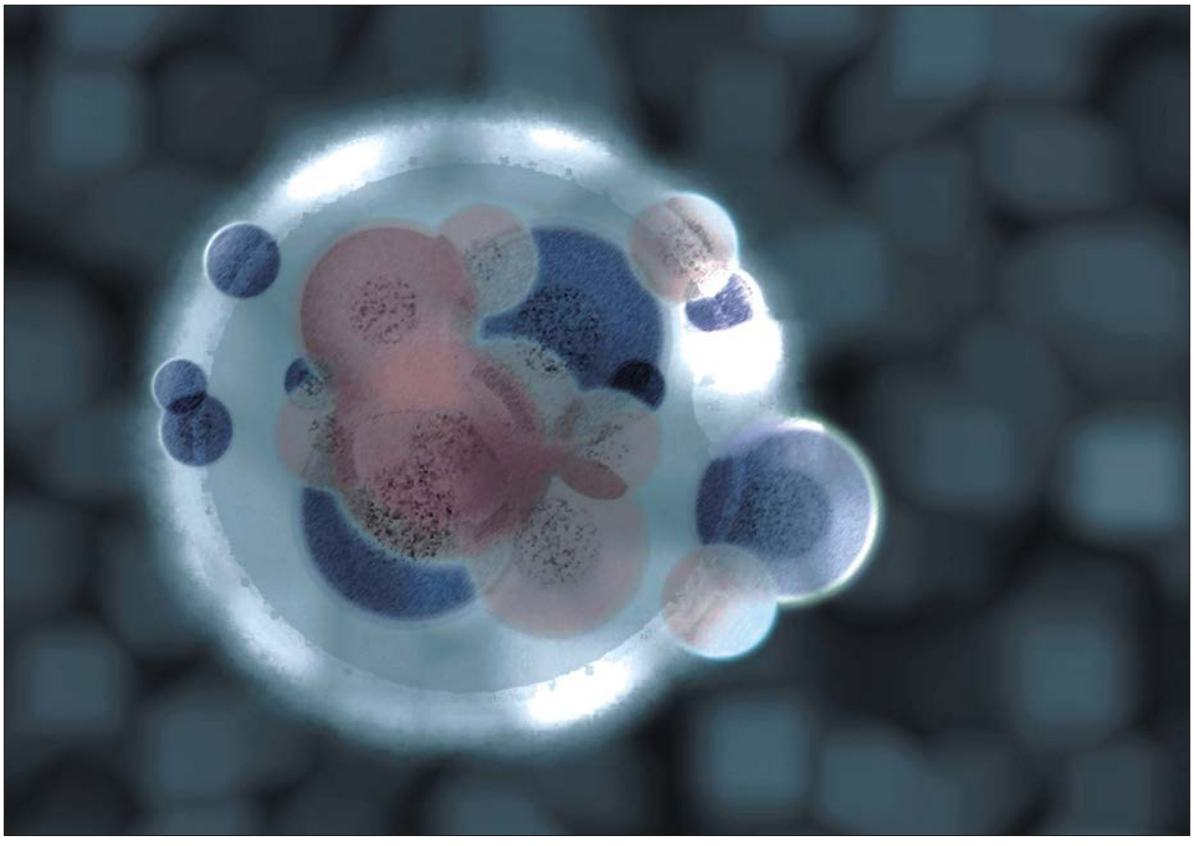

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Antimalarial Activity of Zincke's Salts

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Abstract: A series of Zincke's salts was synthesized from new pyridine precursors and evaluated for their antiplasmodial and cytotoxicity activity. The Zincke's salts were prepared via a nucleophilic aromatic substitution reaction between pyridine derivatives and 1-chloro-2, 4-dinitrobenzene. Antimalarial activity of the compounds was assayed by *in vitro* p-LDH test against a chloroquine resistant *Plamodium falciparum* strain (clone W2). In parallel, the cytotoxicity of the compounds was assessed with the human cell line HeLa by MTT colorimetric method. The Zincke´s salts 5a, c and 7 showed low IC_{50} against *P. falciparum* (ranging from 3.13-4.83 μg mL^{-1}), with a Selectivity Index (SI) higher than 20 which suggests that these compounds may be a promising class of substances with antimalarial activity.

Key words: Antimalarial chemotherapy, cytotoxicity, pyridinium salts, quaternary ammonium salts, selectivity index, Zincke's salts

INTRODUCTION

Currently, almost half the world's population is exposed to malaria which causes over 644,000 deaths each year and kills more children under 5 years old than any other infectious agent (WHO, 2009). The global spread of drug resistance and the collapse of vector control programs resulted in a resurgence in malaria incidence and mortality. Nowadays, epidemiological data suggest that the introduction of new Artemisinin-based Combination Therapies (ACTs) may have reversed this trend (Eastman and Fidock, 2009). However, recent reports indicate that resistance to derivatives of the endoperoxide artemisinin is now emerging (Noedl *et al*., 2008). These observations have motivated the search for new drugs that could be used if artemisinin resistance were to spread.

Quaternary ammonium salts (quats) are generally water-soluble compounds associated with broad biological activity (Pernak *et al*., 2006). These activities include, for example, an inhibition of the growth of bacteria, viruses, fungi and protozoa (Chanawanno *et al*., 2010). Since the early 20th century, numerous studies of the synthesis and antimicrobial characteristics of quats have been completed (Pernak and Chwala, 2003). Pyridinium salts are quats that have been described as a potential class of biocides, drugs and herbicides (Chanawanno *et al*., 2010). Due to their low toxicity to humans and animals, quats are widely used in hospital environments and in the food industry (De Souza *et al*., 2007).

Zincke's salts are quats that had been known for a long time and they are easily prepared from the reaction between pyridine derivatives and 1-chloro-2, 4-dinitrobenzene. They are usually employed as synthetic intermediates in the preparation of chiral pyridinium salts (Viana *et al*., 2008) and their biological activities have been poorly explored. Chiral pyridinium salts are frequently used in asymmetric organic syntheses for the preparation of natural products such as alkaloids (Compere *et al*., 1999). The presence of a nitroaromatic

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group can be promising in these quats due the action of many drugs related to the nitro group (Zhang *et al*., 2012).

In the present study, we describe the synthesis and evaluation of the *in vitro* antimalarial activity of a series of Zincke's salts and their pyridine precursors, as well as one chiral pyridinium salt.

MATERIALS AND METHODS

The general scheme for the synthesis of the new Zincke's salts is depicted in Fig. 1. The four Zincke's salts 5a-d were prepared via a nucleophilic aromatic substitution reaction between 3-pyridinepropanol 1 (or its corresponding ether derivative 3a-c) and 1-chloro-2, 4-dinitrobenzene 4 with yields from 14-84%. Additionally, Zincke's salt 7, obtained from commercial 3-methylpyridine 6, was converted into its corresponding pyridinium salt 9 by treatment with (R)-phenylglycinol 8 with yield of 85% (Genisson *et al*., 1992) (More details on Supplementary Data).

Human red blood cells infected with *P. falciparum* clone W2 (chloroquine-resistant) were maintained in continuous culture (Trager and Jensen, 1976). The synchronization of the parasites was achieved by sorbitol treatment (Lambros and Vanderberg, 1979) and parasitaemias were determined microscopically in Giemsa-stained smears.

Antimalarial activity was assayed by p-LDH test (Jansen *et al*., 2012). The results of three of these drug activity assays were expressed as the mean of the half-maximal inhibitory dose (IC_{50}) and compared with the drug-free controls. Curve-fitting was performed using Origin 8.0 software (Origin Lab. Corporation, Northampton, MA, USA).

The cytotoxicity of the compounds was assessed with the human cell line HeLa (ATCC # CCL2), using a tetrazolium salt MTT colorimetric method. Cytotoxicity was scored as the percentage reduction in absorbance versus untreated control cultures (Pereira *et al*., 2012). All experiments were performed in triplicate. The results were expressed as the mean of LC_{50} (the lethal drug concentration that reduced cell viability to 50%). A Selectivity Index (SI), corresponding to the ratio between the cytotoxic and antiparasitic activities of each compound, was calculated as follows:

$$SI = \frac{LC_{50}\ HeLa}{IC_{50}\ P.\ falciparum}$$

RESULTS AND DISCUSSION

The half maximal inhibitory concentration (IC_{50}) and the lethal drug concentrations (LC_{50}) values were determined for nine pyridine derivatives, six of which are novel compounds (Table 1).

In addition to the Zincke´s salts 5a-d, 7 and chiral pyridinium salt 9, the synthetic intermediates 3a-c were tested in parallel, due to their similarity with marine alkaloid analogues that exhibited antimalarial activity in a previous report (Hilario *et al*., 2011).

Seven of the compounds studied were active against the chloroquine-resistant *P. falciparum* clone W2 as

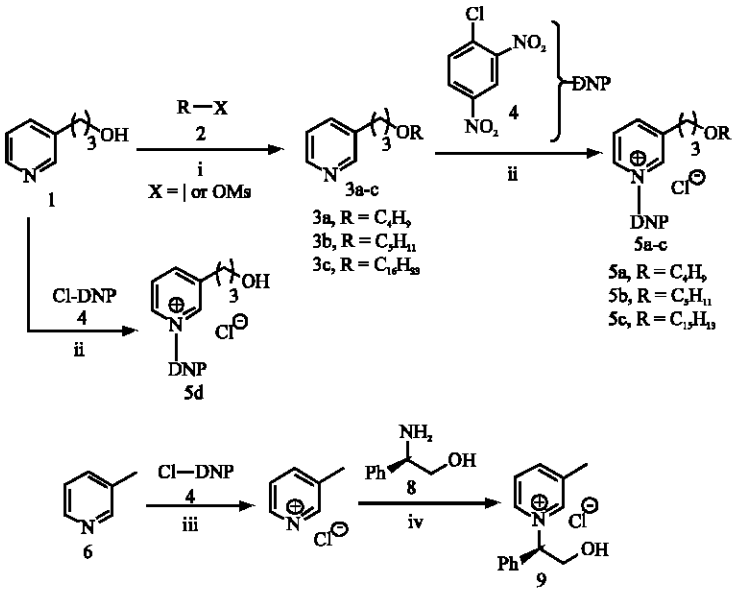
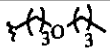
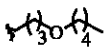
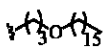
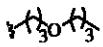
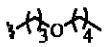
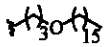
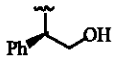


Fig. 1: Reagent, conditions and yields: (i) THF, NaH, rt, 48 h, 59-75%, (ii) MeOH, reflux, 72 h, 14-84%, (iii) Acetone, reflux, 15 h, 63% and (iv) 1-BuOH, reflux, 16 h, 85%

Table 1: Zincke's and chiral pyridinium salts prepared and *in vitro* inhibition of *Plasmodium falciparum* (clone W2) growth

Compounds	R_1	R_2	Yield (%)	IC_{50} (μg mL^{-1}) ±SDa	LC_{50} (μg mL^{-1}) ±SD	SI[b]
3a		-	75	7.37±0.46	115.4±12.5	15.6
3b		-	65	3.99±0.05	15.2±3.4	3.8
3c		-	59	>25	100.2±12.5	4.0
5a		DNP	84	4.78±0.43	140.3±21.8	29.3
5b		DNP	67	5.49±0.26	85.4±16.3	15.5
5c		DNP	14	3.13±0.37	79.3±18.5	25.3
5d		DNP	62	18.38±0.07	92.4±17.7	5.0
7	CH_3	DNP	85	4.83±1.62	97.3±15.5	20.1
9	CH_3		85	>50	122.3±25.6	2.4
Cq[c]	-	-	-	0.134±0.014	>1000	>1000

[a]Values are average±standard deviation; [b]SI = LC_{50} HeLa/IC_{50} *P. falciparum*; [c]Chloroquine

determined by p-LDH assay. The Zincke's salt 5c was the most active (IC_{50} = 3.13 μg mL^{-1}). All the compounds, except 3b, presented LC_{50} higher than 70 μg mL^{-1}. Three Zincke's salts showed a selective antimalarial activity, with SI ranging from 20.1-29.3.

Our preliminary results suggest that the comparison between the compounds 5a and b allow establishing the following observation: the short alkyl chain length present in 5a increased the SI; while the 16-carbon chain length present in 5b decreased the IC_{50}.

Quaternary ammonium compounds exhibit a broad spectrum of antimicrobial activity, acting against gram-positive and gram-negative bacteria, fungi and protozoa (Pernak and Chwala, 2003), with a low toxicity to humans (Francavilla *et al*., 2009). Their antimalarial activity is correlated with the interference of the parasite phospholipid metabolism (Schlitzer, 2007; Tischer *et al*., 2012). Based on this mode of action, the adoption of these compounds in antimalarial chemotherapy combinations could facilitate the access of traditional antimalarials to their targets inside the parasites. This combination may allow treatment of malaria with lower doses of traditional antimalarials, without compromising the efficiency of the treatment.

CONCLUSION

In summary, pyridinium derivatives presented selective antimalarial activities. The work-up procedure of the synthesis is very simple, the yield ranges from moderate to good and the pyridinium compounds are easily purified. The necessary reagents are all inexpensive and readily available. These results indicate that synthetic Zincke's salts are promising antimalarial drug candidates. Further *in vivo* tests are currently ongoing to support these preliminary data.

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