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Identification of Renieramycin A as an Antileishmanial Substance in a Marine Sponge *Neopetrosia* sp.

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Abstract: The newly developed assay system using recombinant *Leishmania amazonensis* expressing enhanced green fluorescent protein (La/*egfp*) has been applied to the screening of Japanese marine sponges for antileishmanial activity. Bioassay-guided fractionation of an active sponge *Neopetrosia* sp. afforded an active compound which was identified as renieramycin A by spectroscopic analysis. It inhibited La/*egfp* with an IC_{50} value of 0.2 μg/mL.

Keywords: leishmaniasis, marine sponge, renieramycin A

Introduction

Leishmaniasis is caused by parasitic protozoans of the genus *Leishmania* spread by the bite of infected sand flies [1-4]. It is endemic in subtropical and tropical countries and approximately 2 million cases are estimated every year [5]. There are several forms of leishmaniasis, of which cutaneous and visceral leishmaniases are the most common. Pentavalent antimony compounds have been used for treatment of leishmaniasis since the 1940s, and more recently amphotericin B and

other antifungal drugs are used as alternatives. However, these drugs have disadvantages including toxic effects [6-8]. Thus, less toxic antileishmanial drugs are urgently required.

In our continuing program on the discovery of drug leads from Japanese marine invertebrates, we screened 120 marine sponges for antileishmanial activity by the newly developed assay system using recombinant *Leishmania amazonensis* expressing enhanced green fluorescent protein as shown in Figure 1 (La/*egfp*) [9], and found promising activity in the lipophilic extract of *Neospongia* sp. collected in southern Japan. Bioassay-guided isolation furnished renieramycin A (**1**) as an active constituent. Here, we report the isolation, identification and antileishmanial activity of **1**.

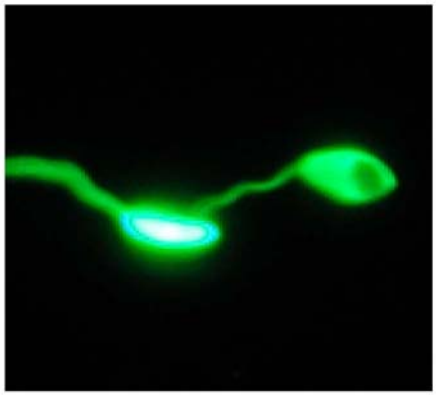
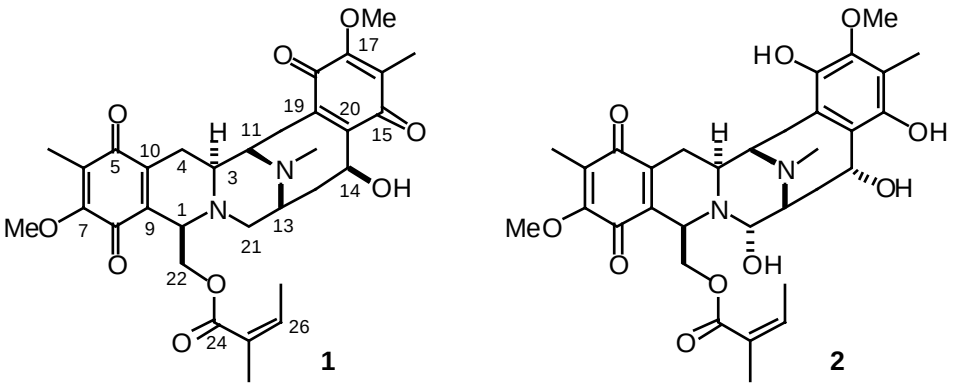


Fig. 1. Fluorescence microscopy image of La/*egfp*.

Results and Discussion

Since this sponge was known to contain highly cytotoxic renieramycin P (**2**: IC_{50} 0.53 nM against P388 cells)[10-12], bioassay-guided fractionation was carried out monitoring both leishmanicidal and cytotoxic activities to distinguish less toxic antileishmanial compounds from those with high toxicity. The organic layer of the extract was fractionated by the modified Kupchan procedure [13] to yield hexane, $CHCl_3$, and 60 % MeOH layers. The $CHCl_3$ layer, which showed the most potent leishmanicidal and cytotoxic activity (IC_{50} 3 and 18 ng/mL, respectively), was separated by ODS flash chromatography using MeOH/H_2O (5:5 and 7:3), CH_3CN/H_2O (7:3 and 85:5), MeOH, and $CHCl_3$/MeOH/H_2O (70:30:5). The fraction eluted with MeCN/H_2O (7:3) which showed less cytotoxicity (IC_{50} values: 450 ng/mL against P388 and 70 ng/mL against La/*egfp*) was purified by reversed phase HPLC using MeCN/H_2O (38:62) with 0.2 M NaCl, and the final purification by reversed phase HPLC using MeCN/H_2O (35:65) containing 0.2 M NaCl afforded renieramycin A (**1**, 0.5 mg).



The FABMS of **1** exhibited an $(M+4H+H)^+$ ion at *m/z* 571, which corresponded to the hydroquinone form; in fact, ESIMS gave an $(M+H)^+$ ion at *m/z* 567. A database search using MarineLit™ suggested this pseudomolecular ion peak coincided with that of renieramycin A [14]. Analysis of 2D NMR data including the HOHAHA [15] and HMBC [16] spectra disclosed three spin systems and two quinone moieties which are the same as renieramycin A (Figure 2). However, some of the chemical shift values obtained in CD_3OD was not consistent with those of the literature. Comparison of ^{1}H-NMR data in the same solvent ($CDCl_3$) with those of the literature enabled us to assign the compound **1** was renieramycin A.

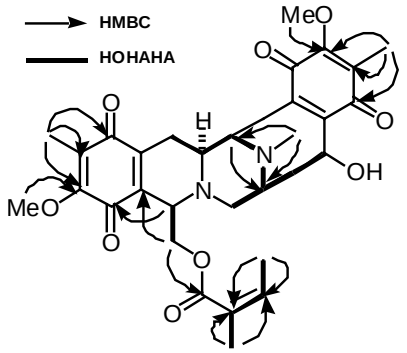


Fig. 2. HOHAHA and HMBC correlations of 1.

Antileishmanial activity of renieramycin A (**1**) was evaluated using La/*egfp*. As shown in Figure 3, renieramycin A showed a dose-dependent inhibition against La/*egfp* with an IC_{50} value of 0.2 μg/mL. On the other hand, it showed cytotoxicity against P388 murine leukemia cells at the ten times higher concentration (IC_{50} 2.2 μg/mL).

Conclusions

Several antileishmanial compounds including cyclic peroxides [17], pyridoacridine alkaloids [18], and manzamine alkaloids [19] have been reported from marine invertebrates. However, the number of antileishmanial compounds isolated from marine source is still limited.

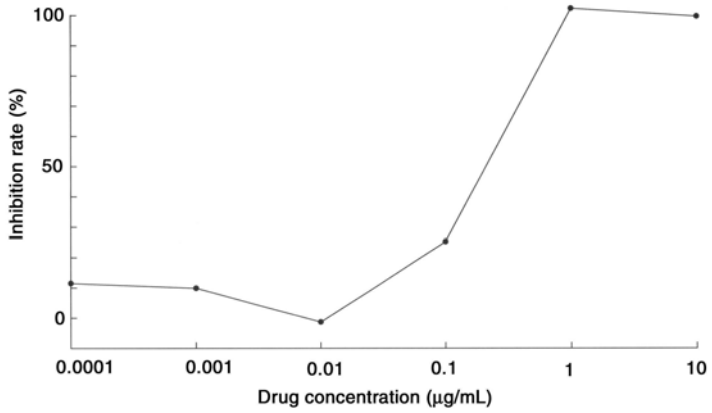


Fig. 3. Inhibition curve of La/*egfp* by renieramycin A

We adopted for the first time the newly developed bioassay using recombinant *Leishmania amazonensis* expressing enhanced green fluorescent protein (La/*egfp*) to the search of leishmanicidal metabolites from marine organisms, and isolated renieramycin A (**1**) from a marine sponge *Neopetrosia* sp. From the less cytotoxic fraction obtained after several steps of chromatographic fractionation, renieramycin A (**1**) was obtained as an active substance. As expected, **1** showed moderate selectivity for inhibition against La/*egfp* proliferation over cytotoxicity against P388 cells.

In this study, we have demonstrated the efficacy of the new assay using La/*egfp* for discovery study of antileishmainal compounds from natural source.

Acknowledgments

We are indebted to the crew of R/V Toyoshio-maru of Hiroshima University for assistance in collection of the sponge samples. We thank Professor Rob W. M. van Soest at University of Amsterdam for identification of the sponge.

Experimental

General

NMR spectra were recorded on a JEOL A600 NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts were referenced to the CD_3OD signals (δ_H 3.3 and δ_C 49, respectively). FABMS spectra were measured on a JEOL JMS700 tandem mass spectrometer using NBA as a matrix. ESIMS data were obtained using JEOL AccuTOF JMS-T100LC.

Animal material

The animal specimens were collected by hand using SCUBA off Kuchinoerabu-jima Island in the Satsunan Islands (30°28'31"N; 130°11'73"E) in July 2001 and identified as *Neopetrosia* sp. by Dr. Rob van Soest, University of Amsterdam. They were immediately frozen and kept at –20 ℃ until processed.

Antileishmanial assay

Fluorescence signals of La/*egfp* promastigotes cultured in 199 medium (NISSUI Pharmaceutical, Tokyo, Japan) in 96-well plates at 25 °C were measured by a fluorescence microplate reader (Fluoro scan Ascent FL., Dainippon Pharmaceutical Co., Osaka, Japan) with excitation at 485 nm and emission at 538 nm. To determine the IC_{50} (0.42 μg/mL) of amphotericin B (ICN, Ohio, USA), La/*egfp* were cultured at 5 x 10^5 cells/mL with various concentrations of the drug, and their fluorescence signals were measured after 72 h incubation.

Isolation

Frozen animals (1.5 kg) were exhaustively extracted with MeOH (2L) and EtOH (2L x 2), and the combined extracts were concentrated and partitioned between H_2O and $CHCl_3$. The organic layer was subjected to the modified Kupchan procedure [7]: first partitioned between *n*-hexane and MeOH/H_2O (90:10), then the MeOH/H_2O (90:10) layer was diluted with H_2O to make MeOH/H_2O (60:40) which was extracted with $CHCl_3$. The $CHCl_3$ layer was separated by ODS flash chromatography using MeOH/H_2O (5:5 and 7:3), CH_3CN/H_2O (7:3 and 85:5), MeOH, and $CHCl_3$/MeOH/H_2O (70:30:5). Fractions eluted with MeCN/H_2O (7:3) was concentrated and separated by reversed phase HPLC [Phenomenex Luna® phenyl-hexyl, 20 x 250 mm] using MeCN/H_2O (38:62) with 0.2 M NaCl. The active fraction was further purified by reversed phase HPLC [COSMOSIL 5C_{18}-ARII, 10 x 250 mm] using MeCN/H_2O (35:65) with 0.2 M NaCl to afford renieramycin A (**1**, 0.5 mg); $[\alpha]_D$ -30 (*c* 0.02, MeOH); ^{1}H- and ^{13}C-NMR see Table 1; FABMS *m/z* 571 $[M+4H+H]^+$; ESIMS *m/z* 567 $[M+H]^+$.

Table 1. NMR Data for 1 and Renieramycin A

#C	δ_C [a]	δ_H [a]	HMBC	δ_H [b]	δ_H [b,c]
1	60.0	3.60	C-8	3.62	3.60
3	*d*	2.65		2.64	2.64
4	*d*	2.63		2.75	2.75
		1.2		1.26	1.26
5	187.1				
6	128.8				
7	157.7				
8	190.5				
9	144.0				
10	*d*				
11	56.9	4.04	C-13	4.04	4.04
13	62.6	3.14		3.18	3.18
14	71.5	3.62		4.43	4.44
15	188.0				
16	130.2				
17	156.9				
18	*d*				
19	*d*				
20	*d*				
21	43.0	3.2		3.18	3.18
		2.7		2.71	2.71
22	64.0	4.45	C-9	4.48	4.47
		4.27	C-24	4.19	4.19
24	168.9				
25	128.3				
26	140.2	5.94		5.92	5.92
6-Me	8.6	1.85 s	C-5, 6, 7	1.93	1.91
7-OMe	61.2	3.95 s	C-7	4.00	4.00
12-NMe	42.5	2.46 s	C-11, 13	2.43	2.43
16-Me	8.3	1.91 s	C-15, 16, 17	1.93	1.92
17-OMe	61.2	3.92 s	C-17	4.01	4.01
25-Me	20.8	1.53 s	C-25, 26	1.57	1.55
26-Me	15.8	1.72 d	C-25, 26	1.80	1.78

a: in CD_3OD, *b*: in $CDCl_3$, *c*: literature data, *d*: not observed

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Samples Availability: Not available.