Design, synthesis and antimalarial activity of novel bis(N-[(pyrrolo[1,2-a]quinoxalin-4-yl)benzyl]-3-aminopropyl)amine derivatives

Jean Guillona,b, Anita Cohenc, Nassima Meriem Gueddouda,a,b, Rabindra Nath Das,a,b, Stéphane Moreaud,a,b, Luisa Ronga,a,b, Solène Savrimumoutoa,b, Louise Basmacian,c, Alix Monnier,a,b, Myriam Monege,a,b, Sandra Rubia,b, Timothée Garnerind,d, Nadine Azasc, Jean-Louis Mergny,a,b, Catherine Mulliéd and Pascal Sonnetd

ABSTRACT
Novel series of bis- and tris-pyrrolo[1,2-a]quinoxaline derivatives 1 were synthesized and tested for in vitro activity upon the intraerythrocytic stage of W2 and 3D7 Plasmodium falciparum strains. Biological results showed good antimalarial activity with IC50 in the μM range. In attempting to investigate the large broad-spectrum antiprotozoal activities of these new derivatives, their properties toward Leishmania donovani were also investigated and revealed their selective antiplasmodial profile. In parallel, the in vitro cytotoxicity of these molecules was assessed on the human HepG2 cell line. Structure–activity relationships of these new synthetic compounds are discussed here. The bis-pyrrolo[1,2-a]quinoxalines 1n and 1p were identified as the most potent antimalarial candidates with selectivity index (SI) of 40.6 on W2 strain, and 39.25 on 3D7 strain, respectively. As the telomeres of the parasite could constitute an attractive target, we investigated the possibility of targeting Plasmodium telomeres by stabilizing the Plasmodium telomeric G-quadruplexes through a FRET melting assay by our new compounds.

Introduction
Malaria remains as one of the most devastating infectious diseases of the developing world. Malaria remains a major cause of public health problem in about 95 countries mainly located in the tropical zone of the globe (notably Africa, South-East Asia and also Eastern Mediterranean region)1; while approximately 3.2 billion people are at risk of being infected with malaria and developing disease. The latest figures on the incidence and mortality of malaria show that, despite progress in the implementation of preventive measures such as insecticide-treated mosquito nets and intermittent preventive treatments, this parasitic disease is still estimated to affect over 214 million people and to account for 438,000 deaths in 2015, of which approximately 80% are concentrated in just fifteen countries, mainly in Africa. The death toll is particularly high in children under five and pregnant women of the World Health Organization (WHO) African region1. Five species of protozoan parasites belonging to the Plasmodium genus, namely, falciparum, malariae, vivax, ovale and knowlesi cause malaria in human beings; from which, P. falciparum is the most dangerous of these species2–4.

The increasing prevalence of multiple drug resistant strains of P. falciparum in most malaria endemic areas has significantly reduced the efficacy of the current antimalarial drugs. Nowadays, the only fully effective antimalarial drugs utilize artemisinin and its derivatives, notably parenteral artesunate now recommended as the first line of treatment of severe malaria for at least 24 h and until oral medication could be tolerated5. Artemisinin and its derivatives are also used in combination with several different partner drugs (including lumefantrine, mefloquine, amodiaquine, sulfadoxine/pyrimethamine and piperaquine) in artemisinin-based combination therapies (ACTs), now recommended as the first line of treatment of uncomplicated Plasmodium falciparum malaria and as the second part of treatment for 3 days of severe malaria in endemic areas6. However, over the last decade evidence has shown that artemisinin resistance has emerged and spread within Southeast Asia, first on the Cambodia-Thailand border in 20096,7, but now across a widening area of the Greater Mekong Subregion. Rapid scientific advances in understanding of this problem have taken place within the last five years8–10 and defined mutations in “K13” gene of P. falciparum associated to reduced ring-stage susceptibility to artemisinins11. Therefore, new antimalarial agents with new mechanisms of action are required to overcome the emergence of resistance and to control an ever-increasing number of epidemics caused by the malaria parasite12. Although current efforts in antimalarial drug discovery are focused on identification of new biological targets, continued research on new 4-aminoquinolone derivatives is still warranted. This is because the hemoglobin degradation pathway in P. falciparum has a proven history as an excellent therapeutic target to which the parasite has difficulty in evolving resistance. In spite of resistance to chloroquine, previous work has shown that modification of the lateral side chain of chloroquine results in aminooquinoline derivatives that avoid the chloroquine resistance mechanism15,14.
A possibility to overcome this multidrug-resistant mechanism is to design new quinoline-based drugs which will not be recognized by the protein system involved in drug efflux. In this regard, two series of compounds that show promise in this regard are the bisquinoline and bisacridine antimalarial drugs A and B (Figure 1)\textsuperscript{15–18}. These drugs show much lower resistance indices than chloroquine, indicating that the bisquinoline or bisacridine structures are less efficiently excluded by drug-resistant parasites.

The pyrrolo[1,2-\textit{a}]quinoxaline heterocyclic framework constitutes the basis of an important class of compounds possessing interesting biological activities. These compounds have been reported as key intermediates for the assembly of several heterocycles including antipsychotic agents, anti-HIV agents, adenosine A\textsubscript{3} receptor modulators\textsuperscript{19}, antiparasitic agents\textsuperscript{20–25} and antitumor agents\textsuperscript{26–30}. In the course of our work devoted to discover new compounds employed in the antiparasitic chemotherapy, we previously identified some series of substituted pyrrolo[1,2-\textit{a}]quinolinemderivatives designed as interesting bioactive isosteres of quinoline derivatives\textsuperscript{20–24}. Thus, taking into account our experience in the field of the synthesis of new antimalarial heterocyclic compounds based on our pyrrolo[1,2-\textit{a}]quinoxaline heterocyclic core\textsuperscript{20–24,27–30}, we decided to incorporate a benzyl group in position 4 of the heterocyclic skeleton of our previously described bispyrrolo[1,2-\textit{a}]quinolines\textsuperscript{30} to broaden the structural diversity of these derivatives, and mainly to increase the aromatic surface of these designed compounds (Figure 1).

Hence, we described here the synthesis of new bis- or trispyrrolo[1,2-\textit{a}]quinolinederivatives 1\textit{a}–\textit{t} (Figure 1) and reported on their in vitro antiplasmodial activity against the chloroquine-sensitive (3D7) and the chloroquine-resistant (W2) strains of the malaria parasite \textit{Plasmodium falciparum}. As these new compounds were designed as quinoline-like bio-isosteres, and as the quinoline skeleton is the fundamental unit of many antiprotozoal drugs, these pyrrolo[1,2-\textit{a}]quinolinederivatives were also investigated on another medically important protozoan, \textit{Leishmania donovani}; in order to evaluate the specificity of their antiparasitic activity. Leishmaniasis is an infectious disease caused by protozoan parasites belonging to the genus \textit{Leishmania}. \textit{Leishmania} parasites exist in two major morphological stages: extracellular flagellated promastigotes in the digestive tract of their sandfly vector, which is the infective stage and immobile intracellular amastigotes in the cells of their host’ mononuclear phagocytic system. Leishmaniasis presents various clinical aspects including cutaneous leishmaniasis, the most common form, muco-cutaneous leishmaniasis and visceral leishmaniasis, the most severe form, lethal in untreated patients. \textit{Leishmania donovani} is one of the major causative agents of human visceral leishmaniasis which represents a public health problem: 0.2 to 0.4 million visceral leishmaniasis cases occur each year and more than 90% of global visceral leishmaniasis cases occur in just five countries: India, Bangladesh, Sudan, Brazil and Ethiopia\textsuperscript{31}. The current treatment of the disease is based on a limited number of chemotherapeutic agents (meglumine antimoniate, sodium stibogluconate, pentamidine, amphotericin B and miltefosine) which present many limits, notably characterized by a high toxicity and cost\textsuperscript{32,33}. In addition, the in \textit{vitro} cytotoxicity of these new molecules was assessed on the human HepG2 cell line, in order to determine their selectivity index. Moreover, as the telomers of the parasite could constitute a target, we also investigated the possibility of targeting \textit{Plasmodium} telomeres by stabilizing the \textit{Plasmodium} telomeric G-quadruplexes through a FRET melting assay by our new synthesized compounds. Indeed, telomerase activity has been identified in gametocytes and during the transition to erythrocytic stage of \textit{P. falciparum}\textsuperscript{34}. The telomeric \textit{G} overhang region of \textit{P. falciparum} is comprised of repeated degenerate unit \(\text{GGGTTYA}^3\) (where \(Y\) may be \(\text{T}\) or \(\text{C}\))\textsuperscript{35} which can fold into intramolecular G-quadruplex\textsuperscript{36}.

**Materials and methods**

**Chemistry**

Commercial reagents were used as received without additional purification. Melting points were determined with an SM-LUX-POL Leitz hot-stage microscope (Leitz GMBH, Midland, ON) and are uncorrected. IR spectra were recorded on a NICOLET 380FT-IR spectrophotometer (Thermo Electron Scientific Instruments LLC, Madison, WI). NMR spectra were recorded with tetramethylsilane as an internal standard using a BRUKER AVANCE 300 spectrometer (Bruker BioSpin, Wissenburg, France). Splitting patterns have been designated as follows: \textit{s} = singlet; \textit{bs} = broad singlet; \textit{d} = doublet; \textit{t} = triplet; \textit{q} = quartet; \textit{dt} = doublet; \textit{ddd} = double double doublet; \textit{dd} = double double; \textit{dt} = double tripletriplet; \textit{m} = multiplet. Analytical TLC were carried out on 0.25 precoated silica gel plates (POLYGRAM SIL G/UV\textsubscript{254}) and visualization of compounds after UV light irradiation. Silica gel 60 (70–230 mesh) was used for column chromatography. Microwave experiments were carried out using a focused microwave reactor (CEM Discover, Saclay, France). High resolution mass spectra (electrospray in positive mode, ESI\textsuperscript{+}) were recorded on a Waters Q-TOF Ultima apparatus. Mass spectra were recorded on an Ultraflex III TOF/TOF system (Bruker Daltonics, Bremen, Germany), equipped with a 200 Hz smartbeam laser (355 nm) and operating in reflectron positive ion mode. Mass spectra were acquired over the m/z range 300–5000 by accumulating data from 1000 laser shots for each spectrum. The instrumental conditions employed to analyze molecular species were the following: ion source 1: 25.08 kV; ion source 2: 21.98 kV; lens: 11.03 kV, pulsed ion extraction: 30 ns, reflector: 26.39 kV, reflector 2: 13.79 kV. Matrix suppression was activated by deflection mode: suppression up to 450 Da. Mass calibration was performed for each sample with a peptide calibration mixture (8206195, Peptide Calibration Standard, Bruker Daltonics). The instrument was controlled using Bruker’s flexControl 3.4 software and mass spectra were analyzed in Bruker’s FlexAnalysis 3.4 software (Bruker Daltonics, Billerica, MA).

**General procedure for the synthesis of 4-(pyrrolo[1,2-\textit{a}]quinolin-4-yl)benzaldehydes and 3-(pyrrolo[1,2-\textit{a}]quinolin-4-yl)benzaldehydes**

To suspension of 4-chloropyrrolo[1,2-\textit{a}]quinoline 7 (3.3 mmol), and Pd(PPh\textsubscript{3})\textsubscript{4} (0.164 mmol) in a mixture of toluene/EtOH (50/3 mL) under nitrogen were added K\textsubscript{2}CO\textsubscript{3} (3.6 mmol) and phenylboronic acid (2.2 mmol). In this regard, the mixture was refluxed for 24 h, and the cooled suspension was extracted with CH\textsubscript{2}Cl\textsubscript{2} (3 × 70 mL). The organic layer was washed with a saturated solution of NaCl (90 mL), and the combined organic extracts were dried over sodium sulfate, filtered, and evaporated under reduced pressure. The crude residue was triturated in ethanol. The resulting precipitate was filtered, washed with ethanol, and purified by column chromatography on silica gel using dichloromethane as eluent gave the pure product 8.

4-(9-Methoxypyrido[1,2-a]quinolin-4-yl)benzaldehyde (8d)

Yellow crystals, Yield: 85%, mp = 162–164 °C; IR \(\nu_{\text{max}}\) (KBr)/cm\textsuperscript{-1} 1705 (CHO); \textsuperscript{1}H NMR \(\delta\) (300 MHz, CDCl\textsubscript{3}) 10.15 (s, 1H, CHO), 8.90 (dd, 1H, \(J = 2.70\) and 1.20 Hz, H-1), 8.17 (d, 2H, \(J = 8.40\) Hz, H-2' and H-6'), 8.08 (d, 2H, \(J = 8.40\) Hz, H-3' and H-5'), 7.70 (dd, 1H, \(J = 8.10\) and 1.20 Hz, H-6), 7.42 (t, 1H, \(J = 8.10\) Hz, H-7), 7.13 (dd, 1H, \(J = 8.10\) and 1.20 Hz, H-8), 7.00 (dd, 1H, \(J = 3.90\) and 1.20 Hz, H-3), 6.90 (dd, 1H, \(J = 3.90\) and 2.70 Hz, H-2'), 4.13 (s, 3H, OCH\textsubscript{3}). MALDI-TOF MS m/z [\textit{M + H}]\textsuperscript{+} Calcd for C\textsubscript{19}H\textsubscript{15}N\textsubscript{2}O\textsubscript{2}: 303.113, Found: 303.127.
4-(3-Ethoxypyrrolo[1,2-a]quinoxalin-4-yl)benzaldehyde (8e)
Yellow crystals, Yield: 68%, mp = 170–172°C; IR ν_{max} (KBr)/cm\(^{-1}\) 1700 (CHO); \(^1\)H NMR δ (300 MHz, CDCl\(_3\)) 10.14 (s, 1H, CHO), 8.00–7.98 (m, 4H, H-2\(_0\), H-3\(_0\), H-5\(_0\) and H-6\(_0\)), 7.94 (dd, 1H, J = 8.10 and 1.40 Hz, H-6), 7.84 (d, 1H, J = 3.15 Hz, H-1), 7.81 (dd, 1H, J = 8.10 and 1.40 Hz, H-9), 7.50 (ddd, 1H, J = 8.10, 8.10 and 1.40 Hz, H-7), 7.43 (ddd, 1H, J = 8.10, 8.10 and 1.40 Hz, H-8), 6.54 (d, 1H, J = 3.15 Hz, H-2), 3.97 (q, 2H, J = 6.90 Hz, OCH\(_3\)), 1.17 (t, 3H, J = 6.90 Hz, CH\(_3\)). MALDI-TOF MS m/z [M+H]\(^+\) Calcd for C\(_{20}\)H\(_{17}\)N\(_2\)O\(_2\): 317.129, Found: 317.135.

3-(Pyrrolo[1,2-a]quinoxalin-4-yl)benzaldehyde (8 g)
Yellow crystals, Yield: 68%, mp = 147–150°C; IR ν_{max} (KBr)/cm\(^{-1}\) 1705 (CHO); \(^1\)H NMR δ (300 MHz, CDCl\(_3\)) 10.18 (s, 1H, CHO), 8.56 (s, 1H, H-2\(_0\)), 8.32 (d, 1H, J = 7.80 Hz, H-6), 8.09 (dd, 1H, J = 2.85 and 1.30 Hz, H-1), 8.08–8.05 (m, 2H, H-4\(_0\) and H-6\(_0\)), 7.93 (d, 1H, J = 7.80 Hz, H-9), 7.75 (t, 1H, J = 7.65 Hz, H-5\(_0\)), 7.56 (t, 1H, J = 7.80 Hz, H-7), 7.53 (t, 1H, J = 7.80 Hz, H-8), 7.02 (dd, 1H, J = 3.90 and 1.30 Hz, H-3), 6.96 (dd, 1H, J = 3.90 and 2.85 Hz, H-2). MALDI-TOF MS m/z [M+H]\(^+\) Calcd for C\(_{18}\)H\(_{13}\)N\(_2\)O: 273.103, Found: 273.127.

3-(8-Methoxypyrrolo[1,2-a]quinoxalin-4-yl)benzaldehyde (8 h)
Beige crystals, Yield: 87%, mp = 135–138°C; IR ν_{max} (KBr)/cm\(^{-1}\) 1705 (CHO); \(^1\)H NMR δ (300 MHz, CDCl\(_3\)) 10.16 (s, 1H, CHO), 8.54 (dd, 1H, J = 1.50 and 1.50 Hz, H-2\(_0\)), 8.30 (ddd, 1H, J = 7.65, 1.50 and 1.50 Hz, H-4\(_0\)), 8.05 (ddd, 1H, J = 7.65, 1.50 and 1.50 Hz, H-6\(_0\)), 8.02 (d, 1H, J = 9.15 Hz, H-6), 7.95 (dd, 1H, J = 2.70 and 1.50 Hz, H-1),
General procedure for bis\([\text{N-}[\text{pyrrolo}[1,2-a]\text{quinoxalin-4-yl}]\text{benzylidene}\]-3-aminopropanylmethylamine and bis\([\text{N-}[\text{4-phenylpyrrolo}[1,2-a]\text{quinoxalin-4-yl}]\text{methylidene}\]-3-aminopropanylpiperazine 9c, bis\([\text{N-}[\text{4-}(\text{indolo}[1,2-d]\text{quinoline-4-yl})\text{benzylidene}]\)-3-aminopropanylpiperazine 9d, and bis\([\text{N-}[\text{4-}(\text{indolo}[1,2-d]\text{quinoline-4-yl})\text{benzylidene}]\)-3-aminopropanylpiperazine 9r

To obtain a solution of diamine (2.7 mmol) in ethanol (15 mL) was added (pyrrolo[1,2-a]quinolin-4-yl)benzaldehyde 8a or (indolo[1,2-d]quinolin-4-yl)benzaldehyde 8i or 4-phenylpyrrolo[1,2-a]quinolin-2-ylcarboxaldehyde 8j (5.4 mmol). The reaction mixture was then heated under reflux for 5 h and then evaporated to dryness under reduced pressure. After cooling, the residue was extracted with dichloromethane (40 mL). The organic layer was dried over sodium sulfate and evaporated to dryness. The products were then used without further purification.

Bis\([\text{N-}[\text{4-}(\text{indolo}[1,2-d]\text{quinoline-4-yl})\text{benzylidene}]\)-3-aminopropanylpiperazine 9c

Yellow crystals, Yield: 96%, mp = 78–80°C; 1H NMR δ (300 MHz, CDCl3) 8.25 (s, 2H, 2 H-3), 7.83 (s, 2H, J = 8.00 Hz, 2 H-2), 7.53 (d, 2H, J = 3.00 Hz, 2 H-6), 7.17 (dd, 2H, J = 9.00 and 3.00 Hz, 2 H-8), 6.99 (dd, 2H, J = 3.90 and 1.20 Hz, 2 H-3), 6.90 (dd, 2H, J = 3.90 and 2.70 Hz, 2 H-2), 3.95 (s, 6H, 2 CH3O), 3.74 (t, 4H, J = 6.90 Hz, 2 CH2), 2.71–2.45 (m, 8H, 4 CH2 pip), 2.50 (t, 4H, J = 6.90 Hz, 2 CH2), 1.97 (qt, 4H, J = 6.90 Hz, 2 CH2).

Bis\([\text{N-}[\text{4-}(\text{8-methoxypyrrolo}[1,2-a]\text{quinoxalin-4-yl})\text{benzylidene}]\)-3-aminopropanylpiperazine 9d

Yellow crystals, Yield: 96%, mp = 61–63°C; 1H NMR δ (300 MHz, CDCl3) 8.40 (s, 2H, 2 H = N), 8.02 (d, 4H, J = 8.20 Hz, 2 H-2' and 2 H-6'), 7.94 (dd, 2H, J = 9.00 Hz, 2 H-6), 7.88 (d, 4H, J = 8.20 Hz, 2 H-3' and 2 H-5'), 7.87 (dd, 2H, J = 2.75 and 1.20 Hz, 2 H-1), 7.26 (d, 2H, J = 7.20 Hz, 2 H-9), 7.05 (dd, 2H, J = 9.00 and 2.70 Hz, 2 H-7), 6.95 (dd, 2H, J = 3.90 and 1.20 Hz, 2 H-3), 6.86 (dd, 2H, J = 4.05 and 2.70 Hz, 2 H-2), 4.09 (s, 1H, NH), 3.73–3.20 (m, 4H, 2 CH2), 2.88–2.31 (m, 4H, 2 CH2), 2.11–1.85 (m, 4H, 2 CH2).

Bis\([\text{N-}[\text{4-}(\text{8-methoxypyrrolo}[1,2-a]\text{quinoxalin-4-yl})\text{benzylidene}]\)-3-aminopropanylpiperazine 9g

Pale-yellow crystals, Yield: 97%; mp = 218–221°C; 1H NMR δ (300 MHz, CDCl3) 8.40 (s, 2H, 2 H = N), 8.06 (d, 4H, J = 8.40 Hz, 2 H-2' and 2 H-6'), 7.99 (dd, 2H, J = 9.00 Hz, 2 H-6), 7.93 (dd, 2H, J = 2.70 and 1.20 Hz, 2 H-1), 7.90 (d, 4H, J = 8.20 Hz, 2 H-3' and 2 H-5'), 7.85 (dd, 2H, J = 8.25 and 1.40 Hz, 2 H-9), 7.52 (dd, 2H, J = 8.25, 8.25 and 1.40 Hz, 2 H-7), 7.44 (dd, 2H, J = 8.25, 8.25 and 1.40 Hz, 2 H-8), 6.96 (dd, 2H, J = 4.05 and 1.30 Hz, 2 H-3), 6.87 (dd, 2H, J = 4.05 and 2.70 Hz, 2 H-2), 3.74 (t, 4HJ = 7.10 Hz, 2 CH2), 2.53 (t, 4H, J = 7.10 Hz, 2 CH2), 2.32 (s, 3H, CH3), 1.96 (qt, 4H, J = 7.10 Hz, 2 CH2).

References

For complete details, please consult the original publication.
Bis[N-[4-(3-ethoxypropyrolo[1,2-a]quinoxalin-4-yl]benzylidene]-3-aminopropyl)methylamine (9g)

Yellow oil, Yield: 97%; ¹H NMR δ (300 MHz, CDCl₃) 8.43 (s, 2H, 2 H-2), 7.46 (m, 4H, 2 H-5), 7.40 (d, 2H, J = 1.50 Hz, 2 H-1), 8.02 (dd, 2H, J = 1.50 and 1.50 Hz, 2 H-1), 7.93–7.86 (m, 4H, 2 H-6' and 2 H-9), 7.63–7.44 (m, 4H, 2 H-5' and 2 H-8), 7.03–7.01 (m, 2H, 2 H-3), 6.93–6.91 (m, 2H, 2 H-2), 3.71 (t, 4H, J = 6.90 Hz, 2 CH₂), 2.86–2.42 (m, 8H, 4 CH₃ pip.), 2.48 (t, 4H, J = 6.90 Hz, 2 CH₂).

Bis[N-[3-(8-methoxypropyrolo[1,2-a]quinoxalin-4-yl]benzylidene]-3-aminopropyl)methylamine (9o)

Yellow oil, Yield: 98%; ¹H NMR δ (300 MHz, CDCl₃) 8.41 (s, 2H, 2 H-2), 8.02 (dd, 2H, J = 1.50 and 1.50 Hz, 2 H-2'), 8.01 (dd, 2H, J = 2.35 and 1.50 Hz, 2 H-4'), 7.97 (d, 2H, J = 7.05 Hz, 2 H-5'), 7.91 (dd, 2H, J = 7.05 and 1.50 Hz, 2 H-6'), 7.59 (d, 2H, J = 7.05 Hz, 2 H-5), 7.31 (t, 4H, J = 6.90 Hz, 2 CH₂), 2.65–2.42 (m, 8H, 4 CH₃ pip.), 2.48 (t, 4H, J = 6.90 Hz, 2 CH₂).

General procedure for tris[N-[pyrrolo[1,2-a]quinoxalin-4-yl]benzylidene]-3-aminopropylamines 9s-t

To a solution of triamine (1.8 mmol) in ethanol (15 mL) was added (pyrrolo[1,2-a]quinoxalin-4-yl)benzaldehyde B1j (5.4 mmol). The reaction mixture was then heated under reflux for 5 h and then evaporated to dryness under reduced pressure. After cooling, the
residue was extracted with dichloromethane (40 mL). The organic layer was dried over sodium sulfate and evaporated to dryness. Products were then used without further purification.

**Tris[N-4-(7-methoxy-2H-pyrrolo[1,2-a]quinoxalin-4-yl)benzylidene]-3-aminopropyl]amine (9b)**

Orange oil, Yield: 82%; ¹H NMR δ (300 MHz, CDCl₃) 8.42 (s, 2H, 3 H-C₃), 7.99 (d, 2H, J = 8.40 Hz, 2 H-2, 2 H-9), 7.88 (d, 2H, J = 1.20 Hz, 2 H-1), 7.69 (d, 2H, J = 1.20 Hz, 2 H-3), 7.65 (d, 2H, J = 1.50 Hz, 2 H-4), 7.45 (d, 2H, J = 8.20 Hz, 2 H-6), 7.25 (d, 2H, J = 1.30 Hz, 2 H-5), 7.21 (d, 2H, J = 1.30 Hz, 2 H-1), 7.16 (s, 2H, 2 NCH₂), 6.94 (s, 2H, 2 NCH₂), 2.61 (s, 6H, 2 NCH₃), 1.92 (q, 6H, J = 6.90 Hz, 3 CH₂).

**Tris[N-3-(8-methoxy-2H-pyrrolo[1,2-a]quinoxalin-4-yl)benzylidene]-3-aminopropyl]amine (9a)**

Orange oil, Yield: 71%; ¹H NMR δ (300 MHz, CDCl₃) 8.40 (s, 3H, 3 H-C₃), 8.27 (dd, 3H, J = 1.50 and 1.50 Hz, 3 H-2'), 7.99 (dd, 3H, J = 7.80, 1.50 and 1.50 Hz, 3 H-4'), 7.98 (dd, 3H, J = 9.00 Hz, 3 H-6), 7.92 (ddd, 3H, J = 7.80, 1.50 and 1.50 Hz, 3 H-5), 7.86 (dd, 3H, J = 2.85 and 1.35 Hz, 3 H-1'), 7.52 (t, 3H, J = 7.80 Hz, 3 H-5'), 7.25 (d, 3H, J = 2.70 Hz, 3 H-9), 7.04 (ddd, 3H, J = 9.00 and 2.70 Hz, 3 H-7), 6.94 (ddd, 3H, J = 4.05 and 1.35 Hz, 3 H-3'), 6.87 (ddd, 3H, J = 4.05 and 2.85 Hz, 3 H-2'), 3.98 (s, 9H, 3 CH₂O), 3.71 (t, 6H, J = 6.90 Hz, 3 NCH₂), 2.61 (t, 6H, J = 6.90 Hz, 3 NCH₂), 1.92 (q, 6H, J = 6.90 Hz, 3 CH₂).

**N, N'-[oxybis(2,1-ethanediyl-2,1-ethanediyl)] bis-3-(8-methoxy-2H-pyrrolo[1,2-a]quinoxalin-4-yl)benzylidene] 9u**

To a solution of 1,11-diamino-3,6,9-trioxaundecane (0.56 mmol) in ethanol (12 mL) was added 3-(pyrrolo[1,2-a]quinoxalin-4-yl)benzaldehyde 8j (1.12 mmol). The reaction mixture was then heated under reflux for 5h and then evaporated to dryness under reduced pressure. After cooling, the residue was extracted with dichloromethane (35 mL). The organic layer was dried over sodium sulfate and evaporated to dryness. Product 9u was then used without further purification. Yellow oil, Yield: 97%; ¹H NMR δ (300 MHz, CDCl₃) 8.39 (s, 2H, 2 H-C₃), 8.29 (dd, 2H, J = 1.50 and 1.50 Hz, 2 H-2'), 8.05–8.78 (m, 6H, 2 H-4', 2 H-6', 2 H-7' and 2 H-1'), 7.59–7.55 (m, 2H, 2 H-5'), 7.17 (d, 2H, J = 2.70 Hz, 2 H-9), 7.06 (dd, 2H, J = 9.00 and 2.70 Hz, 2 H-7), 6.96 (m, 2H, 2 H-3), 6.88 (m, 2H, 2 H-2'), 3.95 (s, 6H, 2 CH₂O), 3.81–3.79 (m, 8H, 4 OCH₂), 3.64–3.62 (m, 8H, 4 OCH₂).

**General procedure for bis[N-(pyrrolo[1,2-a]quinoxalin-4-yl)benzylidene]-3-aminopropyl]piperazine (1c)**

Colorless oil, Yield: 61%; ¹H NMR δ (300 MHz, CDCl₃) 8.04 (dd, 2H, J = 8.25 and 1.35 Hz, 2 H-6), 8.00 (dd, 2H, J = 2.70 and 1.20 Hz, 2 H-1), 7.78 (d, 4H, J = 8.40 Hz, 2 H-2' and 2 H-6'), 7.89 (d, 2H, J = 8.25 and 1.35 Hz, 2 H-9), 7.53 (ddd, 2H, J = 8.25, 8.25 and 1.35 Hz, 2 H-7), 7.50 (d, 4H, J = 8.40 Hz, 2 H-3' and 2 H-5'), 7.45 (dd, 2H, J = 8.25, 8.25 and 1.35 Hz, 2 H-8), 7.01 (dd, 2H, J = 3.90 and 1.20 Hz, 2 H-3), 6.91 (dd, 2H, J = 3.90 and 2.70 Hz, 2 H-2), 3.91 (s, 4H, 2 NCH₂), 2.74 (t, 4H, J = 6.90 Hz, 2 NCH₂), 2.58–2.39 (m, 8H, 4 NCH₃), 2.44 (t, 4H, J = 6.90 Hz, 2 NCH₂), 1.76 (dt, 4H, J = 6.90 Hz, 2 CH₂). ¹³C NMR δ (100 MHz, CDCl₃) 153.2 (C-4), 141.8 (C-3a), 139.0 (C-5a), 138.7 (C-4), 137.6 (C-1), 131.5 (C-8), 130.3 (C-2' and C-6'), 128.8 (C-2), 128.5 (C-9a), 126.6 (C-6), 116.0 (C-11), 115.4 (C-2), 115.0 (C-9), 110.0 (C-3), 54.5 (NCH₂), 50.0 (NCH₂), 48.9 (NCH₂), 27.9 (CH₂). MALDI-TOF MS m/z [M + H⁺] Calcd for C₂₃H₄₃N₈: 658.366, Found: 658.362.

**Bis[N-(pyrrolo[1,2-a]quinoxalin-4-yl)benzylidene]-3-aminopropyl]piperazine (1d)**

Pale-yellow crystals, Yield: 88%; mp = 65–67°C; ¹H NMR δ (300 MHz, CDCl₃) 7.95 (d, 4H, J = 8.10 Hz, 2 H-2' and 2 H-6'), 7.90
Bis[N-[4-[7-methoxyrryrolo[1,2-a]quinoxalin-4-yl]benzyl]-3-amino-propyl]piperazine (1e)

Pale-yellow crystals, Yield: 93%; mp = 51–53°C; 1H NMR δ (300 MHz, CDCl3) 7.97 (d, 4H, J = 8.20 Hz, 2H-2’ and 2H-6’), 7.93 (dd, 2H, J = 2.75 and 1.25 Hz, 2H-1’), 7.79 (dd, 2H, J = 9.00 Hz, 2H-9’), 7.52 (2H, J = 2.70 Hz, 2H-7’), 7.49 (4H, J = 8.20 Hz, 2H-3’ and 2H-5’), 7.13 (2H, J = 9.00 and 2.70 Hz, 2H-8’), 6.98 (4H, J = 3.90 and 1.25 Hz, 2H-3), 6.87 (2H, J = 3.90 and 2.75 Hz, 2H-2’), 3.93 (4H, J = 2.70 Hz, 2H-4’), 3.89 (4H, J = 2.70 Hz, 2H-4’, 2H-8’), 2.43 (4H, J = 6.90 Hz, 2NCH2), 2.71 (2H, J = 6.90 Hz, 2NCH2) ppm. 13C NMR δ (100 MHz, CDCl3) 135.8 (C-5’), 135.8 (C-4’), 132.8 (C-3’), 132.6 (C-4’), 131.2 (C-2’, C-6’), 129.6 (C-3’ and C-5’), 126.4 (C-1’), 122.8 (C-9a), 117.9 (C-9), 115.9 (C-8), 115.6 (C-11), 115.0 (C-2), 109.7 (C-6), 106.7 (NCH2), 57.1 (OCH3), 55.2 (NCH2), 49.5 (NCH2), 43.7 (NCH2), 29.0 (CH3). MALDI-TOF MS m/z [M + H]+ Calcd for C45H50N7O2: 718.416. Found: 718.416.

Bis[N-[4-[9-methoxyrryrolo[1,2-a]quinoxalin-4-yl]benzyl]-3-amino-propyl]piperazine (1i)

Orange-pale oil, Yield: 92%; 1H NMR δ (300 MHz, CDCl3) 8.83 (dd, 2H, J = 2.70 and 1.35 Hz, 2H-1), 7.95 (4H, J = 8.10 Hz, 2H-2’ and 2H-6’), 7.66 (2H, J = 8.10 and 1.20 Hz, 2H-8), 7.48 (4H, J = 8.10 Hz, 2H-3’ and 2H-5’), 7.37 (2H, J = 2.70 Hz, 2H-7), 7.04 (dd, 2H, J = 8.10 and 1.20 Hz, 2H-6’), 7.00 (2H, J = 4.10 and 1.35 Hz, 2H-3’), 6.82 (2H, J = 4.10 and 2.70 Hz, 2H-2’), 4.08 (4H, J = 2.70 Hz, 2CH2), 3.89 (4H, J = 2.70 Hz, 2NCH2), 2.71 (2H, J = 4.10 Hz, 2CH3). MALDI-TOF MS m/z [M + H]+ Calcd for C48H53N8O2: 773.429. Found: 773.446.

Bis[N-[4-[7-methoxyrryrolo[1,2-a]quinoxalin-4-yl]benzyl]-3-amino-propyl]piperazine (1g)

Yellow oil, Yield: 97%; 1H NMR δ (300 MHz, CDCl3) 7.96 (d, 2H, J = 9.00 Hz, 2H-6’), 7.94 (4H, J = 8.10 Hz, 2H-2’ and 2H-6’), 7.89 (dd, 2H, J = 2.75 and 1.20 Hz, 2H-1’), 7.47 (4H, J = 8.10 Hz, 2H-3’ and 2H-5’), 7.32 (2H, J = 2.70 Hz, 2H-9’), 7.04 (dd, 2H, J = 9.00 and 2.70 Hz, 2H-7’), 6.95 (2H, J = 3.90 and 1.20 Hz, 2H-3’ and 2H-5’), 6.87 (2H, J = 3.90 and 2.70 Hz, 2H-2’), 3.96 (2H, J = 6.90 Hz, 2NCH2), 2.25 (2H, J = 6.90 Hz, 2NCH2) ppm. 13C NMR δ (100 MHz, CDCl3) 160.4 (C-8), 153.0 (C-4), 143.3 (C-3a), 138.6 (C-5a), 132.7 (C-6), 132.0 (C-4’), 130.0 (C-2’ and C-6’), 129.6 (C-3’ and C-5’), 126.2 (C-1’), 126.7 (C-9a), 115.5 (C-7), 115.4 (C-11), 114.2 (C-2), 109.5 (C-3), 98.9 (C-9), 58.4 (NCH2), 57.2 (OCH3), 55.1 (NCH2), 54.7 (NCH2), 49.4 (NCH2), 28.3 (CH3). MALDI-TOF MS m/z [M + H]+ Calcd for C47H52N7O2: 773.428. Found: 773.429.
Bis[N-(4-(3-ethoxypropyrolo[1,2-a]quinoxalin-4-yl)benzyl]-3-aminopropyl]piperazine (1k)

Yellow oil, Yield: 76%; 1H NMR δ (300 MHz, CDCl3) 7.93 (dd, 2H, J = 1.20 and 1.20 Hz, 2H-1), 7.99 (dd, 2H, J = 2.70 and 1.20 Hz, 2H-2), 7.99 (t, 2H, J = 1.20 Hz, 2H-2'), 7.89 (m, 4H, 2H-6' and 2H-9), 7.55-7.42 (m, 12H, 2H-4', 2H-5', 2H-7 and 2H-8), 3.88 (s, 4H, 2 CH2N), 2.70 (t, 4H, J = 6.90 Hz, 2 NC3H), 2.40 (t, 4H, J = 6.90 Hz, 2 NC3H), 2.22 (s, 3H, NC3H), 1.72 (qt, 4H, J = 6.90 Hz, 2 CH2), 1.12 (qd, 4H, J = 6.90 Hz, 2 CH2). 13C NMR δ (100 MHz, CDCl3) 160.5 (C-5' and C-2'), 151.5 (C-9), 151.3 (C-1), 143.7 (C-2), 139.8 (C-3), 131.5 (C-6), 131.0 (C-7), 130.0 (C-2'), 129.7 (C-5'), 128.8 (C-6'), 128.6 (C-7'), 128.5 (C-1'), 126.7 (C-9a), 126.6 (C-6), 116.0 (C-1), 115.0 (C-9), 110.1 (C-10), 58.3 (NC3H), 53.3 (NCH2), 49.4 (NCH2), 43.6 (NCH2), 28.8 (CH2). MALDI-TOF MS m/z [M+H]+ Calc for C54H67N9O2: 713.408, Found: 713.447.

Bis[N-(4-(3-ethylpyrrolo[1,2-a]quinoxalin-4-yl)benzyl]-3-aminopropyl]piperazine (11)

Pale-yellow crystals, Yield: 79%; mp = 81–83 °C; 1H NMR δ (300 MHz, CDCl3) 8.27 (d, 2H, J = 1.20 Hz, 2H-1), 8.20 (t, 2H, J = 1.20 Hz, 2H-2), 7.94 (dd, 2H, J = 1.20 and 1.20 Hz, 2H-2'), 7.89 (t, 2H, J = 1.20 Hz, 2H-2'), 7.87 (m, 4H, 2H-6' and 2H-9), 7.55-7.42 (m, 12H, 2H-4', 2H-5', 2H-7 and 2H-8), 3.88 (s, 4H, 2 CH2N), 2.70 (t, 4H, J = 6.90 Hz, 2 NC3H), 2.40 (t, 4H, J = 6.90 Hz, 2 NC3H), 2.22 (s, 3H, NC3H), 1.72 (qt, 4H, J = 6.90 Hz, 2 CH2). 13C NMR δ (100 MHz, CDCl3) 154.6 (C-4), 146.6 (C-3a), 140.2 (C-5a), 137.6 (C-4 and C-5'), 135.7 (C-1'), 131.2 (C-6), 130.9 (C-2' and C-6'), 128.5 (C-3' and C-5'), 128.4 (C-8), 128.0 (C-9a), 126.4 (C-7), 114.0 (C-11), 113.5 (C-3), 113.2 (C-9), 103.2 (C-2'), 68.8 (OCH3), 58.4 (NCH2), 55.1 (NCH2), 49.1 (NCH2), 28.3 (CH3), 16.1 (CH3). MALDI-TOF MS m/z [M+H]+ Calc for C39H37N5O2: 585.40. Found: 585.45.

Bis[N-(3-(pyrrolo[1,2-a]quinoxalin-4-yl)benzyl]-3-aminopropyl)methylamine (1m)

Yellow oil, Yield: 83%; 1H NMR δ (300 MHz, CDCl3) 8.04 (dd, 2H, J = 1.20 and 1.20 Hz, 2H-1), 7.94 (t, 2H, J = 1.20 Hz, 2H-2'), 7.87 (m, 4H, 2H-6' and 2H-9), 7.55-7.42 (m, 12H, 2H-4', 2H-5', 2H-7 and 2H-8), 3.88 (s, 4H, 2 CH2N), 2.70 (t, 4H, J = 6.90 Hz, 2 NC3H), 2.40 (t, 4H, J = 6.90 Hz, 2 NC3H), 2.22 (s, 3H, NC3H), 1.72 (qt, 4H, J = 6.90 Hz, 2 CH2). 13C NMR δ (100 MHz, CDCl3) 154.6 (C-4), 154.5 (C-1'), 146.6 (C-3a), 140.0 (C-5a), 137.6 (C-4 and C-5'), 135.4 (C-1'), 131.7 (C-8), 131.2 (C-7), 130.6 (C-3' and C-5'), 130.4 (C-2' and C-6'), 129.7 (C-9), 129.2 (C-4'), 128.6 (C-3' and C-5'), 128.1 (C-9a), 127.6 (C-3' and C-5'), 126.9 (C-6), 118.7 (C-2'), 115.0 (C-9), 112.8 (C-10), 107.2 (C-3), 58.5 (NC3H), 54.0 (NCH2), 53.2 (NCH2), 49.5 (NCH2), 31.1 (CH3). MALDI-TOF MS m/z [M+H]+ Calc for C38H35N5O2: 567.40. Found: 567.45.

Bis[N-(4-(3-ethoxypropyrolo[1,2-a]quinoxalin-4-yl)benzyl]-3-aminopropyl]piperazine (1q)

Yellow oil, Yield: 86%; 1H NMR δ (300 MHz, CDCl3) 8.55 (dd, 2H, J = 8.20 and 1.20 Hz, 2H-1), 8.51 (dd, 2H, J = 8.70 and 0.90 Hz, 2H-1), 8.09 (dd, 2H, J = 8.00 and 1.65 Hz, 2H-4'), 8.00 (dd, 2H, J = 8.40 Hz, 2H-2' and 2H-6'), 7.95 (2H, J = 7.80 Hz, 2H-8), 7.67–7.60 (m, 4H, 2H-2' and 2H-3'), 7.53 (4H, J = 8.40 Hz, 2H-3' and 2H-5'), 7.48–7.43 (m, 4H, 2H-9 and 2H-10), 7.27 (s, 2H, 2H-7), 3.92 (4H, 2 CH2N), 2.75 (t, 4H, J = 6.90 Hz, 2 NC3H), 2.63–2.41 (m, 8H, 4 NCH2), 2.46 (4H, J = 6.90 Hz, 2 NC3H), 1.76 (bs, 2H, 2 NH). 13C NMR δ (100 MHz, CDCl3) 157.3 (C-6), 142.8 (C-6a), 138.6 (C-11a), 137.7 (C-4a), 134.4 (C-5), 131.9 (C-3), 131.6 (C-11), 130.5 (C-8a), 130.2 (C-2' and C-6'), 129.9 (C-3' and C-5'), 129.7 (C-12), 125.8 (C-4), 125.6 (C-1), 124.1 (C-9), 124.0 (C-10), 116.0 (C-8 and C-11), 103.8 (C-7), 58.5 (NCH2), 54.6 (NCH2), 49.5 (NCH2), 28.2 (CH3). MALDI-TOF MS m/z [M+H]+ Calc for C44H44N9O2: 734.39. Found: 734.43.
Bis[N-(4-phenylpyrrolo[1,2-a]quinazolin-2-yl)methyl]-3-amino-propyl)piperazinone (1r)

Pale-yellow crystals, Yield: 70%, mp = 62–64 °C; 1H NMR δ (300 MHz, CDCl3) 8.04 (dd, 2H, J = 7.80 and 1.50 Hz, 2H-6), 8.03–7.97 (m, 4H, 2 H-2’ and 2 H-6’), 7.98 (d, 2H, J = 1.10 Hz, 2 H-1’), 7.85 (dd, 2H, J = 7.80 and 1.50 Hz, 2H-9), 7.57–7.53 (m, 8H, 2 H-3’, 2 H-4’, 2 H-5’ and 2H-7’), 7.47 (ddd, 2H, J = 7.80, 1.50 Hz, 2H-8), 6.94 (d, 2H, J = 11.0 Hz, 2H-3’), 3.95 (s, 4H, 2 NCH2), 2.75 (t, 4H, J = 6.90 Hz, 2 NCH2), 2.61–2.35 (m, 8H, 4 NCH2 pips.), 2.40 (t, 4H, J = 6.90 Hz, 2 NCH2), 1.93 (bs, 2H, 2 NH), 1.72 (qt, 4H, J = 6.90 Hz, 2 CH2), 13C NMR δ (100 MHz, CDCl3) 157.5 (C-7), 155.8 (C-4), 143.1 (C-5a), 138.7 (C-3a and C-4’), 129.9 (C-5’ and C-4’), 129.2 (C-1’), 128.7 (C-6’), 126.7 (C-9a), 115.4 (C-7), 115.3 (C-1), 114.2 (C-2), 109.6 (C-3), 98.9 (C-9), 71.8 (OCH3), 71.5 (OCH2), 56.7 (OCH2), 54.8 (NCH2), 49.8 (NCH2). MALDI-TOF MS m/z [M + H]+ Calcd for C64H63N6O3: 765.376; Found: 765.457.

In vitro antimalarial activity

The in vitro antimalarial activities were tested over concentrations ranging from 39 nM to 40 μM against culture-adapted Plasmodium falciparum reference strains 3D7 and W2. The former strain is susceptible to chloroquine (CQ) but displays a decreased susceptibility to mefloquine (MQ) while the latter is considered resistant to chloroquine. The parasites were cultivated in RPMI medium (Sigma-Aldrich, Lyon, France) supplemented with 0.5% Albumax I (Life Technologies corporation, Paisley, United Kingdom), hypoxanthine (Sigma-Aldrich), gentamicin (Sigma-Aldrich), and human erythrocytes and were incubated at 37 °C in a candle jar, as described previously. The P. falciparum drug susceptibility test was carried out in 96-well flat bottom sterile plates under a final volume of 250 μL. After a 48 h incubation with the drugs, quantities of DNA in treated and control cultures of parasitemia were compared according to the SYBR Green I (Sigma-Aldrich) fluorescence-based method. Briefly, after incubation, plates were frozen at -20 °C until use. They were then thawed to thaw for 2 h at room temperature and 100 μL of the homogenized culture were transferred to 96-well flat bottom sterile black plates (Nunc Inc) already containing 100 μL of the SYBR Green I lysis buffer (2xSYBR Green, 20 mM Tris base pH 7.5, 5 mM EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100). A negative control, controls treated by solvents (DMSO and H2O, typically) and positive controls (chloroquine and mefloquine) were added to each set of experiments. Plates were incubated for 1 h at room temperature and then read on a fluorescence plate reader (Tecan, Austria) using excitation and emission wavelengths of 485 and 535 nm, respectively. Concentrations inhibiting 50% of the parasite’s growth (half maximal inhibitory concentration IC50) values were then calculated from the obtained experimental results using a regression program available on line.
20 μL of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) solution (5 mg/mL) were added to each well followed by incubation for another 4 h. The enzyme reaction was then stopped by addition of 100 μL of 50% isopropanol − 10% sodium dodecyl sulfate. Plates were shaken vigorously (300 rpm) for 10 minutes and the absorbance was measured in a plate reader at 570 nm in a BIO-TEK ELx808 Absorbance Microplate Reader. Inhibitory concentration 50% (IC50) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of L. donovani promastigotes compared to the control. IC50 were calculated by non-linear regression analysis processed on doseresponse curves, using TableCurve 2D V5.0 software. IC50 values represent the mean value calculated from three independent experiments.

Cytotoxicity evaluation

A cytotoxicity evaluation was realized according to the method of Mosmann41 with slight modification to determine the cytotoxic concentrations 50% (CC50) and using doxorubicin as a cytotoxic reference-compound. These assays were performed toward the human HepG2 cell line (HepG2 CC50). HepG2 (hepatocarcinoma cell line purchased from ATCC, ref HB-8065) is a commonly used human-derived hepatocarcinoma cell line that has shown characteristics similar to those of primary hepatocytes. These cells express many of the hepatocyte-specific metabolic enzymes, thus enabling the cytotoxicity of tested product metabolites to be evaluated. Briefly, cells in 100 μL of complete RPMI medium, [RPMI supplemented with 10% FCS, 1% L-glutamine (200 mM) and penicillin (100 U/mL)/streptomycin (100 μg/mL)] were inoculated into each well of 96-well plates and incubated at 37°C in a humidified 6% CO2. After 24 h incubation, 100 μL of medium with various product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were added and the plates were incubated for 72 h at 37°C. Duplicate assays were performed for each sample. Each plate-well was then microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 μL of MTT solution (0.5 mg/mL in medium without FCS) were then added to each well. Cells were incubated for 2 h at 37°C. After this time, the MTT solution was removed and DMSO (100 μL) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (300 rpm) for 5 minutes. The absorbance was measured at 570 nm with 630 nm as reference wavelength spectrophotometer using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as blank and doxorubicin (Sigma Aldrich) as positive control. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentration was determined from the dose-response curve by using the TableCurve 2D V5.0 software (Systat Software, San Jose, CA).

FRET melting experiments

FRET melting experiments were performed with dual-labeled oligonucleotides mimicking the Plasmodium telomeric sequences FP1T [FAM-5'GGGGTTA]2-GG3'-TAMRA] and FP8T [FAM-5'GGGGTTA]2-GG3'-TAMRA] and the human telomeric sequence F21T [FAM-5'GGGGTTA]2-GG3'-TAMRA]36,43. The oligonucleotides were prefolded in 10 mM lithium cacodylate buffer (pH 7.2), with 10 mM KCl and 90 mM LiCl (K' condition). The FAM emissions were recorded at 516 nm using a 492-nm excitation wavelength in the absence and presence of a single compound as a function of temperature (25 to 95°C) in 96-well microplates by using a Stratagene MX3000P real-time PCR device at a rate of 1°C min⁻¹. The data obtained were normalized between 0 and 1, and the temperature required for half-denaturation of oligonucleotides corresponded to the emission value of 0.5 was calculated as ΔTm. Each experiment was performed in duplicate with 200 nM of labeled oligonucleotide and 0, 1, 2, or 5 μM of compound under K' condition. For each compound, two independent experiments were carried out. The data were plotted using OriginLab Corporation, Northampton, MA).

Results and discussion

Chemistry

The reported bis[N-[pyrrolo[1,2-a]quinoxalin-4-yl]benzyl]-3-aminopropylamine or piperazine derivatives 1a–p were synthesized in seven steps from 2-nitroaniline (Scheme 1). Preparation of 1-(2-nitrophenyl)pypyroles 3a–d was performed according to the Clauson-Kaas reaction run under micro-wave irradiation starting from 2-nitroaniline and 2,5-dimethoxytetrahydrofuran in acetic acid. This pathway partially involved the synthetic methodologies already described by our group20–23,27. The resulting 1-(2-nitrophenyl)pypyrole intermediates 3a–d was subsequently reduced into the attempted 1-(2-aminophenyl)pypyroles 4a–d using a sodium borohydride-copper (II) sulfate in ethanol at room temperature. This NaBH4-CuSO4 system was found to be quite powerful in reducing our aromatic nitro group with excellent yield (73–85%). The reaction of 4a–d with triphosgene in toluene gave the lactams 5a–d22. Reduction of the nitro moiety of 6a–b with iron in hot glacial acetic acid produced the spontaneous ring closure onto the ester to afford the desired lactams 5e–f through a one-pot reduction-cyclization step27,25.

The lactams 5a–f were subsequently chlorodehydroxylated with phosphorous oxychloride, leading to the 4-chloropyrrolo[1,2-a]quinoxalines 7a–f.

Coupling chloro derivatives 7a–f with 3- or 4-formylphenylboronic acid in the presence of Pd(PPh3)4 as a catalyst under Suzuki-Miyaura cross-coupling conditions proceeded to afford the substituted benzaldehydes 8a–h22,27,28,30. Reaction of primary amines, such as 3,3′-diamino-N-methyldipropylamine and N-(3-aminopropyl)-1,3-propanediamine or 1,4-bis(3-aminopropyl)piperazine, with the latter 7a–f gave the di-imines 9a–p, reduced into the bis[N-[pyrrolo[1,2-a]quinoxalin-4-yl]benzyl]-3-aminopropyl]amines 1a–p using sodium borohydride in methanol. The same pathway was used for the synthesis of bisindoloquinoxaline 1q and bis(4-phenyl)pyrroloquinoxaline 1r from aldehydes 8i and 8j, respectively (Schemes 2 and 3).

This strategy was also used to prepare the tris[N-[pyrrolo[1,2-a]quinoxalin-4-yl]benzyl]-3-aminopropyl]amines 1s–t (Scheme 4) and N,N'-[oxysbis(2,1-ethanediyloxy-2,1-ethanediyl)]bis[pyrrolo[1,2-a]quinoxalin-4-yl]benzylamine 1u (Scheme 5).

All these quinoxaline compounds 1a–u were then converted into their ammonium oxalate salts by treatment with oxalic acid in refluxing isopropanol (Table 1).

Biological activity

In vitro antimalarial activity

All new substituted bis- and trispyrrolo[1,2-a]quinoxaline derivatives 1a–u were evaluated for their antimalarial activity in vitro on P. falciparum CQ-resistant strain W2 (IC50 CQ =0.20 μM) and the CQ-sensitive and MQ decrease susceptibility strain 3D7 (IC50 CQ =0.08 μM). As shown in Table 2, they were found to have IC50
between 0.05–4.20 μM on W2, and 0.04–8.96 μM on 3D7 *P. falciparum* strains, respectively.

For the pyrroloquinoxalines unsubstituted on their heterocyclic moiety (compounds 1a–c), compound 1a joined by a bis-(3-aminopropyl)amine linker was found more active (up to 4 to 5 times) on the W2 strain than its counterparts with bis-(3-aminopropyl)methylamine (compounds 1b) or bis-(3-aminopropyl)piperazine linkages (compounds 1c): i.e. IC₅₀ = 0.85 μM for 1a versus 4.20 μM for 1c.
as a general rule against the W2 strain, the introduction of a methoxy substituent in position 7, 8 or 9 of the bis-pyrrolo[1,2-a]quinoxaline skeleton linked in position 4 of the benzyl ring (compounds 1d-i) strongly increased the antiparasitic activity in comparison with their respective unsubstituted bis-pyrrolo[1,2-a]quinoxaline homologs 1b-c: i.e. IC\(_{50}\) = 0.13–0.44 \(\mu \text{M}\) for 1d-i and 3.40–4.20 \(\mu \text{M}\) for 1b-c. Moreover, introduction of an ethoxy substituent in position C-3 of the pyrrolo[1,2-a]quinoxaline moieties joined by a bis-(3-aminopropyl) piperazine linker (compound 1k) was found more active (up to 38 times) than its non-substituted homolog 1c (IC\(_{50}\) = 0.09 \(\mu \text{M}\) for 1k in comparison of 3.40 \(\mu \text{M}\) for 1c). In addition, 1k was found to be 2.2 times more active than the reference drug CQ (IC\(_{50}\) = 0.20 \(\mu \text{M}\)). In contrast, the addition of a phenyl in position 2 of the pyrrolo[1,2-a]quinoxaline moiety (compound 1l) reduced considerably the antimalarial activity on the W2 strain (IC\(_{50}\) = 1.39 \(\mu \text{M}\)).

The bis-pyrrolo[1,2-a]quinoxalines 1m-n, which are linked with polyaminoalkyl chains on position 3 of the benzyl moieties, increased the antimalarial activity up to 35 and 68 times when compared to their non-substituted counterparts linked in position 4 (compounds 1b-c). Thus, compound 1n bearing a diaminopropylpiperazine linker exhibited the most potent antiplasmodial activity (IC\(_{50}\) = 0.05 \(\mu \text{M}\)) against the CQ-resistant strain (W2). Nevertheless, in these subseries in which the polyaminoalkyl side chain was anchored in position 3 of the benzyl ring, the introduction of a methoxy substituent on the pyrrolo[1,2-a]quinoxaline heterocycles (compounds 1o-p) seemed to be less detrimental against drug-resistant strain W2 in comparison with their analogs 1d-i. The bis-pyrrolo[1,2-a]quinoxaline 1r, structural isomer of derivative 1c, was also found to have a significant activity against the W2 strain (IC\(_{50}\) = 0.75 \(\mu \text{M}\)). In addition, the tris[N-(pyrrolo[1,2-a]quinoxalin-4-yl)benzyl]-3-aminopropyl]amines 1s-t showed similar antimalarial activities when compared with their bis-homologs 1d-i (IC\(_{50}\) = 0.19–0.29 \(\mu \text{M}\) for 1s-t versus 0.13–0.44 \(\mu \text{M}\) for 1d-i). Moreover, the replacement of the polyaminoalkyl linker by a 1,11-diamino-3,6,9-trioxaundecane chain (compound 1u) showed the same level of antimalarial activity against the W2 strain with IC\(_{50}\) = 0.25 \(\mu \text{M}\).

Against the CQ-sensitive strain (3D7) and in the subseries of the pyrrolo[1,2-a]quinoxalines unsubstituted on their heterocyclic moieties (compounds 1a-c), compound 1c joined by a
bis-(3-aminopropyl)piperazine linker was found more active than its structural analogs with bis-(3-aminopropyl)amine (compound 1a) or bis-(3-aminopropyl)methylamine linkages (compound 1b): i.e. IC$_{50}$ = 0.80 μM for 1c versus 3.42 and 1.20 μM for 1a and 1b, respectively. Surprisingly, most of the pyrrolo[1,2-a]quinoxalines 1d–g and 1i bearing a methoxy substituent on the heterocyclic skeleton exhibited moderate antimalarial activity against 3D7 strain with IC$_{50}$ ranging from 1.28 to 5.85 μM, in the exception of compound 1h which showed an interesting IC$_{50}$ of 0.25 μM against this Plasmodium strain. As against the W2 strain, replacement of the methoxy function by an ethoxy one in position 3 of the pyrrolo[1,2-a]quinoxaline moieties (compounds 1j–k) increased the anti P. falciparum activity against the 3D7 strain (IC$_{50}$ = 0.26 and 0.19 μM for 1j and 1k, respectively). The introduction of a phenyl ring in position 2 of the pyrrolo[1,2-a]quinoxaline moiety (compound 1l) reduced considerably the antimalarial activity on the 3D7 strain (IC$_{50}$ = 8.96 μM). Similar observations could be made with the structural analogs 1q–r which showed moderate activity against 3D7 strain (IC$_{50}$ = 1.00–7.22 μM). In the subseries in which the polyaminoalkyl side chain was anchored in position 3 of the benzyl ring, the piperazine substituted bis-pyrrolo[1,2-a]quinoxaline 1p was found to be two times more active than the reference drugs chloroquine and mefloquine (IC$_{50}$ = 0.040 μM for 1p versus IC$_{50}$ = 0.080 μM for the references). The tris-pyrrolo[1,2-a]quinoxalines 1s–t showed similar antimalarial activities when compared with their methoxy substituted bis-homologs 1o–p (IC$_{50}$ = 0.20–0.24 μM for 1s–t versus 0.04–0.18 μM for 1o–p). Compound 1u bearing diaminotrioxaundecane linkage exhibited similar activity against the 3D7 strain than those observed for chloroquine and mefloquine (IC$_{50}$=0.08 μM).

In vitro antileishmanial activity against promastigote forms
In order to increase the biological profile of molecules 1a–u, complementary analyses were performed. Notably, P. falciparum belonging to coccidian protozoan parasites, their in vitro biological profile.
activity on flagellate protozoan parasites like *Leishmania donovani* was evaluated (Table 2). In comparison with amphotericin B and pentamidine used as reference drugs (IC\textsubscript{50} = 0.1 μM and 5.5 μM, respectively), most of the tested compounds 1 were found active against the promastigote forms of *L. donovani*. It must be noticed that a majority of these bis-pyrrolo[1,2-α]quinoxaline derivatives 1 (compounds 1a, 1c, 1d–j and 1m–q) with IC\textsubscript{50} ranging from 1.0 to 4.26 μM were found slightly more potent than pentamidine, one of the reference compounds (IC\textsubscript{50} = 5.5 μM). In particular, bis[N-4-(indolo[1,2-α]quinolin-6-yl]benzyl]-3-aminopropyl]piperazine 1q was found to be 9.6 times more active than pentamidine (IC\textsubscript{50} = 0.57 μM versus IC\textsubscript{50} = 5.5 μM). In our previously described antileishmanial series\textsuperscript{21,22}, we have noticed that introduction of a methoxy group in positions 7 and 8 of the pyrrolo[1,2-α]quinoxaline moiety generally increased the antileishmanial activity. In this new series, we also decided to introduce a methoxy or an ethoxy to evaluate the influence of such groups for their *in vitro* antileishmanial activity upon the *L. donovani* strain. Meanwhile, the presence of these alkoxy groups in the 7, 8, 9 or 3 position seems not to be detrimental for the activity against *L. donovani* as illustrated by results obtained for compounds 1d–k and 1o–p (IC\textsubscript{50} = 1.07–4.26 μM) in comparison with unsubstituted compounds 1a, 1c and 1m–n (IC\textsubscript{50} = 1.0–3.71 μM). The same observation could be done for the nature of the polyalkylamine linker between the two benzylpyrrolo[1,2-α]quinoxaline moieties that seemed also not to be crucial, with the exception of piperazine substituted 1g, which showed a better antileishmanial activity in *vitro* compared to its structural methyleamine analog 1f (IC\textsubscript{50} = 4.25 μM versus 1.07 μM) for 1g upon the *L. donovani* strain.

Moreover, in the subseries in which the polyaminoalkyl side chain was anchored in position 3 of the benzyl ring, the various bis-pyrrolo[1,2-α]quinoxalines 1m–p were found less beneficial for the *in vitro* antiparasitic activity with an IC\textsubscript{50} of 2.80–3.71 μM in comparison with their analogs 1a–c and 1f–g. In addition, the tri-substitution of the tris(3-aminopropyl)amine linker by the pyrrolo[1,2-α]quinoxaline core (compounds 1s–t) led to a decrease in the antileishmanial activity (IC\textsubscript{50} > 10 μM) in comparison with their bis-pyrrolo[1,2-α]quinoxaline analogs 1d–e and 1o–p.

**Cytotoxicity and selectivity index**

In order to assess their selectivity of action, the cytotoxicity of the antimalarial compounds 1a–u was also evaluated *in vitro* on one human cell line. The cytotoxic concentrations 50% (CC\textsubscript{50}) on the metabolizing human hepatocyte HepG2 cell line allowed access to the corresponding selectivity indexes (SI) defined as ratio of cytotoxic and antimalarial activities (SI = CC\textsubscript{50}/W2 or 3D7 IC\textsubscript{50}). The SI could validate their real potential as selective antiparasitic agents. The results concerning the cytotoxicity and SI data are presented in Table 2. As expected, most of the active bis- and trispyrrolo[1,2-α]quinoxalines 1 showed significant level of cytotoxicity against the HepG2 cell line (IC\textsubscript{50} = 1.25–3.38 μM). Considering the W2 *P. falciparum* strain, the selectivity indexes varied between 0.37 and 40.6, and SI ranged from 0.53 to 39.25 by taking into account the 3D7 strain. This SI led to the identification of compound 1n with SI of 40.6 on W2 strain, and SI of 39.25 for derivative 1p on 3D7 strain. We could notice that these last bioactive compounds 1n and 1p were bis-pyrrolo[1,2-α]quinoxalines linked by a 1,4-bis(3-aminopropyl)piperazine moiety in position 3 of their benzyl ring. On the other hand, the bis-pyrrolo[1,2-α]quinoxaline 1u is also interesting with SI = 20.26, on the 3D7 malaria strain. However, according to the SI values, these compounds appeared to be toxic; thus, new modulations by replacing the benzyl substituted heterocyclic moiety to synthesize new candidates could be developed for forthcoming pharmacological investigations.

**FRET-melting experiments**

As these new compounds presented the same requirements as those required for G-4 stabilizing ligands, we have also investigated their ability of targeting *P. falciparum* telomeres as a potential strategy to interfere with human protozoan parasite infections. In fact, the telomeres of the parasite could constitute an attractive target. They are composed of repetitions of a degenerate motif (\(5'\)GGGTYYA\(^3\)), where Y could be T or C, which is different from...
Table 2. In vitro sensitivity of compounds 1-a-w on *P. falciparum* and *L. donovani* strains, and cytotoxicity on the HepG2 cell line.

| Compound | W2 (IC₅₀, μM) | 3D7 (IC₅₀, μM) | L. donovani (IC₅₀, μM) | Cytotoxicity HepG2 (IC₅₀, μM) | Selectivity Index (SI)
|----------|---------------|-----------------|------------------------|-----------------------------|----------------------|
| Chloroquine | 0.20 ± 0.03 | 0.08 ± 0.003 | n.d. | 30 | 150
| Mefloquine | 0.03 ± 0.001 | 0.08 ± 0.001 | n.d. | 2.3 ± 0.5 | n.d.
| Pentamidine | n.d. | n.d. | 0.1 ± 0.04 | 8.8 ± 0.6 | n.d.
| Amphotericin B | n.d. | n.d. | 5.5 ± 0.8 | 8.8 ± 0.6 | n.d.
| 1a | 0.85 ± 0.15 | 3.42 ± 0.54 | 1.0 ± 0.2 | 1.81 ± 0.10 | 2.13 ± 0.53
| 1b | 4.20 ± 0.65 | 1.02 ± 0.20 | n.d. | 1.56 ± 0.60 | 0.37 ± 1.30
| 1c | 3.40 ± 0.74 | 0.80 ± 0.12 | 1.14 ± 0.2 | 1.25 ± 0.30 | 0.37 ± 1.56
| 1d | 0.34 ± 0.04 | 4.14 ± 0.03 | 3.89 ± 0.3 | 2.57 ± 0.77 | 7.56 ± 0.62
| 1e | 0.21 ± 0.03 | 2.62 ± 0.25 | 2.52 ± 0.1 | 1.46 ± 0.6 | 6.95 ± 0.56
| 1f | 0.13 ± 0.01 | 5.85 ± 0.07 | 4.25 ± 0.2 | 3.38 ± 0.9 | 26 ± 0.58
| 1g | 0.36 ± 0.04 | 2.22 ± 0.25 | 1.07 ± 0.1 | 2.14 ± 1.40 | 5.94 ± 0.96
| 1h | 0.44 ± 0.11 | 0.25 ± 0.04 | 2.07 ± 0.02 | 2.44 ± 0.4 | 5.54 ± 9.76
| 1i | 0.32 ± 0.04 | 1.28 ± 0.10 | 1.58 ± 0.1 | 1.33 ± 0.3 | 4.16 ± 1.04
| 1j | 0.49 ± 0.16 | 0.26 ± 0.03 | 4.26 ± 0.1 | 1.95 ± 0.3 | 3.98 ± 7.50
| 1k | 0.09 ± 0.01 | 0.19 ± 0.01 | ≥10 | 1.48 ± 0.5 | 16.44 ± 7.79
| 1l | 1.39 ± 0.13 | 8.96 ± 0.69 | n.d. | n.d. | n.d.
| 1m | 0.12 ± 0.01 | 1.07 ± 0.16 | 2.80 ± 0.1 | 1.94 ± 1.0 | 16.17 ± 1.81
| 1n | 0.05 ± 0.01 | 0.37 ± 0.04 | 3.71 ± 0.4 | 2.03 ± 1.2 | 40.6 ± 5.49
| 1o | 0.20 ± 0.06 | 0.18 ± 0.02 | 3.51 ± 0.1 | 1.50 ± 0.9 | 7.28 ± 8.33
| 1p | 0.17 ± 0.03 | 0.04 ± 0.004 | 3.39 ± 0.4 | 1.57 ± 0.8 | 9.23 ± 39.25
| 1q | n.d. | 1.00 ± 0.05 | 0.57 ± 0.1 | 1.56 ± 0.70 | 1.56 ± n.d.
| 1r | 0.75 ± 0.10 | 7.22 ± 0.77 | n.d. | n.d. | n.d.
| 1s | 0.29 ± 0.05 | 0.20 ± 0.04 | ≥10 | 1.66 ± 0.5 | 5.72 ± 8.30
| 1t | 0.19 ± 0.02 | 0.24 ± 0.03 | ≥10 | 1.72 ± 0.6 | 9.05 ± 7.17
| 1u | 0.25 ± 0.08 | 0.076 ± 0.009 | ≥10 | 1.54 ± 0.7 | 6.16 ± 20.26

**Notes:**
- W2 (IC₅₀, μM) values were measured on the chloroquine-resistant and mefloquine-sensitive strain W2 and the chloroquine-sensitive and mefloquine-resistant strain 3D7.
- L. donovani (IC₅₀, μM) values were measured on the promastigotes of *Leishmania donovani* strain. The IC₅₀ (μM) values correspond to the mean ± standard deviation from three independent experiments.
- Cytotoxicity CC₅₀ values correspond to the mean ± standard deviation from three independent experiments.
- Selectivity Index (SI) was defined as the ratio between the CC₅₀ value on the HepG2 cells and the IC₅₀ value against the *P. falciparum* W2 or 3D7 strain.
- Chloroquine and mefloquine were used as antimalarial drug-compounds of reference.
- Pentamidine and Amphotericin B were used as antileishmanial drug-compounds of reference.
- gn.d.: not done.

In the present report, we have described the synthesis and the antimalarial activity of new bis- and trispyrrolo[1,2-a]quinoxaline derivatives 1 in which aromatic nuclei are joined by various aliphatic polyanimes linker. These new compounds were then tested for their in vitro antiparasitic activity on (i) two *P. falciparum* strains (the CQ-resistant W2 and CQ-sensitive 3D7); and (ii) the promastigote forms of *L. donovani* strain. Among these new synthesized molecules, few of them were identified as potential in vitro antiplasmodial hits, displaying IC₅₀ ranging from 0.04 to 0.09 μM on the W2 and 3D7 strains of *P. falciparum*. Thus, the most promising antimalarial results were obtained for the two bis-pyrrolo[1,2-a]quinoxalines 1n and 1p linked by a 3-(aminopropyl)liperase chain in position 3 of their benzyl moieties. These compounds 1n and 1p were identified as the most potent antimalarial candidates with selectivity index (SI) of 40.6 on W2 strain, and 39.25 on 3D7 strain, respectively. In addition, biological results showed activity against the promastigote forms of *L. donovani* with IC₅₀ ranging from 0.57 to 4.26 μM. In parallel, the in vitro cytotoxicity of these new...
molecules was assessed on the human HepG2 cell line. Structure-activity relationships of these new synthetic compounds are here also discussed, as well as their relative ability of targeting *P. falciparum* telomeres as potential mechanism of action. Thus, as the telomeres of the parasite could constitute an interesting target, we have also established the possibility of targeting *Plasmodium* telomeres by stabilizing the *Plasmodium* telomeric G-quadruplexes through a FRET melting assay. These results led us to conclude that the two bis(N-(4-(methoxypyrrolo[1,2-a]quinoxalin-4-yl)benzyl)-3-aminopropyl)piperazines 1e and 1i seemed to be able to discriminate between *Plasmodium* and human telomeric quadruplexes. By taking into account the biological and biophysical results and also the structure–activity relationships of these new series and those previously described, our overall results could be now considered as a promising starting point for the further development and optimization of new and potent antiprotozoan compounds.

**Acknowledgements**

We thank the DGA and ANR (project ANR-12-ASTR-003) for the financial support of this study.

**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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