Published in final edited form as:

Toxicon. 2014 November ; 90: 184–190. doi:10.1016/j.toxicon.2014.08.003.

Acute toxicity of karlotoxins to mice

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Abstract

Karlotoxins, polyketide derivatives produced by the dinoflagellate *Karlodinium veneficum*, are associated with fish kills in temperate estuaries world wide. In this study, the acute effects of 3 pure karlotoxin analogs (KmTx 1, KmTx 3 and KmTx 2) have been examined in mice. Transient lethargy and increased respiratory rates were observed soon after dosing with the karlotoxins by intraperitoneal injection, but no deaths were recorded in animals dosed with KmTx 2 at up to 500 µg/kg or with KmTx 1 or KmTx 3 at up to 4000 µg/kg. Animals dosed intraperitoneally with KmTx 1 and KmTx 3 at 4000 µg/kg showed a pronounced decrease in food and water intake, lasting 3–4 days after dosing, accompanied by a significant decrease in body weight. After this time, the lost body weight was regained and the behavior and appearance of the mice remained normal throughout the following 10 day observation period. No effects were seen in mice dosed orally with KmTx 1 or KmTx 3 at a dose of 4000 µg/kg. It is concluded that contamination of seafood if it were to occur with these karlotoxins is unlikely to pose a major risk of acute intoxication in consumers.

Keywords

Acute toxicity; Karlotoxins; Mice

1. Introduction

The athecate dinoflagellate *Karlodinium veneficum* produces a number of polyketide toxins, collectively known as karlotoxins (Peng et al., 2010; Place et al., 2012; Van Wagoner et al., 2008, 2010). These toxins have a surprising structural similarity to amphidinols produced by dinoflagellates of the *Amphidinium* genus which are characterized by long carbon chains with multiple hydroxyl groups and polyolefins (Satake et al., 1991). The karlotoxins have

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicon.2014.08.003>.

been reported to display a variety of interesting effects on biological systems including cellular lysis (Deeds et al., 2002; Kempton et al., 2002), damage of fish gills (Deeds et al., 2006; Nielsen, 1993), and immobilization of prey organisms (Van Wagoner et al., 2010). The cytolytic activity of the karlotoxins is modulated by membrane sterol composition, which has been proposed as a mechanism for *K. veneficum* avoiding autotoxicity (Deeds and Place, 2006; Place et al., 2006, 2009). The ichthyotoxicity of karlotoxins can be traced to its targeting the gills of fish and especially the chloride cells responsible for osmoregulation in these animals (Deeds et al., 2006).

Originally two families of karlotoxins were described as belonging to the KmTx 1 & KmTx 3 and KmTx 2 groups, which differ from one another in UV absorbance maxima, potency, and geographic distribution (Bachvaroff et al., 2009; Deeds et al., 2004). Although the reports of toxic compounds from *K. veneficum* (originally *Gymnodinium veneficum*) date back to the 1950s (Abbott and Ballantine, 1957), it has only been in recent years that structures were reported for KmTx 1 & KmTx 3 (Van Wagoner et al., 2008, 2010) and KmTx 2 (Peng et al., 2010) including the absolute configuration for the latter compound. With the structures now reported, the difference between the compounds in carbon chain structure is known to be localized to the length of the lipophilic side chain. In KmTx 1 the side chain is 18 carbons in length (C48–C65), whereas in KmTx 2 it is two carbons shorter (C48–C63). KmTx 3 differs from KmTx 1 in having one less methylene group in the saturated portion of its lipophilic arm (Van Wagoner et al., 2010). The karlotoxins, like the amphidinols, have a hairpin like structure with three distinct regions: a polyol arm that exhibits variable hydroxylation and methylation, a hinge region containing two ether rings, and a lipophilic arm that often includes conjugated trienes in amphidinols but in karlotoxins contain a terminal diene, which gives these compounds their distinctive UV spectra. While these substances are known to be highly toxic to fish (Deeds et al., 2006) and mammalian cells (Deeds et al., 2002), little information is available on their toxicity to mammals. In the present study, the acute effects of three karlotoxins KmTx 1, KmTx 2, and KmTx 3 have been investigated in mice through both intraperitoneal injection and oral administration.

2. Material and methods

Samples of purified KmTx 2 were from a culture of CCMP 2064 (Bachvaroff et al., 2008) and samples of purified KmTx 1 and KmTx 3 were from a *K. veneficum* bloom in the Baltimore Inner Harbor in September of 2005 (Adolf et al., 2008). Based on integration of the MS spectra of the purified UV peak, KmTx 3 was a mixture of two congeners KmTx 3 (80% 1347.8 Na adduct) and KmTx 7 (20% 1331.8 Na adduct). Similarly, KmTx 1 was a mixture of two congeners KmTx 1 (70% 1361.8 Na adduct) and 30% KmTx 6 (1345.9 Na Adduct). The purity of all three substances based on UV absorbance exceeded 95%. They were dissolved in absolute ethanol and stored at –20 °C before use. Their planar structures are shown in the Graphical Abstract. The KmTx 1 and KmTx 3 mixtures will be referred to as KmTx 1 and KmTx 3 throughout the manuscript.

2.1. Experiment 1. Acute toxicity of KmTx 2 by intraperitoneal injection

In an initial study, 10 female Balb/c mice, of initial body weight 20 g, were injected intraperitoneally with KmTx 2 at 50, 100, or 500 µg/kg. The ethanolic solutions were diluted in 1% Tween 60 in saline, and 1 ml of the diluted solution injected. After 24 h, 2 mice from each treatment group were killed by cervical dislocation and major abdominal organs taken for histological examination. After fixation, paraffin embedded tissues were stained with hematoxylin and eosin (H&E), according to Clark (1981).

2.2. Experiment 2. Acute toxicity of KmTx 1 and KmTx 3 by intraperitoneal injection

Female Swiss mice, initial body weight between 18 and 22 g, were injected intraperitoneally with the test substances at 500, 1,000, 2000 and 4000 µg/kg. The ethanolic solutions were diluted in 1% Tween 60 in saline, and 1 ml of the diluted solution injected. The appearance and behavior of the mice were closely monitored for 14 h after dosing, and abnormalities were recorded.

2.3. Experiment 3. Effect of intraperitoneal injection of KmTx 1 and KmTx 3 on mice at a dose of 4000 µg/kg

Groups of 4 mice were injected with KmTx 1 and KmTx 3 at a dose of 4000 µg/kg. A further group of 4 mice served as control, being injected with vehicle alone. After 24 h, the mice were weighed and then killed by carbon dioxide inhalation. Blood samples were taken for biochemistry and hematology. At necropsy, the liver, kidneys, spleen, lungs and heart of each animal were weighed, and these tissues, together with the pancreas and sections of the jejunum and peritoneum, were preserved in 4% buffered formalin for subsequent histological examination. The weights of the stomach and the whole of the intestinal tract were also recorded at necropsy, and weighing was repeated after the contents had been rinsed away under running water. From these measurements, the weights of stomach and intestinal contents were calculated. Blood packed cell volumes and hemoglobin levels were measured, and plasma activities of creatine kinase (CK), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as indicators of liver damage and plasma levels of creatinine, sodium, potassium and chloride as indicators of kidney damage were determined (Gribbles Veterinary, Hamilton, NZ).

2.4. Experiment 4. Effect of oral administration of KmTx 1 and KmTx 3 on mice at a dose of 4000 µg/kg

Groups of 4 mice were dosed by gavage with KmTx 1 and KmTx 3 at 4000 µg/kg or with vehicle. The mice were killed and necropsied 24 h later. Organ weights were recorded, and hematological and biochemical parameters were assayed as in Experiment 3.

2.5. Experiment 5. Long term effects of intraperitoneal injection of KmTx 1 and KmTx 3 on mice

Three groups of 4 mice were housed individually. Their body weights and food and water intakes were measured daily. After 5 days, the mice were injected intraperitoneally with vehicle or with KmTx 1 and KmTx 3 at a dose of 4000 µg/kg. Body weights, food intakes and water intakes were measured for a further 14 days, after which time the mice were killed

and necropsied. Organ weights were measured, and hematological and biochemical parameters were assayed as in Experiment 3.

3. Histology

Fixed tissues were cut at 3 μm and stained with hematoxylin and eosin (Clark, 1981) for examination by light microscopy.

4. Statistical analysis

Statistical significance of the data was evaluated by analysis of variance followed by the Studente–Newman–Keuls multiple comparisons test using Instat software (GraphPad, San Diego, CA).

5. Results

5.1. Acute toxicity of KmTx 2 by intraperitoneal injection

No mice died at any of the dose levels employed. At the highest dose (500 $\mu\text{g/kg}$), however, mice became lethargic and displayed an increased respiration rate. No histological changes were recorded in mice dosed at 50 or 100 $\mu\text{g/kg}$, but inflammation of the peritoneum was observed in both animals that were killed 24 h after a dose of 500 $\mu\text{g/kg}$. Pancreatic necrosis (Fig. 1) was also observed in one of these mice.

5.2. Acute toxicity of KmTx 1 and KmTx 3 by intraperitoneal injection

Doses of 500, 1,000, 2000 and 4000 $\mu\text{g/kg}$ were progressively given, and with increasing doses, pronounced behavioral changes were evident. The mice were normal for about 5 min after dosing, but they subsequently became lethargic, with occasional abdominal breathing. The mice were somewhat hunched, but there was no piloerection. The respiration rate was normal to high (a rate of 130–170 per minute), and there was no cyanosis. The appearance of the test mice gradually normalized, and after 2–3 h they were indistinguishable from controls. The test mice were monitored for 14 h after dosing, and, like control mice, they became very active during the evening. Unlike control mice, however, they ate very little food. None of the treated mice died, and next day they were normal in appearance and behavior. All had lost a significant amount of weight, however, particularly at the highest dose level.

5.3. Effect of intraperitoneal injection of KmTx 1 and KmTx 3 on mice at a dose of 4000 $\mu\text{g/kg}$

The behavioral changes in the mice were as described above. The severity and duration of the changes were somewhat lower in mice dosed with KmTx 1 than in those given KmTx 3. At necropsy, there was no ascites or pleural effusion, and no macroscopic alterations in any organ was observed. The relative weights of the liver, kidneys, spleens, heart and lungs of the test mice were not significantly different from those of control mice, but the weights of stomach, intestinal and total gut contents of mice receiving the karlotoxins were significantly lower than those of control mice (Table 1). Blood packed cell volumes and hemoglobin levels were significantly elevated in mice dosed with KmTx 1 and KmTx 3

(Table 1). Plasma activities of AST and ALT were increased in mice receiving both of the karlotoxins (Table 2). Histological examination revealed a moderate, predominantly neutrophilic, peritonitis in all of the animals dosed with the karlotoxin derivatives. This appeared as edema and fibrin leakage from blood vessels, which was most prominent in the pancreas. Milder inflammation and mesothelial hypertrophy were also visible on the hepatic capsule and the intestinal serosa in some sections. Necrotizing pancreatitis was observed in one mouse dosed with KmTx 3, as seen with KmTx 2 (see Fig. 1). No histological changes were observed in the kidneys, lungs, spleen or heart of any of the mice.

5.4. Effect of oral administration of KmTx 1 and KmTx 3 on mice at a dose of 4000 µg/kg

No behavioral changes were seen in mice dosed orally with either of the test compounds, and no changes in body weight were recorded. Organ weights and weights of gut contents were normal, as were packed cell volumes and hemoglobin levels (Table 3). No changes in plasma biochemical parameters were observed (Table 4).

5.5. Long term effects of intraperitoneal injection of KmTx 1 and KmTx 3 on mice

The changes in body weight in control mice and those injected intraperitoneally with the karlotoxins are shown in Fig. 2A. Dosing was on Day 0, after a 5-day acclimatization period. The body weights of the mice dropped precipitately after dosing, with KmTx 3 producing a somewhat greater effect than KmTx 1. After 2–3 days, body weights increased, and the weights of all animals were similar 6 days after dosing. Food intakes of the mice are shown in Fig. 2B. Food intake decreased immediately after dosing with the karlotoxins, and the effect of KmTx 3 was again somewhat greater than that of KmTx 1. Food intakes normalized 4 days after administration of the test compounds.

Water intakes of the mice are shown in Fig. 2C. A sharp decrease in water intake was recorded in the test animals, but water intakes returned to normal 3 days after dosing. At necropsy, 14 days after dosing, adhesions between the liver lobes of 2 mice dosed with KmTx 3 were observed, and one of these animals also showed adhesions between the stomach and spleen. There was no ascites in the animals, or in any other mouse in this experiment. No other macroscopic changes were observed in any animal. Organ weights, weight of stomach contents, blood packed cell volumes and biochemical parameters were entirely normal (Tables 5 and 6).

6. Discussion

None of the purified karlotoxin derivatives were highly acutely toxic. Although pronounced behavioral changes were observed in mice dosed intraperitoneally with these substances, there were no deaths at doses as high as 4000 µg/kg. At the latter dose level, however, the karlotoxins caused a marked decrease in food and water intake, which persisted for 3–4 days after injection (see Fig. 2). In mice killed 24 h after intraperitoneal injection of the karlotoxins, a moderate peritonitis was observed, and it is possible that this toxic change was responsible for the loss of appetite in the mice dosed with KmTx 1 and KmTx 3. The etiology of the peritonitis is unknown, but it may reflect direct necrosis of the smaller blood vessels within the peritoneum. A necrotizing pancreatitis was seen in a two animals (one

treated with KmTx 2 and one with KmTx 1) which again may be due to a direct effect of the toxin within the peritoneal cavity, consistent with the non selective cellular damage previously observed for KmTx 2 exposure in assays using mammalian cell lines (Deeds et al., 2002) and in fish exposed to this substance (Deeds et al., 2006).

Consistent with the anorexic action of the toxins, the weight of gut contents was significantly decreased 24 h after dosing. The increased blood packed cell volumes and hemoglobin levels recorded at this time are most likely attributable to hemoconcentration, due to the observed decrease in water intake of animals injected with the karlotoxins. Plasma activities of ALT and AST were significantly elevated in mice receiving KmTx 1 and KmTx 3, which could indicate damage to the liver and/or kidney. No histological changes were observed in these tissues, however, and the other indicator of renal function (creatinine level) was unchanged. It is possible that the increased activities of these enzymes are due to the observed peritonitis, exacerbated perhaps by hemoconcentration.

The effects of the toxins on body weights and food and water intake were reversible, and the animals appeared entirely normal after they resumed feeding and drinking. Adhesions within the peritoneum of two of these animals were recorded, a commonly found sequel to peritonitis. No other lesions were seen in these animals, and their organ weights, hematology and plasma biochemical parameters were normal.

In the evaluation of the potential risk of harm to human health from toxins present in seafood, toxicity by the oral route is the relevant parameter. At a dose of 4000 µg/kg, neither KmTx 1 nor KmTx 3 caused any perceptible effect in mice. This may be compared with the acute oral toxicities of seafood contaminants that are known to cause toxic effects in humans, such as Pacific ciguatoxin 1 (LD₅₀ 0.22 µg/kg; Lewis et al., 1993), brevetoxin 2 (LD₅₀ 200 µg/kg; Baden and Mende, 1982), palytoxin (LD₅₀ 510 µg/kg; Munday, 2006) and tetrodotoxin (LD₅₀ 332 µg/kg; Sakai et al., 1961). While toxic *K. veneficum* is poorly filtered by juvenile oysters, no karlotoxin was detected in oyster tissues after being fed for 5 days on toxic strains (Brownlee et al., 2008). It must be concluded, therefore, that contamination of seafood if it were to occur with these karlotoxins it is unlikely to pose a major risk of acute intoxication in consumers.

Acknowledgments

The KmTx 2 exposures were done by Jonathan Deeds as part of his Doctoral Thesis and approved by the Institutional Animal Care and Use Committee of the University of Maryland. All experiments with KmTx 1 and KmTx 3 were approved by the Ruakura Animal Ethics Committee. This is contribution number #4934 of the University of Maryland Center for Environmental Science and #14-127 of the Institute of Marine and Environmental Technology.

Abbreviations

KmTx	Karlotoxins
CCMP	Culture collection of marine phytoplankton

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Fig. 1.
Histological section of the pancreas from a mouse injected intraperitoneally with KmTx 2 at 500 µg/kg after 24 h. The local necrosis of the pancreas is evident from the less intense staining of the cells in upper part of the lobe.

Fig. 2.
Changes in body weight, water intake and food intake in control mice and in mice dosed intraperitoneally with KmTx 1 and KmTx 3.

Table 1

Relative organ weights, weights of gut contents, packed cell volumes and hemoglobin levels of control mice and of mice dosed intraperitoneally with KmTx 1 and KmTx 3 at 4000 µg/kg, and killed 24 h later.

Treatment	Relative organ weight (g/100 g body weight)				Weight of stomach contents (g)	Weight of intestinal contents (g)	Total gut contents (g)	Packed cell volume (%)	Hemoglobin level (g/l)
	Liver	Kidneys	Spleen	Heart					
Control	5.00 ± 0.29	1.46 ± 0.09	0.415 ± 0.123	0.528 ± 0.056	0.647 ± 0.228	0.853 ± 0.134	1.399 ± 0.325	43.3 ± 0.5	146 ± 5
KmTx 1	4.88 ± 0.49	1.57 ± 0.22	0.557 ± 0.155	0.561 ± 0.021	0.241 ± 0.076*	0.313 ± 0.087***	0.553 ± 0.157**	48.0 ± 0.9***	158 ± 5*
KmTx 3	5.26 ± 0.43	1.50 ± 0.31	0.487 ± 0.112	0.568 ± 0.021	0.294 ± 0.170*	0.309 ± 0.124***	0.603 ± 0.135**	47.6 ± 0.8***	157 ± 6*

Numbers represent the means ± SD of the values for the 4 animals per group. Values marked with asterisks are significantly different from control.

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$.

Table 2

Plasma biochemistry of control mice and of mice dosed intraperitoneally with KmTx 1 and KmTx 3 at 4000 µg/kg, and killed 24 h later.

Treatment	CK (I.U./l)	AST (I.U./l)	ALT (I.U./l)	Creatinine (µmol/l)	Sodium (mmol/l)	Potassium (mmol/l)	Chloride (mmol/l)
Control	238 ± 104	31.3 ± 11.6	82.0 ± 9.2	8.0 ± 1.1	160 ± 3	110 ± 1	7.73 ± 0.43
KmTx 1	816 ± 546	79.0 ± 24.0 *	384.8 ± 236.7 *	8.0 ± 1.6	158 ± 2	106 ± 4	6.93 ± 0.86
KmTx 3	571 ± 348	106.8 ± 29.0 **	357.0 ± 11.6 *	7.0 ± 0.8	159 ± 4	105 ± 2	7.43 ± 1.71

Numbers represent the means ± SD of the values for the 4 animals per group. Values marked with asterisks are significantly different from control.

Abbreviations CK – creatine kinase; AST – aspartate aminotransferase; ALT – alanine aminotransferase.

* $P < 0.05$;

** $P < 0.01$.

Table 3

Relative organ weights, weights of gut contents, packed cell volumes and hemoglobin levels of control mice and of mice dosed orally with KmTx 1 and KmTx 3 at 4000 µg/kg, and killed 24 h later.

Treatment	Relative organ weight (g/100 g body weight)				Weight of stomach contents (g)		Weight of intestinal contents (g)	Total gut contents (g)	Packed cell volume (%)	Hemoglobin level (g/l)
	Liver	Kidneys	Spleen	Heart						
Control	5.04 ± 0.45	1.41 ± 0.07	0.425 ± 0.114	0.499 ± 0.039	0.698 ± 0.075	0.436 ± 0.085	0.971 ± 0.378	1.407 ± 0.447	45.8 ± 1.2	148 ± 7
KmTx 1	5.01 ± 0.17	1.36 ± 0.15	0.414 ± 0.083	0.566 ± 0.054	0.729 ± 0.050	0.553 ± 0.152	0.842 ± 0.035	1.437 ± 0.122	45.3 ± 2.8	145 ± 1
KmTx 3	4.91 ± 0.44	1.35 ± 0.08	0.524 ± 0.060	0.544 ± 0.027	0.754 ± 0.041	0.470 ± 0.09	0.828 ± 0.054	1.299 ± 0.105	44.6 ± 1.1	143 ± 4

Numbers represent the means ± SD of the values for the 4 animals per group.

Table 4

Plasma biochemistry of control mice and of mice dosed orally with KmTx 1 and KmTx 3 at 4000 µg/kg, and killed 24 h later.

Treatment	CK (I.U./l)	AST (I.U./l)	ALT (I.U./l)	Creatinine (µmol/l)	Sodium (mmol/l)	Potassium (mmol/l)	Chloride (mmol/l)
Control	184 ± 101	25.3 ± 2.2	68.8 ± 18.1	8.00 ± 1.15	159 ± 2	108 ± 1	7.43 ± 0.70
KmTx 1	251 ± 129	29.3 ± 5.9	76.3 ± 18.9	7.77 ± 0.58	159 ± 2	107 ± 1	6.60 ± 1.18
KmTx 3	319 ± 172	27.8 ± 4.0	81.0 ± 9.9	8.50 ± 0.58	157 ± 5	107 ± 3	7.35 ± 1.47

Numbers represent the means ± SD of the values for the 4 animals per group. Abbreviations CK – creatine kinase; AST – aspartate aminotransferase; ALT – alanine aminotransferase.

Table 5

Relative organ weights, weights of stomach contents, packed cell volumes and hemoglobin levels of control mice and of mice killed 2 weeks after dosing intraperitoneally with KmTx 1 and KmTx 3 at 4000 µg/kg.

Treatment	Relative organ weight (g/100 g body weight)					Weight of stomach contents (g)	Packed cell volume (%)	Hemoglobin level (g/l)
	Liver	Kidneys	Spleen	Heart	Lungs			
Control	5.43 ± 0.16	1.46 ± 0.23	0.480 ± 0.077	0.631 ± 0.045	0.875 ± 0.157	0.619 ± 0.207	43.3 ± 2.6	148 ± 6
KmTx 1	5.62 ± 0.51	1.37 ± 0.09	0.568 ± 0.133	0.559 ± 0.044	0.802 ± 0.051	0.611 ± 0.194	44.3 ± 2.0	150 ± 6
KmTx 3	5.55 ± 0.46	1.63 ± 0.14	0.508 ± 0.073	0.607 ± 0.009	0.966 ± 0.129	0.483 ± 0.244	42.0 ± 0.6	140 ± 3

Numbers represent the means ± SD of the values for the 4 animals per group.

Table 6

Plasma biochemistry of control mice and of mice killed 2 weeks after dosing intraperitoneally with KmTx 1 and KmTx 3 at 4000 µg/kg.

Treatment	CK (I.U./l)	AST (I.U./l)	ALT (I.U./l)	Creatinine (µmol/l)	Sodium (mmol/l)	Potassium (mmol/l)	Chloride (mmol/l)
Control	485 ± 470	67.5 ± 44.1	92.3 ± 66.8	7.0 ± 1.2	149 ± 4	102 ± 4	6.13 ± 1.76
KmTx 1	760 ± 237	38.8 ± 17.2	79.0 ± 27.5	7.7 ± 0.6	155 ± 4	105 ± 2	4.83 ± 0.38
KmTx 3	439 ± 345	83.8 ± 48.9	128 ± 38.5	7.0 ± 0.8	157 ± 6	107 ± 3	6.45 ± 0.87

Numbers represent the means ± SD of the values for the 4 animals per group. Abbreviations CK – creatine kinase; AST – aspartate aminotransferase; ALT – alanine aminotransferase.