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Mefloquine in combination with hemin causes severe damage to adult *Schistosoma japonicum in vitro*

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ABSTRACT

In order to explore the interaction of mefloquine with hemin against adult *Schistosoma japonicum in vitro*, the 50% and 95% lethal concentration (LC50 and LC95) of mefloquine and hemin against schistosomes, some factors, such as other iron providing agents, iron chelaters, zinc protoporphyrin-IX, and biological relevant reductants, that might impact on antischistosomal activity induced by interaction of mefloquine with hemin, and preliminary analysis of chemical interaction of both compounds were undertaken. The LC50 and LC95 of mefloquine and hemin alone against schistosomes were determined to be 6.5 μg/ml and 7.8 μg/ml as well as 232 μg/ml and 355 μg/ml, respectively. The LC50 and LC95 of mefloquine in the presence of hemin 100 μg/ml was 0.24 μg/ml and 0.59 μg/ml, respectively. On the other hand the LC50 and LC95 of hemin in the presence of mefloquine 1 μg/ml was 23.2 μg/ml and 77.2 μg/ml, respectively. Meanwhile, mefloquine/hemin combinations showed potential synergistic effects against adult *S. japonicum*, with combination index (CI) values <1. Apart from hemin, zinc protoporphyrin-IX, and other iron providing agents such as ferrous sulfate and ferriammonium sulfate combined with mefloquine exhibited no toxic effect against schistosomes. On the other hand, addition of iron chelators (deferiprone, desferrioxamine mesylate, or 2,2′-bipyridine) to the medium containing mefloquine-hemin resulted in no protective effect on the worms. Furthermore, biological reductants like glutathione, vitamine C or cysteine showed no apparent worm protection effect from toxic mefloquine–hemin even at higher concentrations (242.3–614.6 μg/ml, *i.e.*, 6.4–17.8-fold higher than the concentration of hemin). Chemical interaction of mefloquine with hemin was studied in 40% DMSO–Tris buffer solution. Both UV–Vis spectrum and mass spectrum demonstrated the strong interaction of mefloquine with hemin, which resulted in a reduction of hemin color and emergence of an adduct formed by mefloquine and hemin. The results confirm that mefloquine combined with hemin exhibits potential synergistic effect against adult *S. japonicum in vitro*.

1. Introduction

The antimalarial drug, mefloquine was recently found to be effective against three species of schistosomes (*Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma hematobium*) in experimental studies (Van Nassauw et al., 2008; Keiser et al., 2009; Ingram et al., 2012). Mefloquine belongs to quinoline drugs. The major antischistosomal features of mefloquine can be summarized as follows: (1) mefloquine possesses similar effects against both juvenile and adult schistosomes (Keiser et al., 2009; Xiao et al., 2009a; Xiao, 2013); (2) the *in vitro* effect of meflquine against schistosomes is irreversible (Xiao et al., 2009b), while *in vivo*, the efficacy of the drug is independent to the host immune response (Keiser et al., 2010a); (3) mefloquine exhibits histopathological and ultrastructural damage to adult and juvenile schistosomes (Xiao, 2013), (4) combined treatment with mefloquine and praziquantel, artesuante or artemether shows synergistic effect against schistosomes in experimental therapy (Keiser et al., 2011; Xiao et al., 2011; el-Lakkany et al., 2011), while mefloquine in combination with artesunate also exhibits higher cure rates against infections with *S. haematobium* in school-aged children (Keiser et al., 2010b), and (5) in intermittent preventive treatment of malaria during pregnancy (IPTp), mefloquine shows promising effect against concomitant *S. haematobium* infection in pregnant women in sub-Saharan Africa (Basra et al., 2013). Although we have gained a better understanding of the antischistosomal properties displayed by mefloquine, the exact drug action mechanism against schistosomes is still unclear.

It is well known that malaria parasite, *Plasmodium*, is a protozoa lodging in erythrocytes and digesting hemoglobin. As a consequence of the proteolytic digestion of this heme-containing protein,

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free heme (protein-free ferriprotoporphyrin IX, FP) is released and constitutes a major threat for the parasites due to its toxic action including induction of oxygen-derived free radicals formation, lipid peroxidation, inhibition of enzyme activity, and lysis of different types of cells (Tappel, 1955; Orjih et al., 1981, 1985; Davies, 1988; Gutteridge and Smith, 1988; Van der Zee et al., 1996). In order to detoxify the free heme, the malaria parasites convert this toxic molecule into insoluble crystals, which is a dark brown pigment known as hemozoin and primarily be found in food vacuoles of the parasites (Slater et al., 1991; Pagola et al., 2000). Besides malaria parasites, another hematophagous organism schistosomes, parasitizing in portal vein system of the host also digest hemoglobin, and produce *Schistosoma* pigment filled in the gut of the worms, especially in female ones (Kloetzel and Lewert, 1966; Homewood et al., 1972; Lawrence, 1973). Subsequently, comparative studies on chemical properties and absorption spectrum identify the pigment isolated from *S. mansoni* as hemozoin (Oliveira et al., 2000).

Since the formation of hemozoin is essential to the malaria parasites survival, it is suggested that inhibition of heme crystallization represents an attractive target, and interference with hemozoin formation in malaria parasites may be the basis of the activity of aminoquinoline (such as chloroquine) and quinolinemethanol (such as quinine and quindine) drugs, (Ginsburg and Demel, 1983; Schmitt et al., 1993; Egan et al., 1994; Kaschula et al., 2002; Egan, 2002, 2006). Recent studies *in vivo* indicate that chloroquine, quinine and quindine inhibit heme crystallization in *S. mansoni*, and suggest that interference with hemozoin formation in *S. mansoni* may represent the schistosomicidal action mechanism of these compounds (Oliveira et al., 2004; Corrêa Soares et al., 2009). However, further experimental therapy studies show that chloroquine, lumefantrine and pyronaridine exhibit no killing effect against adult *S. mansoni* and *S. japonicum* (Keiser et al., 2009; Xue et al., 2013), while halofantrine, quinine, quinidine and mefloquine possess killing effect against both species of. schistosomes (Keiser et al., 2009; Xiao et al., 2009a; Xue et al., 2013). These results stimulated us to further study the relationship between the *in vitro* or *in vivo* antischistosomal activity, as well as the inhibition of β-hematine (hemozoin) formation displayed by chloroquine, lumefantrine, pyronaridine, quinine, quinidine and mefloquine. Our results indicated that these six antimalarial drugs significantly inhibit the β-hematine (hemozoin) formation *in vitro*, but exhibit no definite correlation to their *in vitro* and *in vivo* antischistosomal activity (Xue et al., 2013).

According to the aforementioned results, we suppose that free heme is more toxic to kill the *Plasmodium* parasites, but might exhibit less or light toxic effect against adult schistosomes. Since morphological, histopatological and ultrastructure observations manifested that the worm gut is one of the fast and severely destructed sites caused by mefloquine (Xiao, 2013), it is reasonable to suggest that in the gut of adult schistosomes, the weakly alkaline mefloquine might interact with hemozoin or its degraded product to generate a substance which is toxic to the worms. Hence, we carried out a series of *in vitro* test to survey the effect of mefloquine in combination with hemin (the chloride of heme).

2. Materials and methods

2.1. Drugs and chemicals

Mefloquine hydrochloride was provided by Libang Pharmaceutical Co. Ltd (Xian, China); hemin and zinc protoporphyrin-IX were purchased from Sigma, and Sigma-Aldrich, respectively; glutathione was bought from Kyowa (Japan); desferrioxamine mesylate, deferiprone, and 2,2′-bipyridine were provided by Novartis, Aldrich, and Shanghai Runjie Chemical Reagent Company Ltd, respectively. RPMI 1640 was the product of Gibco (USA); penicillin, streptomycin, and amphotericin B were purchased from Life Sciences (USA). The calf serum was provided by Shanghai Shisheng Cell Biotechnology Co., Ltd (Shanghai, China). Other reagents, L-cysteine and vitamine C, were higher pure grade.

2.2. Drug and reagent concentration

A stock solution of mefloquine or deferiprone was prepared by dissolving 1 or 2 mg of each drug in 0.4 or 0.8 ml diemethyl sulfoxide (DMSO) and then adding 0.6 or 1.2 ml normal saline to prepare a stock solution with 1 or 2 mg of the drug per milliliter; 2,2′-bipyridine, cysteine, vitamine C and glutathione were dissolved in normal saline at concentrations of 2, 3, 5 and 6 mg/ml, respectively. Desferrioxamine mesylate was prepared before use with deionized water at a concentration of 5 mg/ml. Fresh hemin and zinc protoporphyrin-IX was prepared by dissolving 5 mg of the substance in 1 ml of 0.1 M aqueous solution of NaOH and adding 3.95 ml of PBS at pH 7.4, followed by 0.05 ml of 1 M HCl to adjust the pH to 7.4.

2.3. Collection of Schistosomes and incubation in vitro

One hundred and twenty mice infected with 80–100 *S. japonicum* cercariae each for 34–38 days were used for the *in vitro* tests. For each test, 5–7 mice were sacrificed by bleeding, and adult schistosomes were collected by perfusion with ice cold Hanks' balanced salt solution (HBSS) containing heparin from the mesenteric veins and livers. The collected worms were rinsed with HBSS three to four times before using them for *in vitro* incubation.

RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 IU/ml penicillin sodium, 100 IU/ml streptomycin, and 0.25 μg/ml amphotericin B (RPMI 1640-10% calf serum) was used to maintain the schistosomes *in vitro*. The volume of the medium added to each of the 12 wells of a Falcon plate was 3.58–4.00 ml, then four pairs of schistosomes were placed in each well. The plate was incubated at 37 °C in 95% air + 5% CO_2 for about 2 h before addition of mefloquine or other reagents at various concentrations. The final volume in each well was 4.0 ml. Control wells contained the worms and medium with a final concentration of 0.16%–1% DMSO alone, *i.e.*, the corresponding concentrations of DMSO used in the highest concentrations of mefloquine or other reagents in a final volume of 4.0 ml. After addition of the aforementioned substances, the plates were incubated continuously for 72–96 h.

2.4. Concentrations used for LC_{50} and LC_{95} determination

For mefloquine and hemin alone, the final concentrations of each compound applied in each test were 2–9 μg/ml, and 120–280 μg/ml, respectively. In mefloquine combined with hemin 100 μg/ml, the concentrations of mefloquine used were 0.01–0.6 μg/ml, while hemin in combination with mefloquine 1 μg/ml, its concentrations were 6.25–48.9 μg/ml. Aforementioned each test was repeated for four times, and all schistosomes were incubated for 72 h. Meanwhile, the number of worms died during the incubation period was recorded.

2.5. The possible influencing factors on interaction of mefloquine with hemin

For understanding the impact of administration scheme, schistosomes were exposed first to mefloquine 0.25–3 μg/ml or hemin 100 μg/ml for 24 h, then the medium with mefloquine or hemin was removed, followed by washing the worms with RPMI 1640 for three times. Afterwards, the worms were transferred