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Toxicity of palytoxin after repeated oral exposure in mice and *in vitro* effects on cardiomyocytes

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ABSTRACT

Palytoxin (PLTX) is a highly toxic hydrophilic polyether detected in several edible marine organisms from intra-tropical areas, where seafood poisoning were reported. Symptoms usually start with gastro-intestinal malaise, often accompanied by myalgia, muscular cramps, dyspnea and, sometimes, arrhythmias. Monitoring programs in the Mediterranean Sea have detected PLTX-like molecules in edible mollusks and echinoderms. Despite the potential exposure of the human population and its high toxic potential, the toxicological profile of the molecule is still an issue. Thus, the effects of repeated oral administration of PLTX in mice were investigated. Seven days of PLTX administration caused lethality and toxic effects at doses ≥30 μg/kg/day. A NOAEL was estimated equal to 3 μg/kg/day, indicating a quite steep dose–response curve. This value, due to the limited number of animal tested, is provisional, although represents a sound basis for further testing. Macroscopic alterations at gastrointestinal level (gastric ulcers and intestinal fluid accumulation) were observed in mice dead during the treatment period. Histological analysis highlighted severe inflammation, locally associated with necrosis, at pulmonary level, as well as hypereosinophilia and fiber separation in myocardium. A cardiac damage was supported by the *in vitro* effect of the toxin on cardiomyocytes, indicating a severe and irreversible impairment of their electrical properties: electrophysiological recordings detected a progressive cell depolarization, arrest of action potentials and beating.

1. Introduction

Palytoxin (PLTX) is a polyhydroxylated polyether (MW = 2680 Da) that was first isolated in 1971 by the group of Prof. Moore in Hawaii (Moore and Scheuer, 1971) and structurally elucidated in 1981 (Moore and Bartolini, 1981; Uemura et al., 1981). Originally described as a zoanthid toxin typical of corals of the genus *Palythoa*, to which the toxin owes its name, the molecule became sadly famous due to poisoning episodes in humans, with lethal outcomes, in tropical and sub-tropical regions after consumption of contaminated fish and crustaceans (Alcala et al., 1988; Noguchi et al., 1987; Onuma et al., 1999; Taniyama et al., 2002). Other cases of intoxications were ascribed to this toxin only on the basis of symptoms, as described in detail by Tubaro et al. (2011a).

The molecular target of PLTX and its analogues seems to be the Na^+/K^+-ATPase, a key enzyme for all eukaryotic cells

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functions. This family of toxins is supposed to convert the pump into a non-specific cationic channel (Habermann, 1989; Kim et al., 1995; Rossini and Bigiani, 2011; Wu, 2009). The ubiquitous distribution of this molecular target makes all the eukaryotic cells potentially sensitive and excitable cells, being Na^+/K^+-ATPase involved in controlling the membrane potential. In accordance, the *in vitro* toxicity of PLTX and analogues has been described in a wide variety of non-excitable and excitable cellular models and tissues preparations, with severe impairment of the cellular ability to maintain ionic homeostasis (Artigas and Gadsby, 2002, 2003a, 2003b, 2004; Bellocci et al., 2008, 2011; Del Favero et al., 2012; Frelin and Van Renterghem, 1995; Ichida et al., 1999; Pelin et al., 2011; Sheridan et al., 2005).

The increasing diffusion of the PLTXs-producing organisms, such as the microalgae of the genus *Ostreopsis* (Del Favero et al., 2012; Rhodes, 2011; Ukena et al., 2001; Usami et al., 1995) in the Mediterranean areas, and the cyanobacteria of the genus *Trichodesmium* (Kerbrat et al., 2011), favoured the entrance of PLTX and/or its analogues into the food web (Mebs, 1998) also in areas far from tropical regions. Indeed, PLTXs have been detected in several edible marine organisms, such as mollusks and echinoderms, from Mediterranean temperate areas (Aligizaki et al., 2008, 2011; Amzil et al., 2012; Ciminiello et al., 2011; ISPRA, 2011), resulting in a widespread possibility that commercialized seafood could be contaminated. In 2009, the CONTAM Panel of the European Food Safety Authority (EFSA) expressed its opinion on PLTX toxicity and, from the few available *in vivo* acute toxicity studies on PLTXs, derived an Acute Reference Dose (ARfD) in humans of 0.2 μg/kg, on the basis of which the maximum concentration of PLTX and related compounds in shellfish was calculated to be 30 μg/kg shellfish meat, considering a body weight of 60 kg and 400 g shellfish consumption (EFSA, 2009).

Acute oral toxicity studies on PLTXs showed similar LD_{50} values in rodents, in the range 510–767 μg/kg body weight (Munday, 2008; Sosa et al., 2009; Tubaro et al., 2011b). Nevertheless, PLTX-induced toxicity after repeated oral administration has never been investigated, although repeated consumption of contaminated seafood at least for a short period of time can be representative of human exposure scenarios. Therefore, the aim of the present study was to investigate the effect of 7-day-repeated administration of doses below the known LD_{50} in order to identify a No Observed Adverse Effect Level (NOAEL). Furthermore, since after acute oral PLTXs administration an increased plasma level of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) was associated to ultrastructural modifications at skeletal and cardiac muscles (Sosa et al., 2009; Tubaro et al., 2011b) and toxic signs in humans (arrhythmias, electrocardiographic changes) suggest cardiac muscle as main PLTXs target (Tubaro et al., 2011a), a preliminary *in vitro* study was carried out on primary cultures of rat cardiomyocytes.

2. Materials and methods

2.1. Toxin and other chemicals

Palytoxin, isolated from *Palythoa tuberculosa*, was purchased from Wako Chemicals GmbH (Neuss, Germany; lot number WKL 7151; purity: higher than 90%). If not otherwise specified, other chemicals were from Sigma Aldrich (Milan, Italy).

2.2. Animals

Female CD-1 mice (18–20 g body weight, 4 weeks old) were purchased from Harlan Laboratories (S. Pietro al Natisone, Italy). Animals were acclimatized for 2 weeks before the experiments. Controlled temperature (21 ± 1 °C) and humidity (60–70%) were maintained in the animal room, illuminated with a fixed artificial light cycle (7.00 a.m.–7.00 p.m.). Animals were caged, in groups of 6 or 8, using dust free poplar chips for bedding and fed with the standard diet for rodents (Harlan Laboratories, S. Pietro al Natisone, Italy). Diet composition, as indicated by the supplier, was: proteins (18.5%), fats (5.5%), fibers (4.5%), hashes (6.0%), non-nitrogen compounds (53.5%), water (12.0%). Water and feed were provided *ad libitum*.

Animals experiments were carried out at the University of Trieste in compliance with the Italian Decree n. 116/1992 as well as the EU Directive (2010/63/EU) and the European Convention ETS 123.

2.3. Experimental design of "in vivo" study

Two set of experiments have been carried out: since all the PLTX doses used in Experiment 1 induced some toxic effects, Experiment 2 was designed in order to identify a no effect level.

2.3.1. Experiment 1

Groups of six mice were treated, once a day for 7 days (days 1–7), at three PLTX doses (30, 90 or 180 μg/kg/day) or vehicle (phosphate buffered saline, PBS; 10 ml/kg/day). PLTX was dissolved in PBS, pH 7.0: to avoid chemical instability, solutions were prepared immediately before administration (9.00–10.00 a.m.) by gavage (10 ml/kg/day, adjusted on the mouse weight). After dosing, signs and symptoms were monitored every hour within the first 3 h post administration, then mice were observed at regular time intervals (every 12 h). Body weight and food consumption were recorded daily in the morning. Twenty-four hours after the last treatment (day 8), three animals from each group were weighed, anesthetized with ketamine hydrochloride (350 mg/kg; Inoketam100; Virbac; Milan, Italy), and bled to death through the abdominal aorta. All animals were necropsied and liver, heart, lungs, kidney, spleen and brain were removed and weighed. The main organs and tissues (see Section 2.5.) were fixed in neutral buffered 10% formalin for the histological analysis. To estimate the reversibility/progression of the PLTX-induced damage, the remaining mice from each dose group were maintained for a 14-day recovery period, with daily observations recorded. At the end of the recovery period the animal were sacrificed, necropsied and the biological samples processed as previously reported. Animals dead during the treatment period or during the 14-days post-exposure period were immediately weighed and necropsied; the main organs and tissues were weighed and/or fixed for the histological evaluation, as reported above.