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Anti-malarial activity of some 7-chloro-2-quinoxalinecarbonitrile-1,4-di-*N*-oxide derivatives

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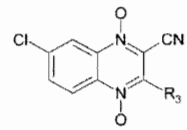
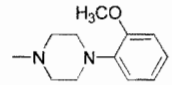
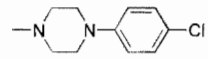
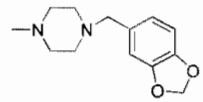
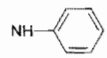
Malaria remains one of the most widespread health threats in the tropics. It is estimated that there are 300 to 500 million cases of malaria every year and around two million deaths [1]. The major concern in the treatment of this plague is the spread of resistance of *Plasmodium falciparum* to the very limited arsenal of antimalarial drugs. This situation appeals for the development of novel antimalarial leads [2].

Therefore, we investigated the *in vitro* antimalarial activities of drugs from our library containing N^1,N^4-quinoxaline dioxide derivatives, which were previously shown to have good antituberculosis activity [3–5]. Structures related to this heterocycle system have important biological activity and their biodisponibility and toxicity depend on the presence of substitutes in the lateral chains [6].

In order to better understand the mechanism of action of the compounds evaluated herein, we also determinated their ability to interfere with the ferriprotoporphyrin (FP) biomineralization process, a fundamental metabolism process of *Plasmodium* [7].

Table: ***In vitro*** **activity of 7-chloro-2-quinoxalinecarbonitrile-1,4-di-*N*-oxide derivatives against** ***Plasmodium falciparum***



Compd.	R_3	IC_{50} (μM)	Index*
4a		0.05	1.25
4b		0.76	19
4c		12.6	315
5		1.28	32
Chloroquine		0.04	1

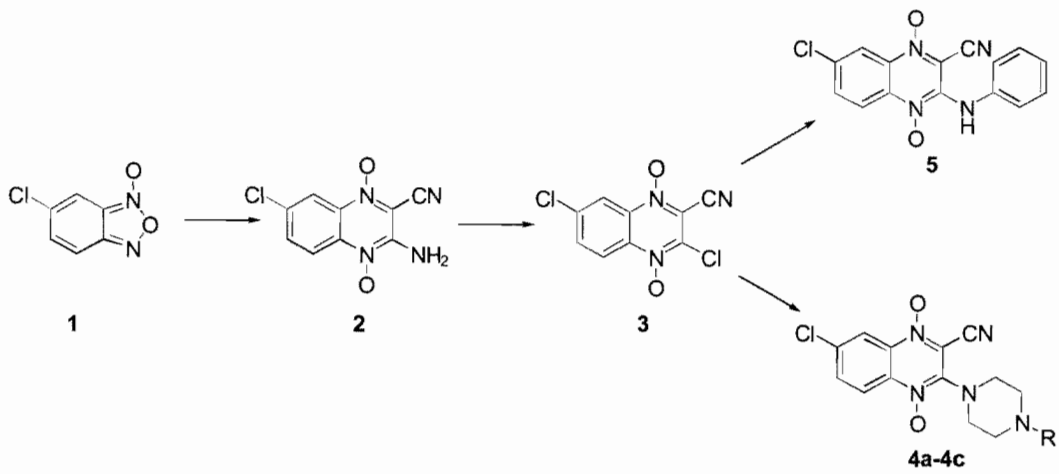
* Index = IC_{50} μM compound/IC_{50} μM reference drug

The results of activity assays, along with the comparative potency index relative to the reference drug (chloroquine), are summarized in the Table.

The relation between structure and activity are related to the presence of Cl on position 7 (frequently found in active compounds as chloroquine), an amine situated in lateral chain and the 1,4-di-*N*-oxide system. The importance of cyano moiety in position 2 will be studied later. From data in the Table, it can easily be deduced that the presence of a piperazine ring is essential for the antimalarial activity.

In **4a**, the 2-methoxy group promoted the highest activity (IC_{50} 1.25 higher than chloroquine). When a chloride replaced this group in position 4, for compound **4b**, the antimalarial activity decreased dramatically (IC_{50} 20 times higher than chloroquine). A weak antimalarial product, **5**, (IC_{50} 30 times higher than chloroquine) was obtained

Scheme



when a phenylamino group replaced the piperazinyl group. When the piperazine ring carried a benzo[1,3]dioxo-5-ylmethyl group (**4c**), the antimalarial activity was totally lost. The oxidative capacity of the dioxo bridge was insufficient for restoration of the antimalarial activity.

The drugs were then tested for their ability to block the heme biomineralization process, but none of them were active. The mechanism of the antimalarial activity observed here is therefore not based on an interference with this target.

It can be concluded that we have discovered a novel antimalarial lead, represented by the 7-chloro-3-[4-(2-methoxyphenyl)-piperazin-1-yl]-quinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivative **4a**.

In vivo antimalarial determinations and studies of cytotoxicity are scheduled.

Experimental

1. Synthesis of 7-chloroquinoxaline-2-carbonitrile 1,4-di-N-oxide derivatives

The compounds under study were previously synthesized [8, 9]. The synthesis was carried out as shown in the Scheme. Compound **1** was formed from the corresponding commercial substituted aniline. Reaction of **1** with malononitrile in the presence of triethylamine as condensing base in DMF at 0 °C yielded the derivative **2**. Compound **3** was synthesized by reaction of **2** with anh. copper(II) chloride and *tert*-butylnitrite in dry acetonitrile, heating at 80–85 °C and under nitrogen atmosphere for 3 h. Amines **4a–4c** and **5** were obtained by reaction of the 3-chloroquinoxaline with the appropriate piperazine or aniline in dry chloroform or dichlorometane, in presence of K_2CO_3, in order to facilitate the reaction.

2. Determination of antimalarial activity

2.1. Assay against Plasmodium falciparum

F32-Tanzania (chloroquine sensitive) strains of *Plasmodium falciparum* kindly provided by Pr. H. Ginsburg (Hebrew University of Jerusalem) were cultured according to Trager and Jensen [10] on glucose-enriched RPMI 1640 medium supplemented with 10% human serum at 37 °C. DMSO (50 µl) was added to the products, which were dissolved in RPMI 1640 medium with the aid of mild sonication in a sonicleaner bath (Branson Ltd.), and then diluted as required in culture medium. The final DMSO concentration was never greater than 0.1%. A total volume of 150 µl of total culture medium with the diluted products and the suspension of human red blood cells in medium (0^+ group, 5% haematocrit) with 1% parasitaemia was placed into the wells of 96-well microtitre plates. All tests were performed in triplicate. After 24 h. of incubation at 37 °C in a candle jar incubator, the medium was replaced by fresh medium with the diluted extract, and incubation was continued for another 48 h. On the third day of the test, a blood smear was taken from each well and parasitaemia was counted. The parasitaemia for each well was obtained and the percentage inhibition of parasitaemia for each concentration of extract was calculated in relation to the control. IC_{50} values were determined graphically by plotting concentration versus percentage inhibition. Each test also included an untreated control with solvent and a positive control with chloroquine (IC_{50} = 40 nM).

2.2. FBIT

The procedure for testing FP biomineralization was described by Deharo et al. [3] and consisted of incubating a mixture in a normal non-sterile, flat bottom, 96-well plate at 37 °C for 18–24 h. This mixture contained: 50 µl of a 10 mg/ml drug solution or 50 µl of solvent (for control), 50 µl of 0.5 mg/ml of haemin chloride (Sigma H 5533) freshly dissolved in DMSO and 100 µl of 0.5 M sodium acetate buffer pH 4.4 (prepared according to Deutscher [11]). The final pH of the mixture was 5–5.2. It is important to adhere to the following order of addition: first the haemin chloride solution, second the buffer, and finally the solvent or the solution of drug. After incubation, the plate was centrifuged at 1600 × g for 5 min. The supernatant was discarded by vigorously flipping the plate upside down twice. The remaining pellet was resuspended with 200 µl of DMSO to remove unreacted FP. The plate was centrifuged once again and the supernatant was discarded in a similar manner. The pellet, consisting of precipitate of β-haematin, was dissolved in 150 µl of 0.1 M NaOH for direct (in the same plate) spectroscopic quantification at 405 nm with a micro-ELISA reader (Titertek Multiskan MCC/340). The data was expressed as the percentage of inhibition of FP biomineralization, calculated by using the following equation:

$$\%\text{inhibition} = 100 \times [(\text{O.D.control} - \text{O.D.drug})/(\text{O.D.control})]$$

Chloroquine was used as control and had an IC_{50} of 28 µM. All of the chemicals used for biological assays were obtained from Sigma Chemicals Co., U.S.A.

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