CHAPTER 3

3 Article for submission

Chapter 3 contains an article published in the European Journal of bioorganic & medicinal chemistry. The article contains the background, aim, experimental details and results, including the physicochemical properties and *in vitro* biological results of synthesised compounds of this study. This article is prepared according to the author's guidelines available in the Author information pack at the journal homepage: www.elsevier.com/locate/bmcl

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Original article

Synthesis and *in vitro* antimalarial activity of a series of bisquinoline and bispyrrolo[1,2a]quinoxaline compounds

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ABSTRACT

Series of bisquinolines **4**–**15** and bispyrrolo[1,2a]quinoxalines **16**–**20** containing various polyamine linkers were synthesized. The aqueous solubility and distribution coefficient were experimentally determined. The compounds were screened for antimalarial activity alongside chloroquine against D10 and Dd2 strains of *Plasmodium falciparum*. The growth inhibitory effects of biscompounds **4**–**9** were assessed against various cancer cell lines. The aqueous solubility was found to increase with an increase in potential protonation sites. Bisquinolines **8** and **9** featuring triethylenetetramine and N,N′-bis(3-aminopropyl)ethylene-diamine linkers, respectively, were the most active of all synthesized compounds. They were found as potent as chloroquine against D10 but significantly more potent against the Dd2 strain, with good selectivity towards parasitic cells. Compound **4** containing a diethylenetriamine bridge displayed the most important anticancer activity of the series, and was a more effective antiproliferative inhibitor than etoposide against all three TK10, UACC62 and MCF7 cancer cell lines.

1. Introduction

Malaria affects an estimated 250 million people and accounts for almost 1 million deaths annually, with over 90% of these fatalities recorded in Africa [1–3]. The disease's incidence has increased during the last decade as a result of the rise in resistance to existing antimalarial drugs such as chloroquine, mefloquine, suphadoxine and pyrimethamine. The emergence of resistant parasite strains severely limits the choice of available antimalarial medicines, and has driven the search for new drugs that might circumvent resistance mechanisms. The aminoquinoline, chloroquine, was for much of the last 40 years the drug of choice for malaria chemotherapy as it was highly effective, save and cheap [4,5]. However, the spread of chloroquine-resistant strains has limited its use to *Plasmodium falciparum* resistance free areas of the world. Chloroquine resistance is associated with reduced accumulation of the drug inside the digestive vacuole, which is connected to a *P. falciparum* chloroquine resistance transporter (PfCRT) or ATP-dependant P-glycoprotein efflux pump (Pgh1) [6]. Despite this drawback, the 4-aminoquinoline pharmacophore remains an attractive scaffold in design of new drugs, since it demonstrates a unique affinity for haematin and is known to be the (Fe^{3+}) ferri-protoporphyrin complex formation template [7,8].

Many studies on bisquinolines have indicated an increase in activity against chloroquine-resistant strains. Piperaquine **A** was the first bisquinoline drug synthesized in 1960 and was active *in vitro* against both CQS and CQR strains, but further development was suspended for toxicity reasons [9,10]. Vennerstrom et al. synthesized a series of bisquinoline analogues linked by various alkanediamines or heteroalkanediamines of which the most promising was WR 268,668 **B**. This compound exhibited potent *in vivo* antimalarial activity but development was stopped due to phototoxicity [11,12]. Girualt et al. [13] synthesized a series of bisquinolines **C**, tri- and tetraquinolines with increased steric hindrance that displayed good activity against resistant *P. falciparum* strains, suggesting that the greater bulkiness results in a weaker efflux by PfCRT. However, the increased rigidity by cyclisation reduced toxicity but did not increase activity in comparison with their linear counterparts. Bisquinoline compounds **D** displayed superior activity compared to chloroquine against the resistant K1 strain [14] (Fig. 1).

Chloroquine and other 4-aminoquinoline antimalarials are weak bases which traverse down the pH gradient to concentrate inside the acidic food vacuole. The protonation of the drug inside the vacuole increases its accumulation *via* a pH-trapping mechanism that

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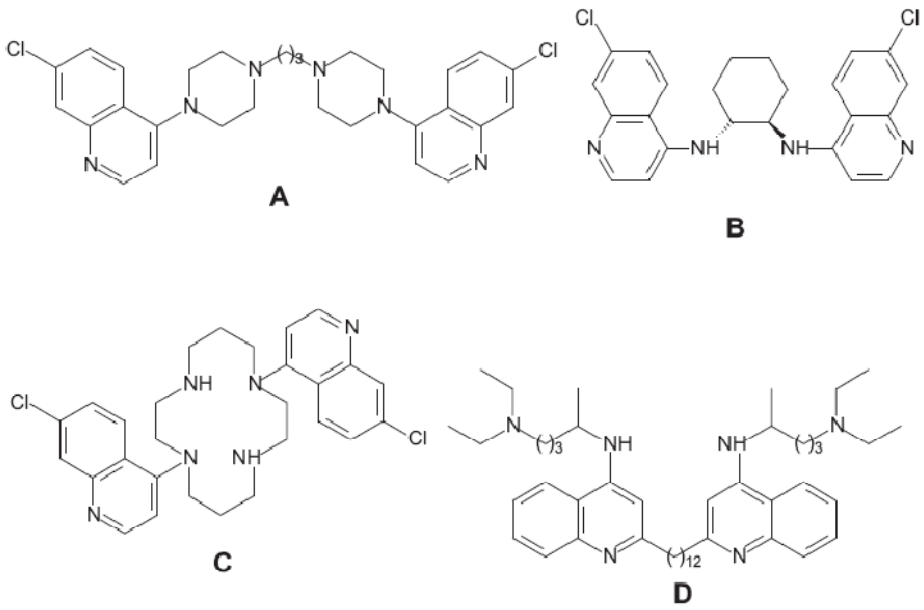

Fig. 1. Structures of some literature reported bisquinolines with various linkers: piperaquine **A** (1-[3-(piperazin-1-yl)propyl]piperazine linker), compound WR 268,668 **B** (cyclohexane-1,2-diamine linker), bisquinoline **C** (1,4,8,11-tetraazacyclotetradecane linker) and chloroquine-like bisquinoline **D** (dodecane-1,12-diamine linker).

appears critical for their activity [5]. This mechanism explains the hyper concentration of chloroquine from nano- in the plasma to millimolar concentrations in the digestive vacuole [15].

The aim of this study was to synthesize a series of bisquinoline and bispyrrolo[1,2a]quinoxaline compounds containing various polyamines, which may act as potential protonation sites in the hope of increasing their accumulation *via* pH-trapping. In this paper, we report the synthesis of these biscompounds, their aqueous solubility, distribution coefficients, cytotoxicity and *in vitro* antimalarial activity in comparison with chloroquine. The most active antimalarial series was also screened for anticancer activity against TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cells.

2. Results

2.1. Chemistry

The synthesis of bisquinolines **4**–**9** and **10**–**15** (Scheme 1), Series 1 and Series 2, respectively, was achieved by using reported methods [16,17].

The bispyrrolo[1,2a]quinoxalines **16**–**20**, on the other hand, were brought about following another general procedure [18] (Scheme 2).

These compound-types were synthesized by aromatic nucleophilic substitution, using polyamine as both reagent and solvent at elevated temperatures and longer reaction times to promote disubstitution. The nucleophilic substitution on an aromatic ring proceeds by the addition/elimination mechanism. The quinoline nitrogen atom creates a decrease in electron density *via* mesomeric and inductive effects at the C-2 and C-4 position of the quinoline ring. The approaching nucleophiles, in this case, the two primary amines at the ends of the polyamine, first add to the electron-deficient alkylhalide forming a resonance stabilized negatively charged intermediate. The halide ion is then eliminated in the second step generating hydrochloric acid from the reaction [19]. The bisquinolines **4**–**9** were obtained as off-white powders upon crystallization in ethyl acetate whereas compounds **10**–**20** were obtained after purification by column chromatography on silica gel and crystallization in ethyl acetate. The yields were in the range of 3–60%. The chemical structures of the compounds are consistent with both the NMR and MS data.

2.2. Experimental aqueous solubility and logD

Series 1 compounds **4**–**9** and Series 2 compounds **10**–**15**, which contain the quinoline moiety showed lipid solubility in same order. On contrary, Series 3 compounds **16**–**19**, which contain the pyrroloquinoxaline moiety and therefore a greater aromatic surface was slightly more lipid soluble. Overall, the series showed comparable aqueous solubility, which was found to increase with the increasing number of protonation sites in the polyamine bridge. However, this effect was overruled as the C–C chain separating two intrabridge N atoms lengthens (Table 1).

2.3. Biological

Compounds **4**–**9** were all active against both the D10 and Dd2 strains. These were significantly more potent than CQ against the resistant Dd2 strain. The compounds **10**, **14**–**17**, and **20** were all found to be less potent than CQ against both strains. The bisquinolines **11**, **12** and **13**, on the other hand, have activity in the same range as CQ against the sensitive D10 strain, while **18** and **19** showed activity comparable to that of CQ against both strains. Compounds **17** and **20** were the only compounds that demonstrated cytotoxicity at the concentrations tested. The Series 1 was the most potent against *P. falciparum*, and was subsequently selected for anticancer activity screening (Tables 2 and 3).

3. Discussion

3.1. Chemistry

These low yields in these nucleophilic substitution reactions may be attributed to multi-substitutions. The chemical structures

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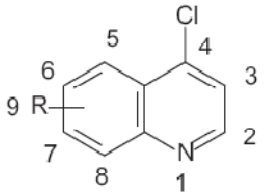
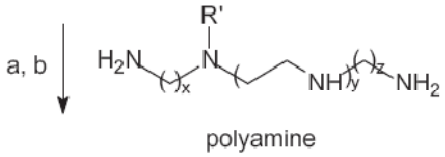
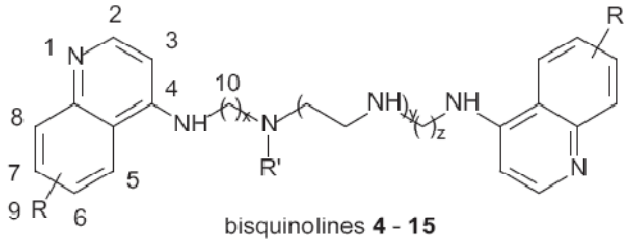


1 R = 7-Cl
2 R = 5-Me





bisquinolines **4** - **15**

Compds	R	R'	x	y	z
4	7-Cl	H	2	0	2
5	7-Cl	H	3	0	2
6	7-Cl	H	3	0	3
7	7-Cl	CH_3	3	0	3
8	7-Cl	H	2	1	2
9	7-Cl	H	3	1	3
10	5-CH_3	H	2	0	2
11	5-CH_3	H	3	0	2
12	5-CH_3	H	3	0	3
13	5-CH_3	CH_3	3	0	3
14	5-CH_3	H	2	1	2
15	5-CH_3	H	3	1	3

Scheme 1. A general reaction scheme to illustrate the synthesis of bisquinolines **4**–**15** from polyamines and appropriate substitued quinoline. Reagents and conditons: (a) 4,7-dichloroquinoline **1**, polyamine, 135–14 °C, 16–20 h; (b) 4-chloro-5-methylquinoline **2**, polyamine, 135–14 °C, 16–20 h.

of the synthesized compounds were confirmed by NMR and MS analyses. The signals for heterocyclic- and linker protons in the biscompounds **4**, **6**–**10**, **11**–**15**, **16** and **18**–**20** showed double proton intensity, revealing that they are symmetrical. Thus, only half of these protons and carbon signals appear in the spectra due to the equivalence. This was not the case for the non-symmetrical compounds **5**, **11** and **17**. The signals of the quinoline moiety were all present in the spectra of all Series 1 and 2 compounds, confirming the presence of this pharmacophore in their structures. Additionally, the spectra of Series 2 compounds show a signal with a three-proton intensity in the 2.43–2.24 ppm region and resonance of C-9 in the 21.27–21.16 ppm region. This confirms the presence of the 5-methyl group on the quinoline ring. The signals relative to the pyrrolo[1,2a]quinoxaline moiety were all present in the spectra of Series 3 compounds, confirming the presence of this pharmacophore in the structures of the quinoxalines **16**–**20**.

3.2. Physicochemical properties

Aqueous solubility and water/lipid partition coefficient are two physicochemical properties that can provide a guideline for predicting a drug's solubility characteristics in other aqueous and organic solvents [22]. These properties influence the distribution of the drug within the body since drugs must possess some lipophilic properties in order to cross biological membranes and hydrophilic properties to be taken up in systemic circulation. The experimental aqueous solubility and log *D*

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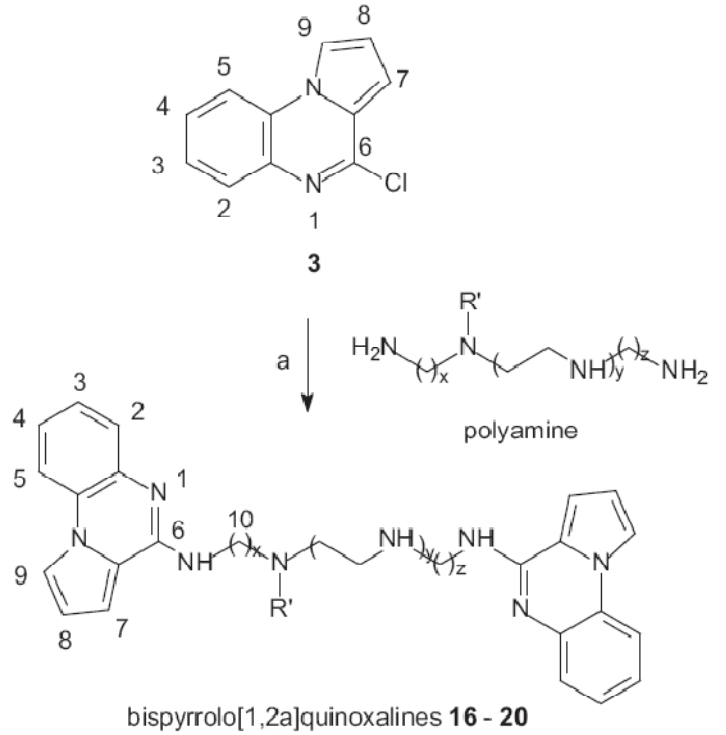

Compds	R'	x	y	z
16	H	2	0	2
17	H	3	0	2
18	H	3	0	3
19	CH_3	3	0	3
20	H	2	1	2

Scheme 2. A general reaction scheme illustrating the synthesis of bispyrrolo[1,2a]quinoxalines **16**–**20** from polyamines and 6-chloropyrrolo[1,2a]quinoxaline **3**. Reagents and conditons: (a) 6-chloropyrrolo[1,2a]quinoxaline **3**, polyamine, K_2CO_3, DMF,13 °C, 16 h.

values of compounds were determined at pH 5.5 to mimic the acidic parasitic digestive vacuole, which is their established site of action. In an acidic environment, the aliphatic nitrogen and aromatic nitrogen of weak base quinoline drugs are fully protonated, contributing to the hydrophilicity of these compounds. This makes them membrane impermeable and trapped, resulting in high concentrations of drug needed for hemozoin formation inhibition.

Comparison between S_W values of compounds **4** and **5** showed a decrease in solubility, which may be attributed to the addition of one carbon atom in the C–C chain separating the nitrogen atoms of the polyamine bridge. Compound **6** was slightly more hydrophilic than **7** presumably as result of increased protonation at the tertiary amine of the bridge in the acidic environment. The aqueous solubility of compound **8** was noticeably higher than that of compound **9** indicating that the addition of two carbon atoms in the polyamine bridge reduces the water solubility. This finding is in accordance with the structures of the linkers since a propyl C–C chain is generally more lipophilic than an ethyl one. Thus, in these two pairs of compounds, the aqueous solubility decreased slightly with the addition of a carbon atom in the C–C chain separating the 2 N in the polyamine bridge. Such was also the case for compounds **4** and **5**, and **10** and **12**.

Series 3 compounds slightly more lipid soluble than those of Series 1 and 2. The poor antimalarial activity within this Series may suggest that, as result of their high lipophilicity, these compounds are trapped in the hydrophilic biolayers and, thus, do not reach the digestive vacuole in sufficient concentration conducive to parasites death. Compounds **16** and **17** showed similar aqueous solubility. The comparison of compounds **18** and **19** again confirms the increase in hydrophilicity of the latter as a result of increased protonation in the polyamine bridge. In summary, the aqueous solubility within the Series increases with the increasing number of protonation sites in the polyamine bridge. However, the effect is overruled as the C–C chain separating the two intrabridge N atoms lengthens.

3.3. *In vitro* antimalarial activity

Series 1 was the most active while 3 was the least active of the three Series. Comparing the antimalarial activity of the

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Table 1
The physicochemical data of synthesized bisquinoline and bispyrrolo[1,2a]quinoxaline compounds. For each compound, aqueous solubility (S_W) was determined in PBS (pH 5.5) at 37 °C after incubation in water bath with stirring for 24 h; distribution coefficient (log D) was obtained after n-octanol/PBS (pH 5.5) partition.

Compds	Yield (%)	Mp (°C)[a]	S_W (mM)[b]	SD	log D[b]	SD	S_{OC} (mM)[d]	SD
4	60	248	32.01	0.13	0.30	0.09	63.91	0.50
5	23	255	23.51	0.25	0.30	0.01	46.67	0.94
6	9	200	28.89	0.24	0.28	0.11	54.26	0.98
7	15	165	32.92	0.33	0.29	0.01	64.76	0.60
8	28	123	38.68	0.10	0.28	0.04	73.30	4.71
9	10	178	18.94	0.53	0.30	0.01	37.50	4.57
10	17	129	29.50	0.02	0.05	0.02	35.17	4.14
11	19	230	28.23	0.08	0.32	0.01	58.80	1.49
12	20	87	27.90	0.34	0.14	0.03	37.50	3.01
13	25	71	31.18	0.68	0.16	0.01	45.19	0.26
14	3	113	38.86	1.67	0.40	0.04	98.39	5.88
15	21	56	36.52	0.43	0.34	0.04	73.75	3.72
16	18	75	27.51	0.45	0.39	0.01	83.57	1.53
17	17	85	28.56	0.21	0.34	0.01	63.03	0.85
18	33	82	24.13	0.32	0.32	0.01	55.55	1.77
19	14	80	30.15	0.56	0.36	0.01	69.29	1.35
20	8	124	33.98	0.38	−0.02	0.03	30.67	7.07
CQ	n.a	n.a	0.03[c]		4.30[c]		n.a	

[a] Melting point (Mp).
[b] Experimental, data represent the mean of three independent measurements.
[c] Reported [20,21].
[d] Solubility in octanol (S_{OC}) calculated from experimental S_W and log D using $\log S_{OC} = \log D + \log S_W$; not available (n.a).

bischloroquinolines to that of CQ, Dunnett's test reveal the mean IC_{50} values of compounds **4**–**9** not statistically significantly different from the mean of CQ as the p-values were greater than 0.05, implying that these compounds were as potent as CQ against the D10 strain. Against Dd2, however, their mean IC_{50} values differed statistically significantly from that of CQ indicating that they were all more potent than CQ. Compounds **8** and **9** stood as the most active against both strains as confirmed by the lowest resistance index value of 0.4.

The potent activity observed with this Series suggests that these compounds possessed a better balance between hydrophilicity and lipophilicity, and that the latter property is adequate enough to allow the intercalation onto the porphyrin rings of haematin within the hydrophobic environment provided by membrane fragments inside the digestive vacuole [23]. These fragments originate from the inner membrane of the transport vesicle and provide ideal conditions for nucleation of hemozoin formation, and favor the iron–carboxylate bond formation in the ferri-protoporphyrin IX dimers [24].

Furthermore, since these compounds only possessed increased activity over CQ against the CQR strain, it may be presumed that the increased protonation sites due to the polyamines may be responsible of this activity. Thus, compounds **4**–**9** were able to cross the lipid membranes of the digestive vacuole and sufficiently accumulate in the acidic medium of the vacuole to overcome chloroquine resistance.

Within the Series 2, compounds **10**, **14** and **15** were as potent as CQ while **11**, **12** and **13** were less potent than CQ against the D10. Against the Dd2 strain, only **11** and **12** were more potent, all others possessed less potency. Thus, compound **12** was the most active against both strains. It possessed more potent antimalarial activity than CQ against the Dd2 strain and showed activity in the same class as CQ against the D10 strain. Compounds **14** and **15** were significantly less potent than CQ against both strains. This poor antimalarial activity could be attributed to the fact that as result of

Table 2
In vitro antimalarial activity of bisquinolines **4**–**15** and bispyrrolo[1,2a]quinoxalines **16**–**20** against D10 and Dd2 strains of *Plasmodium falciparum*, and their cytotoxicity against CHO cells. Cells were incubated with compounds at various concentrations for 48 h; antimalarial activity and cytotoxicity were determined using parasite lactate dehydrogenase and MTT-assays, respectively.

Compds	D10					Dd2					RI[e]	CHO[f]		SI[h]
	n[a]	IC_{50} (nM)[b]	SD	ANOVA p value: Welch	p Value: Dunnett[c] CQ	n	IC_{50} (nM)[b]	SD	ANOVA p value: Welch	p Value: Dunnett[c] CQ		IC_{50} (μM)[b]	SD	
4	3	85.65	3.47		1.00	3	57.02	13.93		0.02[d]	0.7	[g]		
5	3	66.12	4.64		1.00	3	56.35	3.78		0.01[d]	0.9	3.10	0.30	47
6	3	110.46	9.77		0.99	3	72.88	12.07		0.03[d]	0.7	2.61	0.17	24
7	3	37.38	2.46		1.00	3	71.13	20.47		0.03[d]	1.9	7.80	2.43	211
8	3	87.67	4.00		1.00	3	35.49	7.31		0.01[d]	0.4	8.01	3.28	93
9	3	128.59	27.43		0.93	3	49.48	2.45		0.01[d]	0.4	12.97	1.41	102
10	3	740.84	32.78		0.00[d]	3	507.12	58.18		0.00[d]	0.7	[g]		
11	3	213.78	25.95		0.27	3	104.60	11.07		0.11	0.5	[g]		
12	3	128.98	0.98		0.93	3	80.22	21.13		0.40	0.6	132.12	14.92	1024
13	3	184.93	31.12		0.48	3	1914.01	85.44		0.00[d]	10.4	[g]		
14	6	1416.13	312.07		0.00[d]	3	1097.96	220.80		0.00[d]	0.8	n.a	n.a	n.a
15	3	921.93	40.50		0.00[d]	3	608.43	78.37		0.00[d]	0.7	173.99	71.40	187
16	3	554.00	8.05		0.00[d]	3	1903.47	113.57		0.00[d]	3.4	[g]		
17	3	199.00	12.67		0.03[d]	3	1377.75	94.89		0.00[d]	6.9	2.8	0.47	14
18	3	75.34	1.31		0.89	3	356.17	102.69		1.00	4.7	3.8	0.23	51
19	3	111.72	10.40		0.44	3	724.14	109.15		0.18	6.5	4.3	0.73	39
20	3	1800.32	123.54		0.00[d]	3	4166.67	463.25		0.00[d]	2.3	8.1	0.30	4.5
CQ1	9	48.35	14.63	0.00		5	242.30	0.023	0.00		5.0	n.a		n.a
CQ2	2	36.11	0.66	0.00		2	295.90	41.34	0.00		8.2	n.a		n.a
EM1	2	n.a		0.00		2	n.a		0.00		n.a	0.30	0.15	n.a
EM2	2	n.a		0.00		2	n.a		0.00		n.a	0.30	0.10	n.a

[a] Number of replicates.
[b] Data represents the mean of three independent experiments.
[c] Statistical significance at 0.05 level.
[d] p-Value (Dunnett's test) <0.05 indicates a statistically significant difference between IC_{50} values with more than 95% certainty.
[e] Resistance index (RI) = IC_{50}Dd2/IC_{50}D10.
[f] Chinese hamster ovarian (CHO).
[g] Value above maximum tested concentration.
[h] Selectivity index (SI) = IC_{50}CHO/IC_{50}D10; not available (n.a).

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Table 3
Growth inhibitory effect of bisquinolines **4**–**9** upon renal (TK10), melanoma (UACC62) and breast (MCF7) cells proliferation. Cells were incubated with bisquinolines in different concentrations for 48 h, and the growth inhibition effect was determined using SRB assay.

Compds	TK10					UACC62					MCF7				
	GI_{50}[a]	TGI[b]	LC_{50}[c]	LC_{100}[d]	Z′[e] factor	GI_{50}	TGI	LC_{50}	LC_{100}	Z′ factor	GI_{50}	TGI	LC_{50}	LC_{100}	Z′ factor
4	0.27	0.6	0.94	>100	0.92	0.33	0.59	0.86	>100	0.91	0.23	0.55	0.86	>100	0.80
5	1.23	4.25	7.27	>100	0.92	2.07	4.89	7.72	>100	0.91	0.60	2.79	7.21	>100	0.80
6	0.60	2.05	6.18	46.40	0.91	0.38	0.74	3.17	>100	0.88	0.26	0.58	0.90	>100	0.82
7	0.54	1.00	5.61	45.12	0.91	0.36	0.64	0.92	>100	0.88	0.39	0.69	1.20	>100	0.82
8	5.89	5.36	7.71	>100	0.92	2.87	5.30	7.74	>100	0.88	0.76	3.53	7.37	>100	0.85
9	2.10	4.74	7.38	>100	0.92	1.06	4.09	7.11	>100	0.88	0.38	1.65	6.43	>100	0.62
ET	5.89	43.33	92.61	>100	0.78	3.41	4	>100	>100	0.92	0.83	>100	>100	>100	0.80

[a] Concentration resulting in 50% growth inhibition (GI) and signifies the growth inhibitory power of the test agent.
[b] Concentration inducing total growth inhibition (TGI) and signifies the cytostatic effect of test agent.
[c] Lethal concentration (LC) resulting in 50% cells death and signifies the cytotoxic effect of the test agent.
[d] Lethal concentration resulting in 100% cells death and signifies the cytotoxic effect of the test agent, all concentrations are expressed in μM.
[e] Z′ factor, statistical effect size measurement, factor value in 0.5–1 range is interpreted as an excellent assay.

their high lipid solubility, these bisquinolines were trapped in the lipid membranes and do not reach the digestive vacuole in sufficient concentration to inhibit hemozoin formation.

In Series 3, only compounds **18** and **19** possessed activity in the same range as CQ against both strains. Compound **20** was the least active with a significantly higher mean IC_{50} value than CQ against both strains, displayed aqueous comparable to that of all others but was considerably the least lipid soluble. One may presume that this quinoxaline was unable to cross the biological membranes as result of its increased aqueous solubility leading to weaker antimalarial activity.

Overall, these data confirm the 7-chloro substituted quinoline to be a more potent antimalarial pharmacophore than are both the 5-methylquinoline and pyrrolo[1,2a]quinoxaline moieties. This activity is accordance with results from previous studies that the 7-chloro substituent contributes to the haematin inhibitory activity [25]. The activity of compounds **8** and **9** against the CQR strain in conjunction with the lack of potent activity for the same linkers in Series 2 and 3 tend to suggess that increased pH-trapping plays a less significant role in antimalarial activity for compounds without the 7-chloro substituent. Furthermore, it is important to note that increased hydrophilicity does not necessarily increase vaccuolar accumulation or the ability of a molecule to be absorbed, since polarity, flexibility, charge distribution, molecular weight, H-bonding properties, etc., all affect drug absorption through biological membranes [26]. The ideal linker providing optimal configuration for haematin interactions could be identified in the current study as containing 3 C–C chain separating the N atoms of the polyamine bridge with or without an N-methyl substitution.

The bis(7-chloroquinoline)s of the current study were more active than the bisquinolines of Raynes et al. against the D10 strain [27,28]. Compounds **10**–**12** and **17**–**19** were as active as their bis(8-aminoquinolines) but possessed more antiplasmodial activity than their bis(6-aminoquinolines) against the same strain [27]. Compounds **5** and **7** have activity against D10 in the same range as the most active cinchonidine-like bisquinoline synthesized by Ayad et al. [14]. Compounds **4**–**9**, **12**, **18** and **19**, on the other hand, showed higher antimalarial activity than their chloroquine-like bisquinolines containing aliphatic linker of 8 or 10 carbons.

3.4. Cytotoxicity

In general, the Series 1 was more cytotoxic than 2 as result of the presence chlorine substituent. In the bispyrrolo[1,2a]quinoxaline series, compounds **17** and **20** were the most cytotoxic. The high toxicity of compound **20** may be the result of the lengthening of the C–C chain, since an increase in aliphatic chain length is associated with toxicity towards mammalian cells [29]. Overall, the bisquinolines were less cytotoxic than the bisquinoxalines. One can then safely conclude that the 4-aminoquinoline pharmacophore is less toxic to the mammalian cells than is quinoxaline.

Thus, given that cytotoxicity remains a major problem in the development of bisquinolines, the absence thereof in these bis-compounds appears noteworthy.

3.5. In vitro anticancer activity

The bisquinolines **4**–**9** possessed potent anticancer activity with selectivity for fast dividing tumour cells in light of the absence of cytotoxicity towards mammalian cells. This can be ascribed to the polyamines which may be recognized by the polyamine transporter upregulated tumour cells, and thus provide a drug uptake mechanism for these compounds into the cancer cells [30]. Their anticancer activity may be the result of the 4-aminoquinoline pharmacophore interacting favorably with the narrow minor groove of AT-rich sequences leading to DNA strand scission [31].

Furthermore, the small quinoline pharmacophore has the advantage of lowering the DNA binding strength, which reduces non-specific binding to other cell macromolecules like proteins and lipids [32]. The polyamine linkers confer a region of flexibility between the identical pharmacophores, with the ionized secondary amines capable of increasing van der Waals interactions with the highly polar phosphate backbone and bases of DNA [30,31]. Overall compounds **4**, **6** and **7** exhibited the most potent cytostatic and cytotoxic effects on the proliferation of all three cancer cell lines. This potent activity suggests an optimal distance of 2–3 C atoms between two consecutive N atoms of the polyamine bridge possibly allowing optimal interaction with the negatively charged phosphate-groups of the DNA backbone. This ultimately may induce the external binding of these compounds to DNA and pull the AT-rich strains apart and result in the disruption of DNA transcription leading to tumour cell death.

4. Conclusion

Series of bisquinoline and bispyrrolo[1,2a]quinoxaline compounds were synthesized and their structures confirmed by NMR and MS analyses. The increase in potential protonation sites afforded by polyamines increases the aqueous solubility in each series. However, this effect was overruled as the C–C chain separating the two intrabridge N atoms lengthens. The triprotic and tetraprotic polyamine bisquinolines were more hydrophilic than the diprotic ones. The 7-chloro substituted quinoline was also found to be a more antimalarial active pharmacophore than both 5-

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methylquinoline and pyrrolo[1,2a]quinoxaline moieties. The bisquinolines **8** and **9** were significantly more potent than CQ against the resistant Dd2 strain, demonstrated good selectivity towards the parasitic cells and possessed the highest activity of all synthesized compounds. The bis(7-chloroquinoline) also displayed potent anticancer activity against all three cell lines. Compounds **4**, **6** and **7** exhibited a significantly more potent cytostatic effect on the proliferation of all the cell lines compared to etoposide with bisquinoline **4** being the most potent of all. In view of these results, the bis(7-chloroquinoline)s appear to be better anticancer compounds than antimalarials, and may thus be worthwhile further investigated to ascertain whether the good cancer cells antiproliferative activity observed *in vitro* can be carried out *in vivo*.

In conclusion, the formation of biscompounds containing the quinoline pharmacophore with increased protonation sites afforded by polyamine linkers could be a valid strategy to overcome CQ resistance.

5. Materials and methods

5.1. Materials

The reagents diethylenetriamine, N′-(2-aminoethyl)-1,3-propanediamine, bis(3-aminopropyl)amine, 3,3′-diamino-N-methyldipropylamine, triethylenetetramine, N,N′-bis(3-aminopropyl) ethylene-diamine and solvents N,N-dimethylformamide (DMF) were purchased from Sigma–Aldrich (Johannesburg, South Africa). The reagents 4,7-dichloroquinoline, 6-chloropyrrolo[1,2a]quinoxaline and 4-chloro-5-methylquinoline were obtained from Dayangchem (Hangzhou, China). Chloroquine (CQ) diphosphate was generously donated by Cipla Medpro (Cape Town, South Africa). All the reagents and chemicals were of analytical grade.

5.2. General procedures

Thin-layer chromatography (TLC) was performed using silica gel plates (60F254 Merck, Johannesburg, South Africa). Preparative flash column chromatography was done on silica gel (230–240 mesh, G60 Merck). Analytical quantities of samples were weighed on a Sartorius/BP211D balance. Melting points (mp) were determined on a Stuart SMP 10 apparatus and are uncorrected.

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 spectrometer purchased from Bruker (Karlsruhe, Germany) (at a frequency of 600.17 MHz and 150.913 MHz, respectively) in deuterated dimethylsulfoxide (DMSO-d6) and deuterated chloroform ($CDCl_3$). Chemical shifts are reported in parts per million (δ ppm) using tetramethylsilane (TMS) as internal standard. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublet), t (triplet), td (triplet of a doublet) and m (multiplet).

The high resolution electron spray ionisation mass spectra (HRMS ESI+) were recorded on a Waters Synapt G2 spectrometer. The source was electrospray positive, capillary voltage 3 kV, cone voltage 15 V. Introduction with direct injection (1 μl) into a stream of 50% acetonitrile, 0.1% formic acid, using a Waters UPLC at flow rate of 0.2 ml/min. Positive ions $[M+H]^+$ were recorded.

5.3. High performance liquid chromatography (HPLC)

Alignent 1100 series HPLC equipped with an auto sampler, gradient pump, diode array UV detector and a Restek, Bellefonte, PA, Ultra C18, 5 μm, 150 × 4.6 mm column, was used for determination of physicochemical properties. Data analysis was done on Chemstation Rev. A.10.03 data acquisition and analysis software.

The compounds were quantified using a gradient method (A: 0.005 M octane sulphonic acid sodium salt adjusted to pH 3.5 with *ortho* phosphoric acid, B: acetonitrile) at a flow rate of 1 ml/min with 20 μl standard sample injections. The gradient starts with 20% of solvent A and 70% of solvent B (ACN). Solvent A is increased linearly to 95% and solvent B decreased to 5% until 8 min and held there for 3 min, where after the instrument was re-equilibrated to the starting conditions for 2 min. A calibration plot of peak area versus drug concentration for each compound showing excellent linearity ($0.978 < r^2 \leq 1$) over the concentration range (0–800 μg/ml) was employed for the assays. The absorption maximum for the synthesized compounds was at UV 247 nm. This wavelength was therefore used for detection. The peak retention times (t_R) for each compound analysed is reported.

5.4. Experimental procedures

5.4.1. Synthesis of bisquinolines

The synthesis of bisquinoline compounds **4**–**9** (Series 1) was achieved as follows: A mixture of 4,7-dichloroquinoline **1** (5 g, 25 mmol) and polyamine (75 mmol, 3 equiv.) was refluxed for 16–20 h while stirring continuously. For the synthesis of bisquinolines **10**–**15** (Series 2), another literature reported method [17] was adopted with slight modifications. A mixture of 4-chloro-5-methylquinoline **2** (5 g, 28 mmol) and polyamine (75 mmol, 3 equiv.) was heated at 135–145 °C with stirring for 16–20 h. The progress of the reactions was followed by thin-layer chromatography (TLC). After completion, the reaction mixture was allowed to cool to 50 °C and 1 M NaOH (100 ml) was added. The mixture was stirred until room temperature was reached. The product was extracted with boiling ethyl acetate (3 × 100 ml) and the combined organic fractions were thoroughly washed with distilled water (5 × 50 ml) to remove any excess of amine. The organic phase was dried over $MgSO_4$ for 1–3 h, and the solvent was evaporated under reduced pressure to 25 ml. The concentrated solution was then allowed to crystallise at 0–5 °C to afford compounds **4**–**9** as powders.

Compounds **10**–**15**, on the other hand, were purified by silica gel column chromatography eluting with EtOAc:MeOH:NH_4OH (17:2:1) and were also obtained as powders.

5.4.1.1. 7-Chloro-N-{2-[(7-chloroquinolin-4-yl-)amino]ethyl}quinolin-4-amine **4**. Off-white powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.36 (d, J = 5.4 Hz, 2H, H-2), 8.21 (d, J = 6 Hz, 2H, H-5), 7.77 (d, J = 2.4 Hz, 2H, H-8), 7.39 (dd, J = 9.0, 2.4 Hz, 2H, H-6), 6.48 (d, J = 5.4 Hz, 2H, H-3), 3.5–3.36 (4H, H-10), 2.88 (t, J = 6.6, 4H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 151.91 (C-2), 150.16 (C-4), 133.30 (C-7), 127.49 (C-8), 124.03 (C-6), 123.94 (C-5), 98.71 (C-3), 46.99 (C-11), 42.59 (C-10). HRESI m/z: 426.1256 (M + 1) [(M + 1), $C_{22}H_{22}Cl_2N_5$, 426.1252 cald.), t_R = 1.151 min.

5.4.1.2. 7-Chloro-N-{3-[(7-chloroquinolin-4-yl)amino]propyl}quinolin-4-amine **5**. Off-white powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.38 (d, J = 5.3 Hz, 1H, H-2), 8.24 (d, J = 9.0 Hz, 1H, H-5), 7.77 (d, J = 1.8 Hz, 1H, H-8), 7.40 (dd, J = 9.0, 2.1 Hz, 1H, H-6), 6.50 (d, J = 5.4 Hz, 1H, H-3), 3.50–3.32 (4H, H-10, H-14), 2.84 (t, J = 6.4 Hz, 2H, H-13), 2.70 (t, J = 6.5 Hz, 2H, H-12), 1.77 (q, J = 6.6 Hz, 2H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 152.00 (C-2), 150.13 (C-4), 133.21 (C-7), 127.49 (C-8), 124.10 (C-6), 123.95 (C-5), 98.72 (C-3), 47.41 (C-13), 46.97 (C-12), 42.47 (C-14), 40.93 (C-10), 27.93 (C-11). HRESI m/z: 440.1398 (M + 1) [(M + 1), $C_{23}H_{24}Cl_2N_5$, 440.1409 cald.), t_R = 1.154 min.

5.4.1.3. 7-Chloro-N-{3-({3-[(7-chloroquinolin-4-yl)amino]propyl}amino)propyl]quinolin-4-amine **6**. Off-white powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.35 (d, J = 4 Hz, 2H, H-2), 8.20 (d, J = 9 Hz, 2H, H-5), 7.75 (d, J = 2.3 Hz, 2H, H-8), 7.52 (dd, J = 9.0, 2.4 Hz, 2H, H-6),

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6.44 (d, J = 5.4 Hz, 2H, H-3), 3.40–3.32 (4H, H-10), 2.65 (t, J = 6.4, 4H, H-12), 1.82 (q, J = 6.6 Hz, 4H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 151.87 (C-2), 150.17 (C-4) 133.39 (C-7), 127.45 (C-8), 124.12 (C-6), 123.99 (C-5), 98.70 (C-3), 47.38 (C-12), 41.13 (C-10), 28.07 (C-11). HRESI m/z: 454.1568 (M + 1) [(M + 1), $C_{24}H_{26}Cl_2N_5$, 454.1565 cald.), t_R = 1.157 min.

5.4.1.4. Bis({3-[(7-chloroquinolin-4-yl)amino]propyl})(methyl)amine ***7***. White powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.33 (d, J = 5.4 Hz, 2H, H-2), 8.19 (d, J = 9 Hz, 2H, H-5), 7.76 (d, J = 1.5 Hz, 2H, H-8), 7.40 (dd, J = 9.0, 2.1 Hz, 2H, H-6), 6.39 (d, J = 5.4 Hz, 2H, H-3), 3.27 (t, J = 5.4, 4H, H-10), 2.43 (t, J = 6.6 Hz, 4H, H-12), 2.21 (s, 3H, H-13), 1.82 (q, J = 6.6 Hz, 4H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 151.77 (C-2), 150.14 (C-4), 133.45 (C-7), 127.43 (C-8), 124.08 (C-6), 123.96 (C-5), 98.48 (C-3), 55.13 (C-12), 42.00 (C-13), 40.98 (C-10), 25.47 (C-11). HRESI m/z: 468.1727 (M + 1) [(M + 1), $C_{25}H_{28}Cl_2N_5$, 468.1722 cald.), t_R = 1.152 min.

5.4.1.5. 7-Chloro-4-[10-(7-chloroquinolin-4-yl)-1,4,7,10-tetraazadecan-1-yl]quinoline ***8***. Light yellow powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.37 (d, J = 5.4 Hz, 2H, H-2), 8.23 (d, J = 9 Hz, 2H, H-5), 7.76 (d, J = 2.2 Hz, 2H, H-8), 7.39 (dd, J = 8.8, 1.7 Hz, 2H, H-6), 6.45 (d, J = 5.4, 2H, H-3), 3.50–3.30 (4H, H-10), 2.80 (t, J = 6.3 Hz, 4H, H-11), 2.65 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 151.85 (C-2), 150.08 (C-4), 133.29 (C-7), 127.36 (C-8), 124.16 (C-6), 123.87 (C-5), 98.63 (C-3), 48.63 (C-11), 47.20 (C-12), 42.52 (C-10). HRESI m/z: 469.1691 (M + 1) [(M + 1), $C_{24}H_{27}Cl_2N_6$, 469.1674 cald.), t_R = 1.153 min.

5.4.1.6. 7-Chloro-4-[12-(7-chloroquinolin-4-yl)-1,5,8,12-tetraazadodecan-1-yl]quinoline ***9***. White powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.37 (d, J = 5.4 Hz, 2H, H-2), 8.20 (d, J = 9 Hz, 2H, H-5), 7.77 (d, J = 2.1 Hz, 2H, H-8), 7.43 (dd, J = 9.0, 2.1 Hz, 2H, H-6), 6.45 (d, J = 5.4 Hz, 2H, H-3), 3.50–3.31 (4H, H-10), 2.64 (t, J = 6.4 Hz, 4H, H-12), 2.63–2.57 (t, 2H, H-13), 1.76 (q, J = 6.6 Hz, 4H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 152.02 (C-2), 150.05 (C-4), 133.36 (C-7), 127.50 (C-8), 124.13 (C-6), 123.98 (C-5), 98.48 (C-3), 48.97 (C-10), 47.42 (C-12), 41.06 (C-13), 27.98 (C-11). HRESI m/z: 497.1992 (M + 1) [(M + 1), $C_{26}H_{31}Cl_2N_6$, 497.1987 cald.), t_R = 1.150 min.

5.4.1.7. 5-Methyl-N-[2-({2-[(5-methylquinolin-4-yl)amino]ethyl}amino) ethyl]quinolin-4-amine ***10***. Light yellow powder; ^{1}H NMR. (600 MHz, DMSO) δ (ppm): 8.30 (d, J = 5.2, 2H, H-2), 7.68–7.58 (m, H-7), 7.42 (dd, J = 8.6, 1.8 Hz, 2H, H-8), 6.43 (d, J = 5.3 Hz, 2H, H-3), 3.36 (t, J = 9.3 Hz 4H, H-10), 2.90 (t, J = 6.4 Hz, 4H, H-11), 2.43 (s, 3H, H-9). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 149.48 (C-2), 146.31 (C-4), 133.14 (C-5), 130.47 (C-7), 128.51 (C-6), 120.53 (C-8), 98.12 (C-3), 47.20 (C-11), 42.56 (C-10), 21.17 (C-9). HRESI m/z: 386.2339 (M + 1) [(M + 1), $C_{24}H_{28}N_5$, 386.2345 cald.), t_R = 1.146 min.

5.4.1.8. 5-Methyl-N-[2-({3-[(5-methylquinolin-4-yl)amino]propyl} amino)ethyl]quinolin-4-amine ***11***. White powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.29 (dd, J = 9.8, 5.2 Hz, 2H, H-2), 7.65 (dd, J = 8.6, 5.3 Hz, 2H, H-8), 7.41 (t, J = 7.9 Hz, 2H, H-7), 6.43 (d, J = 5.3 Hz, 2H, H-3), 3.33–3.36 (4H, H-10, H-14), 2.84 (t, J = 6.6 Hz, 2H, H-12), 2.72 (t, J = 6.5 Hz, 2H, H-13), 2.43 (s, 3H, H-9), 1.82 (q, J = 6.7 Hz, 2H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 149.53 (C-2), 146.67 (C-4), 133.00 (C-5), 130.38 (C-7), 128.87 (C-6), 120.50 (C-8), 97.94 (C-3), 47.70 (C-13) 47.02 (C-12), 43.69 (C-14), 41.58 (C-10), 27.72 (C-11), 21.16 (C-9). HRESI m/z: 400.2505 (M + 1) [(M + 1), $C_{25}H_{30}N_5$, 400.2501 cald.), t_R = 1.589 min.

5.4.1.9. 5-Methyl-N-[3-({3-[(5-methylquinolin-4-yl)amino]propyl} amino)propyl]quinolin-4-amine ***12***. Off-white powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.31 (d, J = 5.2 Hz, 2H, H-2), 7.67 (d, J = 8.4 Hz, 2H, H-7), 7.42 (dd, J = 8.4, 1.8 Hz, 2H, H-8), 6.37 (d, J = 5.3 Hz, 2H, H-3), 3.29 (t, J = 6.7 Hz, 4H, H-10), 2.66 (t, J = 6.4 Hz, 4H, H-12), 2.55 (s, 3H, H-9), 1.80 (q, J = 6.6 Hz, 2H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 149.53 (C-2), 146.67 (C-4), 133.00 (C-5), 130.55 (C-7), 128.87 (C-6), 120.50 (C-8), 97.94 (C-3), 47.57 (C-12), 41.58 (C-10), 27.72 (C-11), 21.16 (C-9). HRESI m/z: 414.2549 (M + 1) [(M + 1), $C_{26}H_{32}N_5$, 414.2658 cald.), t_R = 1.155 min.

5.4.1.10. Methylbis({3-[(5-methylquinolin-4-yl)amino]propyl})amine ***13***. Yellow powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.26 (d, J = 5.2 Hz, 2H, H-2), 7.66 (d, J = 8.5 Hz, 2H, H-8), 7.41 (d, J = 8.5 Hz, 2H, H-6), 7.18 (t, J = 5.0 Hz, 2H, H-7), 6.32 (d, J = 5.3 Hz, 2H, H-3), 3.26 (t, J = 6.5 Hz, 4H, H-10), 2.50 (s, 3H, H-9), 2.47 (t, J = 6.8 Hz, 4H, H-12), 2.24 (s, 3H, H-13), 1.84 (p, J = 6.8 Hz, 4H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 149.47 (C-2), 146.43 (C-4), 132.97 (C-5), 130.56 (C-7), 128.85 (C-6), 120.46 (C-8), 98.01 (C-3), 55.46 (C-12), 41.86 (C-13), 41.16 (C-10), 25.56 (C-11), 21.22 (C-9). HRESI m/z: 428.2901 (M + 1) [(M + 1), $C_{27}H_{34}N_5$, 428.2814 cald.), t_R = 1.156 min.

5.4.1.11. 5-Methyl-4-[10-(5-methylquinolin-4-yl)-1,4,7,10-tetraazadecan-1-yl]quinoline ***14***. Off-white powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.30 (d, J = 5.3 Hz, 2H, H-2), 7.66 (d, J = 8.4 Hz, 2H, H-7), 7.42 (dd, J = 8.5, 1.2 Hz, 2H, H-8), 6.33 (d, J = 5.3 Hz, 2H, H-3), 3.35–3.26 (4H, H-10), 2.62 (t, J = 6.6 Hz, 4H, H-11), 2.45 (s, 3H, H-9), 2.51–2.49 (2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 149.38 (C-2), 146.64 (C-4), 133.09 (C-5), 130.48 (C-7), 128.74 (C-6), 120.52 (C-8), 98.25 (C-3), 48.81 (C-11), 47.36 (C-12), 42.50 (C-10), 21.22 (C-9). HRESI m/z: 429.2760 (M + 1) [(M + 1), $C_{26}H_{33}N_6$, 429.2767 cald.), t_R = 1.158 min.

5.4.1.12. 5-Methyl-4-[12-(5-methylquinolin-4-yl)-1,5,8,12-tetraaza-dodecan-1-yl]quinoline ***15***. Off-yellow powder; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.25 (d, J = 5.3 Hz, 2H, H-2), 7.61 (d, J = 8.5 Hz, 2H, H-7), 7.37 (dd, J = 8.6, 1.8 Hz, 2H, H-8), 7.29–7.26 (m, 2H, H-6), 6.32 (d, J = 5.3 Hz, 2H, H-3), 3.27–3.18 (4H, H-10), 2.63–2.60 (m, 8H, H-12, H-13), 2.45 (s, 3H, H-9), 1.77–1.71 (q, J = 6.6 Hz, 4H, H-11). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 149.37 (C-2), 146.70 (C-4), 132.86 (C-5), 130.52 (C-7), 128.78 (C-6), 120.45 (C-8), 97.93 (C-3), 49.21 (C-13), 47.63 (C-12), 41.36 (C-10), 27.96 (C-11), 21.20 (C-9). HRESI m/z: 457.3077 (M + 1) [(M + 1), $C_{28}H_{37}N_6$, 457.3080 cald.), t_R = 1.149 min.

5.4.2. Synthesis of bispyrrolo[1,2a]quinoxalines

The bispyrrolo[1,2a]quinoxaline compounds **16**–**20** (Series 3) as follows: A mixture of 6-chloropyrrolo[1,2a]quinoxaline **3** (5 g, 24 mmol) and polyamine (12 mmol, 0.5 equiv.) suspended in 35 ml of DMF was dissolved upon heating in an oil bath at 130 °C. Potassium carbonate (K_2CO_3) (3.8 g, 27 mmol) was added to the mixture and heating with stirring was continued for 16 hours. After completion, the mixture was allowed to cool to 50 °C and distilled water (100 ml) was added. The mixture was stirred until cooling to room temperature. The product was extracted with heated dichloromethane (3 × 100 ml), and the combined organic fractions washed successively with distilled water (5 × 50 ml) to remove any excess amine. The organic layer was dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The residue was redissolved in dichloromethane and purified by flash column chromatography eluting successively with DCM:MeOH (9:1) and EtOAC:DCM:MeOH:NH_4OH (10:4:5:1) to afford target compounds.

5.4.2.1. N-(2-{[2-({pyrrolo[1,2-a]quinoxalin-6-yl}amino)ethyl]amino} ethyl)pyrrolo[1,2-a] quinoxalin-6-amine ***16***. Off-white powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.72 (m, 2H, H-5), 7.64 (dd, 2H, J = 8.0, 1.3 Hz, 2H, H-9), 7.60 (dd, J = 7.9, 1.5 Hz, 2H, H-2), 7.27–7.21 (td, J = 6.0, 1.2 Hz, 2H, H-4), 7.19–7.12 (dt, J = 7.6, 1.4 Hz, 2H, H-3),

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6.64–6.63 (t, $J = 3$ Hz, 2H, H-8), 6.61 (dd, $J = 3.9$, 1.2 Hz, 2H, H-7), 3.79 (t, $J = 5.7$ Hz, 4H, H-10), 3.02 (t, $J = 5.7$ Hz, 4H, H-11). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 149.44 (C-6), 125.32 (C-3), 125.06 (C-4), 122.70 (C-2), 119.36 (C-9), 114.31 (C-7), 113.30 (C-5), 112.27 (C-8), 48.66 (C-11), 40.16 (C-10). HRESI *m/z*: 436.2239 (M + 1) [(M + 1), $C_{26}H_{26}N_7$, 436.2250 cald.), $t_R = 1.151$ min.

5.4.2.2. N-(3-{[2-({pyrrolo[1,2-a]quinoxalin-6-yl}amino)ethyl]amino} propyl)pyrrolo-[1,2a] quinoxalin-6-amine ***17***. Yellow powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.75–7.69 (m, 2H, H-5, H-9), 7.55 (td, 2H, $J = 7.9$, 1.3 Hz, H-4), 7.23–7.20 (m, 2H, H-2), 7.19–7.08 (m, 2H, H-3), 6.82 (t, $J = 3$ Hz, 1H, H-8), 6.75 (m, 1H, H-7), 3.87 (t, 2H, $J = 5.5$ Hz, H-10), 3.78 (t, 2H, $J = 6.2$ Hz, H-14), 3.03 (t, 2H, $J = 5.9$ Hz, H-12), 2.89 (t, 2H, $J = 6.1$ Hz, H-13), 1.96 (q, 2H, $J = 6.6$, H-11). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 125.35 (C-3), 125.03 (C-4), 122.88 (C-2), 119.33 (C-9), 114.37 (C-7), 113.19 (C-5), 112.65 (C-8), 48.98 (C-13), 47.20 (C-12), 39.34 (C-14), 38.9 (C-10), 28.18 (C-11). HRESI *m/z*: 450.2400 (M + 1) [(M + 1), $C_{27}H_{28}N_7$, 450.2406 cald.), $t_R = 1.154$ min.

5.4.2.3. N-(3-{[3-({pyrrolo[1,2-a]quinoxalin-6-yl}amino)propyl]amino} propyl)pyrrolo-[1,2a] quinoxalin-6-amine ***18***. Dark yellow powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.67 (m, 2H, H-5), 7.59 (dd, $J = 7.5$, 0.6 Hz, 2H, H-9), 7.55–7.47 (dd, $J = 7.2$, 0.6 Hz, 2H, H-2), 7.20–7.17 (td, $J = 6.0$, 1.2 Hz, 2H, H-4), 7.12–7.08 (td, $J = 7.2$, 1.2 Hz, 2H, H-3), 6.62–6.58 (dd, $J = 9.2$, 3.3 Hz, 2H, H-7), 6.58–6.57 (t, $J = 3$ Hz, 2H, H-8), 3.73 (t, $J = 6.2$ Hz, 4H, H-10), 2.73 (t, $J = 6.2$ Hz, 4H, H-12), 1.87 (q, $J = 6.2$ Hz, 4H, H-11). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 149.57 (C-10), 125.18 (C-3), 125.04 (C-4), 122.46 (C-2), 119.25 (C-9), 114.25 (C-7), 113.30 (C-5), 112.28 (C-8), 47.67 (C-12), 39.43 (C-10), 28.92 (C-11). HRESI *m/z*: 464.2556 (M + 1) [(M + 1), $C_{28}H_{30}N_7$, 464.2563 cald.), $t_R = 1.193$ min.

5.4.2.4. Methylbis[3-({pyrrolo[1,2-a]quinoxalin-6-yl}amino)propyl] amine ***19***. Dark brown powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.71 (m, 2H, H-5), 7.64 (dd, $J = 8.1$, 0.8 Hz, 2H, H-9), 7.60 (dd, $J = 8.0$, 1.1 Hz, 2H, H-2), 7.25 (dt, $J = 7.0$, 5.3 Hz, 2H, H-4), 7.18–7.14 (dt, $J = 7.2$, 1.2 Hz, 2H, H-3), 6.63–6.61 (t, $J = 2.4$ Hz, 2H, H-8), 6.59–6.57 (dd, $J = 3.6$, 0.6 Hz, 2H, H-7), 3.75 (4H, H-10), 2.61 (t, $J = 6.5$ Hz, 4H, H-12), 2.37 (s, 3H, H-13), 1.94 (q, $J = 6.5$ Hz, 4H, H-11). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 125.13 (C-3), 124.97 (C-4), 122.50 (C-2), 119.71 (C-9), 114.13 (C-7), 113.30 (C-5), 112.16 (C-8), 56.61 (C-12), 42.09 (C-13), 40.16 (C-10), 26.13 (C-11). HRESI *m/z*: 478.2712 (M + 1) [(M + 1), $C_{29}H_{32}N_7$, 478.2719 cald.), $t_R = 1.157$ min.

5.4.2.5. 1,10-Bis({pyrrolo[1,2-a]quinoxalin-6-yl})-1,4,7,10-tetraazadecane ***20***. Off-white powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.51–7,50 (dd, 2H, H-5), 7.49–7.48 (dd, $J = 8.9$, 1.1 Hz, H-9), 7.18–7.14 (td, $J = 7.9$, 1.2 Hz, 2H, H-4), 7.03–7.00 (dd, $J = 3.8$, 1.4 Hz, 2H, H-2), 6.99–6.96 (td, $J = 7.2$, 1.2 Hz, 2H, H-3) 6.77 (dd, $J = 8.0$, 1.1 Hz, 2H, H-7), 6.55 (t, $J = 3.3$ Hz, 2H, H-8), 4.17–4.5 (t, $J = 9.8$ Hz, 2H, H-12), 3.95–3.92 (t, $J = 9.5$ Hz, 4H, H-10, H-11). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 123.40 (C-3), 120.34 (C-4), 120.20 (C-2), 116.04 (C-9), 113.95 (C-7), 112.93 (C-5), 112.74 (C-8), 53.32 (C-11), 53.18 (C-12), 45.74 (C-10)., $t_R = 1.151$ min.

5.4.3. Physicochemical properties

5.4.3.1. Aqueous solubility. The aqueous solubility values (S_W) of compounds **4**–**20** were determined in phosphate buffer (PBS) at pH 5.5. Each compound was added in excess to the PBS and the suspensions were stirred in a water bath at 37 °C for 24 h. An excess of solute was present at all times to provide saturated solutions. After 24 h the solution was filtered and analysed directly by HPLC to determine the concentration of solute dissolved in the buffer. The experiment was performed in triplicate for each compound.

5.4.3.2. Experimental log D. Equal volumes of *n*-octanol and PBS (pH 5.5) were saturated with each other under vigorous stirring for 48 h. An accurately weighed amount (2 mg) of each compound was dissolved in 0.75 ml of pre-saturated *n*-octanol. The solution was then agitated for 10 min in 2 ml graduated tubes (0.5 ml division). Pre-saturated buffer (0.75 ml) was transferred to the tubes containing the above-mentioned solutions. The tubes were then agitated for 45 min and centrifuged at 4000 rpm (1503 g) for 30 min. The *n*-octanol and aqueous phases were allowed to separate at room temperature for 5 min, after which their volume ratio (v/v) were determined. The ratio was found in all cases to be 1. The different phases were diluted with methanol and analysed by HPLC. The concentrations of the derivative in both phases were determined. The log *D* values were calculated as logarithmic ratios of the concentrations in the *n*-octanol phase to the concentrations in the PBS buffer. The experiment was performed in triplicate for each compound.

5.4.4. Biological study

5.4.4.1. In vitro antimalarial activity. The samples were tested in triplicate on one occasion against chloroquine-sensitive (CQS) D10 and chloroquine-resistant (CQR) Dd2 strains of *P. falciparum*. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen [33]. The quantitative assessment of *in vitro* antimalarial activity was determined *via* the parasite lactate dehydrogenase assay using a modified method described by Makler et al. [34]. The test samples were prepared from a 20 mg/ml stock solution in 10% dimethylsulfoxide (DMSO) and sonicated to enhance solubility. Samples were tested as a suspension if not completely dissolved. Stock solutions were stored at −20 °C. Further dilutions were done on the day of the experiment. Chloroquine (CQ) was used as the reference drug in all experiments. A full dose–response was performed for all compounds to determine the concentration inhibiting 50% of parasite growth (IC_{50} value). The bisquinolines **4**–**15** samples were tested at a starting concentration of 100 ng/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 ng/ml. The same dilution technique was used for all samples. Chloroquine (CQ1) was tested at a starting concentration of 100 ng/ml and used as a reference drug for bisquinoline compounds **4**–**15**. Samples that did not show activity at the initial screen were retested at a starting concentration of either 1000 ng/ml or 100 μg/ml.

The bispyrrolo[1,2a]quinoxalines **16**–**20** samples were tested at a starting concentration of 1000 ng/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 2 ng/ml. The same dilution technique was used for all samples. Chloroquine (CQ2) was used as a reference for bispyrrolo[1,2a]quinoxaline compounds **16**–**20**, and tested at a starting concentration of 100 ng/ml against the CQS strain and 1000 ng/ml against the CQR strain. The sample of compound **17** was tested at a starting concentration of 10 μg/ml against the CQR strain. The IC_{50} values were obtained using a non-linear dose–response curve fitting analysis *via* GraphPad Prism v.4.0 software.

5.4.4.2. Cytotoxicity assay. The MTT assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays [35,36]. Mammalian cell line, Chinese Hamster Ovarian (CHO) cells were treated with the compounds, and MTT assay was used to measure all growth and chemosensitivity. The test samples were tested in triplicate on one occasion. The same stock solutions were used for both tests and dilutions were prepared on the day of the experiment. Emetine (EM1) was used as the reference drug for the bisquinoline series

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4–**15** and EM2 was the reference drug for the bispyrrolo[1,2a]quinoxaline series **16**–**20**. The initial concentration of emetine was 100 μg/ml, which was serially diluted in complete medium with 10-fold dilutions to give six concentrations, the lowest being 0.001 μg/ml. The same dilution technique was applied to the all test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability. The 50% inhibitory concentration values (IC_{50} value) were obtained from full dose–response curves, using a non-linear dose–response curve fitting analysis via GraphPad Prism v.4 software.

5.4.4.3. In vitro anticancer activity. The growth inhibitory effects of the compounds were assessed in a panel of three cell lines, recommended by the National Cancer Institute (NCI) for preliminary screens, using a sulforhodamine B (SRB) assay [37]. The panel consists of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells. The SRB assay was developed to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye sulforhodamine B (Acid Red 52) to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions sulforhodamine B binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilised for measurement. The SRB Assay is performed at CSIR in accordance with the protocol of the Drug Evaluation Branch of the (NCI).

The human cell lines TK10, UACC62 and MCF7 was obtained from NCI in the framework of a collaborative research program between CSIR and NCI. Cell lines were routinely maintained as monolayer cell cultures culture at 37 °C, 5% CO_2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50 μg/ml gentamicin. For the screening experiment, the cells (3–19 passages) were inoculated in 96-well microtiter plates at plating densities of 7–10,000 cells/well and were incubated for 24 h. Afterwards, one plate was fixed with TCA to represent a measurement of the cell population for each cell line at the time of drug addition (T0). The other plates with cells were treated with the experimental compounds which were previously dissolved in dimethylsulfoxide (DMSO) and diluted in medium to produce five concentrations (6.25–100 μg/ml or 0.01–100 μM). Cells without drug served as control. The blank contains complete medium without cells. Etoposide (ET) was used as a reference. The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at the wavelength 540 nm using a multiwell spectrophotometer. Optical density measurements were used to calculate the net percentage cell growth.

The optical density of the test well after 48 h period of exposure to test drug is T_i, the optical density at time zero is T0, and the control optical density is C. Percentage cell growth is calculated as: $[(T_i - T_0)/(C - T_0)] \times 100$ for concentrations at which $T_i \geq T_0$, $[(T_i - T_0)/T_0] \times 100$ for concentrations at which $T_i < T_0$.

The results of five dose screening were reported as TGI (total growth inhibition). The TGI is the concentration of test drug where $100 \times (T - T_0)/(C - T_0) = 0$. The TGI signifies a cytostatic effect. The biological activities were separated into four categories: inactive (TGI > 50 μg/ml or TGI > 100 μM), weak activity (15 μg/ml < TGI < 50 μg/ml or 30 μM < TGI < 100 μM), moderate activity (6.25 μg/ml < TGI < 15 μg/ml or 10 μM < TGI < 30 μM and potent activity (TGI < 6.25 μg/ml or TGI < 10 μM). For each tested compound, four response parameters, GI_{50} (50% growth inhibition and signifies the growth inhibitory power of the test agent), TGI (which is the drug concentration resulting in total growth inhibition and signifies the cytostatic effect of the test agent), LC_{50} (50% lethal concentration and signifies the cytotoxic effect of the test agent), LC_{100} (100% lethal concentration and signifies the cytotoxic effect of the test agent), were calculated for each cell line.

5.4.5. Statistical analysis

The following statistical procedures were used to test if there were significant differences between the mean IC_{50} values of the compounds **4**–**20** and the standard CQ. This was done for both the D10 and Dd2 strains. One-way analyses of variances (ANOVA) were done to determine if significant differences existed between the means of the tested compounds and CQ in general. These were done for data on both D10 and Dd2 strains. Levene's tests were performed to assure equality of variances in each ANOVA's case. In the case of inequality of variances, Welch tests were performed. Normal probability plots on the residuals were done to assure that the data were fairly normally distributed [38]. Dunnett's tests were done to determine which of the tested compounds' means differ statistically significantly from the mean of the standard compound. To validate the results of the ANOVAS and Dunnett's tests, non-parametric procedures like Kruskal–Wallis and Dunn tests were done, respectively. These procedures were done using the statistical data analysis software system [39]. All tests were done at a 0.05 significant level.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.07.037.

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