In vitro Antimalarial Activity of Flavonoids and Chalcones

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Malaria is one of the most common infectious diseases in tropical and subtropical countries, including parts of the Americas, Asia, and Africa. Each year, it affects nearly 400-900 million people and causes approximately one to three million deaths annually.[1] Human malaria is caused by *Plasmodium falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*, however, *P. falciparum* is the most prevalent for the disease and it is responsible for about 80% of infections and 90% of deaths.[2] The first effective treatment (17th century) against the *P. falciparum* parasite was the bark of cinchona tree, which contains quinine, a quinoline alkaloid. A number of medicines have been developed to treat malaria with chloroquine and its derivatives[3] as the mainstay therapy. In recent years, *P. falciparum* has become increasingly resistant to conventional antimalarial drugs, and the search for new antimalarial compounds by combining natural sources and synthetic approaches is still underway.[4,5]

As a part of our search[6-13] for novel antimalarial agents from plants or via chemical synthesis, we prepared twenty derivatives of flavonoids and chalcones. Flavonoids comprise a large group of polyphenolic secondary metabolites in plants. They are based on the flavan skeleton, consisting of two aromatic rings (ring A and B) interconnected by a three-carbon-atom, heterocyclic C ring, and classified into six main groups, flavanones, flavones, isoflavones, flavonols, flavanols, and anthocyanins. Many natural and synthetic flavonoids possess antimalarial activity.[14-17] Chalcones have a diverse array of substituents on the two aromatic rings of 1,3-diphenyl-2-propen-1-one, which was derived by the cleavage of the C ring in flavonoids. Depending on the substitution pattern on the two aromatic rings, chalcones have a wide range of biological activities, including antimalarial activity.[18-21] However, there has not been a direct comparison of flavonoids and chalcones and their structure-activity relationships.

In the present study, four derivatives for each of flavones (**1**-**4**), flavanones (**5**-**8**), chalcones (**9**-**12**), dihydrochalcones (**13**-**16**), and 3'-chloro-chalcones (**17**-**20**) (Fig. 1) were synthesized and evaluated for *in vitro* antimalarial activity against *P. falciparum* strain FCR-3 and cytotoxicity against FM3A cells (a mouse mammary tumor cell). The aim of this paper is to derive predictive structure-activity relationships to guide lead compound design.

Among the flavonoids and the related chalcones tested, the most active compounds were 3'-methyl-substituted flavanones (**5**) and 4'-methoxy-substituted dihydrochalcones (**15**), showing 100% inhibition against *P. falciparum* at the final concentration of 5.0 μg/mL and 5.4 μg/mL, respectively (EC_{50} = 1.6 μg/mL and 1.0 μg/mL, respectively) (Table 1). These compounds also showed strong cytotoxicity against FM3A cells, a model of the host, with relatively low EC_{50} values (>4.8 μg/mL and 3.3 μg/mL, respectively) and low selectivity indices (>3 and 3.3, respectively), indicating that these compounds have non-selective antimalarial activity. The selective toxicity index of quinine, a well-known antimalarial drug, was 450 in our previous report.[10] Eight compounds were weakly antimalarial, ten compounds were not cytotoxic, and five compounds showed no activity, but without cytotoxicity.

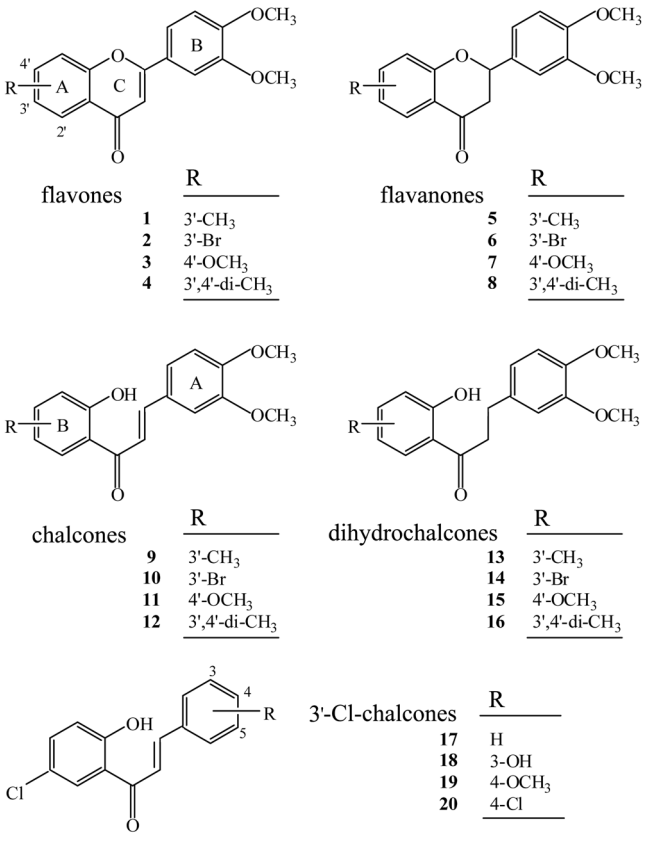

Figure 1. Chemical Structures of Tested Compounds.

Table 1. *In vitro* Antimalarial Activity and Cytotoxicity of tested compounds

Compound	Final Conc. (μg/mL)	*P. falciparum* % Inhibition (EC_{50})	FM3A EC_{50} (μg/mL)	Selectivity Index[a]
1	5.2	NA	NC	–
2	5.3	40	NC	–
3	5.8	37	3.2	–
4	5.2	13	2.0	–
5	5.0	100 (1.6)[b]	>4.8	>3
6	5.3	NA	NC	–
7	5.7	36	NC	–
8	5.2	NA	NC	–
9	5.4	NA	NC	–
10	5.2	22	1.7	–
11	5.4	37	1.4	–
12	5.5	5	NC	–
13	5.2	15	NC	–
14	5.1	NA	NC	–
15	5.4	100 (1.0)[b]	3.3	3.3
16	5.4	29	NC	–
17	5.2	14	1.7	–
18	5.0	NA	1.6	–
19	5.2	1	1.9	–
20	5.3	NA	2.8	–

[a]Selectivity index refers to the ratio of the EC_{50} value for the FM3A cells and the EC_{50} value for *P. falciparum*. [b]Values in parenthesis represent the EC_{50} value (μg/mL). NA = not active. NC = no cytotoxicity.

Antimalarial activity and cytotoxicity of the test compounds were influenced either by the kind or position of the substituents, or by the molecular skeleton. First, C-4'-methoxy substituents (**3**, **7**, **11**, **15**) were effective against *P. falciparum*, showing 36-100% inhibition at a concentration range of 5.4-5.8 μg/mL, and most C-3'-substitutions (**1**, **2**, **5**, **6**, **9**, **10**, **13**, **14**) had weak or no antimalarial activity, except compound **2** (40% inhibition) and **5** (100% inhibition, EC_{50} = 1.6 μg/mL). An α,β-unsaturated carbonyl structure (flavones **1**-**4** and chalcones **9**-**12**) increased the activity when the electron withdrawing or donating group was introduced at the C-3' or C-4' position. However, since the 3'-chloro-chalcones (**17**-**20**) were practically inactive, the substitution pattern in other aromatic rings may also affect antimalarial activity. Displacement of two methyl groups in compounds **4**, **12**, and **16** slightly elevated the activity compared to one methyl substitution (**1**, **9**, **13**) except **5**. Since the potent antimalarial activity of licochalcone A, a natural chalcone, was first reported,[22] over 200 chalcone derivatives have been assayed and their structure-activity relationships proposed,[19,22] but these studies did not include our novel synthetic chalcones.

In the cytotoxicity test, C-3'-chloro-substituted chalcones (**17**-**20**) with practically no antimalarial activity exhibited potent cytotoxicity against FM3A tumor cells (EC_{50} = 1.6-2.8 μg/mL). Together with the record of compound **10**, the chalcone compounds bearing a halogen group at C-3' with substituents in ring A were potent cytotoxic agents.

The introduction of other electron withdrawing or donating groups at C-4' in dihydrochalcones, as well as long-chain electron donating groups at C-3' in flavanones, would be useful for identifying other antimalarial agent.

Experimental Section

Materials. The flavonoid and chalcone derivatives used in this study were >98% pure and were synthesized according to methods described in our previous reports.[23,24] RPMI 1640 culture medium and fetal bovine serum were provided by Gibco (NY, U.S.A), and ES medium was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Giemsa staining reagent was obtained from Merck (Germany). All other chemicals and reagents were of the highest grade available.

Malaria Parasites. *Plasmodium falciparum* (ATCC 30932, FCR-3 strain) was used in this study. *P. falciparum* was cultivated by a modification of the method of Trager and Jensen[25] using a 5% hematocrit of type A human red blood cells suspended in RPMI 1640 medium, and supplemented with heat-activated 10% type A human serum. The plates were placed in a CO_2-O_2-N_2 incubator (5% CO_2, 5% O_2 and 90% N_2 atmosphere) at 37 °C, and the medium was changed daily until 5% parasitemia (which means the existence of 5 parasite-infected erythrocytes in every 100 erythrocytes).

Mammalian Cells. Mouse mammary tumor FM3A cells (wild-type, subclone F28-7)[26] were supplied by the Japanese Cancer Research Resources Bank (JCRB). FM3A cells were maintained in a suspension culture at 37 °C in a 5% CO_2 atmosphere in plastic bottles containing ES medium supplemented with 2% heat-inactivated fetal bovine serum.

***In vitro* Antimalarial Activity Assay.** The following procedures were used to assay antimalarial activity.[12,13] Asynchronously cultivated *P. falciparum* was used. Various concentrations of compounds in DMSO were prepared. Five microliters of each solution was added to the individual wells of a 24-well multi-dish.

Erythrocytes with 0.3% parasitemia were added to each well containing 995 μL of culture medium to give a final hematocrit level of 3%. The plates were incubated at 37 °C for 72 h in a multigas incubator (5% CO_2, 5% O_2 and 90% N_2 atmosphere). To evaluate the antimalarial activity of the test compound, we prepared thin blood films from each culture and stained them with Giemsa. A total of 10000 erythrocytes per thin blood film were examined by microscopy. All of the test compounds were assayed in duplicate at each final concentration (5.0-5.8 μg/mL). Drug-free control cultures were run simultaneously. All data points represent the mean of three experiments. Parasitemia in the control reached between 4% and 5% at 72 h. The EC_{50} value refers to the concentration of the compound necessary to inhibit the increase in parasite density at 72 h by 50% of the control.

Toxicity against a Mammalian Cell Line. FM3A cells grew with a doubling time of about 12 h. Prior to drug exposure, cell density was adjusted to 5×10^4 cells/mL. A cell suspension of 995 μL was dispensed to the test plate,

and the compound at various concentrations suspended in DMSO (5 μL) was added to individual wells in a 24-well plate. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. All of the test compounds were assayed in duplicate at each concentration. Cell numbers were measured using a micro cell counter CC-130 (Toa Medical Electric Co., Japan). All data points represent the mean of three experiments. The EC_{50} value refers to the concentration of the compound necessary to inhibit by 50% the increase in cell density of the control at 48 h. Selectivity refers to the mean EC_{50} value for FM3A cells divided by the mean EC_{50} value for *P. falciparum*.

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