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Tetrodotoxin neutralisation with a monoclonal antibody: *In vitro* Neuro-2a cell-based assay and *in vivo Artemia salina* test

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ABSTRACT

Tetrodotoxin (TTX) is a potent marine neurotoxin causing severe seafood poisoning, with no available antidote worldwide. In this work, the ability of an anti-TTX monoclonal antibody (mAb) to neutralise TTX standard as well as the toxin present in different matrices, including *Lagocephalus sceleratus* tissue extracts, oyster extracts and human urine samples, was demonstrated using a Neuro-2a cell-based assay (CBA). The results showed that the *in vitro* neutralising activity varied depending on the biological matrix, with neutralisation percentages ranging from 22% to 80%. The specificity of the mAb towards TTX was further demonstrated using a *Lagocephalus lagocephalus* tissue extract containing saxitoxin (STX). The toxicological effect of TTX at different concentrations on *Artemia salina* was also evaluated. The *in vivo* neutralising activity was proved by the increase in the survival rate. These results suggest that the anti-TTX mAb could be use as therapeutic agent in tetrodotoxication cases.

1. Introduction

Tetrodotoxin (TTX) is one of the most potent marine neurotoxins (Narahashi, 2001). Structurally, TTX ($C_{11}H_{17}O_8N_3$) consists of a guanidinium moiety connected to a highly oxygenated carbon backbone with a 2,4-dioxaadamantane portion containing five hydroxyl groups and has a low molecular weight (319.27 g/mol) (Chau et al., 2011). TTX was first detected in pufferfish (Tahara and Hirata, 1909). Nevertheless, it has later been found in various organisms, including vertebrates and invertebrates (Hwang and Tsai, 1999; Hwang et al., 2007; Kim et al., 1975; Thuesen et al., 1988). Many bacteria, such as *Pseudomonas sp.*, *Vibrio sp.* and *Alteromonas sp.*, have been observed to produce TTX, however the mechanisms involved in its accumulation in eukaryotes have remained unknown (Magarlamov et al., 2017; Turner et al., 2018). Up to date, more than 30 TTX analogues have been described, with varying toxic potencies (Reverté et al. 2023). In natural environments, TTX coexists with several analogues. TTX and some of its analogues are highly toxic due to their cellular targeting and mode of action (Kao and Walker, 1982). TTX is known to bind to site 1 of voltage-gated sodium channels (VGSCs), leading to a blocking of the inward flow of sodium ions. The guanidinium group present in TTX and its analogues is able to act as a cationic substitute for the sodium ion (Cusick and Sayler, 2013; Hartshorne and Catterall, 1984). As a consequence of the inhibition of sodium influx, the normal neuronal and muscular functions can be altered, leading to paralysis and, in extremely severe cases, to death (Katikou et al., 2022). The first recorded case of tetrodotoxication in humans was in 1774, following the ingestion of pufferfish (Fugu) (Katikou et al., 2022). In recent years, there has been an increasing number of poisoning cases worldwide. In the Eastern Mediterranean, 171 tetrodotoxication incidents have been reported between 2004 and 2023 (Ulman et al., 2024). The clinical symptoms of tetrodotoxication are similar to those observed in poisonings caused by paralytic shellfish toxins (PSTs) (Durán-Riveroll and Cembella, 2017). In fact, although the chemical structures of TTX and STX are considerably different, both

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toxins exhibit similar modes of action (Huang et al., 2012). PSTs toxins can be found in organisms such as bivalve molluscs, pufferfish, crabs and gastropods, being saxitoxin (STX) the main representative toxin of the family. As TTX, in natural environment STX may coexist with its own analogues. The co-occurrence of TTX and STX in numerous marine and freshwater organisms has also been documented (Bane et al., 2014; Nakashima et al., 2004; Soliño et al., 2021; Zhu et al., 2020).

Regarding legislation, the Japanese government has set an official regulatory limit for TTXs at 2 mg TTX/kg pufferfish tissue (Noguchi and Ebesu, 2001). In Europe, the *Tetraodontidae* fish family and all derived products are banned in markets, and similar regulatory requirements exist in other non-EU Mediterranean countries (Regulation (EC) No 853/2004). In 2017, the CONTAM panel of the European Food Safety Authority (EFSA) proposed a provisional guideline value of 44 μg TTX equiv./kg shellfish meat, which was considered not to result in adverse effects in humans (EFSA CONTAM Panel EFSA Panel on Contaminants in the Food Chain et al., 2017). Regarding STX, a regulatory limit at 800 μg STX equiv./kg shellfish meat was set (Regulation (EC) No 853/2004). Furthermore, the EFSA CONTAM panel as well as some other researchers recommend the potential integration of STX, TTX and their analogues within a unified health-based guidance value, taking into account the fact that they exert similar toxic effects via a similar mode of action, as mentioned above, and their toxicities are additive (EFSA CONTAM Panel EFSA Panel on Contaminants in the Food Chain et al., 2017; Finch et al., 2018). There is currently no effective antidote for TTX to treat intoxications. Treatment remains primarily symptomatic and supportive, and may involve mechanical ventilation for oxygen supply, normal saline infusion for distending the intravascular volume and gastric lavage for removal of unabsorbed toxins [14]. It is theoretically possible to achieve scavenging of TTX by immunising individuals, which could provide a new strategy for the prevention of tetrodotoxications (Fukiya and Matsumura, 1992; Kaufman et al., 1991). However, given the time required for vaccines to induce protective immunity and the dependence on the host's ability to mount an immune response (Casadevall, 2002), the only practical method of providing immediate immunity against TTX would be passive immunotherapy, which utilises neutralising antibodies (Rivera et al., 1995; Xu et al., 2005).

This study addresses a critical gap in toxicology by providing experimental evidence on the neutralisation of TTX using antibodies, a strategy with direct implications for mitigating human poisoning. Given the absence of an effective antidote and the increasing number of TTX-related intoxication cases worldwide, establishing the efficacy of antibody-based neutralisation is essential for advancing future therapeutic approaches. Specifically, the efficacy of an anti-tetrodotoxin monoclonal antibody (anti-TTX mAb) to neutralise the toxicity of TTX *in vitro*, using Neuro-2a cells in a cell-based assay (CBA), and *in vivo*, using *Artemia salina* (*A. salina*), was investigated. For the CBA, mouse neuroblastoma Neuro-2a cells, which express VGSCs on their membrane, were used (Alkassar et al., 2022). In the CBA for TTX, the use of O and V, which block the ATP-dependent Na^+ /K^+ pumps and the VGSCs, respectively, leads to an increase in intracellular sodium ions, resulting in Neuro-2a cell death. Exposure of cells, pre-treated with O/V, to different TTX concentrations counteracts this O/V-induced cytotoxicity, thereby increasing cell viability. Therefore, if the mAb is able to neutralise TTX toxicity, pre-incubation of TTX with mAb and subsequent addition to the O/V-pretreated cells should result in cell mortality. *In vitro* neutralisation of TTX toxicity by the anti-TTX mAb was evaluated using brine shrimp *A. salina* metanauplii. *Artemia* has been used as a toxicological model for the study of marine toxins, and it has been demonstrated that metanauplii is the most sensitive stage for this purpose (Zhenxing et al., 2006). Although information and research on TTX in the chemical and biological field is abundant, there is a lack of experiments testing its lethal and sublethal effects in the early development of larvae. Thus, first, the effect of TTX on *A. salina* metanauplii was evaluated. In the application of these tests, the death rate and behavioural parameters are commonly used to evaluate the impact of toxic compounds on the *A. salina* nauplii (Albarano et al., 2022).

In this work, we first determined the concentration of anti-TTX mAb necessary to neutralise standard TTX in the CBA. Then, different matrices, including *Lagocephalus sceleratus* (*L. sceleratus*) tissue extracts, oyster extracts and human urine, were used to demonstrate the ability of the mAb to neutralise TTX in naturally contaminated samples. The specificity of the mAb against TTX was also evaluated using different STX-containing *Lagocephalus lagocephalus* (*L. lagocephalus*) tissue extracts. Afterwards, the toxicological effect of TTX in *A. salina* metanauplii was studied and the neutralising activity of anti-TTX mAb *in vivo* was demonstrated.

2. Materials and methods

2.1. Reagents and materials

Neuroblastoma murine (Neuro-2a) cells were purchased from ATCC LGC Standards (Manassas, VA, USA). Foetal bovine serum (FBS), ouabain (O), veratridine (V), phosphate buffered saline (PBS), penicillin–streptomycin, RPMI-1640 medium, sodium pyruvate, methyl thiazolyl tetrazolium (MTT) were purchased from Merck KGaA (Gernsheim, Germany). Glacial acetic acid (HAc) was obtained from Chemlab (Zedelgem, Belgium). TTX (CAS Number: 4368–28–9) standard (≥98% purity) was purchased from Tocris (Bio-Techne R&D Systems, Madrid, Spain) and the standard solution was prepared at 1 mg/mL in 1% HAc. Anti-TTX mAb was obtained from Deltaclon (Madrid, Spain). *Artemia salina* cysts were purchased from Acuazul (Cadiz, Spain).

2.2. Naturally contaminated samples and TTX/STX extraction

Pufferfish (gonads, liver, skin and muscle), oyster and human urine samples were obtained from previous studies (Alkassar et al., 2024a, b; Rambla-Alegre et al., 2017, 2018). TTX extraction from pufferfish (Reverté et al. 2015) and oyster samples (Campàs et al., 2020) was performed using 0.1% and 1% HAc, respectively. Briefly, after homogenisation, samples were vortexed at 2500 rpm for 2 min and immersed in a water bath set at 100 °C for 10 min with occasional stirring. Then, they were cooled and vortexed again. For the pufferfish liver samples, an additional liquid-liquid partition with hexane (1:1) for 2 h, was performed to remove fats by discarding the hexane layer. Finally, samples were centrifuged, and the supernatant was passed through a 0.2-μm nylon filter and kept at −20 °C until analysis. The final extracts obtained were at 200 mg equiv. of pufferfish tissue/mL and 1000 mg equiv. of oyster tissue /mL, respectively for pufferfish tissue and oyster.

2.3. Cell maintenance and neutralising in vitro assays

The CBA was performed as previously described (Alkassar et al., 2023). Briefly, Neuro-2a cells were trypsinised and suspended in culture medium (containing 5% FBS). Then, Neuro-2a cells were seeded in a 96-well microplate at an approximate density of 35,000 cells/well in 200 μL of culture medium for 24 h at 37 °C in 5% CO_2 humid atmosphere. After 24 h, some Neuro-2a cells were pretreated with 20 μL of a mixture containing O and V at final concentrations of 0.125 and 0.2 mM in PBS, respectively. TTX standard solution or naturally contaminated extracts were dried under N_2 stream at 40 °C using a TurboVap evaporator. The dried extracts were then reconstituted in RPMI medium and pre-incubated with 1 mg/mL of anti-TTX mAb (0.5, 0.25, 0.12, 0.06 and 0.03 mg/mL for the optimisation study) for 30 min under agitation. Subsequently, cells pre-treated with O/V were exposed to 10 μL of the obtained TTX-mAb mixture. In the optimisation study, the neutralising effect was evaluated using 3-fold the half maximal inhibitory concentration of TTX standard (5 ng/mL) of TTX standard. For the neutralising experiment, four tissue extracts obtained from pufferfish specimen *L. sceleratus* (gonads, liver, skin and muscle), three oyster extracts from The Netherlands (EX179, EX177 and EX162) and two human urine

samples (HU1 and HU2) were used. All these samples were previously analysed by CBA (Alkassar et al., 2024a, b; Campàs et al., 2024), and then tested at a specific tissue concentration corresponding to a toxin concentration that results in approximately 50% viability. Cells pre-treated with O/V and exposed to the dried extract in absence of anti-TTX mAb were used as control. After 24 h, cell viability was measured using the MTT assay. Absorbance (ABS) at 570 nm was measured with a Synergy LX microplate reader from BioTek (Agilent Technologies, Inc., Santa Clara, CA, USA). Measurements were performed in triplicate. Calibration curve for TTX in the presence of O/V was constructed and fitted to a sigmoidal logistic four-parameter equation:

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

Where a and y_0 are the asymptotic maximum and minimum values respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point.

Neutralising activity percentages were calculated as follows:

$$\text{Neutralising activity } (\%) = \frac{(\text{ABSno mAb} - \text{ABSmAb})}{\text{ABSno mAb}} \times 100$$

2.4. Neutralising in vivo assays

First, the toxic effects of TTX on *A. salina* at matanauplii stage (≥ 300 μm) were evaluated. The mortality test was based on a concentration and time-dependent approach to determine the optimal exposure conditions. Briefly, dried cysts of *A. salina* were hatched in filtered sea water at 28–30 °C under continuous illumination and aeration. After 48 h of incubation, *A. salina* metanauplii individuals were collected and placed into 96-microplate (1 individual per well) containing 50 μL of autoclaved sea water and 50 μL of TTX standard solution at different concentrations, starting at 1000 ng/mL and ½ serially diluted. A total of 10 individuals was used for each concentration. The culture plate was incubated at 27 °C in the darkness and the survival rates were determined every 6 h up to 36 h. *A. salina* individuals incubated with 100 μL autoclaved seawater were used as a control group (a total of 40 individuals were used for the control group).

Then, to evaluate *in vivo* neutralisation of TTX toxicity, *A. salina* metanauplii individuals were exposed to 50 μL of TTX standard solution at 0.49 ng/mL and 50 μL of anti-TTX mAb at 1 mg/mL. The TTX exposure concentration selected was based on concentration–response experiments and represents a low-level exposure at which clear toxic effects occur, enabling assessment of antibody-mediated protection under biologically relevant conditions. For positive and negative control groups, *A. salina* individuals were incubated with 100 μL of autoclaved seawater and with 100 μL of TTX standard solution at 0.49 ng/mL, respectively (a total of 40 individuals were used for the control groups). The mortality rate in each group was determined at 18 h and 24 h. The mortality rate was expressed as follow:

$$\text{Mortality rate}(\%) = \frac{\text{Number of dead organisms}}{\text{Number of total organisms}} \times 100$$

3. Results

3.1. Neutralisation of TTX toxicity in vitro

First, the viability of Neuro-2a cells in the presence of different anti-TTX mAb concentrations was evaluated (Fig. 1). As expected, a mAb concentration-dependent response was observed, the 50% neutralising concentration being 0.24 mg/mL. At 0.03 mg/mL of mAb, cell viability was practically the same as with only TTX and therefore, mAb was not able to neutralise its effect. On the contrary, at 1 mg/mL of mAb, almost all the effect of TTX was neutralised, the cell mortality observed being due to O/V. Although this concentration did not completely neutralise the TTX (81% of neutralising activity), higher mAb concentrations were not used because of budgetary reasons. Therefore, this concentration was used for the subsequent experiments.

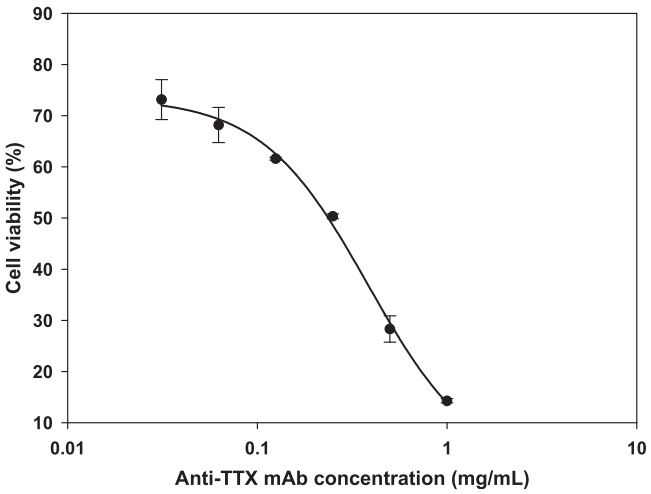

Fig. 1. Viability of O/V-pre-treated Neuro-2a cells exposed to TTX standard at 5 ng/mL and different anti-TTX mAb concentrations.

To evaluate the neutralising activity of TTX present in extracts using the anti-TTX mAb, several naturally contaminated samples, including *L. sceleratus* tissue extracts, oyster extracts and human urine, that had been previously tested positive for TTX and its analogues were used. Fig. 2 displays the percentage of neutralising activity of the mAb (1 mg/mL) against TTX among these different samples. In general, the mAb showed efficacy in neutralising the TTX present in those samples, although the degree of neutralisation varied depending on the sample matrix (between 22% and 80%). Notably, all tissue extracts from *L. sceleratus* and human urine samples showed a high neutralisation level. However, the neutralising performance in oyster extracts was lower: approximately 50% of TTX was neutralised in samples EX179 and EX162, while EX177 showed lower neutralisation efficacy (~22%). These findings demonstrate that the mAb can neutralise TTX across different biological matrices (to a greater or lesser extend), highlighting its potential for being applied in future therapeutic treatments against TTX exposure.

In addition, the specificity of the mAb for TTX and its analogues was

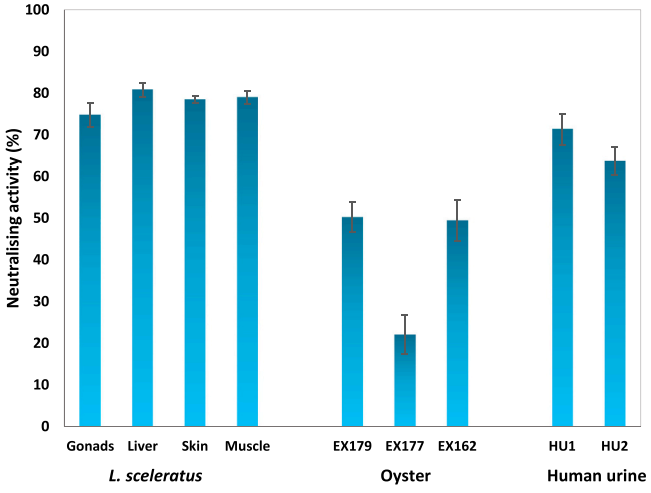

Fig. 2. *In vitro* TTX neutralising activity of anti-TTX mAb (1 mg/mL) in different naturally contaminated matrices.

examined by performing a neutralising experiment with different tissue extracts from a pufferfish sample (*L. lagocephalus*) that tested positive for PSTs and negative for TTX (also diluted down to 5 ng/mL). Fig. 3 shows the results of viability of Neuro-2a cells, pre-treated with O/V and exposed to the different tissue extracts, in the presence and absence of anti-TTX mAb. As we mentioned before, CBAs are based on the mechanism of action of toxins on VGSCs. However, they cannot distinguish between toxins with similar mechanisms of action, such as TTX and STX. In the previous experiment, the presence of the mAb prevented TTX from suppressing O/V-induced cytotoxicity in Neuro-2a cells, leading to a decrease in cell viability (Fig. 2). On the contrary, in the case of STX-containing samples the presence of the mAb (O/V-mAb, Fig. 3) was not able to reverse the protective effect of STX on Neuro-2a cells and no cell death was observed, confirming the specificity of the mAb for TTX and not STX.

3.2. Neutralisation of TTX toxicity in vivo

The time evolution of *A. salina* mortality after exposure to different TTX concentrations is shown in Fig. 4. At low toxin concentrations (≤7.81 ng/mL), TTX induced mortality in metanauplii, with a 100% mortality being induced after only 24 h of exposure. Surprisingly, when metanauplii were exposed to higher TTX concentrations (TTX ≥ 15.6 ng/mL), the mortality rate increased more slowly with time. In general terms, these high TTX concentrations induced 100% of mortality after 36 h of exposure. The exposure of *A. salina* metanauplii to these higher TTX concentrations caused deformities and affected their behaviour, effects not observed at low TTX concentrations (Fig. 5). Specifically, starting at 6 h of TTX exposure, the metanauplii presented an imbalanced and irregular swimming pattern, and their motility was almost completely hampered at the highest concentration tested (1000 ng/mL). It is important to mention that spasms and convulsions were also observed. These results show that *A. salina* metanauplii are highly sensitive to TTX, with low concentrations being sufficient to induce full mortality within 24 h.

Based on this, a TTX concentration of 0.49 ng/mL, the lowest concentration causing 100% mortality within 24 h, and an anti-TTX mAb concentration of 1 mg/mL were selected to evaluate the *in vivo* neutralisation of toxicity in metanauplii. The mortality rate was calculated in two different endpoints, 18 and 24 h. As shown in Fig. 6, *A. salina* incubated in autoclaved seawater (negative control) showed low mortality at both 18 and 24 h, serving as a baseline for normal survival rates. However, larvae exposed to TTX without the mAb exhibited an increase in the mortality rate, being of 100% at 24 h of exposure. As a result of the incubation with the TTX-mAb mixture, the TTX toxicity decreased

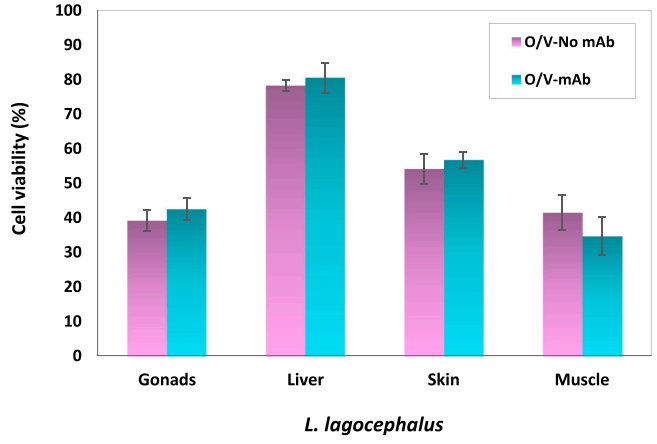

Fig. 3. Viability of O/V-pre-treated Neuro-2a cells exposed to different tissue extracts (gonads, liver, skin and muscle) obtained from PST-containing *L. lagocephalus* in the absence and presence of anti-TTX mAb (1 mg/mL).

dramatically, as can be observed by the decrease in the mortality rate of the metanauplii. These results revealed that the use of the mAb effectively has a protective effect against the TTX toxicity and corresponding lethal effects in *A. salina*. Higher TTX concentrations were not explored due to antibody cost constraints. However, this proof-of-concept design does not limit the validity of the findings, as such studies would be feasible with scalable monoclonal antibody production.

4. Discussion

TTX is highly toxic and poses a serious threat to public health worldwide. By blocking VGSCs and interrupting neuromuscular transmission, TTX causes a series of clinical symptoms, even death [14]. In fact, several poisoning cases and mortalities due to the consumption of shellfish contaminated with TTX (Alkassar et al., 2024a; Rodriguez et al., 2008) or due to the accidental consumption of pufferfish have been reported (Alkassar et al., 2023; Bane et al., 2014; Nakashima et al., 2004; Soliño et al., 2021; Zhu and Sonoyama 2020). The inexistence of an antidote makes this poisoning cases still an issue around the world. Antibody therapy has long been used in clinic to prevent the harmful effects of biological agents (Casadevall, 2002). However, regarding TTX, the only described experiments are those where the neutralisation of TTX standards with antibodies has been tested with mice (Rivera et al., 1995; Xu et al., 2005).

In our study, the neutralising capacity of anti-TTX mAb *in vitro* and *in vivo* was demonstrated. The *in vitro* experiments have not only been performed with TTX standard, but also with different biological matrix types. The differences in TTX neutralisation between the different matrix types or even within the same matrix (but different individuals) are not surprising. In fact, similar results were obtained by Reverté and co-authors (Reverté et al. 2026). In their study, where automated patch clamp was used instead of CBA, the mAb was also able to effectively neutralize TTX present in different tissues of *L. sceleratus* from Greece, but with different neutralisation percentages. The differences observed between biological matrix types could be explained by the differences in matrix components. These matrices may contain proteins, lipids, hormones, metabolites and other molecules, which may interfere with mAb binding to TTX. Moreover, the presence of these matrix components could alter mAb stability and also cause steric impediments. Other parameters that may have an influence on the efficacy of the mAb in biological samples are the presence and/or co-occurrence of several TTX analogues in different percentages, which may have a different cross-reactivities with the mAb. As previously stated, the samples used in this study were previously subjected to analysis and their toxin profiles were determined by LC-MS/MS (Table 1). All samples have a multi-toxin profile with the presence of TTX and several of its analogues such as 4-*epi*TTX, 11-norTTX-6(*S*)-ol, 11-norTTX-6I-ol, 4,9-anhydroTTX, 5-deoxyTTX, 11-deoxyTTX, 5,11-dideoxyTTX and 5,6,11-trideoxyTTX (Alkassar et al., 2024a; Rambla-Alegre et al., 2017, 2018). Furthermore, it is essential to consider the varying degrees of toxicity exhibited by different TTX analogues, which is influenced by their chemical structures. In our previous works (Alkassar et al., 2023; Reverté et al. 2024), toxicity equivalency factors (TEFs) of some TTX analogues were established using the same cell line (Neuro-2a cells), all of them being less toxic than the parent TTX. In addition, Reverté and co-authors found that TTX analogues with lower TEFs exhibited a lower degree of cross-reactivity with the anti-TTX mAb (Reverté et al. 2026). To further understand the neutralising activity in the presence of different biological matrices, TEFs obtained by CBA (Alkassar et al., 2023) and cross-reactivity factors (CRFs) of the different TTX analogues obtained in the study of Reverté et al. (2026) (Table 2) were applied to the toxin profiles obtained by LC-MS/MS analysis (Fig. 7). It is important to note that TEFs and CRFs were only applied to some of the TTX analogues, as they were not available for all of them. As it can be seen in Fig. 7, besides TTX, 5,6,11-trideoxyTTX was present at high concentrations in all samples. This analogue has been observed to be less toxic and less

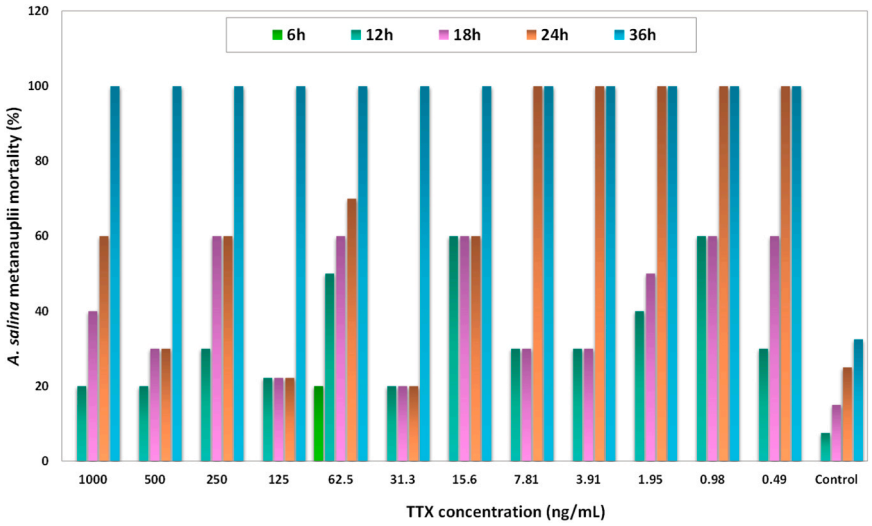

Fig. 4. Mortality of *A. salina* metanauplii exposed to different TTX concentrations and times.

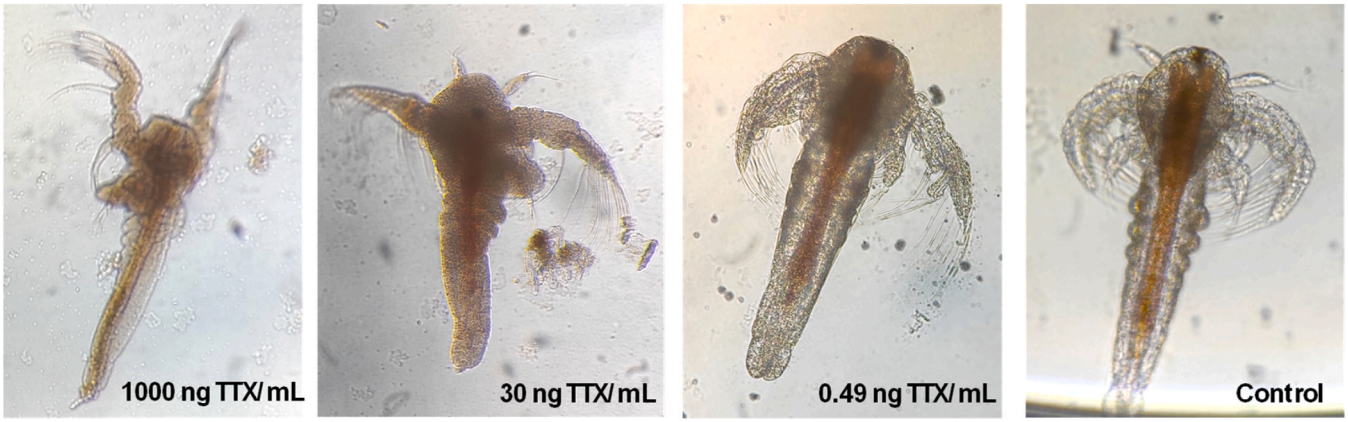

Fig. 5. Optical light microscopy images of *A. salina* metanauplii after 24 h of exposure to different TTX concentrations.

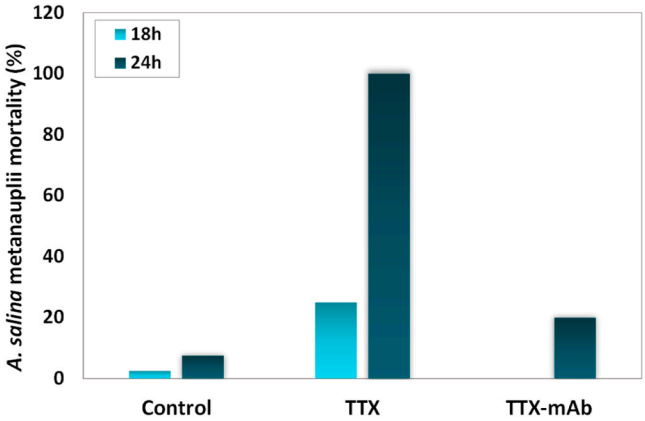

Fig. 6. *In vivo* TTX neutralising activity of anti-TTX mAb (1 mg/mL) on *A. salina metanauplii* using TTX standard at 0.49 ng/mL.

cross-reactive with the mAb (Alkassar et al., 2023; Reverté et al. 2024, 2026, 2015). By applying the TEFs (Figs. 7, 2) and CRFs (Figs. 7, 3) to the TTX analogues present in the different biological samples, the total toxicity of the samples can be attributed mainly to parent TTX. This suggests that the neutralising percentages are mainly due to the binding of the mAb to this parent TTX. The high specificity of the mAb towards TTX was also demonstrated in the experiment with STX-containing pufferfish samples (as demonstrated by HPLC-FLD, Table 1), where the toxicity was not neutralised.

There are a few *in vivo* studies of the toxicological effects of marine toxins in *A. salina*. Zhenxing and co-workers have shown that STX-producing dinoflagellate strains induce lethality to populations of *A. salina* (Zhenxing et al., 2006). In another study (Veeruraj et al., 2016), the lethality of brine shrimp exposed to different tissue extracts obtained from a pufferfish individual containing TTX was evaluated and found to be directly proportional to the toxin concentration in the extracts. In our study, exposure of *A. salina* to TTX resulted in mortalities as well as changes in their physiological behaviour. The deteriorating effect on the mobility of *Artemia* spp. and the loss of their phototactic response due to the accumulation of toxic compounds was observed in other studies (Astuya et al., 2015; Cavion et al., 2022; Miller and McLennan, 1988; Neves et al., 2017; Tapia-Salazar et al., 2022). The unexpected slower mortality rate of metanauplii exposed to high TTX concentrations should be further studied. It is known that *Artemia spp* survive adverse conditions in several ways by the induction of stress proteins, known as heat shock proteins (Hsps) (Frankenberg et al., 2000; Han et al., 2021; MacRae, 2016; Miller and McLennan, 1988; Sung et al., 2008). These proteins play essential roles in immune reactions of animals against toxic compounds and cross-tolerance to environmental perturbations (Sung et al., 2008). The cross-tolerance is a mechanism by which a primary stress transiently increases the resistance to other stressors of

Table 1
TTX and analogues contents in *Lagocephalus sceleratus* (mg TTX or analogue/kg) (Rambla-Alegre et al. 2017), oysters (mg TTX or analogue/kg) (Alkassar et al. 2024a), and human urine (ng TTX or analogue/mL) (Rambla-Alegre et al. 2018) by LC-MS/MS, and PSP contents in *Lagocephalus lagocephalus* (mg TTX or analogue/kg) (Alkassar et al. 2024b) by HPLC-FLD.

	TTX 4-*epi*TTX	11-norTTX-6(*R*)-ol 11-norTTX-6(*S*)-ol	4,9-anhydroTTX	5-deoxyTTX	11-deoxyTTX	5,11-dideoxyTTX 6,11-dideoxyTTX	5,6,11-trideoxyTTX	STX	dcSTX
L. sceleratus gonads	26.1	17.4	0.5	0.9	1.1	0.4	94.3	-	-
L. sceleratus liver	3.0	1.6	0.2	-	0.2	0.2	12.4	-	-
L. sceleratus skin	1.5	1.2	-	-	0.1	-	1.8	-	-
L. sceleratus muscle	1.0	0.8	0.1	-	0.1	0.1	1.2	-	-
Oyster EX179	0.8	-	-	-	-	-	0.4	-	-
Oyster EX177	0.2	-	-	-	-	-	0.1	-	-
Oyster EX162	0.3	-	-	-	-	-	0.2	-	-
Human urine 1	318.6	-	108.3	-	-	-	1008.2	-	-
Human urine 2	832.9	-	184.7	-	-	-	171.6	-	-
L. lagocephalus gonads	-	-	-	-	-	-	-	0.4	0.8
L. lagocephalus liver	-	-	-	-	-	-	-	16.5	41.6
L. lagocephalus skin	-	-	-	-	-	-	-	0.4	0.4
L. lagocephalus muscle	-	-	-	-	-	-	-	0.1	0.2

Table 2
Toxicity equivalency factors (TEFs) and cross reactivity factors (CRFs) of the different TTX analogues obtained by CBA (Alkassar et al. 2023) and magnetic bead-based immunoassay (Reverté et al. 2026), respectively.

	TEF	CRF
TTX + 4-*epi*TTX	1	1
11-norTTX-6(*S*)-ol	0.404	0.103
11-deoxyTTX	0.139	0.068
5,11-dideoxyTTX	0.750	0.015
5,6,11-trideoxyTTX	0.011	0.001

the same or different nature (Völker et al., 1992), thus enabling cells or animals to survive subsequent, more severe stress (Jean et al., 2004). This could be happening in our experiments.

The protective efficacy of the mAb *in vivo* was demonstrated by its ability to inhibit TTX-induced toxicity in *A. salina* metanauplii. Metanauplii exposed to TTX exhibited a rapid increase in mortality. The protective effect was evidenced by an increase in the survival rate if the group treated with the TTX-mAb mixture. Many studies have shown the potential of different antibodies and vaccines against TTX toxicity (Huot et al., 1989; Matsumura et al., 1995; Rivera et al., 1995; Watabe et al., 1989; Xu et al., 2005). However, all of them display effects in mice, and have not been tested in *Artemia* spp, which makes the comparison very

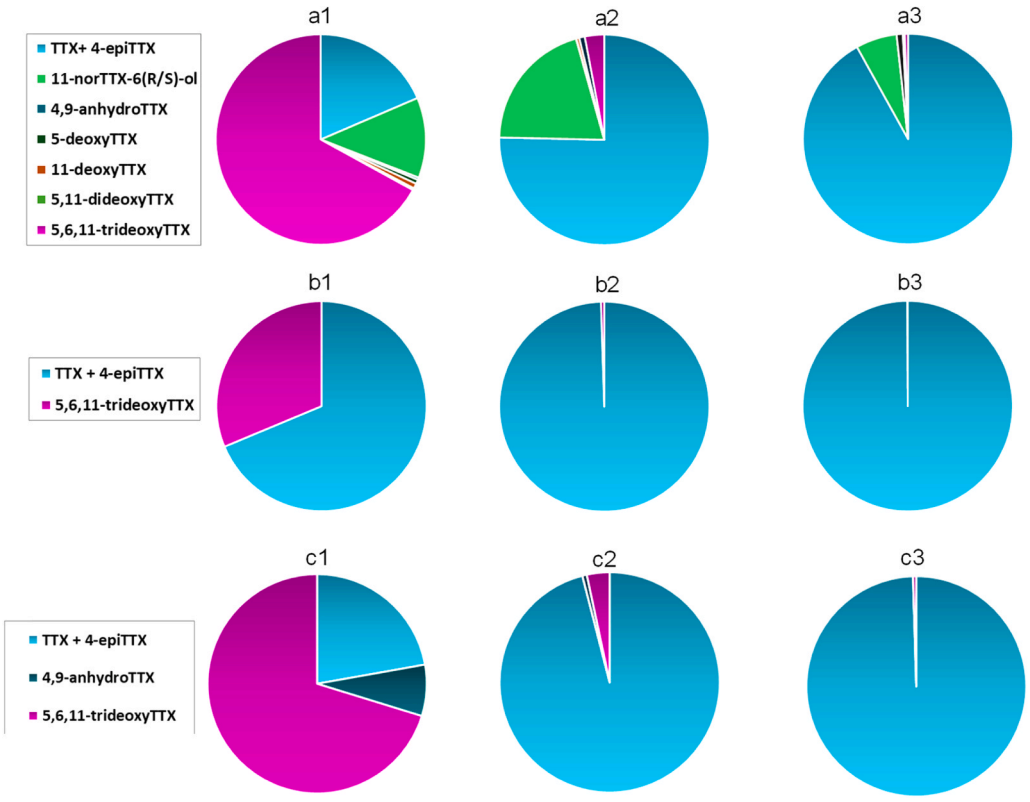

Fig. 7. Graphical representation of multi-toxin profiles of *L. sceleratus* gonads extract (**a**), oyster extract EX179 (**b**) and human urine 1 (**c**), obtained by (**1**) LC-MS/MS analysis, (**2**) after applying the corresponding TEFs, and (**3**) after applying the CRF to the different TTX analogues. The three samples were selected as representative examples of the three matrix types.

difficult. The first mAb against TTX was developed by Watabe et al. in 1989, which was specifically targeted against tetrodonic acid. Unfortunately, this mAb was unable to neutralise TTX administered to mice via injection. Another mAb produced by Huot and coworkers in 1989, with high affinity for TTX but not for STX, was able to show protective ability in rat tibial nerve cells against TTX toxicity (Huot et al., 1989). Matsumura (Matsumura, 1995) produced a mAb that was able to react with the OH group on C-4 and C-9 of TTX, thus confining the effect of the antibody only to TTX and not to any of its analogues. In another study (Rivera et al., 1995), the oral administration of the mAb T20G10 to mice previously exposed to a lethal dose of TTX (which may result in mortality within 25–30 min) demonstrated a prolonged survival rate of over 24 h. In the study of Xu and co-workers, the TTX-neutralizing potential of polyclonal antibodies was demonstrated by the increase in survival animal challenged by lethal doses of TTX pre-incubated *in vitro* or neutralised *in vivo* with those antibodies (Xu et al., 2005). It is important to mention that, although in our study the mAb was able to effectively neutralise TTX both *in vitro* and *in vivo,* the mAb protection was preventive (prophylaxis by neutralization of the toxin exposed simultaneously to the mAb) but not real a therapy of previously poisoned *Artemia*. Nevertheless, antibodies against TTX are not only useful for the detection of this toxin in naturally contaminated samples, but they also hold promise for the development of vaccines with therapeutic applications. Since the lethal dose for TTX in humans is different among sex, weight and intoxication level (EFSA, 2009), the potential efficacy of the proposed treatment remains uncertain, with the possibility that this lethal dose may be too high for any of the antibodies or vaccines to be able to prevent toxicity to a meaningful degree. One potential issue with the efficacy of antibody or treatment in humans is the amount of the treatment compound required to achieve significant effects. If this amount is too high, the option may become unfavourable in practical use. However, it is likely that potential treatments will be adapted to humans in the future, which could be a significant advancement in the treatment of TTX and other poisonings. This could potentially reduce the number of severe cases and fatalities from toxin poisonings.

5. Conclusions

In this study, the neutralisation of TTX *in vivo* and *in vitro* using a mAb has been successfully achieved. The mAb concentration needed to observe *in vitro* TTX neutralisation was optimised. Then, the capacity of the mAb to neutralise TTX present in several biological matrix types (pufferfish tissues extracts, oyster extracts and human urine samples) was evaluated. The neutralising activity exhibited a matrix-type dependence, with higher neutralising percentages observed in the pufferfish extracts. Another remarkable result was the high specificity of the mAb towards TTX, evidenced by a lack of neutralisation of the toxicity in STX-containing *L. lagocephalus*. Moreover, the toxicological *in vivo* study on *A. salina* showed that high TTX concentrations induced important changes in the physiological behaviour, whereas at low concentrations, TTX increased the mortality. The *in vivo* TTX neutralisation using the mAb was evidenced by its ability to inhibit mortality in *A. salina* exposed to TTX. The suppression of TTX-induced toxicity *in vitro* and the improvement of *A. salina* survival rate *in vivo* provide evidence of the promising use of antibodies as therapeutic agents.

CRediT authorship contribution statement

Mònica Campàs: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Alicia Estévez:** Writing – review & editing, Methodology, Conceptualization. **Jorge Diogène:** Writing – review & editing. **Jaume Reverté:** Writing – review & editing, Investigation. **Mounira Alkassar:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

Ethical statement

The collection of all samples was carried out within the framework of nationally or EU-supported monitoring or research projects, in compliance with all the relevant ethical guidelines established by competent authorities. This study employed *Artemia salina*, an invertebrate organism not subject to animal experimentation regulations. As such, the ARRIVE guidelines and regulatory frameworks governing vertebrate and cephalopod research (e.g., EU Directive 2010/63/EU and the U.K. Animals (Scientific Procedures) Act 1986) do not apply, and ethical approval was not required.

Declaration of Generative AI and AI-assisted technologies in the writing process

No Generative AI and AI-assisted technologies were used in the writing process.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Data availability

Data will be made available on request.

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Glossary

CBA: Cell-based assay
CRF: Cross-reactivity factor
EC: European Commission
EFSA: European Food Safety Authority
EU: European Union
FBS: Foetal bovine serum
HAc: Acetic acid
mAb: Monoclonal antibody
MTT: Methyl thiazolyl tetrazolium
O/V: Ouabain/Veratridine
PBS: Phosphate buffered saline
PSTs: Paralytic shellfish toxins
RPMI: Roswell Park Memorial Institute
STX: Saxitoxin
TEF: Toxicity equivalency factor
TTX: Tetrodotoxin
VGSCs: Voltage-gated sodium channels