ARTICLE IV

Antimalarial and Anticancer Activities of Artemisinin-Quinoline Hybrid-Dimers and Pharmacokinetic Properties in Mice

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Antimalarial and anticancer activities of artemisinin–quinoline hybrid-dimers and pharmacokinetic properties in mice

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ABSTRACT

Malaria, one of the three most important life-threatening infectious diseases, is recommended to be treated with ACT (artemisinin combination therapy) against which *Plasmodium falciparum* already displayed resistance. Two artemisinin-4-amino-quinoline hybrid-dimers (**1** and **2**), previously synthesized, possessed low nanomolar *in vitro* antiplasmodial activity, while poorly toxic against mammalian cells. They are here investigated to ascertain whether this antimalarial activity would be carried on *in vivo* against *Plasmodium vinckei*. During the four day treatment, parasitemia of less than 1% were observed on day 5 after doses from 2.5 mg/kg ip and 50 mg/kg po for hybrid-dimer **1**, and from 7.5 mg/kg ip and 25 mg/kg po for hybrid-dimer **2**. Snapshot pharmacokinetic analysis demonstrated that the antiplasmodial activity of these C-10-acetal artemisinin dimers may be due to active metabolites, which were confirmed by *in silico* findings. Hybrid-dimer **1** also displayed potent *in vitro* activity against tumor cells and was found to be more active than etoposide against TK10, UACC62 and MCF7 cell lines (TGI values 3.45 vs. 43.33 μM, 2.21 vs. 45.52 μM and 2.99 vs. >100 μM, respectively). The 1,3-diaminopropane linker, present in hybrid-dimer **1**, was therefore identified as the optimum linker.

1. Introduction

Plasmodium falciparum (P. falciparum), the most virulent of the human malaria parasites causes a major global health problem. Malaria was the underlying cause of death for 1.24 million individuals in 2010, whereof 57% is children below the age of 5 (Murray et al., 2012). The widespread resistance of *P. falciparum* to most antimalarial drugs is a major obstacle in the elimination of the disease. Consequently, there is an urgent need for new drugs.

The discovery and development of artemisinin-based antimalarials have provided a new class of highly effective antimalarials which have become the most important class of drugs now used as artemisinin-based combination therapy (ACT) to overcome the chemoresistance problem. The recently reported potential emergence of resistance to artemisinin (Carrara et al., 2009; Dondorp et al., 2010) is a major threat, thus highlighting the need for new chemotherapeutic approaches to treat *P. falciparum* infections. The major drawback of artemisinin despite its rapid antimalarial action is its very short half-life which is due to the drug's capacity for auto-induction of its metabolism (Ashton et al., 1998; Asimus and Gordi, 2007). Artemisinin has also been shown to influence cytochrome P450 (CYP) mediated metabolism of other drugs, increasing the risk of drug–drug interactions when ACTs are used (Asimus et al., 2007).

When artemisinin is used in a chemical combination with a longer half-life quinoline antimalarial, the risk of treatment failure is reduced and partner drugs are substantially protected against appearance of resistance. Aminoquinoline is the pharmacophore of all classic quinoline antimalarials e.g. mefloquine, amodiaquine, primaquine, etc. which are currently used as part of ACTs. Long-acting quinoline antimalarials possess half-lives of 10 h to 10 days (White, 2004). Therefore by chemically combining artemisinin to aminoquinoline, dual pharmacological action could result in synergism, which in turn would allow lower doses and a potentially wider safety margin. Drug–drug interactions, which are becoming an increasing concern when using ACT in malaria treatment, would then be avoided when adopting hybrid chemotherapy.

The observation that artemisinin dimers possess significant antiplasmodial and anticancer activity prompted various previous efforts in drug discovery (Chadwick et al., 2009; Galal et al., 2009; Posner et al., 1997; Rosenthal et al., 2009; Slade et al., 2009). Dimers were found to be more than 1000-fold more active than

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their monomeric counterparts, and their anticancer activity has been associated with heme catalysed reactive oxygen species (ROSs) and endoplasmic reticulum (ER) stress induction (Stockwin et al., 2009). During the synthetic procedure of the artemisinin–quinoline hybrids another artemisinin moiety coupled to the hybrid complex, forming a dimer. A series of artemisinin–quinoline hybrid-dimers, which contained different linkers, was then synthesized by our research group (Lombard et al., 2010). The nature of the linker plays a crucial role in imparting a hybrid with potent activity (Jeyadevan et al., 2004). Very distinct linkers were therefore used in the formation of the dimers. Hybrid-dimer **1** contained an aliphatic linker (diaminopropane), whereas hybrid-dimer **2** featured a cyclic linker (amino ethyl piperazine) (Fig. 1). These artemisinin C-10 acetal dimers contained both ether and amine bonds, and were isolated in oxalate salt form for stability reasons. These two dimers displayed potent low nanomolar antimalarial activity against the D10 and Dd2 strains of *P. falciparum*, with an *in vitro* selectivity index (SI) > 100. The activity of these two dimers was in the order of that of dihydroartemisinin (DHA) (Lombard et al., 2010). Hybrid-dimers **1** and **2** were subsequently selected for further investigation. At first their *in vitro* antimalarial activity was determined against the 3D7 strain, where after their *in vivo* antimalarial activity against *Plasmodium vinckei* was determined alongside artesunate. In order to assess their dual activity, the antiproliferative ability of these hybrid-dimers was determined against three different cell lines: renal (TK10), melanoma (UACC62) and breast (MCF7) cancer cells in comparison to that of the anticancer agent, etoposide. A snapshot pharmacokinetic (PK) study was also performed on these two hybrid-dimers to get an indication of their PK profiles.

2. Materials and methods

2.1. In vitro antimalarial activity against 3D7 strain

The 3D7 strain of *P. falciparum* was asexually cultured in human blood in complete medium (RPMI 1640 supplemented with 25 mM Hepes, pH 7.4) and 10% AB^+ human serum (Trager and Jensen, 1976). Drug effects were measured in microtiter plates on suspensions of asynchronous *P. falciparum* infected red blood cells (1.5% final haematocrit, 0.6% parasitemia) according to Desjardins et al. (1979). Drugs, previously dissolved in DMSO, were diluted in culture medium so that the final DMSO concentration never exceeded 0.25%. After 48 h incubation at 37 °C parasite growth was assayed by the incorporation of [^{3}H]-hypoxanthine (0.5 μCi/well, 22.2 kBq) in parasitic nucleic acids for 18 h. Analyses of dose-effect curves were performed with the Graphpad Prism analytical software. The results are expressed as IC_{50}, corresponding to the drug concentration leading to 50% parasite growth inhibition. Values are the means of at least two independent experiments (different cell cultures, different drug dilution stocks), each performed in duplicates.

2.2. In vivo antimalarial activity against P. vinckei

In each experiment, female Swiss OF1 mice (Charles River Laboratories, France) were infected on day 0 (D0) by intravenous injection into the caudal vein of 10^7 *Plasmodium vinckei* infected erythrocytes (BY strain) in 200 μl 0.9% NaCl. These injections lead to a parasitemia on day 1 (D1) of between 0.3% and 1.5%. Mice were treated once a day for four days on D1, D2, D3 and D4 intraperitoneally (ip) with 0.8 mg/kg, 2.5 mg/kg, 7.5 mg/kg or 15 mg/kg of the compound, or orally (per os, po) with 2.7 mg/kg, 8.3 mg/kg, 25 mg/kg or 50 mg/kg. Hybrid-dimers **1** and **2** were dissolved in DMSO and were administered in volumes of 100 μl. Each group consists of three mice and the control group received only the vehicle (DMSO). On D5, the ED_{50} (efficient dose) was determined using Giemsa-stained thin blood smears and flow cytometry (Yoyo-1 iodide (491/509) – Invitrogen) (Barkan et al., 2000). The survival of the mice was monitored for up to one month after the end of the treatment.

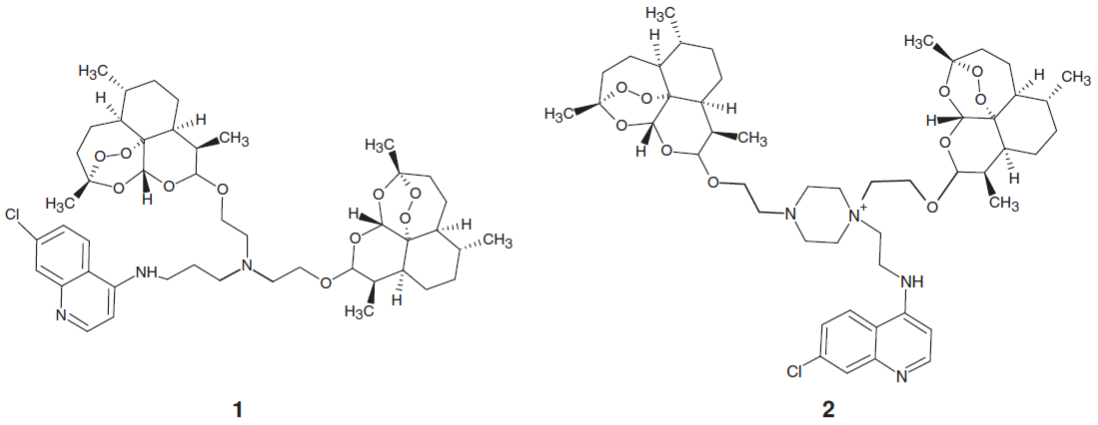
2.3. Anticancer activity

2.3.1. Cell lines

The following three human cancer cell lines were used: renal adenocarcinoma (TK-10), breast adenocarcinoma (MCF-7) and melanoma (UACC-62). All cell lines were obtained from National Cancer Institute (NCI) and routinely maintained as a monolayer cell culture at 37 °C, 5% CO_2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50 μg/ml gentamicin.

2.3.2. Method

The growth inhibitory effects of the compounds were tested in the three-cell line panel by Sulforhodamine B (SRB) assay. The SRB assay was developed by Skehan et al. (1990) to measure drug-induced cytotoxicity and cell proliferation. The primary anticancer



1 **2**

Fig. 1. Structures of artemisinin–quinoline hybrid-dimers 1 and 2.

assay was performed in accordance with the protocol of the Drug Evaluation Branch of NCI (Kuo et al., 1993; Leteurtre et al., 1994; Monks et al., 1991).

For the screening experiment, the cells (3–19 passages) were inoculated in 96-well microtiter plates at plating densities of 7–10,000 cells/well and were incubated for 24 h. After 24 h one plate was fixed with 50% trichloroacetic acid (TCA) to represent a measurement of the cell population for each cell line at the time of drug addition (T0). The other plates were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce five concentrations in the range of 0.01–100 μM. Cells without drug addition served as control. The blank contained complete medium without cells. Etoposide, a clinically active agent, was used as a positive control. The assay prerequisite for the *Z*-factor (Zhang et al., 1999), which is the quality of a screening assay and measure of statistical effect size, was >0.5.

The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% TCA, washed, dried and dyed by SRB (0.4% w/v in 1% acetic acid). Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at the wavelength of 540 nm using a multiwell spectrophotometer. Optical density measurements were used to calculate the net percentage cell growth.

The optical density of the test well after 48-h period of exposure to test drug is *Ti*, the optical density at time zero is *T0*, and the control optical density is *C*. Percentage cell growth was calculated as:

$$[(Ti - T0)/(C - T0)] \times 100 \text{ for } Ti \geqslant T0 \text{ and } [(Ti - T0)/T0] \times 100 \text{ for } Ti < T0.$$

For each tested compound, four response parameters, GI50 (50% growth inhibition), total growth inhibition (TGI), LC50 (50% lethal concentration) and LC100 (100% lethal concentration), were calculated for each cell line.

2.4. Pharmacokinetics

The PK properties of hybrid-dimers **1** and **2** were evaluated in a mouse model. The PK evaluation study was approved by the Ethics Committee of the University of Cape Town, approval number 009/034.

2.4.1. Mouse strain, formulation and mice study protocol

The animals used were male C57/BL6 mice, weighing approximately 25 g each. Hybrid-dimers **1** and **2**, dissolved separately in DMSO and water, were added (1:9, v/v). The concentration of the test compound formulations was prepared at 20 mg/kg for the oral dose, and at 2 mg/kg for the intravenous (IV) experiments.

The test compounds were administered *via* oral and IV routes. Test animals were randomly divided into four groups. Each group consisted of three mice. Group A received hybrid-dimer **1** at an oral gavage dose concentration of 20 mg/kg. Group B received hybrid-dimer **1** IV at a concentration of 2 mg/kg. Group C received hybrid-dimer **2** at an oral gavage dose concentration of 20 mg/kg. Group D received hybrid-dimer 2 IV at concentration of 2 mg/kg. The animals were anesthetized for the IV dorsal penile vein bolus injections.

Blood samples (40 μl) were collected before, and at 10, 20, 30, 40 and 50 min after oral gavage dosing (Groups A and C), and blood samples (40 μl) were collected before, and at 5, 15, 25, 35 and 50 min after IV dosing (Groups B and D). The blood samples were collected on ice into 0.8 ml lithium heparin gel tubes. The samples were centrifuged at 1500 G for 10 min, and the plasma layer was transferred to 1.5 ml microcentrifuge tubes and stored at −80 °C until analysis.

2.4.2. PK sample analysis

An LC/MS/MS system (Shimadzu HPLC and an AB Sciex API 3200 Q-Trap mass spectrometer) was used to analyse the plasma samples. A sensitive and selective assay was developed to determine plasma concentrations of hybrid-dimers **1** and **2**.

2.4.3. LC/MS/MS summary

Twenty microliters of plasma was precipitated with a 100 μl of acetonitrile. The samples were vortexed for one minute, sonicated for 5 min and centrifuged at 13,000 G for 5 min. The supernatant was transferred to a 96 well plate, and 10 μl was injected onto the HPLC column.

Gradient chromatography was performed on a Phenomenex, Gemini-NX (5 μl, C18, 110A, 50 × 2 mm) analytical column using a Shimadzu HPLC. The mobile phase A consisted of acetonitrile and mobile phase B consisted of a mixture of 4 mM ammonium acetate and 0.1% formic acid (1:1, v/v). The organic solvent was increased from 5% to 95% over 4 min, with an equilibration time of 3 min between 4 and 7 min. The flow-rate was set at 0.5 ml/min and 10 μl was injected onto the analytical column. The samples were cooled to 5 °C whilst awaiting injection.

Detection of the two hybrid-dimers was performed on an AB Sciex API 3200 Q-Trap mass spectrometer (ESI in the positive ion mode, MRM). The mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 856.4 to the product ions at m/z 191.2 for hybrid-dimer **1** and monitoring the transition of the protonated molecular ions at m/z 911.4 to the product ions at m/z 274.2 for hybrid-dimer **2**.

Calibration standards (8 levels) were prepared in mouse plasma. The concentration range was between 9.8 to a 1000 ng/ml for both hybrid-dimers. The calibration standards were analysed in duplicate with the study samples. The accuracy and precision statistics of the calibration standards of hybrid-dimer **1** were between 92.0–113%, and 3.6–7.7%, respectively, and the accuracy and precision statistics of the calibration standards of hybrid-dimer **2** were between 95.9–110.6%, and 1.8–13.9%, respectively.

2.4.4. Pharmacokinetic parameters and statistical evaluation

Non compartmental analysis was used to calculate the PK parameters for the two hybrid-dimers (Summit PK software, version 2.0). The following PK parameters were calculated using non-compartmental analysis: Maximum plasma concentration (C_{max} [ng/ml]) and corresponding time (T_{max} [min]), apparent terminal half-life ($t½$ [min]), total plasma exposure (AUC_{0-Inf} [ng.min/ml]), volume of distribution [l/kg], plasma clearance (CL [ml/min/kg]) and percentage oral bioavailability (%BA).

3. Results

3.1. Antimalarial activity

3.1.1. In vitro antimalarial activity of hybrid-dimers 1 and 2

In vitro antimalarial activity was determined against the 3D7 strain after one blood cycle (48 h) contact with *P. falciparum* according to the procedures described by Desjardins et al. (1979). Both compounds, hybrid-dimers **1** and **2**, exhibited potent *in vitro* activity showing half-maximal inhibition concentration (IC_{50}) of 8.7 nM and 29.5 nM, respectively (Table 1). DHA and chloroquine (CQ) were used as standards. The IC_{50} values of the antimalarial activity of dimers **1** and **2** against D10 and Dd2 strains of *P. falciparum*, previously determined (Lombard et al., 2010), are also reported in Table 1.

Table 1
In vitro IC_{50} of hybrid-dimers 1 and 2 against sensitive 3D7, D10 and resistant Dd2 strains.

Compound	IC_{50} (nM) ± SD		
	3D7 ($N = 3$)	D10 ($N = 3$)	Dd2 ($N = 3$)
1	8.7 ± 2.3	5.31 ± 0.67	28.43 ± 1.17
2	29.5 ± 8.5	19.62 ± 1.76	55.68 ± 15.20
DHA	ND	5.11 ± 0.64	2.09 ± 0.33
CQ	20 ± 1.6	21.54 ± 6.73	157.90 ± 52.70

ND = not determined.

3.1.2. In vivo ED_{50} of hybrid-dimer 1 after four injections by intraperitoneal (ip) and oral (po) routes

Compounds were evaluated for their *in vivo* antimalarial activity in *P. vinckei* infected mice (Fig. 2). After ip administration, hybrid-dimer **1** exerted a strong antimalarial effect from 2.5 mg/kg ip, eliminating more than 99% of the parasites ($P \approx 0.03\%$ vs. 86% for the control group on D5). Treatment at 2.5 and 7.5 mg/kg nearly induce clearance of blood parasitemia, but recrudescence led to mice death between D7 and D18.

When the hybrid-dimer **1** was administered to mice orally, it already exhibited a substantial antimalarial activity at 25 mg/kg, eliminating 60% of the parasites ($P = 36.8\%$ vs. 93% for the control group on D5). Treatment at 50 mg/kg demonstrated a potent *in vivo* antimalarial activity and eliminated more than 99% of the parasites when parasitemia decreased to 0.01% of the control value ($P \approx 0.01\%$ vs. 93% for the control group on D5). Similarly to the ip route, recrudescence was observed leading to mice death at D14.

After four days of treatment the ED_{50} values of hybrid-dimer **1** were 1.4 mg/kg and 20 mg/kg for the ip and po route respectively (Fig. 2A). The oral absorption index, defined by the ED_{50} ip/ED_{50} po ratio, is 7% in DMSO.

3.1.3. In vivo ED_{50} of hybrid-dimer 2 after four injections by intraperitoneal and oral routes

Treatment with hybrid-dimer **2** administered at 7.5 mg/kg ip revealed a potent *in vivo* antimalarial activity, eliminating more than 99% of the parasites when parasitemia decreased to 0.02% of the control value ($P = 0.02\%$ vs. 86% for the control group on D5). Although 33% mouse survival was obtained, mice death due to recrudescence was variable. At 15 mg/kg ip, potent *in vivo* antimalarial activity of hybrid-dimer **2** was observed. On D5, parasitemia was 0.05% of the control (parasitemia = 0.04% vs. 86% for the control group). Surprisingly, despite this low parasitemia, all three mice died between D7 and D9 probably due to semi-chronic toxicity.

A four day oral treatment with hybrid-dimer **2** at 25 mg/kg po displayed a strong antiplasmodial effect, decreasing parasitemia to 0.06% of the control group on D5 ($P = 0.05\%$ vs. 93% for the control group). As observed by the ip route, **2** of the three mice died on D8 resulting in 33% mouse survival. Hybrid-dimer **2** exerted potent *in vivo* antimalarial activity at 50 mg/kg po. This treatment decreased parasitemia to 0.02% of the control on D5 ($P = 0.02\%$ vs. 93% for the control group). However, recrudescence was observed leading to mice death at D14/D15.

The ED_{50} values of compound **2**, after a four day treatment, were 2.1 mg/kg and 13 mg/kg for the ip and po route, respectively (Fig. 2B). The oral absorption index, defined by the ED_{50}ip/ED_{50}po ratio is 16% in DMSO.

3.1.4. In vivo ED_{50} of artesunate by intraperitoneal and oral routes

A well-known antimalarial drug, artesunate, was used as reference drug. Results were obtained, using the same four day experimental protocol with different dosages. *P. vinckei* infected mice were treated *via* ip route at 1, 3, 10 and 30 mg/kg/day and po route at 1, 4, 20 and 80 mg/kg/day. Artesunate exerted a very significant antimalarial effect, with ED_{50} ip < 1 mg/kg and ED_{50} po = 1.8 mg/kg (Fig. 2C).

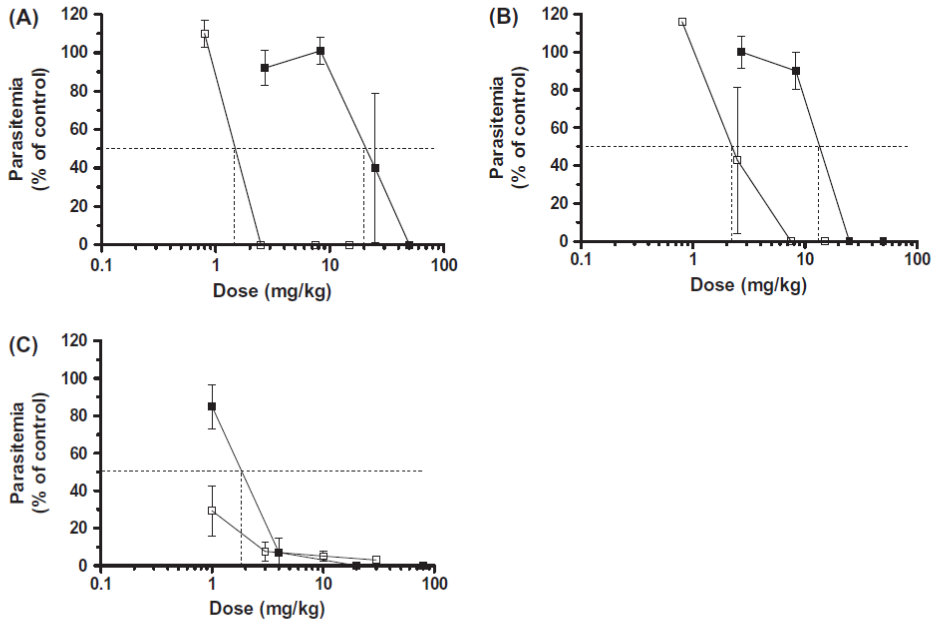


Fig. 2. In vivo antimalarial properties of hybrid-dimer 1 (A), hybrid-dimer 2 (B) and artesunate (C) in *P. vinckei*-infected mice. Treatment consisted in one daily ip (white squares) or per os (black squares) injection for four consecutive days. Parasitemia was monitored at D5 ($N = 3$).

At D5 after ip treatment, artesunate reduced mice parasitemia by 70% at 1 mg/kg. Higher doses were not able to clear parasitemia and 3% remained after treatment with 30 mg/kg ip. None of the tested doses allowed a complete cure and only 50% of survival was observed at 30 mg/kg ip. Similar profile was observed after oral administration, with an ED_{50} of 1.8 mg/kg and a decrease of parasitemia by 93% at 4 mg/kg.

3.2. Anticancer activity

The anticancer activity of hybrid-dimers **1** and **2** was determined against renal (TK10), melanoma (UACC62) and breast (MCF7) cancer cell lines in 5 × 10-fold serial dilutions. Etoposide, a well-known anticancer agent, was used as a reference standard. The ability of each compound to suppress cell growth is presented Fig. 3.

For each tested compound, four response parameters were determined for each cell line: GI_{50} (50% growth inhibition), TGI (drug concentration resulting in total growth inhibition), LC_{50} (50% lethal concentration) and LC_{100} (100% lethal concentration). Results are displayed in Table 2.

3.3. Pharmacokinetics

The PK properties of hybrid-dimers **1** and **2** were determined by performing a snapshot PK study. Three mice were used for each experiment, each receiving 20 mg/kg orally or 2 mg/kg intravenously. The plasma concentration–time profiles and PK parameters were determined.

3.3.1. Plasma concentrations

The plasma concentration profiles for hybrid-dimers **1** and **2** after oral and IV administration are displayed in Fig. 4.

3.3.2. Pharmacokinetic data analysis

The PK parameters of hybrid-dimers **1** and **2** are presented in Table 3.

4. Discussion

4.1. Antimalarial activity

The *in vitro* IC_{50} for hybrid-dimers **1** and **2** against the 3D7 strain were 8.7 and 29.5 nM respectively, compared to chloroquine's activity of 20 nM. Both hybrid-dimers exerted similar low nanomolar *in vitro* antimalarial activity against the 3D7 strain, compared to previously tested D10 and Dd2 strains. The corresponding activity confirmed that the compounds were stable and also highlighted the robustness of the compounds. Although, hybrid-dimer **1** had superior activity against all 3 strains, both compounds' activity was in the same range than that of DHA.

The treatment of mice infected by *P. vinckei* with hybrid-dimers **1** and **2** reveal a strong *in vivo* antimalarial effect. At very low doses both compounds were able to decrease parasitemia to extremely low levels. During the four day treatment, parasitemia of less than 1% were observed on day 5 after doses from 2.5 mg/kg ip and 50 mg/kg po for hybrid-dimer **1,** and from 7.5 mg/kg ip and 25 mg/kg po for hybrid-dimer **2**, whereas artesunate was only able to decrease parasitemia to 3% at 30 mg/kg ip, resulting in 50% survival and obtained complete cure at only 80 mg/kg po. Recrudescence was similarly observed with the treatment of hybrid-dimers **1** and **2** between days 7 and 18, as was usually observed with artemisinin derivatives (=see below).

The C-10 acetal functionality in the synthesized hybrid-dimers, implicated metabolic instability (Posner et al., 2002). It was therefore expected that the two artemisinin moieties would be released quickly after administration, and because artemisinin are very susceptible to auto-induction of its metabolism (Asimus and Gordi, 2007), parasites' recrudescence was expected. The results confirm this expectation. Semi-chronic toxicity was only observed with hybrid-dimer **2** at 15 mg/kg ip.

Although slightly higher, the ip ED_{50} values of dimers **1** and **2** (1.4 and 2.1 mg/kg, respectively) were in the same range than that of artesunate (<1 mg/kg). The oral ED_{50} of dimers **1** and **2** were significantly higher than that of artesunate (20, 13 and 1.8 mg/kg for dimers **1**, **2** and artesunate, respectively). These dimers have very high molecular masses, resulting in lower oral bio availabilities, explaining the significant difference between the oral and ip ED_{50} values. This was confirmed during the snapshot PK study.

Lower doses were required in the *in vivo* antimalarial treatment with hybrid-dimer **1,** with no observed toxicity. The 3C-aliphatic linkage therefore appears to be more active, although the C-10 acetal functionality needs to be replaced by a C-10 non-acetal functionality, in order to improve the biological stability and oral bioavailability.

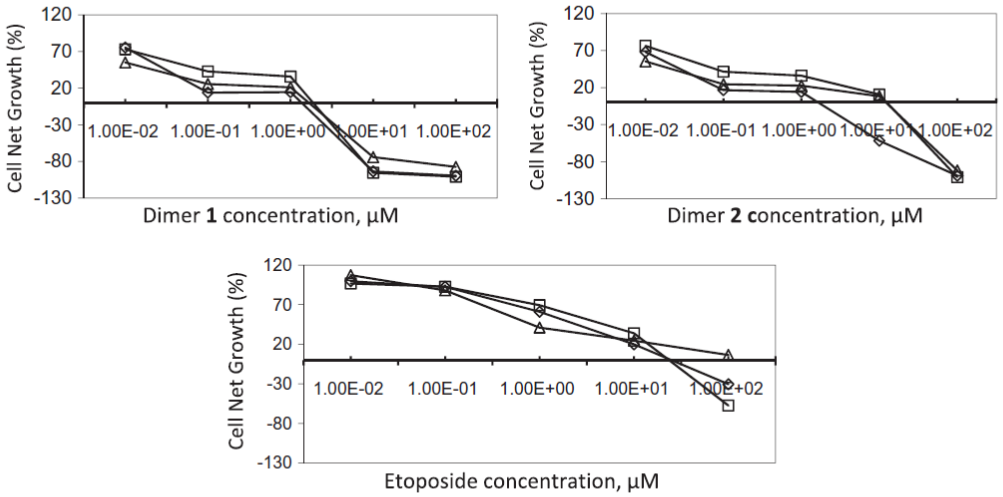


Fig. 3. The anticancer activity of hybrid-dimers 1 and 2 and etoposide, the reference standard. Cancer cell lines: TK10 (□), UACC62 (◇) and MCF7 (Δ) cell lines. Results are the mean ± SD.

Table 2
Anticancer response parameters for dimers 1 and 2 and etoposide.

Compound	Activities (μM)	TK10	UACC62	MCF7
1	GI_{50}	0.08	0.05	0.03
	TGI	3.45	2.21	2.99
	LC_{50}	6.87	6.36	7.71
	LC_{100}	83.66	>100	>100
2	GI_{50}	0.08	0.04	0.03
	TGI	18.50	2.92	17.43
	LC_{50}	58.83	9.74	62.30
	LC_{100}	99.17	>100	>100
Etoposide	GI_{50}	5.89	3.41	0.83
	TGI	43.33	45.52	>100
	LC_{50}	92.61	>100	>100
	LC_{100}	>100	>100	>100

4.2. Anticancer activity

Results of the five dose cancer screening were reported as TGI (total growth inhibition). TGI is the concentration of test drug where $100 \times (T - T0)/(C - T0) = 0$ and signifies a cytostatic effect. The biological activities were separated into 4 categories: inactive (TGI > 100 μM), weak activity (30 μM < TGI < 100 μM), moderate activity (10 μM < TGI < 30 μM and potent activity (TGI < 10 μM).

According to this criterion, the cytostatic effect of hybrid-dimer **1** can be evaluated as potent. TGI was 3.45 μM for cell line TK10, 2.21 μM for cell line UACC62 and 2.99 μM for cell line MCF7. Dimer **1** was 13-fold (TGI, 3.45 vs. 43.33 μM), 20-fold (TGI, 2.21 vs. 43.52 μM) and 33-fold (TGI, 2.99 vs. > 100 μM) more potent than etoposide against cell line TK10, UACC62 and MCF7, respectively (Table 4).

Hybrid-dimer **2**, on the other hand, was less active and its anticancer activity was classified as moderate against renal (TGI = 18.5 μM) and melanoma (TGI = 17.43 μM) cell lines. The breast (MCF7) cell line showed a higher sensitivity towards dimer **2** with a TGI of 2.92 μM, and therefore the activity of dimer **2** could be classified as potent against that cell line. Dimer **2** was 2-fold (TGI, 18.5 vs. 43.33 μM), 15-fold (TGI, 2.92 vs. 43.52 μM) and 5.7-fold (TGI, 17.43 vs. > 100 μM) more active than etoposide against TK10, UACC62 and MCF7, respectively.

The synthesized dimers displayed moderate to potent anticancer activity against the investigated cell lines and inhibited the growth of all three cell lines at very low concentrations (GI_{50} values in the 0.03–0.08 μM range). Hybrid-dimer **1** was able to inhibit all three cell line's growth at 10 μM, whereas hybrid-dimer **2** could only inhibit the UACC62 cell line at 10 μM and the other two cell line's growth at 100 μM. Very low LC_{50} and LC_{100} values were obtained for both compounds. The cytotoxicity was previously determined *in vitro* against Chinese Hamster Ovarian (CHO) cells, and resulted in IC_{50} values (±SD) of 0.68 ± 0.65 and 74.82 ± 18.06 × 10^3 nM for hybrid-dimers **1** and **2** respectively, compared to that of emetine 0.19 ± 0.05 × 10^3 nM. The SI index (128 and 3813 for hybrid-dimers **1** and **2**) confirmed the high selectivity of these compounds (Lombard et al., 2010). Therefore, both

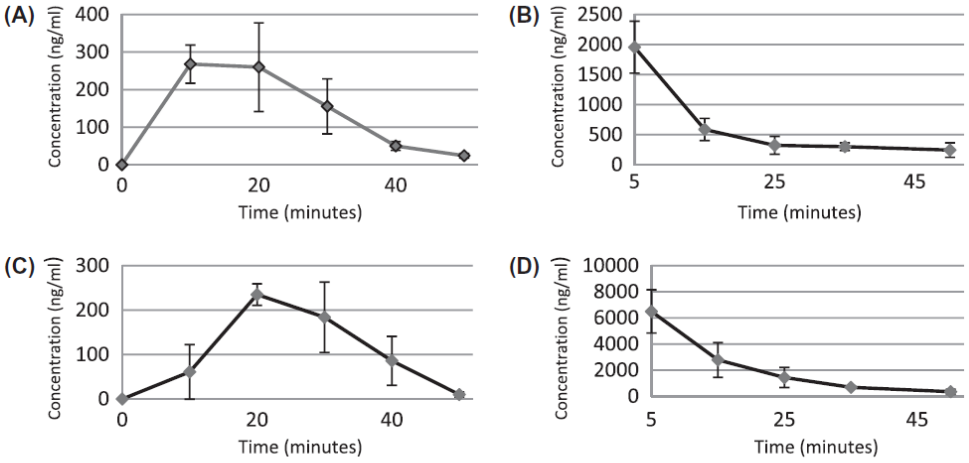


Fig. 4. Plasma concentration profiles for hybrid-dimers 1 and 2. After oral administration of 20 mg/kg the plasma concentration vs. time profiles of hybrid-dimers 1 and 2 are displayed on graphs A and C, respectively, whereas the 2 mg/kg IV data are shown on graph B for hybrid-dimer 1 and graph D for hybrid-dimer 2. Results are the mean of at least three mice per dosage ± SD.

Table 3
PK parameters of hybrid-dimers 1 and 2 after a 20 mg/kg oral and 2 mg/kg IV administration.

PK parameter	Hybrid-dimer 1		Hybrid-dimer 2	
	Mean ± SD (oral)	Mean ± SD (IV)	Mean ± SD (oral)	Mean ± SD (IV)
C_{max} (ng/ml)	279 ± 114	ND	210 ± 61.61	ND
T_{max} (min)	16.7 ± 5.77	ND	20.0 ± 10.0	ND
Apparent Terminal $t½$ (min)	8.54 ± 3.4	37.5 ± 5.8	5.33 ± 2.64	18.4 ± 17.08
AUC_{0-Inf} (ng min/ml)	7202 ± 2869	43129 ± 13041	5171 ± 2386	140991 ± 32067
V_D/F (l/kg)[a]	4.04 ± 2.9	2.63 ± 0.65	34.7 ± 20.2	0.452 ± 0.51
Plasma CL/F (ml/min/kg)[a]	3060 ± 1085	49.2 ± 14.15	4784 ± 2984	14.8 ± 3.72
%BA	1.67 ± 0.67	ND	0.37 ± 0.17	ND

[a] For the oral experiment, apparent oral CL and V_D are reported, ND = not determined.

Table 4
Comparison of TGI values for dimers 1 and 2 against etoposide.

Cell Lines	TGI (μM)			Ratio	
	Dimer 1	Dimer 2	Etoposide	Etoposide/Dimer 1	Etoposide/Dimer 2
TK10	3.45	18.5	43.33	12.6	2.3
UACC62	2.21	2.92	43.52	19.7	14.9
MCF7	2.99	17.43	>100	33.4	>5.7

hybrid-dimers are potential drug candidates to be further investigated *in vivo* in search for potent and safe anticancer drugs.

4.3. Pharmacokinetics

A snapshot PK preclinical screening approach was used to determine the main pharmacokinetic parameters for the two dimers (Liu et al., 2008).

When administered orally, a maximum concentration of 279 ng/ml and 210 ng/ml was reached within 16.7 and 20.0 min for hybrid-dimers **1** and **2,** respectively, whereas Xing et al. (2007) reported the C_{max} and T_{max} for DHA to be 142.2 ng/ml and 48 min, respectively. Although the total plasma exposure for hybrid-dimers **1** and **2** was lower than that of DHA for the oral dose (7202 and 5171 ng min/ml vs. 8748 ng min/ml, respectively) (Xing et al., 2007) when given intravenously their plasma exposure was 6 and 27 times higher, respectively. This confirms that hybrid-dimers **1** and **2** absorption *via* the GI-tract were minimal. The average bioavailability of artemisinin derivatives is between 19 and 35% (Navaratnam et al., 2000) vs. 1.67% and 0.37% for hybrid-dimers **1** and **2,** respectively.

Hybrid-dimer **1** displayed a very low oral volume of distribution (V_D) (4.04 l/kg) whereas the V_D for hybrid-dimer **2** was similar to that of DHA (34.7 vs. 36.3 l/kg) (Batty et al., 2008). Both dimers had much higher plasma clearance when compared to DHA (3.06 and 4.78 l/min/kg vs. 1.19 and 1.02 l/min/kg for DHA) (Batty et al., 2008; Xing et al., 2007). Both dimers resulted in apparent terminal half-lives significantly shorter than that of DHA, which is already rapid (8.54 and 5.33 min for dimers **1** and **2**, respectively; vs. 25 min for DHA) (Batty et al., 2008).

Artemisinin derivatives are between 43 and 81.5% protein bound (Navaratnam et al., 2000), explaining the low V_D for hybrid-dimer **1**. However, one could expect to find a greater amount of dimer **2** in extravascular tissues. The high protein binding of artemisinin and its derivatives could also be related to the biotransformation to DHA, also referred to as artenimol, as DHA is the principal metabolite (Navaratnam et al., 2000). The synthesized dimers, containing a C-10 acetal functionality, were therefore expected to be cleaved resulting in the release of DHA.

A comprehensive *in silico* study using MetaSite and VolSurf+ software was carried out for these **2** artemisinin hybrid-dimers. According to the predictions, DHA formed through O-dealkylation as the 5th and 3rd metabolite for dimer **1** and **2,** respectively. Not only does these data correlate with the experimental data, they implied that the aliphatic linker was more stable. The five metabolites that were predicted to form were either artemisinin dimers (without the quinoline moiety) or artemisinin–quinoline hybrids (without an artemisinin portion). Each metabolite still contained either one or two artemisinin functionalities. Therefore, although the compounds had been metabolized very easily, activity was expected to be attained.

5. Conclusion

The present work describes a potent in *vivo* antimalarial activity of the two artemisinin–quinoline hybrid-dimers in a rodent model, and simultaneously elucidates their PK parameters in mice. It was also showed that both compounds possess significant activity against the *in vitro* proliferation of tumoral cell lines.

These dimers were able to cure the blood parasitemia of mice infected by *P. vinckei* at low doses but recrudescence was usually observed within 7–18 days as also observed with artesunate. Chemical modifications could be introduced to the structure of artemisinin–quinoline hybrid-dimers so as to replace its metabolic unstable C-10 acetal functionality resulting in compounds that will not be easily metabolized. For these dimers to be used as oral antimalarial treatment, modifications to improve bioavailability are necessary.

Furthermore, the attachment of a long acting drug moiety (quinoline) did not appear to increase the half-life of the artemisinin pharmacophore. However, prolonged antimalarial drug activities did occurred and were expected to be due to active metabolites. Consequently, during PK analysis one should not look for only the parent compound, but also for possible active metabolites.

Dimer **1**, featuring the aliphatic 1,3-diaminopropyl linker, displayed potent anticancer activities against all three cell lines, had an overall superior antimalarial activity and PK characteristics, and therefore might be potentially interesting for further research in search for better anticancer drugs.

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