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# Pro-oxidant properties of indolone-*N*-oxides in relation to their antimalarial properties

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ABSTRACT

Indolone-*N*-oxides (INODs) are bioreducible and possess remarkable anti-malarial activities in the low nanomolar range in vitro against different *Plasmodium falciparum* (*P. falciparum*) strains and in vivo. INODs have an original mechanism of action: they damage the host cell membrane without affecting non-parasitized erythrocytes. These molecules produce a redox signal which activates SYK tyrosine kinases and induces a hyperphosphorylation of AE1 (band 3, erythrocyte membrane protein). The present work aimed to understand the early stages of the biochemical interactions of these compounds with some erythrocyte components from which the redox signal could originate. The interactions were studied in a biomimetic model and compared with those of chloroquine and artemisinin. The results showed that INODs i) do not enter the coordination sphere of the metal in the heme iron complex as does chloroquine; ii) do not generate iron-dependent radicals as does artemisinin; iii) generate stable free radical adducts after reduction at one electron; iv) cannot trap free radicals after reduction. These results confirm that the bioactivity of INODs does not lie in their spin-trapping properties but rather in their pro-oxidant character. This property may be the initiator of the redox signal which activates SYK tyrosine kinases.



## 1. Introduction

Molecular and proteic redox systems play an important role in the control of cellular homeostasis and antioxidant defenses. Some drugs containing a redox pharmacophore (quinone and quinoid compounds, *N*-oxide, nitro and thiol derivatives, endoperoxides) may generate an oxidative stress in the cell that can be fatal for the hosted microbes and for the cells. We recently reported that indolone-*N*-oxide derivatives (INODs), which are bioreducible, possess remarkable anti-malarial activities in the low nanomolar range in vitro against different *Plasmodium falciparum* (*P. falciparum*) strains and are also active in vivo [1,2]. Moreover, these compounds are only cytotoxic at very high doses (micromolar range) thus giving a very interesting selectivity index. Early studies showed that INODs have redox potentials near to those of 1,4-quinones and therefore may exert their biological action by oxidizing essential biomolecules [3]. In addition, they contain a nitrone function that may react with the glutathiyl radical formed within the cells upon oxidation of glutathione [4]. To explore the mechanism of action of INODs, we screened for changes in INOD-treated *P. falciparum*-infected red blood cells (RBCs) using a comprehensive proteomic approach. INODs have an original mechanism of action: they damage the host cell membrane, without affecting non-parasitized erythrocytes, with the consequent RBC membrane vesiculation and destabilization responsible for parasite death. The mechanism leading to the selective destabilization of the membrane of parasitized erythrocytes involves the activation of a stress responsive phosphorylation pathway which finally induces the uncoupling of membrane-cytoskeleton interactions. Marked hyperphosphorylation of AE1 (band 3) appears to be the hallmark of the process [5]. We have also studied the kinetics of penetration and biotransformation of these molecules in the erythrocytes. The compounds penetrate very rapidly, accumulate and are rapidly bio-transformed in the RBC cytosol by a thiol-dependent reduction possibly via an enzymatic pathway [6]. Because INODs contain the *N*-oxide functional group, this bioreductive transformation was expected, as it had previously been described for other *N*-oxides derivatives [7]. We recently reported the examination of the antimalarial properties of these compounds in relation to their redox properties using cyclic voltammetry coupled to EPR spectroscopy [8]. Given the redox events occurring in *Plasmodium* infected RBCs, this

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bioreductive transformation may be pivotal for the parasite redox balance and for the antiplasmodial activity. Altogether these studies showed that these compounds target the redox metabolism of the infected host cell.

In a previous study [2] we investigated the in vitro antimalarial properties of compound **1** (6-(4-chlorophenyl)-7*H*-[1,3]dioxolo [4,5-f]indol-7-one-5-oxide) against fresh clinical isolates of *P. falciparum*. This study was carried out to (i) compare the activity of compound **1** with that of chloroquine (CQ) and artemisinin (ART) to assess the potential for cross-resistance, (ii) investigate drug interactions of indolone-*N*-oxides with standard antimalarials and (iii) determine the stage-dependent activity of indolone-*N*-oxides. Compound **1** was equipotent against chloroquine-susceptible and chloroquine-resistant isolates. There was no correlation between responses to chloroquine and compound **1** ($r = 0.015$; $P > 0.05$), but the in vitro responses of compound **1** and dihydroartemisinin were significantly and positively correlated ($r = 0.444$; $P < 0.05$). INODs, as well as artemisinin, inhibited parasite maturation at the ring stage. The antimalarials, chloroquine and artemisinin, also target the redox metabolism of *P. falciparum*. The former belongs to the quinolines that interfere with hemoglobin biocrystallization [9] preventing hemozoin formation, while artemisinin-like derivatives create oxidative stress by generating radical intermediates [10]. Considering i) the mechanisms of action of artemisinin and chloroquine; ii) the redox properties of INODs; iii) and the interactions observed between these molecules in fresh clinical isolates [2], the present study aimed to understand the early stages of the biochemical interactions of the INODs with some RBC components and to compare them with the actions of chloroquine and artemisinin, in biomimetic models. As the lead compound of the INOD series, compound **1** was selected for these studies (Fig. 1). EPR and electrochemical experiments were designed to study i) the bioreductive properties of compound **1** in relation to those of ART and CQ; ii) the interaction with non-protein thiols (L-cysteine and glutathione); iii) the capacity to interact with heme; iv) the capacity to generate and/or trap radicals.

## 2. Material and methods

### 2.1. Chemicals

6-(4-Chlorophenyl)-7*H*-[1,3]dioxolo[4,5-f]indol-7-one-5-oxide (compound **1**) was synthesized in our laboratory as previously reported [1]. Ferrous ammonium sulfate, hemin chloride, hydroxylamine hydrochloride ($NH_2OH.HCl$), sodium acetate, sodium dihydrogen phosphate ($NaH_2PO_4$), di-sodium hydrogen phosphate ($Na_2HPO_4$) and ferrocene were purchased from Prolabo (VWR, France); dimethyl sulfoxide (DMSO), HCl 37%, NaOH, tris-HCl, hydrogen peroxide ($H_2O_2$), L-cysteine (L-Cys), anhydrous acetonitrile (ACN), ferrocene, chloroquine diphosphate salt, artemisinin, and glutathione (GSH) were purchased from Sigma-Aldrich (St. Quentin, France); EDTA and tetrabutyl ammonium perchlorate (TBAP) were purchased from Fluka, RPMI from Cambrex (Verviers, Belgium).

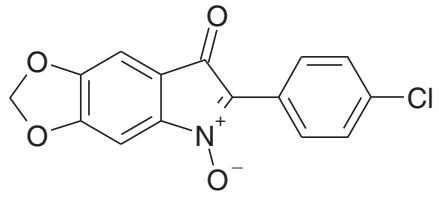


**Fig. 1.** Structure of compound **1**.

### 2.2. Electrochemical and chemical analysis

#### 2.2.1. Electrochemical analysis

Experiments were carried out at 25 °C (thermal bath) in DMSO/Tris–HCl buffer (0.1 M) (80/20, v/v), using a Voltalab 80 PGZ 402 (Radiometer) with a conventional three-electrode system including an Ag/AgCl electrode or an saturated calomel electrode (SCE) as the reference electrode, a platinum electrode (5 × 5 mm) as the counter electrode and a glassy carbon disk (1 mm diameter) as the working electrode. All solutions were deoxygenated by passing a gentle constant stream of pre-purified argon through the solution for 10 min and maintaining a blanket of the inert gas over the solution during the experiment. The glassy carbon electrode was cleaned after each run by electrochemical cleaning to avoid aggressive changes. The electrochemical cleaning process was done in acetate buffer pH 4.5 by polarization for 10 min at −500 mV then at 2000 mV vs. the reference electrode (the electrochemical cleaning process was done according to the Princeton Co protocol). Between experiments, glassy carbon was rubbed on the polishing pad with blue diamond slurry particles (Waters, USA), then washed with methanol and distilled water.

#### 2.2.2. Chemical analysis of the interaction between compound 1 and L-Cys

Liquid chromatography (LC) coupled to mass spectrometry (MS) analysis was carried out with an LC-PDA–$MS^n$ system (Thermo Electron Corporation and Spectra System (SS)) including an automatic injector with an oven (SS-AS3000), a degasser (SS-SCM1000), and a quaternary pump (SS-P1000 XR) coupled to a photodiode array detector PDA (SS-UV6000LP), and an ion trap mass spectrometer (Finnigan LCQ Deca XP Max). Nitrogen was used as a nebulizing and drying gas. Data acquisition was carried out using Finnigan Xcalibur software (version 1.4). The atmospheric pressure chemical ionization (APCI) source was used in the negative ion mode. Mass scans were done in the range *m/z* 50–650. The chromatographic separation was done on an analytical column Luna® C-18 (5 μm, 250 mm × 4.6 mm) using a C-18 pre-column (5 μm, 4.6 mm × 3 mm) (Phenomenex, France). The separation was done in gradient mode using solvent A (water) and solvent B ($CH_3OH$). The gradient program was the following: at $t = 0$–3 min; solvents (95% A/5% B, v/v); at $t = 4$–20 min, solvents (15% A/85% B, v/v); at $t = 22$ min, solvents (95% A/5% B, v/v); the column regeneration time was 10 min and the mobile phase flow rate was 1 ml/min. The analysis was performed at room temperature (RT), with 20 μl of sample injected. Compound **1** was dissolved in DMSO and L-Cys in phosphate buffer and they were mixed to give a final concentration of 0.25 and 2.5 mM for compound **1** and L-Cys, respectively.

### 2.3. Electron paramagnetic resonance (EPR) experiments

EPR spectra were obtained at X-band on a Brüker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity (4119/HS 0205) and a gaussmeter (Bruker, Wissembourg, France). EPR data processing and spectrum computer simulation were performed using WINEPR and SIMFONIA software (Bruker, Wissembourg, France).

#### 2.3.1. Interaction with heme

Compound **1**, artemisinin and chloroquine were each incubated under an argon atmosphere with hemin or heme obtained by reducing hemin with $NH_2OH.HCl$. The analyzed samples were prepared in DMSO by mixing hemin/compound at the stoichiometric ratio 1/4 (0.75/3 mM) or hemin/$NH_2OH.HCl$/compound at the stoichiometric ratio 1/10/4 (0.75/7.5/3 mM). The solutions were stirred at room temperature for 5 h before recording the EPR spectra. The recording was carried out at 107 K using a liquid nitrogen flow in a quartz tube (inner diameter: 4 mm) containing 250 μL analyzed solution. Typical scanning parameters were: scan rate, 0.6 G/s; scan number, 1; modulation amplitude, $5.10^{-4}$ G; modulation frequency, 100 kHz,