Conjugation of *N*-acylhydrazone and 1,2,4-oxadiazole leads to the identification of active antimalarial agents

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ABSTRACT

Malaria, caused by several *Plasmodium* species, is the major life-threatening parasitic infection worldwide. Due to the parasite resistance to quinoline based drugs, the search for antimalarial agents is necessary. Here, we report the structural design, synthesis and antiparasitic evaluation of two novel series of 1,2,4-oxadiazoles in conjugation to *N*-acylhydrazones, both groups recognized as privileged structures, as well as the studies on the antimalarial activity of 16 previous described analogues. By varying substituents attached to the phenyl ring, it was possible to retain, enhance or increase the antiparasitic activity in comparison to the nonsubstituted derivatives. Replacement of substituted aryl rings by ferrocenyl and cinnamyl moieties attached in the *N*-acylhydrazone ablated the antiparasitic response, evidencing the structural features associated with the activity. Active compounds exhibited in vitro potency similar to mefloquine, but not all inhibited β-hematin formation. Additionally, the active compounds displayed low cytotoxicity in HepG2 cells and did not cause hemolysis in uninfected erythrocytes. In *Plasmodium berghei*-infected mice, the compounds reduced parasitemia but exhibited limited efficacy in increasing mice survival when compared to chloroquine, suggesting that pharmacological improvement is still necessary.

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1. Introduction

Malaria represents a major threat to the public human health worldwide, with over 230 million clinical cases and about 660,000 deaths annually. In comparison to the last decade, the mortality is decreasing, however, malaria remains widespread in endemic areas.[1] Current treatment relies on quinolines and artemisinin combinations; even so, there are significant problems of drug resistance.[2,3] It is thus important to identify drug candidates based on new chemotypes. To address this need, phenotype-based antimalarial screening has been employed to identify active compounds chemically distinct from artemisinin and quinolines. For instance, this approach has led to the discovery of the ozonide compounds arterolane and OZ439, the spiroindolone KAE609, the

naphthoquinone decoquinate as well as the thiazole albitiazolium.[4–8] These compounds are considered to be drug candidates and are likely to enter into clinical trials, strengthening the concept that phenotype-based screening is a suitable approach for antimalarial drug discovery.

Oxadiazoles are a class of heterocyclic compounds useful for the development of anti-infectious agents.[9–12] The most notable example is the class of the 1,2,5-oxadiazole-2-oxides, collectively known as furoxans, which have a broad spectrum of antiparasitic activity, including antimalarial property (Fig. 1). Furoxans exert antiparasitic activity in part by releasing endogenous nitric oxide, which is an antimicrobial molecule. Interestingly, furoxan derivatives without the *N*-oxide group, which is essential to confer a nitric oxide release, also exhibit antiparasitic properties. This suggests that furoxan has a multi-target mechanism of action, therefore oxadiazoles are considered potential antiparasitic compounds.[12,13]

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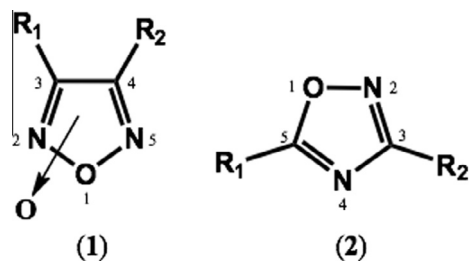
Figure 1. General representation of 1,2,5-oxadiazole-2-oxide (**1**) and 1,2,4-oxadiazoles (**2**).

Previously, *N*-acylhydrazones derived from 3-aryl-1,2,4-oxadiazoles were identified as antiparasitic agents against *Trypanosoma cruzi*, displaying low toxicity in both in vitro and in vivo experiments as well as high efficacy in in vivo models of *T. cruzi*-infection.[14,15] In this line, *N*-acylhydrazones derived from 7-chloroquinoline and 1,3-diiminoisoindoline exhibited strong antimalarial activities.[16,17] Based on these findings, here we investigated the antimalarial activity for *N*-acylhydrazones derived from 3-aryl-1,2,4-oxadiazoles (Fig. 2). First, a series of known *N*-acylhydrazones bearing a 4-hydroxy-3-methoxyphenyl (vanillinyl) portion **SintMed** (**1–8**), planned and synthesized on the basis of molecular hybridization concepts, was investigated. Since the *N*-acylhydrazone-1,2,4-oxadiazole moiety has been identified as important to disclose antiparasitic activity,[14,15] its structural association with the vanillinyl portion was exploited as a promising strategy to increase the molecular recognition by the biological system. Vanillin itself and its derivatives are largely described as biological active compounds,[18] reinforcing the grounds of this strategy. The second series, also previously described in our group, has arisen from the exploitation of the isosteric relation between the vanillinyl group and the 1,3-benzodioxole-5-phenyl (piperonyl) moiety **SintMed** (**9–16**), also a portion of natural occurrence.

In addition, sixteen new *N*-acylhydrazone-1,2,4-oxadiazoles were prepared for the first time and evaluated for their antimalarial activities. Following the defined strategy, the first unexampled series incorporates a cinnamyl moiety attached to the *N*-acylhydrazone, which yielded the compounds **SintMed** (**17–24**). This molecular modification was investigated due to the important role played by the cinnamyl group present in molecules with demonstrated antimalarial activity.[19a,b] Another novel series of *N*-acylhydrazone-1,2,4-oxadiazoles includes a ferrocenyl substitution, a non-natural occurring and unique group, leading to

SintMed (**25–32**). Ferrocenyl has been for long time associated to bioactive molecules, promoting meaningful biological improvements, as demonstrated for ferroquine, a ferrocenyl analogue of chloroquine, which was not only active against chloroquine sensitive *Plasmodium* parasites, but also against resistant strains.[20a,b] Therefore, association of the *N*-acylhydrazone-1,2,4-oxadiazole structure with each of the selected groups can represent a great possibility to disclose new potent active antimalarials agents, whose remarkable property is the structural simplicity and low cost of production.

In vitro screening of compounds **SintMed** (**1–32**) led us to identify some of them having a potent activity and selectivity against the blood-stage *Plasmodium falciparum*, as well as to acquire a better understanding of structure–activity relationships implied in the modification of the *N*-acylhydrazone-1,2,4-oxadiazole basic structure used in our group as a fundamental model in drug design.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **SintMed** (**17–32**), depicted in Scheme 1, is based on a method developed by our group.[15] Briefly, commercially available aryl nitriles were converted into 4-substituted arylamidoximes by refluxing during 4 h in the presence of hydroxylamine with good yields, which constitutes our primary start material. Under inert atmosphere and dry conditions, arylamidoximes react with monomethyl oxalyl chloride, leading to methyl 3-(4-arylsubstituted)-1,2,4-oxadiazole carboxylates, which were converted into the corresponding 1,2,4-oxadiazole hydrazides under mild condition.

Although the condensation of hydrazides with vanillin and piperonal, respectively, under sulfuric acid catalysis at room temperature leads easily to the series **SintMed** (**1–8**) and **SintMed** (**9–16**) as previously reported, this same condition did not work for synthesizing ferrocenyl derivatives **SintMed** (**25–32**). In order to overcome this inconvenience, the reaction between the hydrazides and ferrocenecarboxyaldehyde was carried out in the presence of cerium(III) chloride heptahydrate (CeCl$_3$·7H$_2$O) as a catalyst with isolated average yields of 90%, following a method recently developed in our laboratory.[21]

Compounds **SintMed** (**25–32**) were analyzed by usual ^{1}H NMR, and ^{13}C NMR. Signal assignments for the ferrocene group was assisted by DEPT, HSQC and HMBC experiments, in addition to comparing the findings with related literature.[22,23] ^{1}H NMR analysis of crude ferrocenyl compounds **SintMed** (**25–32**) revealed that only the *E*-isomer was formed, in agreement with previously reported outcomes for other compounds synthesized using this method.[21] Due to the mild condition and low reaction time, cerium(III) chloride catalysis has proved to be highly stereoselective, leading exclusively to the thermodynamically more stable *E*-isomer. Considering that isomeric purity is important for pharmacological evaluation, the new series of cinnamyl derivatives **SintMed** (**17–24**) as well as compounds **SintMed** (**1–16**) were also synthesized in the presence of CeCl$_3$-catalyst and yielded *E*-isomer only. Taking the ^{1}H NMR spectra of representative compounds **SintMed23** and **SintMed31**, the stereoselectivity of the CeCl$_3$-method can be easily corroborated (Fig. 3).

This outcome was analyzed by observing the signal of the iminic proton (–N=CH–), which resonates at $\delta \approx 8.50$–8.30 ppm. In previous reports, it was determined that this value is characteristic for *E*-isomers, while *Z*-isomers usually show signals at $\delta \approx 7.90$–8.00 ppm.[15,21] The presence of *E*/*Z*-isomers can also be established by observing the signal of the amide group (–CONH–), which splits up in two singlets when both isomers are formed. In all cases discussed in this work, only one signal for each of these groups was
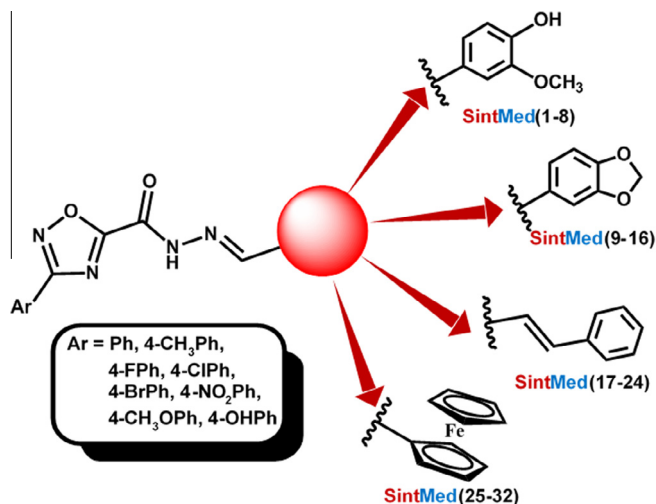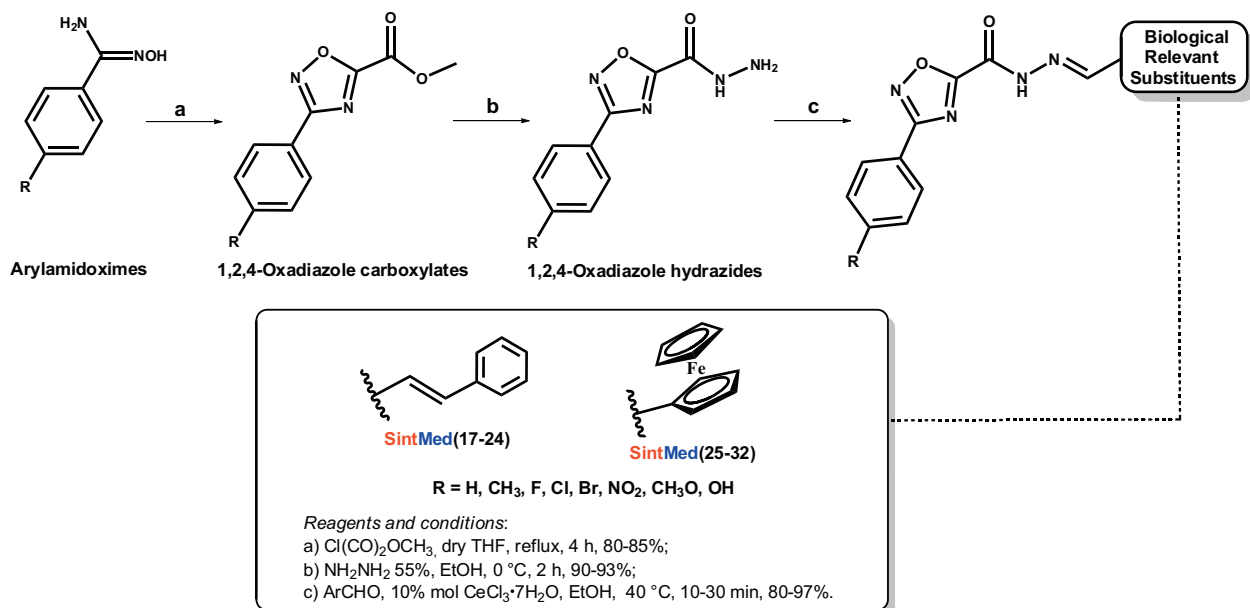

Figure 2. Structure of the *N*-acylhydrazone-1,2,4-oxadiazoles analogues **SintMed** (**1–32**) evaluated as antimalarial compounds.

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Scheme 1. Synthetic route for *N*-acylhydrazone-1,2,4-oxadiazole compounds.

R = H, CH₃, F, Cl, Br, NO₂, CH₃O, OH

Reagents and conditions:
a) Cl(CO)₂OCH₃, dry THF, reflux, 4 h, 80-85%;
b) NH₂NH₂ 55%, EtOH, 0 °C, 2 h, 90-93%;
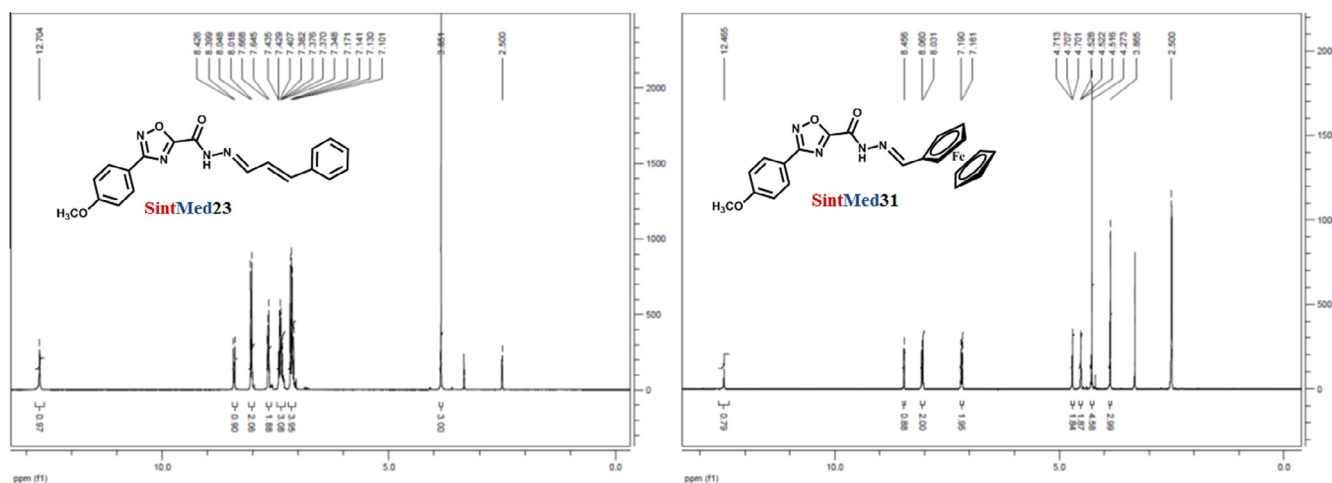c) ArCHO, 10% mol CeCl₃·7H₂O, EtOH, 40 °C, 10-30 min, 80-97%.

Figure 3. ^{1}H NMR spectra of compounds **SintMed23** and **SintMed31** as pure *E*-isomers.

observed, including the known compounds **SintMed** (**1–16**), which were previously described with signals of *Z*-isomers.[15]

2.2. Pharmacological evaluation

Compounds **SintMed** (**1–32**) were initially screened at 10 μg/mL against the chloroquine-resistant W2 strain of blood-stage *P. falciparum* (Table 1). Compounds which have inhibited more than 60% parasite growth at this concentration were considered active and had their IC$_{50}$ values determined. Mefloquine (**Mfq**) was used as the reference antimalarial drug in these assays.

First, the parasite grown inhibited by compounds containing a 4-hydroxy-3-methoxyphenyl moiety **SintMed** (**1–8**) was investigated. The unsubstituted compound at phenyl ring **SintMed1** was inactive, while the attachment of a methyl at position 4 of phenyl **SintMed2** produced an active antimalarial compound. In contrast, attaching fluoro **SintMed3**, chlorine **SintMed4** or methoxy **SintMed7** substituents did not produce active compounds. But attaching a bromo **SintMed5** or a nitro group **SintMed6**

increased the activity of both compounds. In comparison to the unsubstituted compound **SintMed1**, the attachment of a hydroxyl group **SintMed8** disclosed a compound on the edge of the activity parameter. The series bearing the 1,3-benzodioxole moiety **SintMed** (**9–16**) was investigated, based on its close isosteric relation with the previous series **SintMed** (**1–8**). Except for the compound **SintMed16**, substituted with a hydroxy group, and possessing expressive activity, all other members of this series were inactive. Instead of a withdraw, this outcome gives some key clues about the nature of the structural requirements necessary to enhance the antimalarial activity of analogue molecules. The presence of a hydroxyl group linked to a phenyl ring at the *N*-acylhydrazone-1,2,4-oxadiazole skeleton seems to be essential to the emergence of active antimalarial compounds.

This assumption has found support in the results for the series containing cinnamyl **SintMed** (**17–24**) and ferrocenyl **SintMed** (**25–32**) moieties, both inactive against *P. falciparum*. These preliminary observations narrow the structural possibilities that can be pursued in the search for new antimalarials based on the *N*-acylhydrazone-1,2,4-oxadiazole chemotype.

Table 1
Initial anti-*P. falciparum* screening against chloroquine-resistant W2 strain

Compound	R	% inhibition[a]	Compound	R	% inhibition[a]
SintMed1	H	55.4	**SintMed5**	Br	71.1
SintMed2	CH$_3$	61.1	**SintMed6**	NO$_2$	68.7
SintMed3	F	24.7	**SintMed7**	OCH$_3$	42.2
SintMed4	Cl	37.0	**SintMed8**	OH	58.8

SintMed9	H	27.5	**SintMed13**	Br	45.3
SintMed10	CH$_3$	21.9	**SintMed14**	NO$_2$	33.9
SintMed11	F	31.6	**SintMed15**	OCH$_3$	27.5
SintMed12	Cl	53.0	**SintMed16**	OH	71.9

SintMed17	H	51.0	**SintMed21**	Br	58.8
SintMed18	CH$_3$	52.7	**SintMed22**	NO$_2$	25.7
SintMed19	F	34.3	**SintMed23**	OCH$_3$	26.1
SintMed20	Cl	28.8	**SintMed24**	OH	39.1

SintMed25	H	3.3	**SintMed29**	Br	29.8
SintMed26	CH$_3$	35.2	**SintMed30**	NO$_2$	0
SintMed27	F	22.7	**SintMed31**	OCH$_3$	3.0
SintMed28	Cl	0	**SintMed32**	OH	43.8
Mfq	—	100	—	—	—

[a] % of parasite inhibition using the concentration of 10 µg/mL for each compound. Determined 72 h after incubation of W2 strain *P. falciparum* (erythrocytic stage) with the respective compounds. Mfq = mefloquine.

The five active compounds were submitted to the evaluation of their in vitro potency against *P. falciparum*, and the standard drug mefloquine was used as a reference parameter, as shown in Table 2. Mefloquine exhibited an IC$_{50}$ value of 0.04 ± 0.01 µM. In comparison to mefloquine, vanillinyl moiety-bearing derivatives of *N*-acyl-hydrazone-1,2,4-oxadiazole **SintMed2** (4-mehylsubstituted), **SintMed5** (4-bromosubstituted) and **SintMed6** (4-nitrosubstituted) displayed potent anti-*P. falciparum* activities. While compound **SintMed5** was found to be almost as potent as mefloquine with IC$_{50}$ of 0.07 ± 0.12 µM, the other two analogues **SintMed2**

and **SintMed6** were less potent, but still exhibited expressive activities: 0.12 ± 0.16 µM and 0.20 ± 0.16 µM, respectively. Replacement by 4-hydroxyl **SintMed8** decreased potency of the final compound. This revealed the importance of a specific substituent located in the phenyl ring attached to the 1,2,4-oxadiazole group, suggesting that lipophilic groups can be more successful in further planning of antimalarial compounds based on this structural features. In Table 2, the lipophilicity parameters (log*P*) for the five active compounds are given, evidencing that the more potent compounds present higher lipophilicity in the studied

Table 2
In vitro potency, cytotoxicity, selectivity and log*P*

Compounds	*P. falciparum* IC$_{50}$ ± SD (µM)[a]	HepG2 **CC$_{50}$** ± SD (µM)[b]	SI[c]	log*P*[d]
SintMed2	0.12 ± 0.16	46.6 ± 0.5	388	3.00
SintMed5	0.07 ± 0.12	16.9 ± 1.9	241	3.36
SintMed6	0.20 ± 0.16	14.8 ± 0.8	74	2.50
SintMed8	1.9 ± 0.8	>200	—	2.07
SintMed16	1.5 ± 1.09	>200	—	2.62
Mfq	0.04 ± 0.01	11.8 ± 0.3	295	—

[a] Determined 48 h after incubation with the drugs.
[b] Determined 72 h after incubation with the drugs.
[c] SI was determined by the ratio CC$_{50}$/IC$_{50}$. IC$_{50}$ and CC$_{50}$ values were determined from two independent experiments using each concentration in triplicate. IC$_{50}$ = inhibitory concentration to 50%. CC$_{50}$ = cytotoxic concentration to 50%. SD = standard deviation. Mfq = Mefloquine.
[d] Determined using the online version of the Molinspiration Cheminformatics software (http://www.molinspiration.com/).

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group. The only one active compound derived from the 1,3-benzo-dioxole series **SintMed** (**9**–**16**), the 4-hydroxylphenyl substituted analogue **SintMed16**, displayed potency similar to **SintMed8**.

Next, the cytotoxicity against mammalian HepG2 cells was tested and expressed as CC_{50} values, as well as the selectivity index (SI), both results described in Table 2. Comparing to mefloquine, which displayed CC_{50} value of 11.8 ± 0.3 µM, none of the studied compounds was more cytotoxic. Compounds **SintMed8** and **SintMed16** were several folds less cytotoxic than mefloquine, while the other compounds were similarly cytotoxic. Mefloquine had an SI of 295, and compounds **SintMed2** (SI = 388) and **SintMed5** (SI = 241) revealed a similar selectivity, reinforcing the evidences that lipophilic groups are important for the antimalarial activity, especially when the SI of 74 for **SintMed6** is taken into account. The nitro group possesses clearly hydrophilic properties, affecting the activity and selectivity of **SintMed6**. Although SI for **SintMed8** and **SintMed16** could not be precisely determined due to lack cytotoxicity against HepG2 cells, it is estimated that they are selective antiparasitic agents.

In addition to the cytotoxicity in HepG2 cells, the percentage of hemolysis in uninfected erythrocytes was determined. As can be seen in Table 3, except for compound **SintMed5**, which caused hemolysis in the tested concentrations, none of the compounds did it. Again, the nature of the bromine atom attached to the molecule plays an important role for its biological response. The ability of active compounds to inhibit the hemin polymerization into β-hematin was studied. In this assay (Table 3), chloroquine displayed an IC_{50} of 0.84 ± 0.30 mM. Compounds **SintMed2**, **SintMed5** and **SintMed16** inhibited β-hematin in the tested concentrations with IC_{50} similar to chloroquine, while compounds **SintMed6** and **SintMed8** did not. The CQ index indicates the potency of each compound in comparison with chloroquine.

Finally, it was examined the ability of the most potent antimalarial derivatives, compounds **SintMed2**, **SintMed5** and **SintMed6**, to decrease parasitemia and increase survival in *P. berghei*-infected mice. After 1 h of infection with NK65 strain, mice were treated orally by gavage or intraperitoneal injection once a day during 4 consecutive days. As can be seen in Figure 4, untreated infected group shows parasitemia, which usually peaks at 6–8 days post infection. By comparison with the untreated group, chloroquine given orally at 50 mg/kg/day decreases parasitemia ($p < 0.001$) and provides 100% animal survival ($p < 0.05$) after 30 days of infection. Under oral treatment with compounds **SintMed2** and **SintMed5** at 100 mg/kg/day, no parasitemia reduction was detected, and death was observed in less than 30 days (Fig. 4). Even the intraperitoneal administration of compounds **SintMed2** and **SintMed5** at 100 mg/kg/day did not lead to better outcomes (data not shown).

In contrast, intraperitoneal treatment with compound **SintMed6** dosed at 50 mg/kg/day and 25 mg/kg/day decreased parasitemia ($p < 0.001$) in both cases, consisting in a very important experimental result (Fig. 5). Unfortunately, mice survival under such conditions was not so successful, although

Table 3
Hemolytic property, β-hematin formation inhibition and CQ-index

Compounds	Hemolysis $CC_{50} \pm SD$ (µg/mL)[a]	β-Hematin $IC_{50} \pm SD$ (mM)	CQ index[b]
SintMed2	>100	0.6 ± 0.2	1.3
SintMed5	33.0 ± 0.1	0.9 ± 0.03	0.8
SintMed6	>100	>2.0	—
SintMed8	>100	>2.0	—
SintMed16	>100	0.8 ± 0.5	1.0
CQ	>100	0.8 ± 0.3	—

[a] Hemolysis was assayed in uninfected human erythrocytes (type O⁺) after 1 h of incubation with compounds, in triplicate.
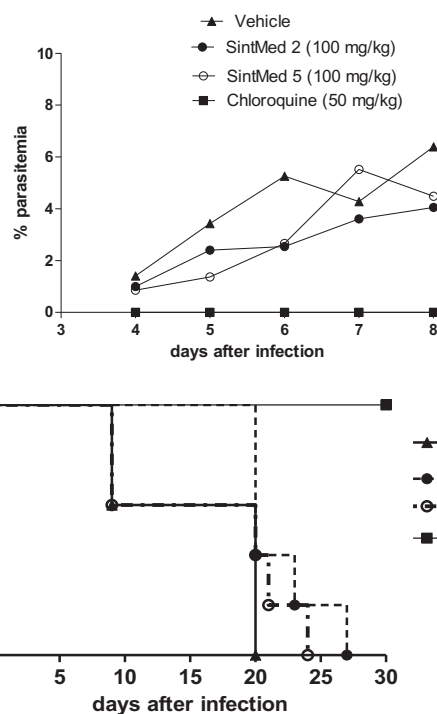[b] Determined by the ratio IC_{50} CQ/IC_{50} test compound. CQ = Chloroquine.

Figure 4. (Top) Blood parasitemia and (Bottom) % survival of *P. berghei*-infected mice. Swiss mice (*n* = 5/group) were infected with NK65 strain and 1 h after, treated once a day for four consecutive days. Vehicle group received 10% DMSO in saline. Values represent the means ± SEM of five mice per group in one experiment of two replicates performed. CQ = chloroquine. i.p. = intraperitoneal injection. p.o. = oral (gavage). Top: vehicle vs CQ (one-way ANOVA, $p < 0.001$). Bottom: vehicle vs CQ (Mantel-Cox test, $p < 0.05$).
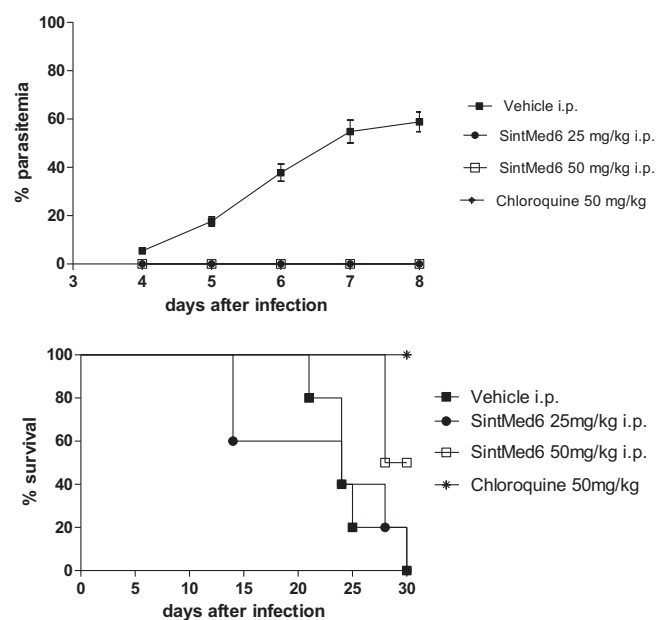

Figure 5. (Top) Blood parasitemia and (Bottom) % survival of *P. berghei*-infected mice. Swiss mice (*n* = 5/group) were infected with NK65 strain and 1 h after, treated once a day for four consecutive days. Vehicle group received 10% DMSO in saline. Values represent the means ± SEM of five mice per group in one experiment of two replicates performed. CQ = chloroquine. i.p. = intraperitoneal injection. p.o. = oral (gavage). Top: vehicle vs CQ (one-way ANOVA, $p < 0.001$), vehicle vs (**SintMed6**) 25 mg/kg (one-way ANOVA, $p < 0.01$), vehicle vs (**SintMed6**) 50 mg/kg (one-way ANOVA, $p < 0.001$). Bottom: vehicle vs CQ (Mantel-Cox test, $p < 0.05$).

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an improvement could be registered, reinforcing our initial assumptions about the importance of the 1,2,4-oxadiazole-*N*-acyl-hydrazone backbone structure as a pattern for the design and development of potential new antiparasitics of high efficacy.

3. Conclusion

Here, new antimalarial compounds were identified on the basis of structural design by employing hybridization between 1,2,4-oxadiazole, *N*-acylhydrazone and natural or synthetic occurring relevant biological groups. Antimalarial activity was observed to be quite dependent on the vanillinyl moiety attached to the iminic structure, whose biological response for the active derivatives seems to be dependent on the lipophilicity of the substituents linked to the 4-position of the phenyl ring attached to the oxadiazole portion. This structural design successfully led to the identification of compounds **SintMed2**, **SintMed5** and **SintMed6** as potent and selective anti-*P. falciparum* agents, as similarly observed in mefloquine-treated parasites. Mechanistically, these compounds did show general inhibitory properties for β-hematin formation, suggesting that they present a mechanism of action similar to that of quinoline based drugs, which are well-known β-hematin inhibitors. Therefore, the collected data suggest that the molecular hybridization is a promising line of research for structural modification targeting antiparasitic agents based on 1,2,4-oxadiazole-*N*-acylhydrazone association.

4. Experimental section

4.1. Chemistry

All solvents and reactants were purchased from Sigma–Aldrich, Fluka, Vetec and Acros Chemicals, and were used without further purification. Reactions' progresses were monitored by thin layer chromatography (TLC), performed onto glass-backed plates of silica gel 60 F254 with gypsum, and all compounds were detected by ultraviolet light (254 nm). Melting points were determined with a capillary apparatus Gehaka PF 1500 Farma and are uncorrected. NMR spectra were recorded at 400 MHz for hydrogen and 100 MHz for carbon, using a Varian UNMRS 400 spectrometer, or at 300 MHz for hydrogen and 75 MHz for carbon nuclei, using a Varian Unitplus 300 NMR. Analyses were determined in DMSO-d_6 with chemical shift values (δ) in parts per million (ppm) and coupling constants (J) in Hertz (Hz), and measured at 25 °C. ^{1}H and ^{13}C assignments were assisted by 2D experiments, such as DEPT full edit, HMBC and HSQC. IR spectra were recorded on a Tensor27 FTIR spectrometer from Bruker with the samples being analyzed as KBr pellets. High resolution mass spectrometry (HRMS) analysis of the pure compounds was performed with an UHPLC-ESI-HRMS on a Micromass LCT Premier time-of-flight mass spectrometer from Waters with an electrospray ionization (ESI) interface (Waters, Milford, MA, USA) equipped with an electrospray interface and coupled to an Acquity UHPLC system (Waters, Milford MA, USA). UHPLC-ESI-HRMS analysis was performed using the following parameters: ESI used capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 20 L/h, and desolvation gas flow 800 L/h. Detection was performed in positive ion mode (PI) with a *m/z* range of 100–1300 Da and a scan time of 0.5 s in the W-mode. The MS was calibrated using sodium formate, and leucine encephalin (Sigma–Aldrich, Steinheim, Germany) was used as an internal reference at 2 µg/mL and infused through a Lock Spray™ probe at a flow rate of 10 µL/min with the help of a second LC pump. The separation was performed on an Acquity BEH C$_{18}$ UPLC column (1.7 µm, 50 × 2.1 mm i.d.; Waters, Milford, MA, USA) using a linear gradient, solvent system: (A) 0.1% formic acid–water, (B) 0.1% formic acid–acetonitrile; gradient: 5–36% B in 1 min, then 36% B to 36% B in 2 min, 36% B to 95% B in 1.5 min; flow rate 1.1 mL/min. The temperature was set to 40 °C. The injected volume was kept constant (1 µL; solution at the concentration of 0.5 mg/mL). Elemental analyses were performed in a Perkin Elmer 2400 Series II elemental analyzer.

4.1.1. Preparation of compounds SintMed (1–32)

To a stirred suspension of 1 mmol of appropriate hydrazide and 1 mmol of aldehyde in 10 mL of ethanol 10 mol % cerium(III) chloride heptahydrate was added and the reaction mixture was stirred at 40 °C during 10–30 min. Reaction's completion was monitored by TLC, using ethyl acetate as eluent system. Once concluded, the heating was put away, and 20 mL of water was added to the medium. After standing at refrigerator, vacuum filtration was carried out, and the solid was washed with cold water/ethanol 1:1 followed by cold water. ^{1}H NMR analysis of all crude products confirmed their purity. Recrystallization from dioxane/water mixture afforded the pure products for biological purposes. Yields, melting points, spectroscopic and spectrometric data are listed below for each new compound.

4.1.1.1. 3-Phenyl-*N′*-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed17. Yield: 90%; Mp 167.6–170–2 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3266 (NH), 3090 (Ar CH), 1700 (C=O), 1625 (C=N, imine), 1529 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.7 (s, 1H, CONH), 8.42 (d, 1H, ^3J = 8.1 Hz, N=CH), 8.10 (d, 2H, ^3J = 7.8 Hz, Ph H-2,6), 7.67–7.59 (m, 5H, Ph H-3,4,5 and Cinnamyl H-2,6), 7.42 (d, 1H, ^3J = 6.6 Hz, CH=C*H*Ph), 7.37 (t, 2H, ^3J = 4.8 Hz, Cinnamyl H-3,5), 7.11 (d, 2H, *H*C=CHPh and Cinnamyl H-4); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.7 (1C, C=O), 168.1 (1C, Oxadiazole C-3), 153.5 (1C, C=NH), 149.4 (1C, Oxadiazole C-5), 141.3 (1C, *C*H=CHPh), 135.6 (1C, Cinnamyl C-1), 132.0 (1C, Ph C-4), 129.4 (2C, Ph C-3,5), 129.2 (1C, CH=*C*HAr), 128.8 (2C, Cinnamyl C-3,5), 127.3 (2C, Cinnamyl C-2,6), 127.2 (2C, Ph C-2,6), 125.4 (1C, Ph C-1), 125.1 (1C, Cinnamyl C-4); UHPLC-TOF-MS for C$_{18}$H$_{14}$N$_4$O$_2$ calcd (found)/Error: 318.1117 (317.1089, [M−H]$^-$)/15.8 ppm.

4.1.1.2. 3-(4-Tolyl)-*N′*-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed18. Yield: 94%; Mp 197.1–199.7 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3232 (NH), 3058 (Ar CH), 1684 (C=O), 1622 (C=N, imine), 1581 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.7 (s, 1H, CONH), 8.41 (d, 1H, ^3J = 6.0 Hz, N=CH), 7.98 (d, 2H, ^3J = 5.7 Hz, Tolyl H-2,6), 7.65 (d, 2H, Cinnamyl H-2,6), 7.43–7.40 (m, 5H, Tolyl H-3,5, CH=C*H*Ph and Cinnamyl H-3,5), 7.13 (d, 2H, C*H*=CHAr and Cinnamyl H-4), 2.39 (s, 3H, CH$_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.5 (1C, C=O), 168.1 (1C, Oxadiazole C-3), 153.4 (1C, C=NH), 149.4 (1C, Oxadiazole C-5), 142.1 (1C, Tolyl C-4), 141.3 (1C, *C*H=CHPh), 135.6 (1C, Cinnamyl C-1), 129.9 (2C, Tolyl C-3,5), 129.2 (CH=*C*HPh), 128.8 (2C, Cinnamyl C-3,5), 127.3 (2C, Cinnamyl C-2,6), 127.1 (2C, Tolyl C-2,6), 125.1 (1C, Cinnamyl C-4), 122.7 (1C, Tolyl C-1), 21.1 (1C, CH$_3$); UHPLC-TOF-MS for C$_{19}$H$_{16}$N$_4$O$_2$ calcd (found)/Error: 332.1273 (331.1189, [M−H]$^-$)/1.8 ppm.

4.1.1.3. 3-(4-Fluorophenyl)-*N′*-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed19. Yield: 94%; Mp 214.4–215.8 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3256 (NH), 3058 (Ar CH), 1685 (C=O), 1607 (C=N, imine), 1584 (C=N, heterocyclic); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 12.7 (s, 1H, CONH), 8.41 (d, 1H, ^3J = 8.0 Hz, N=CH), 8.13 (dd, 2H, $^3J_{FH}$ = 3.2 Hz, $^3J_{HH}$ = 8.8 Hz, 4-FPh H-2,6), 7.68 (d, 2H, ^3J = 7.2 Hz, Cinnamyl H-2,6), 7.49–7.30 (m, 5H, 4-FPh H-3,5, CH=C*H*Ph and

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Cinnamyl H-3,5), 7.18–7.06 (m, 2H, C*H*=CHPh and Cinnamyl H-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 168.5 (1C, C=O), 167.3 (1C, Oxadiazole C-3), 164.2 (1C, d, $^1J_{FC}$ = 249.4 Hz, 4FPh H-4), 153.5 (1C, C=NH), 149.4 (1C, Oxadiazole C-5), 141.3 (1C, C*H*=CHPh), 135.6 (1C, Cinnamyl C-1), 129.8 (2C, d, $^3J_{FC}$ = 9.3 Hz, 4-FPh H-2,6), 129.2 (1C, CH=CHPh), 128.8 (2C, Cinnamyl C-3,5), 127.3 (2C, Cinnamyl C-2,6), 125.1 (1C, Cinnamyl C-4), 122.0 (1C, d, $^4J_{FC}$ = 3.1 Hz, 4-FPh H-1), 116.6 (2C, d, $^2J_{FC}$ = 21.7 Hz, 4FPh H-3,5); UHPLC-TOF-MS for $C_{18}H_{13}N_4O_2F$ calcd (found)/Error: 336.1022 (335.0986, [M−H$^−$])/12.5 ppm.

4.1.1.4. 3-(4-Chlorophenyl)-*N*′-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed20. Yield: 91%; Mp 214.7–216.6 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3226 (NH), 3055 (Ar CH), 1688 (C=O), 1619 (C=N, imine), 1589 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.7 (s, 1H, CONH), 8.40 (d, 1H, ^3J = 8.1 Hz, N=CH), 8.09 (d, 2H, ^3J = 8.7 Hz, 4ClPh H-2,6), 7.69 (d, 2H, ^3J = 5.1 Hz, 4-ClPh H-3,5), 7.65 (d, 2H, ^3J = 8.7 Hz, Cinnamyl H-2,6), 7.42 (d, 1H, ^3J = 6.6 Hz, CH=C*H*Ph), 7.36 (t, 2H, ^3J = 4.2 Hz, Cinnamyl H-3,5), 7.11 (2xs, 2H, C*H*=CHPh and Cinnamyl H-4); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.8 (1C, C=O), 167.3 (1C, Oxadiazole C-3), 153.5 (1C, C=NH), 149.3 (1C, Oxadiazole C-5), 141.3 (1C, C*H*=CHPh), 136.8 (1C, 4-ClPh C-4), 135.6 (1C, Cinnamyl C-1), 129.6 (2C, 4-ClPh C-3,5), 129.3 (2C, 4-ClPh C-2,6), 129.1 (1C, HC=CHPh), 129.0 (2C, Cinnamyl C-3,5), 127.4 (2C, Cinnamyl C-2,6), 125.1 (1C, Cinnamyl C-4), 124.3 (1C, 4-ClPh C-1); UHPLC-TOF-MS for $C_{18}H_{13}ClN_4O_2$ calcd (found)/Error: 352.0727 (351.0622, [M−H$^−$])/7.7 ppm.

4.1.1.5. 3-(4-Bromophenyl)-*N*′-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed21. Yield: 89%; Mp 195.6–198.3 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3426 (NH), 3055 (Ar CH), 1689 (C=O), 1624 (C=N, imine), 1543 (C=N, heterocyclic); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 12.7 (s, 1H, CONH), 8.41 (d, 1H, ^3J = 8.0 Hz, N=CH), 8.02 (d, 2H, ^3J = 8.4 Hz, 4-BrPh H-2,6), 7.84 (d, 2H, ^3J = 8.4 Hz, 4-BrPh H-3,5), 7.66 (d, 2H, ^3J = 7.2 Hz, Cinnamyl H-2,6), 7.42 (d, 1H, ^3J = 7.2 Hz, CH=C*H*Ar), 7.37 (t, 2H, ^3J = 7.6 Hz, Cinnamyl H-3,5), 7.17–7.06 (m, 2H, C*H*=CHPh and Cinnamyl H-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 168.8 (1C, C=O), 167.4 (1C, Oxadiazole C-3), 153.6 (1C, C=NH), 149.3 (1C, Oxadiazole C-5), 141.3 (1C, C*H*=CHPh), 135.6 (1C, Cinnamyl C-1), 132.5 (2C, 4BrPh C-3,5), 129.3 (2C, 4-BrPh C-2,6), 129.2 (1C, CH=CHPh), 128.8 (2C, Cinnamyl C-3,5), 127.4 (2C, Cinnamyl C-2,6), 125.7 (1C, 4-BrPh C-4), 125.1 (1C, Cinnamyl C-4), 124.7 (1C, 4-BrPh C-1); UHPLC-TOF-MS for $C_{18}H_{13}BrN_4O_2$ calcd (found)/Error: 396.0222 (395.0160, [M−H$^−$])/4.1 ppm.

4.1.1.6. 3-(4-Nitrophenyl)-*N*′-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed22. Yield: 91%; Mp 235.5–238.1 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3210 (NH), 3075 (Ar CH), 1708 (C=O), 1620 (C=N, imine), 1569 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.8 (s, 1H, CONH), 8.44 (d, 2H, ^3J = 8.7 Hz, 4-NO$_2$Ph H-3,5), 8.40 (d, 1H, ^3J = 7.8 Hz, N=CH), 8.32 (d, 2H, ^3J = 9.0 Hz, 4-NO$_2$Ph H-2,6), 7.67 (d, 2H, ^3J = 7.2 Hz, Cinnamyl H-2,6), 7.41 (d, 1H, ^3J = 7.2 Hz, CH=C*H*Ph), 7.36 (t, 2H, ^3J = 6.0 Hz, Cinnamyl H-3,5), 7.12 (m, 2H, C*H*=CHPh and Cinnamyl H-4); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 169.1 (1C, C=O), 166.8 (1C, Oxadiazole C-3), 153.6 (1C, C=NH), 149.4 (1C, 4-NO$_2$Ph C-4), 149.1 (1C, Oxadiazole C-5), 141.3 (1C, HC=CHPh), 135.5 (1C, Cinnamyl C-1), 131.2 (1C, 4-NO$_2$Ph C-1), 129.3 (1C, HC=CHAr), 128.8 (2C, Cinnamyl C-3,5), 128.6 (2C, 4-NO$_2$Ph C-2,6), 127.3 (4C, Cinnamyl C-2,6), 125.0 (1C, Cinnamyl C-4), 124.6 (2C, 4-NO$_2$Ph C-3,5); UHPLC-TOF-MS for $C_{18}H_{13}N_5O_4$: [M−H]$^−$ 363.0968 calcd (found)/Error: (362.0908, [M−H$^−$])/5.2 ppm.

4.1.1.7. 3-(4-Methoxyphenyl)-*N*′-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed23. Yield: 96%; Mp 185.8–188.2 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3203 (NH), 3055 (Ar CH), 1698 (C=O), 1618 (C=N, imine), 1597 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.7 (s, 1H, CONH), 8.41 (d, 1H, ^3J = 8.4 Hz, N=CH), 8.03 (d, 2H, ^3J = 8.7 Hz, 4-MeOPh H-2,6), 7.65 (d, 2H, ^3J = 6.9 Hz, Cinnamyl H-2,6), 7.41 (d, 1H, ^3J = 7.2 Hz, CH=C*H*Ph), 7.36 (t, 2H, ^3J = 3.6 Hz, Cinnamyl H-3,5), 7.16 (d, 1H, ^3J = 6.9 Hz, C*H*=CHPh), 7.14–7.10 (m, 3H, 4-MeOPh H-3,5 and Cinnamyl H-4), 3.85 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.4 (1C, C=O), 167.8 (1C, Oxadiazole C-3), 162.0 (1C, 4-MeOPh C-4), 153.5 (1C, C=NH), 149.5 (1C, Oxadiazole C-5), 141.2 (1C, HC=CHPh), 135.6 (1C, Cinnamyl C-1), 129.2 (1C, HC=CHPh), 129.0 (2C, 4-MeOPh C-2,6), 128.8 (2C, Cinnamyl C-3,5), 127.3 (2C, Cinnamyl C-2,6), 125.1 (1C, Cinnamyl C-4), 117.6 (1C, 4-MeOPh C-1), 114.8 (2C, 4-MeOPh C-3,5), 55.4 (C, OCH$_3$); UHPLC-TOF-MS for $C_{19}H_{16}N_4O_3$ calcd (found)/Error: 348.1223 (347.1107, [M−H]$^−$)/10.7 ppm.

4.1.1.8. 3-(4-Hydroxyphenyl)-*N*′-[(1*E*,2*E*)-3-phenylprop-2-en-1-ylideno]-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed24. Yield: 92%; Mp 170.3–171.7 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3426 (OH), 3260 (NH), 3054 (Ar CH), 1690 (C=O), 1624 (C=N, imine), 1531 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.7 (s, 1H, CONH), 10.3 (s, 1H, OH), 8.41 (d, 1H, ^3J = 7.5 Hz, N=CH), 7.93 (d, 2H, ^3J = 9.0 Hz, 4-OHPh H-2,6), 7.65 (d, 2H, ^3J = 7.2 Hz, cinnamyl H-2,6), 7.41 (d, 1H, ^3J = 6.3 Hz, HC=C*H*Ph), 7.36 (t, 2H, ^3J = 6.3 Hz, Cinnamyl H-3,5), 7.11 (d, 2H, ^3J = 7.8 Hz, H*C*=CHPh and Cinnamyl H-4), 6.97 (d, 2H, ^3J = 8.7 Hz, 4-OHPh H-3,5); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.3 (1C, C=O), 168.1 (1C, Oxadiazole C-3), 160.8 (1C, 4-OHPh C-4), 153.5 (1C, C=NH), 149.6 (1C, Oxadiazole C-5), 141.3 (1C, HC=CHPh), 135.6 (1C, Cinnamyl C-1), 129.3 (1C, HC=CHPh), 129.1 (2C, 4-OHPh C-2,6), 128.9 (2C, Cinnamyl C-3,5), 127.4 (2C, Cinnamyl C-2,6), 125.1 (1C, Cinnamyl C-4), 116.2 (2C, 4-OHPh C-3,5), 116.1 (1C, 4-OHPh C-1); LCMS for $C_{18}H_{14}N_4O_3$ calcd (found)/Error: 317.1021 (317;1007, [(M−H$_2$O)+H]$^±$)/12.7 ppm.

4.1.1.9. 3-Phenyl-*N*′-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed25. Yield: 92%; Mp 209.9–211.6 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3240 (NH), 3064 (Ar CH), 1711 (C=O), 1601 (C=N, imine), 1556 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.5 (s, 1H, CONH), 8.47 (s, 1H, N=CH), 8.10 (d, 2H, ^3J = 7.2 Hz, Ar H-2,6), 7.65–7.63 (m, 3H, Ar H-3,4,5), 4.71 (s, 2H, C=NH*Fc* H-2,5), 4.52 (s, 2H, C=NH*Fc* H-3,4), 4.27 (s, 5H, Fc); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.9 (1C, C=O), 168.1 (1C, Oxadiazole C-3), 153.5 (1C, C=NH), 148.8 (1C, Oxadiazole C-5), 132.0 (1C, Ar C-4), 129.4 (2C, Ar C-3,5), 127.2 (1C, Ar C-1), 125.5 (2C, Ar C-2,6), 77.7 (1C, C=NH*Fc* C-1), 70.8 (2C, C=NH*Fc* C-3,4), 69.1 (5C, Fc), 68.0 (2C, C=NH*Fc* C-2,5); UHPLC-TOF-MS for $C_{20}H_{16}FeN_4O_2$ calcd (found)/Error: 400.0623 (401.0713, [M+H$^±$])/3.0 ppm.

4.1.1.10. 3-(4-Tolyl)-*N*′-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed26. Yield: 91%; Mp 220.0–222.2 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3182 (NH), 3100, 3029 (Ar CH), 1682 (C=O), 1598 (C=N, imine), 1547 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.4 (s, 1H, CONH), 8.46 (s, 1H, N=CH), 7.99 (d, 2H, ^3J = 7.5 Hz, Ar H-2,6), 7.43 (d, 2H, ^3J = 7.8 Hz, Ar H-3,5), 4.71 (s, 2H, C=NH*Fc* H-2,5), 4.52 (s, 2H, C=NH*Fc* H-3,4), 4.27 (s, 5H, Fc), 2.41 (s, 3H, CH$_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.7 (1C, C=O), 168.0 (1C, Oxadiazole C-3), 153.5 (1C, C=NH), 148.8 (1C, Oxadiazole C-5), 142.0 (1C, Ar C-4), 129.8 (2C, Ar C-3,5), 127.1 (1C, Ar C-1), 122.6 (2C, Ar C-2,6), 77.7 (1C, C=NH*Fc* C-1), 70.7 (2C, C=NH*Fc* C-3,4), 69.1 (5C, Fc), 67.9 (2C, C=NH*Fc* C-2,5), 21.0 (1C, CH$_3$);

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UHPLC-TOF-MS for $C_{21}H_{18}FeN_4O_2$ calcd (found)/Error: 414.0779 (415.0830, $[M+H^{\pm}]$)/6.5 ppm.

4.1.1.11. 3-(4-Fluorophenyl)-N'-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed27.
Yield: 94%; Mp 209.4–211.5 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3179 (NH), 3100, 3048 (Ar CH), 1681 (C=O), 1607 (C=N, imine), 1550 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.5 (s, 1H, CONH), 8.46 (s, 1H, N=CH), 8.16 (dd, 2H, $^4J_{F,H}$ = 3.0 Hz, ^3J = 5.4 Hz, Ar H-2,6), 7.48 (t, 2H, ^3J = 8.7 Hz, Ar H-3,5), 4.71 (s, 2H, C=NHFc H-2,5), 4.52 (s, 2H, C=NHFc H-3,4), 4.27 (s, 5H, Fc); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 169.0 (1C, C=O), 167.3 (1C, Oxadiazole C-3), 164.2 (1C, d, $^1J_{FC}$ = 249.5 Hz, Ar C-4), 153.5 (1C, C=NH), 148.7 (1C, Oxadiazole C-5), 129.8 (2C, d, $^3J_{FC}$ = 9.15 Hz, Ar H-2,6), 122.1 (1C, Ar C-1), 116.6 (2C, d, $^2J_{FC}$ = 22.3 Hz, Ar C-3,5), 77.7 (1C, C=NHFc C-1), 70.8 (2C, C=NHFc C-3,4), 69.1 (5C, Fc), 68.0 (2C, C=NHFc C-2,5); UHPLC-TOF-MS for $C_{20}H_{15}FeN_4O_2F$ calcd (found): 418.0528 (419.0622, $[M+H^{\pm}]$)/3.6 ppm.

4.1.1.12. 3-(4-Chlorophenyl)-N'-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed28.
Yield: 97%; Mp 217.5 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3181 (NH), 3105, 3029 (Ar CH), 1676 (C=O), 1600 (C=N, imine), 1560 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.5 (s, 1H, CONH), 8.46 (s, 1H, N=CH), 8.11 (d, 2H, ^3J = 8.4 Hz, Ar H-2,6), 7.71 (d, 2H, ^3J = 8.4 Hz, Ar H-3,5), 4.71 (s, 2H, C=NHFc H-2,5), 4.52 (s, 2H, C=NHFc H-3,4), 4.27 (s, 5H, Fc); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 169.1 (1C, C=O), 167.3 (1C, Oxadiazole C-3), 153.6 (1C, C=NH), 148.7 (1C, Oxadiazole C-5), 136.8 (1C, Ar C-4), 129.6 (2C, Ar C-2,6), 129.0 (1C, Ar C-1), 124.4 (2C, Ar C-3,5), 77.7 (1C, C=NHFc C-1), 70.8 (2C, C=NHFc C-3,4), 69.1 (5C, Fc), 68.1 (2C, C=NHFc C-2,5); UHPLC-TOF-MS for $C_{20}H_{15}FeN_4ClO_2$ calcd (found)/Error: 434.0233 (435.0313, $[M+H^{\pm}]$)/0.2 ppm.

4.1.1.13. 3-(4-Bromophenyl)-N'-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed29.
Yield: 95%; Mp 216.0 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3180 (NH), 3099 (Ar CH), 1676 (C=O), 1598 (C=N, imine), 1548 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.5 (s, 1H, CONH), 8.46 (s, 1H, N=CH), 8.03 (d, 2H, ^3J = 8.1 Hz, Ar H-2,6), 7.85 (d, 2H, ^3J = 7.8 Hz, Ar H-3,5), 4.71 (s, 2H, C=NHFc H-2,5), 4.52 (s, 2H, C=NHFc H-3,4), 4.26 (s, 5H, Fc); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 169.1 (1C, C=O), 167.4 (1C, Oxadiazole C-3), 153.6 (1C, C=NH), 148.6 (1C, Oxadiazole C-5), 132.5 (2C, Ar-C3,5), 129.1 (Ar-C2,6), 125.6 (Ar-C4), 124.7 (Ar-C1), 77.7 (Fc–C1 to imine), 70.8 (Fc–C3,4 to imine), 69.1 (Fc), 68.0 (Fc–C2,5 to imino); UHPLC-TOF-MS for $C_{20}H_{15}FeN_4BrO_2$ calcd (found)/Error: 477.9728 (478.9811, $[M+H^{\pm}]$)/1.0 ppm.

4.1.1.14. 3-(4-Nitrophenyl)-N'-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed30.
Yield: 92%; Mp 232.4–234.6 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3181 (NH), 3099 (Ar CH), 1685 (C=O), 1597 (C=N, imine), 1547 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.5 (s, 1H, CONH), 8.49 (d, 2H, ^3J = 9.0 Hz, Ar H-3,5), 8.48 (s, 1H, N=CH), 8.37 (d, 2H, ^3J = 9.0 Hz, Ar H-2,6), 4.72 (t, 2H, ^3J = 1.5 Hz, C=NHFc H-2,5), 4.53 (t, 2H, ^3J = 1.5 Hz, C=NHFc H-3,4), 4.28 (s, 5H, Fc); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 169.4 (1C, C=O), 166.7 (1C, Oxadiazole C-3), 153.7 (1C, C=NH), 149.4 (1C, Ar C-4), 148.5 (1C, Oxadiazole C-5), 131.2 (1C, Ar C-1), 128.6 (2C, Ar C-2,6), 124.5 (2C, Ar C-3,5), 77.6 (1C, C=NHFc C-1), 70.8 (2C, C=NHFc C-3,4), 69.1 (5C, Fc), 68.0 (2C, C=NHFc C-2,5); UHPLC-TOF-MS for $C_{20}H_{15}FeN_5O_4$ calcd (found)/Error: 445.0473 (446.0538, $[M+H^{\pm}]$)/3.1 ppm.

4.1.1.15. 3-(4-Methoxyphenyl)-N'-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed31.
Yield: 93%; Mp 197.7–199.0 °C (from dioxane/H2O); IR (KBr, v_{max} cm^{-1}): 3181 (NH), 3100 (Ar CH), 1682 (C=O), 1611 (C=N, imine), 1550 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.5 (s, 1H, CONH), 8.46 (s, 1H, N=CH), 8.05 (d, 2H, ^3J = 9.0 Hz, Ar H-2,6), 7.17 (d, 2H, ^3J = 8.7 Hz, Ar H-3,5), 4.71 (t, 2H, ^3J = 1.5 Hz, C=NHFc H-2,5), 4.53 (t, 2H, ^3J = 1.5 Hz, C=NHFc H-3,4), 4.27 (s, 5H, Fc), 3.86 (s, 3H, OCH$_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.7 (1C, C=O), 167.8 (1C, Oxadiazole C-3), 162.1 (1C, Ar C-4), 153.5 (1C, C=NH), 148.8 (1C, Oxadiazole C5), 128.9 (2C, Ar C-2,6), 117.7 (1C, Ar C-1), 114.8 (2C, Ar C-3,5), 77.8 (1C, C=NHFc C-1), 70.8 (2C, C=NHFc C-3,4), 69.1 (5C, Fc), 68.0 (2C, C=NHFc C-2,5), 55.5 (1C, OCH$_3$); UHPLC-TOF-MS for $C_{21}H_{18}FeN_4O_3$ calcd (found)/Error: 430.0728 (431.0822, $[M+H^{\pm}]$)/3.5 ppm.

4.1.1.16. 3-(4-Hydroxyphenyl)-N'-(E-ferrocenylmethylidene)-1,2,4-oxadiazol-5-ylcarbohydrazide SintMed32.
Yield: 83%; Mp 221.3–223.0 °C (from dioxane/H$_2$O); IR (KBr, v_{max} cm^{-1}): 3313 (br OH), 3180 (NH), 3100 (Ar CH), 1702 (C=O), 1611 (C=N, imine), 1564 (C=N, heterocyclic); ^{1}H NMR (300 MHz, DMSO-d_6) δ, ppm: 12.4 (s, 1H, CONH), 10.2 (s, 1H, OH), 8.44 (s, 1H, N=CH), 7.93 (d, 2H, ^3J = 6.4 Hz, Ar H-2,6), 6.97 (d, 2H, ^3J = 6.0 Hz, Ar H-3,5), 4.70 (s, 2H, C=NHFc H-2,5), 4.51 (s, 2H, C=NHFc H-3,4), 4.27 (s, 5H, Fc); ^{13}C NMR (75 MHz, DMSO-d_6) δ, ppm: 168.4 (1C, C=O), 167.9 (1C, Oxadiazole C-3), 160.7 (1C, Ar C-4), 153.4 (1C, C=NH), 148.9 (1C, Oxadiazole C-5), 129.0 (2C, Ar C-2,6), 116.1 (1C, Ar C-1), 116.1 (2C, Ar C-3,5), 77.8 (1C, C=NHFc C-1), 70.8 (2C, C=NHFc C-3,4), 69.1 (5C, Fc), 68.0 (2C, C=NHFc C-2,5); UHPLC-TOF-MS for $C_{20}H_{16}FeN_4O_3$ calcd (found)/Error: 416.0572 (417.0664, $[M+H^{\pm}]$)/3.4 ppm.

4.2. Pharmacological evaluation

4.2.1. Cytotoxicity assay
HepG2 cells in RPMI-1640 medium without phenol red (Sigma Chemical Co., MO, USA) plus 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil) and 50 µg mL^{-1} of gentamycin (Novafarma, Anápolis, GO, Brazil) were seeded in 96-well plates (104 cells/well in 100 µL of medium). After 24 h, the compounds, diluted in RPMI-1640 medium without phenol red, were added (100 µL) in five concentrations, in triplicate, and the plates incubated for 72 h at 37 °C and 5% CO$_2$. After this period, 20% of AlamarBlue (Invitrogen, Carlsbad, CA) was added to each well and the plate incubated for 24 h. The absorbance at 570 and 600 nm was measured according to manufacturer's instructions. Gentian Violet (Synth, São Paulo, Brazil) was used as a cytotoxicity control. Controls with untreated cells were read in parallel and triplicates are read in the same plate. The CC_{50} values were calculated using a non-linear regression curve fit in the Graph Pad Prism version 5.03 (Graph Pad Software, San Diego, CA).

4.2.2. Anti-$P. falciparum$ activity
W2 strain $P. falciparum$ was maintained in continuous culture of human erythrocytes (blood group O$^+$) using the RPMI 1640 medium supplemented with 10% of human plasma. Parasites grown at 1% parasitemia and 2.5% hematocrit were distributed into 96-wells culture plate and incubated with the compounds (previously diluted with 4% DMSO and culture medium). After 24 h of incubation, [^{3}H]-hypoxanthine was added, the plate incubated again for 24 h, cooled for 24 h and then parasites were harvested using a cell harvester to quantify the [^{3}H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with [^{3}H]-hypoxanthine uptake in treated versus untreated parasite. Each compound was initially tested at concentration of 10 µg/mL in triplicate and the IC_{50} values were calculated in triplicate concentration using at least five concentrations. Mefloquine (**Mfq**) was used as a standard drug.

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4.2.3. Hemolysis assay

The hemolytic activity of the compounds was assayed in fresh human erythrocytes (type O⁺).[23] Cells were washed three times with phosphate buffered saline, and 100 µL of this suspension (1% hematocrit) was distributed into each well to a 96-well plate. After, 100 µL of each compound (*N*-acylhydrazone), previously dissolved in phosphate buffered saline, were added in triplicate to the plate and incubated for 1 h. Saponin (Sigma–Aldrich, USA) was used as a hemolytic drug at 1% v/v. After incubation, samples were centrifuged (1500 rpm for 10 min) and 100 µL of each supernatant was transferred to another microtiter plate. Released haemoglobin was monitored by measuring the absorbance at 540 nm in a spectrophotometer. The percentage of hemolysis was calculated as previously described.

4.2.4. Inhibition of β-hematin formation

50 µL of a freshly prepared solution of haemin (0.5 mg/mL) dissolved in 0.2 M NaOH was mixed to a 75 µL of 3 M sodium acetate, 25 µL of 17.4 M acetic acid and 50 µL of the tested compounds (*N*-acylhydrazones). All *N*-acylhydrazones were tested at 2.5 mM. After 24 h of incubation at 37 °C, the resulting solution was spun down for 15 min at 3500 rpm, the supernatant was discarded off and the pellet washed with 200 µL of DMSO. This step was repeated once and after a final wash with water, the pellet was dissolved in 0.1 M NaOH (150 µL). Chloroquine (CQ) was included in each experiment as positive control. The absorption at 405 nm was read using a spectrophotometer. Results are expressed as percentage of inhibition of hematin formation in comparison to the negative control (without compound). Experiments were carried out at least twice using compound concentrations in triplicate.

4.2.5. Antimalarial tests against *Plasmodium berghei* in mice

Adult Swiss mice (4–6 weeks) were inoculated by intraperitoneal route with 10⁶ infected red blood cells with NK65 strain *P. berghei*. The infected mice were randomly divided into groups of *n* = 5 and subsequently treated with compounds **SintMed2**, **SintMed5** or **SintMed6** diluted in DMSO 20% given once a day for four consecutive days by oral gavage or intraperitoneal. Two control groups were used in parallel, one treated with Chloroquine (50 mg/kg), standard drug, and one with the vehicle (DMSO 20% in saline solution). Blood smears from mice tails were prepared on days 4, 5, 6, 7 and 8 post-infection and then methanol-fixed, stained with fast panoptic (Laborclin, Pinhais, Brazil) and examined by optical microscopy. The parasitemia was evaluated and the percent inhibition of parasite growth calculated in relation to the untreated control group (considered 100% growth) using the following equation: $[(C - T)/C] \times 100$; where C is the parasitemia where C is the parasitemia in the control group and T is the parasitemia in the treated group.

Acknowledgments

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.09.013. These data include MOL files and InChiKeys of the most important compounds described in this article.

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