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Brief Article

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NO-Donor Dihydroartemisinin Derivatives as Multitarget Agents for the Treatment of Cerebral Malaria

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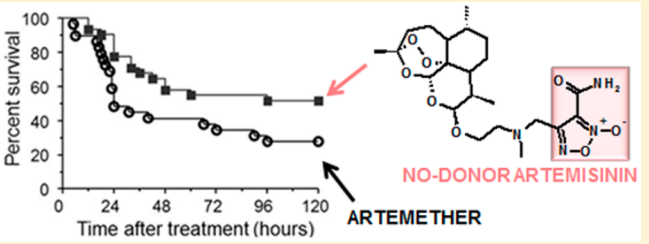
Supporting Information

ABSTRACT: Hybrid products in which the dihydroartemisinin scaffold is combined with NO-donor furoxan and NONOate moieties have been synthesized and studied as potential tools for the treatment of cerebral malaria (CM). The designed products were able to dilate rat aorta strips precontracted with phenylephrine with a NO-dependent mechanism. All hybrid compounds showed preserved antiplasmodial activity in vitro and in vivo against *Plasmodium berghei* ANKA, comparable to artesunate and artemether. Hybrid **10**, selected for additional studies, was capable of increasing survival of mice with late-stage CM from 27.5% to 51.6% compared with artemether. Artemisinin-NO-donor hybrid compounds show promise as potential new drugs for treating cerebral malaria.



INTRODUCTION

Malaria is a world-spread disease caused by *Plasmodium* protozoa transmitted by female *Anopheles* mosquitos. The World Health Organisation (WHO) estimates that 198 million cases of malaria and 584 000 deaths occurred globally in 2013, 90% in the WHO African region, mostly among children in sub-Saharan Africa.[1] *P. falciparum* is responsible for severe malaria (SM), the most aggressive form of the disease characterized by a high incidence of mortality if untreated. A complication of SM is cerebral malaria (CM) that kills 20% of patients, mainly children below 5 years of age, admitted to hospitals and treated with intravenous artesunate, the current mainstay treatment for this deadly condition.[2] In addition 25% of the patients that survive develop cognitive and neurological deficits.[3] Cerebral malaria is characterized by the blockage of the cerebral microvasculature by *Plasmodium*-infected red blood cells with consequent ischemia, hypoxia, disruption of the blood−brain barrier (BBB), edema, and coma.[4] Low availability of nitric oxide (NO) seems to play an important role in the pathogenesis of human and murine experimental cerebral malaria (ECM). It is principally related to the NO-scavenging effects by high concentrations in cell-free plasma of free oxyhemoglobin (HbO^{2+}) derived from hemolysis and to hypoargininemia.[5] Low levels of exhaled NO, low plasma arginine concentration, high levels of free hemoglobin, and endothelial dysfunction were found in patients with SM/CM.[6−9] The same findings are observed in the ECM model,[5] and the resulting widespread vasoconstriction contributes to cerebral hypoxia and acidosis.[10,11] Mice with ECM show impaired response of brain vessels to endothelial and neuronal nitric oxide synthase (eNOS and nNOS) dependent vasodilators.[12] Administration of NO donors can prevent the neurological syndrome and the associated vascular dysfunction,[13−15] and more important, NO donors such as glyceryl trinitrate improve survival of mice with late-stage ECM and reverse ECM cerebrovascular constriction.[16] In a previous study we designed a new series of hybrid compounds in which amodiaquine, an established antimalarial drug included in the World Health Organization Model List of Essential Medicines,[17] was joined with NO-donor furoxan and nitrooxy (ONO_2)[18] moieties and studied them as potential anti SM/CM tools. All the amodiaquine-NO-donor hybrids were able to dilate rat aorta strips precontracted with phenylephrine with a NO-dependent mechanism and displayed high degree of activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. Two of them, tested in vivo on *Plasmodium berghei* ANKA-infected (PbA) mice, showed a trend for prolonged survival of mice with CM. As development of this research, we designed new NO-donor hybrid drugs obtained by joining the dihydroartemisinin scaffold **1** (Chart 1) with NO-donor furoxan and NONOate moieties (Scheme 1,

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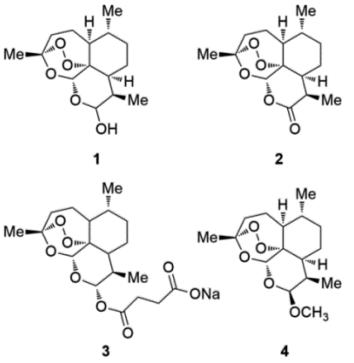
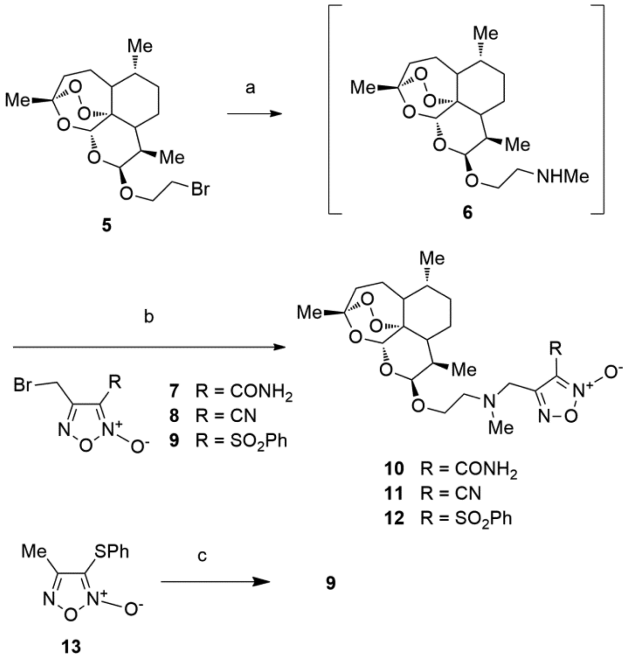
Chart 1. Structures of Dihydroartemisinin (1), Artemisinin (2), Sodium Artesunate (3), and Artemether (4)



Scheme 1. Synthesis of Hybrids 10−12 and Intermediate 9[a]



[a]Reagents and conditions: (a) $MeNH_2$ 33% w/v in abs EtOH, CH_2Cl_2, rt, 72 h; (b) Et_3N, 2-propanol, rt, 18−40 h; (c) (i) NBS/AIBN, CCl_4, reflux, 96 h; (ii) flash chromatography to recover unreacted **13**; (iii) *m*CPBA, 0 °C to rt, 24 h.

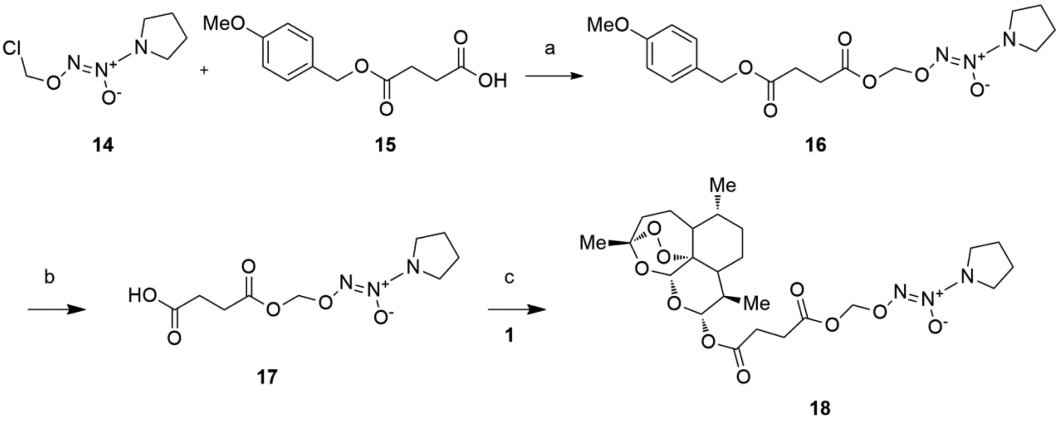
10−**12**; Scheme 2, **18**). Compound **1**, a semisynthetic derivative of artemisinin **2** (Chart 1), causes a rapid decrease in parasites biomass (about 10 000-fold per cycle in vitro) different from amodiaquine which displays a longer parasite clearance time. Dihydroartemisinin is used with other artemisinin derivatives, artesunate (**3**) and artemether (**4**) as first-line drug for the treatment of *P. falciparum* malaria in most endemic areas and for the in vivo treatment of chloroquine-resistant *P. vivax* malaria.[19] In this paper we describe the synthesis of these products, their ability of relaxing rat aorta strips precontracted with phenylephrine, and their *P. berghei*-killing capacity in vitro and in vivo. The ability of **10** to rescue mice with ECM from death in comparison with artemether is also discussed.

RESULTS AND DISCUSSION

Chemistry. The purity of the target compounds was assessed through elemental analysis (C, H, N) and was ≥95%. The hybrid furoxan derivatives **10**−**12** were obtained according to the synthetic pathway described in Scheme 1. 1-Bromo-2-(10b-dihydroartemisinoxy)ethane (**5**) was treated with methylamine (33% in absolute ethanol) to give the substitution product **6**. This intermediate was purified by flash chromatography and, without any additional characterization, was dissolved in 2-propanol and reacted with the appropriate 3-substituted-4-bromomethylfuroxans **7**−**9** to afford the target compounds. Preparation of 4-bromomethyl-3-phenylsulfonylfuroxan (**9**) (Scheme 1), the reagent used to synthesize the hybrid **12**, was time-consuming and laborious. The action of *N*-bromosuccinimide (NBS) and azobisisobutyronitrile (AIBN) on 4-methyl-3-thiophenylfuroxan (**13**), run in refluxing CCl_4, afforded a mixture of the starting material **13**, its isomer 3-methyl-4-thiophenylfuroxan, and related bromomethyl derivatives. Flash chromatography allowed separation of unreacted **13** from a mixture of 3-methyl-4-thiophenylfuroxan and 3(4)-bromomethyl-4(3)-thiophenylfuroxans. The mixture was treated with *m*-chloroperbenzoic acid (*m*CPBA) to give the corresponding phenylsulfonyl derivatives from which **9** was obtained by flash chromatography in 12% overall yield.

The preparation of the hybrid **18** containing the NONOate substructure as NO-donor moiety is depicted in Scheme 2. The coupling of O^2-chloromethyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**14**) with 4-methoxybenzyl ester of succinic acid (**15**) under the action of Cs_2CO_3 gave the adduct **16**. This

Scheme 2. Synthesis of Hybrid 16[a]



[a]Reagents and conditions: (a) Cs_2CO_3, dry DMF, rt, 1 h; (b) PhOH, CF_3COOH cat., 40 °C, 1 h; (c) DCC, DMAP cat., 0 °C to rt, 5 h.

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compound was deprotected by treatment with phenol and a catalytic amount of trifluoroacetic acid to afford the free acid **17**. This acid was then reacted with **1** using dicyclohexylcarbodiimide (DCC) in the presence of 4-dimethylaminopyridine (DMAP) to give the expected product **18**.

Biological Activities. *Vasodilator Activity.* All the hybrid products here described were able to relax rat aorta strips precontracted with phenylephrine in a concentration dependent manner (Figure 1). Their vasodilator potencies, expressed

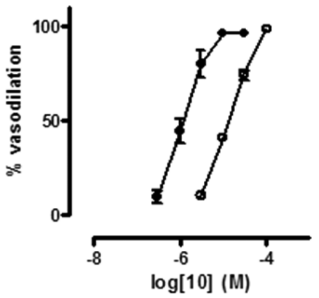

Figure 1. Concentration−response curves for **10** with (○) and without (●) 1 μM ODQ.

Table 1. Vasodilation Potencies of the Synthesized Hybrids 10−12 and 18 on Rat Aorta Strips

compd	EC_{50} (μM)[a]	
		+ODQ, 1 μM[b]
10	1.4 ± 0.2	12 ± 1
11	0.023 ± 0.004	0.25 ± 0.06
12	0.41 ± 0.05	30 ± 17
18	0.0036 ± 0.0004	0.14 ± 0.02

[a]The endothelium deprived aortic strips were allowed to equilibrate for 120 min and then contracted with L-phenylephrine (1 μM). Cumulative concentrations of the vasodilating agent were added. Data are the mean ± SEM of at least three experiments. [b]Effect of 1 μM ODQ was evaluated in a separate series of experiments in which it was added to the organ bath 5 min before the contraction.

as EC_{50}, are reported in Table 1. Analysis of the data shows the potency rank in the order **18** > **11** > **12** > **10**. When the experiments were repeated in the presence of ODQ (1*H*-[1,2,4]oxadiazole[4,3-*a*]quinoxalin-1-one) which is a potent inhibitor of the soluble guanylate cyclase (sGC), an enzyme that mediates a variety of biological responses including the NO-dependent vasodilation, a decrease in the potencies was observed (see Table 1 and Figure 1). This indicates that the products are capable of releasing NO in the vessels, thus suggesting their potential use in the CM therapy.

Antiplasmodial Activity in Vitro and in Vivo. We asked whether the modification of the artemisinin structure by the introduction of the NO-donor moieties might have decreased its antimalarial activity. For this purpose, in vitro and in vivo tests with *Plasmodium berghei* were performed as previously described.[20,21] In vitro, hybrids **10**, **11**, **12**, and **18** were tested and compared with reference drugs **3** and **4**. The hybrid compounds showed in vitro antiplasmodial activity very similar to that of artesunate, in the low nanomolar range. Artemether showed the strongest activity (Figure 2). The evaluation of the in vivo antiplasmodial activity was performed using our

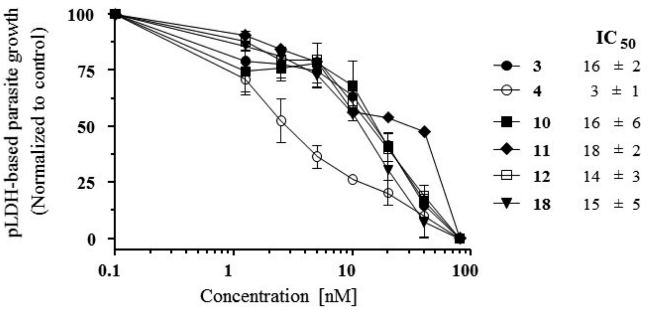

Figure 2. In vitro activity of hybrids **10**−**12**, **18** and reference drugs artesunate (**3**) and artemether (**4**) against *Plasmodium berghei*: curves of *P. berghei* in vitro sensitivity to each drug and their respective IC_{50} values.

previously described protocol, designed to determine the effect of the test drug in mice with established parasitemia.[21] In this protocol, mice were infected with *P. berghei* ANKA and treated at day 5 of infection, before clinical signs of neurological involvement were evident, with parasitemia in the range 5−15%. Drugs were administered once a day for 5 days, and parasitemia was checked every 24 h. There was no difference in the rate of parasite clearance in the first 24 h between artemether and the hybrids **10**, **12**, and **18**, all of them decreasing parasitemia by about 95% with a single dose (Figure 3A). Hybrid **11** showed significantly lower activity, killing about 85% of the parasites with one dose. All drugs were able to bring parasitemia to undetectable levels by day 5 of treatment, and **12** and **18** were also capable of preventing recrudescence (Figure 3B). Overall, these results show that the introduction of the NO-donor moiety did not significantly modify artemisinin's antiplasmodial activity.

Efficacy of Hybrid Compounds in Rescuing Mice with Late-Stage ECM. The main rationale for developing artemisinin-NO-donor hybrid compounds comes from our studies showing that mice with ECM present marked cerebrovascular constriction leading to ischemia and hypoxia linked to low NO bioavailability.[10,11] We have also shown that administration of exogenous NO improves cerebral microcirculatory physiology in *P. berghei* infected mice[13−15] and that glyceryl trinitrate, a drug that generates NO, reverses cerebrovascular constriction and improves survival in mice with ECM.[16] Artemisinin derivatives are potent, fast-acting antimalarial drugs, and intravenous artesunate is the mainstay treatment for human cerebral malaria.[2] We hypothesized that the combination of artemisinin with NO-donors would bring together the potent antimalarial activity of the former with the vascular benefit of the latter and therefore be a more efficacious drug for treating cerebral malaria. We tested this hypothesis using the ECM preclinical model. Mice infected with *P. berghei* ANKA were allowed to develop clinical signs of cerebral malaria and then treated with artemether or hybrid **10**. The objective criterion for treatment was development of hypothermia (rectal temperatures between 30 and 36 °C, Figure 4A), as this is an easily quantifiable clinical sign of neurological involvement in ECM allowing unbiased randomization of animals to the treatment groups.[10] Parasitemia levels were also checked but not considered as a criterion for treatment. Animals in the two groups (artemether or hybrid **10**) showed similar rectal temperatures (Figure 4A) and levels of parasitemia (artemether, 12.0 ± 5.35%; hybrid **10**, 10.8 ± 3.54%; $p = 0.5208$) at the time of treatment. Hybrid **10** was chosen for this study,

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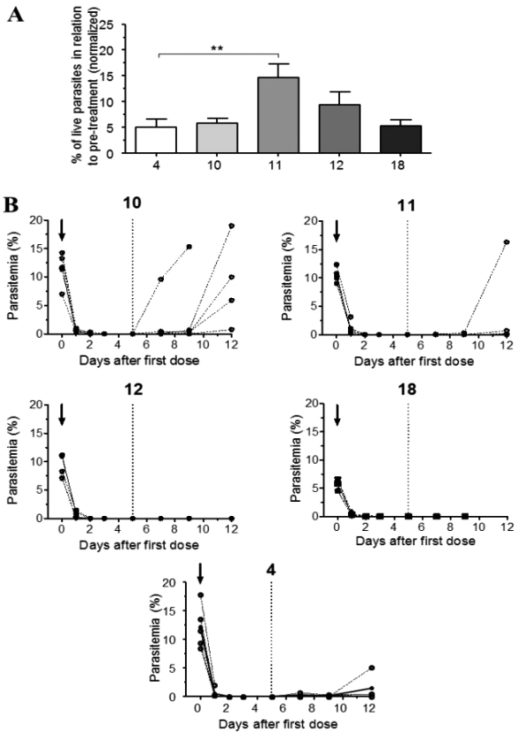

Figure 3. In vivo activity of hybrids **10**–**12**, **18** and artemether (**4**) against *Plasmodium berghei*. (A) Efficacy of the drugs in reducing *P. berghei* parasitemia in C57BL/6 mice with a single dose. Mice showing 5–15% parasitemia were treated ip with 1.4 μmol of each drug, and parasitemia was checked 24 h later. The results are shown as the percentage of parasitemia in relation to parasitemia just before treatment (accounted as 100%): (**) $p = 0.0268$ (one-way ANOVA). (B) Individual curves of parasitemia in *P. berghei* infected mice just before (arrows) and after treatment with daily doses of hybrids **10**–**12** or **4** for 5 consecutive days. The dotted line indicates the time point of 24 h after the last (5th) dose.

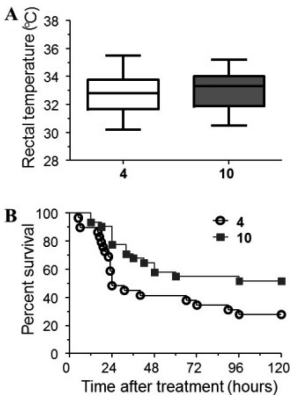

Figure 4. Efficacy of artemether (**4**) and hybrid **10** in rescuing mice with late-stage cerebral malaria. Mice in both groups were treated when presenting similar clinical conditions as determined by body (rectal) temperature (A, no significant differences). Mice treated with hybrid compound **10** ($n = 31$) showed improved survival in relation to artemether-treated mice ($n = 29$) (B, 51.6% versus 27.5%, $p = 0.0264$, log-rank test).

since it behaves as good antiplasmodial and vasodilator agent at low micromolar concentration. It contains the NO-donor methylfuroxan-3-carboxamide substructure present in CAS 1609 (4-hydroxymethyl-3-furoxancarboxamide) that was found to be an in vivo effective, long-lasting orally active, vasodilator agent devoid of tolerance.[22] Derivatives **11**, **12**, and **18** display more potent vasodilating ability, which could potentially reflect a hypotensive response in already compromised mice. Mice with ECM treated with hybrid **10** showed a survival rate of 51.6%, which was markedly higher compared to the survival rate in the group of mice treated with artemether (27.5%) (Figure 4B). When comparing a subgroup of mice treated in better conditions, that is, only those with body temperatures above 32 °C, survival reached 63.6% of those treated with hybrid **10** against 33.3% of those treated with artemether (Figure S1). Also significantly, survival rate in the first critical 24 h was 77% in hybrid **10**-treated mice against 48% in artemether-treated mice (Figure 4B). These data indicate that optimization of the treatment scheme may be able to further increase overall survival rates.

The present study provides solid evidence that artemisinin-NO-donor hybrid compounds show great promise for improving the efficacy of the currently available mainstay treatment for cerebral malaria, which is intravenous artesunate.[2] We have previously shown that artemether is capable of rapidly decreasing parasitemia and reversing vascular congestion in mice with late-stage ECM, clearing vessels from adherent leukocytes 24 h after a single dose.[21] However, artemether had no effect in reversing cerebrovascular constriction.[16] Therefore, even if parasites are cleared and vascular occlusion is reversed, ischemia will persist and hence constitute a critical obstacle for CM patient's recovery. On the other hand, administration of glyceryl trinitrate potentiated the efficacy of artemether, significantly increasing survival of mice with ECM, and the benefit in survival was associated with reversal of cerebrovascular constriction reducing ischemia.[16] These findings are consistent with the demonstration in the present study that hybrid compounds improve survival of mice with late-stage ECM.

CONCLUSION

We successfully developed a series of hybrid compounds in which the dihydroartemisinin scaffold was joined to furoxan and NONOate NO-donor moieties. This new class of compounds, which retains the potent antimalarial activity of the parent artemisinin and the vasoactive properties of the NO-donor moieties, may represent a powerful alternative to increase the efficacy of artesunate in treating cerebral malaria, improving survival and reducing sequelae by restoring proper cerebral blood flow along with rapid parasite killing. Additional studies to define optimal doses and delivery systems, pharmacokinetics and to characterize cerebral and systemic vascular responses to these drugs are warranted.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01036.

Detailed experimental procedures of chemistry and biological studies (PDF)

Molecular formula strings (PDF)

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Author Contributions

#M.B. and P.O.-S. contributed equally.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CM, cerebral malaria; SM, severe malaria; ECM, experimental cerebral malaria; BBB, blood−brain barrier; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; AIBN, azabisisobutyronitrile; NBS, *N*-bromosuccinimide; *m*CPBA, *m*-chloroperbenzoic acid; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; ODQ, 1*H*-[1,2,4]-oxadiazole[4,3-*a*]quinoxalin-1-one; sGC, soluble guanylate cyclase

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