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In vitro Potentiation of Antimalarial Activities by Daphnetin Derivatives Against *Plasmodium falciparum*

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Objective To screen the antimalarial compounds of daphnetin derivatives against *Plasmodium falciparum in vitro*. **Method** *Plasmodium faciparum* (FCC1) was cultured *in vitro* by a modified method of Trager and Jensen. Antimalarial compounds were screened by microscopy-based assay and microfluorimetric method. **Results** DA79 and DA78 showed potent antimalarial activity against *Plasmodium falciparum* cultured *in vitro.* **Conclusion** Though the relationship between the structures of daphnetin derivatives and their antimalarial activities has not been clarified yet, this study may provide a new direction for discovery of more potential antimalarial compounds.

Key words: Daphnetin; Antimalarial; Drug screening

INTRODUCTION

Malaria is one of the most life-threatening and widespread diseases in the world, causing 250-300 million clinical cases and approximately two million deaths annually. The growing resistance of the parasites to known antimalarial agents, such as chloroquine, is responsible for some of the worst cases in the tropical world. Therefore, novel compounds need to be developed by identification of novel chemotherapeutic targets[1]. Iron chelation therapy is considered a suitable treatment for various diseases, including malaria. Iron is needed for catalysis of DNA synthesis and for a variety of enzymes involved in electron transport, *de novo* synthesis of heme, and energy metabolism[2]. Daphnetin (7,8-dihydroxycoumarin) is a Chinese herbal product, which has been used in treatment of cardiovascular diseases for decades in China and proved safe, with few side effects over long-term administration. Recent experimental observations *in vitro* and in rodent showed that this compound has a moderate antimalarial activity[3-5]. But due to the relatively slow antimalarial activity of daphnetin and enhanced activity after prolonged contact, it is necessary to develop more potent antimalarial compounds of daphnetin derivatives. The present study was to detect the antimalarial activities of daphnetin derivatives by traditional and microfluorimetric methods, based on the intercalation of the fluorochrome PicoGreen in *Plasmodium* DNA[6].

MATERIAL AND METHODS

Chemicals

Daphnetin was purchased from Xidian Pharmaceutical Factory (Changchun, China). Twenty-one daphntin derivatives were synthesized by Lin-Qian YU (Ph. D.) and Professor Fan-Jun NAN from Chinese National Center for Drug Screening, Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Graduate School of the Chinese Academy of Sciences (Shanghai, China).

Cultivation of Parasite

Plasmodium faciparum (isolate FCC1) was maintained *in vitro* by a modified method of Trager and Jensen[7]. The culture media consisted of standard RPMI1640 (Gibco) supplemented with 10% rabbit serum (Shanghai Fudan-Yueda Bio-Tech Co.).

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Cultures were maintained in type O+ human red blood cell suspensions at a hematocrit of 5% obtained from Shanghai Blood Center. The parasite density was maintained below 5% parasitemia under an atmosphere of gas mixture containing 5% CO_2, 5% O_2, and 90% N_2 at 37℃ in a water-jacketed incubator. For the traditional method, the samples of stock cultures were adjusted to a final hematocrit of 5% and a parasitemia of 3%. For the microfluorimetric method, they were further diluted with culture medium containing sufficient noninfected type O+ human erythrocytes to yield a final hematocrit of 2% and a parasitemia of 1%. All assays were carried out in microtiter plates. For synchronized assay, asynchronous cultures were pretreated with sorbitol and incubated for 28-30 h to schizont stage parasites.

Microscopy-based Assay

The cultures were adjusted to 5% hematocrit and 3% parasitemia, are 200 μL aliquots were distributed in 96-well microtiter plates. Daphnetin and its derivatives were added at various concentrations. After incubation for 24 h, the parasitermia in each well was assessed by microscopy of a thin blood smear with Giemsa staining.

Fluorimetric Susceptibility Test

Synchronized ring form cultures (hematocrit=2% and parasitemia=1%) were used to test antimalarial compounds in 96-well plates. Cultures of *Plasmodium falciparum* were placed in a humidified, air-sealed container, flushed with the gas mixture described above, and incubated at 37℃. Parasites were allowed to grow for a 48-hour incubation period, after which an aliquot of 150 μL culture was transferred to a new 96-well flat bottom plate. Fifty microliters of the fluorochrome mixture consisting of PicoGreen (0.25 μL, 200-fold dilution in TE buffer) (Molecular Probes, USA), 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5 (TE buffer), and 2% Triton X-100 diluted with double-distilled DNAse-free water, were then added to liberate and label the parasitic DNA. The plates were then incubated for 20 minutes in the dark. The fluorescence signal, measured as the fluorescence was quantitated at 485/20 nm excitation and 528/20 nm emission by luminescence spectrometer LS50B (PERKIN ELMER). Simultaneously, the fluorescence from positive and negative control samples was obtained, stored, and analyzed.

Data Analyses

Data analyses were performed with a preprogrammed calculus sheet on Microsoft Excel 2003 that processes the parasitemia and the fluorescence by related statistical software (STATA 7.0). Analysis of the counts was performed with Graphpad Prism software. The counts were plotted against the logarithm of the drug concentration and curve fitting by nonlinear regression.

RESULTS

Microscopy-based Assay

Among the 21 daphnetin derivatives, only DA79 and DA78 showed potent antimalarial activities against *Plasmodium falciparum in vitro* as daphnetin (7,8-dihydroxycoumarin) as determined by microscopic counting of parasitemia with thin blood smears (Antimalarial activities of other derivatives were lower than those of daphnetin. Data were not shown). Fig. 1 illustrates their dose-response curves.

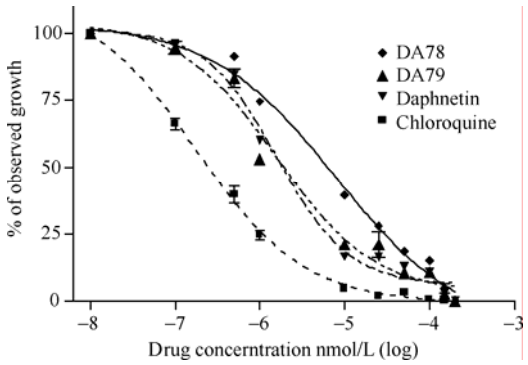

FIG. 1. Dose-response curves of DA78, D79, daphnetin, and chloroquine determined by microscopic counting of parasitemia in thin blood smears. Values were normalized by use of the upper and the lower plateaus of the best-fit curve as 100% and 0% responses, respectively, and plotted as the $\bar{x} \pm s$ errors of the means.

Fluorimetric Susceptibility Test

The fluorescence of 21 daphnetin derivatives and control was obtained. Also, only DA79 and DA78 showed significant antimalarial activities. Fig. 2 shows the tested drug dose-response curve determined by microfluorecence assay.

Correlation Between Microscopy-based Assay and Fluorimetric Susceptibility Test

Preliminary experiments demonstrated that serial dilutions of normal uninfected red blood cells did not emit significant amount of fluorescence when incubated in the presence of PicoGreen, indicating that DNA from contaminating white blood cells and hemoglobin pigment from erythrocytes did not interfere with the detection of plasmodium DNA. The

percentage of infected erythrocytes as determined by microscopic counting was compared with the results obtained by the fluorimetric technique. As shown in Fig. 3, there was a direct relationship between the percentage of infected erythrocytes and fluorescence ($r = 0.9720$, $R^2 = 0.9448$).

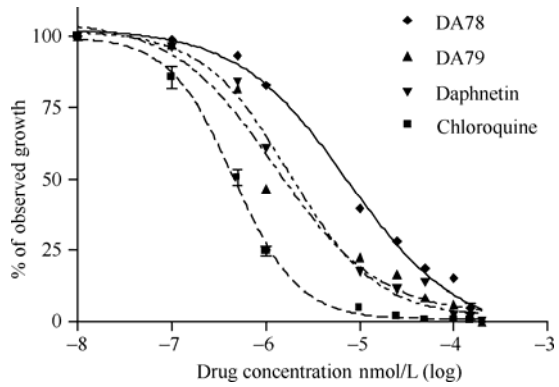

FIG. 2. Dose-response curves of DA78, DA79, daphnetin, and chloroquine determined by microfluorecence assay. Data were analyzed the same as above.

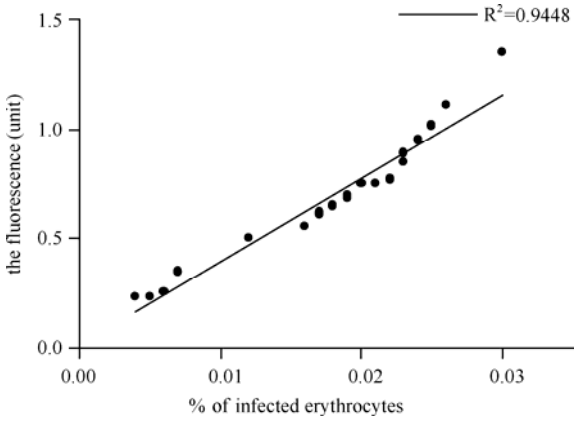

FIG. 3. Correlation between percentage of *Plasmodium falciparum*-infected erythrocytes and the fluorescence detected. A serial three-fold dilution of a synchronized infected culture with noninfected erythrocytes was used ($R^2 = 0.9448$ by linear correlation analysis).

DISCUSSION

Daphnetin is a weak iron chelator with comparable antimalarial activities *in vitro* and *in vivo*. It may specifically interact with some iron-containing enzymes. If it is possible to modify the daphnetin core structure and keep iron-chelating group and increase this specific interaction, new compounds would have more potent and specific activities against malaria. The design of twenty-one daphnetin derivatives based on this hypothesis may keep iron-chelating property and increase the compound stability and PK profile, *etc*.

The antimalarial mechanism of daphnetin and its derivatives is unknown. Daphnetin and its derivatives may act like iron chelators against multiple iron-requiring proteins, such as ribonucleotide reductase (RR), dihydroorotate dehydrogenase (DHOD) or superoxide dismutase (SOD)[8-11]. Daphnetin exerts its action through an effect on parasite DNA synthesis, mitochondria, or mitochondrial protein synthesis[12]. It also inhibit protein kinases, such as tyrosine-specific protein kinase, epidermal growth factor (EGF) receptor, and serine/threonine-specific protein kinases, including cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), which plays a key role in the control of cell proliferation, differentiation and metabolism[13]. The irons withheld by daphnetin in the process of inhibiting the growth of intra-erythrocytic malaria parasites most likely reside within the parasitic compartment of the infected red blood cells as the ferric iron (Ⅲ)[14]. One would thus predict that an effective antimalarial iron chelator would have the ability to cross lipid membranes with a high affinity for iron[15].

Our results indicated that DA79 and DA78 have potent antimalarial activities at the same level of daphnetin. The relationship between the structures of daphnetin derivatives and their antimalarial activities has not been clarified yet, but this study may provide a new direction for discovery of more potent antimalarial compounds. Daphnetin (7,8-dihydroxy-coumarin) is one of the major bioactive components isolated from *Daphne koreane Nakai*, an iron chelator. Its antimalarial activity decreases significantly when its chelating activity is eliminated[16]. The design and characterization of synthetic alternatives to daphnetin have led to the discovery of more effective chelators. The hydroxyl groups increase iron (III)-chelating stability in acid environments and may contribute to an enhanced iron acquisition, which is associated with a reduction in protonated ligand value (pKa, the affinity for protons)[17]. Though the presence of hydroxyl groups may also reduce the affinity for iron (III), its influence on hydrogen ion interaction predominates[18]. The competition between protons and iron (III) cations favors iron (III) chelation in the presence of an intramolecular hydrogen binding to one or more of the complex ligands[19]. In chemistry, the hydroxyl group consists of an oxygen atom joined by a single bond to a hydrogen atom. The hydrogen ion may be removed from this functional group while crossing the membrane into erythrocytes of parasite. Therefore the oxygen group with negative ion can easily bind to the target Fe-containing proteins or enzymes with positive ions of the *Plasmodium*. The

position of hydroxyl group and the antimalarial activity of daphnetin mainly depend on the chelating activity of the hydroxyl group at C7 and C8. Also, their antimalarial activities are based on the three-dimensional conformation. In conclusion, there are several explanations for the relationship between their chemical structures and antimalarial activities.

The microfluorimetric method presented herein for detecting antimalarial compounds has several advantages over the traditional assay. This method is simple and rapid, and is based upon the detection of *Plasmodium* DNA in short-term cultures using 96-well plates, allowing the efficient and quantitative measurements of anti-plasmodial activity in a large number of samples. This method uses PicoGreen, an ultra sensitive fluorophore intercalated into the double-stranded DNA of *Plasmodium* in solution, enabling the detection of 25pg/mL of dsDNA. The PicoGreen assay protocol is straightforward. The parasites are incubated with the test drug for 48 hours, followed by PicoGreen and a 20-minute incubation period prior to the measurement of fluorescence. The replication of the parasite is directly proportional to the amount of fluorescence with a linear relationship between parasitermia of 0.5% to 3%. Therefore, the development of an effective method for measuring antimalarial activity not using radioactive isotopes may stimulate antimalarial drug discovery programs in a number of countries, especially those most affected by this deadly disease.

This study may provide only a new direction. Investigating of new compounds based on the structure of the above two potent antimalarial drugs and further evaluation of their *in vivo* antimalarial effects are needed.

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