Synthesis and Effects on Chloroquine Susceptibility in *Plasmodium falciparum* of a Series of New Dihydroanthracene Derivatives

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To suggest a mechanism of action for drugs capable to reverse the chloroquine resistance, a new set of 9,10-dihydro-9,10-ethano and ethenoanthracene derivatives was synthesized and compounds were tested with the aim to assess their effect on chloroquine susceptibility in *Plasmodium falciparum* resistant strains. With respect to this, reversal of resistance and change in drug accumulation were compared. Structure−activity relationship and molecular modeling studies made it possible to define a pharmacophoric moiety for reversal agents and to propose a putative model of interaction with some selected amino acids.

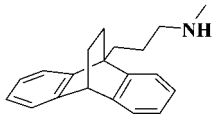
Introduction

Plasmodium falciparum is responsible for the most dramatic consequences of malaria. Chloroquine (CQ) has been one of the most successful antimalarial agents ever developed because of its low cost and high efficacy. Unfortunately, the emergence in the late 1950s and fast spread of resistant strains greatly reduced clinical use of this drug. Thus, contrary to the optimistic forecasts given in the 1960s, increasing prevalence of malaria—40% of the world's population and over one million deaths each year[1]—and difficulties encountered in the efforts to produce vaccines gave interest in the search of compounds capable to reverse resistance of the parasite.

With respect to this, it has been observed that CQ resistant *P. falciparum* strains exhibit reduced drug accumulation as compared with the susceptible ones[2] and that CQ efficiency can be restored when structurally different compounds, known as chemosensitizers, are added to the drug regimen.

Therefore, one possible strategy to counteract the CQ resistance is to potentiate CQ effects by compounds with weak antimalarial activity. Referring to this, several compounds,[2] like verapamil,[3] desipramine,[4] and several antihistamine drugs,[5] demonstrated the promising capability to reverse in vitro CQ resistance in parasite isolates, in animal models,[6,7] and in human infections[8] as well but with side effects.

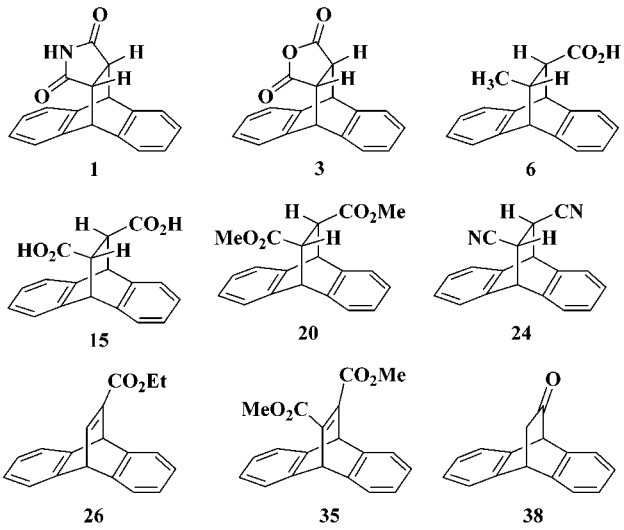
As the 9-γ-methylaminopropyl-9,10-dihydro-9,10-ethanoanthracene (maprotiline) has shown anti-MDR activity on cancer cells[9] and *P. falciparum*,[4] a new set of derivatives with the 9,10-dihydro-9,10-ethano or ethenoanthracene structure (DEEA) was prepared and compounds were tested as chemosensitizers against CQ resistant *P. falciparum* strains; all the more, the semirigid structure of these derivatives could be very convenient for stucture−activity relationship (SAR) mapping. The influence of these compounds on CQ accumulation was also evaluated, and finally, a molecular modeling study allowed us to suggest a possible mechanism of action of the reversal agents on CQ resistance.



Maprotiline

Chemistry

The DEEA derivatives were prepared by the general routes given in Schemes 1 and 2. The starting ethanoanthracene compounds **1**[10] and **3**[11] show an *RS* configuration whereas starting compounds **6**,[12] **15**,[13] **20**,[14] and **24**[10,15] show the *RR-SS* one. Starting compounds **26**, **35**, and **38** are described in the literature.[16−18]



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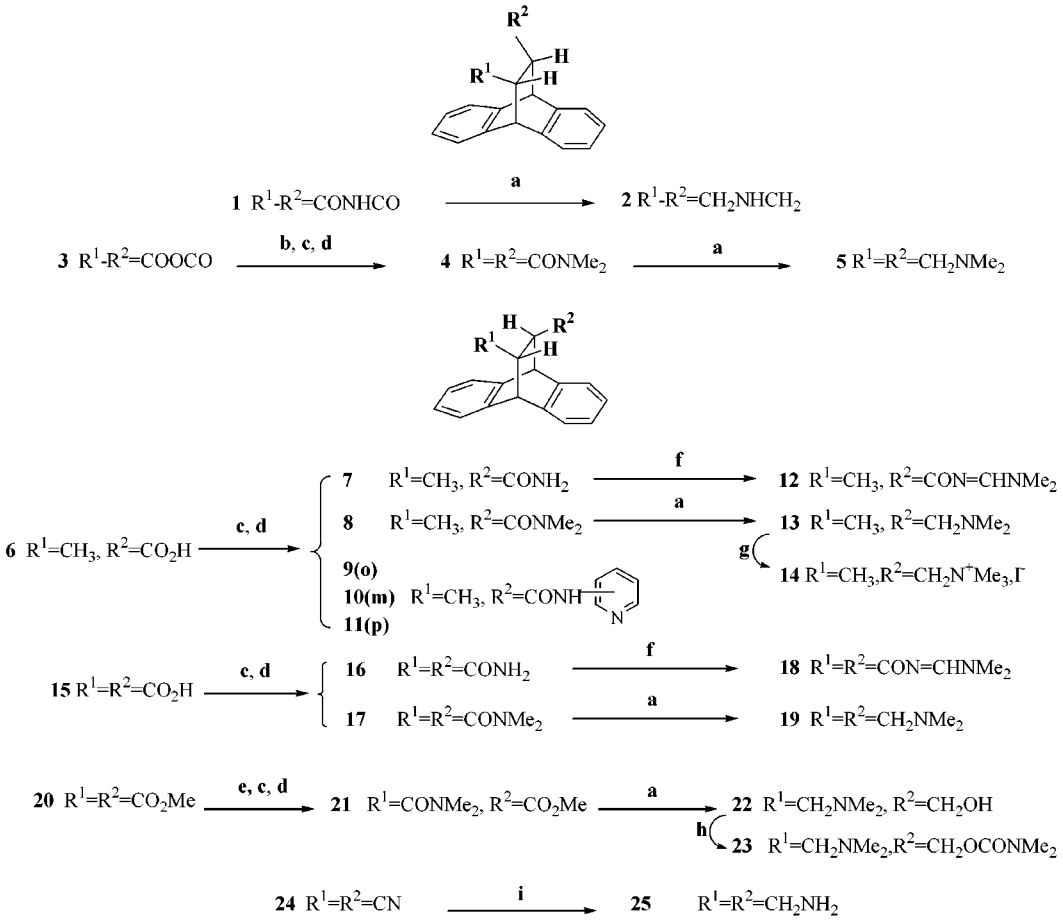
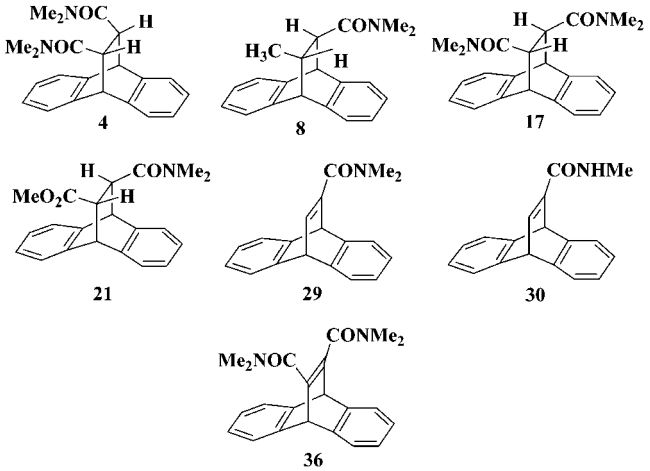
Scheme 1[a]

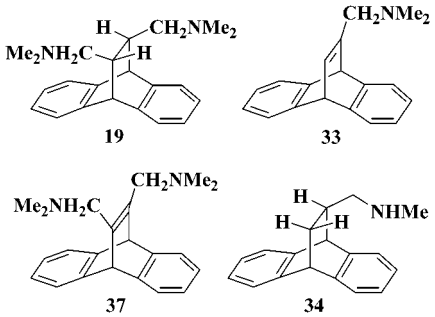


[a] Reagents: (a) $LiAlH_4$, THF; (b) KOH, H_2O; (c) $SOCl_2$; (d) (*o*,*m*,*p*)-aminopyridine, CH_2Cl_2 or Me_2NH, THF or NH_4OH, THF; (e) KOH, MeOH, Et_2O; (f) DMF, DMA; (g) CH_3I, $KHCO_3$, MeOH; (h) NaH, Me_2NCOCl, THF; (i) AlH_3, THF.

The **4**, **8**, **17**, **21**, **29**, **30**, and **36** amides were prepared by condensation of commercially available methylamine or dimethylamine in tetrahydrofuran (THF) on the acid chloride intermediates of the corresponding acids.[12−14,16,17,19]

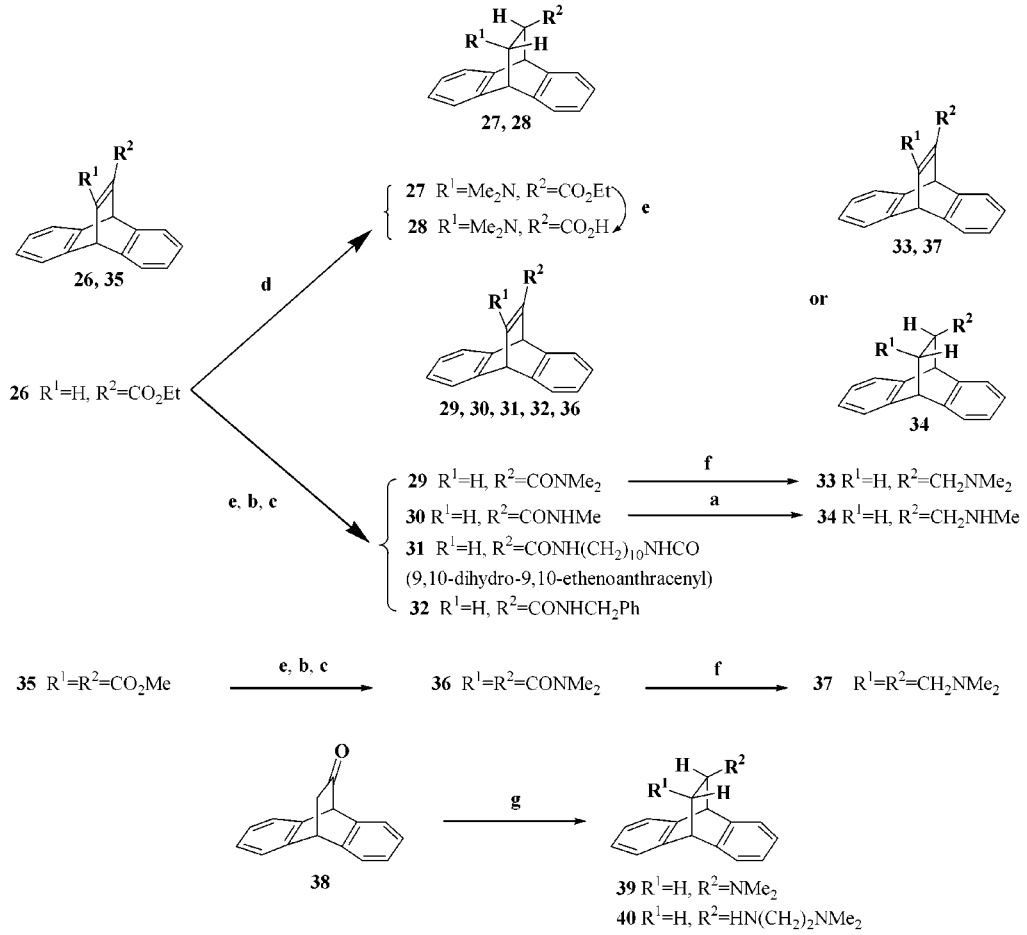


According to Huebner,[16] the reduction of the 9,10-dihydro-9,10-ethenoanthracene amide derivative **29** by $LiAlH_4$ was supposed to lead to the corresponding amine **33**. Yet, in doing that, only a mixture of ethano and ethenoanthracene derivatives was obtained, while the use of AlH_3 led to the pure expected compound. The same method was used to obtain **37** from **36**. In contrast, secondary amide **30** was completely reduced by $LiAlH_4$ in the amino derivative **34**.



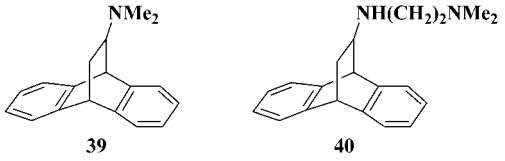
Separation of the two diastereoisomers of amine **19** was achieved by resolution of the brucine salt of the corresponding carboxylic acid **15**.[14] The *RR* and *SS* carboxylic acid previously obtained gave the analogous amides, which were reduced into the *RR* and *SS* amine derivatives **19** as detailed in the Experimental Section. Amine derivatives **39** and **40** were obtained from the ketone **38** by reductive amination with the

Scheme 2[a]

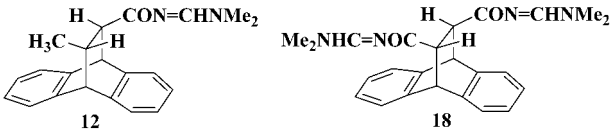


[a] Reagents: (a) $AlLiH_4$, THF; (b) $SOCl_2$; (c) $H_2N(CH_2)_{10}NH_2$, TEA, CH_2Cl_2, or $PhCH_2NH_2$, CH_2Cl_2 or $MeNH_2$, THF or Me_2NH, THF; (d) Me_2NH, EtOH, CH_2Cl_2; (e) NaOH, MeOH, H_2O; (f) AlH_3, THF; (g) Me_2NH, HCl or $Me_2N(CH_2)_2NH_2$, HCl, $NaBH_3CN$, MeOH.

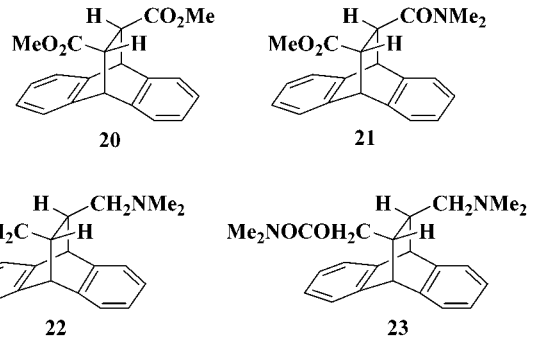
corresponding amine hydrochloride and $NaBH_3CN$.[20]



Carbamidines **12** and **18** were prepared by treating primary amides **7** and **16** with *N*,*N*-dimethylformamide dimethyl acetal.[21]



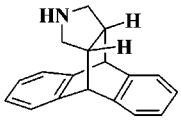
To obtain the nonsymmetrical molecules **21**−**23**, the 9,10-dihydro-9,10-ethanoanthracene-11-carboxy-12-carbomethoxy intermediate was prepared by a controlled saponification of compound **20** with KOH in methanol and diethyl ether at room temperature. The products obtained were separated by successive extractions at different pH values. Compounds **21** and **22** were then prepared with the general procedure detailed in the Experimental Section. The *N*,*N*-dimethylcarbamate derivative **23** was prepared from alcohol **22** by adding *N*,*N*-dimethylcarbamoyl chloride to the corresponding sodium alkoxide.[22]



Results and Discussion

Biological Evaluation. The activities of CQ, verapamil, promethazine, mecamylamine, and DEEA compounds were evaluated in vitro against multidrug resistant Indochina clone W2 of *P. falciparum*. The activity of each compound was expressed (i) by antimalarial activity IC_{50}, e.g., concentration value that leads to a 50% decrease of the parasite growth; (ii) by reversal activity IC_{50}, e.g., concentration value that leads to a 50% decrease of the IC_{50} of CQ; (iii) by reversal percentage at 1 μM concentration of the tested compounds; and (iv) by the values of the CQ accumulation ratio. Hemol-

ysis and cytotoxicity were also evaluated, respectively, on uninfected erythrocytes and on Chinese hamster ovary (CHO) cells. Promethazine and verapamil were selected as reference chemosensitizers. The results are summarized in Table 1.



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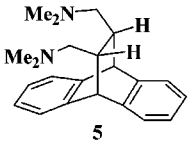

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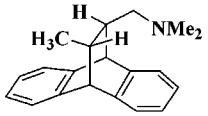

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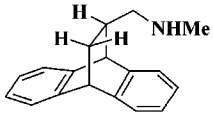

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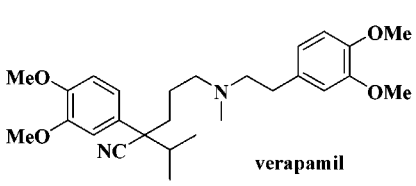
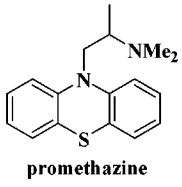


verapamil

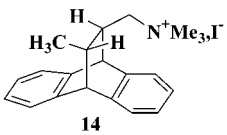


promethazine

Reversal Activity (IC_{50}). Products **2**, **5**, and **34** are more potent than verapamil and promethazine. Two groups of products (**13** and **19** and **37**, **39**, and **40)** are as effective as promethazine and verapamil, respectively. Compounds **22** and **33** have intermediate activity. Except for **13**, **34**, and **40**, potent compounds are less cytotoxic than the reference chemosensitizers.

The reversal activity is under the influence of several features of the synthesized compounds: the presence of at least one amino group, the nature of the group associated with the amino one in case of disubstituted molecules, the length of the chain carrying the functions, and the presence of an ethano bridge vs that of an etheno bridge.

The presence of the amino group suggests that the reversal activity of **2**, **5**, **13**, **19**, **22**, **33**, **37**, **39**, **40**, verapamil, and promethazine depends on their ability to have a protonatable nitrogen at physiological pH and therefore on their pK_a value, which is contained between 10.18 and 8.04 (theoretical values) whereas the pK_a values of the other tested compounds are weaker (Table 2). Substitution of the amine group is also a determining factor. Actually, secondary amines **2** and **34** and tertiary amines **13**, **19**, **33**, **37**, and **39** are more active than the primary **25** and quaternary **14** ones.



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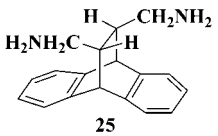
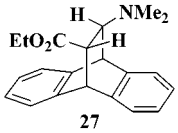


25

For compound **25**, weak lipophilicity can explain the decrease in potency. Indeed, the calculated log*D* values of the potentiating agents are ranged between −1.50 and 2.61 whereas **25** has a log*D* value lower than −1.5 (Table 2). The lower activity of compound **14** is probably due to the charged entity of the quaternary ammonium group, which could be detrimental to cross membranes. Moreover, the reduced activity of **22**, **23**, and **27** and the inefficacy of **28** can be explained by the effect of the function associated with the amino chain.



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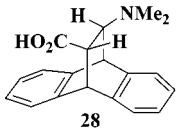


28

Indeed, these functional groups can be ranked in a decreasing order of activity as: amine (**5**, **19**) > hydroxyl (**22**) > carbamate (**23**) > carboethoxy (**27**) > carboxylic acid (**28**).

The double bond in the ethenoanthracene derivatives **33** and **37** vs the ethanoanthracene derivatives **13** and **19** decreases the potentiating activity. The distance of the amino group from the bridge is also a determining factor since **13** or **34** are better than **39** or **40**. There is no difference in efficacy with diastereoisomers *RR* and *SS* of compound **19** and no dramatic changes in potency comparing compounds **5** and **19**. This is in agreement with the nonstereo-dependent activity of verapamil.[23] The lack of aromatic rings drastically decreases activity as shown by the IC_{50} value of mecamylamine. Finally, **2**, **19**, **22**, and **34** are as potent as verapamil and/or promethazine on three other resistant strains of *P. falciparum* (Palo Alto from Uganda; FCR3 from Gambia; Bres 1 from Brazil), which confirms efficacy of these compounds against CQ resistance.[24]

Influence on CQ Accumulation. The in vitro increase of CQ accumulation by compounds synthesized was evaluated on the same clone W2 of *P. falciparum*. Accumulation of [^{3}H]CQ was carried out as previously described.[25] CQ accumulation is expressed as the cellular accumulation ratio, which is the ratio of the amount of radiolabeled CQ in parasites to the amount of [^{3}H]CQ in a similar volume of buffer after incubation.[26] The cellular accumulation ratio of CQ alone (value of 18) and in the presence of DEEA compounds in clone W2 was compared to the cellular accumulation ratio of CQ alone obtained with the susceptible clone 3D7 (value of 191). CQ accumulation results at 1 and 10 μM concentrations of DEEA compounds are gathered in Table 1. The CQ accumulation values at 10 μM are mentioned only to put into evidence the behavior of some molecules for this activity, although this concentration is not relevant for reversal potency.

The ability to increase CQ accumulation has been reported only for desipramine,[4] verapamil, and diltiazem.[27] In the series under investigation, there are four groups of compounds: the first group consists of compounds **2**, **5**, **14**, **19**, **25**, **34**, **37**, and **39**, which have an important effect on the cellular accumulation ratio at 1 μM and which restore completely the accumulation at 10 μM. The second group consists of compounds **13**, **23**, **27**, **32**, **33**, and promethazine. These compounds have an intermediate potency on CQ level with no changes when the concentration of chemosensitizers increases. The third group consists of compounds **11**, **22**, **40**, verapamil, and mecamylamine, which produce an effect on CQ accumulation only at 10 μM. Finally, all other compounds that are ineffective are gathered in the fourth group. The cellular accumulation ratio of CQ at 1 and 10 μM of diastereoisomers *RR* and *SS* of **19** are, respectively, 39/246 and 105/286 and that of **5** is 76/

Table 1. Biological Data

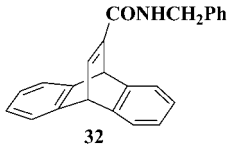
N°	Compounds		Antimalarial activity IC_{50} (μM)	Reversal activity IC_{50} (μM)	Cytotoxicity (μM)	Hemolysis (μM)	Accumulation coefficient at 1μM- 10μM of DEEA	Reversal % at 1μM of DEEA
	R^1	R^2						
								
2	-CH_2NHCH_2-		10	0.2	1,107	>2,000	89-322	80
4	$CONMe_2$	$CONMe_2$	35.5	/	>2,000	>2,000	28-31	0
5	CH_2NMe_2	CH_2NMe_2	2	0.25	1,035	>2,000	76-217	80
								
7	CH_3	$CONH_2$	41	20	>2,000	>2,000	17-22	0
9(o)	CH_3		302	/	205	1827	14-15	0
10(m)	CH_3		164	/	93	>2,000	10-10	0
11(p)	CH_3		833	1494	>500	>2,000	15-101	21
12	CH_3	$CON{=}NHNMe_2$	51	40	>2,000	>2,000	23-19	0
13	CH_3	CH_2NMe_2	30	0.4	711	1402	46-62	60
14	CH_3	$CH_2N^+Me_2$, I^-	16	3.5	>2,000	>2,000	53-192	37
16	$CONH_2$	$CONH_2$	44	50	>2,000	>2,000	22-29	0
17	$CONMe_2$	$CONMe_2$	12.4	9	1,558	>2,000	30-32	3
18	$CON{=}NHNMe_2$	$CON{=}NHNMe_2$	33	30	820	>2,000	16-25	0
19	CH_2NMe_2	CH_2NMe_2	4	0.4	>2,000	>2,000	112-298	80
21	$CONMe_2$	CO_2Me	32	Antagonist	>2,000	>2,000	23-30	0
22	CH_2NMe_2	CH_2OH	36	0.6	>2,000	>2,000	14-56	66
23	CH_2NMe_2	$CH_2OCONMe_2$	21	1.6	758	1563	50-66	25
25	CH_2NH_2	CH_2NH_2	8	12.5	465	>2,000	50-159	7
27	NMe_2	CO_2Et	34	3.1	>2,000	>2,000	62-63	0
28	NMe_2	CO_2H	>50	>100	>2,000	>2,000	18-16	0
34	H	CH_2NHMe	8	0.3	515	1090	68-210	80
39	H	NMe_2	9.3	0.8	>2,000	>2,000	49-219	60
40	H	$NH(CH_2)_2NMe_2$	8	0.8	695	644	16-71	63
								
31	H	$CONH(CH_2)_{10}NHCO$ (9,10-dihydro-9,10-ethenoanthracenyl)	8	Antagonist	>2,000	>2,000	27-34	0
32	H	$CONHCH_2Ph$	50	23	>2,000	>2,000	46	0
33	H	CH_2NMe_2	4	0.5	1,427	1630	31-53	65
36	$CONMe_2$	$CONMe_2$	36.5	>50	>2,000	>2,000	30-23	0
37	CH_2NMe_2	CH_2NMe_2	2.5	0.8	920	>2,000	61-242	55
	Verapamil		13.4	0.8	695	861	16-97	57
	Promethazine		20	0.4	702	618	37-42	67
	Mecamylamine		91.4	6			16-45	15
	Chloroquine		0.9				18 (W2) 191 (3D7)	0

Table 2. Physicochemical Data

compds	pK_a*	log*P*	Log*D* at pH 7
2	10.18	2.98	0
4	−1.34	1.7	1.7
5	9.19	3.41	0
7	−0.89	2.89	2.89
9	5.27	4.17	4.16
10	4.14	3.85	3.85
11	5.27	3.85	3.85
12		3.26	3.26
13	8.99	4.24	2.25
14		1.32	1.32
16	−0.96	0.86	0.86
17	−1.34	1.7	1.7
18		1.6	1.6
19	9.19	3.41	0
21	−1.65	2.47	2.47
22	8.86	2.82	0.96
23	8.99	2.99	1.00
25	10.14	1.89	−3.27
27	7.48	3.60	3.00
28	8.3	2.7	0.10
31	−1.14	9.16	9.16
32	−2.01	4.41	4.41
33	8.04	3.68	2.61
34	10.18	3.66	0.63
36	−2.68	2.15	2.15
37	8.66	3.34	1.26
39	8.67	3.67	2.00
40	9.48	3.17	−1.5
verapamil	8.29 (lit.[a] pK_a = 8.92)	5.52 (lit.[a] log*P* = 3.79)	4.21
promethazine	8.89 (lit.[a] pK_a = 9.1)	3.86	1.96
mecamylamine	10.97 (lit.[a] pK_a = 11.3)	2.13	−0.40
CQ	10.27 (lit.[a] pK_a = 10.16)	4.72 (lit.[a] log*P* = 4.63)	0.17

[a] Craig, P. N. Drug Compendium. In *Comprehensive Medicinal Chemistry*; Hansch, C., Ed.; Pergamon Press: Oxford, U.K., 1990; pp 237−991.

217. These results suggest that the modulation of CQ accumulation by DEEA derivatives is stereospecific contrary to chemosensitization of CQ resistance.



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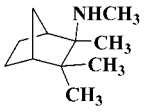


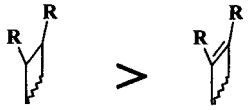
mecamylamine

As antimalarial activity of CQ is dependent on its binding to ferriprotoporphyrin IX (FPIX)[30] and therefore on its access to the parasite digestive vacuole, biological results suggest that DEEA chemosensitizers could allow more or less CQ to reach its target. Indeed, comparison of reversal percentage and CQ accumulation for each compound at 1 μM (Table 3) shows that CQ accumulation correlates well with the reversal activity for derivatives **2**, **5**, **19**, and **34** but correlates to a lesser extent for **13**, **33**, **37**, **39**, and promethazine. In addition, this correlation fails for compounds **22**, **40**, and verapamil, which display a reversal activity without any effect on CQ accumulation. Some other compounds have already been reported as chemosensitizers of CQ resistance without affecting CQ accumulation and suggesting another mechanism than that encountered in MDR cancer cells.[28,29] These data show that compounds **22**, **40**, and verapamil could make the access to FPIX easier for CQ without affecting the global CQ uptake. At the opposite, there are compounds **25** and **27**, which have, respectively, accumulation rates of 50 and 62 at 1 μM but no reversal effects (7 and 0%). Compounds **14** and **23** have a lower reversal effect than that expected regarding only their influence on CQ accumulation rate as shown in Table 3. So, it can be concluded that these derivatives are not capable of facilitating CQ access to FPIX although their influence on CQ accumulation is significant. Finally, these results suggest that chemosensitizers must have at least two sites of action: one that mediates global CQ uptake and one that mediates CQ access to FPIX.

Two putative mediators of CQ resistance were identified as follows: Pgh1[31] and Cg2 protein.[32] Pgh1 is encoded by a MDR gene and is closely related to the human P-glycoprotein,[33] which mediates drug efflux. Pgh1 is localized primarily on the food vacuole membrane and to a lesser extent on the plasma membrane of the parasite[34] suggesting that it could be involved in drug transport across these membranes. Cg2 protein is located at both the parasitophorous vacuole and the food vacuole in association with hemozoin, but a broad range of possible functions could be attributed to this protein. Recently, *pfcrt*, a gene with 13 exons, was identified near *cg2* in chromosome 7.[35] This gene encodes PfCRT, a 424 amino acid protein that appears to be either a channel or a transporter protein lying across the membrane of the parasite digestive vacuole. A set of point mutations in *pfcrt* were associated with CQ resistance.[36] Polymorphisms in *cg2* were highly associated with CQ resistance,[32] but allelic modification experiments have ruled out a role of this gene in CQ resistance.[36] The presence of the *pfcrt* T76 mutation was more strongly associated with CQ resistance than was the *pfmdr1* Y86 one or both mutations.[36]

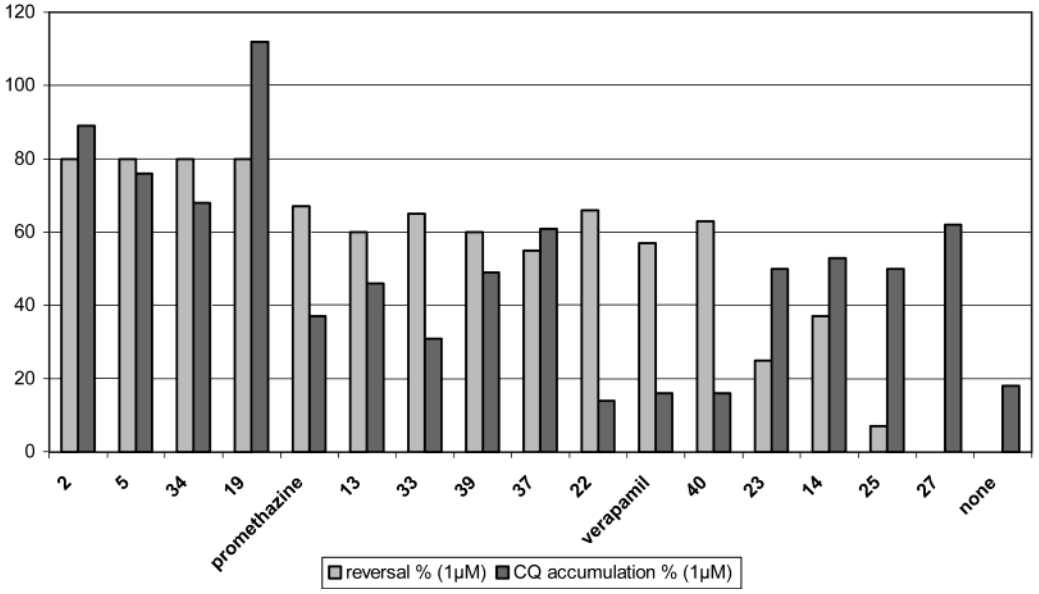
Pharmacophore Determination. Except the presence or not of the double bond in the bridge, the DEEA moiety was kept structurally identical to keep constant the influence of this part of the molecule on biological activity in order to put into evidence only the side chain interactions with a putative receptor. Weak variations on the side chain allowed optimization of the reversal potency as well as identification of important structural features responsible for the biological activity. It seems that the presence of an amino group is a necessary condition but it is not sufficient to modulate CQ accumulation for compounds such as **22**, **40**, verapamil, and mecamylamine. Compound **14** suggests that these modulators could act by ionic interaction on a putative target, but there are no clear relationships between geometrical structure and capability to modulate CQ level. On the contrary, pharmacophore(s) responsible for the reversal activity can be found.

As it is mentioned above, the double bond decreases the biological effect under investigation. So, the pharmacophoric group(s) must be borne on a 9,10-dihydro-9,10-ethanoanthracenic structure.

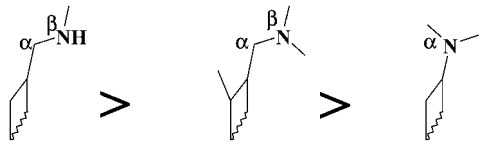


The SAR study also displayed the necessary presence of an amino group. Comparison between **39**/**13** and **34**/

Table 3. Reversal Activity and Influence on CQ Accumulation of DEEA Derivatives at 1 μM

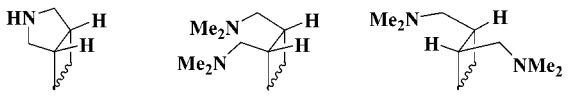


13 indicates that the β position of this amino group in the chain confers a better activity than the α one. The same is true with a secondary amine, which is better than a tertiary one.



34 **13** **39**

Finally, the activity of **2** is better than that of **5** and **19**.



2 **5** **19**

Hence, the most active molecules have at least two hydrogen-bonding acceptor and/or donor groups that influence the reversal of CQ resistance. Consequently, molecule **2** has to be considered as the reference molecule.

Schematic Proposal for a Model of Interaction. Possible ligand/amino acid interactions in a putative receptor responsible for the CQ resistance can be roughly depicted using molecular modeling of the DEEA derivatives studied. These interactions have to explain (i) the activity of mono- and disubstituted derivatives, (ii) the activity of accepting and donating hydrogen-bonding capabilities of derivatives, and (iii) the activity of *RS*, *RR*, and *SS* isomers. The pyrrolidino ring of molecule **2** considered above as a reference can interact with amino acids by hydrogen-bonding and/or ionic interactions. Taking that into account, two different ways of ligand/amino acid interactions were investigated. The first involves only hydrogen bonding, and serine was selected as the target in this case; the second interaction involves on one hand an ionic bond and on the other hand a hydrogen bond. With respect to this, aspartic acid and serine were selected as possible targets.

Models of chemosensitizers selected as the most representative ones were built from the experimental structure of the ethano derivative **19** and of the etheno derivative **33**.[37] The side chain conformation was considered as the sole geometrical variable. For each compound, conformational analysis was performed and the strain energy of the most stable conformers (E_{min}) was calculated as detailed in the Experimental Section.

There are two stable puckered conformations for the reference molecule **2**, depending on whether the nitrogen atom is located above or below the ring mean plane. Consequently, four conformers can be portrayed according to the location in space of the hydrogen branched on the nitrogen atom (Figure 1). These four conformations are equally probable since their strain energies are in the same range (E_{min} (a) = 37.5 kcal/mol; E_{min} (b) = 36.9 kcal/mol; E_{min} (c) = 36.6 kcal/mol; and E_{min} (d) = 37.5 kcal/mol).

However, the fixed position of amino acids in space can be deduced from the various conformations of compound **2**. Conformation **2d** was selected because on one hand it is the only conformation that accounts for the potency of both the *RS* and the *RR* or *SS* isomers and on the other hand it is the only one that permits a good fit of aromatic rings with other DEEA derivatives. Indeed, the 9,10-dihydro-9,10-ethanoanthracenic skeleton was kept structurally identical while there were no geometrical constraints for the side chains. The system ligand/amino acid interactions were optimized keeping the skeleton of DEEA as closely as possible to the initial position. Strain energies of interactions (E_{int}) were estimated, and they are summarized in Table 4.

As mentioned above, it was first considered that **2** makes hydrogen bonds with two serines. But this model does not justify the activity of **13**, **27**, **33**, and **39**. Actually, **27** and **39** have the amino group directly branched on the bridge of DEEA, so that there are too important strains when interaction occurs between the

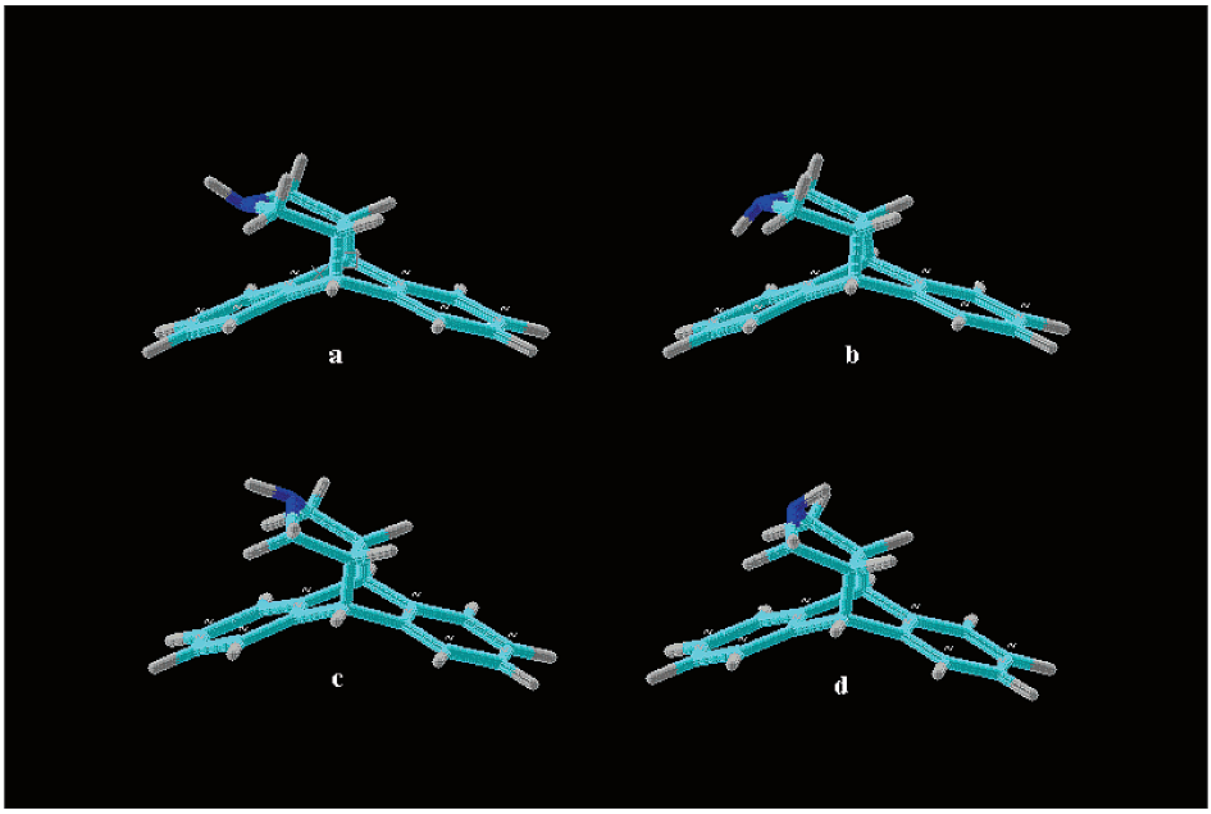


Figure 1. Four conformations of **2**.

Table 4. Energy and Geometrical Parameters of DEEA Chemosensitizers in Relation to the Model of Interactions

compds	E_{int} (kcal/mol)	α (deg)	β (deg)	V (Å^3)
2	−85.4			326
5	−84.0	15.6	18.1	456
13	−72.3	27.9	57.6	393
19	−81.4	28	56.4	457
22	−66.3	6.5	10.6	400
33	−66.7	5.3	8.9	366
34	−76.4	0	6.8	341
37	−64.5	17	11.3	452
39	−61.1	10.2	20	345
40	−70.6	10.6	62.7	407

amino group of the ligand and the hydroxyl group of the serine. In these cases, ligand/amino acid interaction is only stable at a distance between them, which does not permit hydrogen bonding. Moreover, **13**, **33**, and **39** are tertiary monoamine derivatives and only one interaction is not strong enough to fit the ligand firmly into the interaction sites. Hence, an alternative case was considered (Figure 2). In this condition, the hypothetical model of interaction accounts for all chemosensitizer activity even for **13**, **27**, **33**, and **39** because the ionic interaction takes place at a longer distance than the hydrogen bond does, so that this minimizes strains, and interaction energy correlates well with the reversal potency.

Moreover, these results are in agreement with the observed effect of mutations on PfCRT. Indeed, *P. falciparum* CQ resistant strains from South-East Asia carry pfcrt alleles encoding a CVIET haplotype (residues 72−76), whereas CQ susceptible strains from most malaria-infected regions around the world carry a CVMNK haplotype.[38] The nature of mutations 75 and 76 is consistent with the model of interaction defined herein.

Linear regression implying the interaction energy E_{int} between the putative amino acid complex and the chemosensitizers shows a significant correlation with IC_{50}:

$$\log(1/IC_{50}) = -0.0227(\pm 0.004\ 14)\ E_{int} - 1.308(\pm 0.3037)$$

where $n = 10$; $r^2 = 0.789$; $F = 29.92$; $p = 0.0006$; and $s = 0.1065$.

As a result, the distance between C_α of the two amino acids is 9.2 Å. With the side chains kept free, the deviation angles α and β, which are ligand-dependent, can be measured from the initial position of the ligand skeleton (Figure 3, Table 4): $0° < \alpha < 28°$ and $6.8° < \beta < 62.7°$.

Volume V of the ligand (Table 4) also varies in relation to the ligand structure and its active conformation: 326 Å^3 < V < 457 Å^3. Therefore, this volume gives an idea of the putative binding cavity volume.

Although energy values and geometrical data of **14** (E_{int} = −34.3 kcal/mol, α = 28.6°, β = 57.6°, V = 417 Å^3, and IC_{50} (calcd) = 3.32 ± 0.17 μM) and **25** (E_{int} = −107.9 kcal/mol, α = 9.8°, β = 8.2°, V = 351 Å^3, and IC_{50} (calcd) = 0.07 ± 0.06 μM) show that interactions with the hypothetical model of interaction could occur, their biological inefficacy could be attributed to the difficulty to cross the membranes due to their physicochemical properties preventing access to the putative cavity.

Compounds **23** (E_{int} = −49.7 kcal/mol, α = 8.2°, β = 15.5°, and V = 502 Å^3) and **27** (E_{int} = −51.7 kcal/mol, α = 13.1°, β = 55.4°, and V = 433 Å^3), which are weak chemosensitizers, can interact with the binding site but with a higher energy of interaction. The calculated IC_{50}

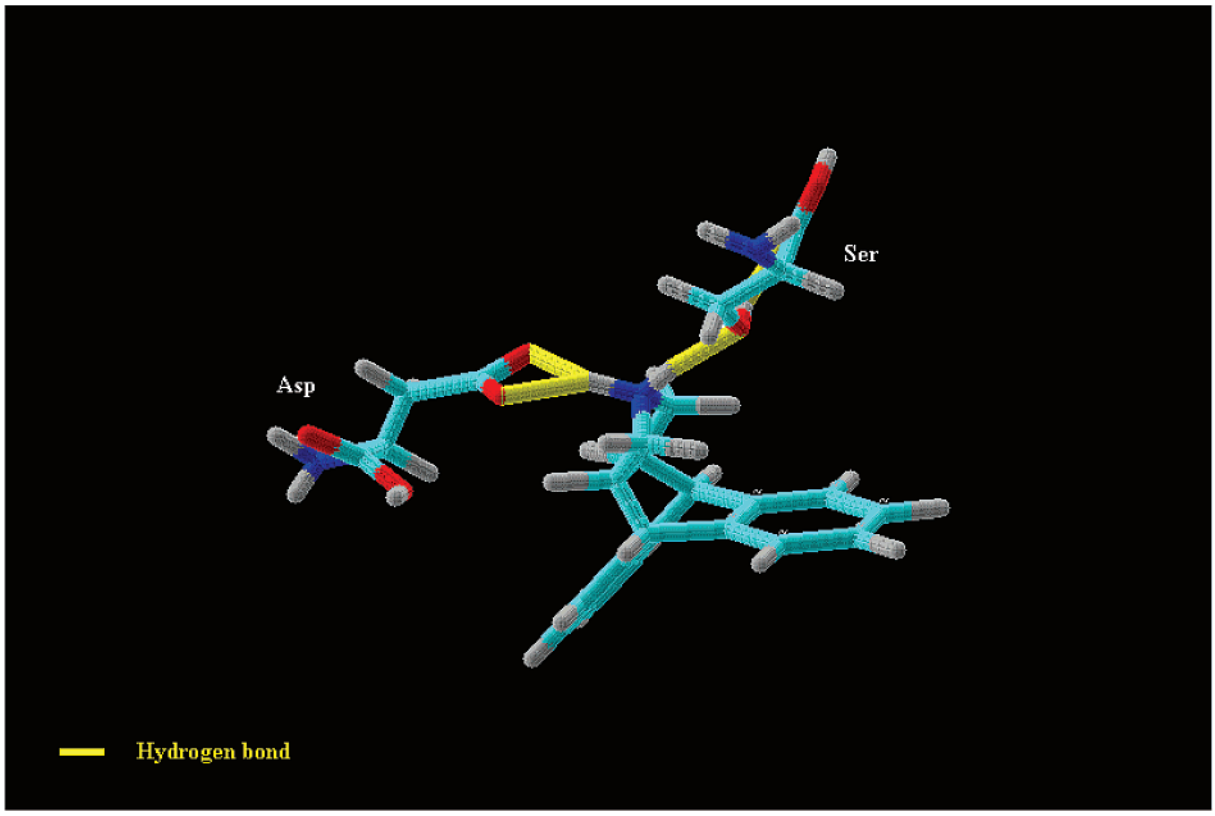


Figure 2. Interactions of the reference molecule **2** with an aspartic acid and a serine in the interaction sites.

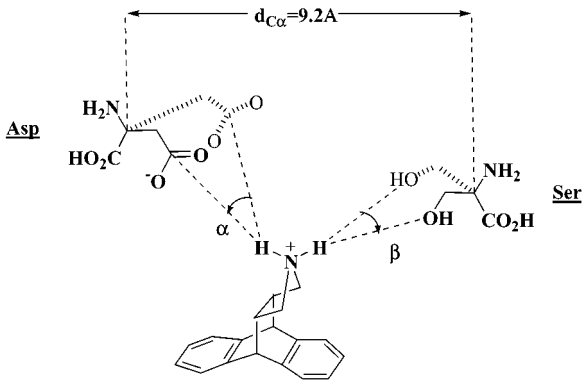


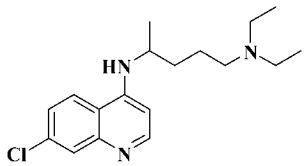
Figure 3. Position of the reference molecule **2** in the interaction site model.

values, which are, respectively, 1.48 (±0.14) μM and 1.34 (±0.14) μM, correlate well with the experimental IC_{50} values and therefore justify the model.

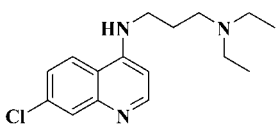
Acid **28** (E_{int} = 0.5 kcal/mol, α = 38.6°, β = 53.3°, and V = 381 Å^3) shows no reversal activity despite the presence of an amino group. Interactions with the amino acids may occur, but the energy of interaction is very high.

Other compounds that do not reverse CQ resistance possess hydrogen bond acceptor and/or donor groups, which cannot make ionic interactions. As these derivatives can only interact with serine in the model under evaluation, this interaction does not minimize strains enough and then high interaction energies ($E_{int} > 0$) ensue. So, these compounds cannot bind the interaction site.

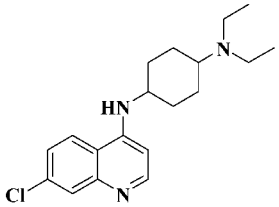
For the compounds studied, variations of side chain were only taken into account, and the cyclic structure was kept unchanged. Hence, no information is available for aromatic rings, as well as optimum number and position. However, it can be noted that a tricyclic-related structure like that of promethazine can be superimposed on the model with a good correlation between energy and reversal activity (E_{int} = −71.2 kcal/mol, α = 25.0°, β = 9.4°, V = 379 Å^3, and IC_{50} (calcd) = 0.48 ± 0.11 μM) as illustrated in Figure 4. Concerning verapamil (E_{int} = −83.7 kcal/mol), the distance between the two nitrogen atoms and the flexibility of the aliphatic chain permit interactions with the proposed model but no further information can be brought up for aromatic rings owing to the multiple conformations allowed. Mecamylamine (E_{int} = −62.1 kcal/mol) with no aromatic ring can also bind to the site, despite the fact that this compound is devoid of reversal activity. This would imply that additional hydrophobic interactions are necessary.



chloroquine



SN 9 584



SN 12 108

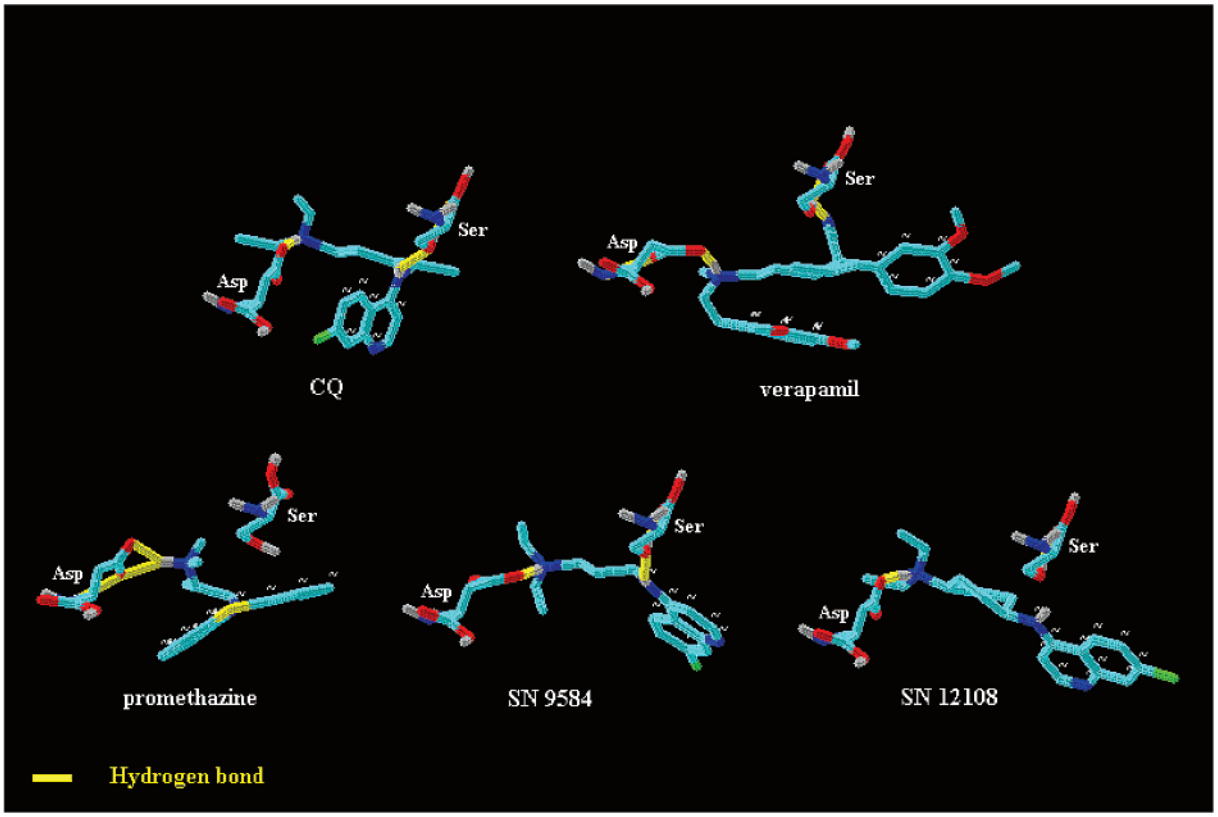


Figure 4. Position of CQ, verapamil, promethazine, SN 9 584, and SN 12 108 in the interaction site model.

CQ ($E_{int} = -103.8$ kcal/mol) acts on the interaction sites, too. This interaction energy does not take into account the position of aromatic rings, as no information is defined for them. In contrast, this value would be in the same range of that of DEEA chemosensitizers, if the quinoline skeleton of CQ is constrained to fit one or the other aromatic rings of DEEA compounds. Therefore, this could suggest competition with reversal agents resulting in release of CQ, which then can reach the FPIX target. Moreover, CQ analogues such as SN 9 584[39] and SN 12 108[40] are active on CQ susceptible and resistant strains of *P. falciparum* as well. Similarly, they could bind to the concerned sites but with high interaction energies (respectively, $E_{int} = -52.2$ kcal/mol and $E_{int} = -41.4$ kcal/mol). This could be due to the amino chain, which is shorter than that of CQ, and to the resulting important constraints, which are detrimental for the binding. Consequently, their antimalarial activity would be retained on resistant strains.

Conclusion

Some of the modulators with the DEEA structure show high ability to reverse CQ resistance in different *P. falciparum* resistant strains. This could be of strong interest, the more so as these compounds are neither cytotoxic nor antimalarial. Thus, their capability to induce resistance is very low. In contrast, their capability to influence more or less CQ accumulation allowed us to display two distinct sites of action, respectively, involved in drug accumulation and reversal of drug resistance.

A SAR study of DEEA compounds including identification of a reference molecule allowed us to define some basic ligand site interactions connected with the reversal activity. These interactions are fully compatible with other structurally unrelated drugs. Nevertheless, because interactions involving aromatic rings still miss, a similar investigation to that which was carried out has to be achieved with benzobarrelene and [2.2.2]-octane derivatives. Finally, it could be suggested that the ligands studied bind in a competitive manner due to the fact that strain energies are quite different depending on the ligand considered.

Experimental Section

Chemistry. General Methods. Melting points were determined on a Büchi apparatus and are given uncorrected. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were performed on a Brüker ARX200 spectrometer with tetramethylsilane (TMS) as internal reference; chemical shifts are given on the δ (ppm) scale with J values in hertz. Liquid chromatography was performed on silica gel 60 (70−230 mesh), and thin-layer chromatography (TLC) was performed on silica gel 60 F_{254}. Rotatory power was measured with a Perkin-Elmer 341 polarimeter.

Preparation of Amide Derivatives. Method A. A mixture of acid derivative (10 mmol) and $SOCl_2$ (10 mL) was heated for 3 h under reflux. After $SOCl_2$ in excess under vacuum was eliminated, the acid chloride obtained was used for the next step without further purification. Acid chloride (10 mmol) and a solution of amine in THF 2 N (25 mL) if commercially available ($MeNH_2$, Me_2NH) or NH_4OH 20% in THF (30 mL) or $PhCH_2NH_2$ (25 mmol) in CH_2Cl_2 were stirred for 1 day at room temperature. The solvent was eliminated under vacuum. The residue obtained was extracted with CH_2Cl_2 and water (pH 10) before the organic phase was dried under drierite. Pure amide derivative was obtained using column chromatography (eluent: ether/methanol).

9,10-Dihydro-12-methyl-9,10-ethanoanthracene-11-carboxamide (7). Yield, 72%; mp 198−200 °C. ^{1}H NMR ($CDCl_3$): 7.39 (m, 4H); 7.24 (m, 4H); 5.81 (br. s, 1H); 5.19 (br. s, 1H); 4.53 (d, $J = 1.8$ Hz, 1H); 4.14 (d, $J = 2.0$ Hz, 1H); 2.26

(m, 1H); 2.23 (d, J = 1.8 Hz, 1H); 1.05 (d, J = 6.5 Hz, 3H). ^{13}C NMR ($CDCl_3$): 176.3 (s); 144.7 (s); 142.5 (s); 140.3 (s); 139.1 (s); 126.4 (d); 126.1 (d); 126.0 (d); 125.9 (d); 125.8 (d); 125.3 (d); 123.3 (d); 123.0(d); 54.4 (d); 50.8 (d); 47.5 (d); 38.9 (d); 21.1 (q). Anal. ($C_{18}H_{17}NO$) C, H, N.

9,10-Dihydro-*N*,*N*-12-trimethyl-9,10-ethanoanthracene-11-carboxamide (8).[12] Yield, 55%; mp 145−147 °C. ^{1}H NMR ($CDCl_3$): 7.58 (m, 4H); 7.13 (m, 4H); 4.14 (d, J = 1.8 Hz, 1H); 3.95 (d, J = 2.1 Hz, 1H); 3.09 (br. s, 3H); 2.89 (br. s, 3H); 2.59 (m, 1H); 2.35 (dd, J = 5.4, 2.0 Hz, 1H); 0.80 (d, J = 6.9 Hz, 3H). ^{13}C NMR ($CDCl_3$): 172.3 (s); 144.3 (s); 142.8 (s); 141.3 (s); 138.6 (s); 126.1 (d); 125.9 (d); 125.7 (d); 125.6 (d); 125.5 (d); 124.6 (d); 122.8 (d); 122.4 (d); 50.8 (d); 49.9 (d); 47.8 (d); 37.3 (q); 36.8 (d); 36.0 (q); 20.7 (q).

9,10-Dihydro-12-methyl-*N*-2′-pyridinyl-9,10-ethanoanthracene-11-carboxamide (9). Yield, 30%; mp 221−222 °C. ^{1}H NMR ($CDCl_3$): 8.22 (d, J = 4.9 Hz, 1H); 8.08 (d, J = 8.4 Hz, 1H); 7.83 (br. s, 1H); 7.62 (m, 1H); 7.29 (m, 4H); 7.14 (m, 4H); 6.97 (m, 1H); 4.52 (m, 1H); 4.06 (d, J = 1.8 Hz, 1H); 5.43 (td, J = 6.6, 2.1 Hz, 1H); 2.23 (dd, J = 2.1, 1.8 Hz, 1H); 0.97 (d, J = 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$): 171.8 (s); 151.5 (s); 147.8 (d); 144.5 (s); 142.4 (s); 140.8 (s); 138.8 (s); 138.4 (d); 126.6 (d); 126.2 (d); 126.1 (d); 125.9 (d); 125.3 (d); 123.3 (d); 123.1 (d); 119.7 (d); 114.1 (d); 55.7 (d); 50.9 (d); 48.0 (d); 37.9 (d); 21.2 (q). Anal. ($C_{23}H_{20}N_2O$) C, H, N.

9,10-Dihydro-12-methyl-*N*-3′-pyridinyl-9,10-ethanoanthracene-11-carboxamide (10). Yield, 38%; mp 222−223.5 °C. ^{1}H NMR (DMSO-d_6): 10.30 (s, 1H); 8.68 (d, J = 2.1 Hz, 1H); 8.23 (dd, J = 4.5, 1.0 Hz, 1H); 7.95 (ddd, J = 8.3, 2.5, 1.5 Hz, 1H); 7.41−7.37 (m, 1H); 7.35−7.26 (m, 3H); 7.15 (m, 3H); 7.07−7.0 (m, 2H); 4.58 (d, J = 1.9 Hz, 1H); 4.10 (d, J = 1.9 Hz, 1H); 2.42 (m, 1H); 2.26 (dd, J = 5.2, 2.0 Hz, 1H); 0.79 (d, J = 6.9 Hz, 3H). ^{13}C NMR (DMSO-d_6): 171.6 (s); 144.9 (s); 144.3 (d); 143.1 (s); 141.5 (s); 141.1 (d); 139.7 (s); 136.3 (s); 126.5 (d); 125.9 (d); 125.7 (d); 125.0(d); 123.9 (d); 123.5 (d); 123.2 (d); 54.1 (d); 50.0 (d); 48.3 (d); 35.8 (d); 20.8 (q). Anal. ($C_{23}H_{20}N_2O$) C, H, N.

9,10-Dihydro-12-methyl-*N*-4′-pyridinyl-9,10-ethanoanthracene-11-carboxamide (11). Yield, 74%; mp 275−276 °C. ^{1}H NMR (DMSO-d_6): 10.49 (s, 1H); 8.38 (d, J = 5.5 Hz, 2H); 7.50 (d, J = 5.5 Hz, 2H); 7.39 (m, 1H); 7.31 (m, 2H); 7.15−7.09 (m, 3H); 7.10 (m, 2H); 4.56 (s, 1H); 4.10 (d, J = 1.3 Hz, 1H); 2.42 (m, 1H); 2.27 (dd, J = 5.1, 1.3 Hz, 1H); 0.78 (d, J = 6.8 Hz, 3H). ^{13}C NMR (DMSO-d_6): 172.2 (s); 150.5 (d); 146.3 (s); 144.9 (s); 143.0 (s); 141.5 (s); 139.5 (s); 126.0 (d); 125.9 (d); 125.7 (d); 125.0 (d); 123.6 (d); 123.2 (d); 113.5 (d); 54.4 (d); 50.0 (d); 48.2 (d); 35.7 (d); 20.7 (q). Anal. ($C_{23}H_{20}N_2O$) C, H, N.

***trans*-9,10-Dihydro-9,10-ethanoanthracene-11,12-dicarboxamide (16).**[10] Yield, 79%; mp 305−306 °C. ^{1}H NMR (DMSO-d_6): 7.64 (s, 2H); 7.28 (m, 2H); 7.18 (m, 2H); 7.03 (m, 4H); 6.85 (s, 2H); 4.66 (s, 2H); 3.14 (s, 2H). ^{13}C NMR (DMSO-d_6): 173.5 (s); 143.5 (s); 141.0 (s); 125.4 (d); 124.5 (d); 123.2 (d); 47.2 (d); 46.7 (d).

***trans*-9,10-Dihydro-*N*,*N*,*N*′,*N*′-tetramethyl-9,10-ethanoanthracene-11,12-dicarboxamide (17).**[12] Yield, 78%; mp 161−163 °C. ^{1}H NMR ($CDCl_3$): 7.32 (m, 4H); 7.10 (m, 4H); 4.34 (s, 2H); 3.69 (s, 2H); 3.21 (s, 6H); 2.90 (s, 6H). ^{13}C NMR ($CDCl_3$): 172.0 (s); 142.7 (s); 139.7 (s); 126.2 (d); 126.1 (d); 125.0(d); 122.5 (d); 47.4 (d); 45.2 (d); 37.4 (q); 36.0(q).

(*RR*) Compound 17. $[\alpha]^{20}_D = -129.75°$ (*c* 2 dioxan).

(*SS*) Compound 17. $[\alpha]^{20}_D = +135.5°$ (*c* 2 dioxan).

9,10-Dihydro-*N*,*N*,*N*′,*N*′-tetramethyl-9,10-ethenoanthracene-11,12-dicarboxamide (36).[17] Yield, 78%; mp 172 °C. ^{1}H NMR ($CDCl_3$): 7.35 (m, 4H); 6.99 (m, 4H); 5.20 (s, 2H); 2.92 (s, 6H); 2.59 (s, 6H). ^{13}C NMR ($CDCl_3$): 168.4 (s); 144.2 (s); 143.5 (s); 125.1 (d); 124.8 (d); 123.6 (d); 123.3 (d); 53.1 (d); 37.9 (q); 34.9 (q).

Method B. Anhydride derivative (36 mmol) was saponified with KOH (0.9 mol) in H_2O (120 mL) under reflux during 3 days. At room temperature, the mixture was filtered and the solution obtained was acidified with HCl 10 N. The acid obtained was filtered and then dried. The corresponding acid chloride and the corresponding amide were prepared as in method A.

***cis*-9,10-Dihydro-*N*,*N*,*N*′,*N*′-tetramethyl-9,10-ethanoanthracene-11,12-dicarboxamide (4).** Yield, 81%; mp 205 °C. ^{1}H NMR ($CDCl_3$): 7.35 (m, 4H); 7.09 (m, 4H); 4.37 (s, 2H); 3. 72 (s, 2H); 3.23 (s, 6H); 2.92 (s, 6H). ^{13}C NMR ($CDCl_3$): 171.9 (s); 142.7 (s); 139.7 (s); 126.2 (d); 126.1 (d); 124.9 (d); 122.5 (d); 47.3 (d); 45.1 (d); 37.3 (q); 35.9 (q). Anal. ($C_{22}H_{24}N_2O_2$) C, H, N.

Method C. A mixture of ester derivative **20** (16.5 mmol) and KOH (16.5 mmol) in MeOH (45 mL) and in Et_2O (50 mL) was left at room temperature during 3 days. Solvents were then eliminated under vacuum. A mixture of 11-carboxy-12-carbomethoxy, 11,12-dicarbomethoxy, and 11,12-dicarboxy-9,-10-dihydro-9,10-ethanoanthracene was obtained. Successive extractions at variable pH separated the expected compounds of the two other products. The 11-carboxy-12-carbomethoxy-9,10-dihydro-9,10-ethanoanthracene was treated as an acid derivative in method A to obtain the corresponding amide derivative.

***trans*-9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethanoanthracene-11-carboxamide-12-carboxylic Acid, Methyl-ester (21).** Yield, 83%; mp 125−130 °C. ^{1}H NMR ($CDCl_3$): 7.31 (m, 3H), 7.12 (m, 5H); 4.76 (d, J = 2.3 Hz, 1H); 4.32 (d, J = 2.1 Hz, 1H); 3.61 (br. s, 4H); 3.52 (dd, J = 5.4, 2.1 Hz, 1H); 3.25 (s, 3H); 2.92 (s, 3H). ^{13}C NMR ($CDCl_3$): 173.5 (s); 171.3 (s); 142.7 (s); 142.1 (s); 140.6 (s); 139.4 (s); 126.4 (d); 126.3 (d); 126.2 (d); 124.9 (d); 124.6 (d); 123.4 (d); 122.9 (d); 52.1 (q); 48.1 (d); 47.2 (d); 46.5 (d); 45.1 (d); 37.4 (q); 36.2 (q). Anal. ($C_{21}H_{21}NO_3$) C, H, N.

Method D. Ester derivative (40 mmol) and NaOH (0.16 mol) in H_2O (48 mL) and MeOH (32 mL) were stirred under reflux during 3 days. The corresponding acid obtained was purified as in method B and treated as in method A, or after acid chloride was obtained, it was treated with decanediamine (7.5 mmol) and triethylamine (TEA, 15 mmol) in CH_2Cl_2 and stirred for 1 day at room temperature. The amide derivative obtained was purified as in method A.

9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethenoanthracene-11-carboxamide (29).[16] Yield, 81%; mp 115 °C. ^{1}H NMR ($CDCl_3$): 7.32 (m, 4H); 7.12 (dd, J = 6.1, 1.9 Hz, 1H); 6.97 (m, 4H); 5.37 (d, J = 1.7 Hz, 1H); 5.21 (d, J = 6.0 Hz, 1H); 2.91 (s, 6H). ^{13}C NMR ($CDCl_3$): 168.9 (s); 146.8(s); 145.3 (s); 145.0 (s); 140.6 (d); 124.8 (d); 124.7 (d); 123.4 (d); 123.1 (d); 53.0 (d); 51.0 (d).

9,10-Dihydro-*N*-methyl-9,10-ethenoanthracene-11-carboxamide (30). Yield, 60%; mp 118−121 °C. ^{1}H NMR (DMSO-d_6): 7.34 (m, 5H); 6.97 (m, 4H); 5.78 (m, 1H); 5.69 (d, J = 1.8 Hz, 1H); 5.16 (d, J = 6.08 Hz, 1H); 2.79 (d, J = 4.90 Hz, 3H). ^{13}C NMR (DMSO-d_6): 165.7 (s); 147.9 (s); 145.3 (s); 144.7 (s); 141.4 (d); 124.9 (d); 124.7 (d); 123.7 (d); 123.2 (d); 51.0 (d); 50.6 (d); 26.3 (q). Anal. ($C_{18}H_{15}NO$) C, H, N.

***N*,*N*′-1,10-Decanediylbis[9′,10′-dihydro-9′,10′-ethenoanthracene-11′-carboxamide] (31).** Yield, 25%; mp 190 °C. ^{1}H NMR ($CDCl_3$): 7.36 (m, 10H); 6.99 (m, 8H); 5.94 (m, 2H); 5.72 (d, J = 1.2 Hz, 2H); 5.16 (d, J = 6.1 Hz, 2H); 3.21 (m, 4H); 1.42 (m, 4H); 1.22 (s, 12H). ^{13}C NMR ($CDCl_3$): 164.9 (s); 148.1 (s); 145.4 (s); 144.7 (s); 141.1 (d); 124.9 (d); 124.6 (d); 123.7 (d); 123.2 (d); 51.0 (d); 50.6 (d); 39.6 (t); 29.4 (t); 29.1 (t); 29.0 (t); 26.7 (t). Anal. ($C_{44}H_{44}N_2O_2$) C, H, N.

9,10-Dihydro-*N*-benzyl-9,10-ethenoanthracene-11-carboxamide (32). Yield, 60%; mp 187−189 °C. ^{1}H NMR ($CDCl_3$): 7.34 (m, 10H); 6.98 (m, 4H); 5.95 (br. s, 1H); 5.75 (s, 1H); 5.19 (d, J = 6.0 Hz, 1H); 4.45 (d, J = 5.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 164.7 (s); 147.8 (s); 145.2 (s); 144.5 (s); 141.4 (d); 128.6 (d); 127.8 (d); 127.5 (d); 124.9 (d); 124.6 (d); 123.7 (d); 123.1 (d); 51.0 (d); 50.5 (d); 43.7 (t). Anal. ($C_{24}H_{19}NO$) C, H, N.

Preparation of Amine Derivatives. Method E. Amide or maleimide derivative (22 mmol) was added to a slurry of $LiAlH_4$ (110 mmol) in anhydrous THF (100 mL). After 1 day at room temperature, H_2O (4.2 mL), NaOH 1.25 N (4.2 mL), and H_2O (8.4 mL) were successively added. The suspension was filtered over Celite. The filtrates were concentrated. The residue was extracted with ethyl acetate/H_2O (pH 1). The aqueous phase was basified (pH 10) and extracted with CH_2-

Cl_2. The organic phase was dried over drierite, filtered, and concentrated under vacuum. Pure amine derivative was obtained using column chromatography (eluent: MeOH/NH_4-OH).

9,10-(3′,4′-Pyrrolidino)-9,10-dihydroanthracene (2). Yield, 80%; mp > 300 °C. ^{1}H NMR (DMSO-d_6): 7.34 (m, 4H); 9.10 (br. s, 1H); 7.18 (m, 2H); 7.00 (m, 2H); 4.34 (s, 2H); 3.35 (m, 2H); 2.71 (br. s, 2H); 1.92 (m, 2H). ^{13}C NMR (DMSO-d_6): 143.3 (s); 140.6 (s); 126.4 (d); 126.3 (d); 126.1 (d); 124.1 (d); 47.2 (t); 44.8 (d); 43.2 (d). Anal. ($C_{18}H_{17}N$) C, H, N.

***cis*-9,10-Dihydro-*N*,*N*,*N*′,*N*′-tetramethyl-9,10-ethanoanthracene-11,12-dimethanamine (5).** Yield, 70%; mp 93 °C. ^{1}H NMR ($CDCl_3$): 7.31 (m, 4H); 7.16 (m, 4H); 4.38 (s, 2H); 2.22 (br. s, 12H); 1.86 (m, 4H); 1.39 (m, 2H). ^{13}C NMR ($CDCl_3$): 150.1 (s); 144.2 (s); 141.3 (s); 125.7 (d); 125.6 (d); 125.4 (d); 123.1 (d); 64.0 (t); 46.3 (d), 45.8 (q); 42.9 (d). Anal. ($C_{22}H_{28}N_2$) C, H, N.

9,10-Dihydro-*N*,*N*-12-trimethyl-9,10-ethanoanthracene-11-methanamine Hydrochloride (13). Yield, 88%; mp 235−237 °C. ^{1}H NMR ($CDCl_3$): 12.3 (br. s, 1H); 7.64 (m, 1H); 7.31 (m, 1H); 7.16 (m, 2H); 7.03 (m, 4H); 4.85 (d, J = 1.7 Hz, 1H); 3.87 (d, J = 2.1 Hz, 1H); 2.68 (s, 6H); 2.50 (d, J = 8.1 Hz, 2H); 1.59 (m, 1H); 1.38 (m, 1H); 0.83 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$): 144.3 (s); 142.2 (s); 140.2 (s); 138.4 (s); 126.7 (d); 126.6 (d); 126.4 (d); 126.2 (d); 126.1 (d); 125.8 (d); 124.0 (d); 123.3 (d); 61.9 (t); 50.5 (d); 45.4 (d); 43.6 (q); 39.8 (d); 20.3 (q). Anal. ($C_{20}H_{23}N$·HCl), C, H, N.

***trans*-9,10-Dihydro-*N*,*N*,*N*′,*N*′-tetramethyl-9,10-ethanoanthracene-11,12-dimethanamine (19).** Yield, 90%; mp 93−94 °C. ^{1}H NMR ($CDCl_3$): 7.28 (m, 4H); 7.10(m, 4H); 4.36 (s, 2H); 2.2 (s, 12H); 1.8 (d, J = 5.6 Hz, 4H); 1.35 (t, J = 5.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 144.3 (s); 141.4 (s); 125.6 (d); 125.3 (d); 123.1 (d); 64.1 (t); 46.4 (d); 45.9 (d); 42.9 (q). Anal. ($C_{22}H_{28}N_2$) C, H, N.

Compound 19, Hydrochloride (*RR*). $[\alpha]^{20}_D = +15°$ (*c* 0.44 ethanol).

Compound 19, Hydrochloride (*SS*). $[\alpha]^{20}_D = -17.4°$ (*c* 0.85 ethanol).

9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethanoanthracene-11-methanamine-12-methanol (22). Yield, 91%; mp 128−130 °C. ^{1}H NMR ($CDCl_3$): 7.25 (m, 4H); 7.12 (m, 4H); 5.79 (m, 1H); 4.10 (s, 1H); 4.05 (s, 1H); 3.70 (m, 1H); 2.79 (m, 1H); 2.29 (m, 1H); 2.18 (s, 6H); 1.64 (br. s, 3H). ^{13}C NMR ($CDCl_3$): 144.1 (s); 144.0 (s); 140.3 (s); 140.1 (s); 126.0 (d); 125.9 (d); 125.7 (d); 125.5 (d); 124.8 (d); 123.0 (d); 122.9 (d); 66.2 (t); 65.7 (t); 49.5 (d); 48.1 (d); 47.3 (d); 46.0 (q); 43.8 (d). Anal. ($C_{20}H_{23}NO$) C, H, N.

9,10-Dihydro-*N*-methyl-9,10-ethanoanthracene-11-methanamine (34).[41] Yield, 75%; mp > 300 °C. ^{1}H NMR (DMSO-d_6): 7.60 (m, 2H); 7.37 (m, 2H); 7.18 (m, 4H); 4.67 (s, 1H); 4.43 (s, 1H); 2.48 (m, 6H); 2.02 (m, 1H); 1.29 (m, 1H). ^{13}C NMR (DMSO-d_6): 144.0 (s); 143.4 (s); 143.2 (s); 139.7 (s); 126.2 (d); 125.9 (d); 125.8 (d); 125.7 (d); 125.6 (d); 123.7 (d); 123.5 (d); 123.2 (d); 53.0 (t); 45.0 (d); 42.8 (d); 35.2 (d); 32.9 (q); 32.4 (t).

Method F. H_2SO_4 96% (0.4 mL) was cautiously added to a slurry of $LiAlH_4$ (23 mmol) in THF (80 mL). After it was stirred for 1 h at room temperature, the amide derivative (12 mmol) in THF (40 mL) was added dropwise at 0 °C and stirred for one more hour. The reaction was then treated as in method E. Cyano derivative (3.9 mmol) was treated in the same way with $LiAlH_4$ (30 mmol) and H_2SO_4 96% (0.5 mL) in THF.

9,10-Dihydro-9,10-ethanoanthracene-11,12-dimethanamine, Dihydrochloride (25). Yield, 87%; mp 250 °C. ^{1}H NMR (D_2O): 7.38 (m, 4H); 7.18 (m, 4H); 4.41 (d, J = 1.2 Hz, 2H); 2.86 (dd, J = 13.2, 3.4 Hz, 2H); 2.49 (dd, J = 13.2, 9.8 Hz, 2H); 1.71 (m, 2H). ^{13}C NMR (D_2O): 145.8 (s); 142.2 (s); 130.4 (d); 130.0 (d); 129.3 (d); 127.3 (d); 47.8 (d); 45.8 (d); 45.3 (t). Anal. ($C_{18}H_{20}N_2$·2HCl), C, H, N.

9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethenoanthracene-11-methanamine, Hydrochloride (33).[16] Yield, 70%; mp 280 °C. ^{1}H NMR (D_2O): 7.32 (m, 4H); 7.13 (d, J = 6.0 Hz, 1H); 6.95 (m, 4H); 5.28 (d, J = 6.1 Hz, 1H); 5.18 (d, J = 1.9 Hz, 1H); 3.84 (s, 2H); 2.53 (s, 6H). ^{13}C NMR (D_2O): 146.1 (s); 145.9 (s); 145.7 (s); 142.8 (s); 126.2 (d); 125.9 (d); 124.3 (d); 124.1 (d); 59.3 (t); 53.6 (d); 51.4 (d); 43.1 (q).

9,10-Dihydro-*N*,*N*,*N*′,*N*′-tetramethyl-9,10-ethenoanthracene-11,12-dimethanamine, Dihydrochloride (37). Yield, 90%; mp 310 °C (decomposition). ^{1}H NMR (D_2O): 7.35 (m, 4H); 6.94 (m, 4H); 5.35 (s, 2H); 4.01 (s, 4H); 2.51 (s, 12H). ^{13}C NMR (D_2O): 145.8 (s); 144.3 (s); 126.6 (d); 124.4 (d); 55.9 (t); 53.5 (d); 43.3 (q). Anal. ($C_{22}H_{26}N_2$·2HCl) C, H, N.

Method G. Ester derivative (9.8 mmol) and a solution of Me_2NH in MeOH 5.6 M (40 mL) in CH_2Cl_2 were stirred at room temperature during 1 day. Solvents were eliminated under vacuum, and the residue obtained was extracted with CH_2Cl_2/H_2O (pH 10). The organic phase was dried under drierite, filtered, and concentrated under vacuum. The solid obtained was crystallized in a mixture of diethyl ether/pentane (20/80, v/v). The product obtained was saponified as the ester derivative in method D, and the corresponding acid obtained was purified as in method B.

9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethanoanthracene-11-amine-12-carboxylic acid, Ethylester (27). Yield, 95%; mp 79−80 °C. ^{1}H NMR ($CDCl_3$): 7.30(m, 3H); 7.10 (m, 5H); 4.56 (d, J = 2.40 Hz, 1H); 4.54 (d, J = 2.40 Hz, 1H); 4.07 (m, 2H); 2.99 (dd, J = 4.45, 2.50 Hz, 1H); 2.67 (dd, J = 4.45, 2.5 Hz, 1H); 2.22 (s, 6H); 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$): 172.7 (s); 142.1 (s); 141.8 (s); 140.5 (s); 126.2 (d); 126.1 (d); 126.0 (d); 125.9 (d); 125.0 (d); 124.3 (d); 124.0 (d); 123.5 (d); 68.6 (d); 60.6 (t); 54.4 (d); 47.5 (d); 47.0 (d); 43.5 (q); 14.2 (q). Anal. ($C_{21}H_{23}NO_2$), C, H, N.

9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethanoanthracene-11-amine-12-carboxylic Acid (28). Yield, 30%; mp 210−212 °C. ^{1}H NMR (DMSO-d_6): 9.7 (br. s, 1H); 7.18 (m, 3H); 6.8 (m, 5H); 4.72 (d, J = 2.2 Hz, 1H); 4.49 (d, J = 2.3 Hz, 1H); 3.57 (br. s, 1H); 2.79 (m, 1H); 2.50 (s, 3H); 2.15 (s, 3H). ^{13}C NMR (DMSO-d_6): 171.9 (s); 142.2 (s); 140.6 (s); 139.9 (s); 137.6 (s); 127.0 (d); 126.8 (d); 126.7 (d); 126.4 (d); 125.7 (d); 124.7 (d); 124.6 (d); 124.5 (d); 47.5 (d); 45.9 (d); 43.8 (d); 41.8 (q); 40.9 (q). Anal. ($C_{19}H_{19}NO_2$), C, H, N.

Method H. Ketone derivative (6.8 mmol), hydrochloride amine (Me_2NH, $Me_2N(CH_2)_2NH_2$, 42.8 mmol), and $NaBH_3CN$ (9.3 mmol) in anhydrous MeOH (20 mL) were stirred at room temperature during 3 days. MeOH was removed under vacuum. The residue obtained was extracted with diethyl ether/eau (pH 1). The aqueous phase was then basified and extracted with CH_2Cl_2. The organic phase was dried under drierite, filtered, and concentrated under vacuum. The product obtained was treated with chlorhydric diethyl ether, and the corresponding hydrochloride obtained was crystallized in MeOH.

9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethanoanthracene-11-amine (39).[42] Yield, 50%; mp 124−125 °C. ^{1}H NMR ($CDCl_3$): 7.24 (m, 4H); 7.08 (m, 4H); 4.51 (d, J = 2.3 Hz, 1H); 4.23 (t, J = 2.5 Hz, 1H); 2.42 (ddd, J = 8.6, 4.3, 2.3 Hz, 1H); 2.21 (s, 6H); 2.04 (ddd, J = 12.0, 8.6, 2.5 Hz, 1H); 1.52 (ddd, J = 12.0, 4.3, 2.5 Hz, 1H). ^{13}C NMR ($CDCl_3$): 143.9 (s); 142.7 (s); 139.8 (s); 125.9 (d); 125.8 (d); 125.5 (d); 125.0 (d); 124.0 (d); 123.3 (d); 123.0 (d); 66.6 (d); 47.1 (d); 44.0 (d); 43.7 (q); 34.3 (t).

***N*′-(9,10-Dihydro-9,10-ethanoanthracen-11-yl)-*N*,*N*-dimethyl-ethylenediamine, Hydrochloride (40).**[43] Yield, 75%; mp 260−262 °C. ^{1}H NMR (D_2O): 7.33 (m, 3H); 7.22 (m, 1H); 7.09 (m, 4H); 4.37 (s, 1H); 3.58 (m, 1H); 3.27 (br. s, 4H); 2.77 (br. s, 7H); 2.17 (td, J = 11.0, 2.7 Hz, 1H); 1.49 (dd, J = 11.0, 2.7 Hz, 1H). ^{13}C NMR (D_2O): 144.2 (s); 143.9 (s); 139.7 (s); 137.0 (s); 128.7 (d); 128.1 (d); 127.6 (d); 127.3 (d); 126.7 (d); 125.7 (d); 125.2 (d); 124.6 (d); 59.0 (d); 53.1 (t); 46.2 (d); 44.0 (q); 43.0 (d); 40.9 (t); 32.5 (t).

Preparation of Carbamidine Derivatives. The primary amide derivatives (3.8 mmol) and dimethylformamide−dimethylacetal (DMF−DMA, 0.27 mol, 17 mL) were heated under reflux during 5 days. DMF−DMA was then eliminated under vacuum. The residue obtained was extracted with CH_2Cl_2/H_2O. The organic phase was dried under drierite, filtered, and concentrated under vacuum. The product obtained was crystallized in methanol. The carbamidine derivative **12** obtained was treated with chlorhydric diethyl ether to obtain the corresponding hydrochloride.

***N*-[(Dimethylamine)methylene]-9,10-dihydro-12-methyl-9,10-ethanoanthracen-11-amine, Hydrochloride (12).** Yield, 68%; mp 146 °C. ^{1}H NMR ($CDCl_3$): 13.87 (m, 1H); 8.39 (m, 1H); 7.60 (m, 1H); 7.25 (m, 2H); 7.06 (m, 5H); 4.94 (s, 1H); 4.01 (s, 1H); 3.68 (s, 3H); 3.23 (s, 3H); 3.15 (br. s, 1H); 2.51 (br. s, 1H); 0.81 (d, J = 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$): 173.6 (s); 153.5 (d); 144.7 (s); 140.9 (s); 140.6 (s); 138.3 (s); 126.2 (d); 125.9 (d); 125.5 (d); 125.3 (d); 124.8 (d); 124.5 (d); 123.2 (d); 53.8 (d); 47.6 (d); 45.8 (q); 41.8 (q); 36.1 (d); 34.6 (d); 20.4 (q). Anal. ($C_{21}H_{22}N_2O$·HCl), C, H, N.

***N*,*N*′-Di[(dimethylamine)methylene]-9,10-dihydro-9,-10-ethanoanthracene-11,12-diamide (18).** Yield, 68%; mp 217 °C. ^{1}H NMR ($CDCl_3$): 8.21 (s, 2H); 7.32 (m, 2H); 7.22 (m, 2H); 7.02 (m, 4H); 4.85 (s, 2H); 3.62 (s, 2H); 3.0 (d, J = 3.3 Hz, 12H). ^{13}C NMR ($CDCl_3$): 186.0 (s); 159.6 (d); 143.7 (s); 141.9 (s); 125.4 (d); 125.3 (d); 124.6 (d); 123.3 (d); 52.0 (d); 47.7 (d); 40.9 (q); 34.9 (q). Anal. ($C_{24}H_{26}N_4O_2$), C, H, N.

Preparation of Quaternary Ammonium Derivative. Amine derivative (1.8 mmol) was treated with CH_3I (3.6 mmol) in methanol (20 mL) at room temperature during 24 h. Methanol was eliminated under vacuum. The residue obtained was washed with acetone and filtered. The solvent was removed under vacuum.

9,10-Dihydro-*N*,*N*,*N*-12-tetramethyl-9,10-ethanoanthracene-11-methanaminium, Iodide (14). Yield, 53%; mp 283−285 °C. ^{1}H NMR (DMSO-d_6): 7.39 (m, 4H); 7.16 (m, 4H); 4.37 (s, 1H); 4.10 (s, 1H); 3.14 (s, 9H); 3.05 (m, 2H); 1.80 (br. s, 1H); 1.52 (m, 1H); 0.88 (d, J = 6.71 Hz, 3H). ^{13}C NMR (DMSO-d_6): 144.9 (s); 142.5 (s); 140.5 (s); 138.6 (s); 126.2 (d); 125.9 (d); 125.7 (d); 125.5 (d); 123.8 (d); 123.2 (d); 69.9 (t); 52.7 (q); 49.6 (d); 47.6 (d); 41.9 (d); 40.9 (d); 19.9 (q). Anal. ($C_{21}H_{26}$-IN), C, H, N.

Preparation of Carbamate Derivative. Alcohol derivative (3.4 mmol), NaH 50% (8.3 mmol), and carbamoyl chloride (3.7 mmol) in THF were stirred at room temperature during 1 day. The mixture was hydrolyzed with water and extracted with CH_2Cl_2. The organic phase was dried under drierite, filtered, and concentrated under vacuum.

9,10-Dihydro-*N*,*N*-dimethyl-9,10-ethanoanthracene-11-dimethylcarbamate-12-methanamine (23). Yield, 25%; mp 78 °C. ^{1}H NMR ($CDCl_3$): 7.30 (m, 4H); 7.12 (m, 4H); 4.42 (s, 1H); 4.24 (s, 1H); 3.87 (m, 1H); 3.60 (m, 1H); 2.90 (s, 6H); 2.19 (s, 6H); 1.87 (m, 2H); 1.64 (m, 2H). ^{13}C NMR ($CDCl_3$): 156.2 (s); 143.7 (s); 143.1 (s); 141.1 (s); 140.5 (s); 125.9 (d); 125.7 (d); 125.5 (d); 125.1 (d); 123.2 (d); 123.1 (d); 67.7 (t); 64.0 (t); 46.2 (d); 46.1 (d); 45.7 (q); 44.0(d); 40.9 (d); 36.2 (q); 35.8 (q). Anal. ($C_{23}H_{28}N_2O_2$), C, H, N.

Computational Chemistry. All molecular modeling was performed using PcModel version 6. 0 molecular modeling software (Serena Software). Models were built from the two experimental structures **19** and **33**[37] for ethano and etheno derivatives, respectively. Only side chains were then modified keeping the bridged tricycle constant. For each compound, we took arbitrarily the *SS* and *SR* diastereoisomers. All calculations were made in vacuo with the RHF calculation option modulated by the nonplanar option for the total Pi system calculation. Geometry optimizations were carried out using the minimizer based on the MMX force field. The conformational analysis of each compound was made using the Dihedral Driver option with a 10° stepwise increment of the dihedral angles. No conformation was eliminated from the search based on energy. Angle files were thus produced, and the MMX energy corresponding to each conformation was evaluated. We thus optimized the geometry in order to obtain for each molecule an estimation of the strain energy of the most stable conformers (E_{min}). Strain energies of interactions between the ligand and the hypothetical model of interaction were obtained using the Dock option. The "Fix Atoms" option of the "Mark" menu was used (when it was necessary) to keep the skeleton of DEEA as closely as possible to the initial position defined by the reference molecule **2d**.

pK_a, log*P*, and log*D* were calculated with Pallas 2.0.[44] Pallas is a tool for making predictions based on the structural formula of compounds. The pK_a values of the compounds are predicted with pKalc.3.1 using approximately 300 Hammett and Taft equations. For log*P* values, the program PrologP 5.1 uses three different systems for the prediction. These systems disintegrate the compound to fragments and express the log*P* value as a superposition of the corresponding fragment constants. The latest superposition method uses about 150 atomic fragments. Prediction of log*D* values with PrologD 2.0 is based on the pK_a and log*P* prediction of the neutral form and on the calculation of the micro and macro dissociation constants of the compound. The linear regression calculation was performed with the Prism 3 software program.[45]

Biology. Strains of *P. falciparum*. A CQ resistant clone W2 (Indochina) and a CQ susceptible clone 3D7 (Africa) were maintained in culture. When required for drug assays, cultures were synchronized by sorbitol lysis.[46] Susceptibilities to CQ, verapamil, promethazine, mecamylamine (hydrochlorides, Sigma), and DEEA derivatives were determined after suspension in RPMI 1640 medium (Life Technologies, Paisley, U.K.), supplemented with 10% human serum (pooled from different A^+ or AB sera from nonimmune donors), and buffered with 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) and 25 mM $NaHCO_3$ (hematocrit of 1.5%, parasitemia of 0.5%).

In Vitro Assay. For in vitro isotopic microtests to determine intrinsic activity, 25 μL/well of antimalarial agents and 175 μL/well of the suspension of parasitized erythrocytes (final parasitemia and hematocrit, 0.5 and 1.5%) were distributed in 96 well plates. To assess synergy between CQ and DEEA compounds, 25 μL of CQ, 25 μL of subinhibitory fixed concentrations of drugs tested, and 150 μL of the suspension of parasitized red blood cells (final parasitemia and hematocrit, 0.5 and 1.5%) were distributed in each well. Parasite growth was assessed by adding 1 μCi of ^{3}H-hypoxanthine with a specific activity of 14.1 Ci/mmol (NEN Products, Dreiech, Germany) to each well. Plates were incubated for 48 h at 37 °C in an atmosphere of 10% O_2, 6% CO_2, 84% N_2, and a humidity of 95%. Immediately after incubation, the plates were frozen and then thawed to lyse the erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter GF/B, Packard Instrument Company, Meriden, CT) and washed using a cell harvester (FilterMate Cell Harvester, Packard). Filter microplates were dried, and 25 μL of scintillation cocktail (Microscint O, Packard) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count, Packard).

The 50% inhibitory concentration (IC_{50}), i.e., the drug concentration corresponding to 50% of the uptake of ^{3}H-hypoxanthine by the parasites in drug-free control wells, was determined by nonlinear regression analysis of log−dose/response curves. Data were analyzed after logarithmic transformation and expressed as the geometric mean IC_{50}, and 95% confidence intervals (95% CI) were calculated.

Evaluation of Drug Interactions. The 50% inhibitory concentration (IC_{50}) of reversal, i.e., the drug concentration of DEEA that leads to a 50% decrease of the IC_{50} value of the CQ used alone, was determined by nonlinear regression analysis of log−dose/IC_{50}.

Measurement of the Uptake of [^{3}H]CQ by Parasitized Erythrocytes. Accumulation of radiolabeled CQ diphosphate [^{3}H]CQ (26 Ci per mmol) (Amersham) was carried out essentially according to the protocol of Bray et al.[30] Infected erythrocytes were suspended in RPMI 1640 medium buffered with 25 mM HEPES and 25 mM $NaHCO_3$, at a parasitemia of 2% and hematocrit of 2%. Eppendorf microfuge tubes were loaded with 400 μL of silicon oil 550, 1 mL of reaction buffer containing [^{3}H]CQ, and reversal drugs on top of the oil, and then with 25 μL of appropriately concentrated cell suspension. Cell suspension was mixed with the reaction buffer and incubated for 1 h at 37 °C in an atmosphere of 10% O_2, 6% CO_2, 84% N_2, and 95% relative humidity. After 1 min of centrifugation at 14 000 rpm, 100 μL of the buffer was processed for scintillation counting. Infected erythrocyte pellets were washed by distilled water and lysed by 5:5:2 ammonia: glacial acetic acid:hydrogen peroxide and left in an oven at 50

°C for 2 h. They were then processed for scintillation counting. CQ accumulation is expressed as the cellular accumulation ratio, which is the ratio of the amount of radiolabeled CQ in parasites (amount of [^{3}H]CQ in parasitized erythrocytes − amount of [^{3}H]CQ in uninfected red cells) to the amount of [^{3}H]CQ in a similar volume of buffer after incubation.[26]

CHO Cell Cultures. CHO cells (ATTC) were maintained in Falcon culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ) and grown at 37 °C in a CO_2 incubator (5% CO_2), as monolayers in Medium 199 (Sigma, St. Louis, MO) supplemented with 5% fetal calf serum (Sigma) and L-glutamine 100 μg/L (Sigma) buffered with $NaHCO_3$ 2.2 g/L (Sigma). Cells were inoculated into 96 well flat-bottomed microtiter plates (Becton Dickinson Labware) at a density of 6×10^4 cells/200 μL culture medium (confluent in 24 h). The cultures were incubated for 24 h. Then, cells in growing phase were exposed for 48 h to several concentrations of antimalarial drugs (25 μL) at 37 °C and at 5% CO_2. Wells without drug served as controls.

Colorimetric MTT (Tetrazolium) Assay.[47] MTT was dissolved in phosphate-buffered saline (PBS) at 1 mg/mL and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. After the incubation of 48 h at 37 °C in a CO_2 incubator (5% CO_2) with the antimalarial drugs (25 μL), the supernatant in each well was removed. To the cells in each well was added 100 μL of the solution of MTT. The plate was gently shaken and incubated for 3 h at 37 °C and 5% CO_2. At the end of the incubation period, the plate was centrifuged at 500*g* for 10 min, and the untransformed MTT was removed. Dimethyl sulfoxide (DMSO, 100 μL) was added to solubilize the produced formazan. The plate was then vigorously shaken to ensure solubilization of the blue formazan. The optical density of each well was measured using an automatic plate reader (Optimax, Molecular Devices, Sunnyvale, CA) with a 570 nm test wavelength and a 630 nm reference wavelength. The 50% inhibitory concentration (IC_{50}), i.e., the drug concentration that reduced cell growth to 50% of that of untreated controls following a 48 h exposure, was determined by nonlinear regression analysis of log−dose/response curves.

In Vitro Toxicity Assay against Uninfected Erythrocytes. Red blood cells (A^+) were obtained from the Blood Transfusion Centre (Military Hospital, Toulon, France). Erythrocytes were washed three times in RPMI 1640 medium (Life Technologies). Erythrocytes were resuspended in RPMI 1640 medium supplemented with 10% human serum and buffered with 25 mM HEPES and 25 mM $NaHCO_3$ to a hematocrit of 1.5%. The suspension was distributed under 200 μL/well into Falcon 96 well plates (Becton Dickinson Labware). Final concentrations of antimalarial drugs (25 μL), which ranged from 2 mM to 1 μM, were distributed in triplicate into plates. A 25 μL amount of water was distributed in triplicate (negative control). In addition, 225 μL of suspension of erythrocytes in saponine solution 5% at a hematocrit of 1.5% was distributed in triplicate (control +). The plate was gently shaken. Plates were then incubated for 42 h at 37 °C in an atmosphere of 10% O_2, 6% CO_2, and 84% N_2 and a humidity of 95% (optimum requirements for isotopic, micro antimalarial drug susceptibility in vitro test). Immediately after incubation, plates were centrifuged at 2200*g* for 10 min and the supernatant of each well was collected and transferred onto a new plate. Serial 4-fold dilutions in glacial acetic acid were performed. A 100 μL amount of a solution of TMB (0.5 mg/mL of glacial acetic acid) was distributed in a new plate. Then, 5 μL of pure and diluted supernatant and 5 μL of hemoglobin standard (30 mg/dL) were added. Then, 100 μL of hydrogen peroxide solution (0.03%) was added in each well. Exactly 10 min after hydrogen peroxide was added, the optical density of each well was measured using an automatic plate reader (Optimax, Molecular Devices) at 455 nm. The data are expressed as hemoglobin concentrations. The 50% hemolytic concentration (HC_{50}), i.e., the drug concentration corresponding to 50% of the lysis of erythrocytes in saponine control wells, was determined by nonlinear regression analysis of log−dose/response curves.

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