



Published in final edited form as:

J Antibiot (Tokyo). 2011 December ; 64(12): 799–801. doi:10.1038/ja.2011.94.

In vitro* and *in vivo* activity of frenolicin B against *Plasmodium falciparum* and *P berghei

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Keywords

frenolicin; malaria; plasmodium; polyketide

Several years ago, the polyketide natural product frenolicin B, produced by the actinomycete *Streptomyces roseofulvus*, underwent preliminary testing for use as an anticoccidial agent in poultry, owing to its promising activity against *Eimeria tenella*.¹ To probe its pharmacophore, a limited structure activity relationship study was undertaken using a series of ~20 semisynthetic derivatives of frenolicin B modified at the 1, 2, 4, 8, 10 and 11 positions.^{2–4} All variants resulted in a loss of potency except for esterification of the phenol at position 11. This esterified derivative likely acted as a pro-drug, increasing the *in vivo* efficacy of frenolicin by a factor of 2.³ Aside from its oral bioavailability in poultry, frenolicin B is tolerated with no acute toxicity in rats fed diets containing 100 mg kg⁻¹ per day, though some chronic effects potentially stemming from chelation of dietary copper were noted after 4 weeks.⁵ To further explore the scope of frenolicin B's antiparasitic activity, we investigated its efficacy against the human malarial pathogen *Plasmodium falciparum*. This Apicomplexan parasite infects ~225 million people and is responsible for ~780 000 deaths annually. We also explored the antimalarial efficacy of frenolicin B *in vivo* using the murine malaria model *P. berghei*.

Frenolicins are produced by *S. roseofulvus* in three isolable forms (Figure 1).⁶ Based on earlier work by Whaley *et al.*⁷ we developed a simple method to convert frenolicin A to deoxyfrenolicin, followed by lactonization to convert deoxyfrenolicin to frenolicin B.⁸ This

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method gives high yields and conversion efficiencies when performed on pure frenolicin A (>80% yield over two steps). Importantly, it can be performed on crude isolate flushed through a silica column. Additionally the method requires no purification of the deoxyfrenolicin intermediate between the de-epoxidation and lactonization steps. Details are provided in the Experimental procedure.

With purified frenolicin B in hand, we tested its effectiveness *in vitro* against three laboratory strains of *P. falciparum*: HB3 (chloroquine and amodiaquine susceptible); Dd2 (highly chloroquine resistant and moderately amodiaquine resistant) and 7G8 (moderately chloroquine resistant and highly amodiaquine resistant).⁹ Parasite growth inhibition by frenolicin B was tested *in vitro* and the ICs of 50 (IC₅₀) and 90 (IC₉₀) percent of parasite population were determined using a standard 72 h antimalarial response assay, which uses SYBR green to detect DNA and estimates parasite multiplication¹⁰ (see Experimental procedure for details). To evaluate whether common liver enzymes would metabolize frenolicin B and affect its antiparasitic activity, the drug was exposed to a panel of human cytochrome P450 enzymes (CYPs) and then retested against each parasite strain by standard growth inhibition assay (see Experimental procedure for details). Parasite *in vitro* growth inhibition by frenolicin B was similar against each of the different strains, with IC₅₀ values in the range of 600–800 nM (Table 1), implying that the natural product's mode of action is distinct from chloroquine and amodiaquine. Additionally, similar or lower IC₅₀ values obtained for frenolicin B after incubation with cytochrome P450 enzymes suggests the drug is not metabolized or if so, its potency is improved by the liver enzymes tested, prompting us to investigate its activity *in vivo*.

We chose to test the compound in a 4-day *P. berghei* suppression model in 8-week-old CD1 mice at doses of 10, 20, 40 and 63 mg kg⁻¹ per day. For each dosing level, a cohort of five mice inoculated with 1×10⁷ *P. berghei* parasites was given a daily oral gavage of frenolicin B for 4 days beginning 1 h after inoculation. On the fifth day the mice were killed, their blood was drawn and parasitemia levels were determined (that is the percent of infected red blood cells; see Experimental procedure for details). Table 2 shows the parasitemia levels of the mice on day 5 versus an uninfected control, as well as the number of mice cured in each group. Mice were designated cured if their parasitemia level was <1.0%. A dose-dependent trend in both the parasitemia levels and number of cured mice could be observed. At the dose of 63 mg kg⁻¹, parasitemias were reduced by >50% and two of five mice were cured. This activity was still considerably lower than piperazine, a drug used in combination therapy with the artemisinin derivative dihydroartemisinin (Table 2). These data illustrate that frenolicin B has a dose-dependent, orally bioavailable activity against *P. berghei* in mice.

In summary we have shown that frenolicin B possesses good antiparasitic activity against *P. falciparum* *in vitro* and orally against *P. berghei* in mice. Additionally, frenolicin B appears to exert its bioactivity via a different mode of action than chloroquine and amodiaquine, making it a potentially useful starting point in the development of new agents to treat drug-resistant strains of malaria.

EXPERIMENTAL PROCEDURE

Isolation of frenolicins from *S. roseofulvus* and conversion to frenolicin B

S. roseofulvus was cultured on semi-solid R5 plates as previously described.¹¹ Optimization of antibiotic production showed a maximum yield of frenolicins after 4 days of growth at 30 °C, with frenolicin B, deoxyfrenolicin and frenolicin A produced in a ratio of ~1:20:5. After growth, the agar was mashed and extracted 1:1 with 1% acetic acid in 99% ethyl acetate overnight, followed by a second overnight extraction. Purification was performed by flushing the crude extract through a silica gel column using 1% acetic acid in 99% ethyl acetate as the mobile phase. The eluate was dried *in vacuo* and subjected to the procedure outlined in Figure 1 to convert all frenolicins present to frenolicin B. It was found that both the de-epoxidation and lactonization reactions described below could be run on this silica gel eluate instead of pure frenolicin A with a minimal loss of yield.

As an example of the reaction run on pure compound, 160 mg frenolicin A (0.46 mmol) was dissolved in 10 ml anhydrous methanol (Acros Organics, Fair Lawn, NJ, USA) and added to a round bottom flask containing 21 mg (20% mol cat.) platinum (IV) oxide (Sigma-Aldrich, St Louis, MO, USA) under nitrogen. Hydrogen gas was bubbled through the mixture for one min before the flask was placed under hydrogen atmosphere and the mixture stirred for 30 min at 0 °C. The mixture was then filtered through celite to remove any accumulated Pt black, the filtrate was dried and redissolved in 50 ml methanol, and then added to a 250 ml round-bottom flask. To this mixture 365 mg pyridine was added (4.6 mmol) before heating at 60 °C overnight while bubbling air through the solution. The solvent was then evaporated yielding 150 mg of material, which showed near complete conversion (>95%) of the frenolicin A to frenolicin B by NMR.

After conversion of the frenolicins in this crude material to frenolicin B through the method outline in Figure 1, frenolicin B was isolated by preparative HPLC on a Beckman 126 solvent module equipped with at Beckman 166 UV detector (Beckman Coulter, Brea, CA, USA). The material was dissolved in 3 ml methanol and injected on a reverse-phase column (250 mm × 22 mm, C₁₈, Vydac, Deerfield, IL, USA). A gradient starting at 15% acetonitrile in water was increased to 40% over 20 min, then increased to 46% over 50 min, yielding 10 mg l⁻¹ of isolated product (see Fitzgerald *et al.*¹² for characterization).

Testing of frenolicin B against *P. falciparum* *in vitro*

P. falciparum parasites were cultured *in vitro* and their dose-response profiles were measured as described elsewhere.^{9,10} Briefly, parasites were maintained in complete RPMI 1640 media (KD Medical, Columbia, MD, USA) containing L-glutamine, 25 mM Hepes, 50 µg ml⁻¹ hypoxanthine, 10 µg ml⁻¹ gentamicin, 0.225% NaHCO₃ and 0.5% Albumax I (Invitrogen, Carlsbad, CA, USA) at 37°C under an atmosphere of 5% CO₂/5% O₂/90% N₂. Frenolicin B was dissolved in dimethyl sulfoxide and serially diluted from 80 to 0.002 µM in 96-well plates. Synchronized ring-stage parasite cultures at 1% parasitemia were incubated with the various drug concentrations for 72 h when cells were harvested and exposed to SYBR Green for parasite growth quantification. Percentage of growth inhibition was calculated by the ratio of SYBR Green signal from each well with the drug over the signal

obtained from wells with no drug (control wells with maximum parasite growth). Growth inhibition curves, IC₅₀ and IC₉₀ values, and statistical analyses were determined using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) and Excel. An *in vitro* method to metabolize frenolicin B and other antimalarials was developed by pre-incubating the drugs with various recombinant cytochrome P450 enzymes and using the supernatant to perform the serial dilutions for the *in vitro* growth inhibition assay described above (O Twu, M Krause and JM Sá, unpublished data to be submitted to *Malaria Journal* in 2011).

Testing of frenolicin B against *P. berghei* *in vivo*

Frenolicin B was dissolved in 100 µl of 0.5% aqueous hydroxyethylcellulose, with 0.1% Tween 80 for dosing by oral gavage in 8-week-old CD1 mice (Charles River Laboratories, Wilmington, MA, USA). Mice were inoculated with 10⁷ parasites by i.p. injection 1 h before first dosing. Following 4 days of daily dosing, parasitemia was measured on day 5. For this, tail vein blood was collected in citrate phosphate dextrose solution/phosphate-buffered saline supplemented with 5% fetal bovine serum. Infected erythrocytes were fluorescently stained using SYBR Green I (Invitrogen) combined with MitoTracker Deep Red (Invitrogen) in 1×phosphate-buffered saline, pH 7.3, containing 5% fetal bovine serum.¹³ Cells were labeled for 40 min at 37 °C and dual-channel fluorescence was detected with an Accuri C6 (Becton-Dickinson, Franklin Lakes, NJ, USA) attached to a Hypercyt sampler (Intellicyt, Albuquerque, NM, USA). SYBR Green was excited with a 488-nm diode laser and emission was detected with a 530 nm filter, whereas a 640-nm laser was used for MitoTracker Deep Red (Invitrogen) with a 675 nm filter. Parasitemias were determined following sample analysis using FlowJo software (Tree Star, OR, USA).

Acknowledgments

This research was partially supported by a grant from the National Institutes of Health to CK (R01 CA 77248) and to DF (R01 AI079709), and by the Intramural Research Program of the NIH, NIAID.

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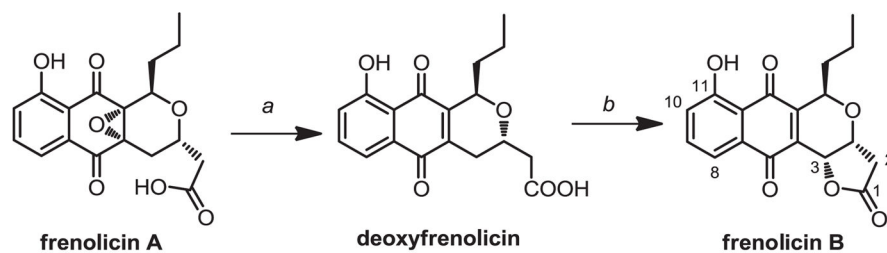


Figure 1.

Conversion of *S. roseofulvus* frenolicins to frenolicin B. *a*: H₂, PtO₂ in methanol at 0 °C for 30 min; *b*: bubbled O₂, pyridine, in methanol at 60 °C overnight.

Table 1

Growth inhibition of three strains of *Plasmodium falciparum* by frenolicin B

Strain	Frenolicin B	P450	1A2	2C8	2C9	2C19	2D6	3A4
<i>IC₅₀ values (nM)^a</i>								
HB3	600±100 (8)	1000±190 (7)	800±260 (4)	600±200 (4)	600±150 (4)	600±160 (4)	700±210 (4)	700±190 (4)
Dd2	800±200 (10)	800±150 (7)	600±130 (4)	490±70 (4)	600±140 (4)	500±100 (4)	600±120 (4)	600±140 (4)
7G8	800±150 (8)	1400±260 (7)	490±30 (2)	400±20 (2)	470±5 (2)	430±30 (2)	520±20 (2)	480±2 (2)
<i>IC₉₀ values (nM)^b</i>								
HB3	1500±240	3100±750	2000±720	1600±580	1700±590	1500±440	2000±740	1800±630
Dd2	2100±490	2800±440	1800±460	2100±660	1600±530	1500±460	2100±670	1800±590
7G8	2000±440	7400±2917	1100±60	850±1	990±7	1000±20	1270±60	960±50

^aValues are reported as IC₅₀±s.e. (number of replicates).^bValues are reported as IC₉₀±s.e.

The number of replicates is the same as in the IC₅₀ values above. The column P450 refers to frenolicin B incubated with a variety of P450 s, whereas the latter columns refer to frenolicin B incubated with a specific P450 isoform.

Table 2*In vivo* efficacy of frenolicin B in mice

Dose (mg kg ⁻¹ per day)	Percentage of parasitemia±s.d.	No. of mice cured/total ^a
Vehicle	31±6.4	0/5
10	39±13	0/5
20	37±6.3	0/5
40	33±19	1/5
63	15±16	2/5
2.5 (piperaquine)	1.4±0.7	2/5
5.0 (piperaquine)	0	5/5

^aEach cohort contained five mice whose parasitemia level was determined after 4 days of daily dosing. Each mouse that had a parasitemia <1.0 % was deemed cured. As a control for the efficacy of the *in vivo* assay, mice were also treated with piperaquine at a dose of 2.5 or 5.0 mg kg⁻¹ per day.

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