RESEARCH ARTICLE

Cowpea Mosaic Virus (CPMV) as a Carrier Vehicle for Antimalarial Drugs, Modification, and Application

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ABSTRACT

Globally more than 45% of the population is under the risk of malaria infection. In this presented work, three different compounds have been tested as anti-malarial agents with and without conjugation to the external surface of the Cowpea mosaic virus (CPMV). The particle developed as a carrier of ferrocene (Fc) due to the resistance to commercially available antimalarial drugs. CPMV-Fc conjugate, in which 174 molecules are covalently bound to external surface carboxylates of the viral nanoparticle (VNP), shows the greatest inhibition toward the proliferation *of plasmodium falciparum* compared to free ferrocene which has no activity as an antimalarial agent. Fc also enhanced the activity of two different compounds of 4-amino chloroquinoline derivatives 3-((7chloroquinolin-4-yl) amino) propionic acid CQp and (7-chloroquinolin-4-yl) alanine chloroquine diphosphate (CQ)-ala, after its conjugation to CPMV-CQp and CPMV-CQ-ala in 82 and 134 molecules per particle respectively to give CPMV-CQ-p-Fc and CPMV-CQ-ala-Fc. The activity of each conjugated ferrocene has been evaluated with and without using biological material. In the case of biological material, these compounds have been tested against both chloroquine-sensitive strain 3D7 and chloroquine resistance strain Dd2 using the chloroquine as a standard antimalarial drug. The CPMV conjugate is targeted to the food vacuole parasite of the *plasmodium falciparum* cell, where the pH is dropped, drug carrier is degraded, and the drug released.

Keywords: Chloroquine, Ferrocene, Viral nanoparticle.

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INTRODUCTION

Ferrocenes (Fc) has played an essential role in different areas of material and synthetic chemistry. While, a slight attention has been given to their biological activities, a lots of publications have shown the bioactivity of ferrocene derivatives as antitumor and inflammatory agents.[1] Over the last 10 years, a significant number of studies has been used and developed a metallocene moiety as antimalarial compounds, ferroquine (FQ) is one of the most successful metallocenes compounds.[2] FQ and other ferrocenyl chloroquine analogs have been shown to be efficacious in vitro in both chloroquine-sensitive and chloroquine-resistant Plasmodium falciparum *(P. falciparum*) across a variety of strains.[3-5] It has been suggested that the Fc ring in FQ may be the reason behind the mode of resistance, but it is not known how this is achieved.[6] ferrocene (Fc) ring has also been incorporated into other known antimalarial agents with variable outcomes.[7] The effect of Fc ring in antimalarial activity still unclear; it may be associated with the physicochemical properties of the ring such as lipophilicity, electronic effects, and size, which may be optimal for transport or delivery processes.[8]

Due to the resistance of malaria against these compounds, looking for new drugs or new ways to eliminate this resistance becomes necessary. It was reported that viruses could provide the best basis for the development of targeted drug delivery vehicles, as they have evolved naturally to deliver cargos to host cells with high efficiency. The plant virus CPMV, which was used in this study as a carrier is an icosahedral virus with an approximate diameter of 30 nm, the structure of CPMV is known to near-atomic resolution.[9,10] The particle has 60 copies of each of two different types of coat proteins that together form the asymmetric unit. The multiple copies of the particle can provide more than one spaced attachment easing the conjugation and production of a wide range of different moieties on the external surface.[11-13]

The present study shows first the activity of each CQ-derivative as a beta-haematin inhibitor and then describes how the CPMV particle enhanced the activity of Fc as an antiplasmodial agent while Fc alone has no antimalarial activity. The effect of Fc on each conjugated CQ-derivative to the external surface of the CPMV particles has been investigated as well.

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EXPERIMENTAL SECTION

Materials and Instrumentation

1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), N hydroxysuccinimide (NHS) were purchased from Sigma. Dimethyl sulfoxide, DNA loading dye 6X, agarose low melting molecular biology grade, standard chloroquine phosphate, and aminoferrocene were purchase from Sigma-Aldrich UK. MilliQ Water was from Elga pure lab Ultra 18.2 mΩcm. Each CQ derivative has been synthesized at the University of Hull. The *Plasmodium falciparum* 3D7 (CQ sensitive) and Dd2 (CQ resistant) strains and their culture media were provided from the Department of Immunology and Infection, Faculty of Infectious & Tropical Diseases, London School of Hygiene and Tropical Medicine. UV-visible spectra were recorded on a Perkin Elmer Lambda 25 spectrophotometer using UVWINLab software. Transmission electron microscopy (TEM) images were obtained on a FEI Tecnai 20 TEM, FEI UK Ltd, using carbon-coated copper EM grids (400 mesh, Agar Scientific).

Cowpea mosaic virus (CPMV)

CPMV particles were supplied by Professor George Lomonossoff (John Innes Centre, Norwich, UK), and the concentration of purified particle was determined photometrically; the molar extinction coefficient of CPMV is $\varepsilon = 8.1$ ml .mg^{-1}. cm at a wavelength of 260 nm.

Electrophoresis Methods

A mixture of 10 µg of CPMV particles suspended in 10 mM sodium phosphate buffer pH 7.0 with 3 µl of loading dye were loaded on 1.2 % (w/v) agarose gel in an electric field of 60 V for 1.5 hour with and without ethidium bromide. For ethidium bromide staining, 0.1 µg/ ml in 1× TBE buffer was added to the gel before loading the CPMV samples. Particles were imagined on a UV transilluminator at 302 nm using Gene Genius Bio Imaging System with software Gene Snap (Syngene). In the case of coat protein staining, gel was stained with Coomassie staining solution (25% (v/v) methanol; 5% (v/v) acetic acid; 0.12% (w/v) Coomassie Brilliant Blue G-250) for 3h followed by destaining solution which contains (25% (v/v) methanol; 10% (v/v) acetic acid in Milli-Q water) overnight. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10 µg of virus in 10 mM sodium phosphate buffer pH 7.0 after mixing with 3 µL of 4× RunBlue LDS Sample Buffer, all samples were heated for 10 minutes at 100°C in order to denature the protein. Modified and unmodified samples were loaded on 12% TEOCI SDS RunBlue precast gels at 180 V for 90 minutes using 500 ml of 20:1 dilution of 20 × RunBlue SDS Running Buffer. The bands that stained with 10 mL of Coomassie staining solution were observed after 15 minutes, and finally, gels were washed with Milli-Q water to be ready for imaging.

The modification of CPMV-ferrocene

CPMV particle (6.5 mg/mL), suspended in 10 mM sodium phosphate buffer(PBS) pH7, was reacted with freshly prepared of EDC (0.008 g, 0.0416 mmol,1000 molar excess) in Phosphate-buffered saline (PBS) buffer at pH 7 (2 mL), and NHS (0.006g, 0.052 mmol, 4000 molar excess) in DMSO 3 mL. The reaction was left to proceed for 2 hours with gentle stirring then aminoferrocene (0.2g, 0.0008 mol, 2000 molar excess) in 5 mL DMSO was added. The reaction was stirred overnight at 4°C with gentle stirring. The final DMSO concentration of the reaction mixture was adjusted to be 20 % by volume. The CPMV-ferrocene conjugate was purified using 100 kDa molecular weight cut-off membranes (Float-A-Lyzer G2) against 10mM sodium phosphate buffer, while, the sample was concentrated on 100 kDa cut-off columns (Millipore).[14] Recovery of particle was 76%.

The modification of CPMV- CQ- derivatives with Fc

A 6000 molar excess of 0.02 g in 3 mL DMSO of each CQ-derivative was added separately to N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.003 g, 0.015 mmol) in 4 mL sodium phosphate buffer at pH 7 in 1000 molar excess, and 4000 molar excess of NHS (0.0028g, 0.026 mmol) in 4 mL sodium phosphate buffer at the same pH was added after 5 minutes. The reaction was left to proceed for 2h with gentle stirring, then 0.7 mg.ml^{-1} CPMV was added to the mixture, and the reaction was left overnight to proceed. The final concentration of DMSO in each reaction mixture was adjusted to be less than 20% by volume. After purification 2000 molar excess of Fc (0.2 g, 0.0008 mol) in 5mL DMSO was added. The reaction was stirred gently overnight at 4°C. The concentration of DMSO in each modified particle was adjusted to be 20 % by volume. 100 kDa molecular weight cut-off membranes (Float-A-Lyzer G2) was used to purify each CPMV-CQ-derivative-Fc conjugate against 10 mM sodium phosphate buffer. Each sample was concentrated on 100 kDa cut-off columns (Millipore). Recovery of the virus was 64 and 67% respectively.[15,16]

Electrochemical measurements

The cyclic voltammetry method was used to evaluate the concentration of ferrocene in the sample and the number of ferrocene moieties per virus particle according to the Randles–Sevcik equation as described elsewhere.[17] Modified particles were generally studied at concentrations rang of 0.5–6 mg.mL^{-1} and the cyclic voltammograms for each sample was recorded at scan rates 10–200 mV/s in duplicate.

In vitro β-haematin or hemozoin formation assay

The activity of tested samples as anti-disease has been evaluated with and without biological material. In the case of non-using organic material, the antimalarial activity of the tested samples was calculated by the method described by Samidha Joshi et al., with a significant modification.[18] each sample was incubated with 2 mM of haematin chloride, and 1M HCl. The final volume was adjusted to be 1ml using sodium acetate buffer, pH 4.5. CQ was used as a control. The reaction mixtures were incubated overnight at 37°C with gentle shaking. After that, samples were centrifuged for 10 min at 14,000 rpm and room temperature. Haemozoin pellet was

repeatedly washed with 15 min incubation at 37°C in a mixture of 2.5% (w/v) SDS in phosphate-buffered saline with regular stirring then finally washed in 0.1M sodium bicarbonate four times until the supernatant been clear. After the final wash, the supernatant was removed while the pellets were dissolved in 1mL of 0.1M NaOH. The determination of hemozoin formation was measured according to the absorbance at 400 nm. The results were confirmed as the percentage of haem crystallization inhibition (I%) compared to CQ control using the following equation:

I% = [(AC−AS)/AC] × 100 Where AC: Absorbance of CQ control and AS is absorbance of test samples

RESULTS AND DISCUSSION

In the first modification, ferrocene was used in 3000 molar excess to ensure maximum coupling. The modified particle was approximately recovered 76% yield according to an initial concentration of wild type CPMV (wt CPMV).

In the case of CPMV-CQp-Fc and CPMV-CQ-ala-Fc, CPMV was modified first with each CQderivative using EDC/NHS protocol; then each particle was modified with Fc. The recovery of modified particles was 64, and 67%, respectively depends on the initial concentration of wt CPMV. Agarose gel electrophoresis and TEM have been used to confirm the integrity of each modified particle. In the electric field, Fc shows an evident migration with its carrier toward the anode. The modified and unmodified CPMV can be noticed in the gel after staining with Coomassie Blue and ethidium bromide Figure 1 A, B.

Slow mobility of the Fc modified CPMV particle (lane 2-4) confirmed the successful single and double modification of the CPMV with Fc and each CQ-derivative. Moreover, the change in the size of the particle after modification can be another reason for the different movements of modified CPMV compared to the unmodified particles. The single and double modifications were also studied by denaturing SDS-PAGE. The result showed that CQ-derivatives and Fc were attached to both subunits (S and L) Figure 1 C. Uranyl acetate stained TEM shows that the CPMV particles still intact after double modification with an average diameter of 30.8 nm Figure 2.

The number of Fc in single and double conjugation to the external surface of CPMV has been calculated electrochemically (results have not been shown). The number of Fc molecules conjugated per CPMV particles was calculated using Randles-Sevčik Eq.[19]

$ip = k\, n^{3/2}\, A\, D^{1/2}\, v^{1/2}\, CFc$

Where ip = current in mA; k = 2.69 x 105 at 298 K (constant); n = 1 and it refers the number of electrons transferred per molecule, while, A is the surface area of the working electrode,

A = 0.07 cm^2; D is the diffusion coefficient of CPMV particles in aqueous buffer solution;

D = 0.16 x 10-6 cm^2/sec.

It was found that 174 Fc per particle was decorated on the external surface of CPMV and the number decreased when CPMV modified in single modification with CQ-derivatives before the conjugation with Fc to be 82 and 134 per particle (CPMV-CQp-Fc and CPMV-CQ-ala-Fc) respectively. This

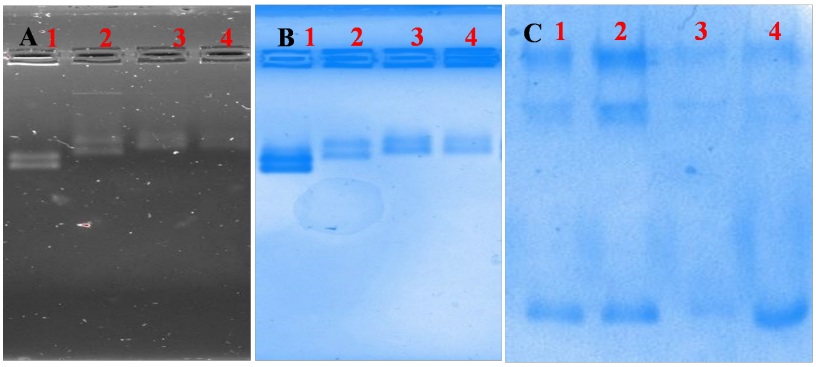

Figure 1: A and B, digital image of agarose gel electrophoresis 1.2 % w/v agarose, 60 V, stained with A: EtBr staining and B :Coomassie staining. C: SDS-PAGE, lane 1: wt CPMV, lane 2: CPMV-Fc lane 3, CPMV- CQp-Fc, lane 4, CPMV-CQ-ala-Fc.

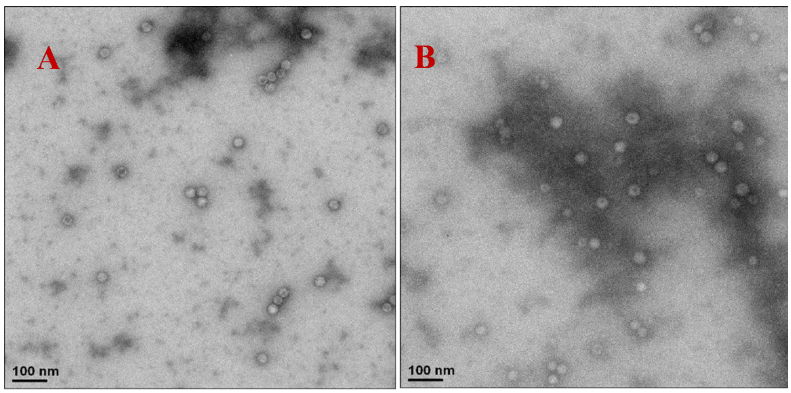

Figure 2: Transmission electron micrographs of uranyl acetate stained A, CPMV-CQp-Fc, and B, CPMV-CQ-ala-Fc.

due to the accumulation of NHS reagent on addressable carboxylates, which was used to activate the carboxyl groups on each CQ-derivative before reacting with wt CPMV.

In vitro test, the activity of each CQ-derivative (CQp / CQ-ala) and Fc was investigated first before conjugation to the external surface of CPMV without biological material using 100µg of each compound as a beta haematin inhibitor. The presented work results displayed that each CQ-derivative has a remarkable inhibition compared to control CQ, whereas Fc has no activity Figure 3.

In the case of combination treatment, Fc was used with each CQ-derivative in a molar ratio 2:1, thus resulting in a good inhibitory of beta- haematin formation as shown above. These results are obtainable as mean ± SD of three independent experiments. The same procedure was used

to identify whether or not these compounds can keep their activity after conjugation to the external surface of CPMV but there were no clear results have been obtained due to the effect of HCL on the stability of CPMV. However, using biological material shown very clear evidence to the main role of viral nanoparticle CPMV as a carrier to each CQ-derivative and ferrocene. Moreover, the role of ferrocene to improve the activity of conjugated CQ derivatives as antimalarial agents has been investigated, single and double modification of CPMV with Fc was tested against both chloroquine sensitive strain 3D7and chloroquine resistance strain Dd2.

Three samples in different concentrations have been prepared including conjugated Fc only and Fc in a combination with each conjugated CQ- derivative as shown in Table 1 compared to standard chloroquine (CQ).

The concentration of stock samples in sodium phosphate buffer pH7 is shown above. All samples had serial two-fold dilutions starting from the top concentration, which is a 10-fold dilution from the stock solutions of each sample.

CPMV-Fc (compound3) showed a very good kill effect against 3D7 and Dd2 strains compared to standard CQ while Fc alone has no antimalarial activity. This may due to the ability of iron as a chelating agent, which was once considered a suitable treatment for various infectious diseases, including malaria or because of the avidity of *Plasmodium* for free iron.[20,21] So providing 174 Fc per particle may increase the avidity of parasite and creating more than one protonation state inside the food vacuole parasite. Moreover, it was reported that the addition of iron to chloroquine molecule which is an important element for the growth of all living organisms may be removed the chloroquine resistance of parasites and enhanced the activity of chloroquine as anti-*Plasmodium*.[22,23]

Therefore, ferrocene (Fc) was used in combination with two different CQ-derivatives after conjugation to the external surface of CPMV as antimalarial agent. The overall results shown that CPMV-CQp-Fc (compound 4) looks to be having perhaps some effect at the highest

concentrations against both strain species but do not drop below 50% against 3D7 strain while the Dd2 values more likely to be accurate. For compounds 5 CPMV-CQ-ala-Fc, it appears the most effective against both strains. The activity of compound 5 was displayed more in the Dd2 line than the 3D7 line Figure 4.

Additionally, the results given a good evidence for the role of Fc to enhance the activity of CQ-derivatives against the viability of the malaria parasite due to its ability to increase CQ accumulation inside the food vacuole parasite (FV) causing heamozion inhibition which is necessary for parasite to survive. The IC_{50} values illustrated in Table 2.

CONCLUSION

In conclusion, the conjugated Fc has a significant effect on the inhibition of *Plasmodium* cells compared to free ferrocene. CPMV nanoparticles as carrier vehicles are provided 174 Fc

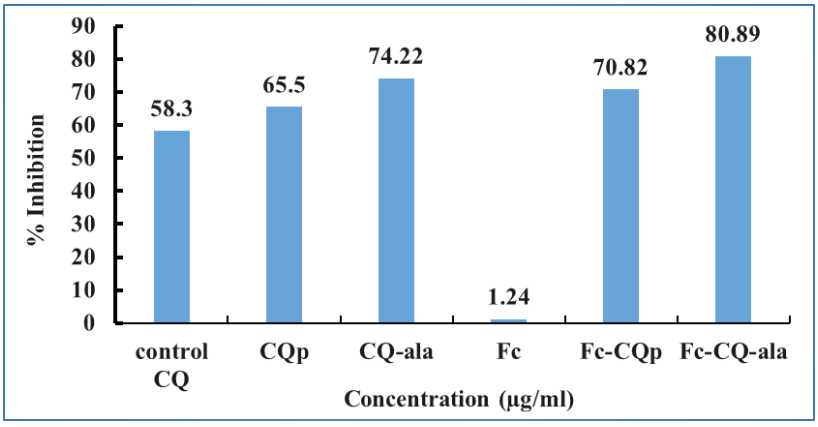

Figure 3: The effect of chloroquinoline derivatives and ferrocene on beta- haematin formation

Table 1: The concentration of each conjugated compound in nM

Compounds	*The concentration of CQp*	*The concentration of CQ-ala*	*The concentration of Fc in each sample*
CPMV-Fc	none	none	10 nM
CPMV-CQp-Fc	3.1 nM	none	3.6 nM
CPMV-CQ-ala-Fc	none	11 nM	10 nM

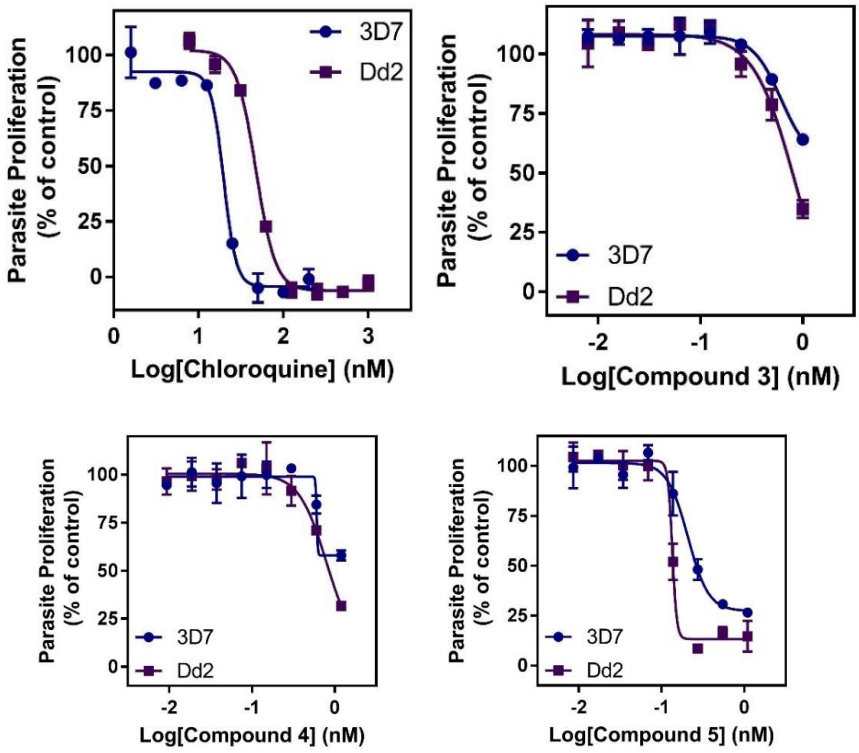

Figure 4: Antimalarial activity analogues of Fc and CQ-derivatives against *P. falciparum* 3D7 and Dd2 strains.

Table 2: *In vitro* activities of the three conjugated compounds tested against *P. falciparum strains*

Compounds	*Strains*	*IC_{50} in nM*
Standard CQ	3D7	19.76
	Dd2	47.58
CPMV-Fc	3D7	0.6142
	Dd2	0.8423
CPMV-CQp-Fc	3D7	0.6056
	Dd2	0.7952
CPMV-CQ-ala-Fc	3D7	0.2087
	Dd2	0.1352

instead of one molecule can diffuse through the membranes of the parasitized erythrocyte, thus allowing higher doses of Fc to accumulate at the target and destroy parasite-resistant. Additionally, it is worth noting that ferrocene has redox chemistry due to its lipophilicity, electron density, relative thermal, and chemical stability. All these properties make it the best chose to enhance the activity of conjugated CQ-derivatives as antimalarial agents. This work has shed light on the ability of ferrocene to improve the activity of synthesized 4- aminoquinoline derivatives in vitro antiplasmodial activity in both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*.

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