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Original article

Synthesis and structure–activity relationship of 3-phenylquinoxaline 1,4-di-*N*-oxide derivatives as antimalarial agents

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Abstract

As a continuation of our research and with the aim of obtaining new antimalarial agents, new series of 3-phenylquinoxaline 1,4-di-*N*-oxide derivatives have been synthesized following the classical Beirut reaction. Antiplasmodial activity was evaluated in vitro against *Plasmodium falciparum* by the incorporation of [^3H]-hypoxanthine. Cytotoxicity was tested in KB cells by AlamarBlue assay. Twenty-one of the 60 compounds that were assayed against 3D7 (CQ-sensitive) showed enough activity to be also evaluated against K1 (CQ-resistant) strain. Ten of them were shown to be more active than chloroquine in the resistant strain. The most interesting compounds are 7-(methyl or methoxy)-3-(4′-fluoro or chloro)phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxides because of their low IC_{50} and their high SI shown for the K1 strain, making them valid new leads.

Keywords: Quinoxaline; *N*-Oxide; Beirut reaction; Antiplasmodial; Malaria; *Plasmodium falciparum*

1. Introduction

In terms of human suffering, malaria remains one of the most important health threats to the developing world. Furthermore, the worldwide burden of malaria is increasing, partly due to the unfortunate spread of resistance to most of the drugs that were once effective and safe [1,2]. For nearly half a century, chloroquine (CQ) has been the primary therapy of choice [1] but today CQ-resistant *Plasmodium falciparum* is observed in nearly all of the malaria-endemic regions, being the cause of the most deadly form of malaria [3]. Thus, renewed efforts are required to develop novel and affordable antimalarials to overcome the detrimental effects of drug resistance [4], particularly against resistant strains of *P. falciparum*.

The quinoxaline derivatives seem to have very interesting biological properties [5–7]. Throughout the past 20 years, many mono- and di-*N*-oxide derivatives of this heterocyclic system have been prepared and their biological activities against *Mycobacterium tuberculosis* [8–18], *Trypanosoma cruzi* [19–21], *Candida* spp. [13,16], *P. falciparum* [22–25] and different human tumor cell lines [26–29] have been reported. More recently we have identified a derivative, 3-(4′-chloro)phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide **5**, with potent activity against a chloroquine-resistant strain of *P. falciparum* (FcB1) [24]. In our continuing efforts to identify new antimalarial drug candidates, we herein describe the

Abbreviations: CQ, chloroquine; ND, non data; *P.f.*, *Plasmodium falciparum*; Qx, quinoxaline derivative; RI, resistance index; SD, standard derivation; SI, selectivity index.

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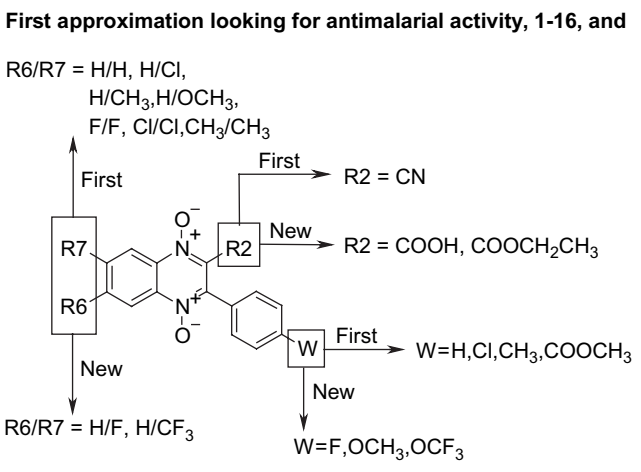
synthesis, the in vitro biological evaluation and the study of structure–activity relationship of quinoxaline 1,4-di-*N*-oxide derivatives designed as potential antimalarial agents (Scheme 1).

According to our previous results, compound 3-phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide **5**, was identified as a lead-compound with promising in vitro anti-*falciparum* activity. In attempts to establish the structural requirements necessary for inhibition of *P. falciparum*, three new series of quinoxaline derivatives were proposed based on structural modifications of the lead-compound **5**. These modifications can be summarized as follows: (a) variations in the electronic pattern of the substituent present in 1,4-di-*N*-oxide quinoxaline ring and in the phenyl group linked to C-4 (series I); and (b) substitution of nitrile subunit, present in the lead-compound **5**, by an ester group in series II and by a carboxylic acid moiety in series III.

In *series I*, the modification made in 4-chloro-phenyl subunit of lead-compound **5** was performed in order to understand the relevance of the electronic parameters in the optimization of the antimalarial activity. It was based on the replacement of the chlorine atom ($\sigma_p = 0.23$), present in the lead-compound **5**, by new substituents such as: a fluorine atom ($\sigma_p = 0.06$; **17**–**22**); a methoxy group ($\sigma_p = -0.27$; **23**–**28**); or a trifluoromethoxy group ($\sigma_p = 0.35$; **29**–**34**) [30].

The introduction of a fluorine atom (**35**–**41**) or a trifluoromethyl (**42**–**48**) group in C-7 (R7) of the 1,4-di-*N*-oxide quinoxaline system was proposed so as to be able to modify the electronic profile of the quinoxaline ring (Scheme 1), improving the interactions of the fluorinated molecule with the receptor site [31]. In addition, the electronegative centre which originates due to the proposed modifications (H *versus* F and CF_3) can introduce new hydrogen bond interactions (as hydrogen acceptor) and alter physicochemical properties such as solubility and log *P*. This could result in an improvement of activity, an increase in stability, or in a reduction of toxicity to mammalian cells, thereby contributing to the optimization of therapeutic efficacy. Trifluoromethyl group was recently described as an important subunit used to replace the chlorine atom in chloroquine (CQ), resulting in a new derivative with faster cell-membrane penetration and intracellular accumulation [32]. In addition, it appears that the introduction of a trifluoromethyl substituent at the R6/R7 position contributes to good physicochemical properties and to therapeutic efficacy in an animal model [33]. Trifluoromethyl group (CF_3) is also present in the current antimalarial drugs, mefloquine and halofantrine; it is also found in the scaffold of tafenoquine (8-aminoquinoline derivative) and arteflene (an endoperoxide which is structurally simpler than artemisinin), promising compounds in advanced stages of clinical development [34].

In *series II and III*, the replacement of carbonitrile moiety by a carboxylic acid subunit (**49**–**53**) or by an ester group (**54**–**63**), respectively, was performed in order to study the structural contribution of the carbonitrile group, present in the lead-compound **5**, to the antimalarial activity. These modifications were proposed in an attempt to study the importance of the pK_a, molecular volume and hybridization of C-atom (from sp to sp^2) in position 2 of the 1,4-di-*N*-oxide quinoxaline scaffold, within the optimization process of prototype **5**.



Scheme 1. Design of new antimalarial agents from our previous results [24].

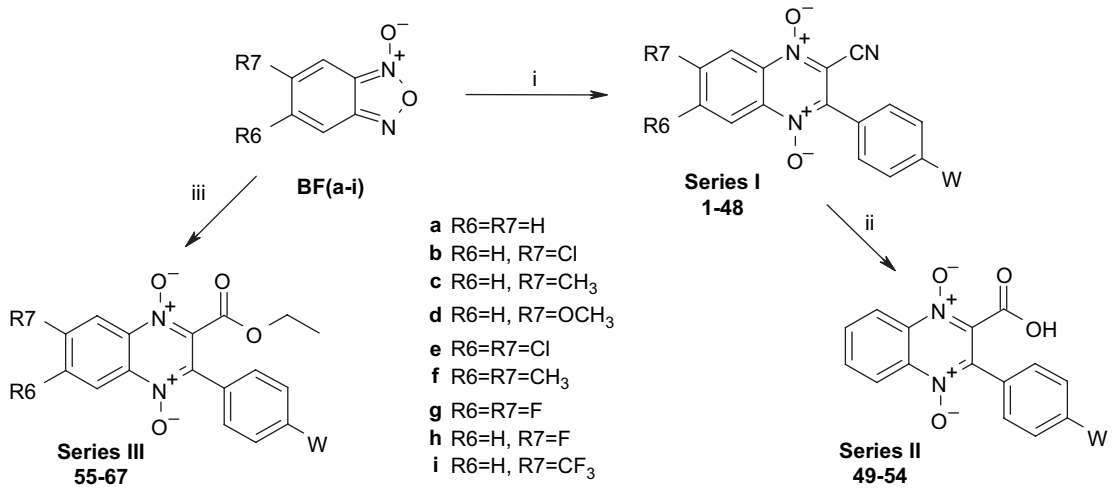
2. Chemistry

The aforementioned compounds were prepared according to the synthetic process illustrated in Scheme 2. The starting compounds, 5-substituted or 5,6-disubstituted benzofuroxanes, BF(a–i), were obtained by previously described methods [27].

The functionalized carbonitrile derivatives **1**–**48** were obtained by the classical Beirut reaction [24,35]. The appropriate benzofuroxane and the corresponding arylacetonitrile were dissolved in dry dichloromethane in the presence of triethylamine, which acted as the catalyst (Scheme 2). When the reaction was finished, the solvent was evaporated to dryness under reduced pressure and a yellow crude solid or brown oil was obtained. The purification methods used for each compound are shown in Section 7.

The synthesis of compounds **54**–**63** was carried out exploring the condensation of benzofurazan oxide with ethyl functionalized benzoylacetate in the presence of potassium carbonate, using acetone as the solvent [36]. The attempts to hydrolyze these esters using different basic conditions in order to obtain carboxylic acids derivatives **49**–**53** were not successful, resulting in decarboxylation. Due to the aforementioned, the syntheses of the acids were then performed; this was carried out by means of hydrolysis of the carbonitrile group, using 2 M KOH in ethanol, and good yields were obtained. Under these conditions, the methyl acetate group of **13** is also hydrolyzed, and the compound obtained has a carboxylic acid group instead of the methyl ester substituent in *para*-position of the benzene ring.

The formation of isomeric quinoxaline 1,4-di-*N*-oxide was observed in the case of monosubstituted benzofuroxanes. According to previous reports [6], we have observed that 7-substituted quinoxaline 1,4-di-*N*-oxide derivatives were



Scheme 2. Synthesis of quinoxaline-2-carbonitrile, ethyl carboxylate and carboxylic acid di-*N*-oxides. Conditions: (i) arylacetonitrile, $CHCl_2$, Et_3N, 24 h; (ii) 2 M KOH in ethanol, 30 min; and (iii) ethyl 4-W-benzoylacetate, K_2CO_3, acetone, 2 h.

prevailing over the 6-isomer, or in the case of the methoxy substituent, only the 7-isomer was formed. In practice, the workup and purification permitted the isolation of the 7-isomer [29].

All of the compounds were chemically characterized by thin layer chromatography (TLC), infrared (IR) and nuclear magnetic resonance (^{1}H NMR) spectra as well as by elemental microanalysis.

3. Pharmacology

3.1. In vitro anti-P. falciparum activity and cytotoxicity in mammalian cells

The modified quinoxaline 1,4-di-*N*-oxide derivatives, **17**–**63**, and the previous analogues, **1**–**16**, were evaluated against 3D7 strain (*P. falciparum* chloroquine-sensitive) and the results are listed in Table 1. Compounds were selected for further cytotoxicity evaluation and tested against *P. falciparum* K1 (chloroquine-resistant strain) if their IC_{50} values were below 2 μM against 3D7 strain. The second cut-off to make these candidates valid new leads against malaria is an SI value above 10.

4. Results

In an attempt to study the pharmacophoric profile of the carbonitrile group in the antimalarial activity of lead-compound **5**, novel series of compounds were designed and synthesized, and the corresponding antimalarial activity was investigated against a CQ-sensitive *P. falciparum* strain (3D7). These series were proposed with substituting the carbonitrile moiety with carboxylic acid subunit in series *II* (**49**–**53**) and with an ester group in series *III* (**54**–**63**). The results are shown in Table 1. Only one of these compounds, **59**, showed an IC_{50} value below 2 μM, the cut-off established for this work.

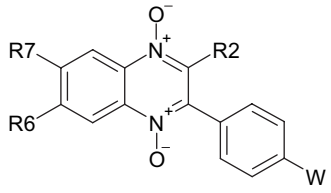
Due to the fact that the replacement of the carbonitrile group by either an ester or a carboxylic acid unit failed to improve the antiplasmodial activity, confirming the pharmacophoric character of the carbonitrile moiety, novel carbonitrile derivatives were designed and obtained for the purpose of investigating the contribution of an aromatic substitution pattern in the optimization of lead-compound **5**. Thus, compounds having structural modifications at positions 6 (R6) and 7 (R7) of quinoxaline scaffold or at *para*-position (W) of phenyl group, linked to C3, were synthesized and evaluated. In this case, 20 of the 48 quinoxaline-2-carbonitrile derivatives assayed against 3D7 strain improved the previously established cut-off value ($IC_{50} < 2$ μM).

Among the 60 assayed compounds against 3D7, 21 of them showed enough activity to qualify for further studies. These derivatives were assayed against a chloroquine-resistant strain, K1, almost all of them showing IC_{50} values below 2 μM. With these values, their resistance indexes (RI) were calculated ($RI = IC_{50}[K1]/IC_{50}[3D7]$) and are shown in Table 2.

The previous lead-compound, **5**, showed an IC_{50} of 1.01 μM for the 3D7 strain and 0.41 μM for the K1 strain. In the sensitive strain, 10 of the 60 tested compounds and, in the resistant strain, six of the 21 assayed compounds in this work improved the activity of the lead-compound, with derivative **42** being the one that showed the smallest value of IC_{50} for both strains (0.33 and 0.18 μM, respectively).

In addition, the cytotoxicity of the 21 most active compounds was tested in KB cells. Only seven of them demonstrated certain cytotoxicity, thus two thirds showed interesting results. With these values, their selectivity indexes (SI) or cytotoxicity:antiplasmodial ratios were calculated with respect to both strains ($SI = IC_{50}[KB]/IC_{50}[P.f.]$). These data are shown in Table 2. Compounds showing SI values above 10 were selected for further in vivo evaluation. According to the established cut-off, this work identified seven derivatives which were selective enough for both strains, and six more

Table 1
Structure and antiplasmodial activity of 3-phenylquinoxaline 1,4-di-*N*-oxide derivatives against *P. falciparum* 3D7



ID	R2	W	R6	R7	$IC_{50} \pm SD$ (μM)
1	CN	H	H	H	2.35 ± 0.04
2	CN	H	H	Cl	>5
3	CN	H	H	CH_3	1.55 ± 0.76
4	CN	H	H	OCH_3	1.47 ± 0.24
5	CN	Cl	H	H	1.01 ± 0.37
6	CN	Cl	H	Cl	>5
7	CN	Cl	H	CH_3	0.48 ± 0.13
8	CN	Cl	H	OCH_3	0.73 ± 0.03
9	CN	CH_3	H	H	3.46 ± 1.08
10	CN	CH_3	H	Cl	>10
11	CN	CH_3	H	CH_3	1.30 ± 0.55
12	CN	CH_3	H	OCH_3	0.81 ± 0.13
13	CN	$COOCH_3$	H	H	6.54 ± 1.03
14	CN	$COOCH_3$	H	Cl	>20
15	CN	$COOCH_3$	H	CH_3	2.15 ± 0.80
16	CN	$COOCH_3$	H	OCH_3	3.16 ± 0.45
17	CN	F	H	H	3.91 ± 0.92
18	CN	F	H	Cl	5.83 ± 0.60
19	CN	F	H	CH_3	0.88 ± 0.13
20	CN	F	H	OCH_3	0.87 ± 0.19
21	CN	F	Cl	Cl	>10
22	CN	F	CH_3	CH_3	1.26 ± 0.16
23	CN	OCH_3	H	H	3.14 ± 0.44
24	CN	OCH_3	H	Cl	>10
25	CN	OCH_3	H	CH_3	2.77 ± 0.65
26	CN	OCH_3	H	OCH_3	2.29 ± 0.12
27	CN	OCH_3	Cl	Cl	>20
28	CN	OCH_3	CH_3	CH_3	3.02 ± 0.31
29	CN	OCF_3	H	H	1.27 ± 0.17
30	CN	OCF_3	H	Cl	0.71 ± 0.34
31	CN	OCF_3	H	CH_3	0.75 ± 0.28
32	CN	OCF_3	H	OCH_3	0.93 ± 0.48
33	CN	OCF_3	Cl	Cl	>10
34	CN	OCF_3	CH_3	CH_3	>5
35	CN	H	H	F	>10
36	CN	F	H	F	5.78 ± 0.20
37	CN	Cl	H	F	4.21 ± 0.57
38	CN	CH_3	H	F	>10
39	CN	OCH_3	H	F	>20
40	CN	OCF_3	H	F	1.70 ± 0.19
41	CN	$COOCH_3$	H	F	>20
42	CN	H	H	CF_3	0.33 ± 0.12
43	CN	F	H	CF_3	0.66 ± 0.03
44	CN	Cl	H	CF_3	1.56 ± 0.19
45	CN	CH_3	H	CF_3	0.72 ± 0.09
46	CN	OCH_3	H	CF_3	2.44 ± 0.30
47	CN	OCF_3	H	CF_3	1.93 ± 0.31
48	CN	$COOCH_3$	H	CF_3	2.03 ± 0.62
49	COOH	H	H	H	ND
50	COOH	Cl	H	H	>10
51	COOH	CH_3	H	H	>10
52	COOH	OCH_3	H	H	ND
53	COOH	COOH	H	H	ND
54	$COOC_2H_5$	H	H	Cl	3.22 ± 0.34
55	$COOC_2H_5$	H	H	CH_3	>10
56	$COOC_2H_5$	H	H	OCH_3	>10
57	$COOC_2H_5$	H	F	F	>10
58	$COOC_2H_5$	H	Cl	Cl	>7
59	$COOC_2H_5$	H	CH_3	CH_3	1.18 ± 0.05
60	$COOC_2H_5$	H	H	H	>10
61	$COOC_2H_5$	Cl	H	H	>10
62	$COOC_2H_5$	CH_3	H	H	>10
63	$COOC_2H_5$	CF_3	H	H	>7
CQ					0.0135

Values shown are the mean $IC_{50} \pm SD$ (μM) from 2 to 3 independent experiments, each one performed in triplicate. CQ = chloroquine.

which were selective for only the resistant strain, with SI values ranging from 10.31 to 50.87.

5. Discussion

As shown in Table 1, the replacement of carbonitrile moiety by either a carboxylic acid group or an ethyl-ester group resulted in an important loss of activity, with the exception of compound **59**. These results appear to indicate that the

Table 2
Antiplasmodial activity, cytotoxicity and selectivity of selected compounds

ID	$IC_{50} \pm SD$ (μM)		SI		RI
	P.f. K1	KB cells	KB/3D7	KB/K1	3D7/K1
3	0.74 ± 0.04	11.65 ± 0.97	7.52	15.76	0.48
4	0.72 ± 0.39	10.46 ± 1.16	7.12	14.47	0.49
5	0.41 ± 0.08	9.73 ± 1.63	9.63	23.57	0.41
7	0.19 ± 0.03	9.25 ± 0.68	19.27	49.46	0.39
8	0.47 ± 0.30	17.51 ± 0.04	23.99	37.53	0.64
11	0.95 ± 0.06	24.98 ± 1.36	19.22	26.19	0.73
12	2.29 ± 0.53	16.95 ± 1.31	20.93	7.39	2.83
19	0.37 ± 0.05	11.19 ± 0.74	12.72	30.44	0.42
20	0.36 ± 0.06	18.54 ± 1.14	21.31	50.87	0.42
22	0.93 ± 0.02	18.84 ± 1.56	14.95	20.36	0.73
29	0.27 ± 0.09	2.76 ± 0.16	2.17	10.31	0.21
30	0.93 ± 0.09	2.35 ± 0.26	3.31	2.53	1.31
31	0.38 ± 0.08	8.12 ± 1.64	10.83	21.41	0.51
32	1.25 ± 0.26	8.25 ± 0.62	8.87	6.58	1.35
40	>8	2.61 ± 0.40	1.54		
42	0.18 ± 0.04	2.82 ± 0.18	8.55	15.67	0.55
43	0.52 ± 0.24	2.85 ± 0.50	4.32	5.48	0.79
44	1.77 ± 0.08	2.76 ± 0.19	1.77	1.56	1.13
45	0.87 ± 0.09	3.17 ± 0.56	4.40	3.64	1.21
47	0.55 ± 0.01	13.45 ± 0.49	6.97	24.45	0.28
59	>10	>60	>50		
CQ	0.682	110	8150	161	50.52
POD		0.003			

Values shown are the mean $IC_{50} \pm SD$ (μM) from 2 to 3 independent experiments, each one performed in triplicate. CQ = chloroquine; POD = podophylotoxin; *P.f.* = *Plasmodium falciparum*; SI = selectivity index; RI = resistance index.

hybridization of the carbon atom linked to position 2 of the quinoxaline nucleus is an important structural requirement for the antiplasmodial activity. These data also demonstrated that the antiplasmodial activity cannot be improved by the use of an acidic substituent at C-2 because when evaluated under the same experimental conditions as those used for **5**, none of the compounds of series *II* (**49**–**53**) was found to be active (Table 1). However, the introduction of a dimethyl substituent in the quinoxaline ring, resulting in compound **59**, appears to compensate the poor activity produced by the ester moiety, thereby maintaining the activity of compound **59** ($IC_{50} = 1.18$ μM) similar to that of the lead-compound ($IC_{50} = 1.01$ μM) for sensitive strain. In spite of the fact that this compound turned out to be the least cytotoxic of all those which were assayed ($IC_{50} > 60$ μM for KB cells), its antiplasmodial activity in resistant strain ($IC_{50} > 10$ μM for K1 strain) was not sufficient enough to merit a more advanced study.

On the other hand, the results obtained clearly demonstrated that it was possible to optimize the antiplasmodial activity of the prototype **5** keeping the carbonitrile group linked to C-2 of quinoxaline scaffold, particularly when a monosubstitution was carried out at quinoxaline scaffold by a methyl group, followed by the introduction of a chlorine atom in the *para*-position of the phenyl ring, linked at C-3 of quinoxaline nucleus, as observed for compound **7** (Table 1).

In short, the results illustrated in Table 1 indicate that the monosubstitution at 1,4-di-*N*-oxide-quinoxaline scaffold by an electron donating group, such as methyl (**3**, **7**, **11**, **15**, **19**, **25**, **31**) or methoxy (**4**, **8**, **12**, **16**, **20**, **26**, **32**), appeared to enhance the antimalarial activity when compared with unsubstituted compound (**1**, **5**, **9**, **13**, **17**, **23**, **29**). In contrast, the monosubstitution at 1,4-di-*N*-oxide-quinoxaline scaffold by a halogen atom, such as chlorine (**2**, **6**, **10**, **14**, **18**, **24**, **30**) or fluorine (**35**–**41**), resulted in decreased activity. Curiously, the disubstitution at R6 and R7 of the quinoxaline ring, generally resulted in a significant loss of activity (Table 1). Despite the electron withdrawing nature of the trifluoromethyl group, the derivatives containing this substituent (**42**–**48**) showed the most interesting values of IC_{50} against *P. falciparum* 3D7; this fact might be due to its larger volume which could contribute even more to hydrophobicity than in the case of fluorine [31].

With regard to the substitution carried out at position *para* of the phenyl ring (W), linked to C-3 of the quinoxaline scaffold, the most active compounds were the ones which presented a chlorine atom at *para* (**7** and **8**). The substitution by an OCF_3 group (**30**, **31**, **32**) also showed promising activity (parameters σ_p and π being very similar). The worst results came from the derivatives with $W = OCH_3$ (**23**–**28**, **39**, **46**) and $COOCH_3$ (**13**–**16**, **41**, **48**); these groups differ from each other considerably with regard to σ_p but their π is very close to zero in both cases.

The most interesting results were obtained from evaluating the selected compounds against K1 strain (CQ-resistant) and mammalian cells (KB), as illustrated in Table 2. While chloroquine shows an IC_{50} 50 times higher in the K1 strain than in the 3D7 strain, we obtained equal or better activity in the resistant strain that in the sensitive strain for almost all of our most active compounds. As observed in Table 2, almost all of the compounds tested against K1 showed a tendency towards lower IC_{50} values against K1 strain than against 3D7 strain. A comparison of the IC_{50} values of the resistant and sensitive strains of *P. falciparum* suggests relatively low levels of cross-resistance to CQ [37]. The resistance index (RI) values, calculated as $IC_{50}(K1)/IC_{50}(3D7)$, were lower than that found for CQ (Table 2). Ten of the derivatives that were studied, bearing an electron withdrawing group on the phenyl substituent (W) such as **5**, **7**, **8**, **19**, **20**, **29** and **31**, or a CF_3 group on the quinoxaline ring (R6/R7) such as **42**, **43** and **47**, showed superior activity for K1 strain in comparison with chloroquine ($IC_{50}(Qx)/IC_{50}(CQ) \leq 1$). In this way, the 2-carbonitrile 1,4-di-*N*-oxide quinoxaline derivatives seem to be a novel promise antimalarial lead-candidates.

With regard to the cytotoxicity of the other compounds, the most cytotoxic derivatives are those which include an OCF_3 group in position W of the phenyl substituent (**29**–**32**, **40**) or a CF_3 group in position R7 of the quinoxaline ring (**42**–**45**), with IC_{50} values ranging from 2.35 to 8.25 μM. Within these derivatives is the compound that shows the lowest IC_{50} values for both 3D7 and K1 strains of *P. falciparum* (**42**). Curiously, the compound that links both aforementioned groups (**47**) presents an SI value of 13.45, very superior to that of its analogues.

By means of the SI value, which relates the cytotoxicity with the antiplasmodial activity of the derivatives studied, we discovered the most interesting structures. Among the seven compounds whose SI is greater than 10 for both strains (**7**, **8**, **11**, **19**–**22**), it should be pointed out that four of them presented an IC_{50} in the K1 strain inferior to that of CQ and in addition, their SI for the same strain was greater than 30. These four compounds present an electron releasing group (methyl, **7** and **19**, or methoxy, **8** and **20**) in position R7 of the quinoxaline ring and a halogen atom (chlorine, **7** and **8**, or fluorine, **19** and **20**) in position W of the phenyl substituent. These compounds are now being considered for further evaluation in vivo against CQ-sensitive and CQ-resistant *Plasmodium berghei* lines.

Although the mechanism of action of our compounds has not yet been investigated, previous results with some quinoxaline derivatives with antimalarial activity correlated with a high inhibitory of β-hematin formation, indicating that this class of compounds may interfere with the heme polymerization process within the food vacuole [24,37].

6. Conclusions

Screening of the in vitro antiplasmodial activity and cytotoxicity of the novel series, quinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives, allowed us to identify interesting antimalarial candidates based on their potency, selectivity and low cytotoxicity, making them valid new leads for

synthesizing new compounds that might improve the previous prototypes. Compounds 7-methyl or 7-methoxy 3-phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide with a chlorine (**7** and **8**) or a fluorine atom (**19** and **20**) in *para*-position (W) of the phenyl substituent were shown to be more active than CQ against K1 strain; in addition, their selectivity indexes for the same strain were greater than 30. These SI values that were obtained suggest parasite-specific effects, perhaps by inhibition of the heme polymerization process, as discussed above. The aforementioned compounds are now being considered for further evaluation in vivo against CQ-sensitive and CQ-resistant *P. berghei* lines.

7. Experimental protocols

7.1. General

The ^{1}H NMR spectra were recorded on a Bruker 400 Ultrashield instrument (400 MHz), using TMS as the internal standard and with DMSO-d_6 and $CDCl_3$ as the solvents; the chemical shifts are reported in ppm (δ) and coupling constants (J) values are given in Hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), t (triplet), q (quadruplet), dd (double doublet) and m (multiplet). The IR spectra were performed on a Thermo Nicolet Nexus FTIR (Madison, USA) in KBr pellets; the frequencies are expressed in cm^{-1}. Elemental microanalyses were obtained on an Elemental Analyzer (Leco CHN-900, Tres Cantos, Madrid, Spain) from vacuum-dried samples. The analytical results for C, H, and N, were within $\pm$ 0.4 of the theoretical values.

Alugram SIL G/UV_{254} (layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG. Postfach 101352. D-52313 Düren, Germany) was used for thin layer chromatography and silica gel 60 (0.040–0.063 mm) for Column flash Chromatography (Merck).

Chemicals were purchased from E. Merck (Darmstadt, Germany), Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma–Aldrich Química, S.A. (Alcobendas, Madrid), Acros Organics (Janssen Pharmaceuticalaan 3a, 2440 Geel, België) and Lancaster (Bischheim-Strasbourg, France).

Benzofurazan oxide (*BF*) was prepared as reported [27].

7.2. General procedure for preparation of 3-arylquinoxaline-2-carbonitrile 1,4-di-N-oxide derivatives (**1–48**) [24]

The corresponding arylacetonitrile (10.6 mmol) was added to a solution of the appropriate benzofuroxane (2.4 mmol) in dry dichloromethane (35 mL). The mixture was allowed to stand at 0 °C. Triethylamine was added dropwise (1 mL), and the reaction mixture was stirred at room temperature in darkness for 24 h. After evaporating to dryness under reduced pressure, a yellow crude solid or a brown oil was obtained. It was then precipitated and washed by adding diethyl ether (or *n*-hexane), affording the target compound. The obtained yellow precipitate was purified by recrystallization from methanol or a mixture of methanol/ethyl acetate. Yields: 8–74%.

7.3. General procedure for preparation of ethyl 3-phenylquinoxaline-2-carboxylate 1,4-di-N-oxide derivatives (**55–67**) [36]

The corresponding functionalized ethyl benzoylacetate (7.34 mmol) and de potassium carbonate (9.55 mmol) were added to a solution of 7.34 mmol of benzofurazan oxide (BF) in 50 mL of acetone. The suspension was stirred at room temperature for 2 h. The quinoxaline 1,4-di-*N*-oxide derivatives were isolated by addition of 50 mL of water followed by extraction with dichloromethane (5 × 40 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The residue was purified by recrystallization from a mixture of methanol/ether/*n*-hexane (2:4:4). Yields: 15–64%.

7.4. General procedure for preparation of 3-phenylquinoxaline-2-carboxylic acid 1,4-di-N-oxide derivatives (**49–54**)

A mixture of carbonitrile derivative (2.6 mmol), ethanol (15 mL) and a solution of 2 M KOH in ethanol (5 mL) was stirred at room temperature for 30 min. The mixture was diluted with 30 mL of water and washed with CH_2Cl_2 (3 × 20 mL). The aqueous layer was neutralized with 3 N HCl and the solid which precipitated was collected by filtration in order to give the desired carboxylic acid in moderate to good yields (45–70%).

7.5. P. falciparum in vitro culture and parasite growth inhibition assays

All parasite clones, isolates and strains were acquired from MR4 (Malaria Research and Reference Reagent Resource Center, Manassas, Virginia, USA). Strains/isolates used in this study were: the drug sensitive 3D7 clone of the NF54 isolate (unknown origin); and the chloroquine, pyrimethamine and cycloguanyl resistant K1 strain (Thailand). In vitro culture of *P. falciparum* was carried out following standard methods [38] with modifications as described [39]. In vitro parasite growth inhibition was assessed by the incorporation of [^{3}*H*]-hypoxanthine based on the method used by Desjardins et al. [40] and modified as described [41]. Briefly, stock drug solutions were dissolved in 100% dimethylsulfoxide (Sigma, Dorset, UK) and 50 μL of a 3-fold dilution series (10.0, 3.33, 1.11, 0.370, 0.124, and 0.0412 μg/mL) of the drugs prepared in assay medium (RPMI 1640 supplemented with 0.5% Albumax II (Invitrogen), 0.2% w/v glucose, 0.03% L-glutamine, and 5 μM hypoxanthine) added to each well of 96-well plates in triplicate. Fifty microlitres of asynchronous (65–75% ring stage) *P. falciparum* culture (0.5% parasitemia) or uninfected erythrocytes (blank) were added to each well reaching a final volume of 100 μL per well, a final hematocrit of 2.5% and final dimethylsulfoxide concentrations $\leq$0.01%. Plates were incubated at 37 °C in 5% CO_2, and 95% air mixture for 24 h, at which point 10 μL (0.2 μCi/well) of [^{3}*H*]-hypoxanthine (Perkin–Elmer, Hounslow, UK), was added to each well. After an additional 24 h incubation period, the

experiment was terminated by placing the plates in a −80 °C freezer. Plates were thawed and harvested onto glass fibre filter mats using a 96-well cell harvester (Harvester 96, Tomtec, Oxon, UK) and left to dry. After the addition of MeltiLex solid scintillant (Perkin–Elmer, Hounslow, UK) the incorporated radioactivity was counted using a Wallac 1450 BetaLux scintillation counter (Wallac).

Data acquired by the Wallac BetaLux scintillation counter were exported into a MICROSOFT EXCEL spreadsheet (Microsoft), and the IC_{50} values of each drug were calculated by using XLFit line fitting software (ID Business Solutions, UK). Chloroquine diphosphate, as a standard drug, and control wells with untreated infected and uninfected erythrocytes were included in all assays.

7.6. In vitro cytotoxicity assay

The AlamarBlue (AccuMed International, USA) method was used to assess cytotoxicity to KB cells as previously described [39]. BrieXy, microtiter plates were seeded at a density of 4×10^4 KB cells/mL in RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal calf serum (complete medium) (Seralab). Plates were incubated at 37 °C, 5% CO_2, 95% air mixture for 24 h followed by compound addition to triplicate wells in a dilution series in complete medium. The positive control drug was podophyllotoxin (Sigma; $IC_{50} = 0.003$ μM). Plates were incubated for a further 72 h followed by the addition of 10 μL of AlamarBlue (AccuMed International) to each well and incubation for 2–4 h at 37 °C, 5% CO_2, 95% air mixture. Fluorescence emission at 585 nm was measured in a SPECTRAMAX GEMINI plate reader (Molecular Devices) after excitation at 530 nm. IC_{50} values were calculated using XLFit (ID Business Solutions, UK) line fitting software.

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Appendix. Supplementary data

Characterization data (IR and ^1H NMR) and elemental analyses of all new compounds are available via the Internet at http://www.sciencedirect.com. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejmech.2007.11.024.

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