

Dual Molecules as New Antimalarials

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Abstract: A new antimalarial pharmacological approach based on inhibition of the plasmodial phospholipid metabolism has been developed. The drugs mimic choline structure and inhibit *de novo* phosphatidylcholine biosynthesis. Three generations of compounds were rationally designed. Bisquaternary ammonium salts showed powerful antimalarial activity, with IC₅₀ in the nanomolar range. To remedy their low *per os* absorption, bioisosteric analogues (bis-amidines) were designed and exhibited similar powerful activities. Finally, the third generation compounds are bis-thiazolium salts and their non-ionic precursors: prodrugs, which *in vivo* can lead to thiazolium drugs after enzymatic transformation.

The compounds are equally effective against multiresistant *Plasmodium falciparum* malaria. These molecules exert a very rapid cytotoxic effect against malarial parasites in the very low nanomolar range and are active *in vivo* against *P. vinckei*-infected mice, with ED₅₀ lower than 0.2 mg/kg. They are able to cure highly infected mice and, retain full activity after a single injection. They also retain full activity against *P. falciparum* and *P. cynomolgi* in primate models with no recrudescence and at lower doses.

Compounds are accumulated in *P.falciparum*-infected erythrocyte, which ensures their potency and specificity. Recently, we discovered that compounds also interact with malarial pigment enhancing the antimalarial effect. It is quite likely that they are dual molecules, exerting their antimalarial activity via two simultaneous toxic effects on the intracellular intraerythrocytic parasites. The current leader compounds are accessible in few steps from commercial products. These crystalline molecules present a remarkable biological activity and low toxicity which is promising for the development of a new antimalarial drug.

Keywords: Antimalarials, *Plasmodium falciparum*, Phospholipid, Phosphatidylcholine, Choline analogues, Malarial pigment, Hemozoin, Bisquaternary ammonium salts, Bis-amidines, Bis-thiazolium salts, Prodrug.

1. INTRODUCTION

Malaria is one of the most important parasitic infections of humans due to its high morbidity and mortality with major consequent impact on economic productivity and livelihood [1]. Approximately 40% of the world population live in areas with the risk of malaria. Each year 300-500 million people suffer from acute malaria and 1.5-2.5 million die from the disease [2-4].

Plasmodium falciparum, the causative agent of the malignant form of malaria, has high adaptability by mutation and is resistant to various types of antimalarial drugs [5]. Thus, cheap malaria treatments such as chloroquine and fansidar (sulfadoxine/pyrimethamine) become ineffective with the selection and spread of mutant drug-resistant parasites and then to multidrug resistance emergence [5-7]. Artemisinin (qinghaosu) has become increasingly important as a malaria treatment but the current routes for its total chemical synthesis [8-11] remain too complex for commercial production [12, 13]. There is no doubt that intensive use artemisinin-classes combinations therapy is an invaluable opportunity that decrease the burden

of malaria and prolong drug life span of effective use. However, in the absence of substitute, the appearance of artemisinin-resistant malaria would lead to untreatable malaria and to a potential humanitarian disaster.

Therefore, new drugs with novel mechanisms of action and that are structurally unrelated to existing antimalarial agents are thus urgently required [14, 15]. The priority is the identification of original target (s) leading to novel antimalarial compounds which would *a priori* not allow cross-resistance with preexisting antimalarials.

During the last 20 years [16, 17] we have developed a new approach to malaria chemotherapy and successively designed and synthesized three generations of compounds. We show here the major structural motif that mediate the antiparasitic activity, and discriminate their specific features, notably concerning the central nervous system and other biscations such as pentamidine. We also briefly explain the current knowledge by which they exert their antimalarial activity. The drugs that we have prepared are dual molecules targeting two mechanisms of action: they interact with the plasmodial phospholipid (PL) metabolism and also with the malarial pigment. They are accessible in few steps from commercial products with a low cost of production. These crystalline molecules, appropriate for an oral administration, present a remarkable biological activity and low toxicity which is promising for the development of a new antimalarial drug.

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2. COMPOUNDS: RATIONALE, DESIGN AND ACTIVITY

2.1. Phospholipid Metabolism as Novel Pharmacological Target

The last two decades have witnessed a very impressive increase in our understanding of the biochemistry and the molecular biology of the malaria parasite, and have focused attention on specific parasite molecules that are keys to the parasite life cycle or the induction of its pathogenesis [18, 19]. Our teams have developed a programme which aim was to discover some metabolic reactions that are specific to the intracellular parasite *Plasmodium* and that can be pharmacologically affected. We became interested in the lipid peculiarities of this intracellular parasite. Indeed, during asexual development within erythrocytes, malaria parasites synthesize considerable amounts of membrane through the phospholipid (PL) metabolism [20-22]. This activity provides an attractive target for chemotherapy because it is absent from mature erythrocytes. Thus, a new antimalarial chemotherapy has evolved, based on choline analogues. It targets the *de novo* phosphatidylcholine biogenesis necessary for membrane synthesis in the erythrocytic stage of the parasite, which is crucial for its development and growth [23].

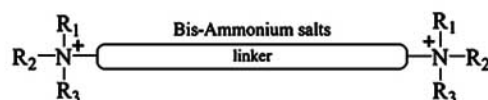
2.2. Quaternary Ammonium Salts are Potent Antimalarials

Mono and bis quaternary ammonium salts were good candidates for mimicking the choline structure. This first compound generation was rationally designed (Fig. 1) [24-26].

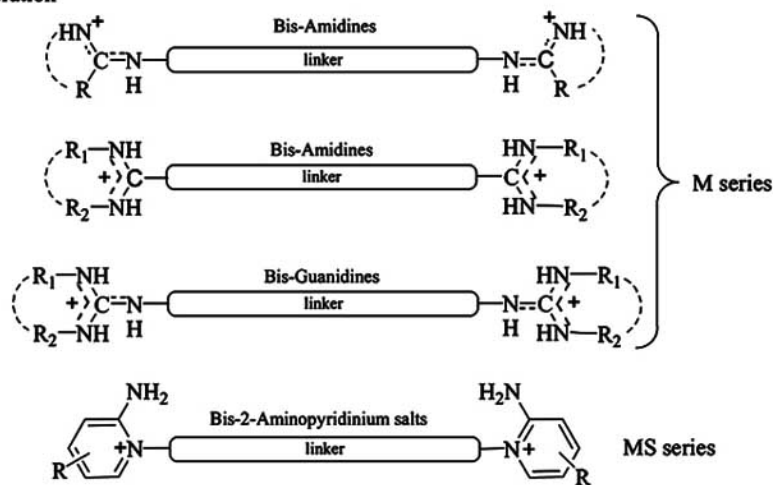
For this series, we made a detailed structure-activity relationship analysis [24] to determine the pharmacophore requirements for an optimal *in vitro* antimalarial activity against the human parasite *P. falciparum*. Size of the polar head (nitrogen substituents), and polar head duplication were sequentially studied.

An increase in the lipophilicity around nitrogen improved antimalarial activity. It appears that the volume of the cationic head meets very strict requirements to adapt to the active site. For N-dodecyl-substituted monoquaternary ammonium salts, the presence of alkyl groups bulkier than methyl on the nitrogen atom was favourable for the activity up to three methylenes. There is a sharp decrease in the IC_{50} for a head volume between 200 and 400 Å³. This active site can then be schematized as a sphere whose volume is similar to that of an N-tripropyl head (i.e. 450 Å³) and therefore whose radius is 4.5 Å. However, the presence of a long hydrophobic alkyl chain was a primordial parameter and N-dodecyl substituent was found to be optimal for antimalarial activity [24]. In parallel studies, we showed that these compounds inhibit choline entry in malaria infected erythrocytes with the lowest K_i values corresponding to an alkyl chain of 10-12 carbons. The increase in the K_i values at 14 carbon chain length (associated with a decreased antimalarial activity) suggests that the end of the hydrophobic alkyl group may butt against a hydrophilic domain and be repelled by it [25]. Thus, for the first generation monoquaternary compounds a suitable very high level of activity has been reached *in vitro* against *P. falciparum*, with maximal activity with E13 (N, N, N-tripropyldodecan-1-ammonium bromide), (IC_{50} = 33 nM) [24, 25].

First Generation



Second Generation



Third Generation

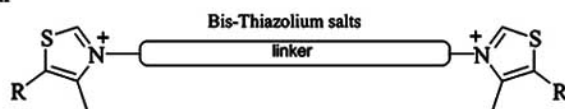
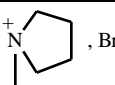
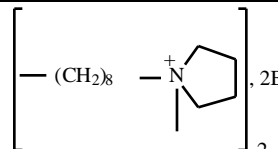
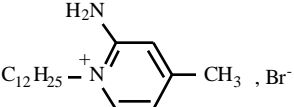
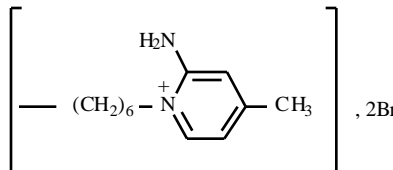
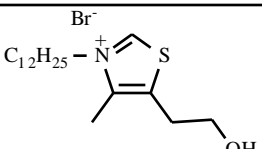
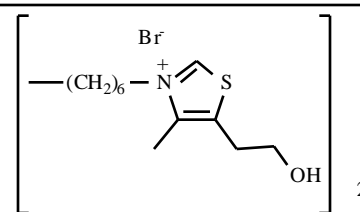


Fig. (1). General structure for the compounds of the three generations.

Table 1. Comparison of Mono and Bis-Cationic Molecules for their *In Vitro* Antimalarial Activities

Generation	Mono cationic compounds	IC ₅₀ (nM)	Bis cationic compounds	IC ₅₀ (nM)
First	$C_{16}H_{33}-N^+$  E26	420	 G25	0.64
Second	$C_{12}H_{25}-N^+$  MS23	1550	 MS1	0.5
Third	$C_{12}H_{25}-N^+$  T1	70	 T3	2.25

IC₅₀ were assessed after 48 h contact of *P. falciparum*-infected red blood cells (Nigerian strains) with the drug (Calas, M.; Ancelin, M. L.; Cordina, G.; Portefaix, P.; Piquet, G. *et al. J Med Chem* 2000, 43, 505-516)

2.3. Effect of Duplicating the Cationic Head

An important and decisive observation was that a molecular variation involving duplication of pharmacophoric groups (“twin-drug”) considerably increased the antimalarial activity and led to very potent drugs. As shown in Table 1, the bis-ammonium salt G25 was more than 600 fold more active than the mono-ammonium E26. The same pattern was observed in the second generation (see below) as illustrated with the molecules MS23 and MS1. It was striking that also bis-thiazolium forms (see below) T3 and T4 (IC₅₀ = 2.25 and 0.65 nM respectively) were much more active than corresponding monoquaternary thiazolium salts (T1 and T2, IC₅₀ = 70 and 75 nM) (Fig. 9). Thus, duplicated molecules exerted an *in vitro* antimalarial activity at (30-1500)-fold more potent than the mono analogues (Vial *et al.* in preparation).

2.4. Effect of Chain Modification

Thus, we focused much of our efforts on duplicated molecules and investigated in the details the optimal structure of the linker (length and composition). We observed that in the case of bis ammonium salts, antimalarial activity increased until 16 methylene groups (IC₅₀ ~ 10⁻⁹ -10⁻¹⁰ M) which was the optimal linker length (Table 2). For the second generation biscationic compounds (see below), a series of compounds with a central bridge arising from 6 to 16 carbon atoms was also synthesized. The optimal linker length for antimalarial activity was observed for a C12 alkyl chain and thus the C12 compound M34 was 300-fold more active than the C16 analog M35. In the case of the third generation compounds, linkers with less than 12 carbons showed a drastic loss of antimalarial activity. By

contrast, the C16 intermediate (T8) didn't show a significant variation respect to the C12 analogue (T4) (Table 2).

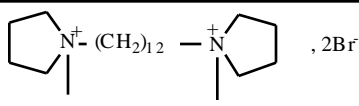
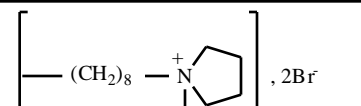
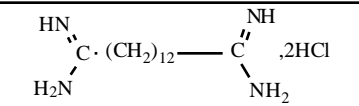
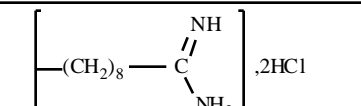
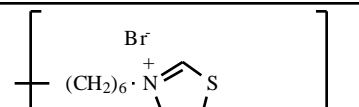
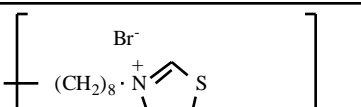
Thus it seems that linker needs lipophilicity, but also that lipophilicity needs a strict spatial distribution. A possible explanation could be that above a certain length of the linker, the alkyl chains could curl up on themselves, leading to expulsion of the quaternary ammonium group out of the anionic site and only the long alkyl chain would be associated in tightly coiled fashion with the hydrophobic region on the target. The reason the target better accommodates C16 drugs for the first generation compounds and C12 for the second and third ones remains unclear but probably reflects the presence of other constraints imposed by additional interactions (probably of - nature) between drug and target.

The chemical nature of the lipophilic chain is also an important point for the antimalarial activity. We modified the lipophilicity of the linker by adding electronegative or electron-rich N-substitutions or its rigification by introducing unsaturations or aromatic groups. All attempts implying a change in the lipophilicity or rigidity of the chain and consequently have led to a decrease in antimalarial activities relative to the corresponding saturated carbon groups [24, 26].

At the evidence, bisquaternary ammonium salts showed powerful antimalarial activity, with IC₅₀ in the very low nanomolar range. Thus, many molecules showed an IC₅₀ lower than 10 nM [26]. When tested, they were equally potent against various resistant isolates [27].

The compounds also exerted potent antimalarial activity *in vivo* both against murine malaria in the rodent model [28] and against human malaria in monkeys [29]. The lead

Table 2. Influence of Linker-Length Modification for the *In Vitro* Antimalarial Activity Against *P. Falciparum*

Generation	C12	IC ₅₀ (nM)	C16	IC ₅₀ (nM)
First	 G24	13	 G25	0.64
Second	 M34	0.3	 M35	100
Third	 T4	0.65	 T8	1.1

compound in the bis-quaternary ammonium salt series was G25 [(1, 16-hexamethylene bis (N-methyl pyrrolidinium) dibromide] with IC₅₀ of 0.64 nM against *in vitro* growth of *P. falciparum*. This compound possesses an intrinsically potent antimalarial activity both *in vitro* against *P. vinckei* in mice [28] (Table 3). G25 also has outstanding *in vivo* antimalarial activity against *P. falciparum* in Aotus monkeys with complete cure at dose as low as 0.030 mg/kg. The capacity to cure parasitemia higher than 10% was remarkable. G25 also cured Rhesus monkeys infected by *P. cynomolgi*, a primate malarial parasite, phylogenetically closely related to the human parasite *P. vivax* that also favors reticulocytes [29].

Despite their potent *in vivo* antimalarial activity after parenteral administration, bisquaternary ammonium salts present very weak oral absorption due to their permanent charge which hampers its permeability through the intestinal barrier. Since oral administration appears to be a prerequisite for a cheap widespread use in the treatment of malaria in endemic countries, the development of bioisosteric groups was necessary.

2.5. Bis Amidines and Bis Guanidines as Quaternary Ammonium Bioisosteres

To remedy the low *per os* absorption of quaternary ammonium, a second generation of compounds consisting in bioisosteric analogues (bis-amidines) was designed. These amidines and guanidines present equilibrium between protonated and unprotonated forms, and thus they can diffuse more easily through the tissues owing to the neutral form. The positive charges are strongly delocalized and, compared to similar quaternary ammonium salts, amidines and guanidines have additional binding opportunities to form hydrogen or ionic bonds. We synthesized more than 50 compounds with a C12 alkyl chain but varying the cationic head. It was striking that *in vitro* antimalarial activity was

strictly correlated to the basicity of cationic heads. This indicates that pKa values of these compounds do not indicate only the percentage of the protonated compound at physiologic pH but especially its ability to form strong bonds with the target (Calas *et al.* in preparation).

The leader compounds MS1 (Table 1), M53 and M60 (Figs. 4 and 5) exhibited as potent antimalarial activity as bisquaternary ammonium salts, with *in vitro* activities against *P. falciparum* between 0.3 and 1.3 nM. After intraperitoneal administration to *P. vinckei*-infected mice, complete cure of the malarial infection do occur with ED₅₀ lower than 3 mg/kg. Unfortunately, *in vivo* antimalarial activity after oral administration was still low, best activity being observed for compound M60 (ED₅₀ of 62 mg/kg) (Vial and Calas, in preparation; Table 3). This observation clearly indicates that their positive charges are still a drawback for the oral absorption.

2.6. Neutral Prodrugs Can Deliver Bis-Thiazolium Salts

Third generation molecules were recently designed to enhance oral bioavailability. The prodrug concept concerns any compound that undergoes biotransformation prior to exhibiting its pharmacological effects. It has been widely applied to overcome problems such as a lack of solubility, lack of bioavailability or lack of stability. It is thus of potential pharmacological interest to overcome the membrane barrier or providing a drug with a more appropriate pharmacokinetic profile. This new approach involved the synthesis of non-ionic prodrugs, which *in vivo* can lead to quaternary ammonium salts.

Our prodrug concept is based on a concept already successfully applied to administer ammonium salts in the oral mode. Indeed, thiamine (B1-vitamin) which is weakly absorbed orally (because of its permanent charge) can be delivered as a neutral thioester (acetiamine, Algo-nevriton®

Table 3. Essential Biological Parameters of Leader Compounds

Compounds	<i>In vitro</i> activity against <i>P. falciparum</i> IC ₅₀ (nM)				<i>In vivo</i> activity against <i>P. vinckei</i> (rodent model)	
	CQ sensitive		CQ resistant		ED ₅₀ ip	ED ₅₀ po
	Nigerian	3D7	FCB1	FCM29	(mg/kg)	(mg/kg)
G25	0.6	0.2	1.5	1	0.22	nd
MS1	0.3	nd	nd	nd	1.6	> 90
M53	0.6	nd	nd	nd	3.4	62
M60	1.3	nd	nd	nd	2.8	85
T3	3.0	2.3	6.3	4.7	0.2	> 10
TE3 (proT3)	2.25	4.8	3.2	5.7	0.25	5
T4	0.65	1.3	2.4	2.0	0.14	nd
TE4a (pro T4)	1.1	4.8	3.6	5.5	0.12	11
TE4c (pro T4)	1.7	nd	nd	nd	0.95	12
TE4g (pro T4)	2.5	7.0	3.2	4.5	3.4	90
Chloroquine (CQ)	20.0	20.0	160.0	400.0	1.1	3.4

ED, Efficient dose; ip., intraperitoneal; po, *per os*; nd, not determined. IC₅₀ were assessed after 48 h contact of *P. falciparum*-infected red blood cells (Nigerian strains) with the drug (Ancelin, M. L.; Calas, M.; Vidal_Sailhan, V.; Herbut, S.; Ringwald, P. *et al. Antimicrobial Agents and Chemotherapy* **2003**, *47*, 2590-2597); ED₅₀ were evaluated against *P. vinckei* in mice after intraperitoneal (ip) or oral administration (po) administration of the compounds once daily for 4 days (Ancelin, M. L.; Calas, M.; Bonhoure, A.; Herbut, S.; Vial, H. J. *Antimicrobial Agents and Chemotherapy* **2003**, *47*, 2598-2605).

or Vitanevri[®]). The bioprecursors undergo an *in vivo* rearrangement under the action of thioesterase, leading to active ionized thiamine [30, 31]. Such a strategy has been used to improve delivery of DOPA in the brain [32].

The third generation compounds are bis-thiazolium salts which are administrated as neutral prodrugs that improve the diffusion across biological membranes (Vial *et al.*, in preparation). Similarly to our previous chemical classes (see above), antimalarial activity was importantly increased by using N-duplicated molecules and best results were obtained for an alkyl chain of 12 carbon atoms (see compounds T4 and T8 in Table 2). Optimal R₂ substituent (Fig. 8) of the thiazole ring was a methyl group. R₃ substituent of the thiazole ring corresponds to a 2-hydroxyethyl (T3) or a 2-methoxyethyl (T4) groups. Bulkier R₃ groups led to a reduction in the antimalarial activity and smaller ones had no effect.

Thiazolium compounds have similar potent antimalarial activity pattern as first and second generation compounds. In this approach, IC₅₀s of our leader compounds, T3 and T4, were 2.25 and 0.65 nM respectively (Table 3). TE bioprecursors appeared to be as active *in vitro* as their corresponding drugs suggesting a quantitative prodrug/drug transformation. TE4a, TE4c and TE4g (Fig. 10), which generate T4 by hydrolysis of their thioester bonds, had an IC₅₀ in the same nanomolar range. TE3 (Fig. 11), a proT3 compound, had an IC₅₀ similar to or higher than that of T3, possibly due to a better cellular penetration of the neutral prodrug (see Table 3). Moreover, a similar compound, with a methyl group instead of RCO (TM1), which cannot undergo enzymatic cleavage leading to thiazolium, is not active until IC₅₀ > 10 μM. This result suggests that the antimalarial activity is due to the quaternary ammonium

compounds and not to the opened-ring derivatives (Vial *et al.* submitted, Table 3).

Evaluation of antimalarial activity in mice infected by *P. vinckei* parasites reveals that the drugs T3, T4 and their respective bioprecursors, TE3 and TE4a (Figs. 10 and 11), had outstanding antimalarial activity after intraperitoneal administration with ED₅₀ of 0.1-0.25 mg/kg, while TE4c and TE4g (Fig. 11), possessing an aromatic ring in the thioester substituent (R) had significant lower activity (ED₅₀ 0.95 and 3.4 mg/kg). The oral ED₅₀ of T3 could not be determined because it was higher than 10 mg/kg (Table 3).

Prodrugs had significant activity after oral administration. TE4g completely cleared the parasitemia, but the ED₅₀ was high (90mg/kg). TE4a, TE4c and TE3 had much higher activity with ED₅₀ of 11, 12 and 5 mg/kg. Importantly, all the compounds exert complete cure and no recrudescence was observed when mice were treated at 4-5 fold ED₅₀. The absolute bioavailability of the TE3 compound after oral administration to Sprague Dawley rats was 16% (Bressolle *et al.* in preparation). Preliminary toxicological tests were performed in mice to determine acute toxicity after a single administration. TE3 promises to have a very good therapeutic index, with an absence of symptoms in mice at doses of 20 mg/kg intraperitoneally and 1000 mg/kg orally.

2.7. Hypothetical Pharmacophore of Our Antimalarial Compounds

Structure-activity relationships (SAR) highlighted the essential features concerning the molecular requirements for antimalarial potency leading to a rough topographic model of the drug pharmacophore and the target binding site [26].

The huge increase in activity with the dimerization of the quaternary ammonium salts respect to the monomer congeners is in agreement with the presence of two active anionic (or electron-rich) pockets in the target able to combine a relatively reduced positively charged polar head. This feature is not unusual for bivalent ligands able to bind adjacent target proteins or subsites, regardless of whether they are identical or not.

These two active sites could be identical or distinct and located either in two regions of a single protein or on two separate protein subunits [26] (Fig. 2). It is likely that these sites could involve either carboxylate anions of Asp or Glu or aromatic rings of some aminoacids. The presence of aromatic side chains also called "aromatic basket" in the binding sites for quaternary ammonium ligands appears to be a common property shared by several acetylcholine muscarinic and nicotinic receptors and also in the potassium channels which bind tetraethylammonium [33].

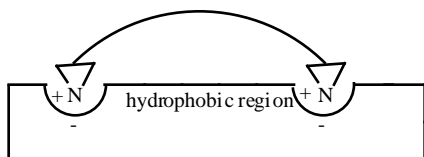


Fig. (2). Active site in the choline carrier.

Between these sites, there is a long hydrophobic domain corresponding to a length of at least 12 methylene groups, the optimal distance to allow simultaneous interaction with the two target binding sites. This is probably a neutral and purely hydrophobic domain. The carbon chains of the bis-ammonium drugs probably form hydrophobic associations with this region, while the quaternary ammonium polar heads could fit in the two anionic pockets.

3. SYNTHESIS OF THE THREE GENERATION COMPOUNDS

One of the main requirements for an antimalarial drug candidate in view of a successful development is its availability and low cost of production. Our three generation compounds are easily accessible from commercial products in a few steps. They are mostly crystalline compounds that are easily purified and thus they become good candidates for a therapeutic formulation.

3.1. First Generation Compounds: Mono and Bis Quaternary Ammonium Salts

First generation compounds most notably contain a quaternary ammonium for high antimalarial activity. We

have prepared several compounds as potential choline analogs which were active *in vitro* over the 10^{-3} - 10^{-9} M concentration range. The lead compound in the monoquaternary ammonium salt series was E13 (N, N, N-tripropyldodecan-1-ammonium bromide), ($IC_{50} = 33$ nM). G25 [1, 16-hexamethylene bis (N-methylpyrrolidinium) dibromide], ($IC_{50} = 0.64$ nM) was chosen as a lead compound for the bisquaternary ammonium salt series because of its intrinsically potent antimalarial activity, both *in vitro* and *in vivo*. E13 was obtained by reaction of 1-bromododecane and tri-*n*-propylamine (Fig. 3). G25 was prepared by reaction between 1, 12-dibromohexadecane and N-methylpyrrolidine, in the presence of NaOH.

For reasons that we will evoke in the paragraph corresponding to the biological activity of our drugs, at this point we concentrated our efforts in the development of compounds with quaternary ammonium bioisosteric groups that led to the second generation compounds.

3.2. Second Generation Compounds: Bis Amidine, Bis Guanidine and Aromatic Amidine Compounds

In the second generation two series of compounds were synthesized. M series includes bis-amidine and bis-guanidine compounds. MS series includes aromatic amidines in which the amidine function is present in the 2-imino-1, 2-dihydropyridine (Fig. 1). Bis 2-aminopyridinium salts, bis-amidines and bis-guanidines, bearing a basic function, protonated at physiological pH, are bioisosteres of quaternary ammonium salts. Their structure includes two basic head groups separated by a linker from 12 to 16 methylene groups.

In bis-amidine series, the amidine function is not conjugated, and N atoms may be substituted by R alkyl groups. M34, M53, M60 and M64 were selected as lead compounds. Preparation of M34 and M53 was performed in three steps from 1, 12-dibromododecane (Fig. 4). Substitution of bromine atoms by cyanide groups led to the corresponding bis cyanide compound which was treated with ethanol furnishing imidate intermediate M24. Reaction of M24 with ammonia and ethylenediamine led to M34 and M53 respectively.

Bis-amidines M60 ($IC_{50} = 1.3$ nM) and M64 ($IC_{50} = 2.0$ nM), with linker on the functional nitrogen atom, were prepared by reaction of 1, 12-diaminododecane with the appropriate imidate (Fig. 5).

In the bis-guanidine series M73 ($IC_{50} = 0.35$ nM) and M75 ($IC_{50} = 0.15$ nM) were the most active compounds. They were prepared by reaction of 1, 12-diaminododecane with the appropriate alkyl iminothiocarbamate (Fig. 6).

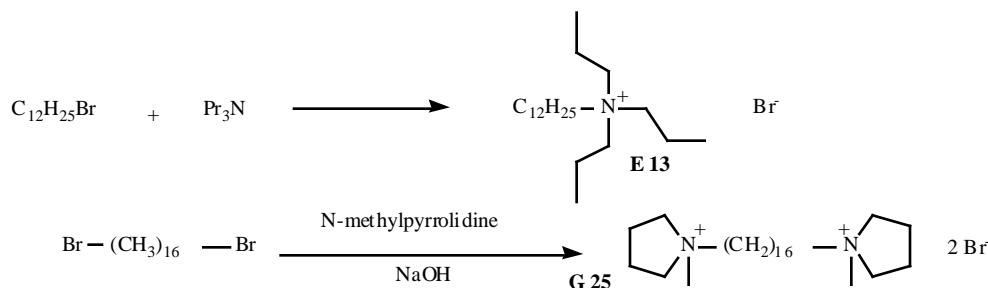


Fig. (3). Synthesis of the first generation leader compounds E13 and G25.

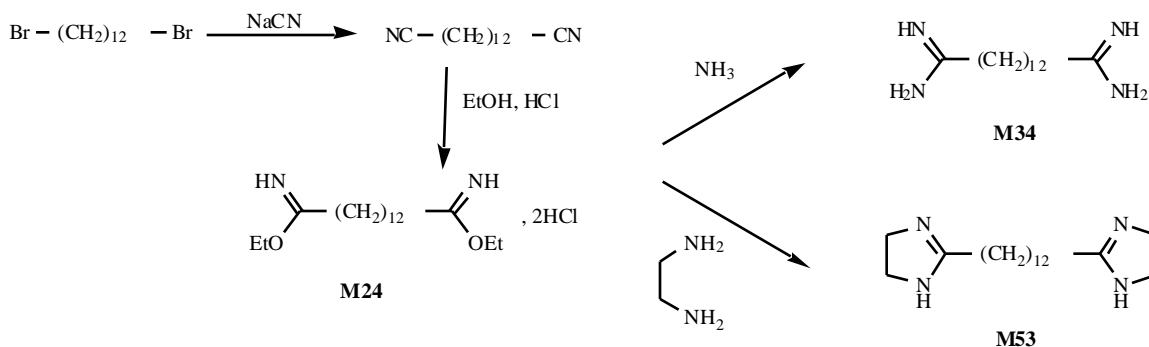


Fig. (4). Synthesis of the second generation leader compounds M34 and M53.

MS series includes aromatic amidines in which the amidine function is present in the 2-imino-1, 2-dihydropyridine group (Fig. 7). In its protonated form, this cationic head is composed of the 2-aminopyridinium cation, which is substituted by various R groups. MS1 ($IC_{50} = 0.3$ nM), the lead compound in this series, was obtained by reaction of 1, 12-dibromododecane with excess of 2-amino-4-methylpyridine.

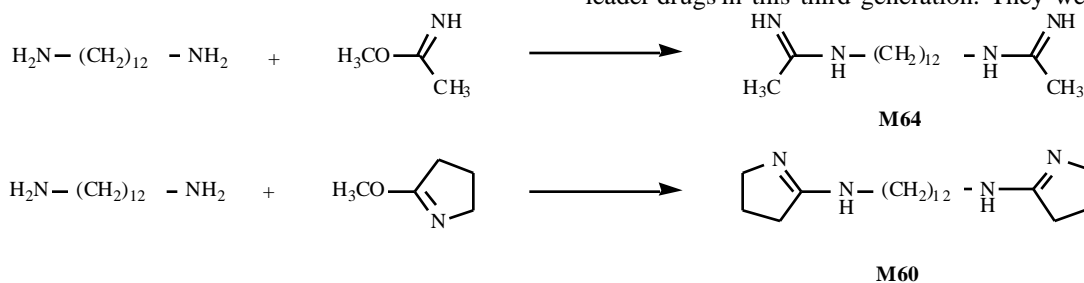


Fig. (5). Synthesis of the second generation leader compounds M60 and M64.

Then, we concentrated our efforts in the design of non-ionic compounds. Thus, a third generation of compounds arose. This approach includes the synthesis of non-charged prodrugs, which *in vivo* can lead to quaternary ammonium salts.

3.3. Third Generation Compounds: Precursors of Thiazolium Salts to Deliver Bis Quaternary Ammonium Salts as Antimalarials

In the latter context, our groups have synthesized a series of cationic choline analogous consisting of mono- and bis-thiazolium salts (T) with the corresponding neutral prodrugs as thioesters (TE). Thiazolium cycles take the place of polar

heads of first and second generation compounds, with the long lipophilic chain on nitrogen atoms. TE prodrugs rapidly permeate the red blood cell membranes and react instantaneously with esterases to release the corresponding drugs (T) that are thus trapped in the erythrocytes (Fig. 8).

Among the 16 thiazolium drugs that we have prepared (Vial *et al.* in preparation), T3 and T4 are considered our leader drugs in this third generation. They were obtained in

good yields in only one step by alkylating the nitrogen of the corresponding thiazole cycle with 1, 12-dibromododecane in refluxing acetonitrile (Fig. 9). In parallel, we have prepared the corresponding mono-quaternary thiazolium salts T1 and T2 by alkylation of the corresponding thiazole cycles with 1-bromododecane.

Then, our efforts were oriented to the synthesis of TE prodrugs with different substituents in order to modify the physicochemical properties and tolerance.

T4 Prodrugs

T4 thioester prodrugs (TE series) were then prepared. We hoped that a judicious choice of the thioester substituent

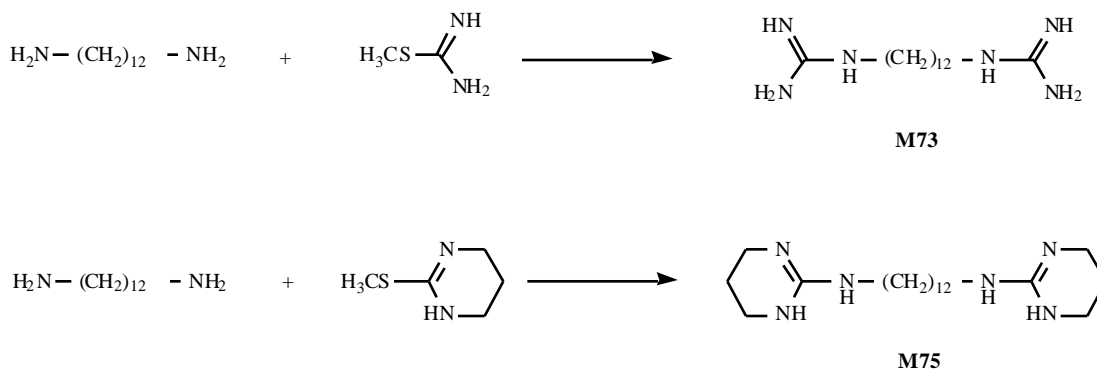


Fig. (6). Synthesis of the second generation leader compounds M73 and M75

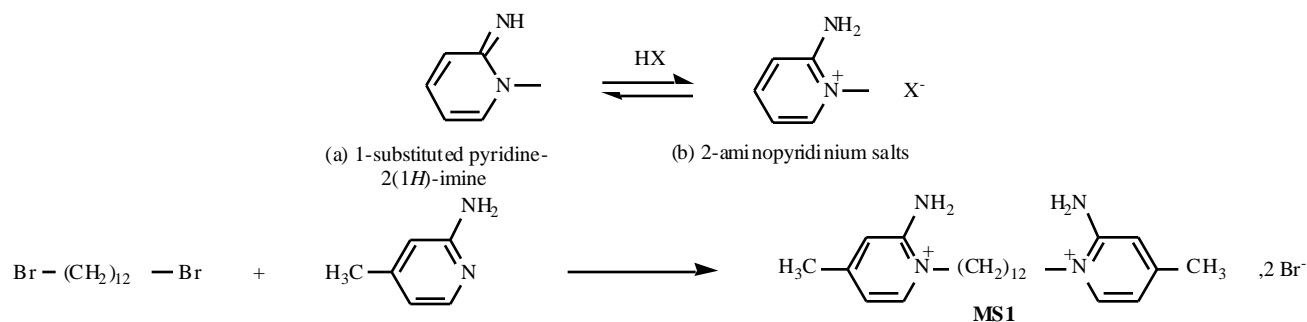


Fig. (7). Synthesis of the second generation leader compound MS1.

could control the prodrug-drug conversion rate, the oral absorption and solubility in water.

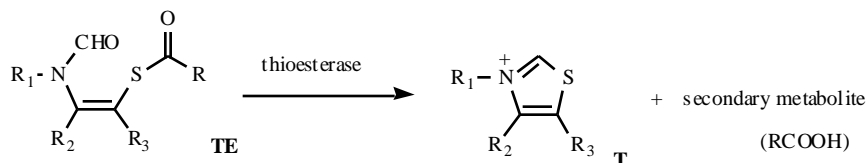


Fig. (8). Prodrug-Drug biotransformation.

An important factor in oral absorption is molecular weight [34]. We tried to get low molecular weight products by reducing the spacer length between the two thiazolium heads but we observed a loss in activity. Thus, we varied the thioester moiety (R, Fig. 10) introducing small substituents. For this proposal we prepared TE4o (R = Me). To reduce the prodrug-drug conversion rate slowing down the thioester hydrolysis, bulky esters (R = *i*Pr, *t*Bu, Ph) were introduced giving TE4a, TE4b, TE4c prodrugs (Fig. 10). Salifiable

prodrugs as TE4g were also synthesized to improve compound solubility in water.

For the synthesis of TE4 prodrugs, we proceeded adding the appropriate acyloyl or aryloyl chloride to a strongly alkaline solution of T4 obtaining the corresponding S-acylated products (Fig. 10).

T3 prodrug

In the context of the search of low molecular weight products we designed the synthesis of a T3 thioester prodrug: TE3. In this molecule, the thioester moiety is the

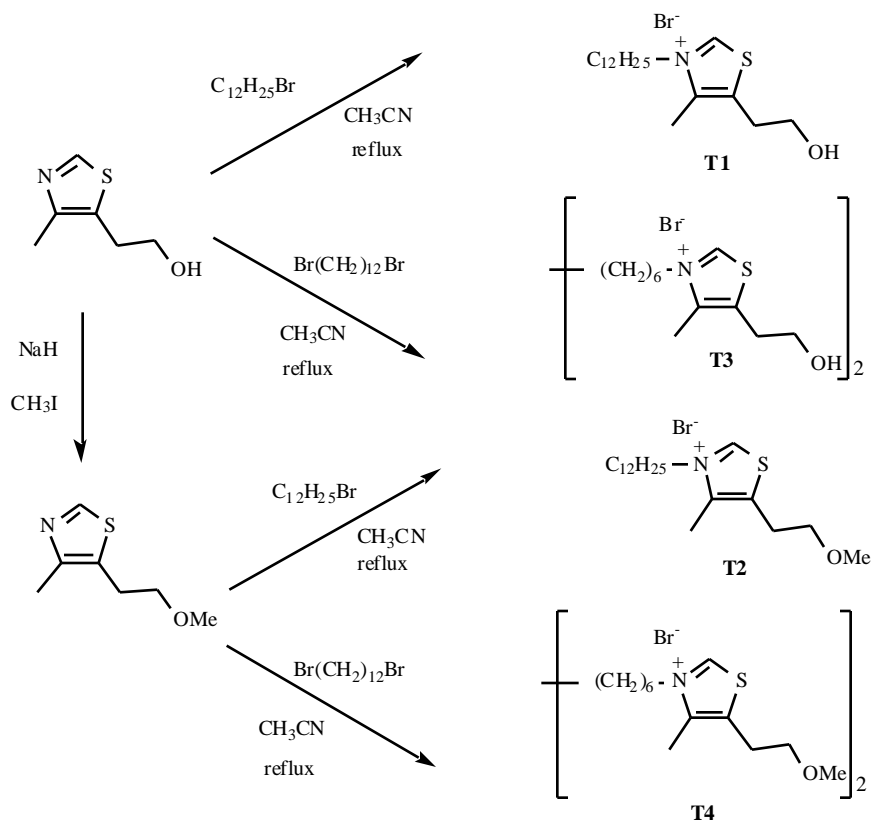


Fig. (9). Synthesis of the third generation compounds T1, T3, T2 and T4.

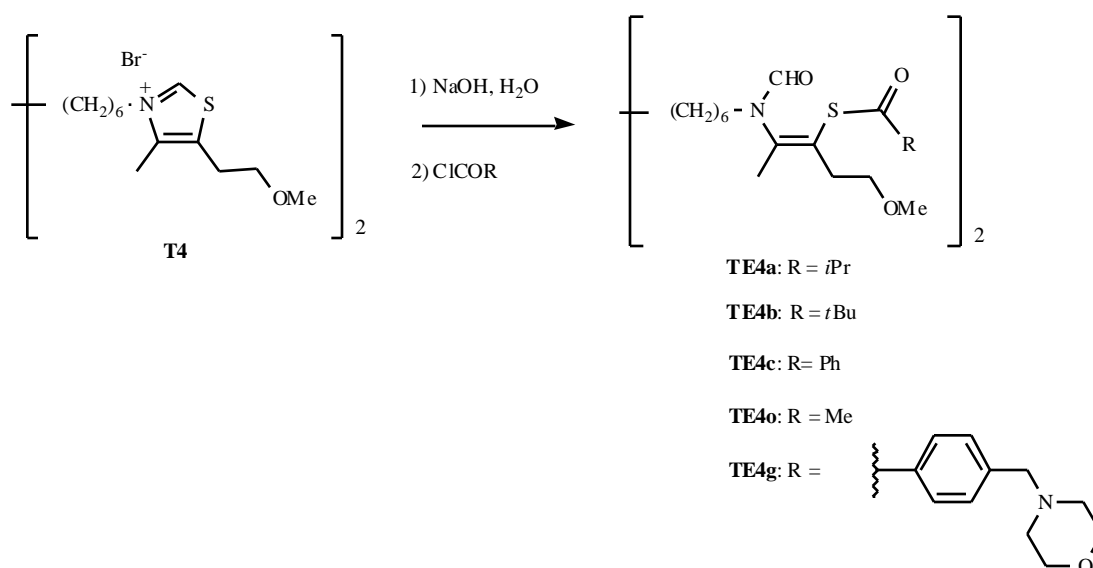


Fig. (10). Synthesis of the third generation TE4 prodrugs.

R₃ substituent of the thiazole ring, avoiding the introduction of additional groups. This derivative is nowadays our leader compound in the third generation. TE3 was obtained one pot from T3. Thus, reaction of T3 alkaline solution with *p*-nitrophenylchloroformate led to the corresponding reactive thioester, which gave, by cyclisation with the hydroxyl group, the prodrug TE3 (Fig. 11).

4. MECHANISM BY WHICH COMPOUNDS EXERT THEIR ANTIMALARIAL ACTIVITY

4.1. Drugs Transport to the Intracellular Parasites

Structural requirements reported for all choline carriers attest that the endogenous choline carrier of erythrocytes would not be able to transport drugs inside the infected erythrocyte. We further evidence this fact by showing that T16, a third generation radiolabeled compound (very similar to T3 and T4) [35], is not transported by the dedicated choline transporter of yeast [36]. On the other hand, biscations [37] including T16 are substrates of the new parasite-induced permeation pathway (NPP), a parasite-induced transport process located on the erythrocytic plasma membrane. The NPP may mediate at least partially the entry of these drugs inside the infected erythrocytes. Then the compounds have to enter the intracellular parasite. We have just demonstrated that the major route of entry of these drugs into the intracellular parasite likely is a poly-specific

organic cation transporter functionally distinct from the known dedicated eukaryotic choline carriers [38].

4.2. Drugs are Naturally Targeted to The Infected Erythrocyte

One prominent feature of bis-quaternary ammoniums is their ability to accumulate by several hundred-fold in the malaria-infected compared to unparasitized erythrocytes. This was observed both with VB5, a tritium-labelled bis-quaternary ammonium salt analog of G25 [29], and with T16. On the other hand, compounds were significantly accumulated neither in uninfected erythrocytes nor in human Jurkat lymphoblasts, indicating that the process causing drug accumulation is linked to the intracellular parasite. The accumulated drug was in part recovered in the *Plasmodium* food vacuole, where T16 was found to associate with heme; finally, this accumulation was shown to be critical for drug accumulation (see below).

4.3. Drugs Specifically Inhibit *De Novo* Plasmodial PC Biosynthesis

Concerning their toxic effect against the malarial parasite, mono quaternary ammonium [25], bisquaternary ammonium [27] and bisthiazolium salts (Vial *et al.* in preparation) exert an early alterations of PC biosynthesis decreasing the choline incorporation into the final product PC. At the same time, compounds have no effect on the biosynthesis of other

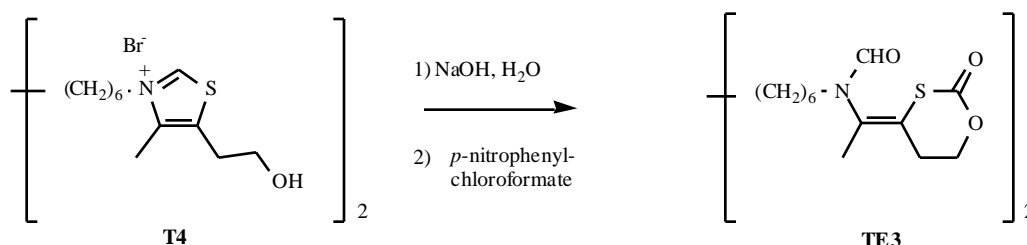


Fig. (11). Synthesis of TE3 prodrug, the third generation lead compound.

macromolecules (including synthesis of others phospholipids). A strong correlation has been demonstrated between the inhibition of the PC biosynthesis (PC₅₀) and antimalarial activity (IC₅₀) [25]. The compounds appear more active against the mature parasites i.e. the most intense phase of PL biosynthesis during the erythrocytic cycle [39].

The mechanisms by which the inhibition of the *de novo* pathway occurs still remain to be fully elucidated. Alkyltrimethyl ammonium salts in which the saturated alkyl chain had from 10 to 16 carbons competitively inhibit choline transport into *P. knowlesi*-infected erythrocyte suspensions. The K_i values were in the very low micro molar range (3 to 8 μM) and closely agreed with previously reported IC₅₀ and PC₅₀ values [25]. Bis-quaternary ammonium compounds likely also interfere with *in vitro* *P. falciparum* growth by affecting its essential *de novo* PC biosynthesis. They selectively blocked *de novo* PC biosynthesis and G25 impaired choline transport into infected erythrocytes. It is worth to note that the bis quaternary G25 inhibits choline transport into *P. knowlesi*-infected erythrocytes but the K_i of 0.4 μM which is similar to the PC₅₀ but far higher than the IC₅₀. These experiments were performed under conditions of short duration and high hematocrite, allowing macromolecule biosynthesis to be evaluated independently of the death of the parasite [27].

The Ward's group in Liverpool has recently characterized an organic cation transporter that mediates the choline entry inside *P. falciparum* parasite. T16, potently inhibits parasite choline uptake with an IC₅₀ of 140 nM. Considering that the drug antimalarial activity relies primarily on their accumulation to high levels in the infected red blood cells, and that compound accumulates in a significant way in the host cell compartment of the infected cell, drugs have likely reached a significant higher concentration critical for the choline entry inside the parasite. Interestingly, increasing the choline concentration in the medium causes a dose-dependent inhibition of T16 uptake that is directly proportional to the inhibition of antimalarial activity [38]. Finally, Ben Mamoun's group (Connecticut, USA) uses the yeast *S. cerevisiae* as a surrogate system to identify the targets of our antimalarial compounds. It was striking that G25 relies for its activity on a functional phosphatidylcholine synthetic machinery as also on a functional PS decarboxylase PSD1 [36]. However, we cannot be sure that antimalarial activity is solely due to the ability of choline analogs to inhibit choline entry into Plasmodium-infected erythrocyte and we cannot at this stage exclude additional inhibition by this compound of the later steps of the CDP-choline pathway.

4.4. Compounds do Interact with Plasmodial Hemoglobin Degradation Metabolite in the Food Vacuoles

P. falciparum has a life cycle divided into three overall stages (mosquito, liver, and blood stages). During the blood stage, the parasite digests haemoglobin to obtain the amino acids it requires. This reaction produces free heme rapidly oxidized into ferriprotoporphyrin IX (FPIX), which is toxic for the parasite. Detoxification of heme occurs in the acidic food vacuole of the parasite via formation of a polymer called hemozoin or malaria pigment [40, 41].

We showed that, in addition to their selective inhibition of *de novo* phosphatidylcholine biosynthesis, the potent antimalarial activity of bis-quaternary ammonium compounds could also be attributed to their compartmentalization into the parasite's food vacuole, where they would bind to ferriprotoporphyrin IX blocking the formation of malaria pigment (hemozoin). Importantly, the use of a protease inhibitor that prevent haemoglobin degradation and free-heme formation, indicates that the binding to ferriprotoporphyrin IX or to hemozoin in the parasite makes a significant contribution to both the accumulation and the antimalarial activity of these dual compounds [35].

5. CHOLINE ANALOGS VERSUS CHOLINERGIC EFFECTS

A crucial problem in drug development is the specificity required to achieve an acceptable therapeutic index. A potential problem with choline analogs concerns involvement of choline as precursor of the neurotransmitter acetylcholine. Thus, quaternary ammonium structure is also probably responsible for the relatively high toxicity of the first generation compounds.

Choline entry in the infected erythrocyte was thus further characterized to identify some specific features [42], notably in comparison with the nervous system where choline is also substrate for various enzymatic reactions [43].

Choline transport into normal erythrocytes does not exhibit Na⁺ requirements. In synaptosomes, choline can be transported by Na⁺ dependent high affinity choline transport (HACT) with a K_t for choline of 1.6 μM, or by low affinity choline transport (LACT) with a K_t for choline of 144 μM. LACT provides choline for phospholipid biosynthesis and is Na⁺ independent [44]. Table 4 shows that choline entry into *Plasmodium*-infected erythrocytes is mediated by carrier(s) that can be distinguished from the choline carrier(s) involved in acetylcholine biosynthesis. HACT was demonstrated to be stereospecific for choline analogues, such as - or - methylcholine. Indeed, the high affinity choline carrier that mediates choline entry into synaptosomes (HACT) has clear stereospecificity, either with or analogs, with the following extents of activity: R(+) > S(-) (by 8-fold) and S(+) > R(-) (by 6-fold). By contrast, for normal and infected erythrocytes, irrespective of the analogue (or) no significant difference was noted in the IC₅₀ (< 2.5x).

The absence of a plateau for antimalarial activity inhibition as a function of the polymethylene chain length of bisammonium cells (up to 16) strongly contrasts with the patterns noted for nicotinic receptors or HACT in synaptosomes, for which inhibition was maximal for 8-12 and 16-18 methylenes, respectively [45, 46]. This indicates that in both cases (nicotinic receptor and HACT), the linker distance is much shorter than in infected erythrocytes. Besides, the cholinergic compound hemicholinium (HC3) showed a 75-fold lower IC₅₀ in synaptosomes than the first generation bisquaternary compound G3 [25] (decamethonium bromide) while their activities of both of them against *P. falciparum* are very close.

The marked differences in response, in terms of pattern and relative affinity as a function of polymethylene chain

Table 4. Effects of Enantiomers of (+) and (-) Methylcholine on the Choline Carrier of Normal (NE) or *P. Knowlesi*-Infected (IE) Erythrocytes and Rat Cortical Synaptosomes (HACT)

Methylcholine	HACT		NE	IE
	IC ₅₀ (μM)	Ki(μM)	IC ₅₀ (μM)	IC ₅₀ (μM)
R(+)	6.4	7.5	420	320
S(-)	52	47.1	1000	620
S(+)	32	32.3	44	110
R(-)	190	190.3	20	50

See Vial, H. J.; Eldin, P.; Martin, D.; Gannoun, L.; Calas, M. *et al. novartis found symp* **1999**, 226, 74-83; discussion 83-78

length and of steric fit at the active site, relative to the nitrogen substitution bulk between antimalarial effect and other cholinergic models (e.g. HACT, nicotinic or muscarinic cholinergic receptor, cholinesterase, etc) reflect considerable differences in these targets. This strongly suggests the possible discrimination between the antimalarial activity of these choline analogues and their toxic effect which is mainly mediated via their cholinergic effect on the central nervous system.

6. PENTAMIDINE-LIKE COMPOUNDS EXERT DIFFERENT ANTIMALARIAL MECHANISMS

Members of other bis cationic molecules (**I**, **IIa**, **IIb**, Fig. 12) have been shown to have significant *in vivo* activity against *Plasmodium falciparum*. Thus, the diamidine furamide [2, 5-bis(4-amidinophenyl)-furan] (**I**, IC₅₀ = 15.5 nM) has been reported recently as an excellent lead for antimalarial drug discovery [47]. Pentamidine congeners (**IIa**, IC₅₀ = 19 nM, **IIb**, IC₅₀ = 4 nM) have also been studied extensively for their inhibition of malaria parasite growth [48]. As furamide (**I**), these compounds are aromatic diamidines, but they possess a flexible aliphatic linker. The piperazine-linked bisbenzamidine [49] (**IIb**) emerged as a highly potent lead with an activity profile superior to pentamidine (**IIa**).

Plausible mechanisms of action of these bioactive bisbenzamidines 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 furamide (**I**), a rigid, curved and planar molecule what confers it the appropriate steric and electrostatic properties to show high-affinity binding to the minor groove of DNA. For pentamidine congeners **IIa** and **IIb**, however, T. L. Huang *et coll.* [49] have reported recently that the DNA binding of benzamidine analogs did not correlate with their antiplasmodial activities (IC₅₀ values). Finally, benzamidines were examined and they were proved, to form complexes with ferriprotoporphyrin IX and therefore to be involved in the inhibition of hemozoin formation in cell-free system [50].

Pentamidine and pentamidine-like compounds possess a common framework with our three generation drugs. They are constituted by two heads, each head with two different pharmacophore groups (see Fig. 13): an ammonium group and a π-electron rich site (benzamidine or a thiazolium cycle), which is only absent in our first generation compounds. Both active heads are separated by a linker. But there is no indication for an interaction of pentamidine-like biscations with the plasmodial phospholipid biosynthesis. In the last years, we have observed that attempts to rigidify the linker of our bis-ammonium salts or to introduce heteroatoms and organic functions induced a decreased antimalarial activity [24, 26]. Thus, the lack of interaction with the choline carrier in pentamidine analogues, would be due to the presence of aromatic rings and heteroatoms (O in pentamidine **IIa** and N in **IIb**) in the linker. These structure features would generate repulsions with the hydrophobic region of the choline carrier.

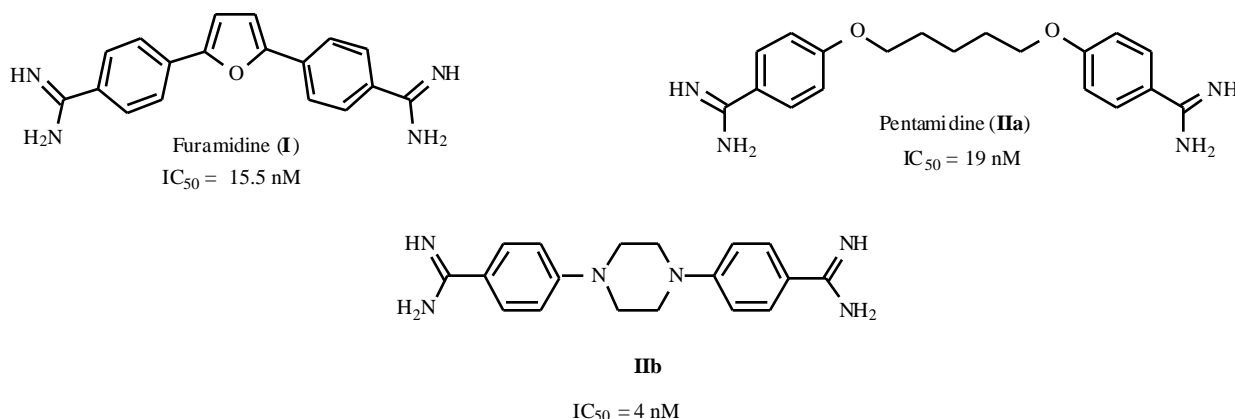


Fig. (12). Furamide and pentamidine analogues.

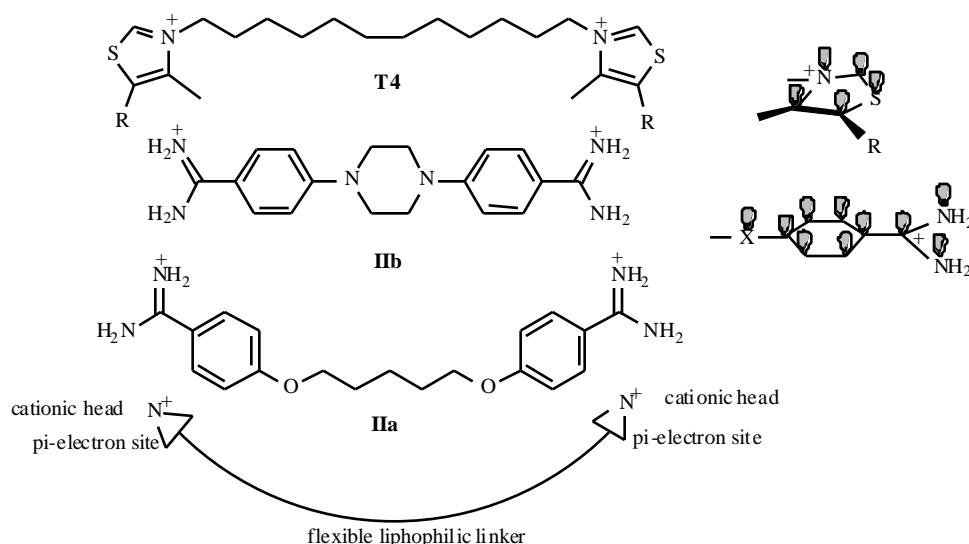


Fig. (13). Common framework between pentamidine-like compounds and our drugs.

Our antimalarial compounds [29, 35] as pentamidine-like biscations [37] bind to ferriprotoporphyrin IX (FPIX). Ferriprotoporphyrin IX possesses two propionate side chains. Linker flexibility in bis cationic drugs could allow the formation of a kind of sandwich-type structure 1:1 (**a**) or 2:1 (**b**) heme-drug [50] which would block FPIX unities avoiding their polymerisation (Fig. 14). Thus, the mechanism of the interaction of antimalarial bis cationic agents with FPIX could involve an ionic interaction between the positive charged ammonium heads of the drug and the heme carboxylates of the target. A second interaction could also be envisaged: formation of a π -complex between drugs and FPIX could increase the interaction drug-target,

analogously to the classical antimalarial quinolines, quinine and chloroquine, which are believed to act via complexation to heme by π -stacking of the coplanar aromatic molecules [51]. An analogous interaction model has been proposed to explain the heme binding affinity of xanthenes, which also exhibit antimalarial activity [52].

Our three generation bis cationic compounds inhibit *de novo* phosphatidylcholine biosynthesis and bind to ferriprotoporphyrin IX. The biological activity observed is the addition of both mechanisms of action. In the case of the first generation, where the π -electron rich site is absent, just the ionic interaction could be responsible of the ferriprotoporphyrin IX binding. Following this assumption,

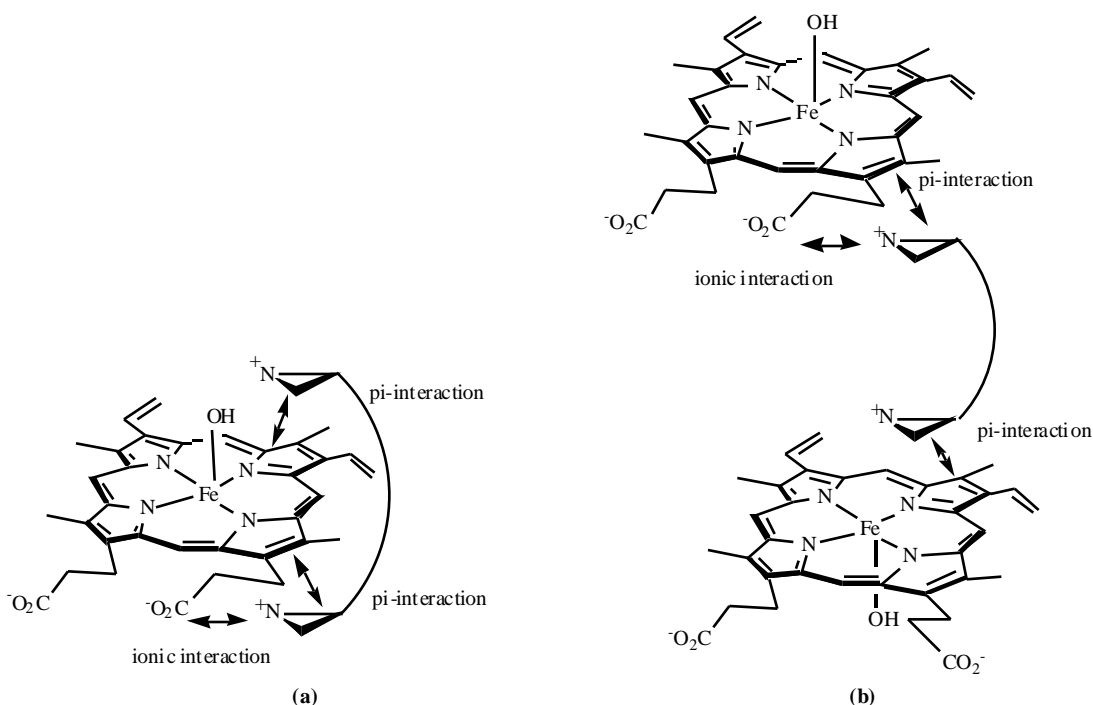


Fig. (14). Hypothetical interaction mechanism between drugs and FPIX. Two sandwich structures with a 1:1 (a) or 2:1 (b) heme-drug stoichiometry are possible.

they would be active mainly by the choline transport mechanism. Second and third generation compounds would strongly interact with both targets since they are capable of giving ionic and π - π interactions.

7. CONCLUSION

Our pharmacological approach is based on the capacity for the compounds to mimic choline and, then, to competitively interact with effectors (transporters or enzymes) that deal with choline-motif containing ligands. This includes the choline carrier, thus inhibiting choline transport within infected red cells, but also possibly enzymes of the *de novo* PC biosynthesis. The target recognizes and interacts with choline or its analogue by electrostatic attraction. In terms of molecular recognition, the competitor must possess a positively charged moiety to bind to the same site as choline. Our compounds also accumulate to high levels in the intracellular parasite where they bind to ferriprotoporphyrin IX, a plasmodial haemoglobin degradation product, which make a significant contribution to both the accumulation and the antimalarial activity of these compounds. Thus, there is evidence that this class of compounds has a dual mode of action on the intracellular parasite, affecting both phosphatidylcholine biosynthetic activity and interacting with the toxic parasite haemoglobin metabolites. This dual activity is unique compared with the other current antimalarial compounds.

Our three generations of compounds are available in few steps from commercial products. They are inexpensive to produce and stable. Most of them are crystalline and therefore adapted for an oral administration.

Both, drugs and prodrugs, had very potent antimalarial activity *in vivo*. Evaluation against the lethal rodent malaria, *P. vinckei*, revealed ED₅₀s as low as 0.1 mg/kg after intraperitoneal administration and complete cure without recrudescence at doses lower than 1 mg/kg; the ED₅₀ proved to be a function of the treatment duration. Use of prodrugs to deliver bithiazolium compounds demonstrated complete protection in murine models, even at high initial parasitemia or using short-course treatment. Moreover, and very significantly, these compounds provided complete cure of *P. cynomolgi* infection and recrudescence in rhesus monkeys, showing valuable pharmacokinetic properties. Efficacy and tolerance studies in mice and primates are very promising, indicating that this approach could be applicable for human infection; detailed toxicological studies are now under way. This class of compounds appears around 10-fold more active than chloroquine [53, 54] or artemisinin derivatives [55, 56] and as active as atovaquone [53] in the rodent model. To our knowledge, this class of compounds appears to represent the most potent antimalarial in the rodent model tested to date. We are also conducting detailed studies to unravel the unique dual aspect of their mechanisms of action.

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ABBREVIATIONS

ED ₅₀	= Efficient dose inhibiting by 50% the parasitemia
FPIX	= Ferriprotoporphyrin IX
HACT	= High affinity choline transport, IC ₅₀
LACT	= Low affinity choline transport
PC	= Phosphatidylcholine
PL	= Phospholipids
T	= Thiazolium salt
TE	= Thioester prodrug

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