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Comparative Study on Schizontocidal Activity of Recrystallized or Crude Daphnetin Against Malaria Parasites[1,2]

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Objective To compare the schizontocidal activity of recrystallized or crude daphnetin against malaria parasites *in vivo*. **Methods** Schizontocidal activity of recrystallized or crude daphnetin at various dosages was assessed in mice infected with *Plasmodium berghei* ANKA using a "4-day suppress assay". **Results** The comparison of the reduction rate of parasitemia caused by either recrystallized or crude dephnetin showed that ED_{50} of crude daphnetin was 18.36 mg/kg, with 95% confidence limit of 5.96-56.54 mg/kg while ED_{50} of recrystallized daphnetin was 11.46 mg/kg, with 95% confidence limit of 8.63-15.22 mg/kg. **Conclution** The results indicate that the efficacy of recrystallized daphnetin is 37.6% higher than that of crude daphnetin.

Key words: Daphnetin; Recrystallization; Schizontocidal activity; *Plasmodium berghei*

INTRODUCTION

Malaria is one of the most geographically widespread and devastating infections in humans. Over the past two decades, global resistance to both insecticides and antimalarials has emerged, the incidence of malaria has increased and the disease has become more widespread. For control of malaria, antimalarial chemotherapy remains the principal means available for reducing the morbidity and mortality of malaria, and the task of developing new antimalarial drugs with new modes of action is of great significance.

Based on the malaria parasite's high replication rate and high iron utilization, iron chelators that are capable of withholding iron from vital metabolic pathways and causing selective "iron starvation" of the parasites have become candidates of antimalarials under investigation[1]. Daphnetin, a novel coumarin which is extracted from plants of the genus *Daphne* as well as several other genera[2-6] has been used clinically in China to treat Burger's disease and other diseases since the 1980s[7]. In the past five years, we have found that daphnetin, a known iron chelator[8] exhibits moderate schizontocidal activity against malarial parasites *in vitro* and *in vivo*. *In vitro*, daphnetin exhibited potent schizontocidal activity comparable to chloroquine(CQ)at the dose range of 1-10 μmol/L. *In vivo*, 50 or 100

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mg/kg.d×4 d daphnetin i.g. and 10, 50, or 100 mg/kg.d×4 d dephnetin i.p. showed an antimalarial efficacy comparable to CQ 10 mg/kg.d×4 d i.g. in mice infected with *P. berghei* ANKA, which was evaluated by both reduction rate of parasitemia on D_4 and average surviving days in 30 days[9]. Although daphnetin alone showed no anti-exoerythrocytic activity *in vivo*, the combination of daphnetin (50 mg/kg.d) with half regular dose of primaquine (5 mg/kg.d) achieved an antimalarial efficacy comparable to that of full dose (10 mg/kg.d) treatment of primaquine[10]. Moreover, the mechanistic study indicated that daphnetin inhibited the activity of superoxide dismutase (SOD), an iron-centered enzyme of vital importance for protecting parasites from oxygen-radicals'damage[11]. For the purpose of improving the efficacy of daphnetin, we developed a purification method of market available crude daphnetin and compared the antimalarial activity of recrystallized daphnetin (RD) with crude daphnetin (CD) against malaria parasites in mice.

MATERIALS AND METHODS

Chemicals and Animals

Crude daphnetin was purchased from Chuncheng Pharmaceutical Factory (Changchun Jilin, China). Each capsule contains crude daphnetin 110 mg. An equal number of male and female mice (20 g±2 g, Kunming outbred strain, from Animal Center, Chinese Academy of Sciences, Shanghai) were used. Standard laboratory chow and water were given.

Recrystallization of Daphnetin

Recrystallization of daphnetin was performed according to the standard method in our laboratory[12]. Briefly, a saturated solution of daphnetin in ethanol was prepared with heating and stirring and then stored in dark at 4℃ for 24 h. The short needle-like crystals of daphnetin thus formed were filtered and dried under vacuum. The pure crystallized daphnetin was identified by HPLC, UV-visible and Mass spectrum.

Instruments

HPLC analysis was performed with a Shimazu LC-6A, consisting of two 1c-6A, SPD-6AV UV-visible detector, a system control and C-R4A data analyzer. The column was Shim-Pack ODS C-18 (5 μm, 150 mm×4.6 mm). The detection wave length was 310 nm. The mobile phase consisted of a 0.005 mol/L tetrabutylammonium dihydrogen phosphate water solution (A) and a 0.005 mol/L tetrabutylammonium dihydrogen phosphate methanol (HPLC grade) solution (B), A:B=45:55, flow rate: 1 mL/min. Mass analysis was performed with a LCQ-MS HPLC-MS, from Phenygun Co. ESI electron ionization was at 4.5KV, the column temperature was 200℃.

Schizontocidal Activity of RD and CD in vivo

Groups of four male and four female mice were infected by i.p. injection of blood with parasites (5×10^6/mouse) from passage mice infected with *Plasmodium berghei* ANKA (obtained from School of Hygiene and Tropical Medicine, London, and maintained in our institute) on Do. The schizontocidal activity of RD and CD at various dosages was tested in infected mice using the "4-days suppress assay" recommended by WHO[13]. Briefly, mice were administered with the suspension of either RD or CD in 1% Gun Tragacanth 4 h post-

infection on Do and once per day for three additional consecutive days (D_1-D_3) intragastrically. Both vehicle and CQ (10 mg/kg.d×4 d) control groups were included in each experiment.

Parasitemia was assessed on D_4 post-infection by preparation of Giemsa-stained slides from tail smears of each mouse.

Statistics

Two-tailed unpaired *t*-test was used to evaluate the results.

RESULTS

The structure of RD was identified by HPLC profile, U.V-visible and MASS spectrum consistent with that of the standard sample of daphnetin as shown in Figs.1 and 2. The RD peak was eluted at 4.62 min under the HPLC condition described above. The molecular weight of RD was 179. The maximum absorbance peak was at 324 nm.

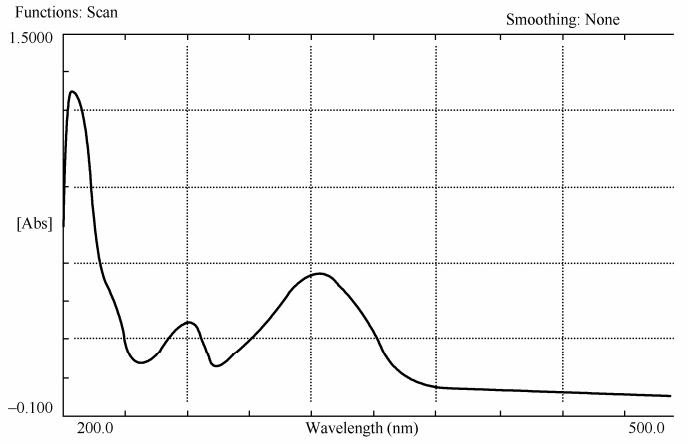

FIG. 1. Typical U. V. spectrum of RD.

The comparison of the reduction rate of parasitemia caused by either RD or CD showed that both drugs exhibited a linear dose-effect relationship between tested dosages and reduction rate of parasitemia while ED_{50} of CD was 18.36 mg/kg, with 95% confidence limit of 5.96-56.54 mg/kg, and ED_{50} of RD was 11.46 mg/ kg, with 95% confidence limit of 8.63-15.22mg/kg. It indicated that the efficacy of RD was 37.58 % higher than that of CD, as shown in Fig. 3.

DISCUSSION

The series of our study on antimalarial effect and its mechanism of action identified daphnetin as a leading compound of iron chelating antimalarial candidates. However due to the fact that market available daphnetin was a herbal product, so its purity was variable, thus affecting the constancy of therapeutic effect against malaria. The applicable approaches to the improvement of its efficacy are to purify the crude products chemically and/or to develop some combination of daphnetin with other antimalarials with different mechanisms.

Our present results indicated that the recrystallized daphnetin exhibited an efficacy by 37.58% higher than that of crude daphnetin. However, due to the low purity of market available capsules of CD, the research on the enhancement of productivity of recrystallization was conducted in our laboratory so as to benefit the economic and cost-effective evaluation.

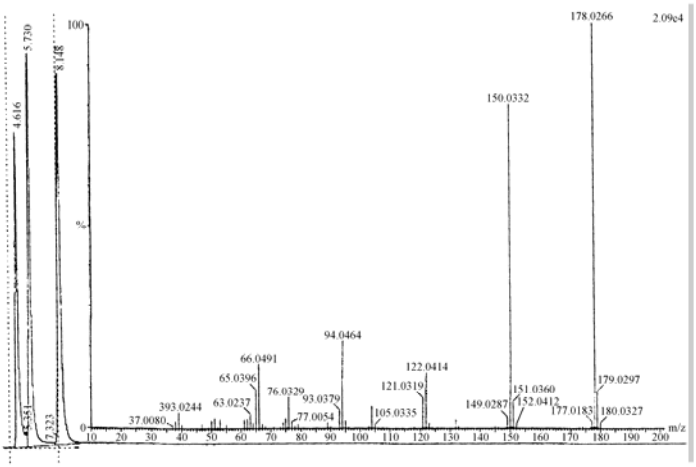

FIG. 2. HPLC profile and Mass spectrum of RD.

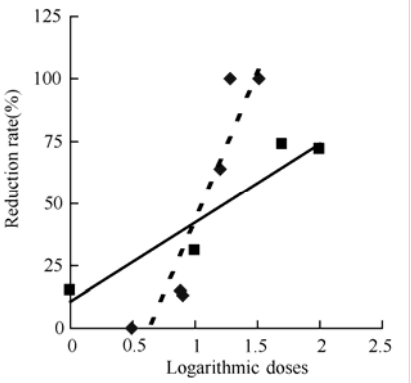

FIG. 3. Effects of RD and CD on mice infected with *P. berghei* ANKA.
RD: Series 1 (◆·····◆), CD: Series 2 (■——■)

There is evidence that withholding iron from vital metabolic pathways of the parasite is a potential antimalarial chemotherapeutic strategy which was first provided by Dr. Simeon Pollack and colleagues 16 years ago. By observation on the growth of *P. falciparum* in cultured erythrocytes in the presence of the iron-chelating agent desferrioxamine (desferriocamine B, deferoxamine, desferal, DFO). Raventos-Suarez *et al.* demonstrated that iron was an essential nutrient for the asexual erythrocytic phase of the parasite while withholding iron inhibited parasite growth and replication[14].

It can be inferred that withholding iron from the parasite by iron chelators conceivably could disrupt the metabolism of the parasite by preventing DNA synthesis, interfering with carbohydrate metabolism, disrupting proteolysis of host hemoglobin, and inhibiting *de novo* synthesis of its normal mitochondrial function and electron transport. The mechanism of antimalarial action of iron chelators appears to be the sequestration of iron necessary for

plasmodial replication rather than a direct toxic effect on the parasite or withholding other essential trace metals. Our mechanistic study indicated the molecular targets of daphnetin were iron-centered macro-molecules biologically significant to malaria parasites, most likely superoxide dismutase (SOD) and ribonucleotide reductase[11].

As a WHO recommendation at the Meeting on Anti-malarial Drug Development held in November 16-17, 2001, Shanghai, China, the combination therapy by using 'novel' antimalarial drugs with different modes of action has been considered as a promosing aproach to improve therapeutic efficacy and delay development of resistance in antimalarial chemotherapy.

Therefore, it can be anticipated that the combined action of two classes of drugs might render the parasites vulnerable at all stages of growth, and increase the antimalarial potential of the drugs. One of our recent studies has revealed an additive effect of each single drug in combination of dephnetin and artemether that could increase the antimalarial efficacy and delay the resistance of parasites to the drugs[15]. Further investigation is in progress to find the best formula of the daphnetin combination for future clinical evaluation.

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