Enhancement of NO Production in Activated Macrophages in Vivo by an Antimalarial Crude Drug, *Dichroa febrifuga*

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The effect of an antimalarial crude drug, *Dichroa febrifuga* Lour. on nitric oxide (NO) production in bacillus Calmette Guérin−induced mouse peritoneal macrophages activated by lipopolysaccharide was investigated. The NO production was significantly enhanced by an oral administration of a MeOH extract of *D. febrifuga*. Febrifugine (**1**) was isolated as the main active compound, and the activation was dose-dependent in the dosage range of 0.1−1 mg/kg/day.

Nitric oxide (NO) is a free radical gas produced by three different isoforms of nitric oxide synthase (NOS). NO has been implicated in a variety of biologic functions including neurotransmission, vascular homeostasis, and antimicrobial and antitumor activities.[1] The activated macrophages (Mϕ) contain a transcriptionally inducible isoform of NOS (iNOS), which concerns a long period of production of a large amount of NO.[1,2] NO produced by Mϕ can exert cytostatic or cytotoxic effects against tumor cells, microbes, viruses, and bacterial protozoa.[3] Recent studies showed that interferon-γ (INF-γ) inhibited the infection of sporozoites into murine hepatocytes by producing NO, and the antiparasitic effect of INF-γ was completely abolished by NOS inhibitors.[4−8] Furthermore, it has been reported that an iron chelator, desferrioxamine B, potentiated the production of NO in cultured Mϕ,[9,10] and the antimalarial effect of quinine (**4**) was enhanced by the simultaneous use of desferrioxamine B.[11,12] Thus, examining the potentiating effect on NO production in activated Mϕ is thought to be a useful method for evaluating an antimalarial effect.

The roots of *Dichroa febrifuga* Lour. (Saxifragaceae) have traditionally been used as an antimalarial drug in China. Quinazolone alkaloids febrifugine (**1**) and isofebrifugine (**2**) were isolated from this plant as toxic principles against the *Plasmodium* parasite.[13,14] However, the mechanism of action of these compounds have not been clarified yet.

In this paper we report the potentiating effect of *D. febrifuga* on the NO production in activated Mϕ, as well as the isolation and identification of the active constituents, febrifugine (**1**), isofebrifugine (**2**), and 4-quinazolone (**3**).

Results and Discussion

The activated Mϕ isolated from bacillus Calmette−Guérin (BCG)-treated ICR mice produced a large amount of NO (32.4 ± 4.1 nmol/mL as NO_2^-) upon stimulation with 10 μg/mL of lipopolysaccharide (LPS) (Figure 1). It is well known that BCG induces INF-γ production in T cells, and the lymphokine leads Mϕ to enhance biosynthesis of iNOS in response to LPS.[15−17] This NO production was significantly enhanced by 97% upon oral administration of the MeOH extract of *D. febrifuga* at a dosage of 20 mg/kg/day for 3 consecutive days before BCG injection (Figure 1). The potentiating activity of the MeOH extract was passed mainly into the alkaloidal fraction and slightly into the acidic/neutral fraction. That is, the former potentiated the NO production by 103% at a dosage of 1 mg/kg/day, and the later potentiated it by 63% at the same dosage (Figure 1).

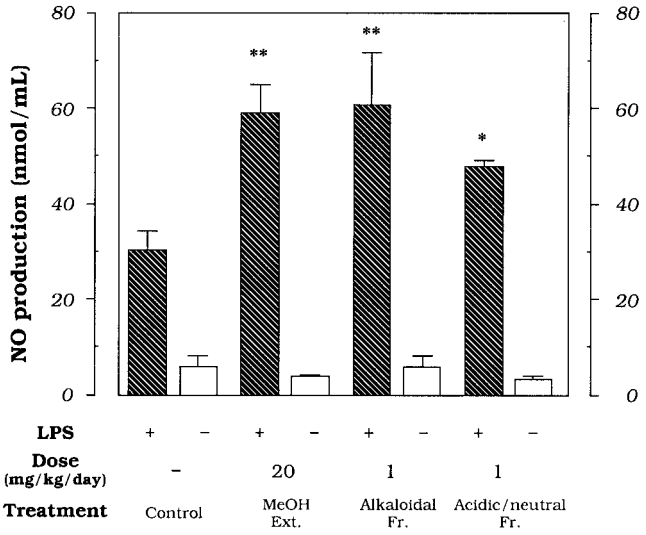

Figure 1. Effects of crude extract and fractions on NO production by BCG-induced peritoneal Mϕ with or without LPS. Test samples were ingested orally once a day for three consecutive days before BCG immunization. NO_2^- content in cultured supernatants was measured at 24 h after LPS challenge. Data are expressed as means ± S. D. Significantly different from control Mf, *$p < 0.05$ and **$p < 0.01$, number of experiments = 3.

The *n*-BuOH-soluble portion of the alkaloidal fraction was chromatographed over Si gel to afford febrifugine (**1**) as the main active compound, together with a minor component, isofebrifugine (**2**). Febrifugine (**1**) and isofebrifugine (**2**) previously isolated from the same source,[18] were identified by analysis of their ^{1}H and ^{13}C NMR spectra, which are being reported for the first

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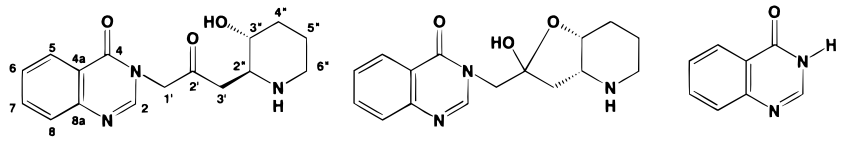
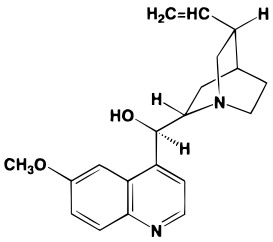


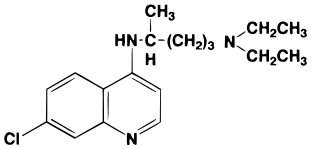
Febrifugine (1)

Isofebrifugine (2)

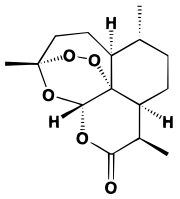
4-Quinazolone (3)



Quinine (4)



Chloroquine (5)



Artemisinin (6)

time. 4-Quinazolone (**3**), an alkaloid having the basic structure of febrifugine (**1**) and isofebrifugine (**2**), was isolated from the *n*-BuOH-soluble portion of the alkaloidal fraction and identified by comparison of its spectral data with those in the literatures.[19,20]

Febrifugine (**1**), isofebrifugine (**2**), and 4-quinazolone (**3**) potentiated the NO production in activated Mϕ by 91% ($p < 0.01$), 22% ($p < 0.05$), and 29% ($p < 0.05$) at doses of 1 mg/kg/day, respectively (Figure 2). Thus, the potentiating activity of febrifugine (**1**) is 3 to 4 times stronger than those of isofebrifugine (**2**) and 4-quinazolone (**3**). Furthermore, febrifugine (**1**) enhanced the NO production in a dose-dependent manner in the dosage range of 50−1000 μg/kg/day (Figure 3).

In this experiment, the number of inflammatory peritoneal exudate cells was reduced from $(5.31 \pm 1.08) \times 10^7$ to $(3.78 \pm 1.04) \times 10^7$ cells/mouse by oral administration of febrifugine (**1**). Isofebrifugine (**2**) also reduced the number of the cells by 30%, but 4-quinazolone (**3**) did not.

Because Mϕ-derived NO mediates cytostatic action toward not only target cells but also Mϕ themselves,[21,22] we examined the correlation between NO production and cell viability in cultured Mϕ with or without LPS. Cumulative NO production was detected 24 h after LPS stimulation and increased over the period of 72 h (Figure 4A). No increment of the NO production was observed without LPS stimulation. On the other hand, Mϕ viability was reduced to 10% after 72 h by LPS treatment, whereas >90% of Mϕ were viable without LPS (Figure 4B). Febrifugine (**1**) enhanced the LPS-mediated NO production (Figure 4A) and slightly increased the cell death by LPS (Figure 4B). Therefore, the reason for the reduction of peritoneal exudate cell number by febrifugine (**1**) is uncertain.

To examine the influence of administration timing on the NO production, 1 mg/kg/day of febrifugine (**1**) was administered orally before (day −2 to 0) or after (day 0 to +2) BCG immunization. As shown in Figure 5, an increase of NO production was observed in both cases, and the effect was more potent in the former case.

We next evaluated the effect of some known antimalarial agents on the NO production in activated Mϕ. As shown in Figure 6, quinine (**4**) and chloroquine (**5**) were not effective upon oral administration at doses of 1 mg/kg/day for 3 consecutive days before BCG immunization. On the other hand, artemisinin (**6**) reduced the NO production by 23% ($p < 0.05$) at the same dosage. Thus, no antimalarial agent tested in this experiment enhanced the NO production.

Recently, Weiss et al.[12] reported that treatment of cerebral malaria with the iron chelator desferrioxamine B, in addition to a standard therapeutic regimen, caused high concentrations of NO_2^- and NO_3^- in the serum and resulted in increased parasite clearance and a faster recovery from coma. These indicate the relationship between antimalarial activity and NO production in activated Mϕ.[10,12,23]

Desferrioxamine B[10] and febrifugine (**1**) enhance NO production in Mϕ by different mechanisms. That is, desferrioxamine B directly stimulates Mϕ and increases NO production,[10] while febrifugine (**1**) enhances NO production in response to LPS.

Febrifugine (**1**) 1.25−5.00 mg/kg/day p.o. has been reported to be effective against malaria parasites in experimental animals, with *p.o.* but to be fatally toxic in the dosage range over 10 mg/kg/day.[24,25] These evidences correspond well with our result. Thus, upon oral treatment at a dosage of 1 mg/kg/day for 3 consecutive days, febrifugine (**1**) apparently enhanced the NO production in activated Mϕ and did not show any significant toxicity to the mice, which was judged by the fact that the weights of mice did not change and the activity of hepatic marker enzymes (aminotransferases) in the serum did not increase (data not shown), Febrifugine (**1**) however, did not enhance NO production and caused diarrhea in mice when administered orally at dosages of more than 5 mg/kg/day (data not shown).

In conclusion, the antimalarial activity of febrifugine (**1**), the main active compound of a traditional antimalarial drug, *D. febrifuga*, is different from those of known antimalarial agents, quinine (**4**), chloroquine (**5**), and artemisinin (**6**). Febrifugine (**1**) enhances NO production in activated Mϕ, which might be the reason for the antimalarial activity, including host defense. Febrifugine (**1**) acts in some processes of immune response because it enhanced the NO production on treatment both before and after BCG immunization. From the facts mentioned above, febrifugine (**1**) might be a promising source for a novel class of antimalarial drugs,

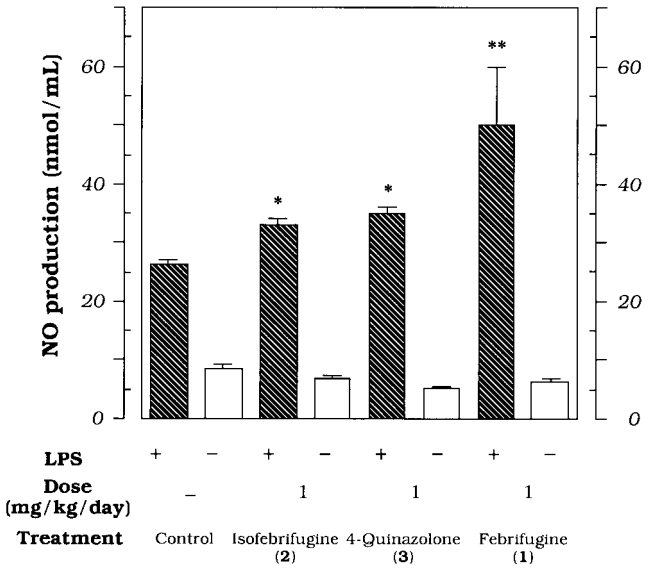


Figure 2. Effects of alkaloids on NO production by Mϕ. Test samples, febrifugine (**1**), isofebrifugine (**2**), and 4-quinazolone (**3**), were administered orally once daily for three consecutive days before BCG immunization. NO_2^- content in cultured supernatants was measured at 24 h after LPS challenge. Data are expressed as means ± S. D. Significantly different from control Mϕ, *p < 0.05 and **p < 0.01, numbers of experiments = 3.

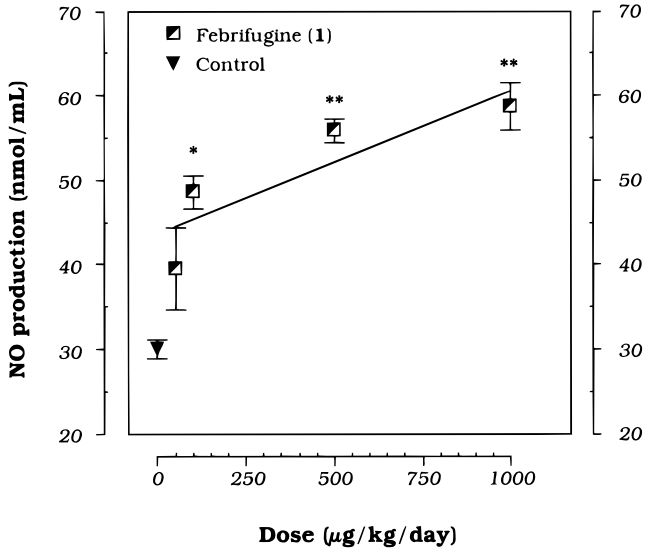


Figure 3. Dose-dependent potentiation by febrifugine (**1**) on NO production by BCG-induced Mϕ. Mice were dosed orally with febrifugine dihydrochloride at doses range of 50−1000 μg/kg/day once daily for three consecutive days before BCG immunization. NO_2^- content in cultured supernatants was measured at 24 h after LPS challenge. Data are expressed as means ± S. D. Significantly different from control Mϕ, *p < 0.05 and **p < 0.01, number of experiments = 3.

and further study about the immunological property of febrifugine (**1**) is under way.

Materials and Methods

General Experimental Procedures. IR spectra were recorded on a JASCO A-100S spectrometer in KBr disks. UV spectra were obtained on a Hitachi U-3200 spectrophotometer. MS and HR EIMS were obtained on JEOL JMX DX-303 and/or AX-500 mass spectrometers using a direct inlet system. Optical rotations were

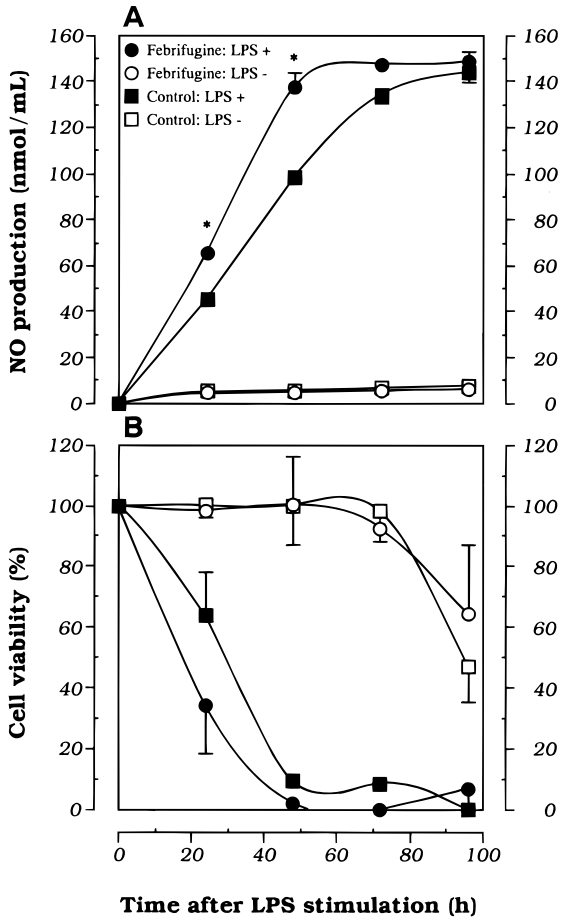


Figure 4. Time course of NO production and Mϕ cell viability in culture of BCG-induced Mϕ. BCG-induced Mϕ from orally applied saline alone or febrifugine (1 mg/kg/day) were cultured with or without LPS (10 μg/mL), and then NO_2^- content in Mϕ cultured supernatants (panel A) and cell viability (panel B) were determined at the indicated time. Data are expressed as means ± S. D. of quadruplicate cultures. Significantly different from control Mϕ, *p < 0.01.

measured on a JASCO DIP-370 polarimeter. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) spectra were measured by Hitachi R-3000 and Varian Gemini 2000 spectrometers with TMS as internal standard, and chemical shifts were recorded in δ units. Wakogel C-200 (Wako Pure Chemical Co.), Silica 60 (Merck), ICN Alumina N, Akt. I (ICN Biomedicals), and Cosmosil 75C_{18}-OPN (Nacalai Tesque Inc.) were used for column chromatography. DC-Fertigplatten Kieselgel 60 F_{254} (0.25-mm thick, Merck) was used for preparative TLC. DC-Alufolien Kieselgel 60 F_{254} (0.2-mm thick, Merck) was used for TLC analyses.

Reagents. LPS (*Escherichia coli* 055:B5) and MTT was obtained from the Sigma Chemical Co. (St. Louis, MO). BCG was from Nippon BCG (Tokyo). Phosphate buffered-saline (PBS), phenol red-free Dulbecco's-modified Eagle's medium (D-MEM), and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Cell culture medium was supplemented with 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 μg/mL penicillin, 100 U/mL streptomycin, and 5% FBS. All other reagents whose suppliers are not indicated were purchased from Wako Pure Chemical Co. (Tokyo). Quinine (**4**) hydrochloride and artemisinin (**6**) were purchased from Sigma Chemical Co. (St. Louis,

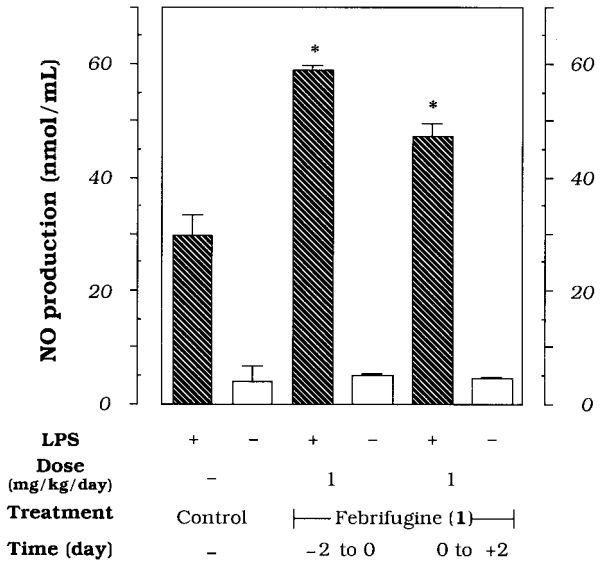


Figure 5. Enhancement of NO production by febrifugine (1). Mice were immunized intraperitoneally with BCG (day 0). Five days after BCG immunization, BCG-induced Mϕ were collected, and NO_2^- content in Mϕ cultured supernatants was measured at 24 h after LPS challenge. Data are expressed as means ± S. D. Significantly different from control Mϕ, *p < 0.01, number of experiments = 3.

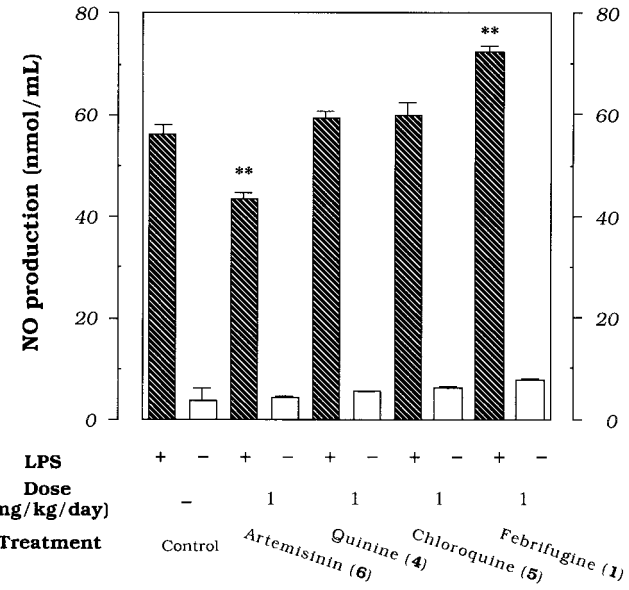


Figure 6. Effects of various antimalarial agents on NO production by BCG-induced Mϕ. Mice were given test compounds orally once a day for three consecutive days before BCG immunization. NO_2^- content in cultured supernatants was measured at 24 h after LPS challenge. Data are expressed as mean ± S. D. Significantly different from control Mϕ, *p < 0.01, number of experiments = 3.

MO). Chloroquine (**5**) diphosphate was from Aldrich Chemical Co. (Milwaukee, WI).

Plant Materials. *D. febrifuga* was obtained from Kinokuni-ya Pharm. Co. Ltd. (Tokyo).

Isolation of Febrifugine (1), Isofebrifugine (2), and 4-Quinazolone (3). The dried roots of *D. febrifuga* (8 kg) were macerated in MeOH (10 L × 2) at room temperature for 1 day. After filtration, the solvent was evaporated under reduced pressure to give MeOH extract (196 g). The extract (160 g) was suspended in 0.1 N HCl (400 mL), and the suspension was extracted with $CHCl_3$ (200 mL × 4). The combined organic layer was washed with 0.1 N HCl (400 mL × 2), dried over $MgSO_4$ and evaporated in vacuo to afford the acid/neutral fraction (15.3 g). The combined 0.1 N HCl layer was adjusted to pH 9 with 20% $NaHCO_3$ and extracted with $CHCl_3$ (300 mL × 4). The combined $CHCl_3$ layer was dried over $MgSO_4$, and the solvent was evaporated to afford the alkaloidal fraction (3.4 g). The alkaloidal fraction was partitioned between *n*-BuOH and H_2O water. The *n*-BuOH-soluble portion (2.6 g) was subjected to Si gel column chromatography, successively eluted with $CHCl_3$, $CHCl_3$−MeOH (19:1, 9:1, 4:1, 2:1, 1:1), and MeOH to give fraction I (0.12 g), fraction II (0.12 g), fraction III (0.21 g), fraction IV (1.39 g), and fraction V (0.13 g). Fraction III was chromatographed on Si gel eluted with *n*-hexane−EtOAc solvent system. From the fraction eluted with *n*-hexane−EtOAc (1:2), 4-quinazolone (**3**) (47.4 mg, 0.0007%) was obtained as white powder after recrystallization from MeOH. Fraction IV was repeatedly chromatographed on Si gel using $CHCl_3$−MeOH−H_2O (90:10:0.85) as a solvent. The fractions containing two alkaloids were purified by preparative TLC and alumina column to give febrifugine (**1**) (white powder, 51.0 mg, 0.0008%) and isofebrifugine (**2**) (white powder, 51.8 mg, 0.0008%).

Febrifugine (1) (free base): white powder; $[\alpha]^{27}_D$ + 13.0° (*c* 0.65, MeOH); UV λ_{max} nm (ϵ) (MeOH) 225 (27 800), 266 (7500), 276 (shoulder), 302 (3500), 313 (2900); IR (KBr) ν_{max} cm^{-1} 3250, 1719, 1664, 1648, 1612; EIMS *m/z* (rel int) 301 (M^+, 3%), 283 (54), 244 (3), 202 (19), 187 (10), 160 (72), 147 (38) 137 (100); HRMS *m/z* 301.1435 [M^+], calcd for $C_{16}H_{19}N_3O_3$; 301.1426; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.36 (1H, ddt, *J* = 5, 11, 13 Hz, H-4″), 1.51 (1H, br dt, *J* = 4, 13 Hz, H-5″), 1.72 (1H, br dt, *J* = 4, 13 Hz, H-5″), 2.06 (1H, br ddd, *J* = 5, 11, 13 Hz, H-4″), 2.55 (1H, br dt, *J* = 4, 13 Hz, H-6″), 2.63 (1H, dd, *J* = 7.5, 16 Hz, H-3′), 2.86 (1H, ddd, *J* = 5, 7.5, 9 Hz, H-2″), 2.95 (1H, dd, *J* = 4, 13 Hz, H-6″), 3.12 (1H, dd, *J* = 5, 16 Hz, H-3′), 3.28 (1H, ddd, *J* = 5, 9, 11 Hz, H-3″), 4.84 (1H, d, *J* = 17 Hz, H-1′), 4.92 (1H, d, *J* = 17 Hz, H-1′), 7.50 (1H, ddd, *J* = 1.6, 8.1, 8.4 Hz, H-6), 7.72 (1H, ddd, *J* = 1.6, 8.0 Hz, H-8), 7.77 (1H, ddd, *J* = 1.2, 8.0, 8.4 Hz, H-7), 7.93 (1H, s, H-2), 8.26 (1H, dd, *J* = 1.2, 8.1 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 25.6 (C-5″), 34.5 (C-6″), 44.0 (C-4″), 46.0 (C-3′), 55.0 (C-1′), 60.2 (C-2″), 72.3 (C-3″), 122.1 (C-4a), 127.0 (C-8), 127.7 (C-6), 127.8 (C-5), 134.8 (C-7), 146.7 (C-2), 148.5 (C-8a), 161.3 (C-4), 203.1 (C-2′).

Isofebrifugine (2) (free base): white powder; $[\alpha]^{27}_D$ + 18.9° (*c* 0.71, $CHCl_3$); UV (MeOH) λ_{max} nm (ϵ) 225 (26 300), 266 (7300), 274 (shoulder), 302 (3500), 314 (2800); IR (KBr) ν_{max} cm^{-1} 3350, 1678, 1648, 1639, 1608, 1459; EIMS *m/z* (rel int) 301 (M^+, 2%), 283 (49), 244 (3), 202 (20), 187 (10), 160 (78), 147 (39), 137 (100); HRMS *m/z* 301.1438 [M^+], calcd for $C_{16}H_{19}N_3O_3$, 301.1426; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.50 (1H, m, H-5″), 1.57 (1H, br ddd, *J* = 4, 5, 11 Hz, H-4″), 1.78 (1H, m, H-5″), 1.88 (1H, d, *J* = 13 Hz, H-3′), 2.08 (1H, dd, *J* = 4, 13 Hz, H-3′), 2.12 (1H, br dd, *J* = 4, 11 Hz, H-4″), 2.54 (1H, dt, *J* = 2, 11 Hz, H-6″), 3.00 (1H, br dd, *J* = 4, 11 Hz, H-6″), 3.30 (1H, dd, *J* = 3, 4 Hz, H-2″), 3.89 (1H, br ddd, *J* = 3, 5, 6 Hz, H-3″), 4.15 (1H, d, *J* = 14 Hz, H-1′), 4.45 (1H, d, *J* = 14 Hz, H-1′), 7.50 (1H, ddd, *J* = 1.5, 7.0, 8.4 Hz, H-6), 7.71 (1H, ddd, *J* = 1.0,

1.5, 8.0 Hz, H-8), 7.76 (1H, ddd, J = 2.0, 7.0, 8.0 Hz, H-7), 8.31 (1H, S, H-2), 8.35 (1H, ddd, J = 1.0, 2.0, 8.4 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 20.1 (C-5″), 26.8 (C-6″), 43.4 (C-4″), 44.6 (C-3′), 50.0 (C-1′), 55.8 (C-2″), 77.0 (C-3″), 105.6 (C-2′), 122.1 (C-4a), 127.1 (C-8), 127.3 (C-6), 127.7 (C-5), 134.6 (C-7), 148.3 (C-8a), 148.5 (C-2), 161.8 (C-4).

Experimental Animals. Male ICR mice (SPF grade), 4−5 weeks old, were obtained from SLC-Japan (Shizuoka, Japan). Animals were housed in plastic cages for at least one week with free access to water and food ad libitum.

Assay for NO production by activated Mϕ. Five days after an intraperitoneal injection of 1 mg of BCG (suspended in pyrogen-free saline, 0.2 mL/mouse) into ICR mice, peritoneal exudate cells (PEC) were washed out with PBS (pH 7.4) through the anterior abdominal wall. After PEC suspended in culture medium at 2.0 × 106 cells/mL were incubated for 2 h in 24-well tissue-culture plates (Becton Dickinson, Oxnard, CA) in humidified CO_2 incubator, nonadherent cells were gently washed out twice with fresh medium. Remaining adherent cells (>90% Mϕ, judged by nonspecific esterase stain) were cultured with the same medium containing 10 μg/mL LPS for 24 h. To measured the amounts of NO_2-derived from NO in culture, 100 μL of culture supernatants were mixed with 100 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylene-diamine dihydrochloride, 3% H_3PO_4).[26] After chromophore was formed at room temperature for 10 min, absorbance was determined at 510 nm using Immunoreader (InterMed, Tokyo).

Mϕ viability. Cell viability was analyzed by a MTT method as described above.[27] In brief, MTT solution (10 μL of 5 mg/mL in PBS) was added to each Mϕ culture (2×10^5 cells/100 μL in 96-well plate). After 4 h of incubation in a CO_2 incubator at 37 °C, the reduced MTT-formazan was solubilized with 100 μL of detergent (10% SDS in 0.05 N HCl) 37 °C for 18 h. Absorbance of MTT-formazan product was read at 590 nm using Immunoreader. Data were expressed as percentages of control.

Test sample treatment. The test samples were suspended in physiological saline and orally ingested by mice once a day for three consecutive days before BCG immunization (three consecutive days after BCG immunization in the case of posttreatment). Febrifugine (**1**), isofebrifugine (**2**), and 4-quinazolone (**3**) were used as hydrochlorides.

Statistical analysis. All values were expressed as arithmetic means ± S. D. of three independent experiments. Statistical significance of differences between groups was determined by Student's *t*-test.

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