_3 , dry MeCN, 90°C ; (iv) diethylenetriamine, 130°C ; (v) ferrocenecarboxylic acid, TBTU, DIPEA, dry DMF; (vi) ruthenocenecarboxylic acid, TBTU, DIPEA, dry DMF; (vii) ferroceneacetic acid, TBTU, DIPEA, dry DMF; (viii) 3-Ferrocenoylpropionic acid, TBTU, DIPEA, dry DMF.

Additionally, complexes **1a**, **1b**, **1c** were characterized using single-crystal X-ray crystallography (Figure 2). Details on the acquisition of the structures can be found in the Supporting Information.

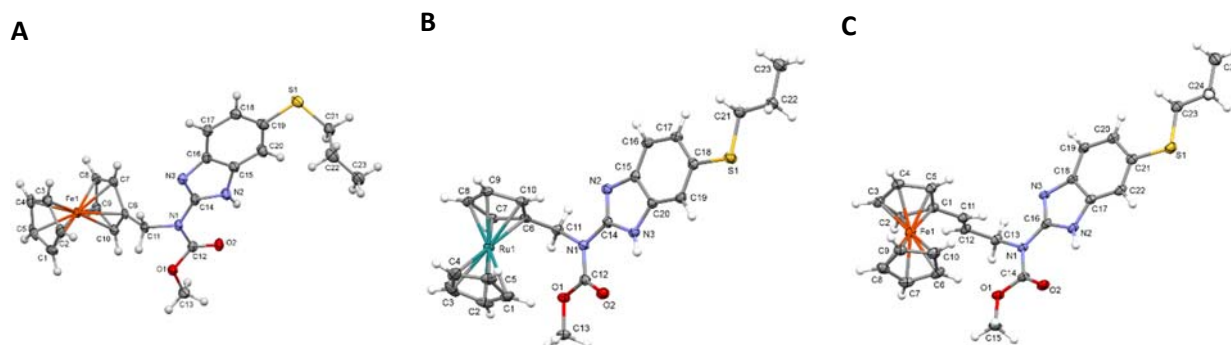


Figure 2. A: molecular structure of **1a**; B: molecular structure of **1b**; C: molecular structure of **1c**. The thermal ellipsoids are drawn at the 30% probability level.

Stability in human plasma

In order to assess the stability of our metallocene-containing **ABZ** derivatives under simulated biological conditions, we first studied their behavior in human plasma. Human plasma stability is a crucial parameter of the stability profile for drug discovery, as compounds unstable in plasma are generally not suitable drug candidates, with the exception of some prodrugs or antedugs.³⁵ In order to do so, a rapid, accurate and economic method based on HPLC and LC-MS technique was chosen, as recently reported by our group.^{26, 36} Caffeine, which is known to be stable in human plasma, was chosen as an internal standard. After incubation in human plasma at 37°C for various times, methanol was added to quench the plasma solution. Afterwards, the recovery of the internal standard, the test compound and the eventual metabolites was performed by liquid extraction with dichloromethane. The obtained residue was analyzed by RP-HPLC (See Supporting Information for details) and the results of these analyses are gathered in Figure 3. **ABZ** and **ABZ-NH₂** proved to be relatively stable after 24 h incubation in human plasma - they are decomposed by only 2% and 17%, respectively. The metallocene-containing **ABZ** derivatives showed moderate to good stability after 6 h but significant decay was observed after 24 h. The ferrocene-containing compound **2d** displayed the best stability of the derivatives in human plasma after 24 h, with only 23% degradation.

Compound **2b** showed the second best stability since it only decomposed by 26% after 24 h incubation. Nearly 50% degradation was measured after 24 h for compounds **1a-1c** and **2a**.

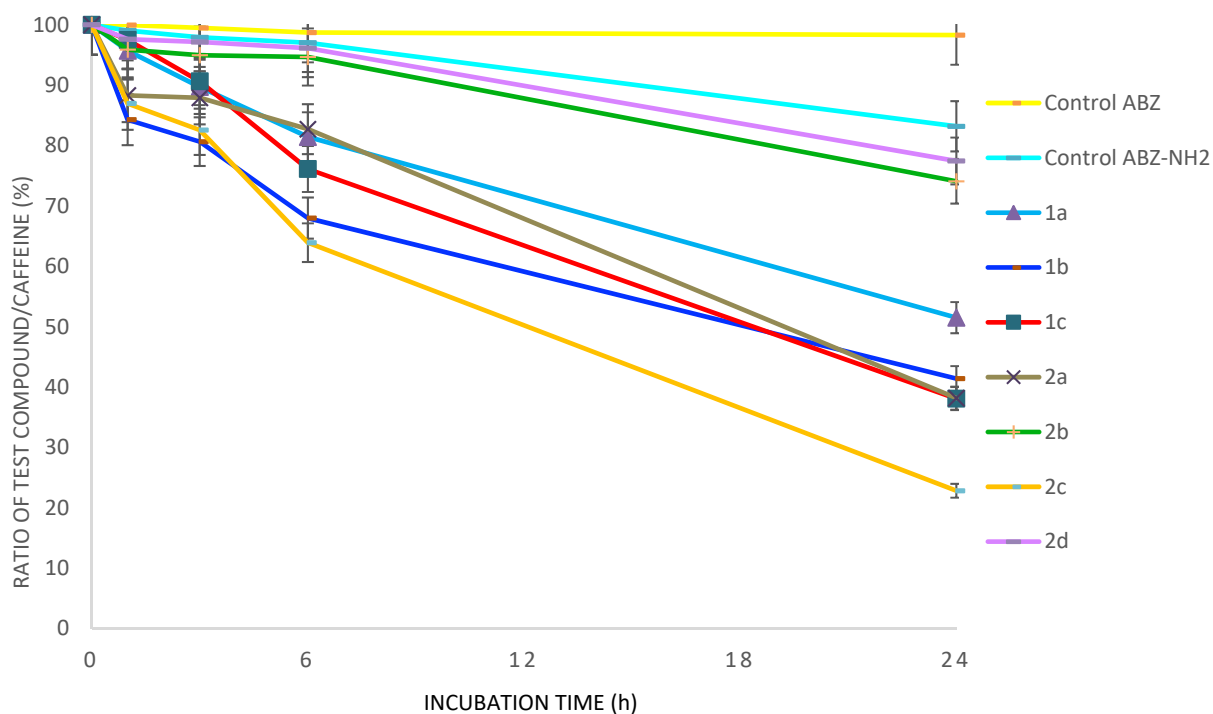
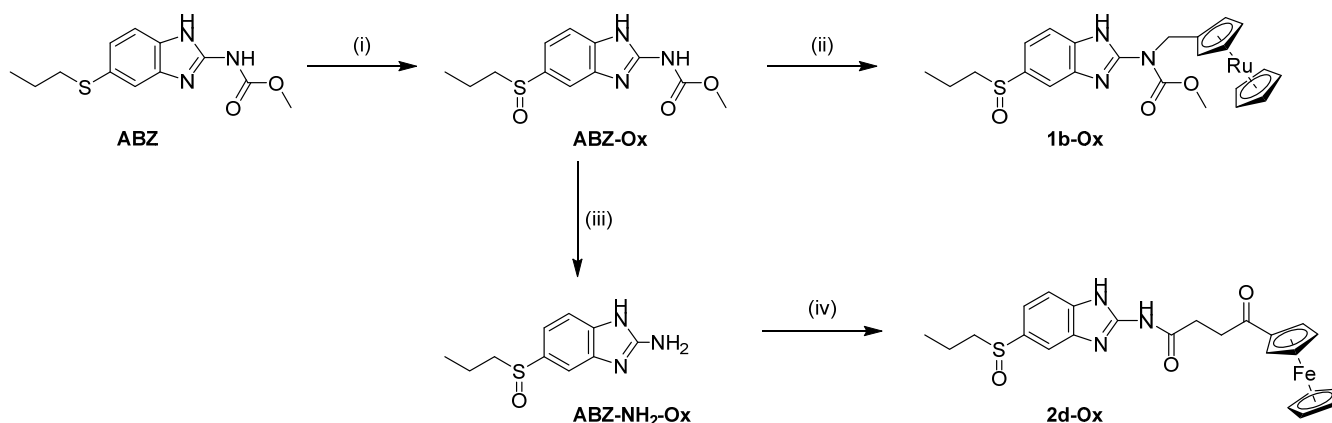


Figure 3. Stability of the metallocene-containing **ABZ** in human plasma. The ratio of the test compound peak area to the internal standard peak area was set as 100% at $t = 0$. This ratio was plotted with regards to the time of incubation.

Compound **2c** was shown to decompose at a highest rate (ca. 77% release after 24 h incubation).

The following order for stability in human plasma can be made from the most stable to the least stable: **2d** > **2b** > **1a** > **1b** > **1c** \approx **2a** > **2c**. We thought the electron-withdrawing functional group attached to the metallocene, as carbonyl, might help to stabilize the organometallic derivatives. To gain further insight on the nature of the decomposition products, LC-MS analyses were performed. In the case of **1b**, the main degradation compound was identified to be the result from the oxidation of **1b**. Hypothesizing that the oxidation site would be the sulfur atom, **1b-Ox** was synthesized from **ABZ** by a two-step oxidation/substitution sequence (Scheme 2). Its identity as the degradation product from **1b** was then confirmed by measuring its retention times under the same HPLC conditions (see Supporting Information). As shown in Scheme 2, the sulfoxide analogue **2d-Ox** was

also synthesized by acylation of **ABZ-NH₂-Ox** that was itself obtained from **ABZ-Ox**. As expected, **2d-Ox** was confirmed to be the major metabolite of **2d** in human plasma. The metabolites of **ABZ** and **ABZ-NH₂** were also confirmed to be **ABZ-Ox** and **ABZ-NH₂-Ox**, respectively (see Supporting Information).³⁷ The metabolites of **1a**, **1c**, **2a** and **2b** were not unambiguously identified, yet, the mass spectra are consistent with oxidized species, presumably also occurring on the sulfur atom. For compound **2c**, despite its rather moderate stability, no degradation peak could be found by HPLC and LC-MS. This study is an excellent indication that these metallocene-containing **ABZ** derivatives were exclusively metabolized to their oxidized species, as neither peaks corresponding to the free metal complexes nor to released **ABZ** could be observed.



Scheme 2. Synthesis of the oxidized analogues of **1b** and **2d**. Reagents and conditions: (i) 37% H₂O₂, glacial acetic acid; (ii) trimethylammoniummethylruthenocene iodide, K₂CO₃, dry MeCN, 90°C; (iii) diethylenetriamine, 130°C; (iv) 3-Ferrocenylpropionic acid, TBTU, DIPEA, dry DMF.

Antiparasitic activities

A broad spectrum screening against a range of helminths was conducted. In a One Health approach, the chosen panel of parasites included species able to colonize both human and animals: *Heligmosomoides polygygrus* (rodents), *Trichuris muris* (mice, analogous to the human parasite *Trichuris trichiura*), *Schistosoma mansoni* (human), *Haemonchus contortus* (ruminants such as sheep) as well as protozoan parasites that can affect humans: *Giardia lamblia* and *Toxoplasma gondii*.

Helminths. Results obtained when testing the derivatives against adult species of the hookworm *H. polygyrus*, the whipworm *T. muris* and the schistosome *S. mansoni in vitro* are summarized in Table 1. Interestingly, compound **ABZ-NH₂** was the most active compound showing high activity against all three species tested at concentrations of 100-200 μ M. Against *S. mansoni*, **ABZ-NH₂** achieved even death of all worms at 200 μ M. On the other hand, **ABZ**, the drug of choice for hookworm and whipworm infections lacked activity against all 3 species tested confirming previous findings.³⁸ Compounds **1b** and **2d** showed activity (resulting in reduced motility) against *T. muris* at the highest concentration tested while **2b** affected *S. mansoni*. None of the compounds showed activity against *H. polygyrus* adults. In the *in vitro* cytotoxicity assay using L6 rat cells, compounds **2a** and **2d** were found to be the least toxic against mammalian cells.

Table 1. *in vitro* activity of derivatives against *Heligmosomoides polygyrus*, *Trichuris muris* and *Schistosoma mansoni*

Compound	<i>T. muris</i> adult			<i>H. polygyrus</i> adult		<i>S. mansoni</i> adult		Cytotoxic L6 cells ^a
	200 μ M	100 μ M	10 μ M	200 μ M	100 μ M	200 μ M	100 μ M	IC ₅₀ (μ M)
1a	31.4 (9.3)			38.3 (22.4)		48.9 (11.8)		14.7
1b	74.5 (9.3)	23 (11.8)		46.7 (11.8)		57.1 (12.5)		4.3
1c	35.3 (37.5)			41.7 (9.3)		56.8 (18.6)		18
2a	19.6 (9.3)			40.0 (0.0)		26.5 (0.0)		> 100 μ M
2b	11.8 (12.5)			31.7 (9.3)		73.4 (9.3)	24.8 (12.5)	3.8
2c	27.5 (9.3)			30.0 (0.0)		20.3 (12.5)		27
2d	78.4 (9.3)	19.2 (0.0)		46.7 (11.8)		59.2 (11.8)		> 100 μ M
ABZ	19.6 (9.3)			28.3 (9.3)		56.8 (18.6)		2
ABZ-NH₂	90.2 (9.3)	84.6 (11.8)	40.0 (12.5)	70.0 (0.0)	33.3 (14.4)	100 (0.0)	52.8 (14.4)	28.9

^a 2 h after adding Resazurin dye. Table reports mean activity values (%) and standard deviation (SD).

Given these promising *in vitro* findings, compounds **ABZ-NH₂**, **1b** and **2d** underwent testing in *T. muris* infected mice. At a 400 mg/kg dose compounds showed moderate worm burden reductions

but, unfortunately, only low worm expulsion rates (Table 2). All compounds were well tolerated by mice despite the low IC₅₀ of **1b** in the cytotoxicity L6 assay.

Table 2. Activity of 1b and two derivatives in *T. muris* infected mice when administered at 400 mg/kg

Compound	Mice cured/investigated	Mean number of worms in intestine (SD)	Mean number of worms in stool (sd)	Worm burden reduction*	Worm expulsion rate **
Control	0/4	114.0 (9.3)	0.5 (0.6)	--	--
ABZ-NH ₂	0/4	48.8 (5.7)	0.3 (0.5)	57	0,5
1b	0/4	36.3 (2.2)	2.0 (1.8)	68,1	5,2
2d	0/4	47.5 (11.2)	0.8 (0.1)	58,1	1,6

* decrease in mean worm burden of treatment groups compared to the mean burden of the control group

** Mean number of worms expelled within 72 hours after treatment

All nine derivatives, including **ABZ**, were tested against *H. contortus* xL3s (a helminth affecting ruminants such as sheep) in the whole-organism assay. The parent compound, **ABZ**, and its derivatives **ABZ-NH₂** and **2a** all decreased L4s development over 7 days using 100 μ M of each compound. **ABZ**,³⁹ which has previously been evaluated, fully inhibited L4 development at this concentration. Compounds **ABZ-NH₂** inhibited L4 development by 35.8% ($p < 0.0001$), whereas compound **2a** reduced this development by 19.1% ($p = 0.001$; Figure 4A). Dose-response evaluations estimated the potencies of individual compounds on xL3 motility after 72 h (Figure 4B). An inhibition of xL3 motility was observed for compounds **1b** and **2b**, but no greater than 27.2% inhibition was observed at 100 μ M, and **ABZ** exhibited xL3 motility by 16.0%. The two reference compounds, monepantel and moxidectin, exhibited the expected reduction in xL3 motility (> 85.0%). All six other compounds were ineffective at the highest concentration tested (100 μ M).

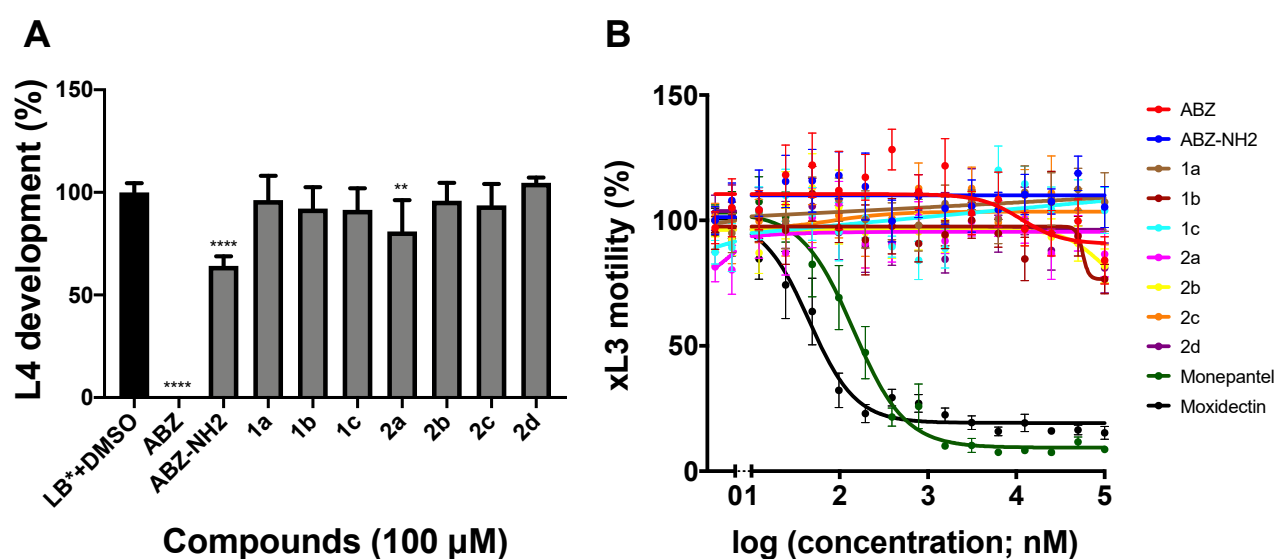


Figure 4: *In vitro* activity of ABZ derivatives against exsheathed third-stage larvae (xL3) *H. contortus in vitro*. (A) Development of xL3 to fourth-stage larvae (L4) at 100 μ M after 7 days. (B)

Dose-response curves of the effects of 9 compounds and two reference compounds (monepantel and moxidectin) on the inhibition of motility at 72 h. Data points represent three independent experiments conducted in triplicate; the mean \pm S EM. ** and **** denote for p values of 0.001 and <0.0001 , respectively.

Giardia lamblia. As expected, cultivation in the presence of 0.1 and 1 μ M **ABZ** resulted in complete inhibition of resazurin reduction, indicating loss of viability in 100% of all parasites, while the presence of 0.01 μ M **ABZ** caused a 70% reduction of viability when compared to the solvent control (Figure 5). Compound **ABZ-NH₂** was largely ineffective at all three concentrations, while for compound **1a** a reduction in viability to approximately 60% was measured at 0.01 μ M, but viability was increased at higher concentrations. For **1c**, **2a**, and **2c**, the presence of these compounds impaired viability to levels of 40-60%, except for **2d**, which resulted in a reduction of $>95\%$ when 1 μ M was applied. Compounds **1b** and **2b** were ineffective under these conditions.

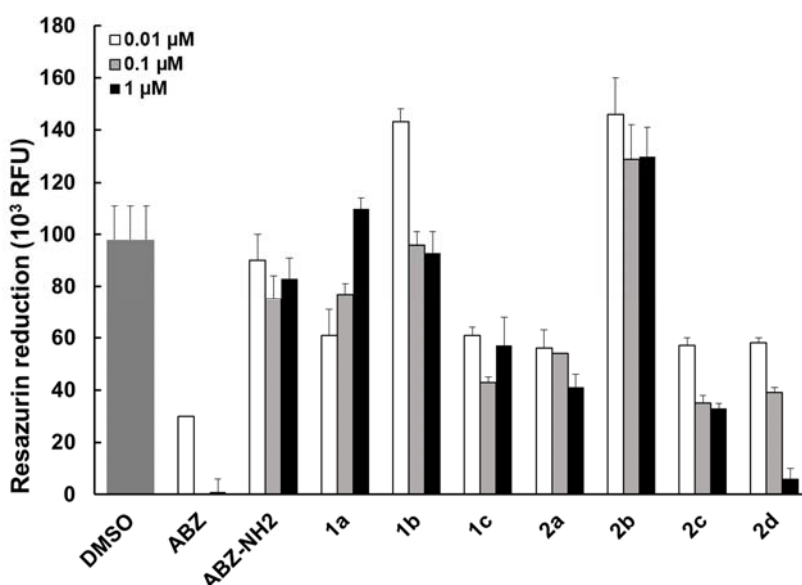


Figure 5. *In vitro* screening of *G. lamblia* trophozoites. The viability of *G. lamblia* trophozoites is measured by Resazurin assay. Compounds were applied at 1, 0.1 or 0.01 μ M for a period of 72 h.

Toxoplasma gondii. As can be seen in Figure 6, compounds **ABZ**, **ABZ-NH₂**, **1a**, **2d** and **2b** severely impair *T. gondii* proliferation when applied at 10 μ M, and when applied at lower concentrations, a clear dose-dependent effect is evident. However, the inhibition seen with **2b** and **2d** at 10 μ M can be partially attributed to the impact these compounds have against HFF host cells (see below).

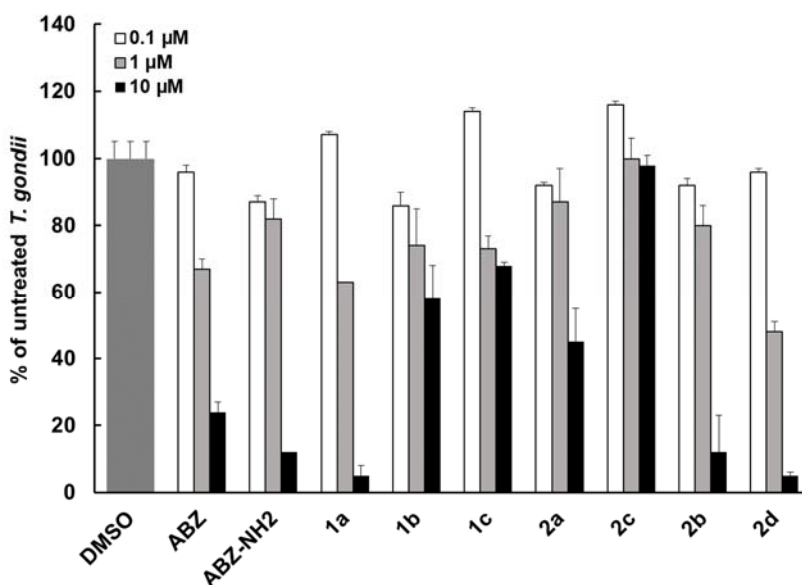


Figure 6. *In vitro* screening of *T. gondii* β -gal tachyzoites cultured in HFF host cells. The proliferation of *T. gondii* tachyzoites was quantified by measuring β -galactosidase activity. Compounds were applied at 0.1, 1 or 10 μ M for a period of 72 h. The compound activity is shown as percentage in relation to the DMSO controls which represent 100% of parasite growth.

In order to discriminate between the activity on the parasite and on the host cell, the toxicity of the compounds was also evaluated on HFF cells alone (Figure 7). With the exception of compound **2b** and **2d** at the highest concentration, none of these compounds presented cytotoxicity against HFF monolayers. Thus, with the exception of **2b**, and **2d**, the effects seen against *T. gondii* can be attributed to mechanisms that are targeting the parasites.

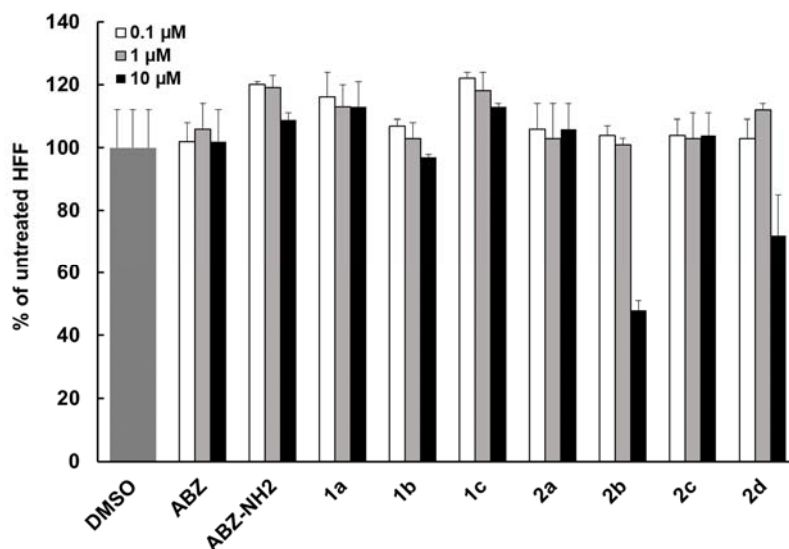


Figure 7. *In vitro* screening of non-infected HFF host cells. The viability of HFF was quantified by measurement of resazurin reduction. Compounds were applied at 0.1, 1 or 10 μM for a period of 72 h. The viability of HFF is shown as percentage in relation to the DMSO controls which represent 100% viability.

CONCLUSIONS

In this study, we synthesized and characterized a series of ferrocene- and ruthenocene-containing organometallic analogues of the anthelmintic drug **ABZ**. In human plasma, the metallocene-containing **ABZ** derivatives showed moderate to good stability after 6 h, with ferrocenyl derivative **2d** displaying the best stability after 24 h incubation. As for **ABZ**, the major metabolites for the compounds turned out to be the corresponding sulfoxides, as demonstrated by the syntheses of the oxidized forms of **1b** and **2d**, namely **1b-Ox** and **2d-Ox**. These findings are a good indication that the active forms were not due to the release of **ABZ** or of a free organometallic moiety. *In vitro* activity of the metallocene-containing **ABZ** derivatives against *H. polygyrus* adult, *T. muris* adult and *S. mansoni* adult showed that **1b** and **2d** displayed over 70% activity against *T. muris* at 200 μM . Compound **2b** also showed over 70% activity against *S. mansoni* at the highest concentration. Encouraged by these promising *in vitro* results, compounds **1b** and **2d** underwent testing in *T. muris* infected mice at a single dose of 400 mg/kg and exhibited interesting, albeit moderate, worm burden reductions and worm expulsion rates. However, the anthelmintic activity against *H. contortus* was negligible for most compounds, compared to the high efficiency of **ABZ** itself. Although not better than **ABZ**, the activity of four analogues (**1c**, **2a**, **2c** and **2d**) against protozoan

G. lamblia was also promising. Finally, against the protozoan *T. gondii*, compounds **1a** and **2d** showed better activity than **ABZ** at 1 μ M, while they mostly showed cytotoxic effects on not infected HFF host cells at 10 μ M. This suggests that the ferrocene-containing compounds **1a** and **2d** are promising anti-toxoplasma candidates for *in vivo* evaluation. Overall, this study demonstrates that organometallic **ABZ** derivatives can outperform **ABZ** as interesting leads for further development not only as a new class of anthelmintic agents but also against other types of parasites due to the attached organometallic portions. Further studies would be warranted to better elucidate the mechanism of action of these organometallic derivatives, investigated the effect on metal-specific properties, like ROS production, redox activation, and lipophilicity, etc. Nonetheless, these compounds present excellent starting points that help advance the field of organometallic medicinal chemistry in the area of neglected tropical diseases.

MATERIALS & METHODS

***In vitro* assays on *H. polygyrus* and *T. muris* adult worms**

Female NMRI mice were infected with 88 *H. polygyrus* L3. Two weeks post-infection, mice were dissected. Seven weeks post-infection of female C57BL/6NRj, *T. muris* adult worms were collected from the intestines. Three to four adult worms were placed in each well of a 24-well plate and exposed to the test drugs at concentrations ranging from 200-10 μ M. The highest percentage of DMSO used in the assays served as a control. The assay was conducted in duplicate. Worms were incubated at 37°C / 5% CO₂ up to 72 h, after which the drug effect was evaluated. The condition of the worms was microscopically evaluated using a viability scale from 3 (normal activity) to 0 (dead) following stimulation with 500 μ l hot water (\approx 80°C).

In vitro* assays on adult *S. mansoni

Mice were dissected seven weeks post-infection with 100 *cercariae*. At least three worms (both sexes) were incubated with 200 and 100 μ M of the derivatives in 2 ml RPMI 1640 (Gibco, USA) supplemented with 5% (v/v) FCS and 1% (v/v) pen–strep at 37°C / 5% CO₂ for 72 hours h. Details on the viability scale used for scoring are published elsewhere.⁴⁰

Animals

Three-week-old female C57BL/6NRj mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and female mice (NMRI strain; age 3 weeks-old; weight ca. 20-22 g) were purchased from

Charles River, Germany. Rodents were kept under environmentally-controlled conditions (temperature: 25°C, humidity: 70%, light/dark cycle 12 h /12 h) with free access to water (municipal tap water supply) and rodent food. Rodents were allowed to acclimatize for 1 week before infection. The current study was approved by the local veterinary agency, based on Swiss cantonal and national regulations (permission no. 2070).

T. muris in vivo studies

C57BL/6NRj mice were orally infected with 200 embryonated *T. muris* eggs. Mice were immunosuppressed with dexamethasone (1 mg/ml) in their drinking water until 2 days prior to treatment. At 42 days post-infection, the stool was collected and checked for the presence of eggs. The compounds were administered orally at a single oral dose of 400 mg/kg. Four untreated mice served as controls. The stool was collected from 0-72 h post-treatment from each mouse to determine the worm expulsion rate. Six to seven days post-treatment, animals were killed, the intestine was dissected and adult worms were collected and counted.

Cytotoxicity testing against L6 cells

Rat skeletal myoblast L6 cells were exposed to the test drugs. Podophyllotoxin (with 0.05 µg/ml as highest concentration) was used as a positive control. After a 70-h incubation at 37°C / 5% CO₂, resazurin dye (Alamar Blue) was added and the plates were incubated for an additional 2 hours. Fluorescence and the cytotoxic concentration (CC50) were measured at 530 nm excitation and 590 nm emission wavelength using a SpectraMax M2 (Molecular Device, Sunnyvale CA, USA; Softmax version 5.4.6). Experiments were conducted in duplicate and repeated once.

In vitro assessment of inhibitory activity on larval development of Haemonchus contortus

All derivatives and **ABZ** were individually tested for their ability to inhibit the motility of exsheathed third-stage larvae (xL3) of *H. contortus* and the development of these larvae to fourth-stage larvae (L4) using established assays.⁴¹ Compounds were tested in an 18 point, two-fold dilution series, starting at 100 µM. The reduction in xL3 motility was measured at 72 h, and inhibition of L4 development was evaluated after 7 days. For the xL3 motility assay, monepantel (Zolvix, Elanco Animal Health, Australia) and moxidectin (Cydectin, Virbac, France) were included as positive controls. Culture medium, i.e. Luria Bertani broth supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B (Gibco, Thermofisher Scientific, USA), with

matched concentrations of DMSO (0.25%), served as negative controls in the assays. Each assay has three technical replicates and was repeated three times on different days. For each compound, dose-response curves were determined by fitting the log₁₀-transformed concentration to a variable slope four-parameter model. The data were analysed with reference to the negative controls using non-parametric one-way ANOVA and Dunnett's multiple comparison tests employing the GraphPad Prism (v.8.3.1).

In vitro activity against the protozoan Giardia lamblia

G. lamblia trophozoites were grown under anaerobic conditions in 10 ml culture tubes (Nunc, Roskilde, Denmark) as described.⁴² Cultures with adherent confluent trophozoite lawns were incubated on ice for 15 min, suspended motile trophozoites were counted in a Neubauer chamber at 200 x magnification), and were distributed into 96 well plates in 0.2 ml Keister-medium containing 1000 trophozoites per well in the presence of the compounds (0.01, 0.1 or 1 µM, all in triplicates) dissolved in DMSO (10 mM stock solutions) or with the corresponding DMSO solvent control. They were then cultured for 72 h at 37°C in an anaerobic chamber (80% N₂, 10% H₂, 10% CO₂). Subsequently, plates were centrifuged (2 min, 1000 x g), medium was removed, and cells were washed once with PBS containing 1 g/l glucose. Then, PBS-glucose containing 10 mg/l resazurin was added, and plates were incubated for up to 5 h at 37°C. Plates were read (excitation 530 nm, emission 590 nm) using an Enspire multilabel reader (2300 EnSpire™ Multilabel Reader, Perkin-Elmer, Turku, Finland) at various time points, and the linear increase of fluorescence was used as a marker for cell viability.⁴³

In vitro cytotoxicity assessment in human foreskin fibroblasts (HFF)

The cytotoxicity of **ABZ** and its derivatives in HFF host cells was assessed as previously described.⁴⁴ Briefly, HFF were grown to confluence in 96 well-plates. The medium was removed, and monolayers received 200 µl fresh medium containing either 0.1, 1 or 10 µM of each compound, or the medium was supplemented with the corresponding concentrations of DMSO. Following cultivation at 37°C / 5% CO₂ for 72 h, the medium was aspirated, and cells were washed once with PBS. Subsequently, 200 µl PBS containing resazurin (10 mg/ml) were added per well. Plates were read (excitation 530 nm, emission 590 nm) using an Enspire multilabel reader (2300 EnSpire™ Multilabel Reader, Perkin-Elmer, Turku, Finland) at different timepoints between 0 and 5h. The cytotoxicity was calculated and is represented as percentage of the DMSO control.

In vitro* activity against the protozoan *Toxoplasma gondii

For the assessment of drug efficacy against *Toxoplasma* tachyzoites, transgenic *T. gondii* β -gal tachyzoites (RH background, constitutively expressing β -galactosidase)⁴⁵ were cultured in human foreskin fibroblasts (HFF) host cells as previously described.⁴⁶ Assays were carried out in 96 well plates. 5×10^3 HFF / well were grown to confluency in 200 μ l phenol-red free culture medium at 37 °C and 5% CO₂. Monolayers were infected with freshly isolated *T. gondii* β -gal tachyzoites (1×10^3 / well) and drugs were added concomitantly with infection. Assessment of drug efficacy was done by exposing parasite cultures to 0.1, 1, 10 μ M (quadruplicates) of each compound (40 mM stock solutions), and respective concentrations of DMSO were added to control wells. After 72 h of culture in the presence of the drugs at 37°C/ 5% CO₂, the medium was removed by aspiration, wells were washed once with 200 μ l PBS and cells were permeabilized with 90 μ l PBS containing 0.05% Triton X-100. After addition of 10 μ L of 5 mM chlorophenol red- β -D-galactopyranoside (CPRG; Roche Diagnostics, Rotkreuz, Switzerland) dissolved in PBS, the absorption shift was measured at 570 nm wavelength at various time points on a EnSpire[®] multimode plate reader (Perkin Elmer). The activity, measured as the release of chlorophenol red over time, was calculated as percentage from DMSO control, which represented 100% of *T. gondii* growth.

ASSOCIATED CONTENT

Supporting Information contains detailed synthetic procedures and characterizations of the compounds and copies of the NMR spectra, details concerning the X-ray analyses and copies of the HPLC chromatograms for the plasma stability assays.

AUTHOR INFORMATION

Corresponding Authors

*e-mail: kevin.cariou@cnrs.fr

* e-mail: jennifer.keiser@swisstph.ch

* e-mail: gilles.gasser@chimieparistech.psl.eu

ORCID:

Yan Lin: 0000-0001-7808-5444

Yih Ching Ong: 0000-0003-0411-1114

Sarah Keller: 0000-0003-2667-9157

Johannes Karges: 0000-0001-5258-0260

Rafika Bouchene: 0000-0003-0688-8365

Eric Manoury: 0000-0001-7991-8890

Aya C. Taki: 0000-0003-3489-4367

Andrew Hemphill: 0000-0002-0622-2128

Robin B. Gasser: 0000-0002-4423-1690

Kevin Cariou: 0000-0002-5854-9632

Jennifer Keiser: 0000-0003-0290-3521

Gilles Gasser: 0000-0002-4244-5097

Author Contribution

All authors were involved with the design and interpretation of experiments and with the writing of the manuscript. K.C., J.K. and G.G. have conceived and supervised the study. Synthesis and characterization of the compounds were performed by Y. L. and R.B. with assistance from Y.C.O., S.K. & J.K. Biological experiments were carried out by J.M., N.A., A.H., A.C.T., R.B.G., J.K.. X-ray crystallography was carried out by O.B. Animal testing was carried out by J.K. All authors have given approval to the final version of the manuscript. The authors thank Sabrina Lebrequier and David-Alexandre Buisson for excellent analytical support (Université Paris-Saclay, Service de Chimie Bio-organique et Marquage, CEA, 91191, Gif-sur-Yvette, France).

Funding Information

This work was financially supported by the Swiss National Science Foundation (Grant Sinergia CRSII5_173718, to G.G.), an ERC Consolidator Grant PhotoMedMet to G.G. (GA 681679) and has received support under the program *Investissements d'Avenir* launched by the French Government and implemented by the ANR with the reference ANR-10-IDEX-0001-02 PSL (G.G.). Y.L. thanks to the China Scholarship Council for financial support. Funding from the Australian Research Council, Yourgene Health and The University of Melbourne is gratefully acknowledged.

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Graphical Abstract

