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REVIEW ARTICLE

Marine tetrodotoxin as a risk for human health

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Abstract

Tetrodotoxin (TTX) is a toxin mainly occurring naturally in contaminated puffer fish, which are a culinary delicacy in Japan. It is also detected in various marine organisms like globefish, starfish, sunfish, stars, frogs, crabs, snails, Australian blue-ringed octopuses, and bivalve molluscs. TTX is produced by marine bacteria that are consumed mainly by fish of the *Tetraodontidae* family and other aquatic animals. TTX poisoning through consuming marine snails has recently begun to occur over a wider geographical extent through Taiwan, China, and Europe. This neurotoxin causes food intoxication and poses an acute risk to public health. The aim of this review is to present the most recent information about TTX and its analogues with particular regard to toxicity, methods of analysis, and risk to humans of exposure.

Keywords: marine shellfish, tetrodotoxin, toxicity, detection, health risk.

Introduction

Tetrodotoxin (TTX) is a strong marine neurotoxin acting by blocking sodium channels of the neuron cell membrane. This toxin is a water-soluble heterocyclic guanidine compound, which is stable in neutral and weak acidic solutions and cannot be inactivated by heat treatment (3, 15, 24). TTX was isolated for the first time in 1909 by the Japanese researcher Yoshizumi Tahara from ovaries of globefish (5, 15). Thirty naturally occurring analogues of tetrodotoxin have been detected and many of them have also been shown to have toxic potential in humans and experimental animals. The main mechanism of tetrodotoxin accumulation in fish is the food chain, which begins with toxin-synthesising bacteria, including *Pseudoalteromonas haloplanktis tetraodonis*, marine microorganisms belonging to the genera of *Vibrio* (*e.g. Vibrio alginolyticus*) and *Shewanella*, (e.g. *Shewanella algae* and *Shewanella putrefaciens*) or *Alteromonas tetraodoni* (3, 26, 47, 51). The main TTX sources for humans are fish from the *Tetraodontidae* family (puffer fish), such as *Takifugu* spp., *Lagocephalus* spp., *Tetraodon alboreticulatus*, *Chelonodon patoca*, *Arothron firmamentum*, and *Canthigaster rivulata,* which are a prized delicacy in Japanese cuisine (3). TTX content is different in various parts of the fish body – the highest level of the toxin being found in the skin and some internal organs like the liver and ovaries. This toxin also occurs in the body of marine gastropods (e.g. *Nassarius glans, Nassarius papillosus, Zeuxis scalaris, Zeuxis samiplicutus, Zeuxis siquijorensis, Niotha clathrata, Charonia sauliae, Charonia lampas, Babylonia japonica,* and *Tutufa lissostoma*), oysters, mussels, fish other than puffer fish (e.g. the members of the Gobiidae subfamily *Yongeichthys nebulosus*, *Parachaeturichthys polynema*, and *Radigobius Caninus* and the sillaginid *Sillago japonica*), and the horseshoe crab (*Carcinoscorpius rotundicauda*) (3, 14, 44, 47, 55, 57, 59, 61, 62). TTX intoxication caused by the consumption of marine gastropods has been observed not only in Japan, but also in Taiwan, China, New Zealand, and Europe, indicating the further spread of organisms containing this toxin around the world (8, 19, 38, 39, 55).

Chemistry of TTX

Tetrodotoxin has the chemical formula $C_{11}H_{17}N_3O_8$ and a molecular mass of 319.1 g/M. Its structural formula is shown in Fig. 1.

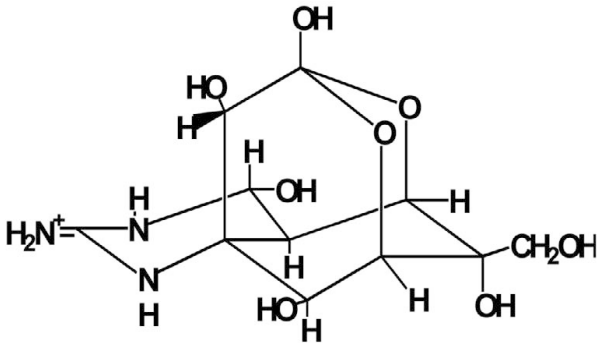

Fig. 1. Structural formula of tetrodotoxin (3)

To date 30 structural analogues of TTX have been studied and it has been proved that their toxicity is connected with their structure (4). Previous studies indicated that the hydroxyl substituents cause that the analogues are more toxic than TTX, while the analogues with deoxy substituents are less harmful. The toxicity of analogues depends on the number and position of hydroxyl substituents in their structure (4). Yotsu-Yamashita *et al.* (67) performed studies assessing the effect of the position of the hydroxyl group in the TTX molecule on the ability of this neurotoxin to penetrate rat meninges. The obtained results revealed that the location of the hydroxyl groups at the C-6 and C-11 carbon atoms has a significant impact on the binding of TTX analogues to the sodium channels through their participation in hydrogen binding. Moreover, Pires *et al.* (52) proved that analogue 11-oxoTTX is 4–5 times more toxic than tetrodotoxin. There are still many analogues which have not been studied for their toxicity, so it is important that further analysis of TTX derivatives be carried out. Such studies are very important in assessment of the risk posed by this kind of toxin to public health.

TTX analogues found in puffer fish can be categorised into following families (65):

1) analogues chemically equivalent to TTX (4-epiTTX and 4,9-anhydroTTX);
2) deoxy analogues (5-deoxyTTX, 11-deoxyTTX, 5,11-dideoxyTTX, 6,11-dideoxyTTX and 5,6,11-trideoxyTTX);
3) 11-CH_2OH oxidised analogue (11-oxoTTX);
4) C11-lacking analogues (11-norTTX-6(*S*)-ol and 11-norTTX-6(*R*)-ol).

Such structures as have been found and analysed for several natural TTX analogues in puffer fish and amphibians are shown in Fig. 2.

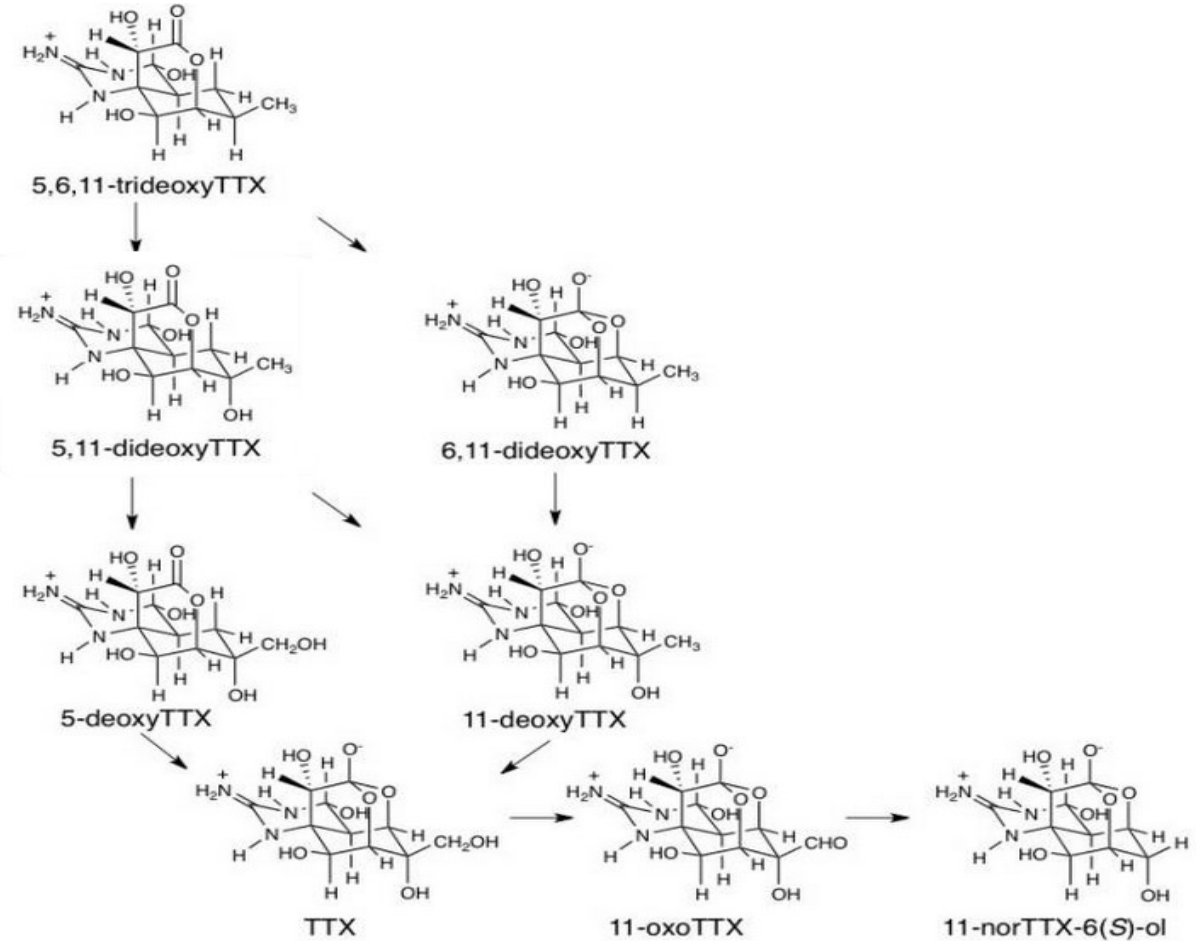

Fig. 2. The structures of natural tetrodotoxin (TTX) analogues (64)

Toxicity

Tetrodotoxin is one of the most toxic substances to humans known. Its lethal dose is 275 times lower than cyanides and 50 times lower than strychnine and curare (15). In mice, the 50% lethal dose (LD_{50}) of TTX administered orally is 334 μg/kg while intravenously it is 8 μg/kg (10). The toxic dose for humans has not been established, but a single dose of 1–2 mg of purified TTX can be lethal (15).

TTX inhibits neuronal firing of action potentials by binding to the voltage-gated sodium channels in nerve cell membranes, and as a result blocks the flow of sodium ions into the neurons (24). For this reason tetrodotoxin is called a sodium channel blocker. The toxin affects action potential generation and impulse conduction, resulting in blockade of the neuron and muscle paralysis. It leads to the following acute symptoms: tingling (paraesthesia) of the tongue and lips, motor paralysis, incoordination, slurred speech, aphonia, hypotension, bradycardia, cardiac dysrhythmia, and unconsciousness (18). The clinical symptoms of TTX intoxication appear very quickly and depend on the toxin amount consumed. In severe cases, death is caused by respiratory and heart failure. The treatment is symptomatic and supportive because there is no specific antidote against TTX, and is based on careful observation and repeated neurological assessment for the early implementation of measures to counter respiratory failure and cardiovascular disorders (45).

Table 1. Cases of TTX poisoning

Country	Source of TTX	Number of cases	Reference
Japan	Puffer fish	1	(49)
Japan	Puffer fish	11	(13)
Thailand	Eggs of horseshoe crab (*Carcinoscorpius rotundicauda*)	71	(31)
Japan	Puffer fish	1	(64)
Bangladesh	Puffer fish	8	(37)
China	Gastropods	30	(59)
Australia	Puffer fish	11	(25)
Taiwan	Gastropods	4	(21)
Taiwan	Puffer fish	6	(18)
Taiwan	Gastropods	1	(23)
Australia	Toadfish	7	(50)
Taiwan	Gastropods	6	(22)
Bangladesh	Puffer fish	36	(1)
Japan	Puffer fish	7	(2)
Taiwan	Puffer fish	5	(20)
Taiwan	Gastropods	1	(29)
Taiwan	Gastropods	3	(30)
Spain	Trumpet shell (*Charonia lampas*)	1	(55)
Japan	Puffer fish, Thread-sail filefish, Marine snail	3	(3)
Spain	Trumpet shell (*Charonia sauliae*)	1	(11)
Taiwan	Gastropods	5	(47)
South Korea	Puffer fish	3	(6)
China	Goby fish	22	(68)

Until the very recent past, TTX poisoning was considered a problem confined to Japan and other Asian countries. Currently, tetrodotoxin poisoning is seen in regions that were previously recognised as safe. It is thought that the rapid growth of the geographical extent of this toxin is due to rising water temperatures around the world (9). The first case of TTX poisoning in Europe was reported in Malaga, Spain, after the consumption of a trumpet shell by a 49-year-old man (54). Table 1 shows the reported cases and outbreaks of TTX poisoning in various parts of the world.

Legislation

Commission Regulation (EC) No 853/2004 establishes detailed regulations for hygiene in foodstuffs in the European Union. Section VIII, chapter V indicates that fishery products derived from poisonous fish of the *Tetraodontidae*, *Molidae*, *Diodontidae*, and *Canthigasteridae* families are banned from the market (7). In Japan, a regulatory limit of 2 mg equivalent of TTX/kg was laid down for tetrodotoxin, while in Europe appropriate criteria have not been determined (4, 19).

Detection of TTX

Bioassays. There are several methods for TTX detection. The first methods used were biological tests, including the mouse bioassay (MBA), tissue culture bioassay, and ELISA, available as commercial kits (63). Bioassays allow the toxicity of the sample to be assessed, but it is not possible to identify individual toxins.

The mouse bioassay was used for the first time as a method for the analysis of TTX by Hashimoto and Migita (16) in 1951 in puffer fish sample examination. Currently, because of limitations in using the MBA due to ethical reasons, its continued use for some marine toxin groups is only as the reference method (46). In mouse bioassays, seafood extracts are given to laboratory animals and then symptoms and time to death are monitored. In addition to the ethical concerns around the use of the MBA method prompted by the killing of experimental animals, other numerous disadvantages of this technique exist which are technical, such as low sensitivity and accuracy. For this reason, in recent years, a serious attempt has been made to introduce other techniques for detection of TTX.

A tissue culture bioassay may be used as an alternative method to the MBA (32, 33). The mechanism of action of TTX is based on the same principle as that of another neurotoxin – STX (saxitoxin). Therefore a cell-based assay is able to detect both TTX and STX. Kogure *et al.* (32) noticed that neuroblastoma Neuro-2a cells can be applied in the identification of TTX. Ouabain or veratridine are added

to the cell cultures, reducing their viability by increasing the flow of sodium ions into the cells, and TTX, which acts as a sodium channel blocker, will nullify the response enabling cell growth to be continued.

Antibody-based techniques like ELISA have been widely used for TTX detection, despite difficulties in toxin-specific antibody production because of the insufficient amount of these compounds available in the world in pure form (43, 58). They were considered to offer large-scale screening capability because of their sensitivity, specificity, rapidity, simplicity, and cost-effectiveness. However, these methods are not useful for conventional screening because they may not be able to detect the majority of TTX analogues.

Chemical methods. High performance liquid chromatography with fluorescence detection (HPLC–FLD) and liquid chromatography–mass spectrometry (LC–MS) are typically used for TTX quantification; however, other techniques such as gas-chromatography–mass spectrometry (GC–MS), infrared (IR) spectrometry, and nuclear magnetic resonance (NMR) spectrometry may be suitable for qualitative determination of TTX. Thin-layer chromatography (TLC) or electrophoresis may be used additionally for TTX detection.

The first analysis of TTX was performed using the GC–MS method by derivatisation of TTX to the C9 base structure and then to a trimethylsilane (42). However, the GC–MS method should not be applied for quantitative analysis because TTX is a non-volatile compound (4). In the NMR technique, clean samples are required to avoid interfering with matrix components.

Reversed-phase (RP) chromatography with a C18 column was applied for a long time in the detection of TTX and its analogues. However, not all of them could be separated using the RP–HPLC method. Other researchers applied normal phase chromatography for the analysis of the TTXs, mainly using hydrophilic interaction liquid chromatography (HILIC) (9, 14, 40, 65). TTX is a polar molecule and it is flushed rapidly from reversed-phase columns but much more slowly in normal phases, which improves separation of its analogues, lowers noise, and heightens sensitivity.

Development of a postcolumn HPLC method with fluorescence detection was intended to obviate the need for the traditional mouse bioassay, and it provides qualitative and quantitative analysis of TTX and its analogues. This method is based on the TTX derivatisation reaction in an alkaline environment, the result of which is a fluorescent compound with excitation and emission wavelengths of 384 and 505 nm, respectively. In the early 1980s, a chromatography technique with a fluorometric measurement of TTX was developed by connecting HPLC and a postcolumn reaction with NaOH to analyse this toxic compound. The most important achievement of this improved analyser was the separation of TTX and 6-epiTTX. So far, a lot of modifications have been made to detect TTX and its analogues under variable HPLC conditions. The LC–FLD method is effective at analysing TTX and many TTX analogues such as 4-epiTTX, 11-oxoTTX, and 4,9-anhydroTTX (4, 28, 34, 35, 46). However, it has the complication of quite large differences in intensity of fluorescence between analogues, *e.g.* 6-epi TTX is 20 times more fluorescent than TTX, while 11-deoxy TTX is 100 times less fluorescent (53). This problem is solved when the LC–MS technique for TTX determination is used.

LC–MS techniques using atmospheric pressure ionisation with an electrospray-ionisation (ESI) in positive ion mode have become a powerful tool for TTX investigation (30, 40, 54, 56, 59, 60). The separation is usually achieved with reversed-phase columns using solvents with an added ion pair reagent, such as ammonium heptafluorobutyrate. The best-known positive ionisation produces a TTX ion of 320 mass-to-charge ratio (m/z) (38). Different TTX analogues can generate the same ion, and therefore liquid chromatography–electrospray ionisation–multiple reaction monitoring mass spectrometry (LC–ESI–MRM-MS) is commonly used to identify them (27, 38, 56, 62, 66).

In 2011, Leung *et al.* (36) analysed urine and plasma of Asian patients and determined the level of TTX by the LC–MS method. This technique, for which an Atlantics dC18 (2.1 mm × 150 mm, 5 μm) column and flow rate of 200 μL/min were specified, allowed the run time to be shortened to 5.5 min. The developed method was validated and applied to determination of TTX in human urine and blood samples (12). In this study the effect of an ion pair reagent such as heptafluorobutyric acid and the optimisation of its concentration at 5 mM was proved. Table 2 provides an overview of LC–MS methods which were used to analyse TTX and its analogues in different matrices.

The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM Panel) have disseminated the opinion that LC–MS-MS methods are the most useful for quantitative and qualitative analysis of TTX (10).

Risk to public health

In 2017, the EFSA CONTAM Panel issued a detailed report regarding the influence of tetrodotoxin and its analogues in marine bivalves and gastropods on human health. Consumption of a dose of 2 mg of TTX (which corresponds to 40 μg/kg body weight in a 50-kg Japanese adult) is considered to be dangerous and can cause serious symptoms in humans, which was described in some case reports of poisoning. However, after reviewing the available literature, the Panel did not find adequate evidence to support a minimum lethal dose for humans of 2 mg, which had been indicated in various publications.

Table 2. Various LC–MS studies on the determination of TTX and its analogues

Mobile phase	Column	Linear range	LOD and LOQ	Recovery	Matrix	Reference
1% acetonitrile, 20 mM ammonium heptafluorobutyrate, 10 mM ammonium formate (pH 4.0)	Reversed phase (250 × 4.6 mm)	0.05–1 nM	LOD – 0.7 pM LOQ – NR	NR	Puffer fish	(56)
20 mM ammonium acetate, methanol (75/25, v/v)	Reversed phase (150 × 4.6 mm)	0.01–1 µg/mL	LOD – 0.1 µg/g LOQ – NR	77.7–80.7%	Puffer fish	(17)
30 mM heptafluorobutyric acid, 1 mM ammonium acetate (pH 5)	Reversed phase (250 × 4.6 mm)	NR	NR	NR	Japanese fire belly newt (*Cynops pyrrhogaster*)	(41)
A: 0.1% formic acid in water B: methanol	HILIC (150 × 4.6 mm)	1–100 ng/mL	LOD – 0.1 ng/mL LOQ – 1 ng/mL	> 95%	Human blood serum	(30)
16 mM ammonium formate buffer (pH 5.5), acetonitrile (3/7, v/v)	HILIC (5 µm, 150 × 2 mm)	NR	LOD – 0.5 nM/g LOQ – NR	NR	Puffer fish	(27)
10 mM/L ammonium formate, formic acid (95/5, v/v), 5 mM heptafluorobutyric acid, 2% acetonitrile	Reversed phase (5 µm, 2.1 × 15 mm)	0–500 ng/mL in urine samples 0–20 ng/mL for plasma samples	LOD – 0.13 ng/mL LOQ – 2.5 ng/mL	75–81%	Human urine and plasma	(12)
A: 10 mM ammonium formate, 10 mM formic acid in water B: acetonitrile, water (95/5, v/v), 5 mM ammonium formate, 2 mM formic acid	HILIC (3.5 µm, 150 × 2.1 mm)	62.5–2,000 ng/mL	LOD – 16 ng/mL LOQ – 63 ng/mL	NR	Puffer fish	(54)
A: 5% acetonitrile B: 95% acetonitrile, 1% acetic acid (pH 3.5)	HILIC (1.7 µm, 100 × 2.1 mm)	5–500 ng/mL	LOD – 0.074 ng/mL LOQ – 0.123 ng/mL	80–92%	Gastropod	(48)

LOD – limit of detection; LOQ – limit of quantification; NR – not reported

The estimated harmfulness for analogues is lower than that set for TTX, but these results are highly doubtful since the current results and determination methods are imprecisely described. After intraperitoneal (i.p.) injection of TTX analogues in mice, most of them cause the same symptoms as TTX, however, no data are available that are precise enough to enable the no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) parameters to be quantified. Nevertheless, LD_{50} and LD_{99} doses after i.p. injection in mice have been determined and on the basis of these values, the approximate effect of analogues in bivalves and gastropods was reported.

In the EFSA report 8,268 analytical results for 1,677 samples of oysters, clams, cockles, mussels, razor clams, and scallops were included (10). These samples were collected between 2006 and 2016 in Greece, the Netherlands, and the UK. The results for TTX were obtained for all of them, disclosing 13.74% and 13.73% occurrence rates for the tetrodotoxin analogues 4-epiTTX and 4,9-anhydroTTX, respectively, and 13.06% for 5,6,11-trideoxyTTX, 11-oxoTTX, mono-deoxyTTX, and 11-norTTX-6-ol. No data were obtained regarding the occurrence of TTX in marine gastropods.

The acute reference dose (ARfD) was used to assess exposure risk for TTX. The average value obtained on the basis of reported consumption of toxin-bearing shellfish with no adverse outcome was not above 0.25 µg TTX/kg except for instances of the intake of large quantities of oysters, which may suggest that marine bivalves do not pose a threat for consumers. The EFSA CONTAM Panel established that a concentration below 44 µg of TTX and/or the same amount of TTX analogues per kg of shellfish meat should not cause adverse effects in humans, in conditions consistent with 400g maximum bivalve consumption, 70kg average adult body weight, and 0.25 µg/kg ARfD. Unfortunately, this value could not be determined for marine gastropods, and neither could the risk of exposure to TTX in consumption of these

organisms be characterised, due to the limited amount of data on such consumption and the paucity of data on the poisonings.

Conclusions

There are still many unexplained issues regarding the influence of TTX and its analogues on human health. Taking into account that no accurate data on the toxicity of analogues are available, further studies are necessary to assess their potentially harmful effects, especially after oral administration. Further research is also required on the relationship between TTX and STX neurotoxins regarding the similarity of their mechanisms of action and induction of similar toxic effects. Detection and quantification of TTX and its analogues should be carried out by EU-accepted and validated methods using reference materials and the highest quality standards to ensure reliable results. It is suggested that further information on the occurrence and factors conducive to the accumulation of TTX in marine organisms is needed. It is also important to provide more results regarding the toxicokinetics of TTX and its analogues. Furthermore, despite the belief that tetrodotoxins cause acute intoxication without chronic effects, this issue requires more research.

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