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BISTRAMIDE A, A NEW TOXIN FROM THE UROCHORDATA *LISSOCLINUM BISTRATUM* SLUITER: ISOLATION AND PRELIMINARY CHARACTERIZATION

D. GOUIFFES,[1] M. JUGE,[1] N. GRIMAUD,[1] L. WELIN,[1] M. P. SAUVIAT,[2] Y. BARBIN,[3] D. LAURENT,[3] C. ROUSSAKIS,[1] J. P. HENICHART[4] and J. F. VERBIST[1]

[1] SMAB (Substances Marines à Activité Biologique), UER des Sciences Pharmaceutiques, 44035 Nantes cedex, France;
[2] Laboratoire de Physiologie Comparée, UA CNRS 1121, Bt 443, Université de Paris XI, Centre d'Orsay, 91405 Orsay cedex, France;
[3] Laboratoire de Pharmacologie, ORSTOM, BP A5, Noumea cedex, Nouvelle-Caledonie;
[4] INSERM U 16, Place de Verdun, 59045 Lille cedex, France

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D. GOUIFFES, M. JUGE, N. GRIMAUD, L. WELIN, M. P. SAUVIAT, Y. BARBIN, D. LAURENT, C. ROUSSAKIS, J. P. HENICHART and J. F. VERBIST. Bistramide A, a new toxin from the urochordata *Lissoclinum bistratum* Sluiter: isolation and preliminary characterization. *Toxicon* **26**, 1129–1136, 1988.—Two cases of human intoxication caused by the lyophilized powder of *Lissoclinum bistratum* Sluiter, a New Caledonian ascidian, are reported. The symptoms observed were caused by a substance designated bistramide A ($C_{30}H_{48}N_2O_6$) of hitherto unknown chemical structure. Preliminary toxicological investigations indicate that bistramide A may effect the central nervous system, leading to paresthesia and loss of muscle tone. A progressive decrease in cardiac rhythm was also observed in animals. Bistramide A (1.4×10^{-6} M) did not alter the resting potential of frog heart and skeletal muscle but reduced the amplitude and duration of cardiac action potential and prolonged the interval between action potentials. Bistramide A also has a marked cytotoxic effect on cancer cells KB ($IC_{50} = 4.5 \times 10^{-8}$ M) and P 388 ($IC_{50} = 2.0 \times 10^{-8}$ M) and on normal endothelial cells ($IC_{50} = 2.2 \times 10^{-8}$ M). However, it has not been possible to relate the cytotoxic property to the symptoms of intoxication. Bistramide A may originate from the urochordate itself or from symbiotic algae.

INTRODUCTION

A FEW minutes after handling the lyophilized powder of a New Caledonia ascidian, *Lissoclinum bistratum* Sluiter, a laboratory worker experienced symptoms of intoxication. These same effects, felt to a lesser degree by a second person in the same room, were described as being "like a state of shock, at first, followed by paralysis or numbness around the mouth and heaviness in the limbs". As no chemical or pharmacological study had been carried out on this urochordate, this experience led us to isolate the substance responsible for the intoxication and then to study it toxicologically and to search for the possible origin of the toxin (urochordate itself or symbiotic algae).

MATERIALS AND METHODS

Isolation of the toxin

Samples of *Lissoclinum bistratum* Sluiter were collected near UA islet in New Caledonia, in February 1986 by divers of ORSTOM Nouméa. Specimens were identified by Dr F. Monniot (Muséum National d'Histoire Naturelle, Paris) who kept one sample. The animals were immediately frozen at −20°C and lyophilized.

TABLE 1. TOXIN ISOLATES

	Fractions (g)		
	A	B	C
Chromatography 1	6	11	2
Chromatography 2	3.5	7.2	0.3
Chromatography 3	2.1	4.8	0.3

Toxin always localized in B fractions.

Chromatography 1: phase — Silicagel-60 70/200 μm — 600 g; solvents — EtOAc: fraction A (4 litres); fraction B (7 litres); MeOH: fraction C (2 litres).

Chromatography 2: phase — Silicagel-60 40/70 μm — 400 g; solvents — EtOAc: fraction A (4 litres); fraction B (6 litres); MeOH: fraction C (2 litres).

Chromatography 3: phase — Lichroprep®-diol 25/40 μm — 300 g; solvents — *n*-hexane/EtOAc (5:5): fraction A (1.5 litres); fraction B (4 litres); EtOAc: fraction C (1 litre).

In order to determine the toxicity, a fraction of the powder was extracted separately using water, alcohol and dichloromethane solvents; the dichloromethane extract was more toxic. The lyophilized powder (2.5 kg; 42% of the fresh weight) was stirred six times in 10 liters of dichloromethane (CH_2Cl_2) at 20°C. This solution was concentrated in a rotary evaporator under reduced pressure at 30°C to obtain a viscous extract (19 g; 0.76% of the dry weight). Thin-layer chromatography of this extract on silica-gel plates with ethylacetate/propanol-2 (9:1, v/v) showed an intense spot (R_f 0.2) under u.v. light at 254 nm and with sulfuric acid spray. This extract was eluted on a chromatograph (Prepamatic LC-2, Jobin-Yvon; u.v. 254 nm detection) using the following phases: silica-gel-60 70/200 μm and 40/70 μm with ethyl acetate and then Lichroprep®-diol 25/40 μm (Merck) with *n*-hexane/ethyl acetate (5:5, v/v) (Table 1).

The purity of the toxin was checked by HPLC (LDC Constametric III, ERC 7510 ERMA OPTICAL RI detector) on 5 μm Hibar Lichrosorb® columns (Merck): diol with *n*-hexane/ethyl acetate (60:40, v/v); Si-60 with dichloromethane/methanol (97:3, v/v); and RP_{18} with methanol/water (75:25, v/v).

Fast atom bombardment mass spectra of bistramide A were obtained on a Kratos MS-50 double-focusing mass spectrometer. The samples were dissolved in thioglycerol, and a small drop of the sample solution was placed on the copper target of the FAB direct insertion probe. The sample was bombarded with 8 keV xenon atoms, and the ions produced were accelerated through 8 kV. A high resolution mass spectrum was recorded on a Kratos MS-50 by using a direct insertion probe in the following conditions: ionizing energy 70 eV, accelerating potential 8 kV, source temperature 250°C. Chemical ionization mass spectra (NH_3) were recorded on a Ribermag R10-10 (combined with a Riber 400 data system mass spectrometer at 70 eV by using direct insertion).

^{1}H-NMR experiments were performed on a sample containing 12 mg of bistramide A in 0.5 ml of $CDCl_3$ using TMS as internal reference. All the NMR spectra were recorded on a Bruker AM 400 wide bore spectrometer operating at 400.134 and 100.614 MHz for ^{1}H and ^{13}C observations respectively. All spectra were acquired using a 5 mm ^{1}H/^{13}C dual tuned probe. Temperature was regulated at 25°C.

Amino acid analysis was done with a Beckmann type 119 CL autoanalyser. A small amount of bistramide A was hydrolysed using HCl at 110°C for 24 hr in a sealed flask. After lyophilization, this hydrolysate was dissolved in a buffer solution with norleucin as internal standard. Reaction of amino functions with ninhydrin was used for the detection.

Toxicological study

Acute toxicity on Swiss, male mice (20 ± 5 g). Extracts were injected i.p. into mice in the following conditions: water extract up to 20 mg/kg of body weight; alcohol extract in an alcohol–trimethylene glycol solution (1:3, v/v) prepared at a concentration of 4 mg/ml and injected in a dose of up to 20 mg/kg of body weight; and dichloromethane extract in a dimethylsulfoxide (DMSO)–H_2O mixture (1:4, v/v) at 2 mg/ml up to 10 mg/kg of body weight. Assessment of LD_{50} of the toxin by i.p. injection was according to the method of LITCHFIELD and WILCOXON (1949). The toxin was administered to five mice in each group in a 1:9 (v/v) DMSO–H_2O solution (0.1 mg/ml) at doses of 0.5, 1, 1.5, 2 and 4 mg/kg of body weight, and a DMSO–H_2O solution (1:4, v/v) was injected into a control group.

Preliminary toxicological screening on rodents. Preliminary screening was performed on the toxin using three animals of one species (either male Wistar CF rats, Swiss male mice, or rabbits) per test. Anesthesia, when performed (see below), was by i.m. injection of ketamine 175 mg/kg (Ketalar®, Parke-Davis, 50 mg/ml). Local anesthetic activity (REGNIER, 1929) was determined by administering the toxin in three drops of 2.5 mg/ml DMSO–H_2O solution (1:9, v/v) placed on the rabbit cornea and by intradermal injection in a DMSO–H_2O

solution (1:9, v/v) at doses of 2 and 20 mg/kg of body weight in rats (250 ± 20 g) (MACK and NELSON, 1953). Analgesic activity according to the tail-flick method (D'AMOUR and SMITH, 1941) was determined in rats (170 ± 20) by i.p. injection in a DMSO–H_2O solution (1:9, v/v) at doses of 1 and 2 mg/kg of body weight. Paralyzing curariform activity was tested on an anesthetized rat (250 ± 20 g) sciatic nerve gastrocnemius preparation (PRESTON and VAN MAANEN, 1953). The nerve, disengaged but left intact *in situ* was stimulated electrically. The contractions of gastrocnemius muscle were recorded after i.v. injection at a dose of 5 mg/kg of body weight. Electrocardiograms were made on anesthetized mice (20 ± 5 g) and rats (250 ± 20 g) before and after administration of the substance by i.p. injection in a DMSO–H_2O solution (5:95, v/v) at a dose of 2 mg/kg of body weight. Hematograms and blood gas levels were measured in anesthetized rats (250 ± 20 g) 15 min and 30 min after i.p. injection of a DMSO–H_2O solution of the toxin (1:9, v/v) at a dose of 1, 10 and 20 mg/kg of body weight.

Electrophysiological studies. Experiments were performed on whole cleaned frog sartorius muscle isolated from the leg and on a spontaneously beating atrium, isolated from the sino-atrial region of the heart of *Rana esculenta*. Transmembrane resting and action potentials were recorded with glass microelectrodes (15–25 MΩ; tip potential ≤ ±3 mV) filled with 3 M KCl and connected to a differential voltage follower by hybrid Ag, AgCl, platinum black electrodes (SAUVIAT and FEUVRAY, 1986). These potentials were displayed on an oscilloscope (Tektronic 5110) and photographed for analysis on an IFELEC X–Y plotter. Results are expressed as mean values of (n) experiments ± S.E. The composition of the standard Ringer solution was (mM): NaCl, 110.5; KCl, 2.5; $CaCl_2$, 2; Hepes buffer, 5; pH, 7.2. Toxin was deluted at 1 mg/ml in DMSO or in ethanol and stored at 4°C. The control solution thus contained the same amount of solvent as the toxin solution.

Cytotoxicity on mammalian cells. Tests on KB and P 388 cancer cell strains were performed according to the NCI protocol (GUERAN *et al.*, 1972). The concentration for 50% inhibition of cell growth (IC_{50}) was used to express cytotoxic activity. Endothelial cells were taken from human umbilical cord and maintained *in vitro* in culture with RPMI 1640 medium. For the test, cells were distributed in microwells (7×10^5 cells/ml), and the toxin was incorporated immediately into DMSO solution. Each concentration was tested twice, and a control DMSO was done each time. Cell growth (IC_{50}) was calculated by colorimetry after 72 hr incubation at 37°C. The reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (M.T.T.) added in microwells led, after 4 hr, to formation of violet crystals dissolved in propyl chloride (MOSMANN, 1983). IC_{50} is expressed as the mean value of seven experiments ± S.E.

Toxicity on other organisms. Gram-negative bacteria tested included *Escherichia coli* (four strains), *Klebsiella pneumoniae* (three strains), *Morganella morganii*, *Proteus mirabilis* (four strains), *Proteus vulgaris*, *Pseudomonas aeruginosa* (three strains) and *Serratia marcescens* (three strains). Gram-positive bacteria were *Staphylococcus aureus* (five strains) and *Streptococcus* group D (two strains). These tests were carried out in microwells, with the toxin incorporated into Mueller–Hinton culture medium at concentrations of 500 [illegible]

were a few isolated cases of myoclonus of the dorsal muscle which varied in intensity according to the animal; at 1.5 mg/kg of body weight (LD_{50}) there was a decrease in motor activity 15 min after i.p. injection, with appearance of myoclonus and spasm, particularly in the dorsal region. Death due to respiratory depression occurred several hr after the dose was administered; at 10 mg/kg of body weight, the same symptoms as for the LD_{50} were observed after 15 min, and respiratory difficulties became more marked after 45 min, with apnea and cyanosis followed by paralysis of the lower limbs. Death of the animals occurred within about 1 hr. The control group injected with DMSO–H_2O solution showed no symptoms of intoxication. Macroscopic examination upon autopsy of animals which had received a lethal dose showed no abnormalities, with neither hemorrhage nor lesions in vital organs.

Preliminary screening on rodents

No local anesthetic activity nor analgesic effect was observed after administration of bistramide A. The muscle contractions of the anesthetized rat sciatic nerve gastrocnemius preparation were not altered. Intracisternal injection of the substance at a dose level of 1.5 mg/kg of body weight (a dose equal to the i.p. LD_{50}) did not cause mortality in mice; but, immobility with loss of muscle tone was rapidly apparent (5 min after injection). However, intracerebroventricular injection of bistramide A (1 mg/kg) caused loss of muscle tone and respiratory disorders followed by the death of the rats within 5 hr. Analysis of blood samples taken 15 min after i.p. injection of the substance revealed no abnormalities (99% O_2 saturation of hemoglobin and no hemolysis). However, 30 min after injection of 10 mg/kg i.p. in the rat, hemoglobin saturation was only 95%. The pO_2 level decreased (11.0 kPa), whereas pCO_2 (6.0 kPa) increased as compared to controls (pO_2 = 13.5 kPa, pCO_2 = 5.7 kPa). Electrocardiograms in mice and rats showed a negative chronotropic effect after 20 min. Cardiac rhythm was reduced by 36% in mice and 41% in rats (Table 2) with T-wave inversion after 10 min of toxin application.

Electrophysiological study

Bistramide A (1.4×10^{-6} M) did not modify the resting membrane potential (RP) of frog skeletal muscle. In 27 impalements of three muscles, RP was -88.8 ± 1.0 mV in the presence and -87.7 ± 1.1 mV in the absence of toxin in Ringer solution. DMSO (1/1000) did not alter, the RP which was -86.6 ± 2.3 mV (15 impalements from two muscles) in Ringer solution. In heart muscle (Fig. 1), the alcoholic solution of bistramide A (1.4×10^{-6} M) did not change the RP, whereas the amplitude of the spontaneous action potential (AP) was reduced by about 12% and the overshoot abolished. Moreover,

TABLE 2. HEART RATES IN MICE AND RATS AFTER i.p. INJECTION OF BISTRAMIDE A (2 mg/kg)

	Time (min)				
	t = 0	t = 3	t = 10	t = 15	t = 20
Rat (n = 6)					
beats/min	536 ± 26	524 ± 43	500 ± 42	472 ± 33	315 ± 44
% decrease		2.5	6.7	12	41
Mouse (n = 4)					
beats/min	400 ± 28	345 ± 10	300 ± 32	275 ± 34	255 ± 30
% decrease		13	25	31	36

Results are shown as means ± S.E.M.

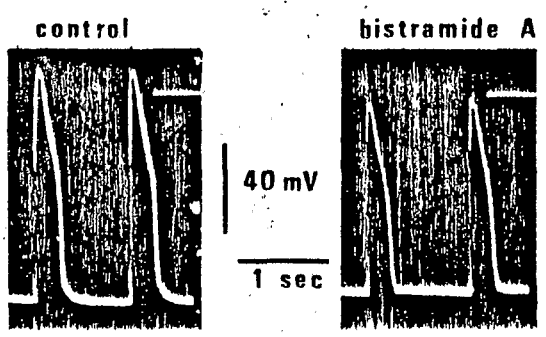

FIG. 1. ACTION POTENTIAL RECORDED ON SPONTANEOUSLY BEATING FROG ATRIUM PREPARATION IN THE CONTROL SOLUTION AND 15 MIN AFTER BISTRAMIDE (1.4×10^{-8} M) APPLICATION ON TWO DIFFERENT FIBRES.
Upper horizontal line indicates 0 mV membrane potential.

bistramide A shortened the AP duration (APD_{10} measured at a membrane potential 10 mV higher than RP) by 11%. In the presence of toxin, the interval between two AP was increased by 8%. Results are summarized in Table 3.

Toxicity on cellular organisms

The IC_{50} for KB, P 388 cancer cells or normal human endothelial cells was of the same order of magnitude: 4.5×10^{-8} M ($3.2 \times 10^{-2} \pm 0.3$ μg/ml), 2.0×10^{-8} M ($1.4 \times 10^{-2} \pm 0.4$ μg/ml) and 2.2×10^{-8} M ($1.6 \times 10^{-2} \pm 0.6$ μg/ml) respectively.

Toxicity on other organisms

Bistramide A showed no antibacterial activity at 500 μg/ml. The LD_{100} of bistramide A was less than 1 μg/ml in *Artemia salina*. Mortality in ichthytoxicity experiments on *Gambusia affinis* was 0% 72 hr after application of 0.1 mg/ml of bistramide A but 100% after 2 hr at 5 mg/ml and 6 hr at 1 mg/ml.

DISCUSSION

Contrary to findings for most other marine toxins, bistramide A was found in large quantities (0.16% of the dry weight of the ascidian in *Lissoclinum bistratum*). The

TABLE 3. EFFECT OF BISTRAMIDE A (1.4×10^{-8} M) ON THE ELECTRICAL ACTIVITY OF SPONTANEOUSLY BEATING FROG ATRIAL FIBRES HEART MUSCLE PREPARATION

	Rp (mV)	Os (mV)	Total AP (mV)	APD_{10} (msec)	AP interval (msec)
Control ($n = 15$)	-88.9 ± 1.0	$+3.2 \pm 0.8$	93.2 ± 1.1	305 ± 3	748 ± 27
Toxin ($n = 21$)	-87.8 ± 1.0		82.1 ± 1.8	271 ± 4	809 ± 14

RP = resting potential Os = overshoot; Total AP = total amplitude of the action potential; APD_{10} = action potential duration measured at membrane potential 10 mV higher than RP; AP interval = interval between two consecutive AP; the Ringer control solution contained ethanol 1/1000. Exposure time was 15 min. Results are shown as means ± S.E.M.

substances previously isolated from the *Lissoclinum* genus were always cyclopeptides (IRELAND and SCHEUER, 1980; IRELAND *et al.*, 1982; WASYLYK *et al.*, 1983; SESIN *et al.*, 1986). Bistramide A is not a cyclopeptide (no amino acid) and its chemical formula ($C_{40}H_{68}N_2O_6$) does not correspond to any known compound.

The acute toxicity of bistramide A is fairly high (LD_{50} = 1.5 mg/kg by i.p. injection in mice). This value is close to that of lophotoxin or brevetoxin B, both marine toxins with LD_{50} values respectively of 8 and 0.25 mg/kg of body weight (i.p. injection in mice). However, the LD_{50} of bistramide A is far higher than those of most other known marine toxins (KAUL and DAFTARI, 1986). The action of bistramide A is very fast, symptoms appearing 15 min after i.p. injection in mice, which suggests that the substance diffuses rapidly and is highly toxic. The symptoms in rodents were identical to those observed with the dichloromethane fraction from which bistramide A was isolated, and similar to those described by the laboratory workers who were intoxicated. The paresthesia around the mouth felt by the intoxicated workers suggested a local anesthetic or analgesic effect, but no evidence of this type of activity was detected experimentally. However, this paresthesia symptom in humans was followed by heaviness in the limbs, whereas mice were affected by a decrease of motor activity which could lead to paralysis and ultimately death after respiratory depression (apnea, cyanosis). These symptoms seem to suggest a curaremimetic action; however, no effects were observed on anesthetized rat sciatic nerve gastrocnemius preparation, which led us to exclude the possibility of any effect on nerve, muscle or end plates. Intracisternal injection (i.c.) led to rapid immobility, with loss of muscle tone, 5 min after injection, which would seem to argue in favor of a central effect of the toxin. However, i.c. injection of the i.p. LD_{50} dose was not lethal in mice while intracerebroventricular injection of a similar dose caused the death of the rats. Intraperitoneal injection of bistramide A in mice and rats caused progressive slowing of cardiac rhythm. At the LD_{50} dose in mice, bistramide A had a negative chronotropic effect which might contribute to its lethal action. However, the product's lethal effect in rodents would seem to be essentially due to respiratory depression. In fact, respiratory disorders occurred early (10 min after administration) in association with myoclonus. The partial blood oxygen pressure (pO_2) measured later in the rat (30 min after i.p. administration) was decreased, whereas pCO_2

Bistramide A has very high cytotoxicity and affects different *in vitro* cell types: murine leukemic cells, human cancer cells and normal human endothelial cells. At the present stage of our studies, we cannot determine whether or not this cytotoxicity is linked to the symptoms caused by the toxin.

It may seem surprising that the toxin, in view of its high concentration in *Lissoclinum bistratum* (0.16% of the dry weight), has no effect on the ascidian itself, whereas it is highly toxic on another invertebrate (*Artemia salina*) and a fish (*Gambusia affinis*). However, since this urochordate is rich in symbiotic algae [*Prochloron* sp. (prochlorophyta)] (GRIFFITHS and LUONG, 1983), it was difficult for us to determine the exact origin of the toxin obtained. Nonetheless, certain features of its structure (ether bridges, hemiacetalic function) are not unlike those of various other marine toxins from microorganisms such as debromoaplysiatoxin from several cyanophytes (MYNDERSE *et al.*, 1977) or pectenotoxins from several pyrrophycophytes (MURATA *et al.*, 1982). Moreover, since prochlorophytes are very similar to bacteria resistant to the toxin, it may be postulated that the toxin is produced by the *Prochloron* in which it accumulates. In this case, one aspect of the symbiosis could be that the *Prochloron* serves to protect the ascidian against predators.

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