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Antimalarial Activities of Peptide Antibiotics Isolated from Fungi

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Malaria caused by *Plasmodium falciparum* is a major public health problem in the developing countries of the world. Clinical treatment of malaria has become complicated due to the occurrence of infections caused by drug resistant parasites. Secondary metabolites from fungi are an attractive source of chemotherapeutic agents. This work reports the isolation and in vitro antiplasmodial activities of peptide antibiotics of fungal origin. The three peptide antibiotics used in this study were efrapeptins, zervamicins, and antiamoebin. The high-performance liquid chromatography-purified peptides were characterized by nuclear magnetic resonance and mass spectral analysis. All three fungal peptides kill *P. falciparum* in culture with 50% inhibitory concentrations in the micromolar range. A possible mode of action of these peptide antibiotics on *P. falciparum* is presented.

Malaria is a major tropical disease that affects more than 300 million people living in the developing countries of the world (21, 24). The widespread occurrence of drug-resistant *Plasmodium falciparum* infection necessitates the urgent development of new chemotherapeutic agents. Secondary metabolites produced by fungi are often novel molecules with large potential for chemotherapeutic applications. Our aim has been to isolate, characterize, and screen fungal metabolites for their antimalarial properties. The secondary metabolites studied in this paper are efrapeptins, zervamicins, and antiamoebin. Table 1 lists the sequences of the peptides used in this study along with the fungal species that produce them. These compounds are peptide antibiotics, 16 amino acids long, that contain the unusual amino acids α-aminoisobutyric acid, isovaline, β-alanine, and hydroxyproline. Efrapeptins are produced by the fungus *Tolypocladium niveum* subsp. *inflatum* and are inhibitors of mitochondrial F_0F_1 ATPase, some bacterial ATP synthases, and photophosphorylation in plants (1, 13, 14). The ATPase of the protozan parasite *Trypanosoma cruzi* is also inhibited by efrapeptins (7). Recently, efrapeptins have also been shown to inhibit exocytosis in an ATP-independent manner in eukaryotic cells (22). Both zervamicins and antiamoebin are uncouplers of mitochondrial oxidative phosphorylation (3, 5, 6, 18). Antiamoebin exhibits trypanocidal activity in a mouse model for trypanosomiasis (19) and has been shown to possess anthelmintic properties (30). All three peptide antibiotics also act as channel-forming ionophores (18). Vial et al. have examined numerous other ionophores for antimalarial activity and found gramicidin D to be the most promising candidate under both in vivo and in vitro conditions (11, 12). This work reports the purification, spectral characterization, and antimalarial activities of efrapeptins, zervamicins, and antiamoebin.

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MATERIALS AND METHODS

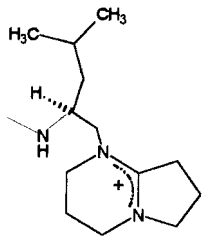
RPMI 1640, chemicals used for culturing the intraerythrocytic stages of *P. falciparum* and diazabicyclononene (DBN) were obtained from Sigma Chemical Company, St. Louis, Mo. [8-^{3}H]hypoxanthine was obtained from Amersham International PLC, Little Chalfont, United Kingdom. Human O^+ blood required for culturing *P. falciparum* was collected from healthy volunteers. Serum from clotted O^+ human blood was prepared in the laboratory. The constituents of the fungal culture medium were obtained from HiMedia Laboratories, Mumbai, India. Silica gel (60 to 80 μm) for medium-pressure liquid chromatography (MPLC) was supplied by Büchi Labortechnik AG, Flawil, Switzerland. The pKb-100 column packed with 5-μm-diameter Supelcosil-DB beads was from Supelco Inc., Bellefonte, Pa. All other chemicals used were from BDH and E. Merck, Mumbai, India. Matrix-assisted laser desorption ionization (MALDI) mass spectra were recorded on a Kompact MALDI-4 (Kratos Analytical Ltd., Manchester, United Kingdom) time-of-flight spectrometer, with a pulsed nitrogen laser (337 nm; 3-ns pulse width). The spectra were recorded in the linear, positive high mass mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in a 1:1 mixture of acetone and water along with 0.1% trifluoroacetic acid was used for obtaining the mass spectra.

Isolation of efrapeptins. Efrapeptins were isolated from the fungus *T. niveum* (*Beauveria nivea*) obtained from the American Type Culture Collection, Manassas, Va. The medium used for fermentation was glucose, 2.5%; meat peptone, 1.0% tryptone, 0.4%; and $MgSO_4 \cdot 7H_2O$, 0.5%. Initially, 200 ml of seed culture was grown for 2 days in the fermentation medium and then subsequently inoculated into 3.2 liters of the same medium. After a fermentation period of 26 days, the medium was centrifuged and the supernatant was extracted with chloroform. The combined organic extract was evaporated under reduced pressure to yield a reddish brown gum (~1 g).

Mycelia were crushed in methanol and left overnight. After evaporating the methanol, the residue, also containing efrapeptins was redissolved in chloroform and mixed with the extracted gum. The reddish brown gum was loaded on a basic alumina column (1 by 25 cm) and eluted gradually with an increasing percentage of methanol in chloroform. Efrapeptins eluted at 5% methanol in chloroform. The presence of efrapeptins was monitored by assaying all fractions for antibacterial activity (by pour plate method) on *Bacillus subtilis* grown on agar plates containing Luria broth (casein enzymic hydrosylate, 1%; yeast extract, 0.5%; and NaCl 0.5%; final pH 7.0 ± 0.2). The residue was concentrated in vacuo and then applied to a reverse-phase C_4 column (particle size, 25 μm; column dimensions, 2 by 25 cm; connected to a Büchi MPLC system). A gradient of methanol-H_2O containing 0.1% trifluoroacetic acid was used as the mobile phase. The elution was monitored at 220 nm. Efrapeptins eluted at 70% methanol–30% H_2O–0.1% trifluoroacetic acid. This fraction was further analyzed on an analytical pKb-100 column (25 cm by 4.6 mm; Supelco) attached to a high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Tokyo, Japan).

Isolation of zervamicins. Zervamicins were isolated from *Emericellopsis salmosynnemata* as described by Argoudelis et al. (3). Zervamicin IIA-IIB was purified from the heterogeneous mixture of zervamicin 1c, zervamicin IIA, zer-

TABLE 1. Amino acid sequences of the peptide antibiotics

X= 

Peptide	Fungal species	Amino acid sequence[a]
Antiamoebin I	*Emericellopsis poonensis*	Ac-Phe-Aib-Aib-Aib-Iva-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Aib-Pro-Phe-OH
Efrapeptin		
C	*Tolypocladium niveum*	Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Aib-X
D		Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X
E		Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X
F		Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X
G		Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X
Zervamicin		
IIA	*Emericellopsis salmosynnemata*	Ac-Trp-Ile-Gln-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phe-OH
IIB		Ac-Trp-Ile-Gln-Iva-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phe-OH

[a] Abbreviations: Ac, acetyl; Pip, pipecolic acid; Iva, Isovaline; β-Ala, β-alanine; Hyp, hydroxy proline; Aib, α-aminoisobutyric acid; X, condensation product of L-leucinol and 1,5-diazabicyclo[4:3:0]nonene (DBN).

vamicin IIB, and zervamicin-Leu by reverse-phase HPLC on a C_{18}, 5 μm, 6- by 150-mm column, using a methanol-water gradient (65 to 75% methanol in 15 min, 75 to 85% in 12 min, 78 to 81% in 60 min) (18). Zervamicin IIA-IIB eluted at 45 min under these chromatographic conditions. The elution was monitored at 226 nm.

Purification of antiamoebin. Antiamoebin was a gift from N. Narasimhachari, Medical College of Virginia Commonwealth University. Purification of antiamoebin I (constituting 98% of the microheterogenous antiamoebins) was effected by reverse-phase HPLC on a lichrosorb RP-18 column (4 by 250 mm; 10-μm particle size) using an LKB HPLC (Pharmacia Biotech, Uppsala, Sweden) system as described earlier (5). For each run, 1 mg of the peptide in 20 μl of methanol was injected and gradient elution (65 to 85% methanol-H_2O in 20 min, 85 to 95% methanol-H_2O in 5 min) was used with a flow rate of 0.8 ml · min^{-1}. Under these conditions antiamoebin I eluted at 21.7 min. The elution was monitored at 226 nm.

In vitro antimalarial activity tests. The *P. falciparum* clone T9/106 (29) was cultivated in vitro using the method described by Trager and Jensen (31). Parasites were maintained in human O^+ cells at 5% hematocrit in RPMI 1640 containing 10% human serum and synchronized using D-sorbitol. The antimalarial activity test was based on previously reported methods (8). All peptides were dissolved in dimethyl sulfoxide (DMSO) and diluted to 10% DMSO with culture medium. A 3.4 mM stock solution of DBN was made in culture medium and the pH adjusted to 7.0 with dilute HCl. For the test, 25-μl aliquots of culture medium were added to the first wells of a 96-well flat-bottom microculture plate, and to the subsequent wells 5% DMSO in culture medium was added. This ensured that the final DMSO concentration in all the wells was maintained at 0.5%. Aliquots of the test solution were added in duplicate to the first wells, and serial two-fold dilutions were made over a 2,048-fold concentration range, from 50 to 0.025 μM. DBN was diluted twofold serially from 3.4 mM to 1.66 μM. Aliquots (200 μl) of a suspension with 1% parasitemia (with parasites predominantly in the ring stages) and 2% hematocrit in culture medium were added to all test wells. Parasitized and nonparasitized erythrocytes and solvent controls were incorporated in all the tests. The plates were incubated at 37°C in a candle jar. After 24 h, each well was pulsed with 25 μl of culture medium containing 0.5 μCi of [8-^{3}H]hypoxanthine and plates were incubated for a further 16 to 18 h. At the time of pulsing, the parasites would be predominantly in the trophozoite stage. The contents of each well were then harvested onto glass fiber filters using a Skaktron automated cell harvester, washed extensively with distilled water, and dried. The incorporated radioactivity was measured as disintegrations per minute using a Wallac 1409 (Wallac Oy, Turku, Finland) liquid scintillation counter. For test samples the percent incorporated with respect to the control was plotted against the logarithm of the drug concentration. The concentration causing 50% inhibition of radioisotope incorporation (IC_{50}) was determined by interpolation. A parallel experiment by microscopy, using Giemsa-stained smears, was also conducted. These wells containing parasites were incubated with the drug for a total period of 36 to 40 h without [^{3}H]hypoxanthine. Stage specificity of drug action was also determined microscopically using Giemsa-stained smears. The data obtained from the parasite counts were subjected to the same analysis as that from the radioactive hypoxanthine incorporation. All data were analyzed using the software Prism (GraphPad Software Inc., San Diego, Calif.).

RESULTS

Isolation of peptide antibiotics and spectral analysis. Yields of efrapeptins were significantly increased by modification of fermentation conditions. It should be noted that in the literature the procedure for the isolation of efrapeptins involved a much shorter fermentation period. The procedure of Linnet and Beechey (20) uses a fermentation period of 3 days, while Jackson et al. (14) report a time of 13 days. However, in our hands peptide yields were extremely low when fermentation was allowed to proceed for periods up to 14 days. After a considerable length of time we realized that longer fermentation periods (up to 26 days) led to a much higher yield of efrapeptins. One hundred fifty milligrams of efrapeptins was obtained from 3 liters of culture.

The microheterogeneous efrapeptin fractions obtained after purification by reverse-phase MPLC contained as many as seven efrapeptin components ranging in mass from 1,592 to 1,676 Da as monitored by electrospray mass spectroscopy (data not shown). The components at 1,606 Da (efrapeptin C), 1,620 Da (efrapeptin D), 1,634 Da (efrapeptin E and F), and 1,648 Da (efrapeptin G) have been characterized by Gupta et al. (13). Each component of the efrapeptin cluster differs from its neighbors by 14 Da, corresponding to the difference of a single

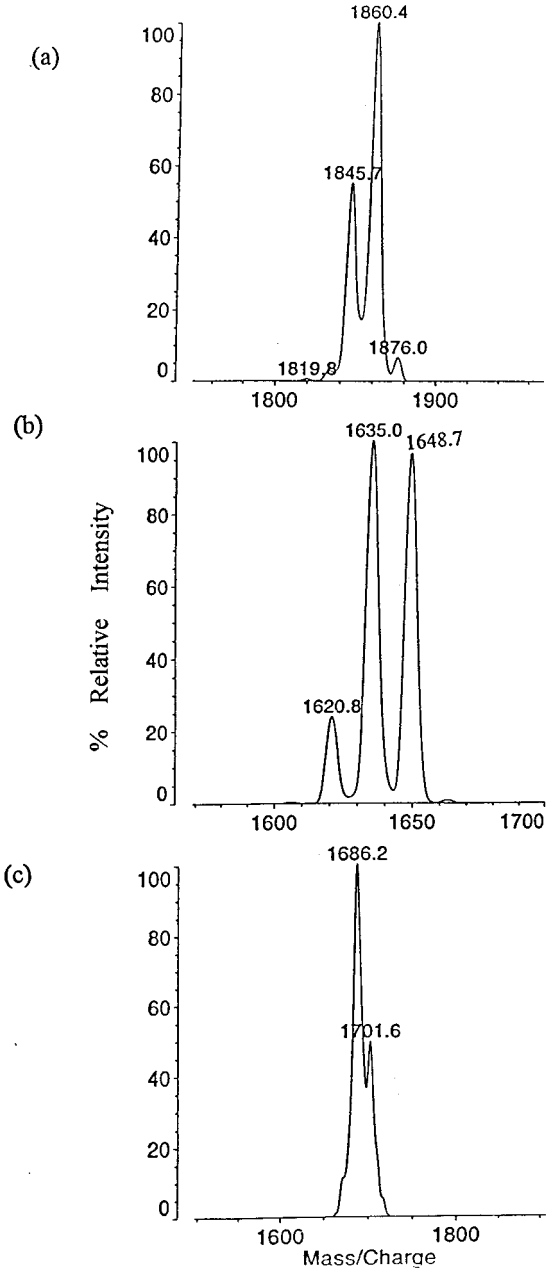


FIG. 1. Mass spectral analysis of peptide antibiotics. MALDI spectrum of zervamicin IIA and IIB (a), efrapeptins (b), and antiamoebin (c). Conditions used for recording the mass spectra are given in Materials and Methods.

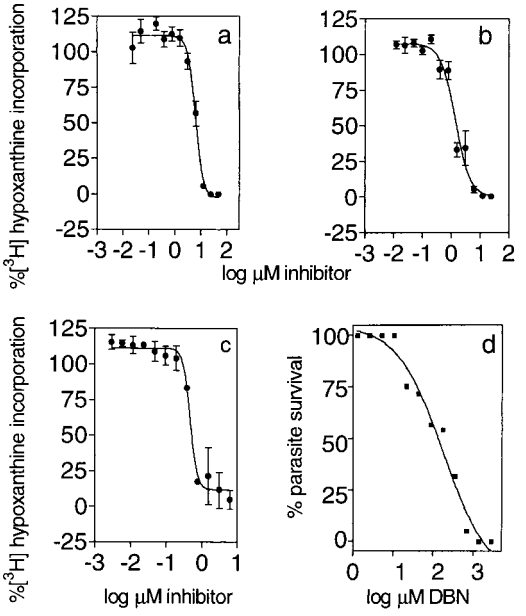


FIG. 2. (a to c) Effect of peptides on uptake of [^{3}H]hypoxanthine by parasitized erythrocytes in culture. (a) Antiamoebin; (b) efrapeptins; (c) zervamicins. The protocol used for measuring [^{3}H]hypoxanthine incorporation is described in the text. (d) Effect of DBN on parasitemia in in vitro cultures. The graphs show means of three experiments, and the error bars represent the standard errors of the means.

CH_2 group. This is consistent with the Gly, Ala, Aib, and Iva replacements noted by Gupta et al. (13). The microheterogeneous composition of efrapeptins (the peptides are labeled efrapeptin C through G) is given in Table 1.

In the present study three new components labeled as efrapeptin A (1,592 Da), efrapeptin H (1,662 Da), and efrapeptin I (1,676 Da) have been identified. Of these, efrapeptins of 1,592 and 1,676 Da are present in very small amounts, and only the efrapeptin of 1,662 Da is present in moderate quantities. Further characterization of the minor components was not undertaken in the present study, because the physicochemical and biological properties of the efrapeptins are not affected by the microheterogeneity. The efrapeptin fraction used in the present study yields three distinct masses by MALDI mass spectral analysis (Fig. 1). The peaks corresponding to 1,635 and 1,648 Da both consist of two isomeric peptides. This sample containing a mixture of efrapeptins was used for the bioassay. The ^{1}H nuclear magnetic resonance spectrum of efrapeptins showed characteristic resonances for the NH, CαH, and CβH protons expected in the peptides and is consistent with the expected amino acid composition (data not shown).

Antiamoebin and zervamicin. The MALDI spectrum (Fig. 1) of the antiamoebin sample gives one major peak of 1,686 Da, which corresponds to that of the antiamoebin sequence given in Table 1. The zervamicin sample yields two peaks of 1,845 and 1,860 Da (Fig. 1), which is consistent with the Aib to Iva replacement seen between zervamicin IIA and IIB peptides. Both peptides have been completely characterized by two-dimensional nuclear magnetic resonance spectroscopy (6, 18). The minor microheterogeneity observed in both samples is commonly observed in fungal peptides produced by nonribosomal polypeptide synthesis (3, 5).

In vitro antiparasitic activity. The uptake of [^{3}H]hypoxanthine by parasitized erythrocytes in microtiter plates was consistent. The mean values for the parasite control wells at the end of the 18-h pulse was generally between 80,000 and 100,000 dpm. Nonparasitized erythrocyte controls contained less than 1.5% of the amount of isotope present in parasitized control cells. The dilution method used in these experiments generated twofold serial dilutions with a 2,048-fold range of concentrations for each compound. The concentration re-

TABLE 2. Effect of peptide antibiotics on growth of *P. falciparum* in culture[a]

Compound(s)	IC_{50} (μM)[b] (^{3}H-hypoxanthine incorporation)	IC_{50} (μM)[c] Smear analysis
Antiamoebin	6.16 ± 0.03	4.72 ± 0.09
Efrapeptins	1.37 ± 0.06	1.21 ± 0.05
Zervamicins	0.48 ± 0.05	0.45 ± 0.05
DBN		174.99 ± 0.18

[a] All results are the means of three experiments ± standard error of mean. The methodology used to estimate IC_{50}s is described in the text.
[b] Concentration of drug that causes 50% inhibition of [^{3}H]hypoxanthine incorporation by *P. falciparum* in culture. All results are the means of three experiments ± standard.
[c] Concentration of drug that causes 50% decrease in parasitemia.

sponse curves for the compounds over this range were characteristically sigmoidal (Fig. 2) after logarithmic transformation of the drug concentration and were interpreted by non-linear regression analysis. An excellent fit of the data to the regression equation (Boltzman sigmoidal) was obtained in each case. The IC_{50}s (Table 2) for efrapeptins, zervamicins, and antiamoebin were 1.37, 0.48, and 6.16 μM, respectively. Data obtained by morphological assessment of Giemsa-stained smears were in close agreement with that from [^{3}H]hypoxanthine uptake (Table 2). In these experiments, parasitemias of 12 to 15% were routinely obtained in the control wells, and drug-treated wells showed corresponding decreases in parasitemia. At high drug concentrations parasites were completely obliterated, and the few survivors were shrunken and pyknotic. However, examination of Giemsa-stained smears of drug-treated cultures showed no deformation of erythrocytes. High concentrations (12.5 μM and higher) of zervamicins did bring about lysis of the erythrocytes. However, pretreatment of erythrocytes with a 3.0 μM concentration of zervamicins did not exhibit visible lysis and the cells remained viable for harbouring parasites. Parasitemias that were obtained with pretreated cells were 9.5 to 11.5% similar to that obtained with untreated cells. Presence of DMSO at a final concentration of 0.5% did not decrease parasitemia or alter parasite morphology. All three compounds were tested on synchronized cultures for their stage specificity and found to be independent of the asexual stage present at the start of the experiment. Efrapeptins were found to kill the parasites even after only 8 h of incubation with the drug.

Efrapeptins (Table 1) have a DBN residue as a C-terminal modification. To check if this basic bicyclic structure is responsible for the peptide's antimalarial property, DBN alone was checked for its effect on parasite growth. As shown in Fig. 2D, a much higher concentration of DBN is required to kill the parasites compared with efrapeptins. The IC_{50} of 175 μM for DBN is 145-fold higher than that obtained for efrapeptins (Table 2).

DISCUSSION

This paper reports the isolation, characterization, and antimalarial properties of antiamoebin, efrapeptins, and zervamicins, which are known inhibitors of mitochondrial activity. During the intraerythrocytic stages of *P. falciparum*, glycolysis is thought to be the main source of ATP, with little or no contribution from mitochondria (26). However, studies on the *P. falciparum* mitochondrion have indicated that this organelle in the parasite maintains a high transmembrane potential (9, 28). The antimalarial drug atovaquone has been shown to rapidly dissipate this potential, indicating that though the mitochondrion in *P. falciparum* does not contribute to the ATP pool, it probably plays a key role in other physiological activities (28).

Antiamoebin and zervamicins are uncouplers of oxidative phosphorylation (5, 6, 18) and work as channel forming ionophores (2, 4). Channel forming activity is mediated by formation of transbilayer helical bundles by these hydrophobic peptides (15, 16, 27). Efrapeptins are specific inhibitors of mitochondrial F_0F_1 ATPase, but at higher concentrations the peptide behaves as a channel-forming ionophore (1). The antiparasitic activity of the channel-forming peptides reported in this work could arise from the dissipation of the parasite mitochondrial membrane potential or alteration of the parasite's plasma membrane potential. Ionophores specific to monovalent cations have been shown to be antiparasitic at concentrations that do not affect lymphoblast and macrophage cell lines, and this has been attributed to enhanced permeability of the erythrocyte membrane after infection (11, 12). The published sequence of chromosome 2 of *P. falciparum* contains the α-subunit of the ATP synthase (10). However, the presence of a functional ATP synthase during the intraerythrocytic asexual stages of the parasite has not been established.

Antiamoebin, efrapeptins, and zervamicins contain unusual amino acids (Table 1), making them less susceptible to proteases and thereby increasing their bioavailability. The antimalarial activity reported in this work indicates that these peptides probably cross multiple membrane barriers to exert their effects on the intraerythrocytic parasite. The antimalarial activity of antiamoebin, an antiprotozoal and anthelmintic peptide antibiotic with low toxicity (25, 30), is particularly promising. However, the toxicities of efrapeptins and zervamicins remain to be determined. Atovaquone, a hydroxynaphthoquinone that dissipates *P. falciparum* mitochondrial membrane potential, exhibits a low IC_{50} of 1 nM in various parasite isolates (23), but there is clinical evidence that a single base change in the *P. falciparum* cytochrome *b* gene can bring about resistance to atovaquone (17). However, parasites treated with the peptide antibiotics studied in this paper, which act at the membrane level, could be less likely to develop resistance to these molecules. High-throughput screening of secondary metabolites from fungi for antiplasmodial activity should lead to the identification of potent chemotherapeutic agents.

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