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Evaluation of amidoxime derivatives as prodrug candidates of potent bis-cationic antimalarials

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ABSTRACT

Plasmodium falciparum is responsible for most of the cases of malaria and its resistance to established antimalarial drugs is a major issue. Thus, new chemotherapies are needed to fight the emerging multi-drug resistance of *P. falciparum* malaria, like choline analogues targeting plasmodial phospholipidic metabolism. Here we describe the synthesis of amidoxime derivatives as prodrug candidates of reverse-benzamidines and hybrid compounds able to mimic choline, as well as the design of a new series of asymmetrical bis-cationic compounds. Bioconversion studies were conducted on amidoximes in asymmetrical series and showed that amidoxime prodrug strategy could be applied on *C*-alkylamidine moieties, like benzamidines and that *N*-substituents did not alter the bioconversion of amidoximes. The antimalarial activity of the three series of compounds was evaluated *in vitro* against *P. falciparum* and *in vivo* against *P. vinckei petteri* in mice.

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Malaria is a widespread life-threatening disease caused by *Plasmodium* parasites, responsible for 219 million cases worldwide in 2017 and 435 000 deaths.[1] Moreover, multidrug resistance, including artemisinin resistance of *P. falciparum* has been reported.[2] To counter chemo-resistance, choline analogues have been developed as a new class of antimalarial drugs.[3] Not only are they structurally different from existing agents, but they also exhibit an innovative mechanism of action, as demonstrated with the bisthiazolium salt **T3** (Figure 1).[4] Indeed, they condemn the parasite to death by multiple ways:[5] i) **T3** highly and specifically accumulates inside *P. falciparum* infected erythrocytes;[6] ii) **T3** competitively inhibits choline transport and enzymes of the phosphatidylcholine *de novo* biosynthesis pathway of *Plasmodium*.[7] Unfortunately,**T3** [named albitiazolium (INN)] will not be further clinically developed due to its low oral bioavailability and high clearance in children.[8] Bis-alkylamidines were also developed as choline analogues,[9] as well as reverse-benzamidines (*e.g.* compound **1**)[10] and hybrid bis-cationic compounds (*e.g.* compound **2**).[11]

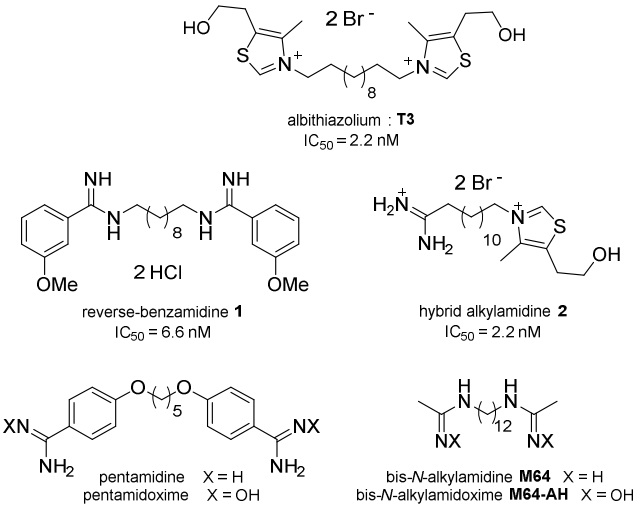

Figure 1. Chemical structures of described drugs and amidoxime prodrugs.

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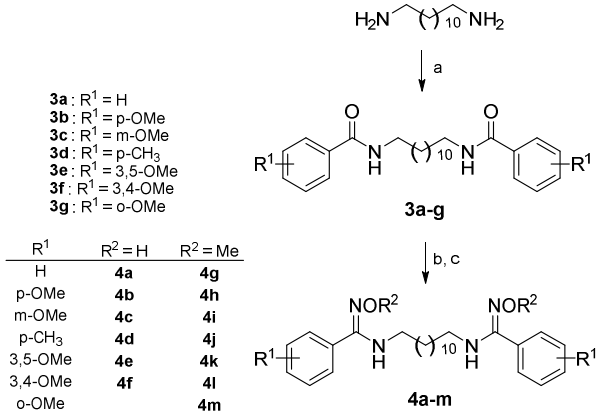
Over the past decade, we focused our effort on the design of orally available bis-alkylamidine-based compounds. Indeed, their bis-cationic character under physiological conditions is necessary for their antimalarial activity but prevents absorption from the gastrointestinal tract. To circumvent the too low oral bioavailability of amidines, the approach "amidoximes instead of amidine drugs" was developed[12] and succeeded for many benzamidine-type compounds, including antiparasitic and antibacterial agents,[13] thrombin inhibitors,[14] and anticancer agent,[15] as well as for antiviral guanidine drug.[16] Therefore, we applied the amidoxime prodrug strategy to bis-*N*-alkylamidines,[17] bis-*C*-alkylamidine[18–20] and bis-*N*-alkylguanidines.[21] The bioconversion of pentamidoxime prodrug (Figure 1) into the corresponding pentamidine drug was described, as well as other benzamidoximes[22] and *N*-hydroxyguanidine or acetamidoxime prodrugs.[16] Similarly, the bis-*N*-alkylamidoxime **M64-AH** could be converted into **M64** drug (Figure 1).[23] Apart from this work on bis-*N*-alkylamidine series and to the best of our knowledge, no data is available on the metabolism of either bis-*C*-alkylamidoxime or *N*-substituted alkyl/benz-amidoximes.

In the present study, we describe the synthesis of amidoxime prodrug candidates of reverse-benzamidines and hybrid compounds as well as the development of a novel series of asymmetrical drugs and their potential prodrugs. *In vivo* and *in vitro* antimalarial activities of the synthesized compounds are reported as well as the bioconversion studies of the asymmetrical amidoxime prodrug candidates into the desired bis-cationic drugs.

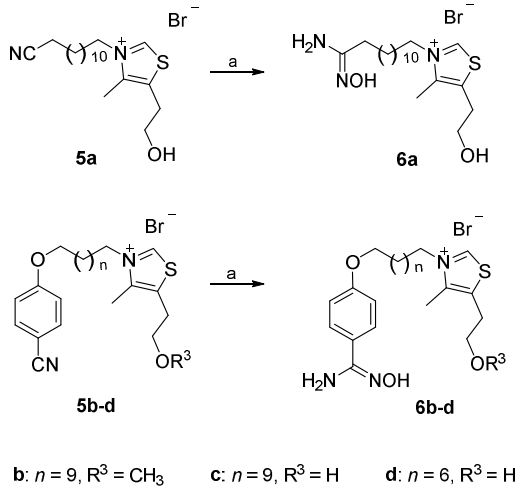
The synthesis of amidoxime derivatives of reverse-benzamidines is presented in Scheme 1. Bis-benzamides **3a-g** were prepared from appropriate benzoyl chlorides and 1,12-diaminododecane in dichloromethane (DCM), in the presence of triethylamine (TEA), at room temperature (RT). By reaction with Lawesson's reagent, **3a-g** afforded bis-thioamides, which reacted with either hydroxylamine or methoxyamine hydrochlorides in the presence of mercury (II) oxide (HgO) and pyridine (Pyr) in tetrahydrofuran (THF) to provide the targeted bis-benzamidoximes **4a-f** and bis-*O*-methyl benzamidoximes **4g-m**.



R^1	R^2 = H	R^2 = Me
H	**4a**	**4g**
p-OMe	**4b**	**4h**
m-OMe	**4c**	**4i**
p-CH_3	**4d**	**4j**
3,5-OMe	**4e**	**4k**
3,4-OMe	**4f**	**4l**
o-OMe		**4m**

Scheme 1. Synthesis of amidoximes in reverse-benzamidine series. Reagents and conditions: a) benzoyl chloride, TEA, DCM, RT, 2 h (80-100%); b) Lawesson's reagent, toluene, reflux, 16 h (60-95%); c) $NH_2OH.HCl$ or $NH_2OMe.HCl$, Pyr/HgO, THF, RT, 24 h (69-95%).

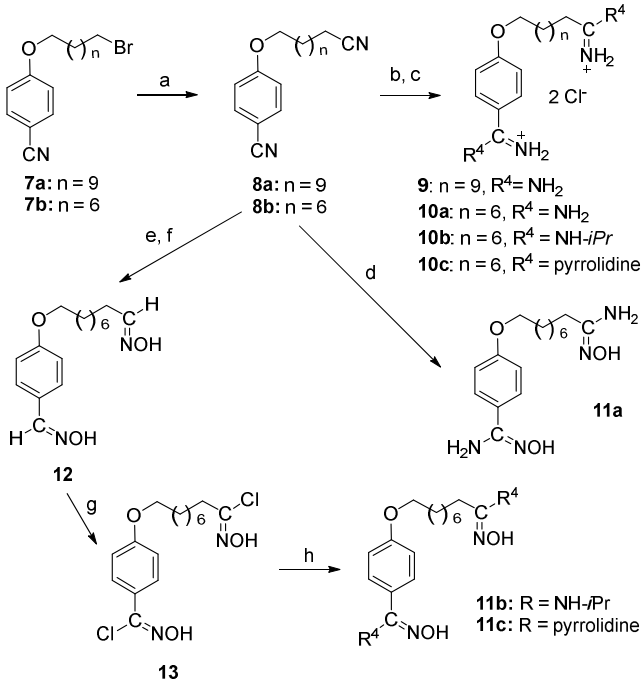
Amidoxime prodrug candidates of hybrid bis-cations were obtained as described in Scheme 2. The nitriles **5a-d**, prepared according to a previous report,[11] reacted with hydroxylamine hydrochloride in the presence of sodium hydroxide in ethanol (EtOH) to generate the amidoximes **6a-d**.



b: $n = 9$, $R^3 = CH_3$ **c**: $n = 9$, $R^3 = H$ **d**: $n = 6$, $R^3 = H$

Scheme 2. Synthesis of hybrid prodrug candidates. Reagents and conditions: a) NH_2OH, EtOH, RT, 4 days (14-53%).

At last, asymmetrical amidines and amidoximes were synthesized according to Scheme 3. Compounds **7a,b**[11] reacted with potassium cyanide to generate the nitriles **8a,b**, which were converted under Pinner's conditions into unstable ethyl imidates,[24] and reacted with appropriate amines to provide the corresponding amidines **9**, **10a-c**. The amidoxime **11a** was obtained from the nitrile **8b** by reaction with hydroxylamine hydrochloride and sodium hydroxide. For *N*-substituted amidoximes **11b-c**, the nitrile **8a** was reduced with diisobutylaluminium hydride (DIBAL-H). The resulting dialdehyde reacted immediately with hydroxylamine hydrochloride and Pyr to generate the dioxime **12**. By reaction with *N*-chlorosuccinimide (NCS) in dimethylformamide (DMF), **12** formed dihydroxamoyle chloride **13**, which reacted immediately with appropriate amines to provide the targeted amidoximes **11b-c**. All the compounds were characterized by ^1H and ^{13}C NMR, MS (ESI), FTIR and the data were consistent with the structures.[25]



Scheme 3. Synthesis of asymmetrical bis-cationic drugs and amidoxime prodrug candidates. Reagents and conditions: a) KCN, EtOH/H_2O, reflux, 24 h (81-85%); b) gaseous HCl, anhydrous EtOH/Et_2O, RT, 24 h; c) NH_3, isopropylamine or pyrrolidine in anhydrous EtOH, RT, 48 h (53-92%, two steps); d) $NH_2OH.HCl$, NaOH, EtOH/H_2O, reflux, 72 h (76%); e) DIBAL-H, CH_2Cl_2, -78°C to -40 °C, 1 h;

f) $NH_2OH.HCl$, Pyr, EtOH, reflux, 24h (46%, two steps); g) NCS, DMF, RT, 2 h; h) isopropylamine or pyrrolidine, TEA, Et_2O, RT, 48h (15-30%, two steps).

Among these three series, seventeen compounds were evaluated for antimalarial activity. They were tested *in vitro* against the Nigerian strain of *P. falciparum*[26,27] and *in vivo* against the *P. vinckei petteri* strain (279BY) in female Swiss mice according to a modified version of the four-day suppressive test.[28] Since the antiplasmodial activity is strongly related to the basicity of the amidines,[9] the bis-amidoximes **4a-m** and **11a-c** were expected to possess very moderate *in vitro* antiplasmodial activity (micromolar range). This assumption was verified by testing *in vitro* one compound in each series, *i.e.* **4b**: IC_{50} = 24 µM, **11a**: IC_{50} = 20 µM (concentration required to inhibit the parasite viability by 50%). *In vivo*, the tested amidoximes in reverse-benzamidine series did not reveal any significant activity at the tested doses.[29]

Table 1 reports the IC_{50} and the ED_{50} (dose required to inhibit the parasitemia by 50%) after intraperitoneal (i.p.) and oral (p.o.) administrations of the amidoxime prodrug candidates of the hybrid drugs (compounds **6a-d**). *In vitro*, the antiplasmodial activities of the amidoxime derivatives of the hybrid drugs were moderate (IC_{50} > 200 nM). Indeed, since the amidoxime polar head is not protonated under physiological conditions, the molecules **6a-d** may not act as bis-cations, but as mono-thiazolium salts, about 100 fold less potent as compared with the duplicated analogues.[30,31] *In vivo*, compound **6c** did not reveal any antimalarial effect at the tested doses while a significant antimalarial activity was detected after *i.p.* administration of **6a** and **6b** (ED_{50} of 20 and 8.8 mg/kg respectively). Unfortunately, **6a** and **6b** did not reveal any oral antimalarial activity at the tested doses. On the contrary, *i.p.* administration of 20 mg/kg of compound **6d** did not result in a clearance of parasitemia, but oral administration of **6d** revealed a significant antimalarial activity (ED_{50} = 85 mg/kg) with a complete clearance of parasitemia (without recrudescence in the following 28 days).[32] This noteworthy oral antimalarial effect may be related to the nature of the linker of **6d**. Indeed, longer linker (12 or 11 methylene units for **6a** or **6c** respectively) may hamper oral bioavailability. On the contrary, the shorter alkyl chain of **6d** decreased molecular flexibility (less rotatable bonds), an important characteristic for good oral bioavailability.[33] In addition, the aromatic ring facilitated the oral efficiency of **6d**[11] and maintained the desired distance between the cationic heads.[30]

Table 1. *In vitro* and *in vivo* evaluation of antimalarial activity of hybrid amidoxime prodrug candidates **6a-d** produced *via* Scheme 2.

Compounds	R^3	Linker		IC_{50} [[nM] [a] *P. falciparum*	ED_{50} [mg/kg] [b] *P. vinckei*	
		n	Aromatic ring		i.p.	p.o.
Artesunate				3.8	1.2	-
Chloroquine				23	1.1	3.4
6a	H	10	-	240 [c]	20	> 180
6b	CH_3	9	Phenyl-O	530	8.8	> 90
6c	H	9	Phenyl-O	315	> 20	> 90
6d	H	6	Phenyl-O	360	> 20	85

[a] IC_{50} are means of at least two independent experiments conducted in duplicate.

[b] Antimalarial activities (Efficient dose 50, ED_{50}) were determined after i.p. or p.o. administration of the compounds once daily for 4 days to infected mice.

[b] Single value determined in duplicate.

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Table 2. *In vitro* and *in vivo* evaluation of antimalarial activity of asymmetrical drugs **9**, **10a-c** and prodrug candidates **11a-c** produced *via* Scheme 3.

Compounds	n	R^4	Calculated values		IC_{50} [nM] [a] *P. falciparum*	ED_{50} [mg/kg] [b] *P. vinckei*	
			pKa	Log *P*		i.p.	p.o.
9	9	NH_2	11.66	4.23	195	2.2	n.d. [c]
10a	6	NH_2	12.40	2.88	51.5	9	> 90
10b	6	NH*i*Pr	12.30	5.24	9.5	2.8	110
10c	6	pyrrolidine	13.00	5.36	16	1.4	51
11a	6	NH_2	6.41	3.31	2000	> 10	> 180
11b	6	NH*i*Pr	3.30	5.63	n.d. [c]	> 20	> 180
11c	6	pyrrolidine	2.21	5.76	n.d. [c]	10	> 100

[a] pKa and Log *P* values were calculated using ACD/pKa DB, version 6.0, Advanced Chemistry Development Inc.

[b] IC_{50} are means of at least two independent experiments conducted in duplicate.

[c] Antimalarial activities (Efficient dose 50, ED_{50}) were determined after i.p. or p.o. administration of the compounds once daily for 4 days to infected mice.

[d] n.d. means not determined.

Regarding these latter interesting results, we consequently designed an original series of asymmetrical drugs/prodrugs sharing simultaneously benz-amidine/-amidoxime and alkyl-amidine/-amidoxime moieties as polar heads, both being possibly *N*-substituted. The evaluation of their antimalarial activity is reported in Table 2. Concerning drugs, compound **9** exhibited weaker *in vitro* antimalarial activity than compounds **10a-c** (IC_{50} in nanomolar range), maybe because of its too long alkyl linker.[30] Nevertheless, when tested *in vivo*, all the amidine drugs **9**, **10a-c** showed potent i.p. antimalarial activities (close to values Artesunate and chloroquine reported in Table 1). Remarkably, a complete clearance of parasitemia could be recorded after oral administration of the two amidine drugs **10b** and **10c** (ED_{50} of 110 and 51 mg/kg). Therefore, pyrrolidine and isopropylamine introduced on amidine moieties led to favourable pharmacological profile. Since **9** had the weakest *in vitro* activity, we focused our effort on testing amidoximes **11a-c** with 8 methylene bridges. Unfortunately, the asymmetrical amidoximes **11a-c** did not reveal any antimalarial activity at the tested doses *in vivo*, whether after *i.p.* or *p.o.* administration, except **11c**, with ED_{50} i.p. of 10 mg/kg. It is worth mentioning that all these new compounds in hybrid (**6a-d**) and asymmetrical series (**9**, **10a-c** and **11a-c**) were well tolerated by mice in the herein reported experiments. No sign of clinical toxicity was observed after i.p. or p.o. administration of drugs once daily for four consecutive days.

In addition, high pressure liquid chromatographic (HPLC) methods were developed to detect and determine each amidine (**10a-c**) and amidoxime (**11a-c**) in human matrices. The stability of amidoximes **11a-c** in human plasma and blood was confirmed by using different conditions (see SD for details). The bioconversion of pentamidoxime (used as reference) and amidoximes **11a-c** into pentamidine and amidines **10a-c**, respectively was studied using a modified version of the conditions described by Clement and co-workers.[22,34] Briefly, human liver microsomal preparations containing amidoximes (0.5 mm) as substrate plus $NADH,H^+$ cofactor solution were incubated for two hours at 37 °C, then assayed by HPLC. The disappearance of the prodrug candidates and the formation of biotransformation products were monitored. The results are presented in Table 3. Liver microsomes effectively reduced both amidoxime functions of pentamidoxime, **11a**, **11b** and **11c**, mainly leading to respectively pentamidine,[35] **10a**, **10b** and **10c** as predominant metabolites (from 82% to 90 % of the amidoxime disappearance depending on the prodrugs).

Table 3. Metabolism of pentamidoxime and asymmetrical amidoximes **11a-c** by human liver microsomes.

Compounds	t_R (min) [a]	Rate of the total prodrug disappearance [b]
Pentamidoxime	29.0	3%
Monoamidine	27.1	15%
Pentamidine	25.1	82%
11a	15.6	7%
Monoamidine	16.5	7%
10a	17.3	86%
11b	13.3	6%
Monoamidine	12.1	4%
10b	10.0	90%
11c	12.3	14%
10c	11.8	86%

[a] Retention time (t_R) of metabolites analyzed by HPLC-UV (for **11a** and **11b**) or by LC-MS (**11c**) after incubation of pentamidoxime,**11a**, **11b**, or **11c** with human liver microsomes.
[b] The prodrug disappearance is expressed in % of control.

In conclusion, we have designed a new series of asymmetrical compounds sharing a *C*-alkylamidine cationic head and a benzamidine cationic head as well as an optimal length of the alkyl chain comprised of eight methylene linked to an aromatic ring. We could reach highly potent compounds **10b-c**. Amidoxime derivatives were synthesized in three series of compounds. Disappointingly, amidoximes did not reveal any oral antimalarial activity at the tested doses in reverse-benzamidine series and in asymmetrical series. Nevertheless, asymmetrical amidoximes **11a-c** were efficiently converted into the corresponding amidine drugs **10a-c** by liver microsomes. Noteworthy, the conversion was not altered using *C*-akylamidine function nor *N*-substituents. The observed lack of oral activity of amidoximes (reverse benzamidine or asymmetrical series) might be solved by specific *O*-substituents. However, the amidoxime prodrug approach improved significantly oral antimalarial activity of one new hybrid amidoxime **6d**, with an i.p./p.o. index > 0.23, paving the way to finding orally potent prodrugs.

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Supplementary data

^1H and ^{13}C NMR, MS (FAB or ESI), FTIR data of new compounds and biological protocol are given.

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25. For example: 4-[8-(5-(2-hydroxyethyl)-4-methyl-thiazol-3-ium)-octyloxy]-benzamidoxime bromide (**6d**)**.** White amorphous powder. ^{1}H NMR (300 MHz, [D6]DMSO): δ = 10.03 (1H, s), 9.40 (1H, s), 7.58 (2H, d, *J* = 8.7 Hz), 6.90 (2H, d, *J* = 8.7 Hz), 5.69 (2H, m), 5.20 (1H, t, *J* = 5.0 Hz), 4.50 (2H, t, *J* = 7.3 Hz), 3.97 (2H, t, *J* = 6.4 Hz), 3.64 (2H, q, *J* = 5.4 Hz), 3.02 (2H, t, *J* = 5.6 Hz), 2.46 (3H, s), 1.70-1.95 (4H, m), 1.34 ppm (8H, m); ^{13}C NMR (75.47 MHz, CD_3OD): δ = 160.3, 153.9, 141.9, 135.9, 133.7, 127.1 (2C), 124.8, 113.9 (2C), 67.5, 59.9, 53.2, 29.4, 29.0, 28.7, 28.6, 28.5, 25.7, 25.4, 10.3 ppm; FT-IR (cm^{-1}) ν 3307, 3065, 2929, 2858, 1631, 1520, 1260, 1057, 840; MS $C_{21}H_{32}N_3O_3S$ (ESI+) *m/z* (%) : 203.6 ($[(M+H)/2]^+$, 100%), 406.2 (M^+, 42%), 204.1 ($[(M+2H)/2]^+$, 39%), 407.3 ($[M+H]^+$, 12%); HRMS calcd for $C_{21}H_{32}N_3O_3S^+$ 406.2164, found 406.2183.
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29. **4a**-**c**, **4g**, **4i**, **4l**: ED_{50} i.p. > 20 mg/kg, ED_{50} p.o. > 90 mg/kg; **4f**: ED_{50} i.p. = 9.1 mg/kg, ED_{50} p.o. > 90 mg/kg.
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Graphical Abstract

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Amidoxime prodrugs

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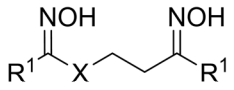
R^1 = primary or *N*-substituted amine



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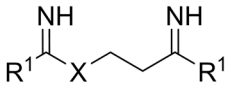
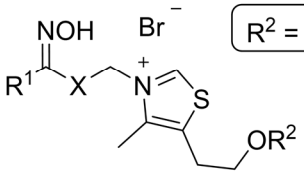
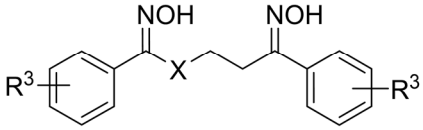




R^2 = H or Me



R^3 = H or mono- or di-substituents

X = Alkyl or phenoxy-alkyl chain