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An *in silico* perspective on the toxicodynamic of tetrodotoxin and analogues – A tool for supporting the hazard identification

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ABSTRACT

Tetrodotoxin (TTX) is a potent neurotoxin naturally found in terrestrial and marine animals targeting the voltage-gated sodium channels. Historically, TTX has raised food safety concerns mainly in the Asian countries due to the consumption of the typical pufferfish-derived delicacy *fugu*. However, the diffusion of TTX is getting wider today, reasonably threating in a close future a broader number of consumers than before. The understanding of TTX group toxicity is still incomplete as most of the analogues and metabolites found together with TTX are largely understudied. This prevents the establishment of a solid background for risk assessment and additional data have been claimed to timely foster the assessment of TTX toward a group-based approach. However, the high costs in sourcing TTX analogues make practically unfeasible the wide-scale assessment using experimental trials. The toxicological assessment *in silico* may succeed in extending data on compounds poorly affordable, hierarchizing compound to focus experiments and supporting the hazard identification. Therefore, the present work investigated the toxicodynamic of TTX, analogues and metabolites by using a molecular modeling approach. In the framework of the hazard identification, the model analyzed TTX analogues never tested before assessing qualitatively their potential toxicity in comparison to TTX. While the analogues from TTX bearing species appeared to be less toxic than TTX, some human metabolites showed a better interaction with the toxin binding site. Such results suggest that human metabolism may partially fail in preventing the interaction with the biological target. Therefore, the identification and assessment of human metabolites should be done to support the decision making process from a more informed perspective.

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin thought produced by a variety of bacteria (Jal and Khora, 2015) naturally occurring in some marine and terrestrial animals (Kasteel and Westerink, 2017). In the marine species TTX can be found mostly in pufferfish and in several marine invertebrates such as snails, worms, crabs, starfish, blue-ring octopus and sea slug (Puilingi et al., 2015). In terrestrial animals it is distributed mainly in newts, frogs and toads (Bane et al., 2014). From a food safety perspective, TTX severely threatens human health and it has been responsible of many intoxications and fatalities over the years (Lago et al., 2015). The food-related menace has been historically confined to the Japan and other Asian countries due to the consumption of *fugu* – a typical delicacy derived from pufferfish (Coleman et al., 2016; Suzuki, 2016). Nevertheless, nowadays the geographical constraints and the TTX-bearer species tropism are changing, also due to the ongoing climate changes (Botana, 2016). As examples, some TTX-bearer species have been recently found adapted in Mediterranean areas (Bentur et al., 2008; Katikou et al., 2009), thus indicating the capability of this toxins chemotype to spread over a wider geographical area. Moreover, TTX has been recently found contaminating bivalve mollusks like mussels and Pacific oysters harvested in Europe (Turner et al., 2014), indicating the capability to migrate in a broad range of animal species – including some intended for a wide human consumption. Therefore, in a close future, TTX may enter the human food chain in geographical areas where the risk has been considered negligible so far, threatening a wider range of consumers than before. Nevertheless, TTX and the structurally related molecules (referred to as congeners) are not included yet in the list of marine toxins to be tested neither at EU level nor at international level (EFSA, 2017). However, the ongoing

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changing scenario implies the need to collect additional data for timely setting appropriate regulations and recommendations to protect the consumer's health (EFSA, 2017).

From the chemical point of view, TTX is a heat-stable, water soluble and low-molecular weight molecule formed by a guanidinium moiety linked to 2,4-dioxaadamantane skeleton bearing five hydroxyl groups (Fig. 1) (Kudo et al., 2016). The chemical features are determinant for the mechanism of toxic action that consists in binding the outer vestibule of the voltage-gated sodium (Na_V) channels α-subunit (Moczydlowski, 2013). The mammalian Na_V channels are composed by the heterotetrameric α-subunit and one or more smaller accessory β-subunits. In particular, the α-subunit is formed by four homologous domain containing six α-helical transmembrane segments forming the ions funnel (Lee and Ruben, 2008). The positively charged guanidinium moiety of TTX strongly binds *via* ionic interactions the negatively charged residues of the selectivity filter of Na_V channels impairing the ions flowing. Such a molecular event completely and reversibly blocks the inward Na^+ current reducing the membrane excitability of cells of heart myocytes, skeletal muscle, and peripheral and central nervous systems (Lago et al., 2015). In humans, the intoxication may cause a wide array of symptoms ranging from generalized malaise to the more severe muscle paralysis and cranial nerve dysfunction. Death can also occur in the most critical intoxications due to respiratory failure and cardiovascular collapse (William and Shepherd, 2001).

Recently, the safety of seafood has been questioned due to the contamination by marine toxins (e.g. (Botana et al., 2016)). In fact, while regulations usually require the monitoring of only one (or few) reference compound(s) belonging to a group of structural congeners, many analogues can be found together. Typically, such molecules neither are regulated nor commonly assessed for toxicity. This is the case of TTX as dozens of analogues have been identified, but only a minority has been investigated in toxicological assays. This scenario definitely prevents the in-depth understanding of the group toxicity raising reasonable concerns on the safety of consumers being potentially exposed to mixtures of many TTX congeners. Therefore, the toxicological assessment of a broader set of TTX analogues has been claimed for reaching a deeper understanding of the overall TTX-related hazard for health, also in the light of fostering the setting of proper group guidance values (Bane et al., 2014; EFSA, 2017).

Furthermore, the detoxification of TTX by human metabolism is not well understood due to the shortage of toxicokinetic and toxicodynamic data. In particular, from toxicodynamic point of view, the effects of metabolic transformation of TTX in interacting with NaV channels are still unknown. Therefore, those metabolites mediating the toxic stimulus in living organisms have not been identified yet, making the understanding of the mechanisms of TTX toxic action incomplete.

Studies dealing with the toxicology of the TTX analogues are very limited in number mainly because such molecules are not commercially available and the chemical features make challenging the synthesis (Bane et al., 2014). As a consequence, a wide-scale toxicity screening of TTX congeners, including the human metabolites, is not feasible in practice. In this respect, the *in silico* analysis already proved to be an effective analytical tool to extend investigation on molecules that are commercially unavailable (e.g. (Dellafiora et al., 2017; Ehrlich et al., 2015)). Indeed, computational analysis may effectively support the toxicological assessment at the early step of the hazard identification process (e.g. (Rybacka et al., 2015)) and they may provide the evidence-based rationale to prioritize molecules for experimental trials (Raies and Bajic, 2016). Specifically, it has been already shown that the molecular modeling can be used to study the TTX-Na_V channels interaction (Durán-Riveroll et al., 2016; Fozzard and Lipkind, 2010; Lipkind and Fozzard, 1994; Tikhonov and Zhorov, 2012). Therefore, it may succeed also in extending analysis on the toxic potential of other TTX congeners.

The present study investigated the structural basis of the TTX-Na_V channels interaction by using a reliable 3D molecular model relying on docking simulations and pharmacophoric analysis. In the framework of the hazard identification process, the model analyzed a wide set of TTX analogues never tested before assessing qualitatively their potential toxicity in comparison to the reference compound TTX. More specifically, the model has been trained and validated using data from the literature assessing the reliability in sensing the effects on the channel binding caused by chemical modifications of TTX. Then, the model has been challenged with 10 TTX analogues never tested before and a set of human metabolites (13 in total including phase I and phase II metabolites) that have been hypothesized according to the possible sites of metabolism on the TTX scaffold.

2. Materials and methods

2.1. Homology modeling of the Na_V channel toxin receptor site

The primary sequences of the 9 isoforms of human Na_V channels have been retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). In order to find out homologous proteins with known structures to be used as template for the homology modeling, the Na_V channel sequences have been used as entries to query the Basic Local Alignment Search Tool (BLAST) algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The "protein BLAST" program has been used and the PDB (Protein Data Bank) database (http://www.rcsb.org/pdb/home/home.do) has been queried (last database access in 6th of July, 2017). All the results have been ranked according to the E-value as the lower, or the closer it is to zero, the more significant the match and the higher the confidence in asserting the homology relationship are. The first-ranked protein found was the voltage-gated sodium channel from *Periplaneta americana* (PDB code 5X0M; from here on referred to as Na_V Pa) (Shen et al., 2017). Therefore, it has been selected as the template for the homology modeling, which has been carried out using the modeller software (version 9.1) (Sali and Blundell, 1993), as previously reported (Dellafiora et al., 2015a), interfaced in the UCSF Chimera software (version 1.11) (Pettersen et al., 2004). Notably, previous eukaryotic Na_V models have been successfully derived from bacterial structures (e.g. (Yang et al., 2012)) and structures of portions of human channels are available as well (e.g. (Wang et al., 2012)). However, Na_V Pa channel may provide a good model for human Na_V channels too because of the high structure coverage and identity (*vide infra*). Additionally, the regions involved in the toxin binding are both heterotetrameric (unlike the bacterial channels that are homotetrameric) showing the same overall structure, with a high conservation in terms of structure topology and organization at the level of the α-subunit (where is locate the TTX site of the interaction) (Zakon, 2012). In addition to that, human channels and that from *Periplaneta americana* have greater identity and similarity values in the region forming the TTX binding site than those found comparing human sequences with the bacterial structures (additional details can be found in Supporting material, Fig. 1S).

The isoform 4 ($Na_V1.4$) has been chosen amongst the 9 human isoforms to derive the 3D Na_V channel model as it showed the highest identity and sequence coverage percentage with the template structure (i.e. 42% and 87%, respectively), showing 58% of similarity in the covered sequence (calculated with EMBOSS Matcher algorithm). Moreover, it is known to have a high affinity for the TTX molecule (Guo et al., 1987; Lee and Ruben, 2008). The