Synthesis and Hemozoin Inhibitor of Side-Chain Modified Copper-Chloroquine Derivatives

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Abstract:

This study uses copper (I) as a transition metal to improve the activity of 4-aminoquinoline as an antimalarial agent. This chloroquine derivative was synthesised and tested for in vitro antimalarial activity using a simple colourimetric method compared to the conventional purification method to measure hemozoin formation. This compound has been characterised by the combination of NMR and IR spectroscopic methods. Copper-chloroquine (Cu-CQp) might strongly exhibit antimalarial activity after showing significant inhibition of hemozoin formation compared to commercial chloroquine (CQ). This is possibly due to its lipophilicity, which enhances cell permeation. The highest activity was shown by the Cu-CQp complex in comparison to that of commercial CQ. Cu-CQp complex and CQ were used in a range of concentrations from 10–50 µM.

Keywords: chloroquine, hemozoin, malaria, trans-metals

1. Introduction:

Malaria is a common problem around the world, particularly in developing countries. The parasite causes haemoglobin degradation in the host erythrocytes so that it can consume the final product, which includes amino acids to build its protein during proliferation, which is necessary for the parasite to survive [1, 2]. The parasite can detoxify free heme in different ways, such as degradation with reduced glutathione and neutralisation with protein-rich histidine [3–6]. It has been shown that hemozoin is identical to b-hematin (BH) both chemically and structurally [7]. Recently, many reports show that the best target for antimalarial selection is destroying or reducing BH formation.

Currently, 4-aminoquinoline derivatives in drug form, for example, chloroquine (CQ), have been successfully used for malarial treatment, but the most dangerous parasite, called Plasmodium falciparum (P. falciparum), has improved its resistance against these types of drugs [8]. Therefore, new antimalarial drugs are needed to control malaria [9], and the most encouraging method includes the use of transition metals [10]. Research has been conducted to develop the effectiveness of known drugs like chloroquine, one of those involved in the modification of drug structures with transition metals [11]. However, many studies have demonstrated that numerous transition metals can inhibit the resistance of malaria against chloroquine-sensitive and chloroquine-resistant strains of P. falciparum. It is well known that several metal complexations of chloroquine and its derivatives have remarkable properties as a drug compared to chloroquine (CQ), which has been used recently for chemotherapy treatment. A reliable improvement in strengthened metal-chloroquine conjugation suggests that metals conjugated with chloroquine are an essential source for generating a new area of antimalarial development structurally and/or chemically.
[11, 12]. Recently, a good example of metal-chloroquine conjugation as an antimalarial agent is gold chloroquine (Au-CQ), which is more active than CQ against chloroquine-resistant strains of *P. berghei* and *P. falciparum* in vivo and in vitro [23]. It was also published that the main reason for the increase in antiplasmodial activity in vitro against two different *P. falciparum* strains (D10 and W2) in comparison to the activity of the CQ alone was the coordination of gold (I) and thiosemicarbazone [13]. To remove or reduce the CQ-resistance of *P. falciparum* parasites, copper (Cu) was used to modify the short side chine of CQP (chloroquine-propionic acid), and the role of Cu to develop the activity of CQP was investigated against the activity of malaria [14]. As heme polymerisation is an important step in the formation of BH, a quantity of heme is needed to study the mechanism action of BH formation. Several papers have described how BH formation can be measured in vitro and how it can be characterised using IR or X-ray diffraction [15]. Another method is to use toxic material, such as pyridine combined with laborious purification steps [16]. In this study, simple colourimetric and conventional purification methods have been used to identify whether or not CQ-metal has any speciality in the field of malaria treatment.

2. Material and methods:

2.1 Reagents

All chemicals were purchased from Sigma-Aldrich UK, except for CQP, which was synthesised and fully characterised in the labs at Hull University as part of a PhD project.

2.1.1 Chemical synthesis of copper-chloroquine complex (Cu-CQP)

An ethanolic solution of anhydrous copper (I) chloride (0.19 g, 1.99 mmol) was added to an ethanolic solution of the compound 3-(7-chloroquinolin-4-yl) amino propionic acid (CQP) (0.5 g, 1.99 mmol). The mixture was refluxed for 2 h until the solution became brown. Then, the precipitate was filtered and dried under vacuum to form Cu-CQP compound as a dark brown solid (0.39 g, 43%).

IR: ionised carboxylic COO− cm⁻¹, NH stretch at 3,340 cm⁻¹ and C=O stretch at 1,730 cm⁻¹. 1H NMR (400 MHz, DMSO-d6): δH/ppm 3.45 (t, 2H, CH2), 4.3 (t, 2H, CH2), 6.25 (d, J=6.12 Hz, 1H, ClQ-C3-H), 7.60 (d, J=7.2 Hz, 1H, ClQ-C6-H), 7.55 (d, J=7.8 Hz, 1H, ClQ-C5-H), 7.90 (d, J=6.8 Hz, 1H, ClQ-C8-H), 8.12 (d, J=7.2 Hz, 1H, ClQ-C2-H) and 8.15 (s, 1H, NH).

2.1.2 Beta-hematin preparation

A stock solution of heme was freshly prepared by dissolving hemin chloride in 0.2 M NaOH; to remove the remaining hemin crystal a stock solution centrifuged for 15 min at 7,000 g. The concentration was evaluated from the absorbance at 385 nm using ε₉₀₀ = 58,400 in 0.1 M NaOH [20]. All absorption spectra were recorded on a double-beam spectrophotometer. The formation of BH was performed following the published method with slight modifications [17, 18]. Briefly, heme (10 ml) was heated in an acetate buffer (0.56 M, pH 5.5) in the absence of CQ or CQP at 70 °C or at 75 °C with a range of concentrations of CQP and Cu-CQP (10–50 μM). At the identified times, 2 ml of heme solution was withdrawn and BH formation was calculated using two methods:

1- Simple colourimetric method

After heating the mixture of heme solution and heme control separately in an acetate buffer, the absorbance of each sample was investigated at 400 nm and 700 nm to confirm hemozoin formation. Consequently, heme fractions were calculated after they were altered to BH using Eq (1).

\[
\text{Fractions} = \frac{(A \text{ at } 400 \text{nm} - A \text{ at } 700 \text{nm}) \text{control} - (A \text{ at } 400 \text{nm} - A \text{ at } 700 \text{nm}) \text{sample}}{(A \text{ at } 400 \text{nm} - A \text{ at } 700 \text{nm}) \text{control}}
\]
2- Conventional purification method

After 2 hrs of heating, the heme solution was centrifuged at 7,000 g for 15 min. A 2 ml mixture of 0.2 M, pH 9.2 sodium bicarbonate and 2.5% sodium dodecyl sulphate were used to suspend the pellets. The insoluble material was improved by centrifugation after 1 h shaking at room temperature. This step was repeated twice. The final product of BH was decrystallised after incubation in 2% sodium dodecyl sulphate and 0.1 M NaOH.

To evaluate the crystallised heme (BH), heme concentration (C) was calculated from the absorbance of the solution at 400 nm with an extinction coefficient of 105 [19]. Fractions were calculated using Eq (2):

\[ \text{Fractions} = C \text{ before converting to BH} - C \text{ after converting to BH} \]

3. Results and discussion

In this paper, a copper-chloroquine was obtained by conjugating CQp with an anhydrous copper (I) chloride to give a new compound of Cu-CQp, which is more active than commercial CQ and CQp alone in in vitro treatment as a BH inhibitor. The disappearance of characteristic 1HNMR of a singlet proton at 11.66 ppm, corresponding to 1H and COOH of CQp and OH broadband of IR spectra for the same compound at 3,010 cm⁻¹, provided good evidence for the production of the Cu-CQp compound. Additionally, the functional group of CQp with copper (I) ion, which was determined using the IR spectra, contained an intense band of asymmetric and symmetric stretching vibrations of the ionised carboxylic COO group involved in the coordination of Cu⁺¹ (the bands at 1,545.4 and 1,326.1 cm⁻¹). This is in agreement with other published values [20]. After synthesis and characterisation, Cu-CQp was tested as a BH inhibitor using two different methods as described in the experimental section. In vitro tests using colourimetric and traditional purification methods were used to confirm whether or not Cu-CQp can work as the same mechanism of CQ that Saroj et al. suggested [21]. Before testing CQp and metal-CQp against the growth of heme crystallisation compared with commercial chloroquine, the effect of temperature on the rate of BH formation was studied. In the colourimetric method, all samples were withdrawn after 30-second intervals, and their absorbance at 400 and 700 nm was recorded using the double beam spectrophotometer. The results display the best incubation time that indicates the beginning of BH formation in each sample, nucleation time and the growth rate of heme crystals. A noticeable difference was found between CQp and Cu-CQp activity as a BH inhibitor compared to commercial CQ, as shown in Figure 1.

![Figure 1: Effect of time on BH growth at 73 °C in the presence of 10 µM of CQ, CQp and Cu-CQp](image-url)
As shown above, the quantity of heme-converted BH significantly changed when CQp was used as a heme crystallisation inhibitor compared to CQ, while the most significant inhibition was indicated after using Cu-CQp as an inhibitor agent of BH formation in comparison with both CQ and CQp. Moreover, there was a remarkable difference in the amount of BH crystallisation after treatment with Cu-CQp, and a noticeable change was observed after using commercial CQ as a BH inhibitor compared with untreated BH. This change increased when the concentration of each CQ-derivative increased from 10–50 µM. The reason might be associated with the ability of CQ-derivatives to conjugate with heme and block its active site to produce BH [22], as shown in Figure 2. The absorbance at 400 and 700 nm was recorded, and the fraction (f) of heme converted to BH was calculated. The results represent data from three independent experiments.

![Figure 2: Effect of CQ, CQp and Cu-CQp concentrations on BH crystal growth at 75°C using the simple colourimetric method](image)

Similarly, the results are a very good indicator of the role of Cu-CQp as an inhibitor of heme polymerisation growth, as shown in Figure 3. The fact that the free Cu of CQp obtained fairly low antimalarial activity compared to metal-CQp, suggested that the ability of the metal to bind CQp might play an important role in exhibiting antimalarial activity, rather than CQp or Cu alone [14].
Figure 3: Effect of CQ, CQp and Cu-CQp concentrations on BH crystal growth at 75 °C using the conventional purification method.

The growth rate of BH approximately matches in the two methods, thus, providing an acceptable relationship between the colourimetric and purification methods. Each experiment was performed in triplicate upon addition of CQ, CQp and Cu-CQp. As expected, when increasing the dosage of each compound, crystal growth began late, especially with Cu-CQp, thus, suggesting it is dosage-dependent.

4. Conclusion

In conclusion, the resistance of malaria against traditional antimalarial drugs has put pressure on public health systems to develop new therapeutics. The use of metal chloroquine as a new agent against BH formation may eliminate chloroquine resistance to traditional drugs. Additionally, using the colourimetric method provides useful information assimilated from BH crystal growth experiments and suggests that using an acetate buffer at high temperatures is convenient, safe and reproducible; it also outperforms other methods for monitoring BH formation.

5. References

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