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Palytoxin toxicity after acute oral administration in mice

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ABSTRACT

The acute oral toxicity of palytoxin (PLTX), a highly toxic compound associated with seafood intoxication in tropical and subtropical areas, was investigated in mice. After gavage administration (300–1697 μg/kg) to groups of five female CD-1 mice, signs of toxicity and lethality were recorded for 24 h. The LD_{50} was 767 μg/kg (95% confidence limits: 549–1039 μg/kg) and the main symptoms observed were scratching, jumping, respiratory distress and paralysis. Hematoclinical analyses showed increased levels of creatine phosphokinase and lactate dehydrogenase at doses of 600 μg/kg and above, and aspartate transaminase at 848 μg/kg and above. Histological analysis revealed acute inflammation of the forestomach in mice surviving up to 24 h after administration (424–1200 μg/kg). Other histological alterations were observed in the liver and pancreas, while cardiac and skeletal muscle cells revealed only ultrastructural alterations visible by transmission electron microscopy. Ultrastructural and hematoclinical findings suggest an involvement of skeletal and/or cardiac muscle as targets of PLTX, according to the observed human symptoms. A NOEL of 300 μg/kg can be estimated from this acute oral toxicity study.

1. Introduction

Palytoxin (PLTX) is one of the most toxic non-proteinaceous compounds known. It has a complex and unique structure, including a long polyhydroxylated and partially unsaturated aliphatic backbone with 64 chiral centers (Moore and Bartolini, 1981; Uemura et al., 1981). The toxin was first purified in 1971 from the soft coral *Palythoa toxica* in Hawaii (Moore and Scheuer, 1971), and subsequently detected in other *Palythoa* species, such as *P. tuberculosa* (Kimura et al., 1972), *P. mammillosa* or *P. caraibeorum* (Attaway and Ciereszko, 1974). In addition, PLTX-like compounds are found in microalgae belonging to the genus *Ostreopsis*, which are thought to be the progenitor organisms (Usami et al., 1995). In the past few years, the presence of *Ostreopsis ovata* in the Mediterranean Sea has been linked to cases of breathing difficulty in humans (Gallitelli et al., 2005; Ciminiello et al., 2006; Durando et al., 2007). PLTX has also been detected in crabs (Alcala et al., 1988) and in several species of fish (Onuma et al., 1999; Taniyama et al., 2002) from tropical and subtropical areas, suggesting its entrance into the food chain (Gleibs and Mebs, 1999). Recently, the presence of PLTX-like compounds has been reported in shellfish and seems to cause positivity of the mouse bioassay for lipophilic toxins (Aligizaki et al., 2008; Deeds and Schwartz, in press).

PLTX has been considered the causative agent of several cases of human poisoning, with some lethalities, following the consumption of seafood (Deeds and Schwartz, in press). The most frequent symptoms seem to start with general malaise characterized by nausea, vertigo and diarrhoea, followed by weakness, myalgia, myoglobinuria and abnormalities of the electrocardiogram (Alcala et al., 1988; Onuma et al., 1999; Taniyama et al., 2002). Nevertheless, the unequivocal presence of PLTX has not always been demonstrated due to lack of residual tissue for analysis (Kodama et al., 1989; Llewellyn, 2001). Not all clinical reports provide information on analysis of seafood samples (Okano et al., 1998; Shinzato et al., 2008). A direct examination of ingested seafood samples was reported by Taniyama et al. (2002), who described a lethal case of intoxication after the consumption of fish (*Epinephelus* sp.) contaminated by PLTX or a PLTX-like compound. In this case the main symptoms involved the skeletal muscles, with pain, high levels of serum creatine phosphokinase (CPK) and myoglobinuria.

Although lethal seafood poisonings have been ascribed to PLTX, an early toxicological characterization classified the molecule as "relatively non toxic" after intragastric administration to rats: lethal dose (LD_{50}) was greater than 40 μg/kg, compared to LD_{50} lower than 1 μg/kg after parenteral administration (Wiles et al., 1974). However, the uncertainty concerning the toxicological data

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is increased by the doubtful purity of the toxin preparation used in this study. At that time the structure of palytoxin was not completely elucidated and the reported molecular weight was 3300 Da instead of 2681 Da. More recently, the LD_{50} of palytoxin after intragastric administration was reported as 510 μg/kg in mice, but histological or biochemical information was lacking (Rhodes and Munday, 2004). In a more recent study PLTX was not lethal to mice at the oral dose of 200 μg/kg (Ito and Yasumoto, 2009).

In contrast, PLTX is highly toxic after intraperitoneal (i.p.) injection, with an LD_{50} in mice less than 1 μg/kg (Levine et al., 1987; Rhodes et al., 2002; Riobó et al., 2008). The main symptoms after i.p. administration were stretching of the hind limbs, muscle spasms and uncoordination, respiratory distress, dispnea and progressive muscular paralysis (Ito et al., 1996; Rhodes et al., 2002; Riobó et al., 2008).

The spread of toxin-producing organisms to temperate climates and the discovery of PLTX-contaminated shellfish in the Mediterranean Sea (Aligizaki et al., 2008; Ciminiello, personal communication) prompted us to better define the toxic effects of PLTX after oral exposure in mice. To this aim, an acute toxicity study was carried out, which include histological analysis of the main tissues, ultrastructural analysis of cardiac and skeletal muscle and the evaluation of some biochemical parameters indicative of cardiac, skeletal muscle, renal and/or hepatic injuries.

2. Materials and methods

2.1. Toxin

Palytoxin, isolated from *P. tuberculosa*, was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan; lot number WKL7151, purity >90%).

2.2. Animals and experimental conditions

Female CD-1 mice (18–20 g body weight, 4 weeks old), bedding and food were purchased from Harlan Italy (S. Pietro al Natisone, Italy). Animals were acclimatized for 1 week prior to use. Animal room controls were set to maintain temperature (21 ± 1 °C) and humidity (60–70%). The room was illuminated with a fixed artificial light cycle (7.00 a.m.–7.00 p.m.). Animals were caged using dust free poplar chips for bedding and fed with the standard diet for rodents containing 18.5% protein. Food and water were provided *ad libitum*. Animals were fasted overnight (16 h) before treatment. PLTX, dissolved in phosphate buffered saline (PBS), pH 7.0, was administered by gavage at 10 ml/kg. Control animals received the vehicle alone at the same dose. The toxin or vehicle was administered to groups of five mice between 9.00 and 10.00 a.m. After dosing, food was returned within 2 h; during the observation period food and water were offered *ad libitum*.

Experiments were carried out at the University of Trieste in conformity with Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC) concerning animal welfare.

2.3. Experimental design

Groups of five mice were treated at each dose level of PLTX (300, 424, 600, 848, 1200 or 1697 μg/kg) or vehicle. After gavage, the mice were observed for 24 h and symptoms and mortality were recorded. After 24 h surviving animals were anesthetized by i.p. injection of ketamine hydrochloride (350 mg/kg; Inoketam100; Virbac; Milan, Italy) and bled to death through the abdominal aorta. Blood samples were collected in heparinized syringes and used for the determination of plasma levels of aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), and creatinine, as well as for determining the leukocyte formula. All animals were weighed prior to necropsy. Heart, liver, lungs, kidneys, spleen, stomach, duodenum, jejunum, colon, rectum, pancreas, thymus, brain, cerebellum, spinal cord, uterus, ovaries and skeletal muscle (soleus) were collected and fixed in neutral buffered 10% formalin. Heart and skeletal muscle were also fixed, as reported in Section 2.7 for examination by transmission electron microscopy (TEM).

Mice that died during the observation period were necropsied immediately and blood was collected for haematological analyses.

2.4. Influence of fasting on PLTX acute oral toxicity

To evaluate the influence of fasting on PLTX acute oral toxicity, a second experiment was performed. Two doses of the toxin (848 and 1200 μg/kg) or vehicle (10 ml PBS/kg) were administered to groups of three mice fasted for either 3 or 16 h. Food was returned within 2 h post-exposure; during the observation period food and water were offered *ad libitum*. Mice were observed for 24 h, as described above. Animals that died during the observation period and those surviving for 24 h were necropsied and the main organs and tissues were analyzed by light microscopy.

Table 1
Mortality induced by acute oral administration of PLTX to mice.

Dose (μg/kg)	Mortality[a]	Survival times (h:min)
0	0/5	–
300	0/5	–
424	0/5	–
600	2/5	1:30–9:54
848	3/5	5:56–8:36–11:34
1200	4/5	1:24–3:56–4:00–6:24
1697	5/5	0:40–2:16–2:37–4:46–9:02

[a] Died animals/treated animals.

2.5. Haematology and clinical chemistry

Plasma levels of ALT, AST, LDH, CPK and creatinine were measured by colorimetric methods using diagnostic kits from Roche S.p.A. (Milan, Italy). A drop from each blood sample was smeared onto slides and stained with Giemsa stain according to standard methodology. Neutrophils, eosinophils, basophils, lymphocytes and monocytes were counted on the basis of cell morphology and staining, and their percentages determined by the leukocyte formula.

2.6. Light microscopy

Heart, liver, lungs, kidneys, spleen, stomach, duodenum, jejunum, colon, rectum, pancreas, thymus, brain, cerebellum, spinal cord, uterus, ovaries and skeletal muscle (soleus) were embedded in paraffin and sectioned (5 μm). Sections were deparaffinized, rehydrated and stained with haematoxylin–eosin, following standard techniques for histological analyses by light microscopy. Histopathological examination by light microscopy was carried out in a blinded way with respect to treatment.

2.7. Transmission electron microscopy

Blocks of heart and skeletal muscle (soleus) were promptly fixed in 3% glutaraldehyde (Serva, Heidelberg, Germany) in 0.1 M cacodylate buffer, pH 7.3, for 3 h at 4 °C, rinsed three times (10 min each) in the same buffer and post-fixed in 1% osmium tetroxide for 1 h at 4 °C. Samples were then dehydrated in a series of increasing concentrations of ethanol solutions and fixed in Dow Epoxy Resin 332 (Lockwood, 1964). Ultrathin sections (0.12 μm) were cut by a Leica Ultracut UCT8 ultratome (Leica; Mikrosysteme Aktiengesellschaft, Wirn, Austria), double stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined using a Philips EM 208 transmission electron microscope (Eihdhoven, The Netherlands). Examination by transmission electron microscopy was carried out in a blinded way with respect to treatment.

2.8. Statistical analysis and determination of LD_{50} values

The LD_{50} values, based on 24 h mortality data, were calculated according to the Finney method (Finney, 1971) at a 95% confidence level. Data are expressed as mean ± standard error (SE) and significant differences between control and experimental groups were calculated using the Student's *t*-test, accepting *p* values lower than 0.05 as significant.

Table 2
Symptoms induced by acute oral administration of PLTX to mice and their frequency.

Dose (μg/kg)	Scratching[a]	Jumping[a]	Respiratory distress[a]	Paralysis[a]	Spasms[a]
0	0/5	0/5	0/5	0/5	0/5
300	2/5	0/5	0/5	0/5	0/5
424	1/5	0/5	0/5	0/5	0/5
600	3/5	1/5	1/5	2/5	2/5
848	5/5	2/5	0/5	1/5	2/5
1200	3/5	2/5	3/5	2/5	4/5
1697	3/5	3/5	5/5	3/5	3/5

[a] Animals showing the symptom(s)/treated animals.