Articles

Antimalarial, Antitrypanosomal, and Antileishmanial Activities and Cytotoxicity of Bis(9-amino-6-chloro-2-methoxyacridines): Influence of the Linker[#]

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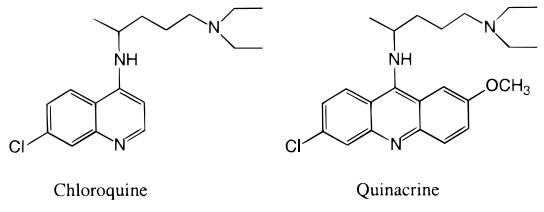
Forty bis(9-amino-6-chloro-2-methoxyacridines), in which acridine moieties are joined by alkanediamines, polyamines, or polyamines substituted by a side chain, were synthesized and tested for their in vitro activity upon the erythrocytic stage of *Plasmodium falciparum*, trypomastigote stage of *Trypanosoma brucei*, and amastigote stage of *Trypanosoma cruzi* and *Leishmania infantum* as well as for their cytotoxic effects upon MRC-5 cells. Results clearly showed the importance of the nature of the linker and of its side chain for antiparasitic activity, cytotoxicity, and cellular localization. Among several compounds devoid of cytotoxic effects at 25 μM upon MRC-5 cells, one displayed IC_{50} values ranging from 8 to 18 nM against different *P. falciparum* strains while three others totally inhibited *T. brucei* at 1.56 μM.

Introduction

Malaria, trypanosomiasis, and leishmaniasis are major diseases in developing countries which continue to infect several hundreds of millions of people and which are responsible for a mortality rate in excess of 1 million per year. Present chemotherapies are proving inadequate, are toxic, or are becoming ineffective due to an increase in resistance. The trivalent arsenical drug melarsoprol is still widely used against the second stage of African trypanosomiasis in which *Trypanosoma brucei rhodesiense* or *gambiense* has invaded the central nervous system. The toxicity of this treatment is responsible for the death of approximately 5% of its recipients. Benznidazole, the only commercial drug still available for treatment during the chronic stage of Chagas' disease, is no longer efficient, while its safety continues to be debated. Against *Leishmania* parasite, amphotericin B and pentamidine are toxic in therapeutic doses, while resistance to pentavalent antimonials is increasing. The effectiveness of chloroquine (CQ, Chart 1), the cheap, antimalarial mainstay for more than 50 years, is also being undermined by the evolution of resistant parasites.[1,2] CQ is believed to exert its activity by inhibiting hemozoin formation in the digestive vacuole of the malaria parasite,[3,4] though this has recently been questioned by Ginsburg and co-workers who have suggested that inhibition of the degradation of ferriprotoporphyrin IX by glutathione-dependent redox processes could be a second mode of action of CQ.[5] Biochemical studies indicate that isolates of the CQ-resistant parasite accumulate less drug in the food vacuole than their more sensitive counterparts. However, opinion remains divided upon the mechanistic explanation for the reduction: (1) a rapid CQ efflux mechanism by CQ-resistant parasites,[6] (2) an elevated pH in the food vacuole of the CQ-resistant parasites,[7] (3) resistance being linked to a carrier-mediated CQ uptake,[8] (4) resistance being linked to a reduced CQ affinity to ferriprotoporphyrin.[9] CQ resistance may

Chart 1. Structure of Antimalarial Molecules



[#] Abbreviations: CQ, chloroquine; DAPI, 4,6-diamidino-2-phenylindole; MF, mefloquine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue); PyBrop, bromotris(pyrrolidinophosphonium) hexafluorophosphate.

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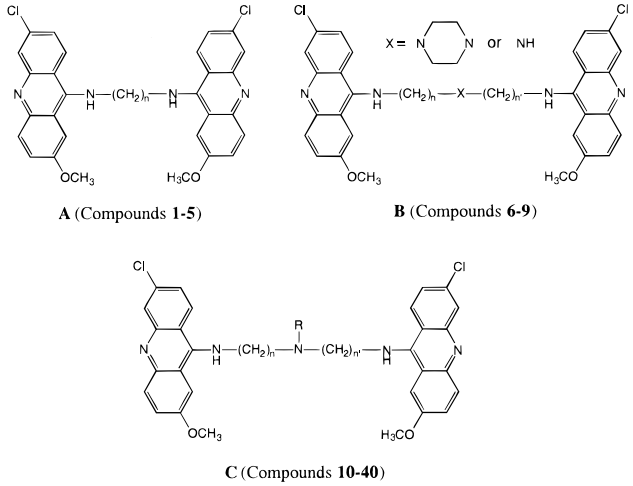
involve several mechanisms; however its reversal by molecules such as verapamil, desipramine, and chlorpromazine suggests that an enhanced CQ efflux by a multidrug-resistant mechanism may be implicated.[6,10] One possibility to overcome this mechanism is to design quinoline-based drugs which are not recognized by the proteins involved in drug efflux. In this regard, bulky bisquinolines were synthesized and suggested to be extruded with difficulty by a proteinaceous transporter.[11] They were also discovered to inhibit the growth of CQ-sensitive and CQ-resistant parasites with similar efficiency though it was not proved that steric hindrance was the deciding factor for this.[11−14] Further development of the most promising molecule Ro 47-7737[13] (Chart 1) as that of other bisquinolines, described earlier, piperaquine, hydroxypiperaquine, and dichloroquinazine,[15] was suspended for reasons of toxicity.

Acridine derivatives have been considered as potential antiprotozoal agents, e.g: 9-anilinoacridine topoisomerase II inhibitors show high levels of antileishmanial and antitrypanosomal activity.[16] Furthermore, quinacrine, the 9-amino-6-chloro-2-methoxyacridine analogue of CQ (Chart 1), which was used clinically before CQ, is still an approved treatment of giardiasis. Sharing the same features as a weak diprotic base, it accumulates in the acidic food vacuole (pH = 5) of *Plasmodium* and prevents hematin polymerization.[4] Until now, bisacridines have been poorly studied for their antiparasitic activity, yet they, along with bisquinolines, may represent an alternative method to avoiding the efflux mechanism. In addition bisacridines offer the advantage of being viewed precisely by cellular fluorescence imaging. However, the presence of an acridine moiety in a molecule can lead to stronger interactions with DNA and generate cytotoxicity, especially in the case of bisintercalation. Bisintercalation requires a minimal distance between the acridine rings. Bis(9-amino-6-chloro-2-methoxyacridines) corresponding to a spermine or spermidine linker were found to bisintercalate, while the removal of a single carbon (symmetrical linker $-NH-(CH_2)_3-NH-(CH_2)_3-NH-$) suppressed bisintercalation.[17] It was also reported that acridine substituents play a role in intercalation and DNA recognition[18,19] and that an increase of rigidity can favor binding affinity in relation to the subsequent decrease of the loss in entropy.[20,21] Besides, for unsubstituted bisacridines, an evolution from monofunctional to bifunctional interaction was found by lengthening the methylene chain from four carbons to six.[19,22,23]

We have therefore undertaken the synthesis and an in vitro antiparasitic activity study upon *Plasmodium falciparum* of three series of bisacridines in which aromatic rings are joined by alkanediamines, polyamines, or polyamines substituted by a side chain (Chart 2). In the latter case, the length of the linker proved unfavorable to bisintercalation, while the nature of the side chain could contribute to either a diminishing or canceling of the interaction with the phosphate groups of human DNA and consequently to a decrease of cytotoxicity. Antiparasitic efficacy of these compounds against *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania infantum*, as well as the evaluation of their cytotoxicity, is also reported.

Chart 2. Bisacridines of the A, B, and C Series

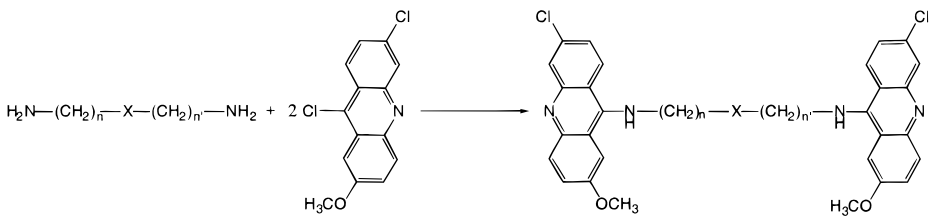


Chemistry

In preference to the generally adopted method, employing phenol, as described by Canellakis et al,[24] bisacridines **1**−**9** (A and B series, Chart 2) were synthesized according to the method described for 4-aminoquinolines preparation,[25] by reacting polyamines with 3 equiv of 6,9-dichloro-2-methoxyacridine in DMF at reflux, in the presence of potassium carbonate as an inorganic base (Scheme 1). Chromatography on silica gel columns was the selected method for purification.

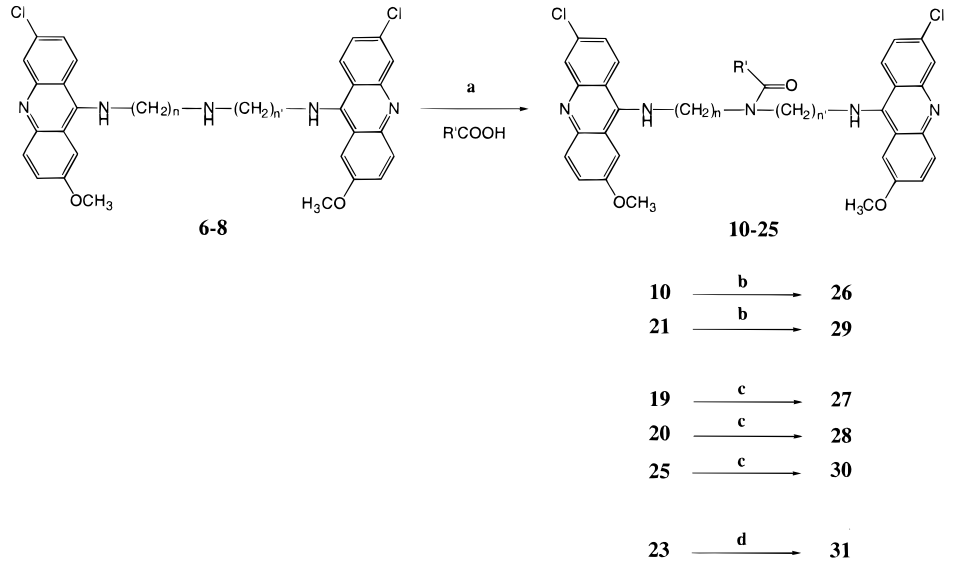
In the C series (Chart 2), the side chain was fixed by coupling various carboxylic groups with the central, secondary amino group of compounds **6**−**8**, using PyBroP reagent.[26,27] Thus, bisacridines **10**−**25** were synthesized, as shown in Scheme 2, by coupling bisacridines **6**−**8** with appropriate *N*-Boc- and/or *N*-Fmoc-amino acids. *N*-Boc-amino protecting groups of compounds **10** and **21** were removed by treatment with a 1:1 mixture of TFA/CH_2Cl_2 to give deprotected analogues **26** and **29,** respectively (Scheme 2). *N*-Fmoc-amino or Fmoc-ester protecting groups of compounds **19**, **20**, and **25** were removed by treatment with a 20:80 piperidine/DMF mixture to give deprotected analogues **27**, **28,** and **30,**

Scheme 1. Synthesis of Bisacridines **1**−**9**[a]



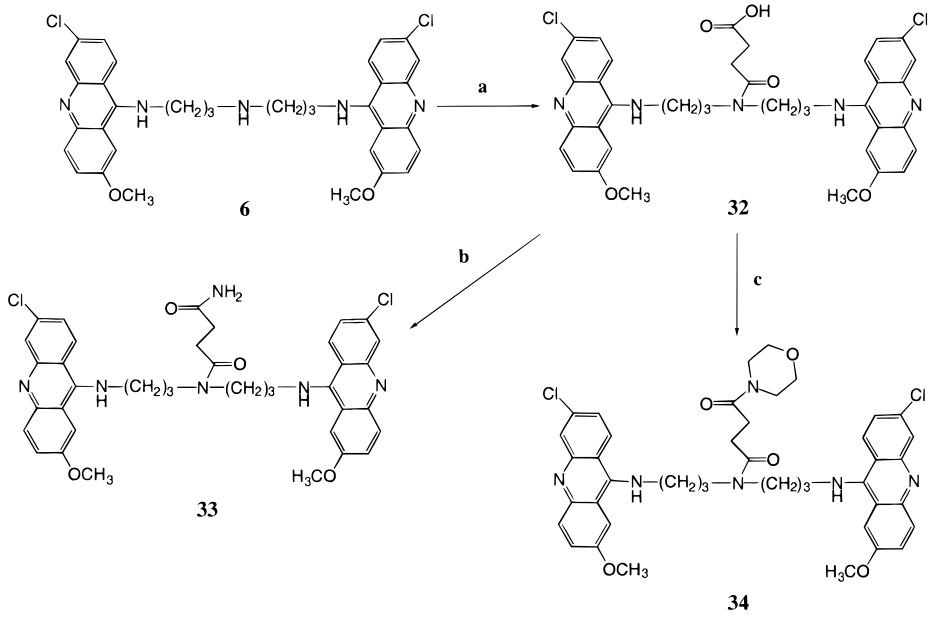
[a] Reagents: K_2CO_3, DMF.

Scheme 2. Synthesis of Bisacridines **10**−**31**[a]



[a] Reagents: (a) PyBrop, DIEA, DMF; (b) TFA/CH_2Cl_2 (1:1); (c) piperidine/DMF (20:80); (d) K_2CO_3, H_2O, MeOH.

Scheme 3. Synthesis of Bisacridines **32**−**34**[a]



[a] Reagents: (a) succinic anhydride, pyridine; (b) Boc_2O, NH_4HCO_3, pyridine, DMF; (c) morpholine, PyBrop, DIEA, DMF.

respectively (Scheme 2). Acetyl protection of compound **23** was removed by treatment with an aqueous solution of K_2CO_3 to give deprotected compound **31** (Scheme 2). Thick-layer chromatography was used for purification.

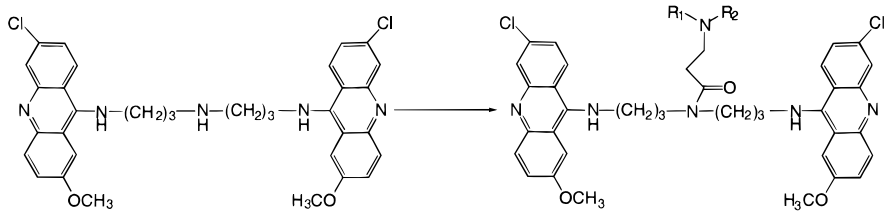
Compound **32** was synthesized, as shown in Scheme 3, by reaction of bisacridine **6** with succinic anhydride, while its amide analogue **33** was prepared by treating compound **32** with ammonium hydrogenocarbonate. The morpholinoamide derivative **34** was synthesized by coupling the carboxylic acid **32** with morpholine using the coupling method described in Scheme 2.

In the case of bisacridines **35**−**40**, fixation of the amino side chain was accomplished in two steps: the reaction of secondary amino compound **6** with 3-chloropropionyl chloride, using 1-ethylpiperidine as a base, followed by substitution of the remaining chloro group by the appropriate amine (Scheme 4).[27] As a tertiary amine 1-ethylpiperidine was preferred to pyridine which was previously reported to lead to a nonconvertible pyridinium salt.[28]

Biological Results

In Vitro Antimalarial Activity upon *P. falciparum*. Initially all of the compounds were tested for their antimalarial activity upon the CQ-resistant strain FcB1R (IC_{50} CQ = 138.6 nM, IC_{50} MF = 6.9 nM). In the A series (Table 1), increasing the length of the alkanediamine linker proved unfavorable toward activity (IC_{50} values increased from 16 to 176 nM from 6 to 12 carbons). Values in the B series (Table 1), were consistent regardless of the distance between the nitrogen atoms of the linker (IC_{50} around 60 nM), except for the piperazine derivative **9** which proved more efficient (IC_{50} = 17 nM). In comparison with all other compounds of the A and B series, this derivative also showed a unique and contrasting behavior at the

Scheme 4. Synthesis of Bisacridines **35**−**40**[a]



6 **35-40**

[a] Reagents: 3-chloropropionyl chloride, 1-ethylpiperidine, THF, then R_1R_2NH, THF.

Table 1. In Vitro Sensitivity of *P. falciparum* FcB1R Strain to Bisacridines **1**−**9** (A and B Series)

molecule	series	central amine	*n*	*n′*	IC_{50} (nM)[a]
1	A		4		18 ± 7[b]
2	A		6		16 ± 10[b]
3	A		8		19 ± 10[b]
4	A		10		57 ± 30[b]
5	A		12		176 ± 96[b]
6	B	NH	3	3	55 ± 9[c]
7	B	NH	2	2	63 ± 10[b]
8	B	NH	3	4	61 ± 3[b]
9	B	piperazine	3	3	17 ± 10[d]

[a] IC_{50} CQ = 138.6 ± 8.4 nM; IC_{50} MF = 6.9 ± 0.3 nM. [b−d] *n*, number of experiments: [b]*n* = 3; [c]*n* = 5; [d]*n* = 4.

cellular localization level. In confocal microscopy, while infected erythrocytes incubated with 10 μM quinacrine displayed a weak fluorescence associated with the parasite food vacuole and the erythrocytic and parasitic membranes (data not shown), a high fluorescence, associated exclusively with the parasite, was observed with 1 μM compound **9** (Figure 1A). No labeling of the food vacuole and of the normal or infected erythrocyte cytosols/membranes was recorded. Fluorescence was mainly concentrated in structures which colocalized with propidium iodide or DAPI labeling, suggesting a concentration of compound **9** in the parasite nuclei (data not shown). In murine muscle L-6 cells, which were used as a control, no nuclear labeling was observed; the fluorescence of compound **9** was only associated with

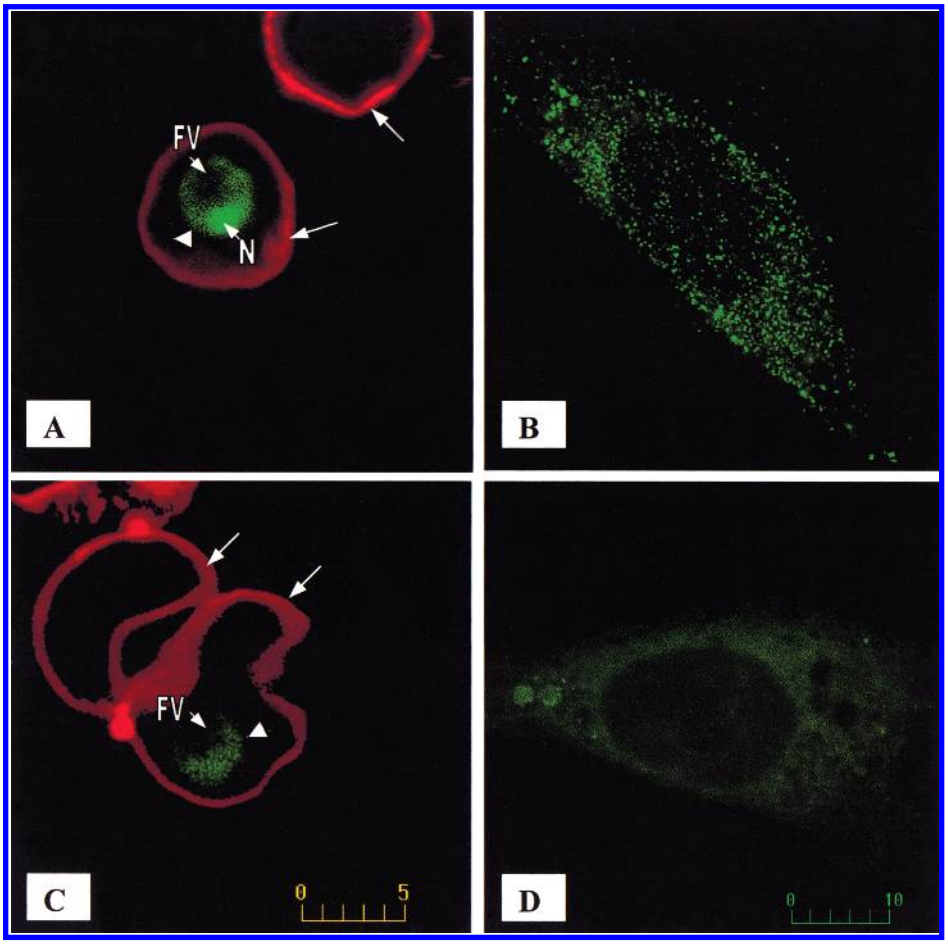


Figure 1. Labeling of *P. falciparum*-infected erythrocytes (A, C) and mouse L-6 cells (B, D) using compounds **9** (A, B) and **1** (C, D). Infected cultures of *P. falciparum* were incubated with 1 μM bisacridine derivative and then with 10 μg/mL nonexchangeable lipid DIL C_{18}(3) (see Experimental Section). DIL C_{18}(3) permitted the visualization of the membranes of the infected and noninfected erythrocytes (arrow). Bisacridine derivatives accumulated specifically in the intracellular parasites (arrowhead). Food vacuole (FV) was not labeled. For compound **9**, a high fluorescence was associated with a structure which colocalized with the parasite nucleus (N). L-6 cells were only labeled with bisacridine derivatives. Bar scale is in μm. A and B are images of one section, C and D are 3D reconstructions of a sequential image collection.

Table 2. In Vitro Sensitivity of *P. falciparum* FcB1R Strain to Bisacridines **10**−**40** (C Series)

molecule	n	n'	R[a]	IC_{50} (nM)[b]
10	3	3	CO-CH(NHBoc)-CH_3	31 ± 2[c]
11	2	2	CO-CH(NHBoc)-CH_3	109 ± 3[d]
12	3	4	CO-CH(NHBoc)-CH_3	330 ± 59[d]
13	3	3	CO-CH(NHBoc)-CH_3 (D enantiomer)	33 ± 2[d]
14	3	3	CO-CH_2-NHBoc	92 ± 19[e]
15	3	3	CO-CH(NHBoc)-CH_2-$CH(CH_3)_2$	27 ± 4[d]
16	3	3	CO-CH(NHBoc)-$CH(CH_3)_2$	25 ± 7[d]
17	3	3	CO-CH(NHFmoc)-CH_3	232 ± 85[d]
18	3	3	CO-CH_2-NH-CO-CH(NHBoc)-CH_3	440[f]
19	3	3	CO-CH(NHBoc)-$(CH_2)_4$-NHFmoc	273 ± 75[d]
20	3	3	CO-CH(NHBoc)-CH_2-NHFmoc	71 ± 3[d]
21	3	3	CO-CH(NHBoc)-CH_2-NHBoc	55 ± 3[d]
22	3	3	CO-CH(NHBoc)-CH_2OBn	138[f]
23	3	3	CO-CH(NHBoc)-CH_2OAc	63 ± 15[d]
24	3	3	CO-CH(NHBoc)-CH_2-$COOC_6H_{11}$	31 ± 4[c]
25	3	3	CO-CH(NHBoc)-CH_2-COOFmoc	230 ± 16[d]
26	3	3	CO-CH(NH_2)-CH_3	>500[d]
27	3	3	CO-CH(NHBoc)-$(CH_2)_4$-NH_2	>500[d]
28	3	3	CO-CH(NHBoc)-CH_2-NH_2	307[f]
29	3	3	CO-CH(NH_2)-CH_2−NH_2	>500[d]
30	3	3	CO-CH(NHBoc)-CH_2-COOH	>500[d]
31	3	3	CO-CH(NHBoc)-CH_2OH	247 ± 92[d]
32	3	3	CO-$(CH_2)_2$-COOH	270[f]
33	3	3	CO-$(CH_2)_2$-$CONH_2$	40 ± 10[e]
34	3	3	CO-$(CH_2)_2$-CO-morpholine	436 ± 56[d]
35	3	3	CO-$(CH_2)_2$-morpholine	26 ± 2[e]
36	3	3	CO-$(CH_2)_2$-piperidine	67 ± 11[d]
37	3	3	CO-$(CH_2)_2$-pyrrolidine	91 ± 19[d]
38	3	3	CO-$(CH_2)_2$-(4-methylpiperazine)	62 ± 8[d]
39	3	3	CO-$(CH_2)_2$-piperazine	>500[f]
40	3	3	CO-$(CH_2)_2$-NH$(CH_3)_2$	39 ± 5[d]

[a] Ac, acetyl; Bn, benzyl; Boc, *tert*-butoxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl. [b] IC_{50} CQ = 138.6 ± 8.4 nM; IC_{50} MF = 6.9 ± 0.3 nM. [c−f] n, number of experiments: [c] $n = 5$; [d] $n = 3$; [e] $n = 4$; [f] $n = 2$.

vesicles (Figure 1B), which co-localized with lysosome-specific probe labeling (data not shown). The other members of the A and B series displayed a specific parasite labeling similar to that for compound **9** (Figure 1C). However, a weaker fluorescence was associated with the parasite nucleus while fluorescence was mainly associated with the parasite cytoplasm. When compared with compound **9**, the A and B series revealed a marked difference in L-6 cells localization, showing a diffuse labeling within the cell cytosol and no concentration in lysosome-like vesicles (Figure 1D).

In the C series (Table 2), activity proved to be highly dependent upon the nature of the Boc- and/or Fmoc-protected amino acid residue fixed to the central nitrogen atom of the linker and upon the distance between the terminal nitrogen atoms. A high level of activity (IC_{50} values about 30 nM) was observed for the alanine derivatives **10** and **13** ($n = n' = 3$), for both enantiomers, while their analogues **11** ($n = n' = 2$) and **12** ($n = 3$, $n' = 4$) were less active (IC_{50} of 109 and 330 nM, respectively). *N*-Boc-amino acids with longer hydrophobic side chains, leucine (compound **15**) and valine (compound **16**), were the most active, while the polarity of the side chains proved consistently unfavorable since the protected derivatives **10** and **19**−**25** were more active than their corresponding deprotected counterparts **26** and **27**−**31**. Also, the succinimide derivative **33** revealed a better activity than its carboxylic analogue **32**. Addition of a second amino acid residue (compound **18**) was unfavorable. Amino derivatives **35**−**40** displayed IC_{50} values between 26 and 91 nM, with the exception of the piperazine derivative **39** (IC_{50} > 500 nM) which via its terminal nitrogen atom could play the role of hydrogen-bond donor. In this series, the absence of a protonable amino group led to the less active morpholine compound **34**. Five compounds among the most active upon the CQ-resistant strain FcB1R (compounds **9**, **10**, **13**, **15**, and **16**) were subsequently evaluated for their efficiency in inhibiting the growth of seven strains showing different degrees of resistance to CQ or MF (Table 3). An inverse correlation was observed for compounds **10**, **13**, **15**, and **16**, as recently reported for bisquinolines by Vennerstrom and coworkers,[29] the more the parasites appeared resistant to CQ, the more they seemed sensitive to the compounds under investigation. On the contrary, piperazine compound **9** displayed quite similar efficiency (IC_{50} values between 8 and 18 nM), whatever the CQ resistance of the strain. No such correlation was observed with the resistance to MF.

In Vitro Antitrypanosomal Activity upon *T. cruzi, T. brucei,* and *L. infantum*. Compounds of the series A and B (Table 4), with the exception of the piperazine derivative **9** and to a lesser extent compounds **4** and **5**, were found to be very cytotoxic upon human diploid embryonic lung cells (MRC-5 cells) and upon mouse primary peritoneal macrophages used in the *T. cruzi* and *L. infantum* tests as well as upon *T. brucei* which is known to be highly sensitive to toxic molecules. In confocal imaging of *T. cruzi* epimastigotes, compound **9** was found to be mainly associated with a structure which colocalized with kinetoplast DNA (Figure 2), while compound **1** displayed a more diffuse fluorescence associated with both the kinetoplast and the cytosol (data not shown).

In the C series (Table 5), only five compounds (**25**, **29**−**32**) were devoid of cytotoxicity toward MRC-5 cells and mouse macrophages. Four of these molecules possess a polar group (amine, alcohol, or carboxylic acid)

Table 3. Efficiency of Five of the Most Active Compounds against FcB1R, To Inhibit Growth of Parasites Expressing Different Degrees of Resistance to CQ

P. falciparum strain	IC_{50} (nM)[a]						
	CQ	MF	**9**	**10**	**13**	**15**	**16**
3D7	20 ± 4	40 ± 10	13 ± 3	22 ± 10	57 ± 6	48 ± 6	49 ± 10
F32a	22 ± 2	62 ± 6	8 ± 2	66 ± 5	89 ± 11	52 ± 4	57 ± 2
GP1	39 ± 2	17 ± 3	11 ± 5	35 ± 12	66 ± 5	63 ± 6	83 ± 6
FCR3	102 ± 6	36 ± 15	18 ± 6	nd[b]	32 ± 8	17 ± 6	22 ± 8
FCM29	216 ± 25	8 ± 3	13 ± 6	11 ± 4	11 ± 4	13 ± 5	16 ± 6
W2	219 ± 16	30 ± 3	12 ± 2	10 ± 5	18 ± 8	15 ± 7	40 ± 11
K1	267 ± 42	12 ± 1	16 ± 5	12 ± 2	23 ± 5	14 ± 7	36 ± 6

[a] Parasites were considered resistant to CQ for IC_{50} > 100 nM. IC_{50} values are the mean ± standard deviation of three independent experiments. [b] nd, not determined.

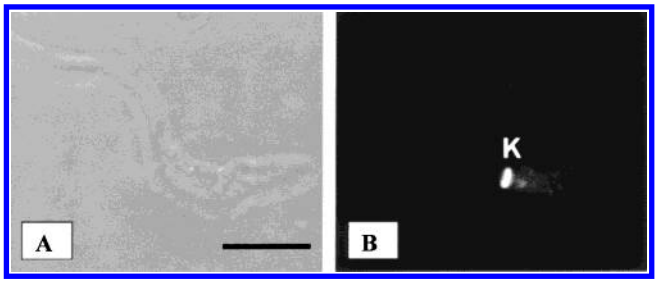


Figure 2. Labeling of *T. cruzi* epimastigotes with compound **9**: A, phase contrast; B, labeling with 1 μM compound **9.** Fluorescence was mainly associated with a structure which colocalized with kinetoplast DNA (K). Bar = 3 μm.

in the side chain linked to the spacer. They were highly active against *T. brucei* (IC_{50} < 1.56 μM or close to 1.56 μM for compound **29**) and only displayed a marginal activity against *P. falciparum*, *T. cruzi*, and *L. infantum*. Compound **29** inhibited trypanothione reductase (TR) from *T. cruzi* (IC_{50} = 20 μM), while IC_{50} values for the other compounds were equal or superior to 50 μM in the presence of 50 μM trypanothione disulfide (data not shown); this result was not really surprising since compound **29** displays the structural requirements for TR recognition, e.g. the presence of aromatic rings and of a protonable side chain.[27,28]

Discussion

Bisquinolines were used to overcome CQ resistance since they were likely to be extruded with difficulty by a proteinaceous transporter.[11] They were also discovered to inhibit the growth of CQ-sensitive and CQ-resistant parasites. However, further development was suspended for reasons of toxicity. Monoacridine derivatives were reported to display high levels of antileishmanial and antitrypanosomal activity[16] while quinacrine, the acridine analogue of CQ, was in clinical use before CQ. Bisacridines could constitute an alternative to bisquinolines and avoid the efflux mechanism related to CQ resistance. Compared with bisquinolines, bisacridines are sterically more hindering, yet they have similar pK_a values (pK_a of acridine rings = 6.5) which permit ionization and consequent accumulation in the food vacuole of *Plasmodium*. However their potential interaction with DNA is greater, especially in the case of bisintercalation which can generate cytotoxicity. Bisintercalation requires a minimal distance between the acridine rings, different if the "excluded site model"[30,31] (two aromatic rings have to be separated by at least two base pairs) is observed: ≥10.2 Å or not (two aromatic rings on either side of a base pair): ≥6.8 Å. These minimal distances were compared by Le Pecq and coworkers, with the distances calculated for bis(9-amino-6-chloro-2-methoxyacridines) bound with amino linkers.[17] Compounds corresponding to a spermine or spermidine linker displayed distances respectively of 16.1 and 11.2 Å and were found to bisintercalate, while the removal of a single carbon (symmetical linker $-NH-(CH_2)_3-NH-(CH_2)_3-NH-$) reduced the distance to 9.9 Å and suppressed bisintercalation. The latter named linker was therefore introduced into the majority of compounds **10**−**40** and was substituted by a variety of side chains in order to decrease possible interaction with DNA. Bisacridines **1**−**9**, differing by the length, the nature of the linker, and the absence of a side chain, were also prepared and studied in comparison.

For compounds **1**−**9**, no clear relationship appears between potential bisintercalation and cytotoxic effects upon MRC-5 cells. Among the compounds unable to bisintercalate (compounds **1**, **6**, and **7**), cytotoxicity of **1** upon MRC-5 cells cannot be related to a definite target since this compound shows a diffuse labeling in murine L-6 cell cytosol (Figure 1D) while that of **6** and **7** might be explained by the possible inhibition of the polyamine metabolism.[32] With compounds capable of bisintercalation (**2**−**5** and **8**−**9**), surprisingly no localization in the nucleus of murine L-6 cells was observed by confocal analysis. As with compound **1**, a diffuse labeling of the cytoplasm was observed for compounds **2**−**5** and **8**, in contrast to mepacrine which accumulated in the nucleus (data not shown). Compounds **4** and **5** displayed the lowest cytotoxic effects upon MRC-5 cells, while an absence of cytotoxicity was noted for compound **9**. This difference in cytotoxicity could reflect differences in their cellular distribution. In fact, introduction of a piperazine leads to the capture of compound **9** in lysosome-like vesicules of L-6 cells (Figure 1B). The high level of activity of compound **9** upon *P. falciparum* could result from its greater accumulation in the parasite nucleus (Figure 1A) and its bisintercalating properties. The difference in composition of plasmodial and mammalian DNA may contribute to such a sizable accumulation.[33,34] However, cytoplasmic labeling in the parasite was also observed for compound **9**, as well as for compounds **2**−**5** and **8**, suggesting that these compounds might also interfere with cytoplasmic targets.

Each of the compounds **1**−**8** shows a high level of activity upon *T. brucei*, yet none of which can be retained due to their toxicity found upon murine macrophages and of the known sensitivity of *T. brucei* to toxic compounds. Therapeutic indexes of compounds **4** and **5** proved to be too weak for potential drug use in large numbers of patients benefitting only from a minimal medical supervision. Thus from series A and B, compound **9** is the sole representative worthy of note as a trypanocidal lead and especially as an antimalarial lead whose interest is enhanced by its consistent efficiency whatever the CQ sensitivity of the strain tested (Table 3).

With the exception of **11** and **12**, compounds **10**−**40** are derived from compound **6** with their side chain bound by an amide bond. As expected, the nature of their respective side chains had a strong influence on both cytotoxicity and activity against parasites. When bisacridines **10**−**40** were compared with starting compound **6**, a global decrease in cytotoxic effects upon MRC-5 cells was observed while activity upon macrophages remained high. Only compounds **25** and **29**−**32** cause no or low levels of cytotoxicity upon both cell lines, and from among them, four include the presence of a polar group (alcohol, amino, or carboxylic). The relative polarity of this group renders likely a decreased cellular availability that would be responsible for this low cytotoxicity and also for the weak activity against all parasites, with the exception of *T. brucei*. *T. brucei* differs from *P. falciparum*, *T. cruzi*, and *L. infantum* by an extracellular growth that confers differences in biochemical pathways, especially in polyamine metabolism.[35] Trypanothione reductase from *T. cruzi*, which is inhibited by compound **29** (IC_{50} = 20 μM), cannot be

Table 4. In Vitro Cytotoxicity of Compounds **1**−**9** upon MRC-5 Cells and in Vitro Activities toward Trypomastigote Forms of *T. brucei* and Amastigote Forms of *T. cruzi* and *L. infantum*

molecule	cytotoxicity (%)				*T. brucei* (%)				*T. cruzi* (%)[a]				*L. infantum* (%)[a]			
concn (μM)	25	12.5	6.25	3.13	12.5	6.25	3.13	1.56	12.5	6.25	3.13	1.56	12.5	6.25	3.13	1.56
1	100	100	100	100	100	100	100	100	T	T	T	90	T	T	T	T
2	100	90	80	20	100	100	99	90	70	40	0	0	40	0	0	0
3	100	100	100	20	100	100	100	100	T	98	90	70	T	95	90	70
4	90	80	0	0	100	100	100	100	99	70	40	0	T	90	70	20
5	100	20	0	0	100	100	100	70	90	40	0	0	T	80	40	0
6	100	100	100	100	100	100	100	100	T	T	T	60	T	T	T	40
7	100	100	100	100	100	100	100	100	T	T	T	T	T	T	T	T
8	100	100	100	100	100	100	100	100	T	T	T	T	T	T	T	T
9	0	0	0	0	100	90	80	60	0	0	0	0	0	0	0	0
Mel[b]	$CC_{50} = 12.5\ \mu M$				$IC_{50} = 200$ nM											
Benz[b]	$CC_{50} > 50\ \mu M$								$IC_{50} = 3130$ nM							
Amp B[b]	$CC_{50} = 25\ \mu M$												$IC_{50} = 50$ nM			

[a] T, toxic upon mouse primary peritoneal macrophages used in the test. [b] Mel, melarsoprol; Benz, benznidazole; Amp B, amphotericin B. These reference drugs are included in each primary in vitro screening batch.

Table 5. In Vitro Cytotoxicity of Compounds **10**−**40** upon MRC-5 Cells and in Vitro Activities toward Trypomastigote Forms of *T. brucei* and Amastigote Forms of *T. cruzi* and *L. infantum*

molecule	cytotoxicity (%)				*T. brucei* (%)[a]				*T. cruzi* (%)[b]				*L. infantum* (%)[b]			
concn (μM)	25	12.5	6.25	3.13	12.5	6.25	3.13	1.56	12.5	6.25	3.13	1.56	12.5	6.25	3.13	1.56
10	100	100	100	0	100	100	100	100	T	90	40	0	T	95	60	40
11	100	0	0	0	100	100	100	99	60	40	0	0	90	60	20	0
12	100	100	0	0	100	100	100	100	90	70	20	0	70	40	20	0
13	100	84	84	81	nd	100	100	100	T	T	95	60	T	T	99	95
14	100	100	20	0	100	100	100	100	80	40	0	0	80	60	40	0
15	55	49	47	42	nd	100	100	100	T	T	80	40	T	T	95	90
16	60	59	54	43	nd	100	100	100	T	T	90	70	T	T	99	95
17	100	100	100	100	100	100	100	100	T	T	0	0	T	T	40	0
18	100	71	41	31	nd	100	100	100	T	40	0	0	T	T	0	0
19	100	90	80	20	100	100	100	100	T	80	20	0	T	40	0	0
20	68	60	57	55	nd	100	100	100	T	90	20	0	T	95	70	20
21	70	50	48	43	nd	100	100	100	T	80	40	0	T	99	95	80
22	100	100	100	100	100	100	100	100	T	80	40	0	T	90	70	40
23	100	90	40	20	100	100	100	100	70	40	0	0	90	80	60	0
24	100	100	100	40	100	100	100	100	98	70	20	0	T	80	60	20
25	0	0	0	0	100	100	100	100	40	0	0	0	20	0	0	0
26	100	100	40	20	100	100	100	100	0	0	0	0	0	0	0	0
27	100	100	40	0	100	100	100	100	99	90	40	0	40	0	0	0
28	39	37	0	0	nd	100	100	99	T	T	40	0	T	T	0	0
29	0	0	0	0	nd	90	60	20	0	0	0	0	0	0	0	0
30	0	0	0	0	100	100	100	100	80	0	0	0	0	0	0	0
31	0	0	0	0	100	100	100	100	80	20	0	0	40	0	0	0
32	0	0	0	0	100	95	80	60	0	0	0	0	0	0	0	0
33	100	100	100	100	100	100	100	100	T	T	T	95	T	T	T	T
34	60	20	0	0	100	100	100	100	60	0	0	0	0	0	0	0
35	100	100	100	80	100	100	100	100	T	T	60	40	T	T	T	T
36	78	71	57	42	nd	100	100	100	T	T	T	40	T	T	T	T
37	76	65	39	37	nd	100	100	100	T	T	T	0	T	T	T	T
38	80	74	57	40	nd	100	100	100	T	T	T	0	T	T	T	T
39	15	0	0	0	nd	100	99	95	90	0	0	0	T	0	0	0
40	54	40	39	30	nd	100	100	100	T	T	T	40	T	T	T	T

[a] nd, not determined. [b] T, toxic upon mouse primary peritoneal macrophages used in the test.

retained as the target since this compound is totally inactive upon *T. cruzi*. Compounds **10**, **13**, and **15**−**16** display high levels of activity upon *T. cruzi* and *L. infantum* at 1.56 μM. They also show an intriguing inverse correlation toward *P. falciparum* growth inhibition for the seven strains studied and varying in their sensitivity to CQ. This might suggest either the presence of more sensitive target(s) in CQ-resistant parasites compared with CQ-sensitive ones or a higher accumulation inside the CQ-resistant parasites due to mechanism(s) which might be involved in CQ resistance. However, interest in these four compounds is rendered questionable by their cytotoxic effects upon MRC-5 cells and macrophages.

Conclusions

From the hypothesis that bulky structures are extruded with difficulty by CQ-resistant strains of *P. falciparum*, a series of bisacridine derivatives (aliphatic di-, tri-, and tetramine) was prepared. A side chain with a variety of amino acid residues was attached to the polyamine linker, both to improve the weak solubility of this type of compound and to reduce the possible interaction with human DNA. The introduction of a piperazine moiety to the linker led to a unique behavior: a strong and selective activity upon *Plasmodium*, a localization outside of the food vacuole and associated mainly with the parasite nucleus along with a total absence of cytotoxic effects upon MRC-5 cells and

murine macrophages. Although no structure−activity relationship was evident in the C series with respect to bisintercalation, the presence of a polar group in the side chain attached to the linker afforded a reduction in antimalarial activity while favoring a specific activity upon *T. brucei*.

Compounds **9**, **25**, **30**, and **31**, which satisfy both of the conditions required for antiparasitic drugs, e.g. safety and low cost, can be considered as good, new lead structures against *P. falciparum* and *T. brucei*.

Experimental Section

In Vitro *P. falciparum* Culture and Drug Assays. *P. falciparum* strains were maintained in continuous culture on human erythrocytes as described by Trager and Jensen.[36] In vitro antiplasmodial activity was determined using a modification of the semiautomated microdilution technique of Desjardins et al.[37] *P. falciparum* CQ-sensitive (3D7/unknown origin, F32a/Tanzania, GP1/Thailand), moderately CQ-resistant (FCR3/Gambia), and CQ-resistant (FcB1R/Colombia, FCM29/Cameroun, K1/Thailand, W2/Indochina) strains were used in sensitivity testing. FcB1R, F32a, GP1, and FCM29 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, for 24 h. The growth inhibition for each drug concentration was determined by a relative comparison of the radioactivity incorporated in 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 are expressed as the mean determined from several independent experiments. The DMSO concentration never exceeded 0.1% and did not inhibit the parasite growth.

Cytotoxicity Test upon MRC-5 Cells. A human diploid embryonic lung cell line (MRC-5, Bio-Whittaker 72211D) and primary peritoneal mouse macrophages were used to assess the cytotoxicity for 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/well. MRC-5 cells were seeded at 5 000 cells/well. After 24 h, the cells were washed and 2-fold dilutions of the drug were added in 200 μL of 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 (25, 12.5, 6.25, and 3.13 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 assay[38] and scored as a percent (%) reduction of absorption at 540 nm of treated cultures versus untreated control cultures. For macrophages, scoring was performed microscopically.

In Vitro Activity against *T. brucei* Trypomastigotes. Bloodstream forms of *T. brucei* were cultivated in HMI-9 medium.[39] In a 96-well microplate, 10 000 hemoflagellates were incubated at different drug concentrations (12.5, 6.25, 3.13, and 1.56 μM) for 4 days. Parasite multiplication was measured colorimetrically (490 nm) following addition of MTT, which converts to an aqueous soluble, formazan product.[38]

In Vitro Activity against Intracellular *T. cruzi* Amastigotes. Primary mouse peritoneal macrophages were seeded in 96-well microplates at 30 000 cells/well. After 24 h, about 10 0000 trypomastigotes of *T. cruzi* were added per well together with 2-fold dilutions of the drug. The cultures were incubated at 37 °C in 5% CO_2−95% air for 4 days. Following fixation in methanol and Giemsa staining, the drug activity was semiquantitatively scored as a percent (%) reduction of the total parasite load (free trypomastigotes and intracellular amastigotes) compared with untreated control cultures. Scoring was performed microscopically.

In Vitro Activity against Intracellular *L. infantum* Amastigotes. Primary mouse peritoneal macrophages were seeded in 16-well Lab-Tek culture slides at 30 000 cells/well. After 24 h, amastigotes of *L. infantum* (derived from the spleen of an infected donor animal) were added at an infection ratio of 10/1 together with 2-fold dilutions of the drug. The cultures were incubated at 37 °C in 5% CO_2−95% air for 7 days. Treatment of uninfected control cultures was also included to determine a selectivity index. Drug activity was semiquantitatively scored as a percent (%) reduction of the total parasite load or the number of infected macrophages in Wright stained preparations. Scoring was performed microscopically.

Confocal Microscopy. *P. falciparum*-infected red blood cells were maintained for 1 h under normal culture conditions, at 37 °C, in the presence of 1 μM bisacridine derivative. Following two washes (2000*g*, 5 min), cells were incubated in culture medium without serum for 15 min in the presence of 10 μg/mL DIL C_{18}(3) (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Inc., Eugene, OR). After three further washes, cells were mounted on poly-L-lysine-coated slides and immediately observed using a confocal laser scanning microscope (MRC 1024, Bio-Rad). In some experiments, following incubation with a bisacridine derivative, cells were fixed with 2% (v/v) formaldehyde in phosphate-buffered saline (PBS), washed twice, and incubated for 15 min with 20 μg/mL propidium iodide or 2 μg/mL DAPI (Sigma). Cells were observed after two further washes; no difference in labeling with bisacridine derivatives was noted between either living or fixed cells, or cells not incubated with DIL C_{18}-(3). For the labeling with mepacrine, infected erythrocytes were incubated with 10 μM drug for 1 h, washed twice, and immediately observed. Murine muscle L-6 cells were grown in 8-well Lab-Tek culture slides (Nunc, Inc., Naperville, IL) in DMEM supplemented with 5% (v/v) fetal calf serum, at 37 °C, in 5% CO_2−95% air. Cells were incubated for 1 h in the presence of 1 μM bisacridine derivative and washed three times prior to observation. For double-labeling experiments using a lysosome-specific probe, cells were incubated simultaneously with a bisacridine derivative and LysoTracker Red DND-99 diluted according to the manufacturer's recommendations (Molecular Probes). *T. cruzi* epimastigotes (Y strain) were maintained and grown in liver infusion medium, containing 10% (v/v) fetal calf serum, at 28 °C.[40] Cells were washed twice in PBS and incubated with 1 μM bisacridine derivative in PBS, for 1 h. After three washes with PBS, cells were fixed with 1% (v/v) formaldehyde in PBS and incubated for 15 min with 2 μg/mL DAPI. Cells were observed following three further washes.

Assays for TR Inhibition. Recombinant *T. cruzi* trypanothione reductase was produced from the SG5 *Escherichia coli* strain with the overproducing expression vector pIBITczTR. TR activity was measured at 21 °C in 0.02 M Hepes buffer, pH 7.25, containing 0.15 M KCl, 1 mM EDTA, and 0.2 mM NADPH with an enzyme concentration of 0.02 U mL^{-1}. The reaction was promoted by the addition of the enzyme, and the subsequent NADPH oxidation was followed at 340 nm. IC_{50} of the different compounds was evaluated in the presence of 50 μM $T(S)_2$ and 1% DMSO.

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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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