Expedited Articles

Antiplasmodial Activity and Cytotoxicity of Bis-, Tris-, and Tetraquinolines with Linear or Cyclic Amino Linkers

Sophie Girault,[†] Philippe Grellier,[‡] Amaya Berecibar,[†,§] Louis Maes,[∥] Pascal Lemière,[†] Elisabeth Mouray,[‡] Elisabeth Davioud-Charvet,[†] and Christian Sergheraert*[,†]

UMR CNRS 8525, Université de Lille II, Institut de Biologie et Institut Pasteur de Lille, 1 rue du Professeur Calmette, B.P. 447, 59021 Lille Cedex, France, Laboratoire de Biologie Parasitaire, IFR CNRS 63, Muséum National d'Histoire Naturelle, 61 rue Buffon, 75005 Paris, France, and Tibotec, B-32800 Mechelen, Belgium

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Bisquinoline heteroalkanediamines were structurally modified in order to study the effects of enhanced bulkiness and rigidity on both their activity on strains of *Plasmodium falciparum* expressing different degrees of chloroquine (CQ) resistance and their cytotoxicity toward mammalian cells. While cyclization yielded molecules of greater rigidity that were not more active than their linear counterparts, they were characterized by an absence of cytotoxicity. Alternatively, dimerization of these compounds led to tetraquinolines that are very potent for CQ-resistant strains and noncytotoxic.

Introduction

Fifty years after its discovery, chloroquine (CQ, **1**, Chart 1) is still a mainstream drug in the fight against malaria, but its efficacy is being eroded by the emergence of resistant parasites.[1,2] CQ is believed to exert its activity by inhibiting haemozoin formation in the digestive vacuole of the malaria carrying the parasite *Plasmodium*.[3,4] Discussion of the exclusivity of this mechanism has recently been renewed by Ginsburg and co-workers who have proposed that inhibition of ferriprotoporphyrin IX degradation by glutathione-dependent redox processes could be an additional mode of action of CQ.[5] Biochemical studies have indicated that isolates of the CQ-resistant parasites accumulate less drug content than their more sensitive counterparts; however, a mechanistic explanation for this observation remains the subject of continuing debate.[6−9] Although CQ resistance may involve several mechanisms, its reversal by molecules such as verapamil, desipramine, and chlorpromazine suggests that an enhanced CQ efflux by a multidrug-resistant mechanism may be implicated.[6,10] A strategy having the potential to overcome this mechanism is the design of quinoline-based drugs that will not be recognized by the proteins involved in drug efflux. In this regard, bulky bisquinolines **2**−**4** (Chart 1) likely to be extruded with difficulty by a proteinaceous transporter[11] have been synthesized and were discovered to inhibit the growth of both CQ-sensitive and CQ-resistant parasites with similar efficacy.[11−13] However, further development of the most promising molecule, **4** (Ro 47-7737),[13] as well as that of other bisquinolines such as piperaquine, hydroxypiperaquine, and dichloroquinazine,[14] has been suspended for reasons of toxicity. Compared with the numerous bisquinolines above, Ro 47-7737 differed by the absence of a proton-accepting side chain and by a greater rigidity likely to reduce its efflux from the parasite while favoring its affinity for ferriprotoporphyrin IX by a decrease in the cost of entropy. With the aim of maintaining both steric hindrance and a reduction of the degrees of freedom while introducing proton-accepting and/or substitution sites, we have designed new bis-, tris-, and tetraquinolines whose 4-amino group belongs to tri- and tetraazamacrocycles (cyclams) (A, C, and D series of Charts 2 and 3). Series B was synthesized as a linear counterpart (Chart 2).

We report here the synthesis and the antiparasitic activities on *Plasmodium falciparum* of these new bis-, tris-, and tetraquinolines. Cytotoxic effects upon human MRC-5 cells (diploid embryonic lung cell line) and mouse peritoneal macrophages are also discussed.

Chemistry

New bis- and trisquinolines were obtained by reacting polyamines with 5 equiv of 4,7-dichloroquinoline in DMF at reflux in the presence of an inorganic base potassium carbonate (Scheme 1). Thick-layer chromatography was used for purification. 1,4,8,11-Tetraazacyclotetradecane was reacted as described above, and after purification, it afforded trisubstituted and disubstituted compounds **5** and **6** (A series of Chart 2). Forcing conditions and a greater excess of 4,7-dichloroquinoline did not yield the tetrasubstituted variant. 1,4,7-Triazacyclononane afforded compound **7**, the disubstituted analogue of **6**, and the monosubstituted compound **8** (A series of Chart 2) but not the trisubstituted derivative. This may have been the result of steric

* To whom correspondence should be addressed. Phone: (33) 3 20 87 12 11. Fax: (33) 3 20 87 12 33. E-mail: christian.sergheraert@pasteur-lille.fr.
[†] Institut de Biologie et Institut Pasteur de Lille.
[‡] Muséum National d'Histoire Naturelle.
[§] Present address: Pfizer Global Research and Development, 3-9 rue de la Loge, 94265 Fresnes Cedex, France.
[∥] Tibotec.

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Chart 1. Chloroquine (**1**, CQ) and Biologically Active Bisquinolines **2**−**4**[11−13]

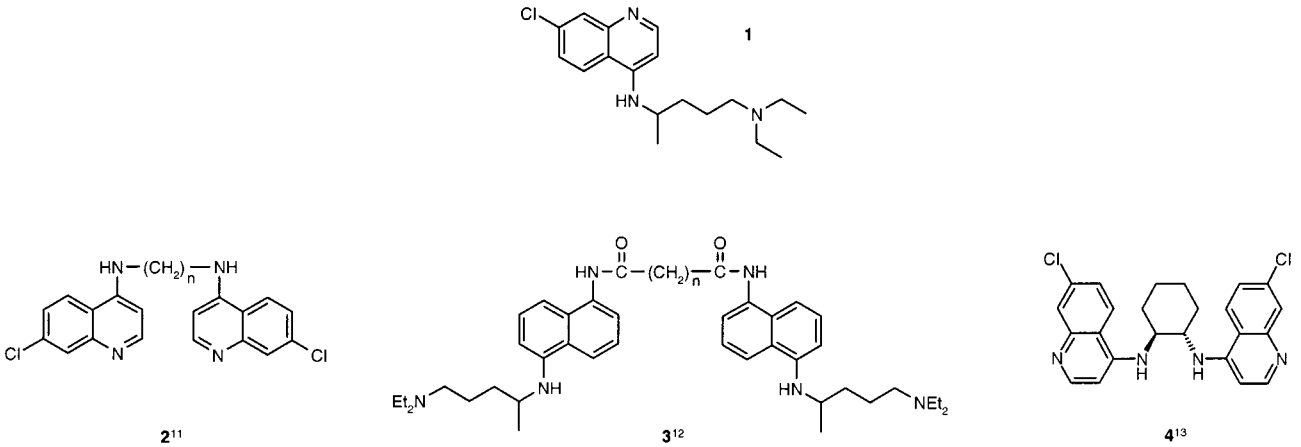
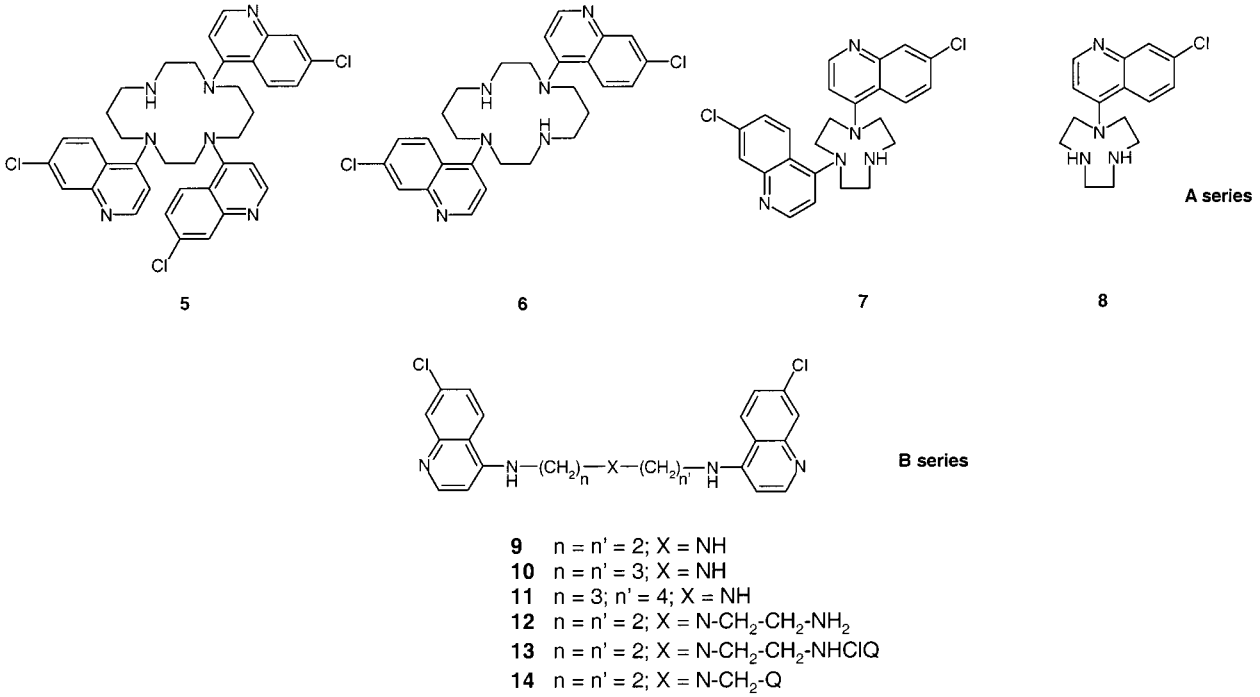


Chart 2. Compounds **5**−**14** (A and B Series)[a]

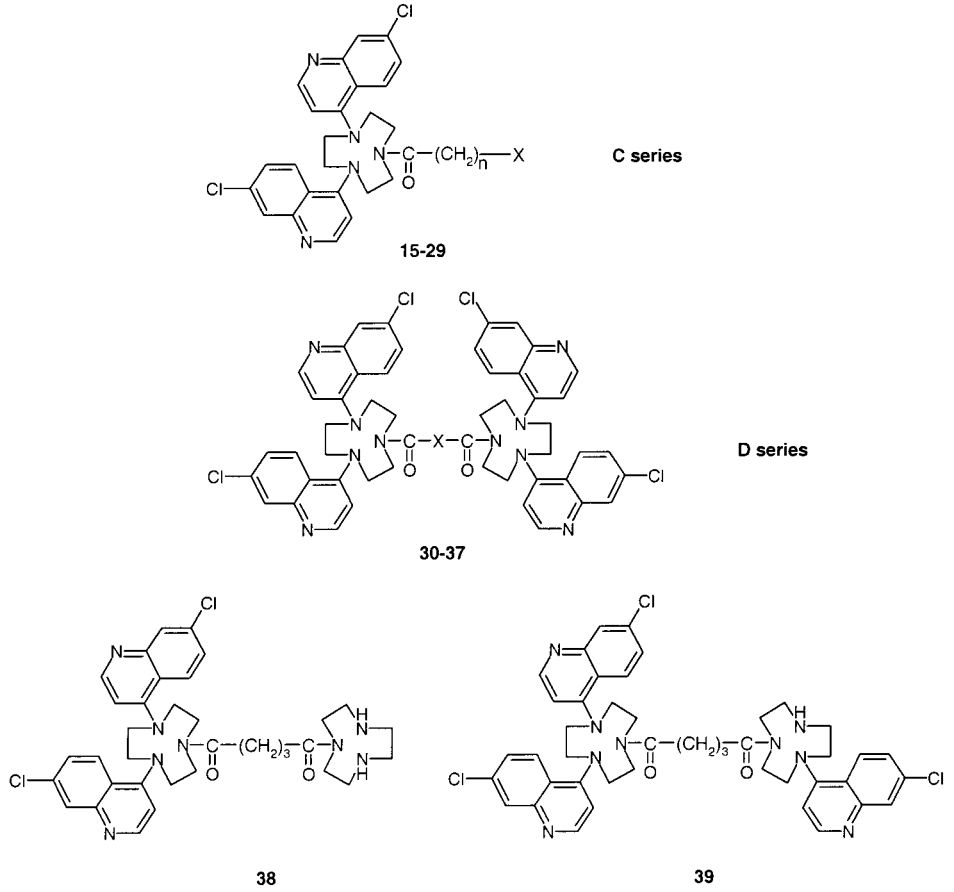


[a] ClQ: 7-chloroquinol-4-yl. Q: quinol-4-yl.

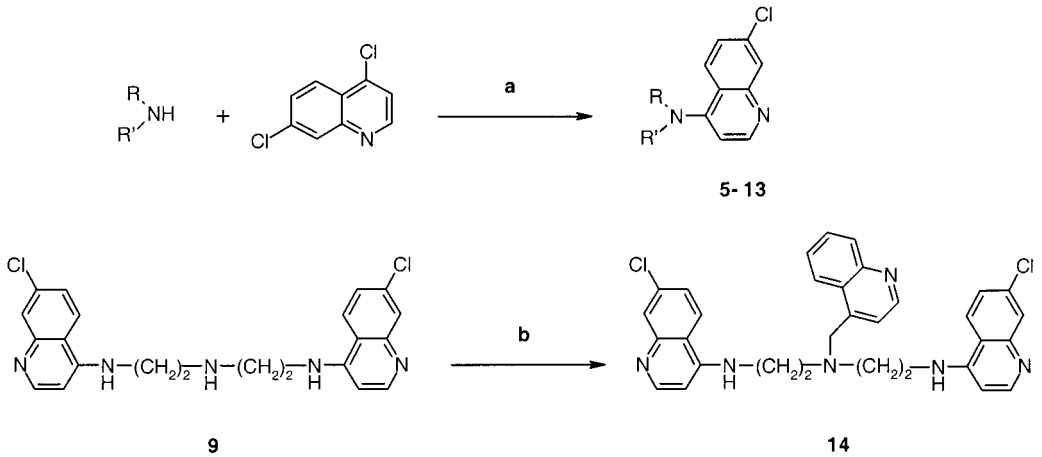
hindrance or of the greater rigidity provided by the ring. Under the same conditions, linear amines diethylenetriamine, *N*-(3-aminopropyl)-1,3-propanediamine, and spermidine were only substituted on primary amino groups, yielding, respectively, amines **9**, **10**, and **11** (B series of Chart 2). Compounds **12** and **13** (Chart 2) were prepared by di- or trisubstitution of tris(2-aminoethyl)-amine, while addition of a third quinoline moiety to compound **9** by reductive amination with 4-quinolinecarboxaldehyde (Scheme 1) afforded trisubstituted compound **14** (Chart 2). Use of *N*-methylpyrrolidinone (NMP) at reflux as solvent had been previously recommended for the obtention of bisquinolines via a displacement reaction with 4,7-dichloroquinoline, alkane- or heteroalkanediamines, and triethylamine.[11,15] Bisquinolines were then isolated by the addition of water and diethyl ether or ethyl acetate to the cooled reaction mixtures, which initiated product precipitation. Under these conditions, yields ranged from 49 to 87% for alkanediamines and only from 12 to 86% for heteroalkanediamines. This decrease explained the low yields that we obtained with linear polyamines and a fortiori with cyclic polyamines, especially since product precipitation in NMP, as with our series of compounds, was not observed. In general, whatever the choice of the solvent (DMF or NMP), it was very difficult to know the fate of polyamines.

Acids **15** and **16** were synthesized, as described in Scheme 2, by reaction of compound **7** with succinic and glutaric anhydride, respectively, while the amide derivative **17** of acid **15** was prepared by treatment with ammonium hydrogen carbonate. In the case of compounds **18**, **20**, **21**, and **25**, the side chain was introduced by coupling various carboxylic acids with the free, secondary amino group of compound **7**, using bromotris-pyrrolidinophosphonium hexafluorophosphate (PyBroP) as coupling reagent (Scheme 2).[16,17] For compounds **26**−**28**, transformation to the amino side chain was achieved in two steps: reaction of secondary amino compound **7** with the appropriate bromo acid followed by substitution of the remaining bromo group by piperidine (Scheme 2). The benzylhydroxyl protecting group

Chart 3. Compounds **15**−**37** (C and D Series), **38**, and **39**



Scheme 1. Synthesis of Compounds **5**−**14**[a]

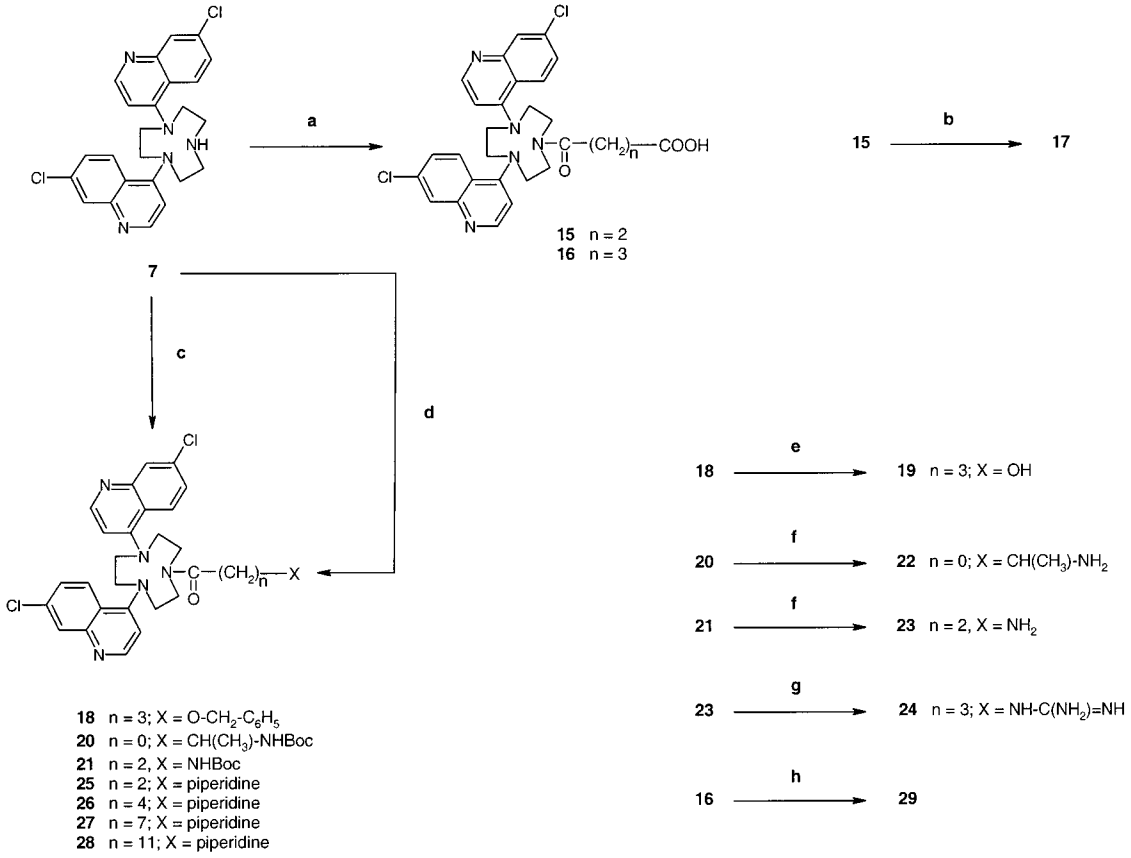


[a] Reagents: (a) K_2CO_3, DMF; (b) 4-quinolinecarboxaldehyde, molecular sieves, absolute EtOH, then $NaBH_4$, absolute EtOH.

was removed from compound **18** by treatment with ammonium formate and Pd/C to give alcohol **19** (Scheme 2). *N*-Boc-amino protecting groups of compounds **20** and **21** were removed by treatment with a 1:1 mixture of TFA/CH_2Cl_2 to give deprotected analogues **22** and **23**, respectively (Scheme 2). The guanidinium derivative **24** was synthesized by treating primary amino compound **23** with 3,5-dimethylpyrazole-1-carboxamide, employing sodium hydrogen carbonate as base (Scheme 2).[18] Compound **29** was synthesized in two steps: (i) formation of the acyl chloride corresponding to acid **16** and (ii) reaction of this latter with compound **9** (Scheme 2).

Bis derivatives of 1,4,7-triazacyclononane and compounds **30** and **32**−**35**, were synthesized as described in Scheme 3 by coupling various diacids with the free, secondary amino group of compound **7**, using the method displayed in Scheme 2. Compound **31** was prepared by coupling acid **15** and amine **7** (Scheme 3). In the case of compound **36**, the synthesis required four steps: (i) reaction of the anhydride, obtained by treatment with DCC[19] of *N*-(*tert*-butoxycarbonyl)iminodiacetic acid, with compound **7**,[19] (ii) formation of the corresponding acyl fluoride by treating acid with cyanuric fluoride,[17] (iii) reaction of the latter with a second

Scheme 2. Synthesis of Compounds **15**−**29**[a]



[a] Reagents: (a) anhydride, pyridine; (b) Boc_2O, NH_4HCO_3, pyridine, DMF; (c) acid, PyBroP, DIEA, DMF for compounds **18**, **20**, **21**, and **25**; (d) (i) bromo acid, PyBroP, DIEA, DMF, (ii) piperidine, 1-pentanol for compounds **26**−**28**; (e) ammonium formate, Pd/C, MeOH; (f) TFA/CH_2Cl_2 (1:1); (g) 3,5-dimethylpyrazole-1-carboxamide, $NaHCO_3$, EtOH; (h) (1) oxalyl chloride, Et_3N, dry CH_2Cl_2, (2) compound **9**, dry CH_2Cl_2.

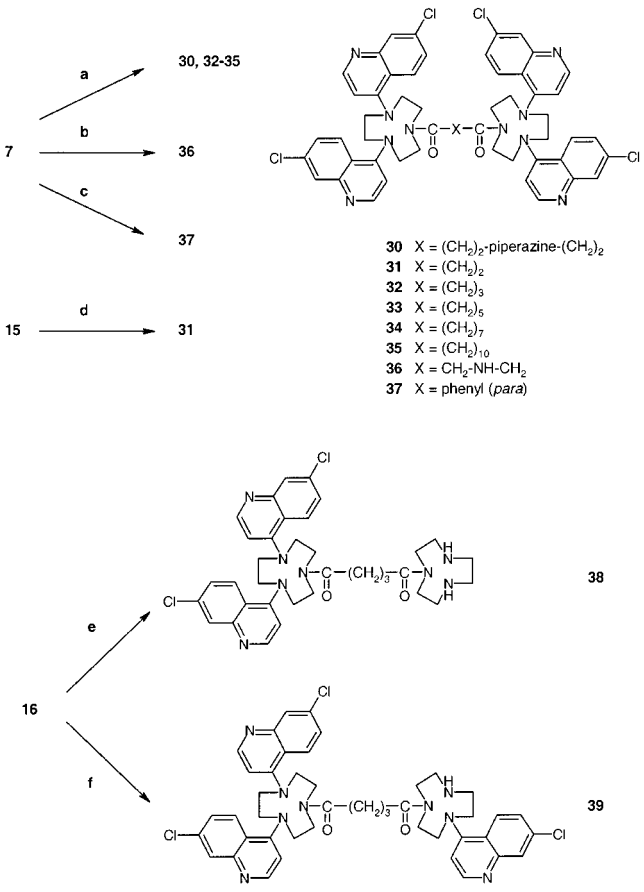
molecule of **7**, and (iv) deprotection of the *N*-Boc-amino protecting group by treatment with a 1:1 mixture of TFA/CH_2Cl_2 to give deprotected derivative **36** (Scheme 3). The phenyl linker of compound **37** was introduced by reaction of terephthaloyl chloride with compound **7**, using diisopropylethylamine as a base (Scheme 3). Compounds **38** and **39** were synthesized by coupling acid **16** with 1,4,7-triazacyclononane and its monoquinoline derivative **8**, respectively, using PyBroP as coupling reagent (Scheme 3).

Biological Results

All of the compounds were first tested for their antimalarial activity upon the CQ-resistant strain FcB1R (Tables 1 and 2, IC_{50} = 110 nM for CQ) and for their cytotoxicity toward human MRC-5 cells and mouse peritoneal macrophages (Tables 3 and 4). Bisquinoline derived from 1,4,7-triazacyclononane, compound **7**, displayed an activity similar to CQ (IC_{50} = 112.8 nM), while its monosubstituted derivative (compound **8**) and the di- and trisubstituted 1,4,8,11-tetrazacyclotetradecane derivatives (compounds **6** and **5**) yielded IC_{50} values greater than 1 μM. While its activity was similar to that of CQ, compound **7** was found to inhibit haem polymerization to a lesser extent (IC_{50} = 170 μM) than CQ (IC_{50} = 65 μM). Among the bisquinolines derived from linear amines (compounds **9**−**11**), compounds **10** and **11** were more active than compound **7** but displayed a high toxicity toward both human MRC-5 cells and mouse peritoneal macrophages while no cytotoxicity was observed for compound **7** (Table 3). Compound **11** inhibited haem polymerization in the same range as CQ (IC_{50} = 83 μM). Compounds **12**−**14**, corresponding to compound **9** substituted at the central nitrogen atom of the linker, were less active than the parent molecule itself (IC_{50} was 605.7 nM, >1 μM, and >1 μM, respectively). Acylation of the remaining cyclic nitrogen of compound **7** led to a comparative weakening in the antimalarial activity (IC_{50} values mostly about 1 μM) irrespective of the nature of the terminal group: the free carboxylic group (compounds **15** and **16**) or carboxamide group (compound **17**), benzyl-protected (compound **18**) or free alcohol group (compound **19**), *N*-Boc-protected (compounds **20** and **21**) or free amino group (compounds **22** and **23**), and guanidinium group (compound **24**). Alternatively, the presence of a terminal piperidine proved favorable to activity independent of the chain length (compounds **25**−**28**, IC_{50} between 38 and 132 nM), yet cytotoxic effects toward human MRC-5 cells and mouse peritoneal macrophages were observed from a concentration of 8 μM.

Whatever the nature of the linker, the presence of an additional chloroquinoline disubstituted 1,4,7-triazacyclononane (compounds **30**−**37**, D series) removed entirely any cytotoxic properties, except in the case of compound **34**, from a concentration of 32 μM (Table 4). Antimalarial activity clearly depended on the length and the nature of the linker; most of the compounds were

Scheme 3. Synthesis of Compounds **30**−**39**[a]



[a] Reagents: (a) diacid, PyBroP, DIEA, DMF; (b) (1) *tert*-butyl-2,6-dioxo-4-morpholinecarboxylate, dry THF, (2) cyanuric fluoride, pyridine, dry CH_2Cl_2, (3) compound **7**, pyridine, dry CH_2Cl_2, (4) TFA/CH_2Cl_2 (1:1); (c) terephthaloyl chloride, DIEA, dry CH_2Cl_2; (d) compound **7**, PyBroP, DIEA, DMF; (e) 1,4,7-triazacyclononane, PyBroP, DIEA, DMF; (f) 1-(7-chloroquinol-4-yl)-1,4,7-triazacyclononane, PyBroP, DIEA, DMF.

more active than both CQ and the starting molecule **7** (IC_{50} between 18 and 103 nM), while a phenyl ring (compound **37**, IC_{50} = 266.2 nM) was found to be less favorable. The presence of one (compound **36**) or two proton-acceptor sites (compound **30**) in the linker, likely to increase vacuolar pH, was also found to be unfavorable to antimalarial activity when compared with a simple polymethylene chain (n = 2 to n = 10). The partial or total absence of chloroquinoline moieties on one of the cyclononanes led to a substantial decrease in activity (compare compound **32**, IC_{50} = 18.3 nM, with its analogues **38** and **39**, IC_{50} > 1000 nM and IC_{50} = 274.7 nM, respectively). In addition, the presence of the second triazacyclononane seems to be crucial for potent antimalarial activity because compound **29**, a linear analogue of compound **32**, displayed a much lower activity (IC_{50} = 152.2 nM) compared with compounds **7** and **9** (see IC_{50} values).

The most active and least cytotoxic compounds of each series (bis- and monoderivatives of 1,4,7-triazacyclononane **7**, **25**, and **30**−**35**) were subsequently evaluated for their capacity to inhibit the growth of other strains expressing different degrees of CQ resistance (Table 5). With the exception of compound **7** they all yielded similar IC_{50} values whatever the CQ resistance of a particular strain. Since a clear correlation between

Table 1. In Vitro Sensitivity of *P. falciparum* FcB1R Strain to Compounds **5**−**29** (A−C Series)

compd	series	n	n'	X[a]	IC_{50} [b] (nM)
5	A				>1000[c]
6	A				>1000[c]
7	A				112.8 ± 24.9[d]
8	A				>1000[c]
9	B	2	2	NH	142.1 ± 10.2[c]
10	B	3	3	NH	75.4 ± 22.6[c]
11	B	3	4	NH	57 ± 6[c]
12	B	2	2	N−CH_2−CH_2−NH_2	605.7 ± 22.7[c]
13	B	2	2	N−CH_2−CH_2−NH−ClQ	>1000[c]
14	B	2	2	N−CH_2−Q	>1000[c]
15	C	2		COOH	>1000[c]
16	C	3		COOH	940[e]
17	C	2		$CONH_2$	980[e]
18	C	3		O−CH_2−C_6H_5	1000[e]
19	C	3		OH	930[e]
20	C	0		$CH(CH_3)$−NHBoc	462.6 ± 41.7[c]
21	C	2		NHBoc	> 1000[e]
22	C	0		$CH(CH_3)$−NH_2	228.3 ± 33.1[c]
23	C	2		NH_2	> 1000[c]
24	C	2		NH−$C(NH_2)$=NH	> 1000[c]
25	C	2		piperidine	38.6 ± 17.7[f]
26	C	4		piperidine	132.2 ± 68.0[c]
27	C	7		piperidine	81.1 ± 24.1[c]
28	C	11		piperidine	108.8 ± 69.8[c]
29	C	3		C(O)-N((CH_2)$_2$-NH−ClQ)$_2$	152.2 ± 9.2[g]

[a] ClQ: 7-chloroquinol-4-yl. Q: quinol-4-yl. [b] IC_{50} = 110 nM for CQ. [c] Number of expts n = 3. [d] Number of experiements n = 5. [e] Number of experiements n = 2. [f] Number of experiements n = 6. [g] Number of experiements n = 4.

Table 2. In Vitro Sensitivity of *P. falciparum* FcB1R Strain to Compounds **30**−**37** (D Series), **38**, and **39**

compd	series	n	n'	X	IC_{50} [a] (nM)
30	D			CH_2−CH_2−piperazine−CH_2−CH_2	76.9 ± 9.4[b]
31	D			$(CH_2)_2$	38.1 ± 14.3[c]
32	D			$(CH_2)_3$	18.3 ± 9.3[c]
33	D			$(CH_2)_5$	32.7 ± 13.5[b]
34	D			$(CH_2)_7$	22.6 ± 7.2[b]
35	D			$(CH_2)_{10}$	79.1 ± 27.6[b]
36	D			CH_2−NH−CH_2	102.6 ± 20.1[b]
37	D			phenyl (para)	266.2 ± 30.1[b]
38		3			>1000[d]
39		3			274.7 ± 32.7[b]

[a] IC_{50} = 110 nM for CQ. [b] Number of experiments n = 3. [c] Number of experiments n = 6. [d] Number of experiments n = 2.

the inhibition of parasite growth and that of haem polymerization was found for many bisquinolines,[15] inhibition of haem polymerization was evaluated for CQ and the two most active compounds **25** (C series) and **32** (D series). When tested at 65 μM, the IC_{50} value of CQ in the haem polymerization assay of compounds **25** and **32** displayed 70% and 66% inhibition, respectively.

Discussion

Although the relationship between the P-glycoprotein homologue 1 protein (Pgh1) of *P. falciparum* and resistance to CQ and mefloquine (MF) remains debated, there is now evidence that mutation occurring on Pgh1 can confer resistance to MF and can influence the parasite resistance to CQ along with the structurally unrelated compound artemisinin.[10] Bisquinolines likely to be extruded with difficulty by a proteinaceous transporter[11] were synthesized with the aim of avoiding CQ resistance. New products were discovered that inhibit the growth of CQ-sensitive and CQ-resistant parasites. However, further development was suspended for rea-

Table 3. In Vitro Cytotoxicity of Compounds **5**−**29** on MRC-5 Cells and Mouse Peritoneal Macrophages

	cytotoxicity on MRC-5 cells (%)				cytotoxicity on mouse peritoneal macrophages[a]		
compd	concn = 32 μM	concn = 8 μM	concn = 1 μM	concn = 0.5 μM	concn = 32 μM	concn = 8 μM	concn = 2 μM
5	18	13	0	0	T	–	–
6	98	0	0	0	T	–	–
7	0	0	0	0	–	–	–
8	0	0	0	0	–	–	–
9	81	0	0	0	T	–	–
10	100	98	98	0	T	T	T
11	100	100	0	0	T	T	–
12	51	0	0	0	–	–	–
13	0	0	0	0	–	–	–
14	0	0	0	0	–	–	–
15	0	0	1	0	–	–	–
16	0	0	0	0	–	–	–
17	0	0	0	0	–	–	–
18	14	0	0	0	T	–	–
19	0	0	0	0	–	–	–
20	0	0	0	0	–	–	–
21	100	0	0	0	T	–	–
22	88	0	0	0	T	T	–
23	0	0	0	0	–	–	–
24	0	0	0	0	–	–	–
25	91	87	0	0	T	T	–
26	92	92	0	0	T	T	–
27	91	91	0	0	T	T	T
28	89	92	11	0	T	T	T
29	0	0	0	0	–	–	–

[a] The letter T means that the compound is toxic at this concentration; – denotes no toxicity.

Table 4. In Vitro Cytotoxicity of Compounds **30**−**39** on MRC-5 Cells and Mouse Peritoneal Macrophages

	cytotoxicity on MRC-5 cells (%)				cytotoxicity on mouse peritoneal macrophages[a]		
compd	concn = 32 μM	concn = 8 μM	conc = 1 μM	concn = 0.5 μM	concn = 32 μM	concn = 8 μM	concn = 2 μM
30	0	0	0	0	–	–	–
31	0	0	0	0	–	–	–
32	0	0	0	0	–	–	–
33	0	0	0	0	–	–	–
34	0	0	0	0	T	–	–
35	0	0	0	0	–	–	–
36	0	0	0	0	–	–	–
37	0	0	0	0	–	–	–
38	0	0	0	0	–	–	–
39	0	0	0	0	–	–	–

[a] The letter T means that the compound is toxic at this concentration; – denotes no toxicity.

sons of toxicity, including that of the most potent candidate, Ro 47-7737, unique for the steric hindrance attributed to its cyclohexyl ring. Decreasing the degrees of freedom of a drug is a well-known method of enhancing acutely its affinity for a target by reducing the loss in entropy. Simultaneously, interactions with other undesired targets responsible for certain levels in toxicity can be limited. We have therefore sought to augment the rigidity of bisquinolines linked via short alkanediamines already reported as revealing a promising level of activity.[11] The new structures were obtained by introducing chloroquinoline moieties to two azamacrocycles, retaining one or two amino groups for substitution. Bisquinoline obtained from the smallest cycle (compound **7**), noncytotoxic at 32 μM, was found to be more active than its tetraazamacrocycle homologue (compound **6**) toward the CQ-resistant strain FcB1R and was therefore selected for optimization. While compound **7** inhibited haem polymerization (IC_{50} = 170 μM) with less potency than CQ, it revealed nonetheless a CQ-like behavior because a correlation was observed between the growth inhibition of 12 different strains of *P. falciparum* and the degree of resistance to CQ (data not shown). In the absence of a possible rational design, acylation of the remaining amino group of compound **7** by a number of different substituents with linkers of varying length (C series) and the dimerization of the resultant molecules (D series) were carried out, and the impacts of these modifications were measured against both CQ-resistant strains and haem polymerization. In the C series, the presence of a terminal amino group (compound **23**) or a guanidino group (compound **24**), which likely allows interaction with haem propionate groups, greatly reduces antimalarial activity. The same effect is observed with the other terminal groups, whatever their nature, and in the presence of a protective group, except for that with a hydrogen bond acceptor such as piperidine (compounds **25**−**28**). In the latter case, the length of the linker proves to be insignificant for antimalarial activity. However, these derivatives are toxic on MRC-5 cells and mouse peritoneal macrophages at concentrations of 8 and 2 μM, respectively. Molecular variations based on the dimerization of compound **7** (D series) led to an increase of the antimalarial activity (6-fold for compound **32**) except for the case of a rigid spacer (compound **37**). Furthermore, the absence of in vitro cytotoxic effects was noted. The presence of one (compound **36**) or two proton-acceptor sites (compound **30**) was found to be unfavorable for antimalarial activity when compared with a simple polymethylene chain (n = 2 to n = 10). These results confirm those previously reported, proving that an increase in the alkalinity of the food vacuole by quinoline drugs and alkylamines does not correlate well with their antimalarial activity.[20] The most active compounds (**30**−**35**) inhibited, in the same range, the growth of parasites expressing different degrees of resistance to CQ and to MF, since the F32 and the W2 strains are respectively 10 and 5 times more resistant to MF than to the FcB1R strain (data not shown). The four isoquinoline moieties are vital for antimalarial activity, as proved by the superior IC_{50} values of compounds **39** and **38** when compared with that of compound **32**. The fact that the most active compounds in the C and D series (compounds **25** and **30**−**35**) displayed similar IC_{50} values irrespective of the CQ

Table 5. Efficiency of Compounds **7**, **25**, and **30**−**35** To Inhibit Growth of Parasites Expressing Different Degrees of Resistance to CQ

	IC_{50}[a] (nM) *P. falciparum* strain			
compd	W2	FcB1R	D6	F32
CQ	175 ± 31	110 ± 26	54 ± 12	19 ± 4
7	170.1 ± 19.7	112.8 ± 24.9	74.5 ± 7.3	64.6 ± 2.2
25	50.2 ± 7.0	38.6 ± 17.7	18.9 ± 4.9	16.1 ± 2.5
30	111.9 ± 13.4	76.9 ± 9.4	59.7 ± 4.4	68.3 ± 3.1
31	29.5 ± 5.7	38.1 ± 14.3	21.9 ± 5.8	23.7 ± 2.2
32	29.2 ± 2.6	18.3 ± 9.3	18.5 ± 3.9	17.9 ± 2.4
33	47.8 ± 6.4	32.7 ± 13.5	29.3 ± 8.1	34.5 ± 1.7
34	67.2 ± 4.9	22.6 ± 7.2	41.7 ± 8.2	46.3 ± 4.6
35	179.9 ± 5.5	79.1 ± 27.6	123.2 ± 38.0	132.6 ± 4.1

[a] Parasites were considered resistant to CQ for IC_{50} > 100 nM. IC_{50} values are the mean ± standard deviation of three independent experiments except for the FcB1R strain (see Tables 1 and 2).

resistance of the strain, while inhibiting haem polymerization in the same range as CQ (compounds **25** and **32**), might suggest that their greater bulkiness results in a weaker efflux by a parasite transporter, although a mechanism of action differing from that of CQ should not be excluded.

In conclusion, an increase in rigidity by cyclization yielded molecules that were not more active than their linear counterparts but yet differed by an absence of cytotoxic effects. Dimerization led to tetraquinolines that are both very potent for CQ-resistant strains and noncytotoxic.

Materials and Methods

Biological Evaluation. 1. In Vitro *P. falciparum* Culture and Drug Assays. *P. falciparum* strains were maintained continuously in culture on human erythrocytes as described by Trager and Jensen.[21] In vitro antiplasmodial activity was determined using a modification of the semiautomated microdilution technique of Desjardins et al.[22] *P. falciparum* CQ-sensitive (F32/Tanzania and D6/Sierra-Leone) and CQ-resistant (FcB1R/Colombia and W2/Indochina) strains were used in sensitivity testing. FcB1R and F32 were strains obtained by limit dilution. Stock solutions of chloroquine diphosphate and test compounds were prepared in sterile, distilled water and DMSO, respectively. Drug solutions were serially diluted with culture medium and added to asynchronous parasite cultures (0.5% parasitemia and 1% final hematocrite) in 96-well plates for 24 h at 37 °C prior to the addition of 0.5 μCi of [^{3}H]hypoxanthine (1−5 Ci/mmol; Amersham, Les Ulis, France) *per* well. The growth inhibition for each drug concentration was determined by comparison of the radioactivity incorporated into the treated culture with that in the control culture (without drug) maintained on the same plate. The concentration causing 50% inhibition (IC_{50}) was obtained from the drug concentration−response curve, and the results were expressed as the mean ± the standard deviations determined from several independent experiments. The DMSO concentration never exceeded 0.1% and did not inhibit the parasite growth.

2. Haem Polymerization Assay. The drug effects on haem polymerization were assessed according to Raynes et al.[23] The haemozoin content was determined using the procedure of Chou and Fitch.[24] Briefly, a 50 μL aliquot of insoluble trophozoite material of the FcB1R strain (approximately equivalent to 4×10^7 parasites) was added to 900 μL of a haem/acetate mixture (0.3 mM bovine haematin, 60 mM sodium acetate, pH 5). A total of 50 μL of drug solution at different concentrations was mixed with the other components. Samples without drug constituted controls. All of the samples contained the same amount of DMSO (1%). After incubation for 4 h at 37 °C, the samples were centrifuged at 27 000 *g* for 15 min at 4 °C. The pellet was resuspended in 1 mL of buffer A (68 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 5 mM glucose, 50 mM sodium phosphate, pH 7.4) and repelleted. This second pellet was resuspended with 2.5% SDS in buffer A and sonicated for 10 min. The polymerized haem was collected by centrifugation at 27 000 *g* for 30 min at 20 °C. The pellet was then washed four times before being resuspended in 900 μL of 2.5% SDS in buffer A, and 100 μL of 1 M NaOH was added to dissolve the polymerized haem. After incubation for 1 h, the concentration of haemozoin was determined by measuring the absorbance at 404 nm.[25] The amount of haemozoin formed during the incubation period was corrected for the endogenous haemozoin of the trophozoite preparation, and the concentration of drug required to produce 50% inhibition of haem polymerization (IC_{50}) was determined. Data presented are the mean of two independent experiments each performed in duplicate.

3. Cytotoxicity Test on MRC-5 Cells and Mouse Peritoneal Macrophages. A human diploid embryonic lung cell line (MRC-5, Bio-Whittaker 72211D) and mouse primary peritoneal macrophages were used to assess the cytotoxic effects toward host cells. The peritoneal macrophages were collected from the peritoneal cavity 48 h after stimulation with potato starch and seeded in 96-well microplates at 30 000 cells *per* well. MRC-5 cells were seeded at 5000 cells *per* well. After 24 h, the cells were washed and 2-fold dilutions of the drug were added in 200 μL standard culture medium (RPMI + 5% FCS). The final DMSO concentration in the culture remained below 0.5%. The cultures were incubated with four concentrations of compounds (32, 8, 1, and 0.5 μM) at 37 °C in 5% CO_2−95% air for 7 days. Untreated cultures were included as controls. For MRC-5 cells, the cytotoxicity was determined using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue)) assay[26] and scored as a percent reduction of absorption at 540 nm of treated cultures versus untreated control cultures. For macrophages, scoring was performed microscopically.

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Supporting Information Available: Details of chemical procedures and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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