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Synthesis and Structure-Activity Relationship of 3-furyl and 3-thienylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives against *Plasmodium falciparum*

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Abstract: The aim of this study was to identify new active compounds against *Plasmodium falciparum* based on our previous research carried out on 3-phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives. Antimalarial activity was evaluated *in vitro* against *Plasmodium falciparum* (3D7 and K1 strains) by the incorporation of [³H]hypoxanthine. Cytotoxicity was tested in KB cells by Alamar Blue assay. Twelve compounds were synthesized and evaluated for antimalarial activity. Eight of them showed a IC₅₀ < 1 μM against 3D7 strain. Derivative **1** demonstrated high potency (IC₅₀= 0.63 μM) and good selectivity (SI=10.35), thereby becoming a new lead-compound.

Introduction

Malaria is by far the world's most important tropical parasitic disease. Mortality, currently estimated at over a million people per year, has risen in recent years, probably due to increasing resistance to antimalarial medicines. It exacts a heavy toll of illness and death - especially amongst children and pregnant women. It also poses a risk to travellers and immigrants, with imported cases increasing in non-endemic areas.¹ A major drawback in the chemotherapy of malaria is the rapid and widespread development of drug resistance in *Plasmodia* to most existing antimalarials.^{2,3} This is the reason research was aimed at the development of new antimalarials.⁴

According to the activity results, our group had previously synthesized 3-phenylquinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives,⁵ and in attempts to establish the structural requirements for inhibition of *P. falciparum*, we decided to synthesize new compound series with a structural modification from our previous lead-compounds (figure 1). The change that was carried out was the substitution of the phenyl subunit in position 3 by 2-furyl (series A) or 2-thienyl (series B) moieties,

following the classical bioisosteric replacement used in Medicinal Chemistry⁶ in order to determine the influence of the size and nature of aromatic rings in this position.

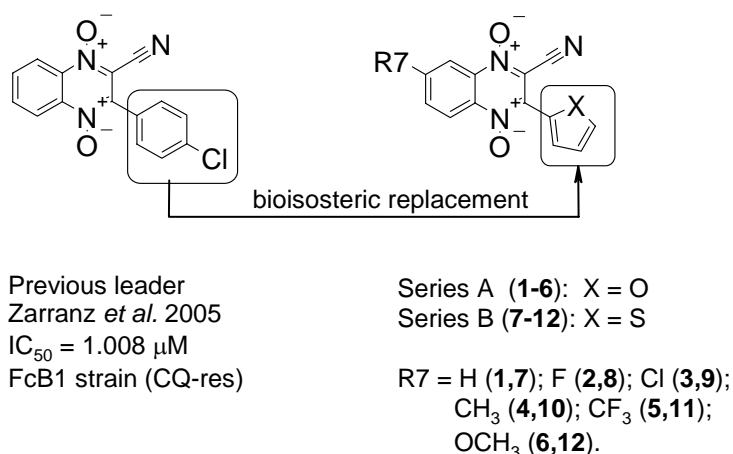


Figure 1. Design of new derivatives as antimalarial drugs from our previous leader

Results and Discussion

The most potent quinoxaline derivative from our previous *in vitro* studies was 3-(4'-chlorophenyl)quinoxaline-2-carbonitrile 1,4-di-*N*-oxide⁵ which was subjected to a structural change in order to obtain new active compounds: replacement of the benzene in position 3 of the quinoxaline subunit by a heteroaromatic 5-member ring, 2-furane or 2-thiene.

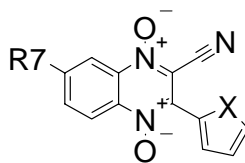
Twelve new compounds were synthesized and evaluated for antimalarial activity against *Plasmodium falciparum*. The modified quinoxaline 1,4-di-*N*-oxide derivatives **1-12** were evaluated against 3D7 strain (CQ-sensitive) and the results are listed in table 1. Compounds were selected for further assays if their IC₅₀ values were less than 1 μM when tested against 3D7 strain. The chosen compounds were tested against K1 strain (CQ-resistant), and their cytotoxicities were determined in mammalian KB cells (table 1).

Upon carrying out the bioisosteric replacement of phenyl subunit with a heteroaromatic 5-member ring, the importance of the heteroatom is clearly observed; only two of the 2-thienyl derivatives (series B) show an IC₅₀ < 1 μM whereas all of the 2-furyl derivatives (series A) show IC₅₀ values ranging from 0.49 to 0.93 μM.

All of the derivatives that were tested showed superior activity for K1 strain in comparison with chloroquine (IC₅₀(**1-6**) < IC₅₀(CQ)). In this way, the 3-(2'-furyl)quinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives appear to be a novel and promising antimalarial candidates.

The activities of these derivatives are also affected by the different substituents on the quinoxaline nucleus: regarding R7 position, it has been observed that the presence of a trifluoromethyl (**5**, **11**) or a methoxy (**6**, **12**) group results in 2 of the most potent compounds in each series. The 7-chloro derivatives (**3**, **9**) show contrasted effects: while the furyl derivative (**3**) is the most potent against 3D7 and the second most active against K1, the thienyl derivative (**9**) is inactive in 3D7.

Table 1. Structures, *in vitro* activity against *P.falciparum* (3D7 and K1 strains) and cytotoxicity in mammalian KB cells.



Comp.	R7	X	3D7 IC ₅₀ (μ M) ^a	K1 IC ₅₀ (μ M) ^a	KB IC ₅₀ (μ M) ^a
1	H	O	0.75 \pm 0.31	0.63 \pm 0.18	6.52 \pm 0.86
2	F	O	0.77 \pm 0.22	0.59 \pm 0.06	5.59 \pm 0.44
3	Cl	O	0.49 \pm 0.28	0.34 \pm 0.04	3.48 \pm 0.78
4	CH ₃	O	0.93 \pm 0.41	0.56 \pm 0.01	4.38 \pm 0.50
5	CF ₃	O	0.53 \pm 0.25	0.37 \pm 0.12	3.00 \pm 0.43
6	OCH ₃	O	0.63 \pm 0.14	0.28 \pm 0.07	3.17 \pm 0.67
7	H	S	2.97 \pm 0.33	ND ^b	ND
8	F	S	>10	ND	ND
9	Cl	S	7.41 \pm 0.20	ND	ND
10	CH ₃	S	2.86 \pm 0.46	ND	ND
11	CF ₃	S	0.89 \pm 0.36	ND	1.80 \pm 0.47
12	OCH ₃	S	0.80 \pm 0.10	ND	3.80 \pm 0.22
CQ ^c			0.0135	0.682	110

^aFigures are the mean IC₅₀ values (μ M) \pm standard deviation from independent experiments each performed in triplicate.

^bNo data

^cChloroquine is used as a standard drug.

Unfortunately, the most active compounds against *P. falciparum* (**3**, **5** and **6** in series A and **11** and **12** in series B) are also cytotoxic in KB cells, with IC₅₀ values lower than 4 μ M. The most cytotoxic derivatives are those which include a CF₃ group in position R7 of the quinoxaline ring (**5**, **11**), and the least cytotoxic compound is 3-furyl derivative nonsubstituted in R7 position (**1**).

The most interesting results were obtained from calculating the Resistance and Selectivity Indexes (RI and SI) of selected compounds (IC₅₀ < 1 μ M), as illustrated in table 2.

As observed in table 1, all of the 3-furyl derivatives showed a tendency towards lower IC₅₀ values against K1 strain than against 3D7 strain. While chloroquine shows an IC₅₀ 50 times higher in the K1 strain than in the 3D7 strain (RI =50), we obtained RI values lower than 1 for all of our most active compounds (table 2). This fact suggests low levels of cross-resistance to CQ.⁷

With regard to the selectivity of the compounds, the SI value required for a compound to be selected for *in vivo* assays is 10. Four of the tested derivatives showed a SI value approximate to the established cut-off with respect to K1 strain (**1**, **2**, **3** and **6**); in addition, the 3-(2'-furyl) derivative nonsubstituted in R7 position (**1**) was the most selective compound with respect to 3D7 strain.

The potency, low cytotoxicity and selectivity of 3-(2'-furyl)quinoxaline 1,4-di-N-oxide (**1**) makes it valid lead for synthesizing new compounds that possess better activity.

Table 2. Resistance index (RI) and Selectivity indexes (SI).

Comp.	RI ^a (K1/3D7)	SI ^b 3D7	SI ^b K1
1	0.84	8.69	10.35
2	0.76	7.26	9.47
3	0.69	7.10	10.24
4	0.60	4.81	7.82
5	0.71	5.66	8.11
6	0.44	5.03	11.32
CQ ^c	50.52	8150	161

^aResistance Index. calculated as (3D7 IC₅₀)/(K1 IC₅₀).

^bSelectivity Index calculated as (KB IC₅₀)/(*P. falciparum* IC₅₀).

^cChloroquine is used as a standard drug.

Conclusions

Screening of the *in vitro* antimalarial activity of these novel series has evidenced that the 3-(2'-furyl)quinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives (**1-6**) appear to be novel and promising antimalarial candidates improving the activity of chloroquine in K1 strain. The nonsubstituted derivative in position R7 is especially noteworthy as it showed the lowest cytotoxicity and the highest SI values.

Experimental Section

All of the synthesized compounds were chemically characterized by thin layer chromatography (TLC), infrared (IR), nuclear magnetic resonance (¹H-NMR), mass spectra (MS) and elemental microanalysis (CHN). Alugram SIL G/UV254 (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). The ¹H NMR spectra were recorded on a Bruker 400 Ultrashield instrument (400 MHz), using TMS as the internal standard and with DMSO-d⁶ and CDCl₃ 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. The mass spectra were measured on an Agilent Technologies Model MSD/DS 5973N (mod. G2577A) mass spectrometer with direct insertion probe (DIP) (Waldbronn, Germany) and the ionization method was electron impact (EI, 70 eV). 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 ± 0.4 of the theoretical values. Chemicals were purchased from Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma-Aldrich Química, S.A., (Alcobendas, Madrid), Acros Organics (Janssen Pharmaceuticaaan 3a, 2440 Geel, België) and Lancaster (Bischheim-Strasbourg, France).

Synthesis of 3-(2'-furyl) and 3-(2'-thienyl)quinoxaline-2-carbonitrile 1,4-di-N-oxide derivatives (1-12)

The 3-arylquinoxaline-2-carbonitrile derivatives were obtained by the classical Beirut reaction.⁹ The method used for their synthesis has been reported previously.^{5,8} The appropriate benzofuroxane and the corresponding arylacetonitrile were dissolved in dry dichloromethane in the presence of triethylamine, which acted as the catalyst.

3-(2'-furyl)quinoxaline-2-carbonitrile 1,4-di-N-oxide (1)

IR (KBr). ν/cm^{-1} : 2233, 1336. **¹H-RMN** (400 MHz, CDCl₃). δ/ppm : 6.80 (td, $J=1.77$, 3.65 Hz, 1H, H_{4'}); 7.86 (d, $J=0.74$ Hz, 1H, H_{3'}); 7.91 (tdd, $J=1.40$, 7.13, 8.61 Hz, 1H, H₇); 8.01 (tdd, $J=1.52$, 7.11, 8.63 Hz, 1H, H₆); 8.38 (dd, $J=0.90$, 3.64 Hz, 1H, H_{5'}); 8.62 (d, $J=8.58$ Hz, 1H, H₈); 8.70 (d, $J=8.64$ Hz, 1H, H₅). *Anal.* calcd for C₁₃H₇N₃O₃ (253.22): C, 61.66; H, 2.79; N, 16.59. Found: C, 61.67; H, 2.86; N, 16.92. **MS** (EI, 70 eV). m/z (%): 253 ([M]⁺, 51), 237 (25), 191 (52), 175 (36), 105 (100).

3-(2'-thienyl)quinoxaline-2-carbonitrile 1,4-di-N-oxide (7)

IR (KBr). ν/cm^{-1} : 2225, 1352, 1322. **¹H-RMN** (400 MHz, CDCl₃). δ/ppm : 7.38 (td, $J=4.29$, 5.10 Hz, 1H, H_{4'}); 7.87 (dd, $J=0.96$, 5.12 Hz, 1H, H_{3'}); 7.92 (ddd, $J=1.28$, 7.10, 8.48 Hz, 1H, H₇); 8.03 (ddd, $J=1.37$, 7.11, 8.60 Hz, 1H, H₆); 8.61 (dd, $J=1.32$, 8.59 Hz, 1H, H₈); 8.69 (dd, $J=0.96$, 4.26 Hz, 1H, H_{5'}); 8.74 (dd, $J=1.22$, 8.63 Hz, 1H, H₅). *Anal.* calcd for C₁₃H₇N₃O₂S (269.28): C, 57.99; H, 2.62; N, 15.60. Found: C, 57.72; H, 2.63; N, 15.75. **MS** (EI, 70 eV). m/z (%): 269 ([M]⁺, 100), 253 (43), 224 (54).

Plasmodium falciparum in vitro culture and parasite growth inhibition assays

In vitro evaluation of the antimalarial activity was carried out at the London School of Hygiene and Tropical Medicine. Biological tests have been performed according to the previously described method.¹⁰

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 cycloguanil resistant K1 strain (Thailand). *In vitro* culture of *P. falciparum* was carried out following standard methods¹¹ with modifications as described.¹² *In vitro* parasite growth inhibition was assessed by the incorporation of [³H] hypoxanthine based on the method used by Desjardins¹³ and modified as described.¹⁰ 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 $\mu\text{g}/\text{mL}$) 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% CO₂, and 95% air mixture for 24 h, at which point 10 μL (0.2 $\mu\text{Ci}/\text{well}$) of [³H]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 IC₅₀ 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. Generally, compounds showing a IC₅₀ value greater than 2 µM in 3D7 strain are not further evaluated.

In vitro cytotoxicity assay

Concurrent with the determination of IC₅₀ in K1 strain, compounds are tested for cytotoxicity (IC₅₀) in KB cells at concentrations less than or equal to 100 µg/mL. After 72 hours of exposure, viability is assessed on the basis of the Alamar Blue method (Accumed International, USA) as previously described.^{10,12} BrieXy, microtiter plates were seeded at a density of 4·10⁴ 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% CO₂, 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). Plates were incubated for a further 72 h followed by the addition of 10 µL of Alamar-Blue (AccuMed International) to each well and incubation for 2–4 h at 37 °C, 5% CO₂, 95% air mixture. Fluorescence emission at 585 nm was measured in a SPECTRAMAX GEMINI plate reader (Molecular Devices) after excitation at 530 nm. IC₅₀ values were calculated using XLFit (ID Business Solutions, UK) line fitting software. The Selectivity Index [(SI=(KB IC₅₀)/(3D7 or K1 IC₅₀))] was also determined; it is considered significant when SI >10.

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