Articles

Potent Antimalarial Activity of 2-Aminopyridinium Salts, Amidines, and Guanidines

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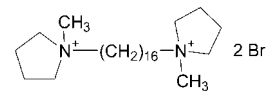
We describe the design, synthesis, and antimalarial activity of 60 bis-tertiary amine, bis-2(1*H*)-imino-heterocycle, bis-amidine, and bis-guanidine series. Bis-tertiary amines with a linker from 12 to 16 methylene groups were active against the *in vitro* growth of *Plasmodium falciparum* within the $10^{-6}-10^{-7}$ M concentration range. IC_{50} decreased by 2 orders of magnitude for bis-2-aminopyridinium salts, bis-amidines, and bis-guanidines (27 compounds with $IC_{50} < 10$ nM). Increasing the alkyl chain length from 6 to 12 methylene groups led to increased activity, while beyond this antimalarial activity decreased. Antimalarial activities appear to be strictly related to the basicity of the cationic head with an optimal pK_a over 12.5. Maximal activity occurs for bis-2-aminopyridinium, two C-duplicated bis-amidines, and three bis-guanidines, with IC_{50} values lower than 1 nM. In comparison to similar quaternary ammonium salts, amidinium compounds have distinct structural requirements for antimalarial activity and likely additional binding opportunities on account of their hydrogen-bond-forming properties.

Introduction

Malaria is a public health problem today for 40% of the world's population. It causes from 1.5 to 2.7 million deaths each year, and the disease is estimated to be responsible for 45 million "disability-adjusted life years".[1–3] The latest analysis indicates that the disease is an even greater problem than previously thought because there were more than a half billion cases of deadly *Plasmodium falciparum* malaria in 2002.[4] The lack of vaccine and the absence of a widely accessible vector control strategy mean that drugs remain the key control measure for malaria. The rapid extension of multidrug-resistant parasites, particularly *P. falciparum*, responsible for the most lethal form of malaria, calls for new pharmacological approaches, leading to original molecules with novel mechanisms of action.

P. falciparum intraerythrocytic growth is associated with a dramatic increase in the total membrane, essentially composed of phospholipids (PLs) but not cholesterol. The mature erythrocyte is devoid of any lipid biosynthetic activity;[5] therefore, the PL biosynthetic machinery is crucial for parasite membrane biogenesis, which is a prerequisite for its development and growth.[6,7] We have identified a family of antimalarial compounds that target the membrane biogenesis of parasites in its erythrocytic stage through the blockage of *de novo* biosynthesis of phosphatidylcholine (PC), the major malarial phospholipid.[8] These choline antagonists consist of mono-[9,10] or bis-[11,12] quaternary ammoniums, which are potent inhibitors of the PL-related choline carrier in eukaryotic cells, such as erythrocytes,[13] and the acetylcholine-related choline carrier in the central nervous system.[14,15] These compounds, which mimic the choline structure, subsequently lead to selective inhibition of *de novo* malarial PC synthesis,[9,16,17] while choline antagonizes the *in vitro* antimalarial activity of the compounds (unpublished). The exact steps that mediate selective inhibition of this metabolic pathway have yet to be clarified. Recently, we have shown that compounds also interact with hemin and/or hemozoin, the hemoglobin-degradation product inside the parasite food vacuole, likely contributing to antimalarial activity.[18]

The structure of these antimalarial compounds has been optimized using pharmacochemistry principles [structure−activity relationship (SAR)]. The most active compounds ($IC_{50} \sim$ nanomolars) are bisquaternary ammonium salts, whose linker between the two nitrogen atoms is composed of at least 12 methylene groups and the cationic head volume is roughly 500 Å^3. The bis-quaternary ammonium salt **G25** ($IC_{50} = 0.65$ nM) and the bis-thiazolium are the lead compounds in this new antimalarial family.[12,19]



G25 $IC_{50} = 0.65$ nM

The antimalarial activity appears to be related to the capacity of these compounds to mimic the choline structure. The compounds thus inhibit choline influx and induce a very

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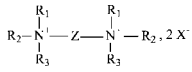
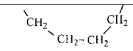
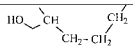
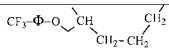
Table 1. Structures and Antimalarial Activities of Bis-tertiary Amines and Their Structurally Related Bis-quaternary Ammonium Salts



Name	R_1	R_2	R_3	Z	X	IC_{50} (nM)
C1 [a]	CH_3	CH_3	H	-$(CH_2)_6$-	Cl	130 000
G1 [a]			CH_3		Br	700 000
C2	C_2H_5	C_2H_5	H	-$(CH_2)_{12}$-	Cl	540
G2 [a]			C_2H_5		Br	45
C3	C_2H_5	C_2H_5	H	-$(CH_2)_{16}$-	I	110
G3 [a]			C_2H_5		Br	1.6
C4			H	-$(CH_2)_{16}$-	Cl	110
G25 [a]			CH_3		Br	0.64
C5			H	-$(CH_2)_{16}$-	I	160
G5			CH_3		Br	1.5
C6			H	-$(CH_2)_{16}$-	I	5 400
G6			CH_3		I	29
C7	C_2H_5	C_2H_5	H	-$(CH_2)_2$-O-$(CH_2)_{12}$-O-$(CH_2)_2$-	Cl	120
G7			C_2H_5		Br	3.3

[a] The activity of these compounds has already been reported.[10,12]

specific and early alteration of malarial *de novo* biosynthesis of PC, while the lethal effect is closely correlated with the blockage of PC biosynthesis.[16] Finally, the pharmacological approach has also been fully validated *in vivo* against rodent malaria[11] and also against *P. falciparum* and *Plasmodium cynomolgi* malaria in monkeys.[17,20] The potency of these compounds seems to be related to their high accumulation inside infected erythrocytes, which ensures both potency and selectivity. Their dual mechanism of action should allow the developed molecules to be active against polypharmacoresistant isolates of *P. falciparum* and limit the risk of emergence of cross-resistance.

Despite this potent antimalarial activity, one major drawback of quaternary ammonium salts for their therapeutic use is their very weak oral absorption because of the permanent positive charge of the quaternary ammonium moiety. One way to circumvent this poor oral absorption would be to replace the quaternary ammonium heads by bioisosteric groups that are highly basic, ionized at physiological pH, and capable of creating bonds with the target(s) similar to or stronger than those of bis-quaternary ammonium salts. Tertiary amines, amidines, or guanidines are thus potential bioisosteric substitutes for cationic heads and should more easily cross the intestinal barrier.

In this study, we designed 60 new compounds, classified into four groups: (a) 7 bis-tertiary amines and three bis-quaternary ammonium salts (Table 1), (b) 22 bis-aromatic 2(1*H*)-imine-heterocycles and one pyridin-2(1*H*)-imine (Tables 2 and 3), (c) 20 bis-amidines (Tables 4 and 5), and (d) 7 bis-guanidines (Table 6), to which one intermediate imidate **I1** was added (Table 4), and we evaluated their *in vitro* antimalarial activity against the human *P. falciparum* parasite.

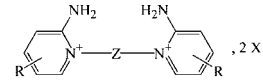
Results

Chemistry. 1. Bis-tertiary Amines and Bis-quaternary Ammonium Salts (C and G Series, Table 1). Except for commercial **C1**, tertiary amines **C2**, **C3**, **C4**, **C5**, and **C6** were prepared by the reaction of α,ω-dihalogenoalkane (bromide or iodide) with an excess of an appropriate secondary amine in refluxing solvent, as shown in Scheme 1. **C7** was obtained by the reaction of 1,12-dibromododecane with diethylethanolamine and KOH in dimethylsulfoxide (DMSO).

Alkylation of **C6** and **C7** with iodomethane and bromoethane generated quaternary ammonium salts **G6** and **G7**, respectively. The reaction of *N*-methyl-2-hydroxymethylpyrrolidine and 1,16-dibromohexadecane led to **G5**.

2. Bis-2(1*H*)-imino-heterocycle Salts (H Series, Tables 2 and 3). Mono- and bis-2-aminopyridinium salts and bis-2-

Table 2. Structures and Antimalarial Activities of Mono and Bis-2-aminopyridinium Salts



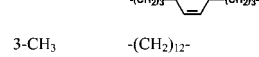
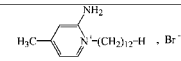
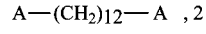
Name	R	Z	X	IC_{50} (nM)	pKa
H2	H	-$(CH_2)_{12}$-	Br	2.3	13.5
H3	4-CH_3	-$(CH_2)_8$-	Br	7.2	n.d.
H4	4-CH_3	-$(CH_2)_{12}$-	Br	0.5	13.5
H5	4-CH_3	-$(CH_2)_{16}$-	Br	3.8	13.6
H6	4-CH_3		I	2.9	12.6
H7	3-CH_3	-$(CH_2)_{12}$-	Br	1.8	13.3
H8	5-CH_3	-$(CH_2)_{12}$-	Br	0.5	13.0
H9	5-Cl	-$(CH_2)_{12}$-	Br	1.4	12.8
H10	4-C_6H_5	-$(CH_2)_{12}$-	I	4.5	12.6
H11	5-C_6H_5	-$(CH_2)_{12}$-	I	13	12.8
H1				1550	n.d.

Table 3. Structures and Antimalarial Activities of Bis-2(1*H*)-imino Heterocycles



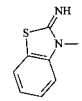
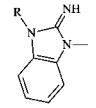
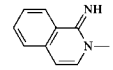
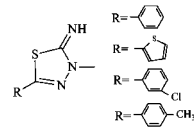
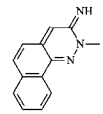
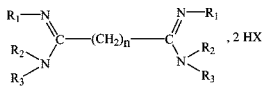
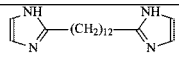
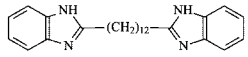
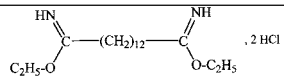
A-	Name	X	IC_{50} (nM)	pKa
	**H12**	Br	800	10.1
	**H13**	C_2HO_4 (oxalate)	4000	n.d.
 R= CH_3	**H14**	C_2HO_4	65	11.9
R= CH_2-C_6H_5	**H15**	C_2HO_4	450	9.4
	**H16**	Br	260	11.1
 R= H	**H17**	Br	145	11.7
R= C_6H_5	**H18**	Br	12.3	11.3
 R=	**H19**	Br	760	8.0
R=	**H20**	Br	8150	7.8
R=	**H21**	Br	18500	7.7
R=	**H22**	Br	5600	8.1
	**H23**	Br	50	11.7

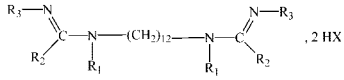
Table 4. Structures and Antimalarial Activities of C-Duplicated Bis-amidines and Imidate (**I1**)

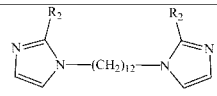


Name	R_1	R_2	R_3	n	X	IC_{50} (nM)	pKa
A1	H	H	H	12	Cl	0.3	13.6
A2	H	H	H	16	Cl	100	13.2
A3	H	CH_3	CH_3	12	Cl	6.3	12.8
A4	H	$-(CH_2)_4-$		12	Cl	0.8	13.2
A5	H	$-(CH_2)_5-$		12	Cl	4.9	12.6
A6	$-(CH_2)_2-$		H	12	[a]	2	13.0
A7	$-(CH_2)_2-$		CH_3	12	Cl	43	11.0
A8	$-(CH_2)_3-$		H	12	Cl	2.3	13.1
A9	$-CH_2-CH(CH_3)-$		H	12	Cl	1	n.d.
A10	$-CH_2-C(CH_3)_2-$		H	12	Cl	1	n.d.
A11			H	12	Cl	3	n.d.
A12					[a]	1 650	9.8
A13					[a]	1 800	9.0
I1						52 000	7.6

[a] These compounds are in the form of free bases.

Table 5. Structures and Antimalarial Activities of N-Duplicated Bis-amidines



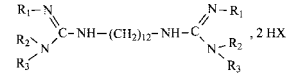
Name	R_1	R_2	R_3	X	IC_{50} (nM)	pKa
A14	H	CH_3	H	[a]	2	12.6
A15	H	$-(CH_2)_3-$		[a]	3.8	12.1
A16	H	$-(CH_2)_4-$		[a]	3	12.7
A17	H	$-(CH_2)_5-$		[a]	2.2	n.d.
A18	$-(CH_2)_3-$		H	[a]	4	n.d.
A19				R_2 = H	2000	n.d.
A20				R_2 = CH_3	270	n.d.

[a] These compounds are in the form of free bases.

amino-aryl salts represented our H series. A total of 10 bis-2-aminopyridinium salts (Table 2) consist of two pyridinium moieties, substituted by a methyl, phenyl, or chloride atom, bound to one another by an alkyl chain of 6−16 methylene groups, for **H6** inclusion of a phenyl cycle. A total of 12 analogues, in which pyridinium moieties were replaced by other differently substituted heterocycles [(benzo)thiazole, benzimidazole, pyrimidine, isoquinoline, thiadiazole, and pyridazine] (Table 3) were also synthesized. Lastly, one monopyridinium salt, **H1** (Table 2), was also prepared.

All symmetrical biscations were prepared by the reaction of α,ω-dihalogenoalkane (bromide or iodide) with excess nucleophilic compound (2-aminopyridine eventually 3, 4, or 5 substituted or 2-amino nitrogenous heterocycle) in refluxing

Table 6. Structures and Antimalarial Activities of Bis-guanidines



Name	R_1	R_2	R_3	X	IC_{50} (nM)	pKa
A21	H	H	H	Br	0.35	n.d.
A22	H	CH_3	CH_3	I	1.6	14.1
A23	C_6H_{11}	C_6H_{11}	H	Cl	21	n.d.
A24	CN	CH_3	CH_3	I	8 500	n.d.
A25	$-(CH_2)_2-$		H	Br	1.7	13.5
A26	$-(CH_2)_3-$		H	I	0.5	n.d.
A27	$-CH_2-C(CH_3)_2-CH_2-$		H	I	0.6	n.d.

solvent as described in Scheme 2. **H6** was obtained by the reaction of 1,4-bis(3-bromopropyl)benzene[21] with an excess of 2-amino-4-methylpyridine.

These compounds can occur in protonated form (Scheme 2a), with an aromatic character (where the positive charge is shared throughout the aromatic heterocycle), or neutral form (Scheme 2b).

3. Bis-amidines (A Series, Tables 4 and 5). Bis-amidine compounds (A series) with a linker attached to the functional carbon atom (C-duplicated compounds, Table 4) were prepared in three steps from alkane dibromide (Scheme 3): (a) substitution of bromine atoms by cyanide groups, (b) addition of EtOH on cyanide, leading to imidates (**I1** or **I2**), and then (c) reaction with an appropriate amine (leading to **A1**−**A5**) or diamine (leading to **A6**−**A11**).

1,12-Bis(imidazol-2-yl)dodecane **A12**[22] was prepared by cyclocondensation of tetradecanedial with a bisulfite combination of glyoxal (hydrate) and ammonium bicarbonate in water and isopropanol. 1,12-Bis(benzimidazol-2-yl)dodecane **A13**[23] was synthesized by acid condensation of *o*-phenylenediamine with tetradecanedioic acid.

Bis-amidines, with a linker on the functional nitrogen atom (N-duplicated compounds, Table 5) and with R_1 = H (**A14**−**A17**), were prepared by the reaction of 1,12-diaminododecane with an appropriate imidate (Scheme 4).

A19 and **A20** (Table 5) were prepared by N-alkylation of imidazole and 2-methylimidazole, respectively, with 1,12-dibromodecane in the presence of KOH (Scheme 4).

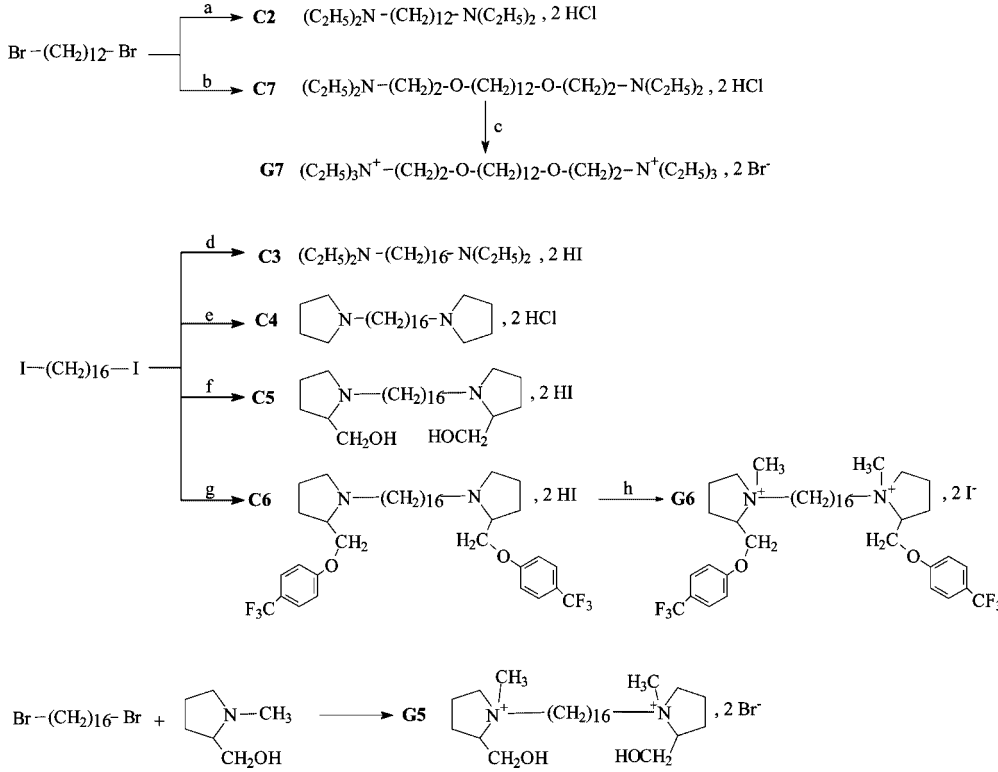
A18 (Table 5) was obtained by alkylation of pyrrolidin-2-one and 1,12-dibromododecane in the presence of sodium and the reaction of 1,12-di(pyrrolidinyl-2-one)dodecane obtained with isocyanatidosulfuryl chloride (Scheme 5).[24]

4. Bis-guanidines (A Series, Table 6). Bis-guanidine compounds **A21**, **A23**, **A25**, **A26**, and **A27** (Table 6) were prepared by the reaction of 1,12-diaminododecane with an appropriate alkyl imidothiocarbamate $CH_3-S-C(=NR_1)NHR_2$ or $C_2H_5-S-C(=NR_1)NHR_2$ (Scheme 6).

A22 and **A24** (Table 6) were prepared by the route shown in Scheme 7. The addition of 1,12-diaminododecane on CS_2 generated 1,12-diisothiocyanatododecane. The latter, treated with dimethylamine, was converted to 1,12-bis(thioureido)dodecane, which was alkylated by iodomethane to give the imidothiocarbamate **I3**. **A22** and **A24** were obtained by the reaction of **I3** with ammonia in MeOH or $NC-NH_2$, respectively.

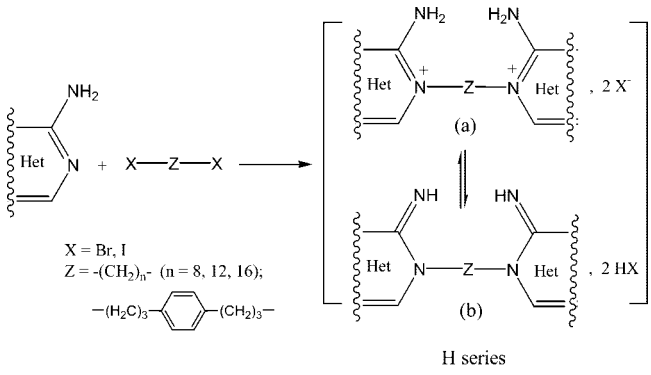
Antimalarial Activity. 1. Bis-tertiary Amines, C Series (Table 1). A total of 7 bis-tertiary amines, whose functions were separated by an alkyl chain of 6−16 methylene groups and whose N substituents were either low alkyl groups (methyl or ethyl) or formed a pyrrolidine cycle with N, possibly substituted,

Scheme 1[a]



[a] Reagents and conditions: (a) $(C_2H_5)_2NH$, NaOH, HCl; (b) $(C_2H_5)_2N-CH_2CH_2OH$, HNa/THF, HCl; (c) C_2H_5Br in EtOH; (d) $(C_2H_5)_2NH$; (e) pyrrolidine, NaOH, HCl; (f) 2-hydroxymethylpyrrolidine; (g) 2(*p*-trifluoromethylphenoxymethyl)pyrrolidine; (h) CH_3I in EtOH.

Scheme 2[a]



[a] (a) Protonated form: salt whose cationic moiety possesses an aromatic character. (b) Neutral form: bis-2(1*H*)-imino heterocycle.

were synthesized. One compound possessed two oxygen atoms in the interchain of 16 methylene groups. Their antimalarial activities were evaluated *in vitro* by adding the compounds to a *P. falciparum* suspension for a complete 48 h parasite cycle before assessing the parasite viability. Among these 7 bis-amines, 6 were active in the micromolar range, with IC_{50} values ranging from 5.4 to 0.11 μM. The activity appeared to be related to the presence of a long alkyl chain (number of methylene groups $\geq$ 12) between the two nitrogen atoms. Progressive elongation of this linker from 6 to 16 carbon atoms (**C1/C2/C3**) increased the antimalarial potency. The presence of oxygen atoms in the linker did not affect the activity against the malarial parasite (**C5/C7**).

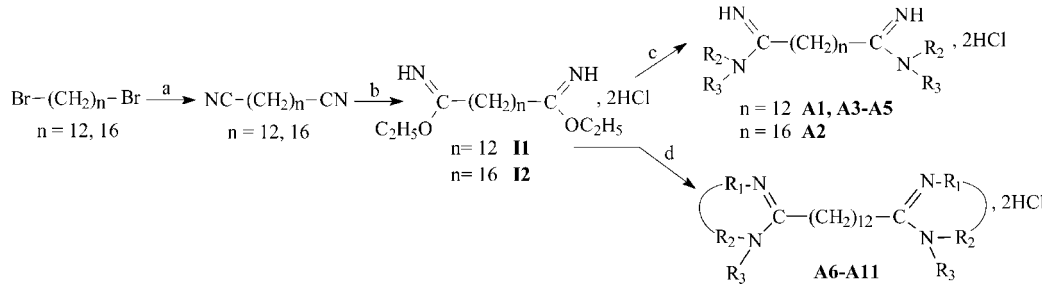
However, except for the poorly active **C1** compound, the antimalarial activity of tertiary bis-amines was 1−2 orders of magnitude lower than that of their structurally related bis-quaternary ammonium salts (with an additional methyl or ethyl group on each nitrogen atom), regardless of the nitrogen substituents and inter-nitrogen chain (Table 1). This was probably due to the less lipophilic environment of the cationic heads of amines (three substituents) in comparison to those of ammonium compounds (four substituents). Indeed, this lipophilic environment around nitrogen atoms is required for the antimalarial activity.[12]

2. Bis-2-amino-*N*-heterocycle Salts, H Series (Tables 2 and 3). Because bis-tertiary amines had much lower antimalarial activity than their corresponding quaternary ammonium, we synthesized bis-2-aminopyridinium salts (Table 2) and bis-2-amino-*N*-heterocycle salts (Table 3), which represented our H series (Scheme 2). In these series, the antimalarial activity of bis-cationic compounds was far more potent (by 3 orders of magnitude) than monocationic compounds (compare **H1** and **H4**). Consequently, we have chosen to focus on duplicated molecules in the following part of this work.

Bis-2-aminopyridinium salts, differently substituted (Table 2), were very potent against *P. falciparum in vitro* growth, with an IC_{50} ranging from 0.5 to 13 nM. A variation in the number of methylene groups in the spacer indicated maximal activity for 12 carbon atoms (compare **H3**, **H4**, and **H5**), with an IC_{50} of 0.5 nM for the **H4** compound. Inclusion of an aromatic cycle in the linker, corresponding to a linker length of 10 methylene groups (**H6**, IC_{50} = 2.9 nM), did not change the antimalarial activity. Thus, we kept the linker to the 12 methylene groups that appeared to be optimal for antimalarial activity.

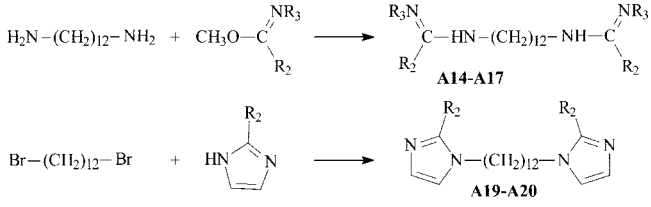
We then substituted the pyridine cycle by other aromatic heterocycles [(benzo)thiazole (**H12** and **H13**), isoquinoline (**H16**), benzimidazole (**H14** and **H15**), pyrimidine (**H17** and **H18**), thiadiazole (**H19**, **H20**, **H21**, and **H22**), and benzo[*h*-]cinnoline (**H23**), Table 3]. The highest activity, was obtained for **H18** (IC_{50} = 12.3 nM), which contained a 5-phenyl pyrimidinium as the cationic head. Other aromatic heterocycles

Scheme 3[a]



[a] Reagents and conditions: (a) NaCN; (b) EtOH, Et_2O, HCl gas; (c) amines (ammonia, dimethylamine, pyrrolidine, or piperidine); (d) diamines (ethylenediamine, *N*-methylethylenediamine, 1,3-diaminopropane, 1,2-diaminopropane, 1,2-diamino-2-methylpropane, or 1,2-diaminocyclohexane).

Scheme 4



resulted in a dramatic decrease of *in vitro* antimalarial activity ($IC_{50} \geq 50$ nM). The antimalarial activity thus did not seem to be related to the cationic head volume but rather to the pK_a (see below).

3. Bis-amidines (Tables 4 and 5) and Bis-guanidines (Table 6), A Series. We also synthesized A series involving bis-amidines and bis-guanidines, possessing various substituents on nitrogen atoms and 12 or 16 methylene groups as the linker.

Two bis-amidine types were designed, i.e., those with the linker on the carbon atom (named C-duplicated compounds, **A1**−**A11**, Table 4), and on the nitrogen atom (named N-duplicated compounds, **A14**−**A18**, Table 5). In each of these two series, two compounds had an amidine function included in the heteroaromatic cycle (imidazoles **A12**, **A19**, and **A20** and benzimidazole **A13**, Tables 4 and 5). The last four compounds were not very active, with an IC_{50} in the micromolar range. On the other hand, bis-amidine and bis-guanidine compounds had very potent *in vitro* antimalarial activities against *P. falciparum*, with an IC_{50} in the very low nanomolar range when the linker contained 12 carbon atoms. Increasing the length of the linker chain from 12 to 16 methylene groups significantly decreased the antimalarial activity (compare **A1**, 0.3 nM with **A2**, 100 nM).

When the bis-amidines had the optimal linker (12 C), the antimalarial activities all appeared to be more potent because the substitution degree on the nitrogen atoms was low for both the C- and N-duplicated amidine series [compare **A1** (no substitution, ~0.3 nM) with **A3**−**A6**, **A8**−**A11**, and **A14**−**A18** (1 or 2 substitutions, 0.8–6.3 nM) and with **A7** (3 substitutions, 43 nM), Tables 4 and 5].

When equally substituted, bis-guanidines (Table 6) appeared to be slightly more active, or as active as bis-amidines (compare **A21/A1**, **A22/A3**, **A25/A6**, and **A26/A8**). **A23**, which has a very bulky cationic head, had lower activity, while **A24**, whose nitrogen is substituted by an electron-withdrawing CN group, was poorly active.

In this study, we also measured antimalarial activity of the synthesis intermediate imidate **I1** (Table 4). This compound was 10^4-fold less active than amidines and guanidines.

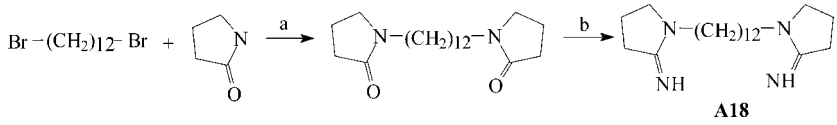
4. Relation between Compound Basicity and Antimalarial Activity. We determined the pK_a of most of the compounds using potentiometric titration. Bis-2-amino-pyridinium salts had a pK_a in the 12.6–13.5 range (Table 2); bis-amidines had a pK_a ~ 11.0–13.6 (Tables 4 and 5); and bis-guanidines had a pK_a ~ 13–14 (Table 6). The pK_a values of other bis-2-amino-*N*-heterocycle salts in Table 3 were lower (pK_a ~ 8–11). All of these biscations were fully protonated at pH 7.4. Figure 1 shows the antimalarial activity versus the pK_a for compounds belonging to A (bis-amidines and bis-guanidines) and H (bis-2-amino-*N*-heterocycle salts) series that had the same inter-nitrogen spacer, $(CH_2)_{12}$, and that differed only by the polar head. It was striking that the *in vitro* activity against the human *P. falciparum* parasite was strictly related to the pK_a values of the molecules. Biscations, with a linker of 12 carbon atoms, possessing a pK_a of more than 12, had an IC_{50} of below 13 nM, regardless of the nitrogen substitution.

Discussion

From a molecular standpoint, our pharmacological approach is based on the capacity for biscations to mimic the choline structure. The SARs assessed for more than 280 quaternary ammonium salts allowed us to optimize their structures for potent antimalarial activity, with IC_{50} against *P. falciparum* in the low nanomolar range. The hydroxyethyl group of choline is not a prerequisite for antiplasmodial activity. On the other hand, the antimalarial activity appeared to be dependent upon nitrogen substitution with one long lipophilic chain, while duplication of the quaternary ammonium increased the inhibitor potency by at least 2 orders of magnitude. Bis-quaternary ammonium salts with two cationic heads separated by a lipophilic spacer (at least 12 methylene groups) are among the most potent antimalarials with this molecule type (**G25** as a leader).[12]

To remedy the low oral absorption of quaternary ammonium salts, inherent to the permanent charge on nitrogen atoms, the

Scheme 5[a]



[a] Reagents and conditions: (a) Na; (b) O=C=N−SO_2Cl and then NaOH.

quaternary polar heads were replaced by bioisosteric groups, which could, at physiological pH, induce the same drug−target interactions as quaternary ammonium salts. These new analogues are tertiary amines, 2-aminopyridinium salts, 2-amino-*N*-substituted aryl salts, amidines, and guanidines. Their functions are ionized at physiological pH, potentially mimicking the cationic head of the choline. Because of the equilibrium between protonated and unprotonated forms, these compounds should diffuse more easily through the cellular membrane and tissue because of the neutral form.

Bis-tertiary amines, whose inter-nitrogen linker contains at least 12 carbon atoms, had significant antimalarial activity but 1−2 orders of magnitude lower than their corresponding bis-quaternary ammonium salts (Table 1). The amine cationic head was probably not lipophilic enough to completely match the target.

Then, we replaced the quaternary ammonium heads by strongly basic bioisosteric groups, ionized at physiological pH, which could form the same bonds with the target as bis-quaternary ammonium salts. The structure of amidine systems is midway between that of tertiary amines and quaternary compounds, at least regarding the ionization and electronic properties, but differ from the latter in shape (planar for amidines and guanidines and tetrahedral for ammoniums). Nevertheless, we investigated amidinium systems for their additional distinctive features. The driving force for the primary docking of amidinium cations with an anionic counterpart of the target is assumed to be an electrostatic interaction, but hydrogen-bond formation could also strengthen the affinity.[25–27]

Among these compounds, 2-amino-pyridinium salts (H series), amidines, and guanidines (A series) showed very potent antimalarial activities, with an IC_{50} in the very low nanomolar range. A total of 27 molecules had an IC_{50} of less than 10 nM against the human parasite *P. falciparum*. Maximal activity was obtained for two bis 2-aminopyridiniums (**H4** and **H8**, with IC_{50} of 0.5 nM), two C-duplicated bis-amidines (**A1** and **A4**, with IC_{50} of 0.4 and 0.8 nM, respectively), and three bis-guanidines (**A21**, **A26**, and **A27**, with IC_{50} of 0.35, 0.5, and 0.6 nM, respectively). The compounds were as potent as bis-quaternary ammonium salts.[12]

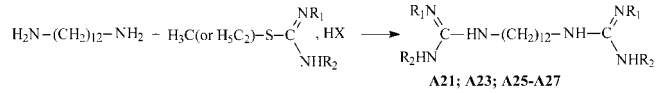
Dimerization of the cationic head produced effectors with enhanced (by 2 orders of magnitude) antimalarial activity relative to the monomeric congener. This feature, which was also found in a previous study, suggests a bivalent interaction between the compound and the target.[12]

The length of the linker between the two cationic heads proved to be a critical parameter for antimalarial activity. The optimal length, in the A and H series, was 12 methylene groups (estimated to be around 16 Å in the extended state of the molecule). This differed from 16, as observed with bis-amines (Table 1) and bis-quaternary ammonium salts.[12]

Most compounds with a $(CH_2)_{12}$ linker, belonging to the bis-2-aminopyridinium series, with a small substituent (methyl group or chloride atom) on the pyridinium cycle (Table 2), and to the A series (amidines and guanidines, Tables 4−6) are potent *in vitro* antimalarials (IC_{50} < 10 nM).

With the pyridinium cycle, amidine or guanidine function replaced by other aromatic heterocycles, including (benzo)thiazole, isoquinoline, (benz)imidazole, pyrimidine, thiadiazole, and benzo[*h*]cinnoline (all compounds in Table 3 and **A12** and **A13** in Table 4), led to much less active compounds. As illustrated in Figure 1, the basicity (pK_a values) of the compound polar heads appeared to be strictly correlated with the antimalarial activity and is likely a crucial parameter for antimalarial activity.

Scheme 6



A21; A23; A25-A27

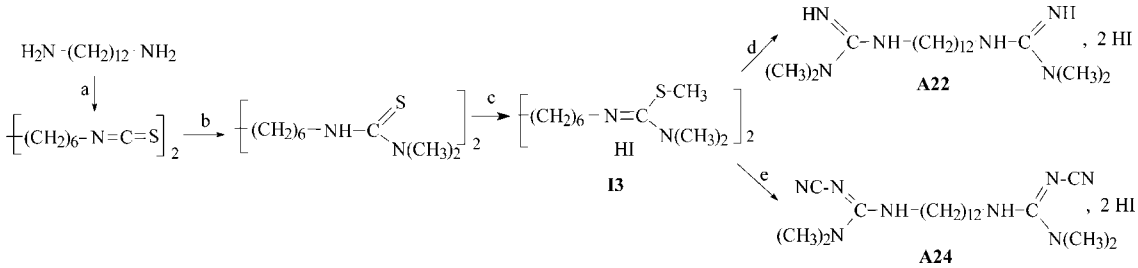
The antimalarial activity of the compounds occurred at lower compound concentrations as their basicity increased. *In vitro* antimalarial activity in the low nanomolar range appears to be restricted to compounds with a pK_a between 12.5 and 14.5. The positive charge of the H and A compound series was strongly delocalized, and the ability of these groups to form bonds (hydrogen or ionic bonds) was higher than that of tertiary amines and quaternary ammoniums. The pK_a values of these compounds thus do not only indicate the percentage of the protonated compound at physiologic pH but likely also its ability to form strong (hydrogen and electrostatic) bonds with the target.[27–29]

In contrast to compounds containing a quaternary ammonium, the polar head of amidines, guanidines, and 2-aminopyridiniums are planar and thus do not occupy the same bulk at the active site. Hydrogen-bond formation may influence the positioning of the interacting molecule, thus explaining the difference in optimum linker length in the two biscation types. However, both types of compounds exerted early effects on PC biosynthesis of *Plasmodium* (unpublished results) and seemed to share the same mechanism of action.

In general, symmetrical biscations, such as amidines, guanidines, and heterocyclic amidines, are known to have various pharmacological effects (antimicrobial[30] and antifungals[31,32]). They act at different targets, including receptors (GPCR muscarinic receptors[33] and channel receptors[34]) and enzymes (protein kinase C). Bis-aromatic benzamidines (pentamidine **I** and its analogues as **II** and **III**, Figure 2), with different linkers connecting the aromatic groups, were extensively studied for their physiological effects on the central nervous system[35] and their inhibition of various microbes and parasites (*Pneumocystis carinii*,[36–38] *Leishmania*,[39] *Trypanosoma brucei*,[40] and *Babesia canis*[41]), including the malarial parasite *P. falciparum*.[42] A and H series compounds are structurally different from pentamidine analogues, for which the cationic heads include an additional phenyl cycle conjugated with the amidine function, and the linker is often shorter and rigid.[43,44] The structure−antiplasmodial activity relationships of the benzamidines and our compounds differed substantially.[45] This suggests that the therapeutic targets also differed. Plausible mechanisms of action of these bioactive benzamidines include initial binding to AT-rich sites of DNA followed by inhibition of one or more of several DNA-dependent enzymes or direct inhibition of the transcription process. This concerns furamidine (**II**), a rigid, curved, and planar molecule; therefore, it has the appropriate steric and electrostatic properties for high-affinity binding to the minor groove of DNA. For pentamidine congeners **II** and **III**, however, Mayence et al.[44] recently reported that the DNA binding of benzamidine analogues was not correlated with their antiplasmodial activities. Finally, benzamidines, particularly pentamidine, which is concentrated 500-fold by erythrocytes infected with *P. falciparum*,[42] were shown to form complexes with ferriprotoporphyrin IX and, therefore, to be involved in the inhibition of hemozoin formation in a cell-free system.[46] Such an accumulation remains to be clarified for our compounds series. Besides, there was no indication of an interaction of pentamidine-like biscations with plasmodial phospholipid biosynthesis.

In conclusion, we have shown that a series of novel bis-amidines, bis-guanidines, and 2-aminopyridinium salts,

Scheme 7[a]



[a] Reagents and conditions: (a) CS2, DCC; (b) $(CH_3)_2NH$ in MeOH; (c) CH_3I; (d) NH_3 in MeOH; (e) N′C−NH_2 in MeOH.

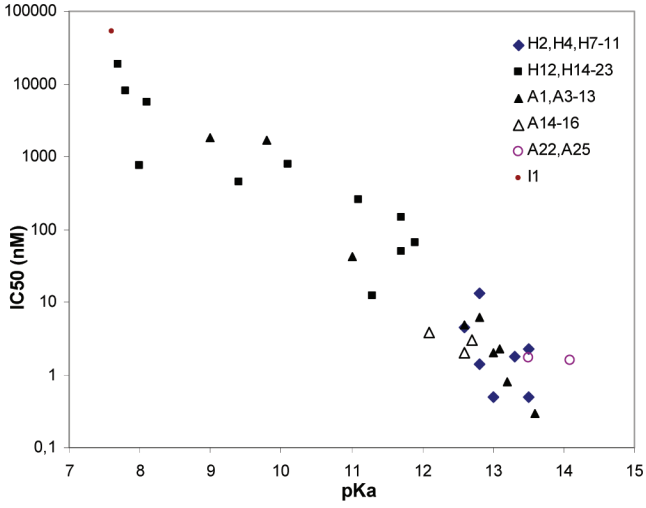


Figure 1. Antimalarial activity (IC_{50}) of bis-2-aminopyridinium salts (◆), bis-2(1*H*)-imine heterocycles (■), C-duplicated bis-amidines (▲), N-duplicated bis-amidines (△), bis-guanidines (○), and one imidate (●) (Tables 2−6) possessing the same inter-nitrogen spacer, $(CH_2)_{12}$, as a function of basicity (pK_a) (35 compounds).

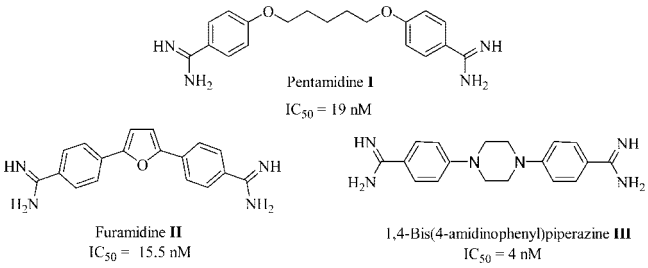


Figure 2. Pentamidine and analogues.

bioisosteres of bis-quaternary ammonium salts, with a lipophilic linker [$(CH_2)_{12}$] and whose pK_a values were over 12, are potent antimalarial compounds, which exert their activity in a very low nanomolar range. Among them, 27 compounds had an IC_{50} < 10 nM and 15 molecules (**H4**, **H7**−**H9**, **A1**, **A4**, **A6**, **A9**, **A10**, **A14**, **A21**, **A22**, **A25**−**A27**) had an IC_{50} of less than or equal to 2 nM. In comparison to similar quaternary ammonium salts, these compounds have binding opportunities on account of their hydrogen-bond-forming capacity with the target(s). These compounds, which can exist in the neutral form and in equilibrium with the cationic form, could be more bioavailable.

Experimental Section

Chemistry. *N*,*N*,*N*′,*N*′-Tetramethylhexane-1,6-diamine, **C1**, was commercially available (Sigma-Aldrich).

All key target compounds possessed purity superior to 98% (results of combustion analyses for C, H, and N were within ±0.4% of the theoretical value).

General Procedure for the Synthesis of Bis-tertiary Amines (Table 1). A mixture of α,ω-dihalogenoalkane (0.15 mol) and excess (4−10 equiv) of secondary amine in 75 mL of EtOH was refluxed for 12 h. The solvent and amine excess were removed. When the amine was isolated with the dihydroiodate, the residue was directly recrystallized in acetone. When the amine was isolated as its dihydrochloride, the residue was diluted with 30 mL of water and 10% NaOH was added until basic pH. Diamine was extracted with 100 mL of diethyl ether, in which HCl gas was added. The resulting solid was recrystallized in acetone.

General Procedure for the Synthesis of Bis-2-aminopyridinium Salts (Table 2) and Bis-2(1*H*)-imino-nitrogenous Heterocycle Salts (Table 3). Procedure A. A mixture of α,ω-dihalogenoalkane (0.1 mol) and an excess (about 3 equiv) of a nucleophilic compound (2-aminopyridine, possibly 3, 4, or 5 substituted, or 2(1*H*)-imino-nitrogenous heterocycle) in butanone (100 mL) was refluxed in an argon atmosphere for 48 h. After the solution was cooled, the sought-after compound was precipitated. It was recrystallized in an *i*PrOH/MeOH mixture (9:1).

Procedure B. A mixture of α,ω-dihalogenoalkane (0.1 mol) and an excess (about 3 equiv) of a nucleophilic compound [2(1*H*)-imino-nitrogenous heterocycle] in sulfolane (100 mL) was refluxed in an argon atmosphere for 1 week. A total of 60 mL of CH_3CN was added. After the solution was cooled, the sought-after compound was precipitated. It was recrystallized in an *i*PrOH/MeOH mixture (9:1).

When the compound was isolated as a dioxalate (**H13**−**H15**), the salt was diluted in K_2CO_3 (10% aqueous). This solution was gently heated (50 °C), and the free diamine was extracted with AcOEt. This compound was diluted in a mixture of *i*PrOH/MeOH (1:1), and 2 equiv of oxalic acid in solution in *i*PrOH was added. The precipitate is filtered and recrystallized in an *i*PrOH/MeOH/$(i\text{Pr})_2O$ mixture.

Determination of Ionization Constants (pK_a). The compound pK_a values were obtained, at room temperature, using potentiometric titration.[47] A Jenway 3040 ion analyzer pH meter (calibrated according to the instructions of the manufacturer) equipped with an Ingold pH electrode was used. The method consisted of measuring the pH values within a potentiometric titration.

An appropriate amount of basic compound (30–50 mg) was dissolved in water or a water−EtOH medium (≤20% EtOH) to overcome the solubility problems associated with the compounds and then titrated to pH 2.0 with 0.1 N HCl. During titration, the titrant was added in increments of 0.1 mL after each stable reading and the corresponding pH values were recorded. The resulting values were plotted (pH versus added HCl), and the pK_a values were relatively accurately estimated from the graphs.

***In Vitro* Antimalarial Activity.** *In vitro* antimalarial activity was measured using the [^{3}H]-hypoxanthine incorporation assay with the Nigerian strain of *P. falciparum*, as previously described.[19] Results were expressed as the concentration resulting in 50% inhibition (IC_{50}).

Acknowledgment. This study was supported by the European Community (QLK2-CT-2000-01166 and PCRDT FP6, Integrated Projects Antimal "Development of New Drugs for the treatment of Malaria"), Ministère de l'Education Nationale et de la Recherche Scientifique (PAL+), the UNDP/World Bank/WHO special program for Research and Training in Tropical Diseases, and Sanofi-Aventis Laboratories (Montpellier, France).

Supporting Information Available: General methods, experimental procedures and details for the synthesis and analytical data of all compounds; assessment of biological activity, *in vitro* and *in vivo* antimalarial activity and acute toxicity in mouse. This material is available free of charge via the Internet at http://pubs.acs.org.

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