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The Bis(Indolyl)Imidazole Alkaloid Nortopsentin A Exhibits Antiplasmodial Activity

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A library of enriched marine natural product fractions was screened for their antiplasmodial activity using a SYBR green I fluorescence-based assay. Fractions derived from a sponge of the genus *Spongosorites* exhibited potent inhibition of *Plasmodium falciparum* growth. This genus of sponge has been reported to contain the nortopsentin and topsentin class of bis-indole imidazole alkaloids. This is the first report of nortopsentin A inhibiting parasite growth at the trophozoite stage at submicromolar 50% inhibitory concentrations (IC_{50}).

Over 300 million clinical cases of malaria occur annually, resulting in more than 1 million deaths (1). Unfortunately, because of the prevalence of drug-resistant malaria parasite strains, existing drugs are increasingly losing their efficacy. Therefore, there is an urgent need to identify new drug leads to reduce the global malaria burden. Marine biological diversity provides an excellent opportunity to identify novel drug leads from secondary metabolites of marine organisms. Although research aiming to discover novel marine-derived antiplasmodial agents is relatively recent, over 60 active compounds have been reported to date (2). One of the more important leads is manzamine A, a structurally complex β-carboline alkaloid isolated from a *Haliclona* sp. Manzamine A cleared 90% of the parasite burden in *P. berghei*-infected mice with a single intraperitoneal dose (3, 4). Unfortunately, toxicity issues have limited the clinical development of the compound.

A library of enriched chemical fractions derived from marine organisms collected at depths greater than 50 m was prepared at Harbor Branch Oceanographic Institute using medium-pressure liquid chromatography on an ISCO Combiflash purification system. These materials were screened for their ability to inhibit the chloroquine-sensitive *P. falciparum* strain 3D7 and the chloroquine-resistant strain Dd2. The enriched fractions were solubilized in ethanol and, when necessary, further diluted in culture medium for assay. The inhibitory properties of the library were evaluated at 5 μg/ml using a SYBR green I assay (5, 6) with the chloroquine-sensitive *P. falciparum* strain 3D7 (1% parasitemia)

Received 15 October 2012 Returned for modification 31 October 2012
Accepted 7 February 2013
Published ahead of print 12 February 2013
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doi:10.1128/AAC.02091-12

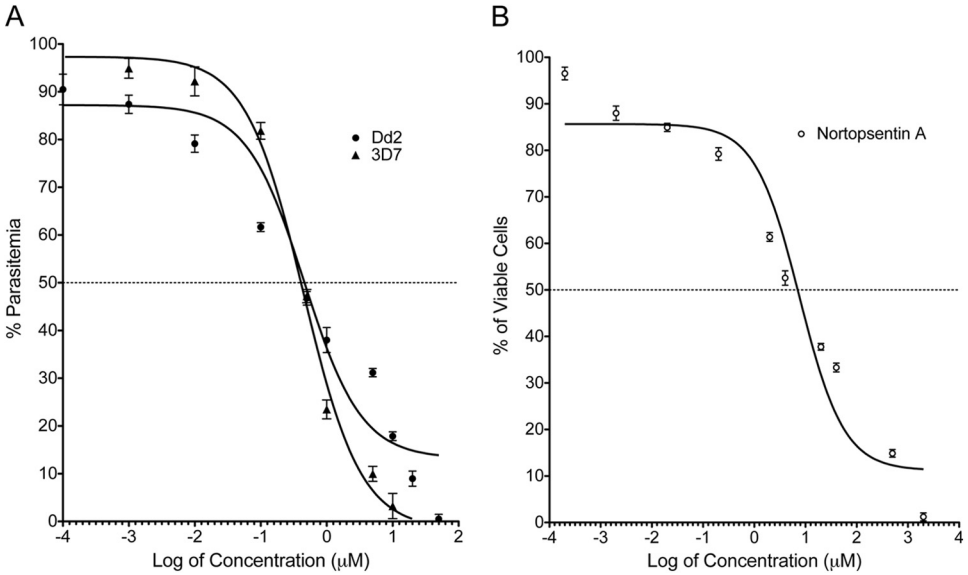
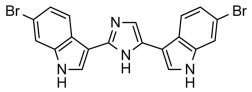
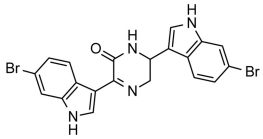
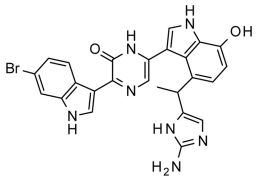
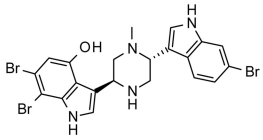
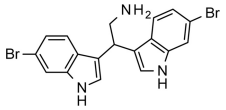
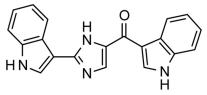
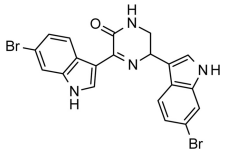


FIG 1 *In vitro* antiplasmodial and cytotoxic analysis of nortopsentin A. (A) Antiplasmodial activity of nortopsentin A in chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) strains of *P. falciparum*. Asynchronous cultures were exposed to different concentrations of inhibitor for 72 h. (B) Cytotoxicity of nortopsentin A. Various concentrations of nortopsentin A were incubated with NIH 3T3 fibroblasts for 48 h to determine cytostatic activity. Data points are averages of at least 3 independent experiments, each done in triplicate. Error bars represent standard deviations.

TABLE 1 Structure-activity relationship of bis(indolyl)imidazole alkaloids[a]

Compound	Structure	IC_{50} (μM) for: *P. falciparum* Dd2	IC_{50} (μM) for: NIH 3T3 cells	Selectivity index
Nortopsentin A		0.46	6.6	14.3
Hamacanthin A		3.1	18.9	6.1
Dragmacidin D		4.3	17.9	4.2
Dragmacidin		6.8	4.5	0.7
Bis(2,2)-6-bromo-indol-3-yl-ethyl amine		8.7	15.9	1.8
Deoxytopsentin		10.3	12.7	1.2
Hamacanthin B		>20	19.1	<0.9

[a] Data shown are the result of at least 3 independent experiments, each done in triplicate.

grown in 100 μl RPMI 1640 medium supplemented with 25 mM HEPES, 11 mM glucose, 25 mM $NaHCO_3$, 100 μM hypoxanthine, 0.5% Albumax I, 25 μg/ml gentamicin, and human A^+ red blood cells (RBC) (2% hematocrit) at 37°C in an atmosphere of 5% CO_2 and 95% air for 72 h. Among the fractions that exhibited >95% inhibition at 5 μg/ml were those from a *Spongosorites* sp. (organism number 5-IV-05-1-002, collected off Lucaya, Bahamas, at a depth of 168 m). Earlier liquid chromatography-mass spectrometry (LC-MS) analysis indicated that nortopsentin A was present as the major component of the active fraction from this organism (7). Nortopsentin A is a bis(indolyl)imidazole alkaloid with anti-inflammatory and antitumor properties that has been previously identified from related specimens of *Spongosorites* (8, 9) and has never been tested for antiplasmodial activity.

To determine if nortopsentin A showed antiplasmodial activity, 50% inhibitory concentrations (IC_{50}s) in both chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *P. falciparum* strains were screened with serial dilutions of compound. The IC_{50}s in 3D7 and Dd2 were 460 nM and 580 nM, respectively, using SYBR green I assays with *Z* factors of >0.9. Although a nortopsentin A structure having aromatic systems in conjugation may emit autofluorescence, our tests show that it did not exhibit any autofluorescence at the concentrations used in this study. To determine the selectivity of this class of inhibitor against malaria, cytotoxicity against NIH 3T3 fibroblasts was evaluated using an MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay (CellTiter 96 aqueous nonradioactive cell proliferation assay; Promega). The test revealed that nortopsentin A has a selectivity index greater than 14, with an IC_{50} in fibroblasts of 6.6 μM (Fig. 1; Table 1).

To gain some insight into structure-activity relationships, a collection of six additional bis(indolyl) natural products that vary in the linking unit between the indole rings was screened using the above-mentioned SYBR green I and MTS assays. The antiplasmodial activities, cytotoxicities, and selectivity indices with respect to these structures are shown in Table 1. Nortopsentin A was the most potent and had the greatest selectivity of all the compounds tested. Deoxytopsentin, which has a keto group adjacent to the imidazole linker, was approximately 10-fold less potent, with a reduced selectivity of 1.2. A series of analogs which contain a six-membered-ring nitrogen heterocycle as the linker unit but which vary in the degree of unsaturation of the linking heterocycle [rang-

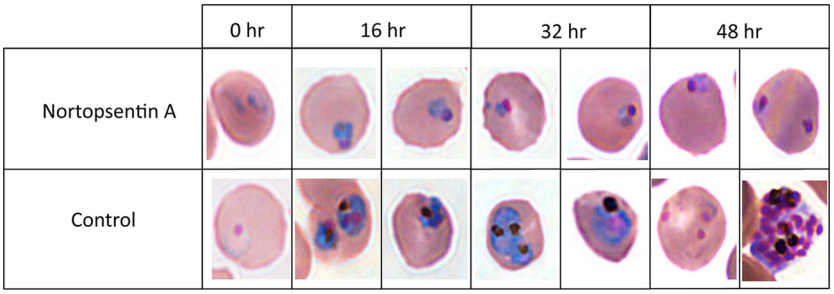


FIG 2 Effect of nortopsentin A on intraerythrocytic development. The development of synchronized *P. falciparum* 3D7 in the presence of 3 μM nortopsentin A was assessed. Giemsa-stained thin blood smears were examined at 0, 16, 32, and 48 h to determine developmental stages. As expected, the control progressed from ring to trophozoite to schizont and to rings and segmenters throughout the 48-h time course. The nortopsentin A-treated culture shows halted progression at the trophozoite stage.

ing from 2-(1*H*)-pyrazinones to 5,6-dihydro-2-(1*H*)-pyrazinones to a fully saturated piperazine] were tested. In this series, the activity against the malaria parasite was all in the low-micromolar range, but the selectivity was lost with increasing saturation and may correlate to loss of planarity of the molecules. Hamacanthin A was an exception to this, as it retained some selectivity for the parasite. In hamacanthin B, where the linkage to the 5,6-dihydro-2-(1H)-pyrazinone changes to the 5 position, the activity against the malaria parasite is lost (>20 μM), while cytotoxicity to the NIH 3T3 cell line is retained. The overall trend observed in the series of compounds tested is consistent with the hypothesis that optimal activity and selectivity may correlate to planarity of the molecule and distance between the two indole rings. Additional modeling and testing of new analogs would be required to confirm this hypothesis.

To better understand the mechanism of action of nortopsentin A against the malaria parasite, a sorbitol-synchronized *Plasmodium falciparum* 3D7 culture was split into two groups, one treated with a 3 μM concentration of the compound and one maintained without compound. Samples were collected at 16-h intervals for 48 h for Giemsa staining of thin smears. As can be seen in Fig. 2, the control culture matured from the ring stage (0 h) through the early trophozoite stage (16 h) to the mature-trophozoite/early-schizont stage (32 h) and finally to the segmenter/ring stage (48 h). However, the development of the nortopsentin A-treated culture was blocked at the early trophozoite stage. Previous studies of this alkaloid have shown that topsentins can act as DNA-intercalating agents and halt DNA synthesis (10). Our results correlate well with this suggested mechanism of nortopsentin A. The DNA synthesis in *Plasmodium* begins at the trophozoite stage of asexual, erythrocytic growth (11, 12). If nortopsentin A binds to DNA during the ring stage of development, DNA synthesis in the trophozoite stage will likely be inhibited, and progression of the cell cycle would cease. Further studies are needed to fully understand the mechanism by which nortopsentin A inhibits the growth of *P. falciparum*.

The fact that only a limited number of clinically relevant drugs are available for malaria therapy is of serious concern. To overcome drug resistance issues, it is important to identify chemical entities with structural features distinct from known antimalarials. This study has identified a novel chemical scaffold from a marine macroorganism as an antiplasmodial agent. Further structure-activity studies are needed to improve the potency and selectivity of this class of compound for it to be seriously considered a candidate for lead optimization.

ACKNOWLEDGMENT

This work was supported by a grant from the National Institutes of Health (R21 AI078376).

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