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Heme-dependent Radical Generation: Possible Involvement in Antimalarial Action of Non-peroxide Microbial Metabolites, Nanaomycin A and Radicicol

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Antimalarial screening was performed for microbial metabolites that simulate artemisinin in their mode of action, a potent antimalarial component of an herbal remedy with a characteristic peroxide structure. Nanaomycin A was identified in this screen as an antimalarial compound, together with radicicol and several other compounds already reported (J. Antibiotics 51: 153~160, 1998). Nanaomycin A inhibited *in vitro* growth of the human malaria parasite *Plasmodium falciparum* with an IC_{80} value of 33.1 nM. It was as potent as radicicol and about 1/10 as potent as artemisinin. Studies on the mode of action suggested that the antimalarial action of the two non-peroxides, nanaomycin A and radicicol, involved heme-dependent radical generation, as is for the peroxide artemisinin. Namely, the inhibition of *in vitro* growth of malaria parasite by nanaomycin A or radicicol was reversed by tocopherol, a radical scavenger added to the assay mixture. Secondly, in a reaction system established for radical detection, in which a test radical donor and β-alanylhistidine as a radical recipient were incubated with and without hemin, the two compounds caused heme-dependent decreases of β-alanylhistidine, as did artemisinin. Among the 14 microbial metabolites identified during this screening, a correlation was observed between antimalarial activity and heme-dependent radical generating activity.

Artemisinin is an endoperoxide sesquiterpene lactone isolated from a traditional Chinese herbal remedy. Artemisinin and its chemically synthesized derivatives are currently in clinical practice as effective antimalarial agents. According to the recent studies by MESHNICK *et al.*, the antimalarial action of artemisinin appears to be mediated by free radicals.[1~3] Artemisinin contains an endoperoxide bridge (Fig. 1), which was suggested to be essential for antimalarial activity,[3,4] because derivatives devoid of this bridge were inactive as antimalarial agents. This moiety reacts with intraparasitic heme irons to generate *O*-radicals and *C*-radicals,[4~6] the latter of which were capable of alkylating proteins.[7,8] This reaction, and therefore the peroxide moiety, appears to be crucial in the antimalarial activity of artemisinin.

In view of the potent antimalarial activity and peculiar structure of artemisinin, we have established a screening system for antimalarial compounds of microbial origin with artemisinin-like mode of action. In the course of a screening run using this system, we found that radicicol, heptelidic acid and other fungal metabolites exhibited *in vitro* antimalarial activity against *Plasmodium falciparum*.[9,10] Radicicol was also effective *in vivo* against *P. berghei* in mice.[10] In our continual efforts in search for antimalarial microbial metabolites, we found that nanaomycin A, an antifungal quinone[11~17] from *Streptomyces*, behaved just like artemisinin in our primary screen employed. It was reasoned that nanaomycin A would be active as an antimalarial agent and that nanaomycin A and radicicol possess a similar mechanism as that of artemisinin in their antimalarial actions.

In this paper we will show antimalarial activity of

nanaomycin A and related homologues. Evidence will be presented in support of a hypothesis that the mechanism of antimalarial action of nanaomycin A and radicicol involves, in common with artemisinin, reactive radicals generated from the non-peroxide microbial products in the presence of hemin. A preliminary account of these results has appeared.[18]

Materials and Methods

Antimalarial Compounds and Reagents

Nanaomycins were isolated as described.[11~13] Radicicol was isolated from *Humicola* sp. FO-4910, as reported.[10] Artemisinin was purchased from Sigma Co., U.S.A. All the test compounds used in this work were 95% pure or higher. NBD-F (4-fluoro-7-nitrobenzo-2,1,3-oxadiazole) was purchased from Dojin Chemical, Tokyo. The other reagents of reagent grade were all from Kanto Chemicals, Tokyo.

In Vitro Antimalarial Assay

Teikyo strain and NIHJ strain of the human malaria parasite *Plasmodium falciparum* were used. The Teikyo stain was originally isolated from a patient in Thailand, and maintained in Department of Parasitology, Teikyo University School of Medicine, Tokyo, Japan. The NIHJ strain was obtained from NIH, Tokyo, Japan. *In vitro* antimalarial activity was assayed according to the method reported previously.[10] Briefly, either of the two strains was grown on human red blood cells, which 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. Test compounds were dissolved in methanol at 1 mg/ml, diluted serially with growth medium, and a 2 μl aliquot was added to the *P. falciparum* culture at day 0 through 3. Incubation was carried out at 37°C under N_2-CO_2-O_2 (85 : 5 : 10) atmosphere. 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 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 4, as compared with the control (no drug) cultures. Mean values of duplicate assays are shown. Methanol at 1% (v/v) gave no effect on parasite growth.

Reaction Mixture

The reaction mixture for detection of radicals was a modification of the method of YANG *et al.*[8] Unless stated otherwise, it contained in a final volume of 1.0 ml: test substance (5 mg/ml-ethanol) 200 μl, 2.5 mM β-alanylhistidine (β-Ala-His) in sodium phosphate buffer 100 μl, 10 mM hemin in dilute NaOH (*ca.* 0.001 N) 20 μl, and 20 mM sodium phosphate buffer (pH 7.2) 680 μl. Control reaction mixtures were made by no addition of β-Ala-His, or no addition of hemin. After incubation at 37°C, 100 μl aliquots were taken at intervals indicated, diluted with water to 1000 μl, and stored frozen until use for assay.

Fluorometric Determination of β-Ala-His

The amounts of residual β-Ala-His in the reaction mixtures were determined by fluorometry using the fluorogenic reagent NBD-F.[19] The fluorogenic reaction mixture contained in the final volume of 200 μl: A solution of 0.1 M borax (pH 9.3) 50 μl, 10-fold diluted reaction mixture 50 μl, and 0.4 mg of NBD-F/ml-acetonitrile (freshly prepared) 100 μl. The reaction mixtures were covered with an aluminum sheet, heated at 60°C for 90 seconds, and then cooled in ice, and mixed with 0.2 N HCl 800 μl. A 200 μl volume of the acidic assay mixture was put into 96-well microplate for fluorometric record at 470 nm (excitation) and 530 nm (emission), with a fluorometer (Cytofluor II, model 4000, Perseptive Biosystem, U.S.A.). Duplicate reactions for radical generation were conducted and each reaction sample was subjected to duplicate fluorometric assays. Mean values are expressed in percent of control.

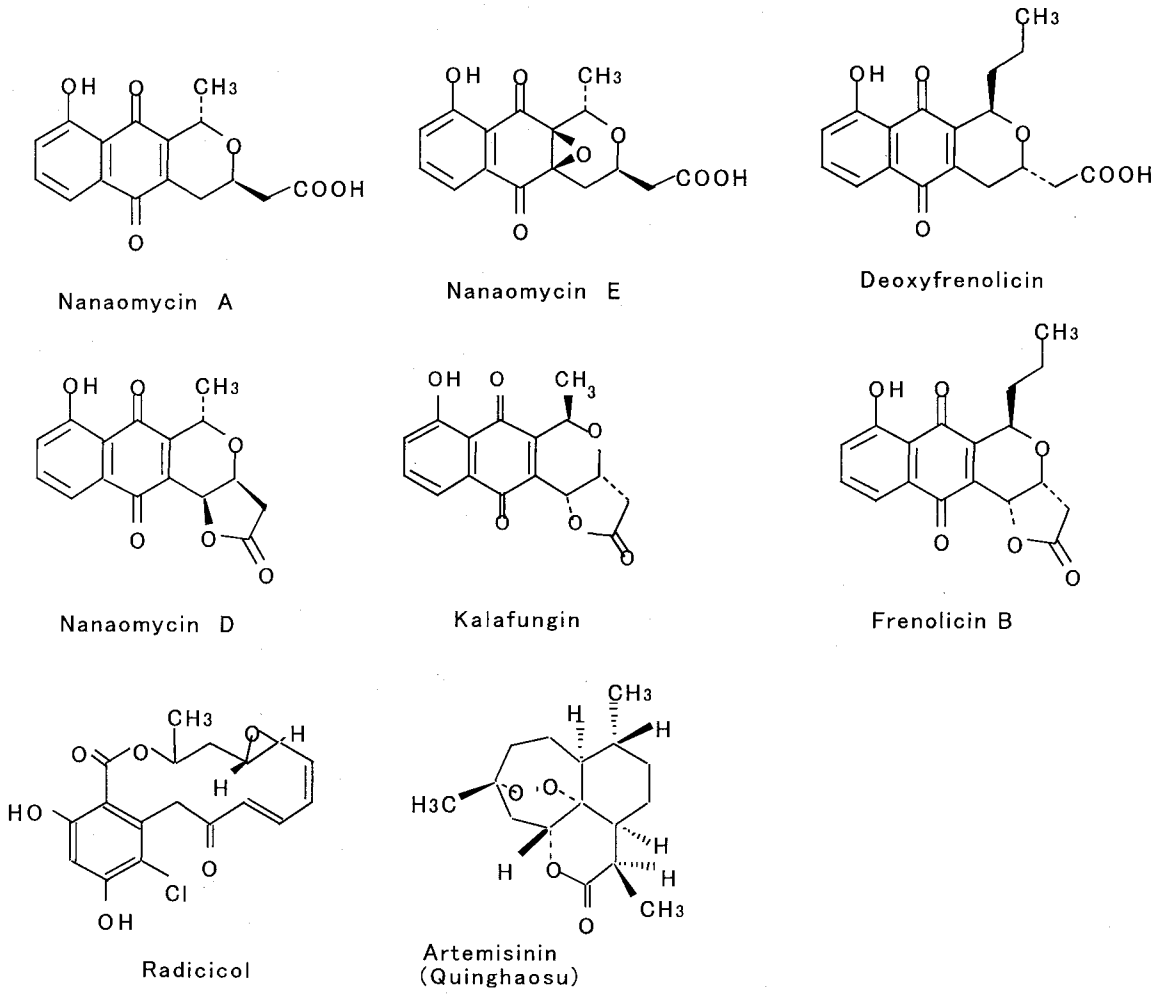
Results

Antimalarial Activity of Nanaomycin A and Related Compounds

As reported previously, our screening system focuses on microbial metabolites that simulate artemisinin in mode of action. Candidate cultures were selected when they showed growth inhibition against an indicator bacterium on hemin-supplemented nutrient broth, but did not on hemin-free medium, as did artemisinin.[9] Some of the candidate cultures produced quinone-like compounds. The heme-dependent growth inhibition by the quinone-like compounds present in a culture extract was reversed by tocopherol, a radical scavenger added to the agar medium. Later, one of the products of a candidate culture was found to be nanaomycin A.

Nanaomycin A was originally discovered by ŌMURA *et al.* as an antifungal compound of the naphthoquinone group produced by *Streptomyces rosa* subsp. *notoensis* KA-301[11]

Fig. 1. Structures of nanaomycin-related compounds, radicicol and artemisinin.



(Fig. 1). Later, the same authors reported additional components of the nanaomycin family, such as nanaomycins B, D, and E,[12,13] their biosynthesis and its regulation.[14~16]

Fig. 2 shows antimalarial activity of nanaomycin components against NIHJ strain of the human malaria parasite *Plasmodium falciparum*. Nanaomycin A was as potent as radicicol (IC_{80} value of 32.3 nM) (see also Fig. 3). The extent of antimalarial activity was about 1/10 or less than that of artemisinin. As illustrated in Fig. 1, compounds of the nanaomycin family can be divided into carboxylic acid components (nanaomycins A and E) and a lactone component (nanaomycin D). The former group of nanaomycins appears to be more potent than the latter. Table 1 summarizes the antimalarial activities of nanaomycin components and nanaomycin-related compounds. It appears that Teikyo strain is more susceptible than NIHJ strain to antimalarial compounds. Nanaomycin A and radicicol were most potent against both strains among the compounds tested in this study.

Effect of Tocopherol on Antimalarial Activity of Nanaomycin A and Radicicol

In vitro and *in vivo* antimalarial activities of artemisinin are antagonized by radical scavengers.[1,3] In an approach toward the understanding of the mechanism of antimalarial action of nanaomycin A and radicicol, the effect of tocopherol, a radical scavenger, on antimalarial activity of the two compounds was studied. The human malaria parasite *P. falciparum* (NIHJ strain) was grown in human red blood cells. To the culture, a test compound was added. Artemisinin was used as reference. Tocopherol was further supplemented, and the effect on parasitemia was examined

Fig. 2. *In vitro* antimalarial activity of nanaomycins and related compounds on *Plasmodium faciparum* (NIHJ strain).

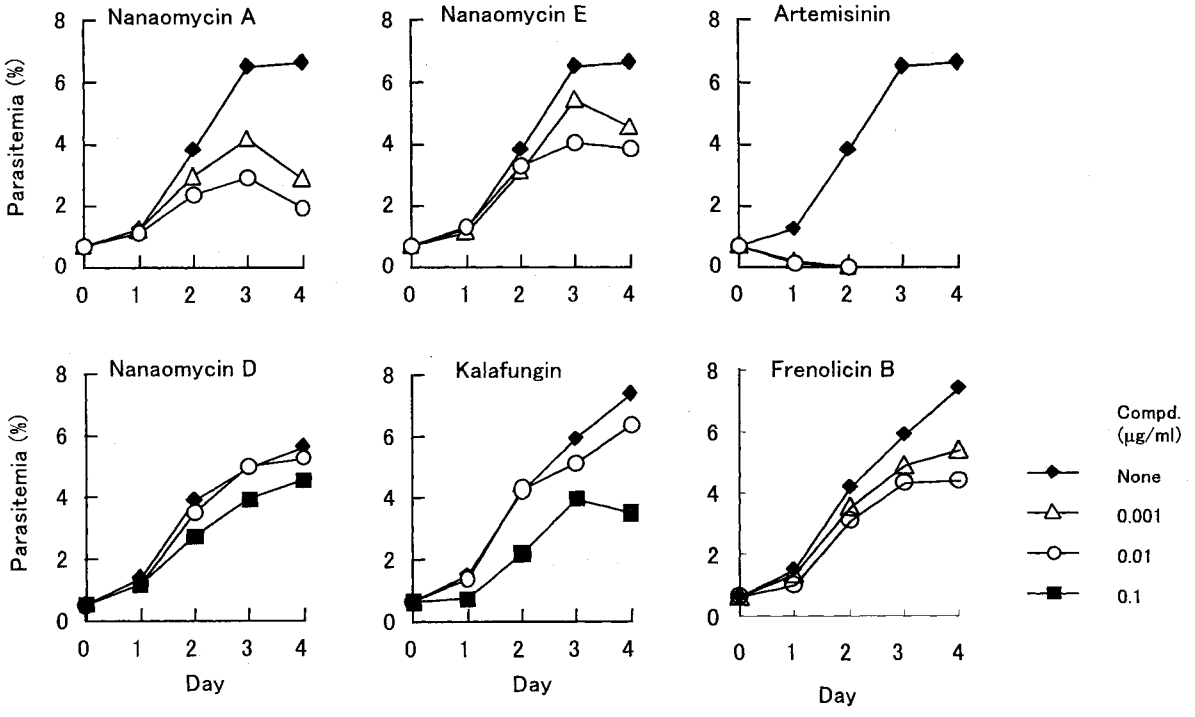
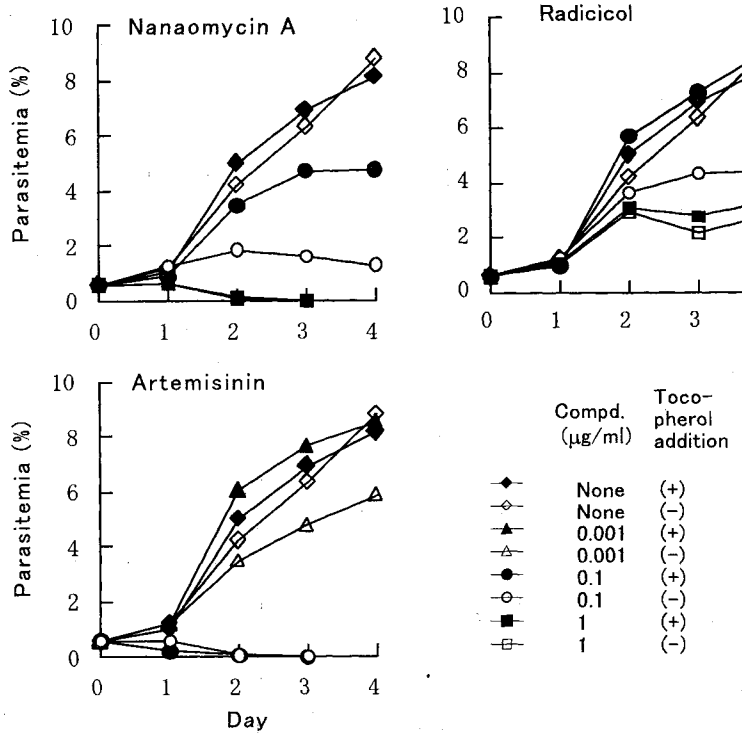


Fig. 3. Reversal effect of tocopherol on antimalarial activity of nanaomycin A and radicicol.

Antimalarial activity of the indicated compounds was assayed as described in Materials and Methods. When indicated, α-D-tocopherol (10 μg/ml in final) was added at day 0 through 3.



microscopically after Giemsa staining. Fig. 3 shows that the antimalarial activities of nanaomycin A and radicicol were reversed to different extents by tocopherol added to the cultures. Artemisinin behaved in a similar manner. It is suggested that the antiparasitic effect of the two compounds is mediated by radicals and that the reversal was caused by the radical scavenging effect of tocopherol.

Reaction System for Radical Detection

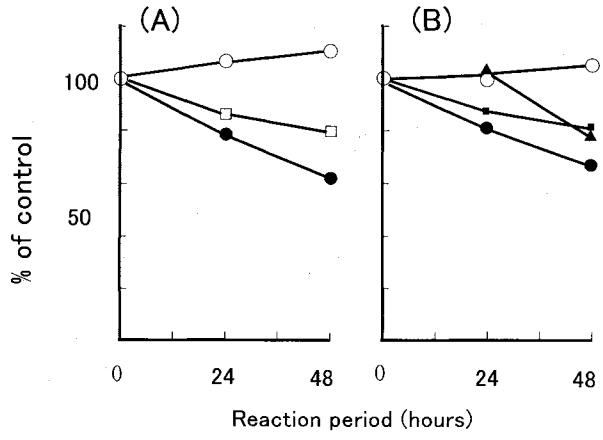
MESHNICK *et al.*[3] proposed that the mechanism of antimalarial action of artemisinin involved heme-dependent generation of *O*- and *C*-radicals. The best way in demonstrating the possible involvement of radicals would be to detect them in reaction mixture. These authors demonstrated heme-dependent decreases of reactive SH-groups of membrane proteins occurring in the presence of artemisinin. In this study, artemisinin as radical donor and human serum albumin or the membrane fraction of red blood cells as radical recipient were incubated for 12 hours, and the decreases of reactive SH groups were determined by SH-reactive DTNB (dithio-bis-nitrobenzoate).[2~4] Unfortunately we failed to reproduce their observations. In addition, the above assay method suffers a defect that the structure determination is difficult for a reaction product formed by the reaction of artemisinin radicals with protein-SH groups. Therefore we attempted an alternative approach.

In our reaction mixture,[18] artemisinin was used as radical donor, and a low molecular weight amine such as amino acids and peptides as radical recipient. They were incubated in the presence and absence of hemin. The amounts of residual amino group were determined by fluorometry with NBD-F as fluorogenic reagent.[9] Fig. 4A demonstrates that by reactions with artemisinin under physiological conditions in the presence of hemin, β-alanylhistidine (β-Ala-His) and 3-amino-1-propanol (β-alaninol) decreased obviously, whereas other amino acids tested (L-lysine, L-glutamic acid, L-arginine, glycine, and histamine) did not decrease under the conditions employed. The decrease of β-alanine was not steadily reproducible. Without artemisinin β-Ala-His remained unchanged in the presence of hemin (not shown).

The decrease of β-Ala-His was more rapid with 3~4 mM of artemisinin than with 1 mM artemisinin (data not shown). Hemin (0.1 mM) could be replaced by Fe (II) (0.5 mM $FeSO_4$) and Fe (III) [0.5 mM Fe $(NH_4)(SO_4)_2$] (Fig. 4B). We believe that the decreases of β-Ala-His and of 3-amino-1-propanol were due to reactions with radicals that were generated from artemisinin in the presence of heme irons.

Fig. 4. Amines and irons as reactants in radical generation from artemisinin.

In A, 4 mM artemisinin as radical donor was incubated with none (○), 0.25 mM 3-amino-1-propanol (□), 0.25 mM β-alanylhistidine (●), as radical recipient in the presence of 0.1 mM hemin. In B, 4 mM artemisinin and 0.25 mM β-alanylhistidine were incubated with none (○), 0.5 mM $Fe(SO_4)$ (▲), 0.5 mM $(NH_4)Fe(SO_4)_2$ (■), or 0.1 mM hemin (●). After the incubation periods indicated the amounts of a residual amine were determined by the NBD-F method.



An observation in support of this interpretation is that the addition of vitamin C, a water-soluble radical scavenger, to the reaction mixture diminished the decrease.

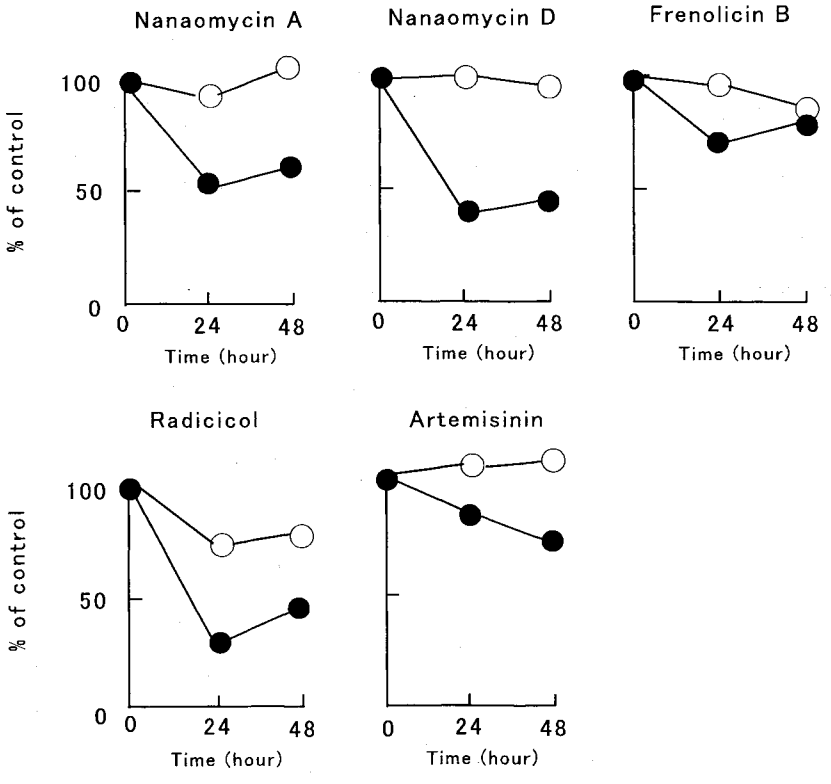
Suggested Radical Generation from Non-peroxide Microbial Metabolites

Based on the results described above, the reaction condition was set up, in which artemisinin or microbial products were used as radical donors, and β-Ala-His as radical recipient. The reactants were incubated in the presence or absence of hemin. The results are shown in Fig. 5. When artemisinin was used as radical donor, it caused 40% reduction of β-Ala-His in the presence of hemin, but did little changes in its absence. A small increase was observed occasionally. When non-peroxide microbial products were incubated individually in place of artemisinin, nanaomycins A and D, and radicicol did as artemisinin did, although heme-independent decreases in smaller amounts of radicicol was also evident. The decrease in peptide amine at 24 hours caused by frenolicin B was smaller than that by nanaomycin A.

Table 1 summarizes the changes in amount of β-Ala-His in the reaction mixture, occurring in the presence of

Fig. 5. Heme-dependent decreases of β-alanylhistidine caused by antimalarial microbial metabolites of non-peroxide structure.

Nanaomycin A or others (each 3 mM) was incubated with 0.25 mM β-alanylhistidine in the presence (●) and absence (○) of 0.1 mM hemin. The structures of the test compounds are shown in Fig. 1.



nanaomycins and related compounds and the other microbial metabolites so far identified in our antimalarial screening. *In vitro* antimalarial activity of these compounds is also shown for comparison. In addition to nanaomycins, some other compounds such as gliotoxin caused heme-dependent decreases in β-Ala-His, but others did not. Included in the latter were aspochalasins F, G, dehydrocurvularin and ergosterol.

Those compounds that showed high activities in β-Ala-His decreases at 24 hours of incubation were nanaomycins A and D, radicicol and heptelidic acid. Those with potent antimalarial activity against the Teikyo strain (0.01 μg/ml) included all of them. Nanaomycins A-beta, E, and E-alpha were also high in either of the two activities, but were not assayed in a paired way. Whereas, frenolicin B, aspochalasins F, G and dehydrocurvularin were not potent in neither activity.

Discussion

The results presented demonstrate good *in vitro* antimalarial activity of nanaomycins and related compounds. The extent of *in vitro* activity of nanaomycin A was about the same or somewhat higher than that of radicicol (Fig. 3). Among compounds of the nanaomycin group, nanaomycins of the carboxylic acid type (nanaomycins A and A-beta, deoxyfrenolicin) were more potent in antimalarial activity than the lactone type of nanaomycins (nanaomycin D, kalafungin, and deoxyfrenolicin). The above structure-activity relationship is contrasted with their anticoccidial activity, where frenolicin B was the most potent among nanaomycin and related compounds studied.[17]

The mode of antimalarial action of nanaomycin A and of radicicol was suggested strongly to resemble that of artemisinin in that it involved heme-dependent radical generation. This was shown by the following results. First,

Table 1. Heme-dependent decreases of β-alanylhistidine and antimalarial activity of non-peroxide microbial metabolites.

Active Substance (3 mM)	Hemin addition (0.1mM)	Changes (%) in amount of β-alanylhistidine at 24th h	Changes (%) in amount of β-alanylhistidine at 48th h	*In vitro* antimalarial activity # IC_{80} (μg/ml) vs NIHJ	Teikyo
Nanaomycin A	(+)	-45	-17	0.01	0.01
	(--)	-5	+17		
Nanaomycin A-beta	(+)	nd	nd	0.1	0.01
	(--)	nd	nd		
Nanaomycin E	(+)	-24	-19	0.1	nd
	(--)	+27	-3		
Nanaomycin E-alpha	(+)	nd	nd	0.1	0.01
	(--)	nd	nd		
Deoxyfrenolicin	(+)	+6	-4	1.0	nd
	(--)	+19	+23		
Nanaomycin D	(+)	-62	-51	1.0	0.01
	(--)	+5	-2		
Frenolicin B	(+)	-24	-12	nd	1
	(--)	-2	-8		
Kalafungin	(+)	nd	nd	1.0	nd
	(--)	nd	nd		
Radicicol	(+)	-72	-57	0.01	0.01
	(--)	-28	-20		
Heptelidic acid	(+)	-92	-70	nd	0.01
	(--)	-30	-12		
Gliotoxin	(+)	nd	-77	nd	0.1
	(--)	nd	-18		
Aspochalasin F	(+)	-23	+8	nd	>5
	(--)	-5	-2		
Aspochalasin G	(+)	+6	-9	nd	>5
	(--)	+22	+21		
Dehydrocurvularin	(+)	+30	+39	nd	>5
	(--)	+18	-26		
Artemisinin	(+)	-16	-32	0.001	0.001
	(--)	+9	+15		

For structure, see Fig. 1 and refs. 9 and 10. nd, not determined.

NIHJ represents a strain obtained from NIH, Tokyo, Japan, and Teikyo means a strain isolated and maintained in Department of Parasitology, Teikyo University School of Medicine, Tokyo, Japan (see Materials and Methods). A part of the activities against Teikyo strain shown here were cited from a previous paper (ref. 10)

the antibacterial activity of the two compounds was heme-dependent, and was reversed by the addition of tocopherol, as was with artemisinin. Secondly, the *in vitro* antimalarial activity of the three compounds was reversed by the addition of tocopherol (Fig. 3). Thirdly, heme-dependent radical generation from the same compounds was detected (Fig. 5). It is assumed that this mechanism is involved in the antimalarial actions of these compounds.

In support of the above interpretation, *in vitro* antimalarial activity of a compound appears to correlate with its heme-dependent radical generation. Namely, nanaomycin A, radicicol and heptelidic acid, which were potently active *in vitro* against malaria parasites, were suggested in this study to be potent heme-dependent radical generators (Table 1). The results shown in Table 1 indicate a contrast between nanaomycin A (and radicicol) and artemisinin. Nanaomycin A and radicicol exhibited high β-Ala-His decreasing rates but comparatively moderate

antimalarial activities, while artemisinin did comparatively a lower β-Ala-His decreasing rate and a higher antimalarial activity. The difference may result from different transport of these compounds into red blood cells. The good accumulation inside the red blood cells is demonstrated for artemisinin and a more lipophilic derivative, artemether.[3] Nanaomycin A and radicicol are more hydrophilic than artemisinin, and would be less permeable through the membrane of red blood cells. Synthesis of more permeable derivatives of nanaomycin A and radicicol would allow more insight into this point.

In the radical detection study using β-Ala-His, the fluorescence increased in the later period of reactions when nanaomycin A and radicicol were used (Fig. 4). The mechanism of this increase is not known. One plausible explanation is that the alkylated products in the early and later reactions were different, giving different intensities of fluorescence, as reported for primary and secondary amines.[19] Structure elucidation of reaction products is required to verify this possibility.

MESHNICK and others[1~5] assume that the heme-dependent *O*-radical generation and *C*-radical generation are an inter-linked process.[20] Endoperoxide moiety plays a key role in forming free radicals.[21] On the other hand, nanaomycin A and radicicol do not possess a peroxide moiety. Instead they both have reactive *O*-functions, such as epoxide, lactone with and without conjugation with diene groups. Assuming that these groups play an important role in free radical formation, the free radical formation from non-peroxide compounds would be elicited by initial attacks by heme ions toward reactive *O*-functions, leading to the formation of *O*-radicals, which are rearranged to produce a variety of *C*-radicals.

The results presented in this paper suggest that heme-dependent radical generation can occur in certain types of compounds of microbial origin without an endoperoxide bridge in their chemical structure. It is reasonable to assume that some of them are active as antimalarial compounds. We believe that antimalarial compounds with artemisinin-like mode of action exist among microbial metabolites more than have been expected. Clinically useful antimalarial compounds may be found among them.

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