Antimalarial Activity of Radicicol, Heptelidic Acid and Other Fungal Metabolites

YOSHITAKE TANAKA, KAZURO SHIOMI, KISEKO KAMEI†, MASAKO SUGOH-HAGINO, YUMI ENOMOTO, FANG FANG, YUUICHI YAMAGUCHI, ROKURO MASUMA, CHENG GANG ZHANG††, XIAN WU ZHANG†† and SATOSHI ŌMURA*

Research Center for Biological Function, The Kitasato Institute, Minato-ku, Tokyo 108, Japan
†Department of Parasitology, Teikyo University School of Medicine, Itabashi-ku, Tokyo 173, Japan
††The Institute of Applied Ecology, Shenyang Academy of Sciences, China

(Received for publication July 22, 1997)

In the course of our screening program for artemisinin-like antimalarial compounds from microorganisms, seven fungal metabolites such as radicicol and heptelidic acid were identified as active compounds. Some of them exhibited antimalarial activity *in vitro* against the human malaria parasite *Plasmodium falciparum* to the extent of approximately 1/10 as potent as artemisinin. Radicicol was moderately active *in vivo* against *Plasmodium berghei* in mice.

Malaria is one of the most important health problems in the modern world. Due to the prevalence of chloroquine-resistant human malaria parasites, new antimalarial drugs are urgently needed. Artemisinin, or qinghaosu in Chinese, is a currently most promising antimalarial agent. It is an endoperoxide sesquiterpene lactone isolated from an old Chinese herbal remedy. It is rapid-acting with low toxicity and very few serious adverse reactions. Artemisinin and its ether derivatives have already been put into clinical practice on a massive scale of several million doses in China, Thailand, Vietnam, Brazil, and African countries[1]. Unfortunately, artemisinin is poor in solubility, fairly high in the incidence of recurrence, and the supply in adequate amounts is limited because it is a compound of plant origin. Therefore, new and effective antimalarial agents are still required to secure the control of malaria with more certainty.

We have established a new screening system for antimalarial compounds from microorganisms. Emphasis was placed on compounds of artemisinin-like bioactivity, *i.e.* heme-dependent radical generating activity[1], and on compounds with a peroxide moiety. Details of this screening system will be reported elsewhere.

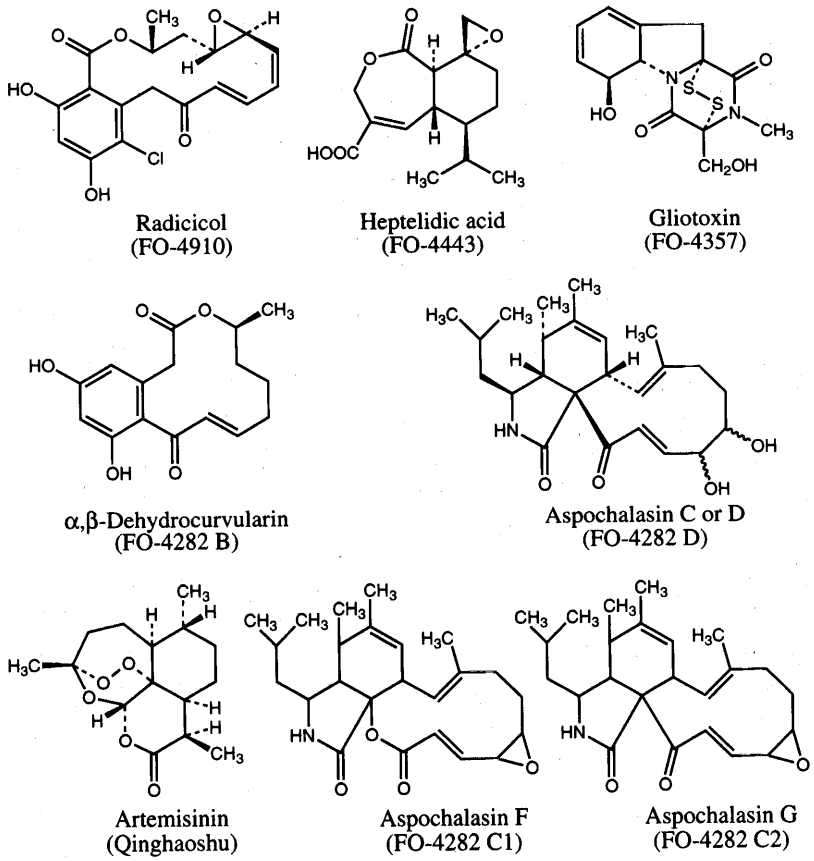
In the course of our antimalarial screening by this method, four fungal isolates were selected as candidate producers of potential antimalarial compounds. Two new active substances isolated from the culture of strain FO-4282 were described[2]. The other three cultures were strains FO-4910, FO-4443, and FO-4357, which were picked up because of potent artemisinin-like growth inhibition patterns against the indicator bacterium L or R in the presence of hemin. These cultures were grown and the active principles in the cultures were isolated and characterized. This paper describes the isolation, characterization and antimalarial activity of them. The results demonstrate that radicicol is an antimalarial compound with moderate *in vivo* activity in mice.

Materials and Methods

Microorganisms

Strains FO-4910, FO-4443, FO-4357, and FO-4282, the active substance-producing cultures, were isolated from soil samples. They were grown at 27°C on YpSs agar slants, and preserved under oil sealing. Newly isolated unidentified soil bacterial strains L and R were used to monitor the heme-dependent growth inhibition of active substances in the cultures. Artemisinin was used

Fig. 1. Structure of active microbial metabolites isolated as potential antimalarial compounds.



as positive control. Taxonomic studies on strain L or R are now underway. Artemisinin showed potent heme-dependent antibacterial activity against the two indicator bacteria L and R, and its reversal upon supplementation with α-tocopherol, a radical scavenger (see Detection of Heme-dependent Antibacterial Activity below and Table 4). Active cultures studied here were picked up as candidates because they showed heme-dependent growth inhibition patterns similar to that shown by artemisinin. Bacterium R was used for monitoring the active substance from FO-4910.

Fermentation

Spores and mycelia on an agar slant of strain FO-4910 were scraped off to inoculate into a test tube (i.d. 2 × 20 cm) containing 10 ml of a seed medium (GP, in Table 1). After incubation with reciprocal shaking (300 rpm) at 27°C for two days, 2 ml of this seed culture was transferred into conical flasks (500-ml volume) containing 100 ml of a second seed medium (F7, Table 1), incubated with rotary shaking (210 rpm) at 27°C for two days. A 2% (v/v) volume of this second seed culture was transferred into conical flasks (5-liter volume) containing 800 ml of F7 medium, which were incubated at 27°C with rotary shaking for 6 days. Cultivation of other cultures were carried out under conditions listed in Table 3.

In the study of cultivation conditions shown in Table 2 inorganic phosphate ion- or ammonium ion-trapping agents were added at 0.5% to the media tested. Allophane is an aluminosilicic clay (Shinagawa Chemicals, Tokyo) capable of trapping inorganic phosphate in fermentation media, while zeolite and magnesium phosphate are ammonium ion-trapping agents. When added to a fermentation medium, they often increases antibiotic yields by decreasing ammonium or phosphate contents in the culture[3].

Detection of Heme-dependent Antibacterial Activity

Heme-dependent antibacterial activity in fermentation broths and in purification steps was determined by a newly established paper disc assay, using strains L or R, as described[2]. This method is based on the finding of MESHNICK *et al.*[1] that the antimalarial activity of artemisinin involves heme-dependent radical generation[1]. Briefly, strain L-seeded nutrient agar (none), and the

Table 1. Composition of fermentation media.

GP medium	%
Glucose	2.0
Yeast extract	0.2
$MgSO_4.7H_2O$	0.05
Polypepton	0.5
KH_2PO_4	0.1
Agar	0.1
	pH 6.0

F7 medium	%
Sucrose	2.0
Glucose	1.0
Corn steep powder	0.5
Meat extract	0.5
$MgSO_4 \cdot 7H_2O$	0.05
KH_2PO_4	0.05
$CaCO_3$	0.3
Agar	0.1
Trace salts solution[a]	1.0 (v/v)
	pH 6.5

F8 medium	%
Soluble starch	3.0
Soybean flour	2.0
Glycerol	1.0
Dry yeast	0.3
KCl	0.3
$CaCO_3$	0.2
$MgSO_4 \cdot 7H_2O$	0.05
KH_2PO_4	0.05
	pH 6.5

F8M medium	%
Soluble starch	2.0
Soybean flour	1.0
Glycerol	0.5
Dry yeast	0.3
KCl	0.3
$CaCO_3$	0.2
$MgSO_4 \cdot 7H_2O$	0.05
KH_2PO_4	0.0
	pH 6.5

[a] Trace salts solution contained each at 1.0 g/liter: $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CuSO_4 \cdot 5H_2O$.

same nutrient agar supplemented with hemin (100 μg/ml) (+H), and additionally with tocopherol (400 μg/ml) (+H+T), were incubated at 37°C for 24~40 hours. Artemisinin showed no growth inhibition on L-agar, but gave a large inhibition zone in the presence of hemin (+H agar). The inhibition zone became smaller and hazy upon supplementation with α-tocopherol, a radical scavenger (+H+T agar) (see also Table 4). It was assumed that when a microbial metabolite showed heme-dependent growth inhibition against bacterium L, or in other words, when it exhibited an artemisinin-like growth inhibition pattern of +/−, +++, + on the three agar media, it was an indication that the substance had an activity to generate radicals in a heme-dependent manner.

Color Reaction

The peroxide nature of active compounds was detected by color reaction on TLC with *N,N*-dimethylphenylenediamine[4]. Artemisinin, giving a pink color, was used as positive control.

In Vitro Antimalarial Activity

A clinical strain of the human malaria parasite *Plasmodium falciparum* was isolated from a patient in Thailand, and maintained in Department of Parasitology, Teikyo University School of Medicine, Tokyo. Human red blood cells were suspended at 10% hematocrit units in RPMI-1640 medium (Gibco) containing 10% human serum, 25 mM HEPES buffer, and 32 mM $NaHCO_3$. A 0.2 ml aliquot was added into 96-well microplate.

Initial parasitemia was at about 1%. Test compounds were dissolved in methanol or acetone at 1 mg/ml, diluted serially with growth medium, and 2 μl aliquots were added to the *P. falciparum* culture at intervals indicated. Incubation was carried out at 37°C under N_2-CO_2-O_2 (85 : 5 : 10) atmosphere[5,6], with medium changes everyday. Parasite growth was measured under a microscope after Giemsa staining, and expressed as % parasitemia (% of parasite-infected red blood cells). The antimalarial effect of test compounds was evaluated by reduction of the rate of parasitemia, and expressed by the IC_{80} values (μg/ml). The IC_{80} value represents the concentration of a test compound which caused 80% net reduction in parasitemia at day 5, as compared with the control (no drug) cultures. Mean values of duplicate assays are shown in Table 4.

In Vivo Antimalarial Activity

In vivo antimalarial effect was measured in mice infected with *P. berghei*. The concentration of parasite-infected mouse erythrocytes was adjusted to 1,000

infected erythrocytes/ml, and a 0.1 ml aliquot was injected into the tail vein of the mice. A group was composed of 5 mice. Two days after infection, test compounds (oil suspension) were injected to each group of mice intramuscularly. Blood samples were taken from tails and parasitemia was recorded as in the *in vitro* assay.

Analytical Procedures

HPLC analyses were carried out using a Senshu HPLC system (model SSC-6530) equipped with a variable wavelength UV detector (model SSC-6500), with ODS columns (i.d. 20 × 250 mm). Open column chromatography was performed using silica gel (Wakogel C-300, Wako Pure Chem. Ind.) and ODS gel (ODS-7515-12, Senshu Scientific Co.) with mixtures of chloroform and methanol (0 : 100 ~ 100 : 0) and methanol or acetonitrile, respectively, as eluents. UV spectra were measured with a Shimadzu spectrophotometer, model UV-240. FAB-MS were recorded by a JEOL spectrometer, model JSM-A-505 HA. All NMR spectra were recorded on a JEOL JNM-EX270 spectrometer.

Results and Discussion

Taxonomy of Strain FO-4910

Strain FO-4910 was isolated from a soil sample collected in Oklahoma, U.S.A. On potato-dextrose agar and yeast extract soluble starch (YpSs) agar, this strain grew rapidly to form pale brown to gray colonies with diameter of 36 ~ 47 mm after incubation for 7 days at 25°C. Reverse color of colonies was brownish gray to charcoal gray. Soluble pigment was not produced. Conidia, moderate to abundantly born on the colony surface, were solitary, and were produced directly on the vegetative hyphae. They were smooth, dark brown, unicellular, thick-walled, globose to subglobose, and 7 ~ 12 μm in size. These characteristics indicate that the strain FO-4910 belongs to the genus *Humicola*.[7].

Isolation and Characterization of Active Substance from *Humicola* sp. FO-4910 (Radicicol)

The isolation procedures for radicicol from the FO-4910 fermentation were as follows. The activity was monitored using bacterium R, because it was susceptible to the active substance of FO-4910. The broth of 10.7 liters was centrifuged and separated into a supernatant liquid and a mycelial cake. The mycelial cake was extracted with acetone and concentrated *in vacuo*. The combined mycelial extract and culture filtrate were extracted with ethyl acetate, and the organic layer was concentrated *in vacuo*. The resulting yellow crude material (8.3 g) was applied to a silica gel column, which was eluted with $CHCl_3$ - methanol (99 : 1) to afford fr. I (1.3 g) and fr. II (2.2 g). Fr. I was purified finally by ODS column chromatography, eluted with 70% methanol, to give a pure material (261 mg).

The pure substance was a yellow powder with the UV absorption maximum in methanol observed at 265 nm. The molecular formula was deduced to be $C_{18}H_{17}O_6Cl$ (*m/z* 363.0620 $(M-H)^-$; calcd for $C_{18}H_{16}O_6Cl$, 363.0635) by HR-FAB-MS. The active substance from FO-4910 was identified as radicicol by NMR studies. It was reported as an antifungal antibiotic[8], and as an inhibitor of protein tyrosine kinase[9] and angiogenesis[10].

Fermentation, Isolation and Characterization of Active Substances from FO-4443 (Heptelidic Acid), FO-4357 (Gliotoxin), and FO-4282 (Dehydrocurvularin)

Before beginning the isolation process, preliminary optimization of fermentation conditions was carried out, which included monospore isolation of active cultures (FO-4910, FO-4443, and FO-4282) and selection of optimum media for seed growth and production of active substances. Table 2 shows that the addition of ion-trapping agents affected these cultures in production of active substances either positively (allophane for FO-4282) or negatively (allophane and natural zeolite for FO-4443 and FO-4357). The culture media giving the highest titers were used for fermentation.

The isolation procedures for heptelidic acid from the FO-4443 fermentation, and the isolation and identification of gliotoxin from FO-4357 are summarized in Table 3. They are briefly described as follows.

The culture filtrate (65 liters) of FO-4443 was extracted with chloroform at pH 3 and back-extracted at pH 7. The crude extract (2.53 g) was purified by using a silica gel column, eluted with $CHCl_3$ - methanol (100 : 1), and then by ODS column chromatography, which was eluted stepwise with 0 ~ 100% CH_3CN, to obtain active material (576 mg). A part (50 mg) was used to purify finally by HPLC (mobile phases, 20% and 50% CH_3CN both in 0.1 M citric acid-0.2 M Na_2HPO_4 buffer (pH 4); column, Capcell pak C18, i.d. 20 × 250 mm; flow rate, 7 ml/minute; detection at 210 nm). The active peak fractions collected and concentrated were extracted with chloroform at an acidic pH to give a pure substance (14 mg). FAB-MS revealed the quasi-molecular ion peak $(M+H)^+$ of 281. The active substance from FO-4443 was identified as

Table 2. Effect of additives on production of active compounds.

Additive (0.5%)	Inhibition zone (mm) against bacterium L on a hemin-supplemented agar [a]		
	FO-4443 F8M, 4 days (x10 concd)	FO-4357 F7, 5 days (x100 dil)	FO-4282 F8, 5 days
None	16.5	16.0	12.8
+ Allophane	-/+	12.1	21.9
+ Zeolite	nd	11.0	nd
+ $Mg_3(PO_4)_2.8H_2O$	nd	18.0	nd

[a] Strain FO-4443 was cultivated for 4 days in 100 ml of F8M medium in 500-ml volume of conical flask, to which the indicated additives were added. The other cultures were cultivated in similar manners as indicated. Active substances were extracted with an equal volume of ethyl acetate, and the organic layer, with or without concentration and dilution, was used for a paper disk assay (thick disk of 8 mm diameter).

Table 3. Summary of fermentation, isolation, and characterization of active substances from FO-4443, FO-4357, and FO-4282.

	FO-4443	FO-4357	FO-4282
Fermentation			
Seed	1) GP, 2 days 2) F7, 2 days	GP, 2 days	F7, 2 days
Production	F8M, 4 days	F8, 4 days	F8 (+)[a], 3 days
Isolation	EtOAc ext. SiO_2 column HPLC on ODS	EtOAc ext. SiO_2 column HPLC on ODS	EtOAc ext. SiO_2 column HPLC on ODS
Culture broth	70 liters	1.7 liters	55 liters
Pure substance obtained	150 mg	20 mg	35 mg
Characterization			
UV (nm)	end	270	end
MS $(M+H)^+$	281	327	291
Identified as	Heptelidic acid	Gliotoxin	Dehydrocurvularin
References	11-14		2

[a] F8 (+) represents F8 medium supplemented with 0.5% of allophane.

heptelidic acid by NMR studies. Heptelidic acid (a.k.a. avocetin and konigic acid) was reported as a fungal metabolite active against anaerobic bacteria[11~13]. The inhibition of glyceraldehyde-3-phosphate dehydrogenase was also reported[14].

The cultured broth of 1.7 liters of FO-4357 was centrifuged and separated. The mycelial acetone extract and culture filtrate were combined and extracted with ethyl acetate. After concentration, the resulting residue (734 mg) was purified by chromatography on silica gel, eluted with chloroform-acetone, then on a Sep-Pak plus C18 cartridge (Waters). Active material (93 mg) was further purified by HPLC (mobile phase, 35% CH_3CN; column, ODS-5251 (Senshu Scientific Co.), i.d. 20 × 250 mm; flow rate, 7 ml/minute; detection at 210 nm), to give 20 mg of pure substance. This substance showed a UV absorption maximum at 270 nm and the quasi-molecular ion peak $(M+H)^+$ of 327. The active substance was identified as gliotoxin by comparison with their TLC and HPLC.

The structure of these active metabolites are depicted in Fig. 1. For the assays described below the active compounds isolated from *Aspergillus* sp. FO-4282 [aspochalasins C, F, and G, and dehydrocurvularin (ref. 2 and Table 3)] were also employed.

Heme-dependent Growth Inhibition of Active Substances against Bacterial Strains L and R and Their Color Reaction

In this study active cultures were selected and active

Table 4. Heme-dependent growth inhibition and *in vitro* antimalarial activity of active microbial metabolites.

Compound	Response to coloring reagent	Solution conc. (mg/ml)	Inhibition zone vs bacterium L (mm)[a]			*In vitro* antimalarial activity IC_{80} (μg/ml)		Cytotoxic activity against melanoma B16 cells IC_{50} (μg/ml)
			None	+H	+H+T	Merozoites	Schizonts	
Radicicol (FO-4910)	–	1	11[b]	17[b]	15[b] (h)	0.01	0.01	> 10
Heptelidic acid (FO-4443)	–	1	15	24	–	0.01	0.01	10
Gliotoxin (FO-4357)	–	0.01	–	20	–	0.1	0.1	1
Aspochalasin C or D (FO-4282 D)	++	1	–	–	11	>5	ND	–
Aspochalasin F (FO-4282 C1)	++	1	–	–	–	>5	ND	>25
Aspochalasin G (FO-4282 C2)	++	1	14	18	13 (h)	>5	ND	>25
α,β-Dehydrocurvularin (FO-4282 B)	++	1	–	–	–	>5	ND	ND
Artemisinin (Qinghaosu)	++	1	–	26	20 (h)	<0.01	<0.01	>10

[a] Thick disks (8 mm in diameter) were used; H, hemin; T, tocopherol; –, No inhibition zone; (h), hazy zone; ND, not determined.
[b] Growth inhibition against strain R.

metabolites in the cultures were purified by monitoring heme-dependent growth inhibition against an indicator bacterial strain L or R, and/or positive color reaction with a peroxide-reactive reagent, *N,N*-dimethylphenylenediamine. Radicicol, heptelidic acid, and gliotoxin were obtained in this study by heme-dependent growth inhibition characteristics. On the other hand, four other compounds were isolated by intense color characteristics, which included the two new aspochalasin components F and G, and aspochalasin C and dehydrocurvularin described in the previous paper[2].

Heme-dependent growth inhibition and color reaction of the seven substances were measured. Table 4 shows that radicicol, heptelidic acid, and gliotoxin, and aspochalasin G as well, inhibited the indicator strain L or R in a heme-dependent manner as did artemisinin. The other three compounds exhibited intense colors (pink, violet, and/or blue-red) upon spraying on TLC with the color reagent employed.

The potent growth inhibition in the presence of hemin, and its reversal upon supplementing with tocopherol, a radical scavenger, suggest strongly that the growth inhibition against the indicator bacterium involved radical generation. Therefore, it is assumed they are heme-dependent radical generators under the assay conditions employed, although these compounds were not structural analogues of artemisinin, nor possessed an endoperoxide moiety in the structure. Studies on radical generation from these microbial metabolites are now underway.

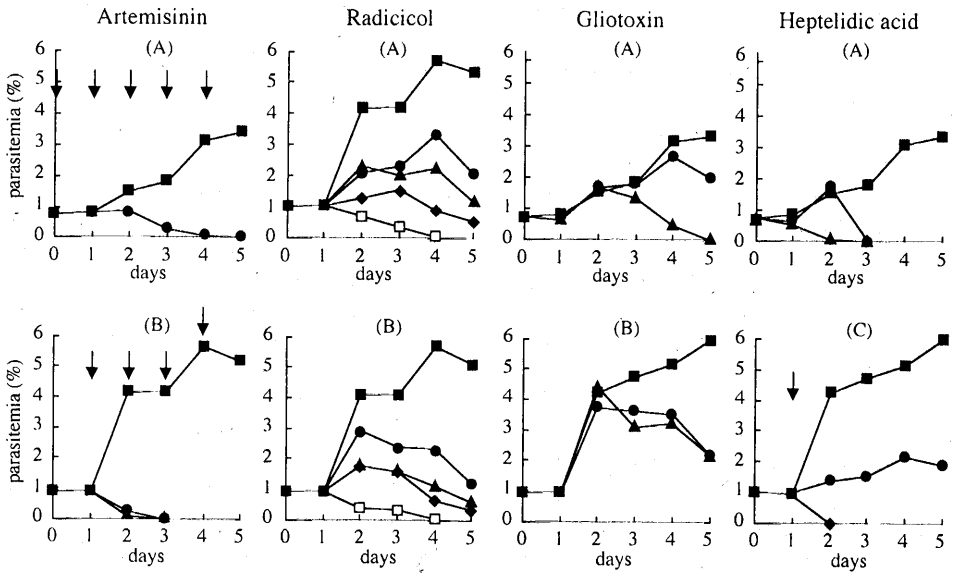
In Vitro Antimalarial Activity

In vitro antimalarial activity of the active microbial metabolites was determined against human malaria parasite, *P. falciparum*. The test compounds dissolved in methanol or acetone were added from day 0 (regime A) or day 1 (regimes B and C) to examine the effect against the early (merozoites) and late (schizonts) phases respectively in the schizogony of human malaria parasite (see footnote to Fig. 2).

Artemisinin was potently active against both phases of parasite at 0.01 μg/ml (34 nM) (Fig. 2). Among the microbial compounds isolated, radicicol was approximately 1/10 as potent as artemisinin in both regimes A and B. Heptelidic acid was also active against *P. falciparum*, although less potent than artemisinin. The cytotoxicities of radicicol and heptelidic acid were low (Table 4). Gliotoxin was active but the cytotoxicity was high. Other compounds tested were active only marginally. It is noteworthy that potent antimalarial activity was found among those compounds detected and isolated by heme-dependent antibacterial activity, *i.e.* those showing artemisinin-simulating and heme-dependent radical generating activity. Comparing radicicol and gliotoxin in Table 4, the *in vitro* antimalarial activity did not appear to correlate with the potency of heme-dependent growth inhibition.

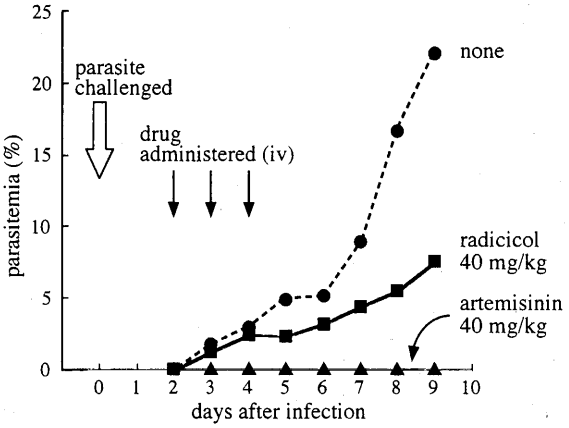
Fig. 2. *In vitro* antimalarial activity of radicicol, heptelidic acid, and gliotoxin against human malaria parasite, *Plasmodium falciparum*.

■ 0 μg/ml, ● 0.01 μg/ml, ▲ 0.1 μg/ml, ◆ 1 μg/ml, □ 10 μg/ml.



(A), (B), and (C) represent regimes of drug addition. Artemisinin was used as control. Under the culture conditions employed, malaria parasites at day 0 were merozoites. Most of them grew up to schizonts by day 1, after which active schizogony followed. At day 5, very little gametocytes were observed.

Fig. 3. Antimalarial activity of radicicol against *Plasmodium berghei* in mice.



In Vivo Antimalarial Activity of Radicicol

Purified samples of radicicol and heptelidic acid were obtained in 100 mg amounts. They were used to determine *in vivo* antimalarial activity against *P. berghei* in mice. The results shown in Fig. 3 indicate that radicicol is moderately efficacious in suppressing the proliferation of *P. berghei* in mice at 40 mg/kg (i.v.). Artemisinin at the same dose level resulted in complete suppression of parasitemia. Heptelidic acid, on the other hand, caused hemolysis at 40 mg/kg. Radicicol was reported as a fungal metabolite with antifungal activity, and as an inhibitor of protein tyrosine kinase[8,9]. The potent *in vitro* antimalarial activity of radicicol is a new finding demonstrated in this study for the first time.

Although the moderate *in vivo* efficacy of radicicol dose not appear to warrant its application as an antimalarial compound *per se*, it is useful as a new lead for chemical modification to generate newer derivatives and for a search of analogous compounds from microorganisms. Unlike the restricted supply of artemisinin, the supply of radicicol by fermentation is practically limitless.

Acknowledgment

This work was supported in part by a grant from Ministry of Health and Welfare for research and development of tropical medicines. We thank Ms. AKIKO NAKAGAWA and Ms. CHIKAKO SAKABE, School of Pharmaceutical Sciences, Kitasato University for measurements of mass spectra. We are also grateful to Mr. MASAKI CHIBA and Ms. TSUYAKO WATANABE of Research Center for Biological Function, The Kitasato Institute for measurements of antimicrobial activity. We are indebted to Mr. YASUYUKI KIMURA, Miss MIYUKI ISHII, Miss MASAKO IWABUCHI, and Miss MIKA HISAMATSU for their helpful assistance.

References

1) MESHNICK, S. R.; T. E. TAYLOR & S. KAMCHONWONGPAISAN: Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol. Rev. 60: 301～315, 1996
2) FANG, F.; H. UI, K. SHIOMI, R. MASUMA, Y. YAMAGUCHI, C. G. ZHANG, X. W. ZHANG, Y. TANAKA & S. ŌMURA: Two new components of aspochalasins produced by *Aspergillus* sp. J. Antibiotics 50: 919～925, 1997
3) MASUMA, R.; Y. TANAKA & S. ŌMURA: Production of nanaomycin and other antibiotics by phosphate-depressed fermentation using phosphate trapping agents. J. Antibiotics 39: 1557～1564, 1986
4) KREBS, K. G.; D. HEUSSER & H. WIMMER: Spray Reagents (No. 78). *In* Thin-Layer Chromatography. 2nd Ed. *Ed.*, STAHL, E., pp. 854～909, Springer-Verlag, Berlin etc., 1969
5) ZHANG, Y. A.; K. S. O. ASANTE & A. JUNG: Stage-dependent sensitivity to chloroquine of *Plasmodium falciparum in vitro*. Acta Leidensia 55: 218, 1987
6) MONS, B.: *In vitro* cultivation of the erythrocytic stages of *Plasmodium berghei*. Acta Leidensia 54: 11～61, 1986
7) BARRON, G. L.: The Genera of Hyphomycetes from Soil. pp. 207～208, Robert E. Krieger Publishing Co., Malabar, 1968
8) AYER, W. A.; S. P. LEE, A. TSUNEDA & Y. HIRATSUKA: The isolation, identification, and bioassay of the antifungal metabolite produced by *Monocillium*. Can. J. Microbiol. 26: 766～773, 1980
9) KWON, H. J.; M. YOSHIDA, Y. FUKUI, S. HORINOUCHI & T. BEPPU: Potent and specific inhibition of $p60^{v\text{-}src}$ protein kinase both *in vivo* and *in vitro* by radicicol. Cancer Res. 52: 6926～6930, 1992
10) OIKAWA, T.; H. ITO, H. ASHINO, M. TOI, T. TOMINAGA, I. MORITA & S. MUROTA: Radicicol, a microbial cell differentiation modulator, inhibits *in vivo* angiogenesis. Eur. J. Pharmacol. 241: 221～227, 1993
11) ARIGONI, D.: Stereochemical aspects of sesquiterpene biosynthesis. Pure Appl. Chem. 41: 219～245, 1975
12) ITOH, Y.; K. KODAMA, K. FURUYA, S. TAKAHASHI, T. HANEISHI, Y. TAKIGUCHI & M. ARAI: A new sesquiterpene antibiotic, heptelidic acid. Producing organisms, fermentation, isolation and characterization. J. Antibiotics 33: 468～473, 1980
13) STIPANOVIC, R. D. & C. R. HOWELL: X-ray crystal structure determination, and biosynthesis of the antibiotic, heptelidic acid. Tetrahedron 39: 1103～1107, 1983
14) ENDO, A.; K. HASUMI, K. SAKAI & T. KANBE: Specific inhibition of glyceraldehyde-3-phosphate dehydrogenase by konigic acid (heptelidic acid). J. Antibiotics 38: 920～925, 1985