



## SYNTHESIS AND ANTIPLASMODIAL EVALUATION OF A NEW TRIOXAQUINE

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### ABSTRACT

The ultimate objective of this work was to develop a novel antimalarial hybrid drug based on quinoline and trioxane pharmacophoric scaffold that is effective against drug-resistant malaria, especially in the face of emerging resistance against artemisinin based combination therapy (ACT). The synthetic design involved introduction of a linker to 4,7-dichloroquinoline and subsequent coupling with artesunate to form the dual drug. The structure's molecular formula  $C_{30}H_{38}N_3ClO_7$  was confirmed by electron spray ionization mass spectrum that showed a molecular ion peaks at  $m/z$  588.24 and 590.24 amu with a relative abundance of 100% and 38.8% respectively against an exact value of 587.24 amu. Through biological studies, it was established that the drug's antiplasmodial activity ( $IC_{50}$ ) against chloroquine (CQ)-sensitive (CQS, D6) and CQ-resistant (CQR, W2) isolates (CQS, 6.89 ng  $mL^{-1}$ ; CQR, 3.62 ng  $mL^{-1}$ ) was comparable ( $p > 0.05$ ) to that of artesunate (CQR, 6.67 ng  $mL^{-1}$ ; CQR, 4.04 ng  $mL^{-1}$ ), currently the most potent antimalarial in the artemisinin family. The drug had a good safety profile, with low percentage inhibition of human HeLa cell proliferation (29%), and  $IC_{50}$  values  $> 10\ 000$  ng  $mL^{-1}$ . The findings validate the concept of "covalent bitherapy" as a feasible strategy in antimalarial drug development.

**KEYWORDS:** Malaria, Drug resistance, Hybrid drug, Covalent biotherapy.

### 1. INTRODUCTION

Malaria is the most widespread parasitic disease caused by protozoa parasite of the genus *Plasmodium*, with malaria endemic regions encompassing approximately 40% of the global human population.<sup>[1,2]</sup> *Plasmodium falciparum* is the most prevalent and virulent of malaria parasites, responsible for about 90% of all annual global malaria-associated deaths.<sup>[3]</sup>

Efforts to develop an effective malaria vaccine are largely unsuccessful and thus chemotherapy may remain the main malaria control strategy for a long time.<sup>[4]</sup> Currently, artemisinin-based combination therapy (ACT) is the World Health Organization (WHO) gold standard against *P. falciparum* malaria, in which the regimen uses a double or triple combination therapy geared towards delay of resistance, or circumventing it altogether.<sup>[5,6]</sup> However, *P. falciparum* is increasingly becoming resistant to most antimalarial drugs in clinical use.<sup>[1,2]</sup>

This dire situation is being aggravated by reports from Southeast Asia confirming that resistance to artemisinin-based combination therapy (ACT), the world's most effective antimalarial therapy, is now established in western Cambodia, Thailand, Vietnam, eastern Myanmar and northern Cambodia.<sup>[7]</sup> Recent reports of high failure rates associated with ACT could greatly disrupt the current malaria elimination and eradication efforts, and again foster an increase in malaria cases and deaths.<sup>[8]</sup>

Resistance to antimalarial drugs arises when spontaneously occurring mutants with gene mutations or amplifications which confer reduced drug susceptibility are selected, and transmitted.<sup>[9]</sup> Simultaneous use of two or more antimalarials with different modes of action thus do not share the same resistance mechanisms reduces the chance of selection.<sup>[9]</sup> It is already an established fact that combinations of chemotherapeutic agents can accelerate therapeutic response, improve cure rates and protect the

component drugs against resistance.<sup>[6]</sup> Therefore, use of combination therapy also known as drug co-formulation is the currently accepted practice in management of infectious diseases.

In the last two decades, only a few compounds belonging to a new class of antimalarial drugs, including amino alcohols, sesquiterpene trioxanes and naphthoquinones reached clinical application.<sup>[10]</sup> Thus, search for novel malaria therapies is more urgent than ever. Different approaches to antimalarial drug discovery have been deployed which includes optimization of therapy with available drugs including combination therapy, developing analogues of the existing drugs, evaluation of potent agents from natural products especially plants, use of compounds originally developed against other diseases, evaluation of drug-resistance reversers, also known as chemosensitizers as well as search for new chemotherapeutic targets.<sup>[2,11]</sup>

One of the challenges of the future malarial chemotherapy is to develop compounds that are innovative with respect to the chemical scaffold and molecular target.<sup>[12]</sup> Development process of new molecules is not only laborious but also costs billions of dollars and takes over a decade to have a single molecule to clinical application.<sup>[13]</sup> Recently, through rational drug design approach, single hybrid molecules with dual functionality and/or targets have been developed as novel antimalarial drugs, a concept termed as covalent biotherapy.<sup>[6]</sup> The concept is premised on chemical combination of two distinct pharmacophores into a single molecule (termed as hybrid, chimeric or conjugate molecule, or simply dual-drug).<sup>[14]</sup> Covalent bitherapy is an emerging strategy within medicinal chemistry and drug discovery, and some of hybrid drugs have been demonstrated to be potent antimalarials, possessing no or minimum toxicity.<sup>[15,16]</sup> However, so far no hybrid antimalarials have reached clinical application stage. It is envisaged that building on pharmacophores of existing drugs already indicated for human application to develop hybrid drugs may not only shorten the drug development period and lower cost but will also reduce drug development attrition rates while achieving the ultimate objective of either delaying or circumventing development of resistance altogether.<sup>[17]</sup>

In this study, artesunate was coupled to an aminoquinoline moiety with an aim of validating the concept of covalent bitherapy. The hybrid molecule offers a simpler and more effective way of delivering two drugs, especially when differences like elimination times occur.<sup>[18]</sup> There are numerous advantages of employing hybrid molecules over multi-component drugs in malaria therapy. Compared to the latter, hybrid drugs may be less expensive since in principle, the risks and costs involved may not be different from any other single entity and also there is a lower risk of drug-drug adverse interactions compared to multi-component drugs.<sup>[19]</sup> It is also expected that the hybrid drug would be

metabolized by the liver enzymes to release both pharmacophores thus giving rise to dual action, increased activity and prolonged half-life.<sup>[6]</sup>

The ultimate object of this work was to develop a novel antimalarial hybrid drug based on quinoline and trioxane pharmacophoric scaffold of quinolines and artemisinins respectively, based on covalent bitherapy strategy especially in the face of emerging ACT resistance.

## 2. MATERIALS AND METHODS

### 2.1 Materials

The progress of all reactions was monitored on Merck precoated silica gel plates 60 F-254 using ethyl acetate and methanol solvent system. Spots were visualized with ultraviolet light lamp (254 nm). Column chromatography was performed with Fluka silica gel 60 (5-40  $\mu$ m mesh, Merck, Germany) using ethyl acetate and methanol solvent system. The stationary phase was impregnated with a few drops of ammonia. The melting points were determined using an electro-thermal melting point apparatus with a thermometer range of 0–360°C and was uncorrected. Infrared spectra were obtained in potassium bromide pellets using IR-840 Shimadzu spectrometer. The <sup>1</sup>H and <sup>13</sup>C NMR analysis were done using mercury Vx Bruker NMR spectrometer operating at 200 MHz and 400 MHz for <sup>1</sup>H NMR, 50MHz and 100 MHz for <sup>13</sup>C NMR in deuteriated chloroform with TMS as the internal standard. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), and m (multiplet). An electron impact mass measurement was recorded on a GC-mass spectrometer machine (VG-12-250).

### 2.2 Synthesis of Amine-functionalized Quinoline Moiety

The synthesis of the amine-functionalized quinoline moiety was achieved by using the general method reported by Antinarelli *et al.*<sup>[20]</sup> Briefly, to 4,7-dichloroquinoline (1 mmol, 0.198g) was added ethylene diamine (4.5 equiv, 0.3mL) in a round-bottomed flask. The flask was then fitted with a condenser and the suspension was heated at 80°C for 1 h before elevating the temperatures to 110°C for 4-6 h with continued stirring with a magnetic stirrer to drive the reaction to completion (Scheme 1). The reaction mixture was cooled to room temperature and diluted with dichloromethane (20 mL). The organic layer was successively washed with 5% NaOH (30 mL), water and finally with brine. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the solvent removed under reduced pressure in a rotary evaporator to afford compound 1 which was used in the next step without further purification.

### 2.3 Coupling Procedure

The coupling procedure is as summarized in Scheme 2. In brief, artesunate (1.2 mmol, 0.460g) was dissolved in dichloromethane (5mL). To this mixture in a round bottomed flask, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (1.5 mmol, 0.270g),

1-hydroxy- benzotriazole (HOBt) (1.0 mmol, 0.162g) and diisopropylethylamine (DIPEA) (1.0 mmol, 0.208g) were added and the mixture stirred at 0°C for 30 min. To this mixture, 7-chloroquinoline-4- ethylene diamine (1) (1.0 mmol, 0.222g) was added and the reaction stirred to room temperature overnight (15-16h). Upon completion of the reaction, the mixture was quenched with saturated NaHCO<sub>3</sub> (20mL). The organic phase was separated and the aqueous phase back-extracted with CH<sub>2</sub>Cl<sub>2</sub> (20mL × 3). The combined organic layers were dried using anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and the solvent removed *in vacuo*. Purification using silica gel column pre-treated with ammonia yielded the desired hybrid drug. The reaction was optimized by using different coupling agents and solvents.

## 2.4 Biological Assays

### 2.4.1 *In vitro* Drug Sensitivity Assay

Laboratory-adapted *P. falciparum* cultures of the international reference isolate D6 (CQ-sensitive) and W2 (CQ-resistant) were used in this study. The strains have been cultured and maintained at the Malaria Laboratories of Kenya Medical Research Institute (KEMRI), Nairobi. The culture medium, a variation of that described by Trager and Jensen<sup>[21]</sup> consisted of RPMI 1640 supplemented with 10% human serum, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 25 mM NaHCO<sub>3</sub>. Human type O<sup>+</sup> erythrocytes (<28 days old) served as host cells and the cultures were incubated at 37°C in an atmosphere of 3% CO<sub>2</sub>, 5% O<sub>2</sub> and 92% N<sub>2</sub> obtained from BOC Nairobi, Kenya.

Aliquots (25 µL) of the culture medium were added to all the wells of a 96-well flat-bottom micro- culture plate. The hybrid drug solution in volumes of 25 µL was added in triplicate, to the first wells and a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, United Kingdom) was used to make serial two-fold dilutions. The highest concentration of 100 ng mL<sup>-1</sup> was serially diluted 2-fold in complete medium to give eleven doubling concentrations with the lowest concentration being 0.0975ng mL<sup>-1</sup>. The same dilution technique was used for the reference drugs CQ and artesunate samples except 4,7-dichloroquinoline whose starting concentration was 1000 ng mL<sup>-1</sup>. To compare the antiplasmodial activity of the hybrid drug (100 ng mL<sup>-1</sup>) with that of the combination of its precursors, wells of the combination of the latter were also included in the test in the ratio of 1:1 (mixture of 50 ng mL<sup>-1</sup> of artesunate and 50 ng mL<sup>-1</sup> of 4,7-dichloroquinoline). Subsequent serial dilutions and semi-automated micro-dilution assays were done. Since it is impossible to determine a single IC<sub>50</sub> of two drugs in combination, the counts per minute (cpm) values for the wells of hybrid drug and for the drugs in combination at a given concentration were used to determine percentage *in vitro* inhibition of parasite proliferation, relative to drug-free parasitized erythrocyte wells.

The *in vitro* semi-automated micro-dilution assay technique that measures the ability of the drugs to inhibit the incorporation of [G-<sup>3</sup>H] hypoxanthine into the malaria parasite was used to assess the hybrid drug's antiplasmodial activity.<sup>[22,23]</sup> Briefly, A suspension (200 µL, 1.5% v/v) of parasitized erythrocytes (0.4% parasitaemia) in culture medium and growth rate (>3-fold per 48h) were added to all test wells. Drug free wells of both parasitized and non-parasitized erythrocytes were also included as controls. The plates were incubated at 37°C in an airtight gas chamber under micro-aerobic environment of 3% CO<sub>2</sub>, 5% O<sub>2</sub> and 92% N<sub>2</sub>. After 48 h each well was pulsed with 25 µL of culture medium containing 0.5 µCi of [<sup>3</sup>H] hypoxanthine and the plates were incubated for a further 18 h. The contents of each well was harvested onto glass fiber filters, washed thoroughly with distilled water, and dried, and the radioactivity cpm was measured by liquid scintillation.

### 2.4.2 Cytotoxicity Assays

Rapid colorimetric assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra- zolium bromide (MTT).<sup>[24,25]</sup> This assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of pale yellow MTT and thereby form dark blue formazan crystals which are largely impermeable to cell membranes, resulting in their accumulation within healthy cells. The amount of generated formazan is directly proportional to the number of cells.<sup>[24]</sup>

The human cervical adenocarcinoma (HeLa) cells that were previously misidentified as human laryngeal carcinoma (Hep2) cells<sup>[26,27]</sup> were used for this study. Cells were maintained in Eagles Minimum Essential Medium (MEM) containing 10% foetal bovine serum (FBS). A cell density of 20,000 cells per well in 100 µl were seeded on 96-well plates and incubated for 12 h at 37 °C and 5% CO<sub>2</sub> to attach to the surface. Samples of the test drug and controls were added to the cultured cells in rows H - B over a concentration range of 0.14 to 100 µg mL<sup>-1</sup>, whereas wells 1-8 row A served as untreated controls and wells 9-12 as blank (1% DMSO v/v). The plates were incubated for 48 hours at 37 C and 5% CO<sub>2</sub>, followed by addition of 10 µl MTT viability indicator reagent, and were incubated for additional 4 hours at the same conditions. All media was removed from the plates and 100 µL DMSO was added to dissolve the formazan crystals. The plates were read on a Multiskan EX Labsystems scanning multi-well spectrophotometer at 562 and 690 nm as reference. The results were recorded as optical density (OD) per well at each drug concentration. The data was transferred into the software Microsoft Excel 2007 and expressed as percentage of the untreated controls.

### 2.4.3 Data and Statistical Analysis

Nuclear magnetic resonance (NMR) spectra for both <sup>13</sup>C and <sup>1</sup>H was analyzed using characteristic chemical shifts in ppm while for infrared (IR) characteristic peaks in

wavenumbers generated were used. In mass spectra, the peaks for the molecular ion and major fragments were analyzed. Data for *in vitro* drug assays were transferred into graphic programme (Microsoft Excel 2007) and results expressed as the drug concentration required for 50% inhibition of ( $G^3H$ ) hypoxanthine incorporation into parasite nucleic acid using non-linear regression analysis of the dose-response curve. Percentage cytotoxicity (PC) as compared to the untreated controls was calculated using the following equation. Percentage cytotoxicity =  $[(A - B)/A] \times 100$ .

Where A is the mean OD of the untreated cells and B is the mean OD at each drug concentration.<sup>[25]</sup> The drug

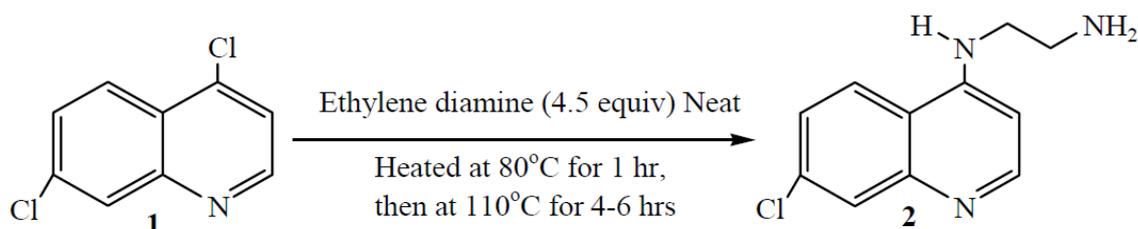
concentration required for 50% inhibition of cell growth was determined using nonlinear regression analysis of the dose-response curve.

For comparison of chemosuppression, two-tailed student's *t*-test was used (Microsoft<sup>®</sup> Excel 2004), with  $p < 0.05$  being considered significant.

### 3. RESULTS AND DISCUSSION

#### 3.1 Synthesis and Structural Elucidation of the Dual-drug

Amine-functionalized quinoline was obtained by condensation of ethylene diamine with 4,7-dichloroquinoline (Scheme 1).

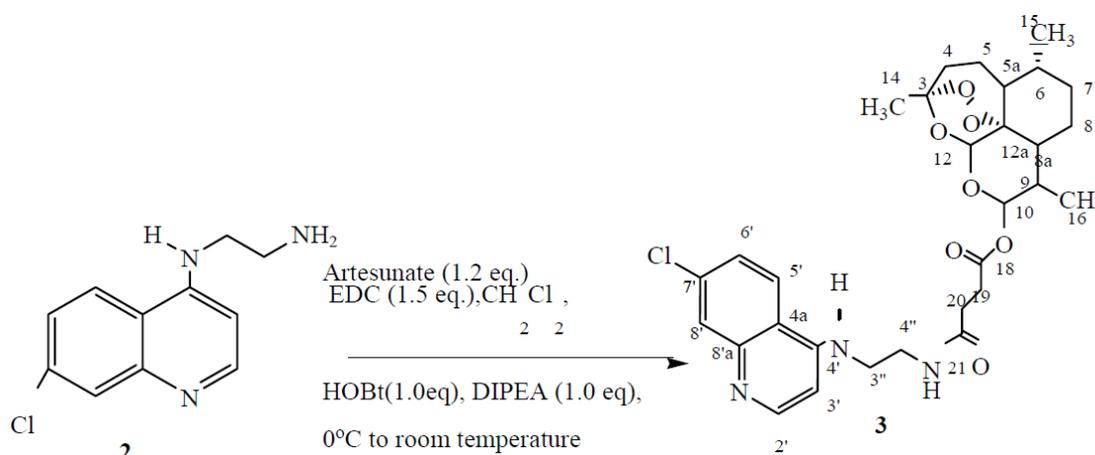


Scheme 1: Introduction of a linker (ethylene diamine) to 4,7-dichloroquinoline.

The product was isolated as a yellow powder (0.186g, yield 84%,  $R_f$  0.6  $SiO_2$ , 1:3, EtOAc: MeOH, mp 142-145°C) and identified as 7-chloro-4-(1,2-diaminoethyl)quinoline (2)<sup>[28]</sup> with molecular formula of  $C_{11}H_{12}N_3Cl$ .

IR:  $V_{max}$  KBr ( $cm^{-1}$ ) 3361(N-H), 1585(C=C) and 1326(C=N)  $^1H$ -NMR (200 MHz,  $CDCl_3$ ): 8.52 (1H, d,  $J = 5.6$  Hz, H-2), 7.95 (1H, d,  $J = 1.8$  Hz, H-8), 7.75 (1H, d,  $J = 9.2$  Hz, H-5), 7.35 (1H, dd,  $J = 2.1$  and 8.8 Hz, H-6), 6.40 (1H, d,  $J = 5.4$  Hz, H-3), 3.36 (2H, m, H-1'), and

ppm 3.13 (2H, t,  $J = 4.8$  Hz, H-2')<sup>13</sup> C-NMR (50 MHz,  $CDCl_3$ ): 152.3 (C-2), 150.2 (C-4), 149.3 (C-8a), 135.1 (C-7), 128.9 (C-5), 125.5 (C-6), 117.6 (C-4a), 99.5 (C-3), 44.9 (C-1'), and ppm 40.4 (C-2'). HRMS ESI [M]:  $m/z$ , Calc for  $C_{11}H_{12}N_3Cl$  is 221.07, found 221.8. Artesunate was then linked with 7-chloro-4-(1,2-diaminoethyl)quinoline (2) to yield the hybrid molecule (3) (Scheme 2).



Scheme 2: Coupling reaction.

The product was isolated as an off white powder (0.449g, 71.4% yield,  $R_f$  0.8,  $SiO_2$ , 2:1, EtOAc: MeOH, mp 184-186°C).

IR:  $V_{max}$  KBr ( $cm^{-1}$ ) 3361(N-H), 1747(C=O), 1699(N-C=O), 1585(C=C) and 1326 (C=N)  $^1H$ -NMR (400 MHz,

$CDCl_3$ ) :  $\delta = 8.50$  (1H, d,  $J = 3.2$  Hz, H-2'), 7.91 (1H, d,  $J = 3.6$  Hz, H-5'), 7.89 (1H, d,  $J = 12$  Hz, H-8'), 7.39 (1H, dd,  $J = 2.8$  and 12 Hz, H-6'), 6.31 (1H, d,  $J = 3.6$  Hz, H-3'), 5.59 (1H, d,  $J = 12$  Hz, H-10), 5.44 (1H, s, H-12), 2.55 (1H, m, H-9), 1.34 (3H, s, H-14), 0.87 (3H, d,  $J = 9.2$  Hz, H-16), 0.74 ppm (3H, d,  $J = 9.6$  Hz, H-15)<sup>13</sup>

C-NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 11.8 (C-15), 20.0 (C-16), 20.3 (C-8), 24.5 (C-5), 25.8 (C-14), 28.2 (C-20), 30.2 (C-19), 31.0 (C-9), 34.4 (C-7), 36.4 (C-4), 37.4 (C-6), 42.8 (C-4''), 44.9 (C-8a), 45.4 (C-3''), 51.5 (C-5a), 52.5 (C-5'a), 80.4 (C-12a), 91.1 (C-12), 92.4 (C-10), 98.5 (C-3'), 104.4 (C-3), 117.3 (C-4'), 122.4 (C-5'), 125.7 (C-6'), 127.7 (C-8'a), 135.2 (C-7'), 150.3 (C-8'),

151.6 (C-2'), 171.9 (C-18), 178.1 ppm (C-21). HRMS ESI [M+H]<sup>+</sup>: m/z, Calc for C<sub>30</sub>H<sub>38</sub>N<sub>3</sub>ClO<sub>7</sub> is 587.24, found 588.24.

Optimization reactions gave yields ranging from trace to 71% (Table 1).

**Table 1: Optimization reactions for N-(7-chloroquinolin-4-ylamino)ethyl-artesunate-19- carboxamide.**

Entry	Coupling Reagent	Solvent	Additives (eq)	Yield (%)
1	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride(EDC)	Tetrahydrofuran (THF)	-	11
2	EDC	Acetone	-	Trace
3	EDC	THF	4- Dimethylaminopyridine DMAP (0.2)	17
4	EDC	Dichloromethane (CH <sub>2</sub> Cl <sub>2</sub> )	-	30
5	EDC	CH <sub>2</sub> Cl <sub>2</sub>	1-Hydroxy-benzotriazole HOBT (0.2)	71
6	Dicyclohexylcarbodiimide (DCC)	CH <sub>2</sub> Cl <sub>2</sub>	-	8
7	DCC	CH <sub>2</sub> Cl <sub>2</sub>	HOBT (0.2)	13
8	Carbonyldiimidazole (CDI)	CH <sub>2</sub> Cl <sub>2</sub>	-	14
9	CDI	CH <sub>2</sub> Cl <sub>2</sub>	HOBT (0.2)	51

### 3.2 Biological Evaluation of the Hybrid Drug

The ability of the hybrid drug to inhibit *P. falciparum* growth *in vitro* was assayed against chloroquine CQ -

sensitive (CQS, D6) and CQ-resistant (CQR, W2) isolates (Table 2).

**Table 2: IC<sub>50</sub> of dual-drug, artesunate, chloroquine (CQ), 4,7-dichloroquinoline (DQ) against CQ-sensitive (D6) and -resistant (W2) *P. falciparum* isolates.**

Drug	IC <sub>50</sub> (ng mL <sup>-1</sup> ) ± SD (D6, CQS <sup>a</sup> )	IC <sub>50</sub> (ng mL <sup>-1</sup> ) ± SD (W2, CQR <sup>b</sup> )
Dual-drug	6.89 ± 0.35	3.62 ± 0.32
Artesunate	6.67 ± 0.07	4.04 ± 0.19
CQ	8.02 ± 0.23	65.35 ± 1.07
4,7-DQ	> 1000	> 1000

<sup>a</sup>CQS, chloroquine sensitive *P. falciparum* isolate; <sup>b</sup>CQR, chloroquine resistant *P. falciparum* isolate.

To validate the concept of covalent bitherapy *in vitro*, the precursors of the hybrid drug were tested in combination in the same wells and percentage inhibition of parasite growth determined (Table 3).

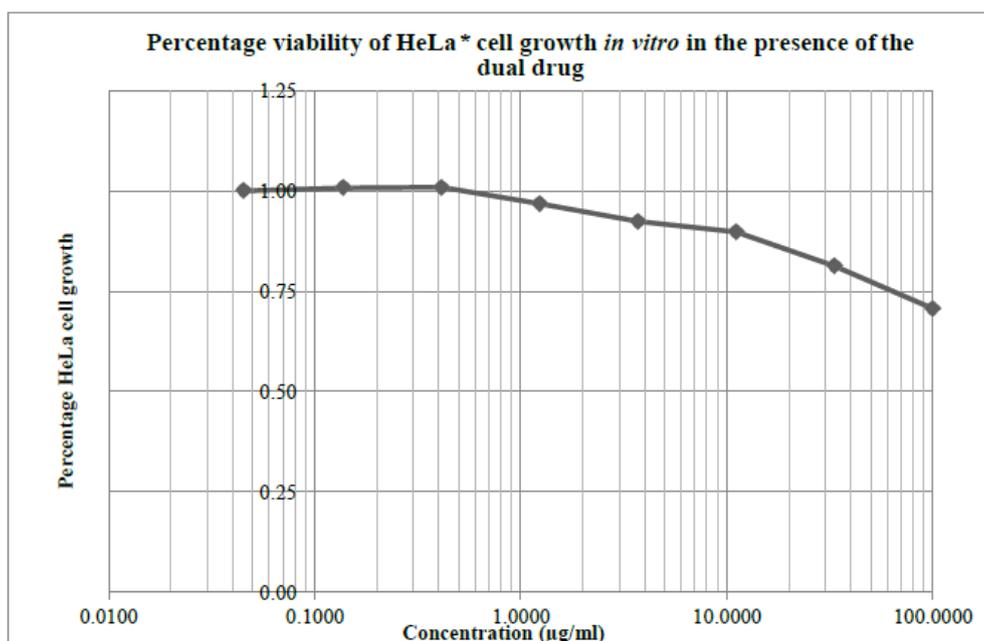
**Table 3: *In vitro* percentage inhibition of parasite growth by the hybrid drug and precursors (artesunate and 4,7-dichloroquinoline) in combination**

Hybrid drug concentration (ng mL <sup>-1</sup> )	Percentage inhibition	Artesunate (ng mL <sup>-1</sup> ) + 4,7-dichloroquinoline (ng mL <sup>-1</sup> )	Percentage inhibition
100	100.0	50 AS <sup>a</sup> + 50 DQ <sup>b</sup>	99.0
50	100.0	25 AS + 25 DQ	99.0
25	99.0	12.5 AS + 12.5 DQ	98.0
12.5	98.0	6.25 AS + 6.25 DQ	73.0
6.25	86.0	3.125 AS + 3.125 DQ	NI <sup>c</sup>
3.125	42.0	1.5625 AS + 1.5625 DQ	NI
1.5625	6.6	0.78125 AS + 0.78125 DQ	NI
0.78125	NI	0.3906 AS + 0.3906 DQ	NI

<sup>a</sup>AS, artesunate; <sup>b</sup>DQ, 4,7-dichloroquinoline; <sup>c</sup>NI, no inhibition

Cytotoxicity studies showed a good safety profile for the drug since at 10 000 ng mL<sup>-1</sup> (the highest concentration used), the drug showed low percentage inhibition of

human HeLa cell proliferation (29%), and IC<sub>50</sub> values >10 000 ng mL<sup>-1</sup> (Figure 1 and Table 4).



\*Human cervical adenocarcinoma (HeLa) cells that were previously misidentified as human laryngeal carcinoma (Hep2) cells<sup>[26,27]</sup> were used for this study.

**Figure 1: Percentage HeLa cells viability grown in the presence of hybrid drug *in vitro* as determined by MTT assay.**

Even at 10 000 ng ml<sup>-1</sup>, the drug showed low inhibition of cell proliferation (29%), and IC<sub>50</sub> values >10 000 ng ml<sup>-1</sup>, the highest concentration used. These values were comparable to that of artesunate (percentage inhibition,

18%) and chloroquine (43%) (p>0.05) at the same concentrations, whose IC<sub>50</sub> values were also >10, 000 ng ml<sup>-1</sup>.

**Table 4: Percentage inhibition of HeLa cell line by the dual drug, artesunate, 4,7- dichloroquinoline and chloroquine.**

Drug concentration (µg ml <sup>-1</sup> )	Percentage (%) inhibition			
	Dual drug	Artesunate	4,7-dichloroquinoline	Chloroquine
100.00*	29	18	37	43
33.33	19	10	19	33
11.11	10	9	08	17
3.70	08	6	05	15
1.23	07	NI	NI	09
0.41	03	NI	NI	07
0.14	NI	NI	NI	08

\*Even at the highest concentration used (100 µg ml<sup>-1</sup>, all the drugs had low percentage inhibition ranging between 18-43%

Like the reference drugs, the IC<sub>50</sub> value of the dual drug was above 10 000 ng ml<sup>-1</sup>, the highest concentrations used (Table 5).

**Table 5: IC<sub>50</sub> values for dual drug, artesunate, 4,7-dichloroquinoline and chloroquine (CQ) against HeLa cell line, CQR\* parasites (W2) and the selectivity index (SI).**

Drug	HeLa cells	IC <sub>50</sub> (ng ml <sup>-1</sup> )	
		CQR* parasite (W2)	Selective Index (SI)
Dual drug	>10,000	3.62	>2762
Artesunate	>10,000	4.04	>2445
4,7-dichloroquinoline	>10,000	>1000	NA
Chloroquine	>10,000	65.35	>153

\*CQR, chloroquine resistant *P. falciparum* isolate (W2)

In this study, quinoline pharmacophore, 4,7-dichloroquinoline was covalently linked to artesunate via a diaminoalkyl linker. Variations in the length of the linker between the trioxane and the 4- aminoquinoline moiety have been shown to affect the activity of the hybrid molecule with lower activity if the alkyl chain is longer than two carbon atom units<sup>[6]</sup>, which informed the choice of a two carbon unit linker in the study. The pharmacophore (1,2,4-trioxane group) which confers artemisinins with their unique activity, has not been synthesized to date, therefore artesunate provided the trioxane moiety which was successfully conjugated to the quinoline in a 2-step process. The structure was elucidated through spectroscopic methods and molecular formula ( $C_{30}H_{38}N_3ClO_7$ ) confirmed by electron spray ionization mass spectrum that showed a molecular ion peaks at  $m/z$  588.24(M)<sup>+</sup> and 590.24 amu (M+2H)<sup>+</sup> with a relative abundance of 100% and 38.8% respectively against an exact value of 588.24 amu. The ease of synthesis of the dual drug should also be considered an additional advantage since it would make it affordable and without perturbations to drug supply, if the drug is shown to be efficacious and safe in clinical studies.

The hybrid drug was subjected to *in vitro* studies to assess its activity against CQ-sensitive (CQS, D6) and CQ-resistant (CQR, W2) *P. falciparum* strains. It was established that the drug was active *in vitro* independently of the susceptibility of the *P. falciparum* strains to CQ. The drug's activity ( $IC_{50}$  of 6.89 ng/ml) against CQS (D6) was comparable to that of artesunate ( $IC_{50}$  of 6.67 ng/ml), ( $p > 0.05$ ) which is the most potent drug in the artemisinin family available. The hybrid drug is significantly ( $p < 0.05$ ) more potent on CQR parasites compared with CQS parasites ( $IC_{50}$ , 3.62 ng/ml vs 6.89 ng/ml) and about 18 times more active than CQ against CQR parasites (Table 2). This finding is very significant in that new drugs are urgently needed in areas where malaria is endemic and where CQ resistance is widespread.<sup>[9,29]</sup> However there is no significant difference between the activity of the hybrid drug and artesunate on CQR parasites ( $p = 0.27$ ) with an  $IC_{50}$  3.6 ng/ml vs 4.04 ng/ml respectively, while the activity of 4,7-dichloroquinoline was  $> 1000$  ng/ml for both isolates. The fact that the drug was active *in vitro* may suggest that it is a conjugate hybrid (i.e., have a distinct linker that is not found in either of the individual drugs but separates the molecular frameworks which contain the pharmacophores) with a metabolically stable linker. This is significant in that with respect to overcoming resistance, it is desirable for a hybrid drug to resist metabolic cleavage.<sup>[18]</sup>

To validate the concept of dual molecules, the individual precursors (artesunate and 4,7- dichloroquinoline) of the hybrid drug were tested in combination in the same wells. The percentage inhibition of parasite growth by the hybrid drug and precursors (Table 3) demonstrated that the hybrid drug is more potent than a combination of its precursors at comparable concentrations. This is evident

at lower concentrations of the dual drug (6.25 ng ml<sup>-1</sup>, % suppression 86%; 3.125 ng ml<sup>-1</sup>, % suppression 42%; 1.5625 ng ml<sup>-1</sup>, % suppression 6.6%), where it exhibited significant activity ( $p < 0.05$ ) while the combination of its precursors had no activity at all, suggesting that the linker between the two molecules improves the dual drug's antiplasmodial activity, findings that are consistent with those of Benoit-Vical and colleagues.<sup>[15]</sup>

Benoit-Vical *et al.*<sup>[15]</sup> developed several second generation trioxaquinines which revealed *in vitro* antiplasmodial activities ( $IC_{50}$  values) ranging from 4–32 nM, activities that were independent of the CQ sensitivities of the *P. falciparum* isolates tested. These activities were comparable to those of artesunate, and to test the concept of dual activity of the molecules, the quinoline and trioxane precursors were tested individually and in combination, and their activities compared with that of the conjugates.<sup>[15]</sup> Irrespective of the *P. falciparum* strain used, the trioxane entity alone had  $IC_{50}$  values ranging from 200–600 nM, while the  $IC_{50}$  values of the quinoline motif alone ranged from 120 nM to 2  $\mu$ M. In combination in the same well however, both entities had  $IC_{50}$  values ranging from 40-180 nM. The fact that the trioxaquinines  $IC_{50}$  values were low (4-32 nM) implies that the link between both pharmacophores of the trioxaquinines is essential for their activity.<sup>[15]</sup>

In another study, it has been demonstrated that a hybrid, artemisinin covalently linked to quinine via an ester linkage had superior activity to that of artemisinin alone, quinine alone, or a 1:1 mixture of artemisinin and quinine<sup>[30]</sup> results that are consistent with our findings, further confirming that the linker between both pharmacophores of the trioxaquinines is essential for their activity. In a similar study consistent with our work, N'DA and Breytenbach<sup>[14]</sup> synthesized artemisinin-quinoline hybrids and tested them *in vitro* against CQS (D10) and CQR (Dd2) *P. falciparum* strains. Their  $IC_{50}$ s range (0.021-0.034 nM) was comparable to that of CQ (0.035 nM) against D10 strain (CQS) but more active than CQ against the CQR strain (Dd2) with values of 0.009-0.184 nM and 0.255 nM respectively. However all the synthesized molecules were less active than dihydroartemisinin which was the reference drug and had formed the trioxane moiety in the hybrid drug.<sup>[14]</sup>

The Dual drug showed a good safety profile which is significant since CQ and artesunate (its precursors) are conventional drugs indicated for treatment of human malaria, and are reputed for their good safety profile. The selectivity index of the dual drug was determined by finding the ratio between the  $IC_{50}$ s of HeLa cell line and that of the parasite (W2). The high value obtained ( $> 2762$ ) indicates that the high antiplasmodial activity was due to the dual drug's activity and not due to cytotoxicity.

#### 4. CONCLUSIONS

In conclusion, there is a challenge and pressing urgency to synthesize potent and cost-effective chemotherapeutic agents for treatment of malaria after widespread development of resistance to CQ and ominous signs of losing the artemisinins to resistance too. Recent reports have confirmed that resistance to the world's most effective antimalarial drugs, artemisinin derivatives, is now established in western Cambodia, Thailand, Vietnam, eastern Myanmar and northern Cambodia<sup>[31]</sup> If these parasites are not contained, it is possible that resistance will spread into malaria endemic regions of Africa and could derail the global drive to control and eventually eliminate malaria altogether. Thus the urgency for search for novel antimalarial drugs and targets can never be gainsaid. We have successfully synthesized a quinoline-artesunate hybrid drug through a quick two-step procedure. The *in vitro* antiplasmodial activities indicate that the molecule was active against both CQS and CQR strains of *P. falciparum*. Although the drug did not show remarkably better activity than artesunate, it should be noted that the objective of combining drugs is not premised on enhanced activity alone, but also delaying of development of resistance due to individual drugs having different molecular targets, a trait that may be possessed by the hybrid drug. The safety of the drug as determined by cell cytotoxicity indicates that the drug is safe. This is not surprising since both artesunate and quinoline moieties (the precursors of the hybrid drug) are components of antimalarial drugs indicated for human use. The high *in vitro* antiplasmodial activity of the hybrid drug against sensitive and resistant parasites strains, together with a dual mode of action (artemisinin-like and CQ-like), and low toxicity makes this molecule a promising candidate for covalent bitherapy strategy. The data reported here indicate that quinoline-artesunate hybrid drug is a potent antiplasmodial drug, and if the findings can be replicated in clinical studies, then the drug should be considered as model for new candidates in the arsenal of drugs for fight against drug-resistant malaria. Thus the safety aspect coupled with the drugs ease of synthesis gives a higher degree of confidence that preclinical development of the drug is feasible.

#### Declarations

#### Competing Interests

The authors' declare that they have no competing interests.

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