Heme-dependent Radical Generation from Antimalarial Fungal Metabolites, Radicicol and Heptelidic acid

Sir:

Artemisinin is an endoperoxide sesquiterpene lactone isolated from a traditional Chinese herbal remedy (Fig. 1). Artemisinin and its chemically synthesized derivatives are currently in clinical practice as effective antimalarial agents. According to recent studies by MESHNICK *et al.*, the antimalarial action of artemisinin is mediated by free radicals, which are generated from artemisinin in a heme-dependent manner.[1~3] The endoperoxide bridge was suggested to be essential for radical generation and antimalarial activity.

In the course of our screening program for antimalarial compounds from microorganisms, we have found that radicicol produced by *Humicola* sp. FO-4910, heptelidic acid produced by an unidentified fungus FO-4443 and other fungal metabolites of non-peroxide structures exhibited *in vitro* antimalarial activity against the human malaria parasite *Plasmodium falciparum*.[4,5] These compounds exhibited heme-dependent growth inhibition against an indicator bacterial strain, and the inhibition was diminished by the additional supplement of a radical scavenger, tocopherol. Artemisinin behaved in the same manner under the identical conditions. These results suggest that the growth inhibition by the non-peroxide microbial metabolites involves heme-dependent radical generation. In this paper we describes evidence in support for this notion that radicicol and heptelidic acid reacted with amine groups when incubated in the presence of hemin under physiological conditions. The result is implicated in the mechanism of antimalarial action of the non-peroxide microbial metabolites.

MESHNICK *et al.* proposed that the antimalarial action of artemisinin involves heme-dependent generation of *O*- and *C*-radicals, which oxidize and alkylate SH, NH_2 and OH groups of amino acid residues of membrane proteins. In proposing this mechanism they observed heme-dependent decreases in reactive SH-groups of membrane proteins when incubated with artemisinin and determined by the SH-reactive DTNB [5,5'-dithiobis (2-nitrobenzoate)].[1,2] They also demonstrated the incorporation of radioactivity of labeled dihydroartemisinin into protein fractions.[6] In an attempt to examine possible radical generation from the non-peroxide microbial metabolites, we employed a different approach, in which were used artemisinin (peroxide control) or non-peroxides as potential radical donors and amino acid-related compounds as radical recipients, and the amounts of residual amine group of radical recipients were monitored by fluorometry.

The reaction mixture was a modification of the method of YANG *et al.*[6] It contained in a total volume of 1.0 ml: 20 mM test substance (in ethanol) 200 μl, 2.5 mM β-alanylhistidine (β-AH) or 3-amino-1-propanol (β-alaninol) in sodium phosphate buffer 100 μl, 10 mM hemin in 0.001 N NaOH 20 μl, and 20 mM sodium phosphate buffer (pH 7.2) 680 μl. Control reaction mixtures were made by no addition of β-AH, or no addition of hemin. During incubation at 37°C for 48 hours, 100 μl aliquots were withdrawn at intervals indicated, diluted with water to 1000 μl, and stored frozen until use for fluorometric assay.

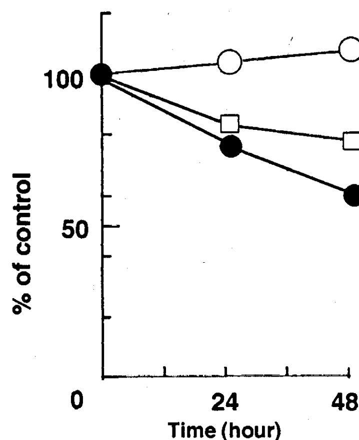
The amounts of residual amine were determined by fluorometry using amine-reactive NBDF (4-fluoro-7-nitrobenzo-2,1,3-oxadiazole, Dojin Chemical, Tokyo).[7] The fluorogenic reaction mixture contained in the final volume of 200 μl: A solution of 0.1 M borax (pH 9.3) 50 μl, 10-fold diluted reaction mixture 50 μl, and 0.4 mg/ml of NBDF in 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 acidified mixture was put into a 96-well microplate for fluorometric record at 470 nm (excitation) and 530 nm (emission), with a fluorometer (Cytofluor II, model 4000, Perseptive Biosystem, USA). Duplicate reactions for radical generation were conducted and each reaction sample was subjected to duplicate fluorometric assays. Mean values are shown in Fig. 1.

Fig. 1A shows that when incubated in the presence of hemin, artemisinin reacted with the amine groups of β-alanylhistidine (β-AH) and of 3-amino-1-propanol, and the contents of NBDF-reactive amines of the latter compounds apparently decreased. Whereas, no decrease of amine was observed in the absence of either artemisinin or hemin (see Fig. 1B for artemisinin).

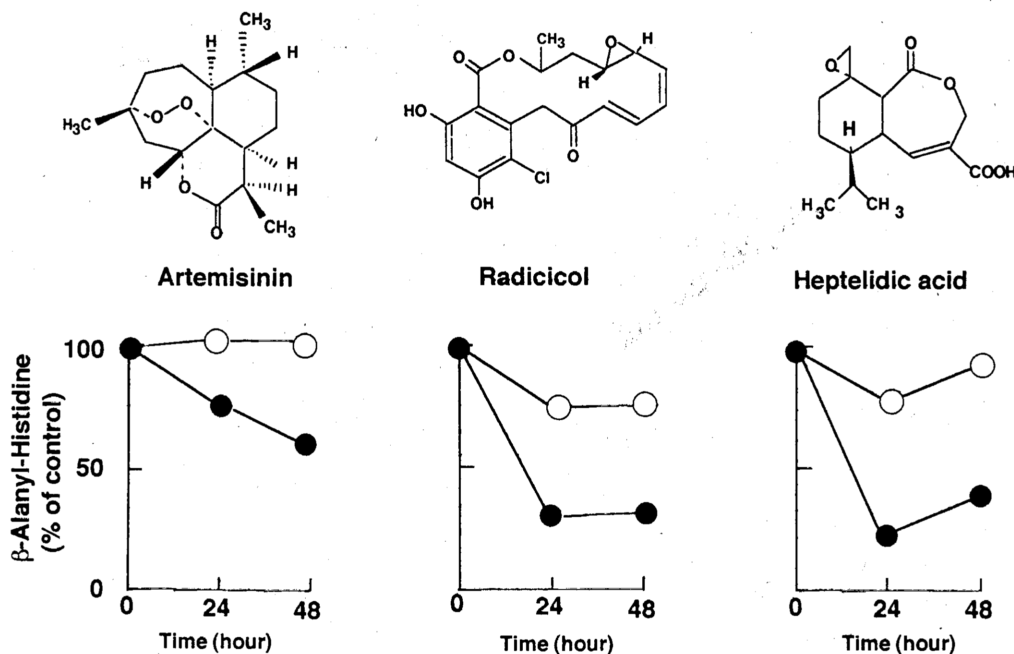
Non-peroxide fungal products were incubated individually with β-AH with and without hemin at physiological pH. The results are shown in Fig. 1B. Under the conditions where artemisinin (control) caused 40% decrease of β-AH at 48th hour of incubation in the presence of hemin, radicicol and heptelidic acid caused considerable decreases of NBDF-reactive amine of β-AH. Although heme-independent decreases were ob-

Fig. 1. Heme-dependent decrease of amino acid derivatives by radicicol and heptelidic acid.

A. 4 mM artemisinin as radical donor was incubated with 0.25 mM β-alanylhistidine (●), 3-amino-1-propanol (□), or none (○) as radical recipient in the presence of 0.1 mM hemin.



B. 4 mM non-peroxide fungal metabolite (radicicol or heptelidic acid) was incubated with 0.25 mM β-alanylhistidine in the presence (●) and absence (○) of 0.1 mM hemin. The structures of the fungal metabolites are also shown.



served, the heme-dependent declines of β-AH were far more significant. An analysis by TLC revealed the formation of new amine derivatives in the reaction mixture as detected by ninhydrin reagent. These results, together with the heme-dependent antibacterial activity reported previously[5] suggest that radicicol and heptelidic acid are heme-dependent radical generators under the reaction conditions employed. This is the first example in which artemisinin-like action mechanism is suggested for an antimalarial microbial metabolite.

Fig. 1B shows that the fluorescence with radicicol and heptelidic acid increased in the later period of reaction. The mechanism of this increase is not known. A simple explanation is that the alkylated products in the early and later periods of reaction were different, giving different intensities of fluorescence, as reported for

primary and secondary amines.[7] MESHNICK *et al.*[1~3,8] and others[9] assume that the heme-dependent *O*-radical generation and *C*-radical generation are a linked process. Endoperoxide moiety plays a key role in forming free radicals. Radicicol and heptelidic acid do not possess a peroxide moiety, but instead they have reactive *O*-functions, such as epoxide, lactone and ketone with and without conjugated diene. Assuming that these functional groups play a positive role, free radical formation from non-peroxide compounds would likely be elicited by the initial attack by heme ions upon reactive *O*-functions, leading to the formation of *O*-radicals and *C*-radicals. This hypothesis needs to be verified. Details of reaction conditions and characterization of reaction products will be reported in a forthcoming paper.

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