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**HEALTH SCIENCES**

# *In vitro and in silico* assessment of cytotoxicity and chromosome instability induced by saxitoxin in human derived neural cell line

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**Abstract:** In freshwater, saxitoxins (STX) are produced by different cyanobacteria genera, including *Raphidiopsis*. Data regarding cytogenotoxicity effects of STX on human cells are scarse, this merit further studies of its toxicology. This study assessed the cytotoxicity and the chromosome instability of STX on SHSY-5Y human cell line. The CBMN assay allows the detection of chromosome breaks and abnormal chromosomal segregation. Additionally, *in silico* systems biology approach, used to search for known and predicted interaction networks, was applied to study the interactions between STX and SHSY-5Y cellular components. The results of the CBMN assay demonstrated that STX concentrations of 2.5 – 10 µg/L induced cytostasis and chromosome instability in a dose-response relationship. Apoptosis was detected after exposure of SHSY-5Y cultured cells to STX concentration of 10 µg/L. The results of the systems biology analysis revealed the interaction of STX with proteins related with acetylcoline pathway, cell cycle regulation and apoptosis. Furthermore, combining the *in vitro* and *in silico* approachs, it was possible to suggest a mechanism of action of STX in SHSY-5Y cells. Overall, the data demonstrated the cytotoxicity and mutagenicity of environmentally relevant concentrations of STX. These results should be considered when setting up guidelines for monitoring STX in water supply.

**Key words:** STX, micronucleus, cytostasis, apoptosis, systems biology.

## INTRODUCTION

The contamination of water resources from human activities, such as domestic and industrial sewage discharges, has accelerated the process of artificial eutrophication in supply waters. Eutrophication, mainly due to excessive input of phosphorus and nitrogen, causes changes in water quality. In this sense, the reduction of dissolved oxygen, decrease in biodiversity, aesthetic deterioration of the environment, fish death and increased occurrence of cyanobacteria and microalgae blooms stand out, with negative consequences on the efficiency and cost of water treatment, when it comes to public supply source (Su et al. 2016, Glibert 2017, Ho et al. 2019).

Cyanotoxins are metabolites synthesized by cyanobacteria that can affect aquatic biota, resulting in toxic effects also in terrestrial mammals (Sivonen & Jones 1999, Buratti et al. 2017). Human exposure to cyanotoxins, in general, can occur through primary contact with water containing the toxins, by inhalation, oral drinking and intravenous (in the treatment of hemodialysis) and by marine and freshwater food such as fish, crustacea and mollusks, that have been in contact with cyanobacteria, accumulating toxins in their tissues (Drobac et al. 2013, Calado et al. 2020, Nielsen & Jiang 2020).

*Raphidiopsis raciborskii* produces extremely aggressive toxins that can cause both human health problems and damage to the environment. Its high competitiveness in eutrophic environments, combined with its ability to form toxic blooms in nitrogen and phosphorus limited waters (Miotto et al. 2017) make this species one of the most studied cyanobacteria from both an ecological and public health point of view. There are two well-known toxins, cylindrospermopsin, an alkaloid with action on the liver and kidneys, and saxitoxin (STX), also known as PSP (*Paralytic Shellfish Poisons*), which acts on the neuromuscular system (Pratheepa & Vasconcelos 2017, Yang et al. 2021). Regarding STX, most toxicological data was obtained with STX produced by marine organisms as dinoflagelates (Cusick & Sayler 2013) and little information is available for those produced by freshwater cyanobacteria.

The use of a nervous tissue cell line (SH-SY5Y), with the potential to differentiate into cells with a phenotype similar to mature neurons, may be a suitable model to assess the cytotoxic and genotoxic effects of STX (Xicoy et al. 2017). Additionally, the *in vitro* micronucleus test, which was used in this study, is regulated by OECD guideline 487, and the advancement of mammalian cell culture technologies makes it possible to more accurately assess the consequences of human exposure to these toxins, especially, at the DNA level. In addition, systems biology, through interaction networks, can help in understanding the interactions between environmental compounds, such as cyanotoxins, and cellular components, prospecting molecular pathways. The characteristic of network modeling is the analysis of cells in terms of their underlying network structure at many different levels of detail (Xia 2010).

The purpose of this study was to apply the cytokinesis-block micronucleus cytome (CBMN-Cyt) assay alongside with *in silico* tools to assess the cytotoxic and genotoxic profile of STX synthetized from a *Raphidiopsis raciborskii* culture. Combining *in vitro* and *in silico* methodologies could shed more light on the mechanisms of action underlying the cytogenotoxicity of STX in environmentally relevant concentrations.

## MATERIALS AND METHODS

### Source of Saxitoxin (STX)

The strain used in the experiment was *Raphidopsis raciborskii* (T-3), formelly belonging to the genus *Cylindrospermopsis*. Saxitoxins extracts were obtained from 150 mL cultures of *Raphidiopsis raciborskii* cells from the Culture Collection of the Laboratory of Cyanobacteria and Phycotoxins (LCF). Filaments were grown in ASM-1 medium (Gorham et al. 1964) at 25 ± 1°C under 12hs:12hs; dark/white cycles provided by fluorescent light (intensity: 127 µE $m^{-2}$ $s^{-1}$). STX concentration in filaments were detected by HPLC– FLD and analyzed and compared with the commercial standards of STX variants (NRC, Canada). For extraction of toxins, filaments of *R. raciborskii* were centrifuged at 10,000 x g at 4 °C during 10 min and the debris resuspend in a 10 mL of 0.05M HCL. This suspension was then sonicated (three cycles of 1 min each) in order to break the cellular walls and release the toxins. This acid extract was then filtered through a cellulose acetate membrane and the remaining solution containing saxitoxins was quantified by HPLC. The method used followed the protocol described by Rourke et al. (2008). In this investigation we assessed environmentally relevant concentrations of STX, based on the Brazilian Ministry of Health (Regulation number 2914/2011).

## Cell line and culture conditions

Neural SH-SY5Y human derived cell line was kindly provided by Dr. Juliana da Silva from laboratory o Genetic Toxicology of Ulbra-Brazil. SH-SY5Y cells were maintained as a monolayer in 75 $cm^2$ flasks in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®, Waltham, MA), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco®, Waltham, MA) and antibiotics (1% of penicillin/streptomycin solution and 0.1% of gentamycin solution) (Gibco®, Waltham, MA), and kept in a humidified 5% $CO_2$ atmosphere at a temperature of 37 ºC until confluence was reached. SH-SY5Y cells were seeded at a density of $1 \times 10^4$ into 24-well plates and cultured for 24 h.

## CBMN-Cytome (Cyt) assay

The CBMN-Cyt assay was carried out as recommended by Fenech (2007) with modifications. After incubation, cells were treated with different concentrations (1.25 – 10 µg/L) of *Raphidiopsis* extracts containing saxitoxin variants as well as negative control (0.05M HCL) and positive control (bleomycin 3 µg/mL) for 24 h of treatment. After treatment, SH-SY5Y cells were washed twice in Dulbecco's phosphate saline buffered (DPBS, Sigma-Aldrich, St. Louis, MO) and cytochalasin B (Cyt B; CAS 14930–96–2; Sigma-Aldrich, St. Louis, MO), was added to a final concentration of 3 µg/ml in complete fresh medium.

After two cell cycles, Cyt B was removed and the cells were collected and harvested by cytocentrifugation (Cientec, Belo Horizonte, MG, Brazil); 160 µL of cell suspension was transferred to cytocentrifuge cups and centrifuged for 5 min at 700 rpm to produce 1 spot per slide. Slides with the cells were stained with Instant Prov (Newprov, Pinhais, PR, Brazil). After staining, slides were air-dried and examined under 400x magnifications using a light microscope. Two independent experiments, in duplicate, were performed.

The cytotoxicity of STX was assessed by means of the cytokinesis-block proliferation index (CBPI) (cytostatic effect) as well as the analysis of apoptotic and necrotic cells (cell death). CBPI was estimated by scoring 500 cells with one to four nuclei and was calculated using the formula [M1+2(M2)+3(M3)+4(M4)]/500, where M1 - M4 represent the number of cells with one to four nuclei, respectively. The cytostasis per- centage was calculated using the formula $100 - 100\ \{(CBPI_T - 1)/(CBPI_C - 1)\}$, where $CBPI_T$ represents the mean CBPI of each concentration used in the treatment of culture and $CBPI_C$ represents the mean CBPI of the control culture (OECD 2016). Apoptotic and necrotic cells were estimated by scoring 500 cells. The parameters of chromosomal instability — micronuclei (MNi), nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs) — were counted in 1000 binucleated cells (BNC) per experimental point and were scored according to Fenech (2007).

## Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) software, version 18.0 (SPSS Inc., Chicago, IL). Normality was evaluated with the Kolmogorov–Smirnov test and homogeneity of variance was evaluated with the Levene's test. Since NBUDs values were not normally distributed, even after data transformation, the non-parametric Mann–Whitney U test with $p<0.05$ was used to quantitatively determine the difference between negative control and treated groups. For the DNA damage, CBPI, MNi and NPBs values, analysis of variance (one-way ANOVA with Dunnett's multiple comparison test) was used to compare treated groups with the negative control. The results were expressed as means and standard deviations (±SD) or percentages.

### System biology analysis

The constructing of the compound protein network for systems biology analysis uses an opensource database, based on experimentation and research findings. However, the information obtained in this way, data without processing, reflect non-specific findings, needing refinement. To develop a relation between the most relevant proteins for the SH-SY5Y cell line, we selected 777 overexpressed proteins, obtained from the Oxford database (Rouillard et al. 2016). We selected these proteins due to the understanding that overexpression can be related to the high degree of interaction between one node and another and correlate with an *in silico* model. The networks corresponding to the proteins were obtained by the online chemical interaction search software STITCH 5.0 (Szklarczyk et al. 2016) and the protein interaction search tool STRING 10.5 (Szklarczyk et al. 2019), both following the parameters of Minimum required interaction score 0,400; Max number of interactors to show on the first shell 20; Max number of interactors to show on the Second shell 5 and Active interaction sources of experiments, databases, co-expression, neighborhood. We modeled the interaction networks in order to incorporate the desired proteins and compounds by the fusion tool present in Cytoscape 3.8 (Shannon et al. 2013), to allow the analysis of complex regions by the application "complex molecular detection" (MCODE 1.5), which evaluates the network as a function of regions with a high degree of connectivity to each other, forming complexes of functional nodes called clusters and that may be associated with biological process (Deng et al. 2019, Bader & Hogue 2003). With all the information obtained and properly exported to Cytoscape, the individual networks were merged, overlaying the common interactions, creating a Compound protein interaction network. The degree of functional enrichment for a given cluster and category was quantitatively computed (P value) by hypergeometric distribution, and multiple test correction was also assessed by applying the false discovery rate (FDR) algorithm (Benjamini & Hochberg 1995) which was fully implemented using the BiNGO software with a significance level of $P < 0.05$.

Centralities implemented were degree and betweeness to undirected networks, nodes with a relatively higher degree were termed hubs, and nodes with higher betweenness were named bottlenecks. Hub nodes interact with many other nodes in the network, and thus often occupy central positions in the network. By contrast, a bottleneck node does not necessarily have many interactions, but it has a high degree of betweenness centrality, meaning that it will often be a linker between different clusters (Barabási & Oltvai 2004, Yu et al. 2007). Therefore, a node hub-bottleneck N-HB can be considered a key regulator of biological processes and essential for the successful transfer of information through the network, where N-HB perturbations are more likely to cause network fragmentation.

## RESULTS

### *In vitro* cytotoxicity and chromosome instability

Considering cell death, STX (10 µg/L) significantly increased the frequency of apoptotic cells in relation to the negative control (Figure 1). None of STX treatments were able to significantly enhance the frequencies of necrotic cells. Additionally, the mitotic status was evaluated with the cytokinesis-block proliferation index (CBPI). CBPI was significantly reduced after exposure of SH-SY5Y cells to STX concentrations of 2.5, 5 and 10 µg/L, whereas the percentage of cytostasis was about 13, 15 and 26% at STX

concentrations of 2.5, 5 and 10 µg/L, respectively (Figure 2).

We next investigated the effects of STX on markers of chromosomal instability (Figure 3). STX significantly increased the frequencies of MNi and NBUDs at the concentrations of 2.5, 5 and 10 µg/L. None of the tested concentrations were able to significantly increase the frequencies of NPBs in comparison with the negative control group.

### *In silico* analysis of STX integration to SH-SY5Y protein model

The compilation of 777 individual protein networks, fused into a single network, produced the protein-protein interactions (PPI) network, with 814 nodes and 4114 interactions. The PPI is a complex network, divided into two regions that do not interact with each other, because they have different final biological functions.

The "a" macroregion of PPI is divided into broad biological functions, such as cell cycle maintenance, chromosomal regulation, cell division, cytosolic organization, cytoskeleton structuring and processes related to DNA transcription and translation. The "b" macroregion is more specific for RNA-related processes such as RNA transcription by polymerase II, RNA metabolic processes (general), gene expression, regulation of nucleoside organization. After introducing the STX isolated network into the PPI, the nodes are rearranged, increasing its complexity and changing its global topology into a compound - protein interaction network (Figure S1 - Supplementary Material).

Modularity analyses afforded to visualize ten clusters above the cutoff score (data not shown). Next, gene ontology analysis was carried out for all clusters, and identified the biological processes associated to these proteins' modules (Table I). Cluster 1 had the highest score, presenting key biological processes such as: RNA processing, spliceosome assembly and ribonucleoprotein complex biogenesis.

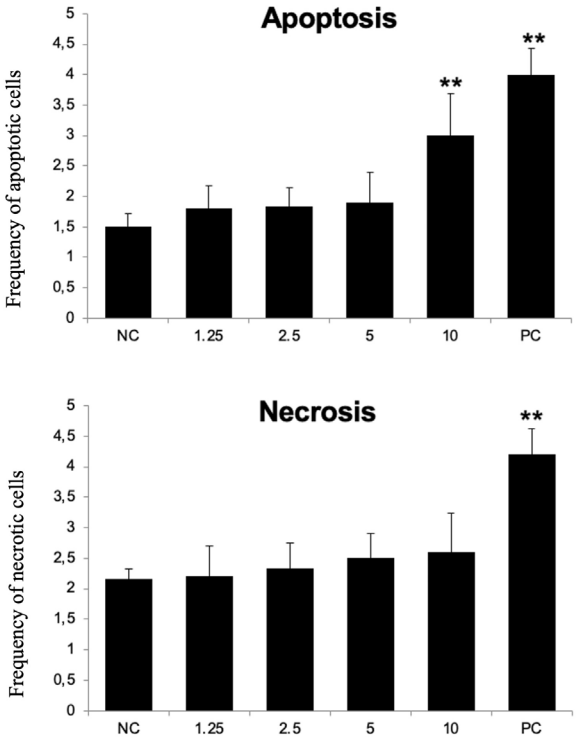


**Figure 1. Effects of STX on the induction of apoptotic and necrotic cells. Cultured SHSY-5Y cells were exposed to different concentrations (1.25 – 10 µg/L) of STX for 24 h. **Significantly different from the negative control (NC) ($p < 0.01$). PC: positive control (Bleomycin 3 µg/mL).**

The presence of STX in compound-protein interaction network, in module 13, presents ontologies of neural membrane organization, synapse regulation and processes related to neuronal organization. The proximity relationship between modules 12 and 13 demonstrates the indirect interaction of STX in compound-protein interaction network. In module 12, the main ontologies are wound recovery, regeneration, nervous system development and neurogenesis regulation. Modules 12 and 13 share some proteins in common, thus, one impacts the biological action resulting from another. Proteins associated with ontological processes shown in

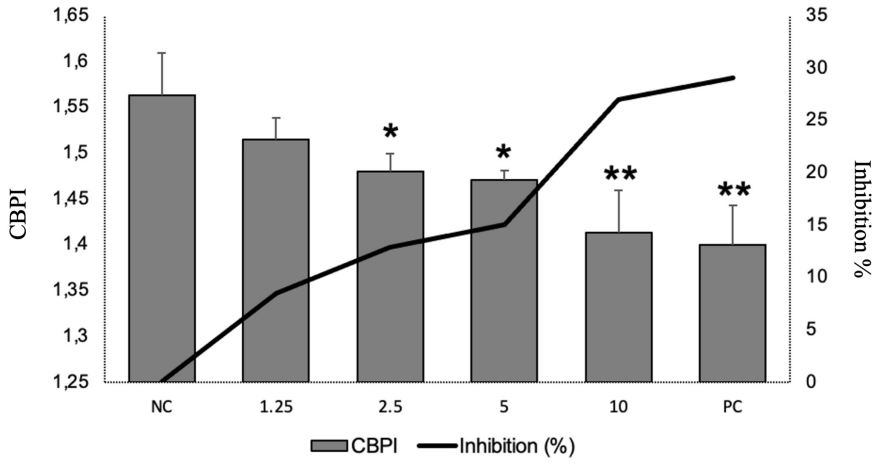


**Figure 2. Effects of STX on the cytokinesis-block proliferation index (CBPI) and cytostasis (% of inhibition). Cultured SHSY-5Y cells were exposed to different concentrations (1.25 – 10 µg/L) of STX for 24 h. *Significantly different from the negative control ($p < 0.05$). **Significantly different from the negative control ($p < 0.01$) **Significantly different from the negative control ($p < 0.001$) (one-way ANOVA; Dunnett's multiple comparison test). NC: Negative control. PC: positive control (Bleomycin 3 µg/mL).**

Table I were evaluated for their relevance to the performance of the network as a whole. Thus, it is possible to identify how these processes are mediated with the incorporation of STX in the PPI and the active mechanism that triggers from this topological change.

The main proteins – CHAT, ACHE, BCL2, BAX, BAD, CDK5, CDK8, P53, P21, ERCC2 – were identified with the profile of compound-protein interaction network N-HB (Node Hub Bottleneck) and were selected based on the relationship established with their biological processes (Table I). This process refines the analysis in order to obtain only the most relevant results for the construction and suggestion of a possible mechanism of action.

## DISCUSSION

In this study we used the *in vitro* CBMN-Cyt test in SH-SY5Y human derived neural cells to assess the cytogenotoxicity of relevant environmental concentrations of STX, obtained from a *Raphidiopsis raciborskii* culture. We next applied computational biology tools to study the possible mechanisms underlying cytotoxicity and chromosome instability induced by STX. The undifferentiated SH-SY5Y cells are considered the closest to a phenotype of immature catecholaminergic neurons (Xicoy et al. 2017), being a suitable model for the study of the cytotoxicity and genotoxicity of neurotoxins.

The results regarding CBMN-Cyt assay demonstrated the cytostatic effect and apoptosis of cells exposed to the STX concentrations of 2.5 - 10 µg/L and 10 µg/L, respectively. Chromosome instability was revealed by the significant enhancements of MNi and NBUDs frequencies in cells treated with STX concentrations of 2.5, 5 and 10 µg/L. MNi are originated from chromosome breaks (clastogenic effect) and/or chromosome loss (aneugenic effect), whereas NBUDs are the result of amplified DNA or chromatin which were eliminated throughout the S-phase of mitosis (Fenech 2006). NPBs are biomarkers of chromosome rearrangements that arises from DNA mis-repair and/or telomere end-fusions events (Fenech 2000). Thus, the results of this study point to the cytotoxicity and mutagenicity of STX in neural cells.

Melegari et al. (2015) exposed Neuro 2A (N2A), a neuroblastoma mouse cell line, and Vero cell line, derived from Vero green monkey kidney cells to several concentrations of STX to

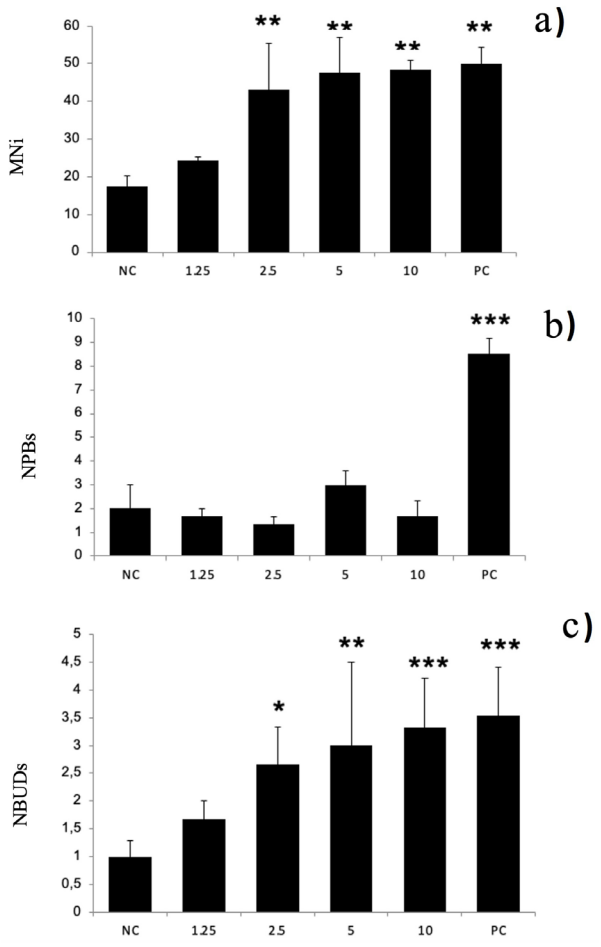


**Figure 3. Effects of STX on the frequencies of a) MNi: micronuclei; b) NPBs: nucleoplasmic bridges and c) NBUDs: nuclear buds. Cultured SHSY-5Y cells were exposed to different concentrations (1.25 – 10 µg/L) of STX for 24 h. NC: Negative control. PC: positive control (Bleomycin 3 µg/mL). Significantly different from the negative control group *p<0.05; ** p<0.01; *** p<0.001.**

determine cell viability, induction of apoptosis and formation of micronuclei (MN) following 24 h of incubation. With increasing STX concentration there was evidence of DNA fragmentation indicating apoptosis induction in Vero cells with a 50% increase in DNA fragmentation compared to control at the highest STX concentration tested (3 nM). In the other hand, the results demonstrated no significant changes in the frequency of micronucleate binucleated cells in N2A and Vero cells exposed to STX, indicating the absence of genotoxicity under the test conditions. Cultured cells from the brain of *H. malabaricus* were treated with different concentrations of STX. The results revealed the induction of reactive oxygen species (ROS), quantified by the specific activity of glutathione peroxidase and lipoperoxidation level, cytotoxicity and genotoxic potential of STX in the comet assay (Da Silva et al. 2014).

It is well documented that the exposure to STX is associated with oxidative stress, as observed by the induction of ROS and/or the depletion of antioxidants in aquatic organisms (Estrada et al. 2007, Nogueira et al. 2004, Clemente et al. 2010, Choi et al. 2006). Ramos et al. (2014) evaluated STX produced by *Raphidiopsis raciborskii* in rats. The animals received, for 30 days, water with STX at concentrations of 3 and 9 µg/L. The results of this study showed that, at both concentrations, STX induced lipid peroxidation in the liver of animals, reduced levels of antioxidant enzymes, and induced oxidative stress, through increased levels of reactive oxygen species (ROS) in the cortex and hippocampus. Some tissues, such as the brain, are more vulnerable to oxidative stress, due to high oxygen consumption and, consequently, the generation of large amounts of ROS (Migliore & Coppedè 2009). Thus, the induction of ROS in SHSY-5Y cultured cells exposed to STX is possibly the cause of the increments in the frequencies of MNi and NBUDs as observed in this study.

In order to shed more light on the mechanisms underlying chromosome instability, cytostasis and apoptosis in SHSY-5Y cultured cells exposed to STX, we applied a systems biology approach. The main cellular response resulting from the administration of STX is the blockage of $Na^+$ channels (Valério et al. 2010). The *in silico* study identified proteins related to this process by the relationship of these channels with acetylcholine, ACH. We determined as relevant nodes, the modulating proteins of ACH, CHAT (choline acetyltransferase)

**Table I. Specific gene ontology classes derived from protein-protein interactions within compound-protein interaction (CPI).**

| Cluster | GO-ID | Corrected p Value | Description |
|---|---|---|---|
| 1 | 375 | 3,71E-19 | RNA splicing, esterification reactions |
| | 377 | 2,67E-16 | RNA splicing, transesterification reactions with bulged adenosine as nucleophile |
| | 398 | 2,67E-16 | nuclear mRNA splicing, via spliceosome |
| | 8380 | 2,58E-14 | RNA splicing |
| | 6397 | 2,11E-12 | mRNA processing |
| | 6396 | 3,08E-10 | RNA processing |
| | 22613 | 4,25E-07 | ribonucleoprotein complex biogenesis |
| | 245 | 2,96E-06 | spliceosome assembly |
| 2 | 7269 | 1,54E+01 | neurotransmitter secretion |
| | 1505 | 2,96E+01 | regulation of neurotransmitter levels |
| | 23061 | 2,96E+01 | signal release |
| | 7268 | 4,54E+01 | synaptic transmission |
| | 50807 | 4,54E+01 | regulation of synapse organization |
| 4 | 6120 | 1,43E-24 | mitochondrial electron transport, NADH to ubiquinone |
| | 42773 | 2,20E-23 | ATP synthesis coupled electron transport |
| | 42775 | 2,20E-23 | mitochondrial ATP synthesis coupled electron transport |
| | 55114 | 3,24E-13 | oxidation reduction |
| | 16310 | 8,63E-10 | phosphorylation |
| 5 | 7399 | 7,11E+01 | nervous system development |
| | 48699 | 1,60E+02 | generation of neurons |
| | 22008 | 1,60E+02 | neurogenesis |
| | 30182 | 3,75E+02 | neuron differentiation |
| 7 | 31124 | 8,49E-06 | mRNA 3'-end processing |
| | 6378 | 9,11E-05 | mRNA polyadenylation |
| | 6379 | 9,11E-05 | mRNA cleavage |
| | 6139 | 2,89E-03 | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 8 | 9755 | 3,38E-04 | hormone-mediated signaling pathway |
| | 6887 | 1,09E-03 | exocytosis |
| | 32870 | 2,50E-01 | cellular response to hormone stimulus |
| | 6397 | 7,96E-01 | mRNA processing |
| 9 | 398 | 7,89E-07 | nuclear mRNA splicing, via spliceosome |
| | 10467 | 1,73E-05 | gene expression |
| 10 | 8154 | 1,72E-01 | actin polymerization or depolymerization |
| | 7010 | 1,71E+02 | cytoskeleton organization |
| 11 | 23033 | 1,15E+02 | signaling pathway |

**Table I. Continuation.**

| Cluster | GO-ID | Corrected p Value | Description |
|---|---|---|---|
| 12 | 42246 | 1,27E+00 | tissue regeneration |
| | 42060 | 1,73E+00 | wound healing |
| | 31099 | 3,69E+00 | regeneration |
| | 7399 | 2,05E+01 | nervous system development |
| | 50767 | 2,05E+01 | regulation of neurogenesis |
| 13 | 50804 | 1,39E+00 | regulation of synaptic transmission |
| | 31644 | 1,85E+00 | regulation of neurological system process |
| | 42416 | 4,09E+00 | dopamine biosynthetic process |
| | 61024 | 1,28E+01 | membrane organization |

**Clusters 5 and 10 correspond to macro-region "b" and have not changed with the introduction of STX in the PPI network. Clusters 3 and 6 do not have ontological representation as the connections did not result in biological activity.**

and ACHE (acetylcholinesterase). CHAT is responsible for the synthesis of acetylcholine, catalyzing the process of incorporation of the acetyl group into the coenzyme acetyl-coA, synthesizing acetylcholine. CHAT expression is particularly high in cholinergic neurons and, in the case of the SH-SY5Y cell line, it is identified as an overexpressed protein (Matsuo et al. 2011, de Medeiros et al. 2019). ACHE is a primary cholinesterase, responsible for the breakdown of ACH and other choline esters, regulating ACH levels. The dysregulation in the expression of CHAT and ACHE in SH-SY5Y cell line results in higher levels of the neurotransmitter ACH (de Medeiros et al. 2019, Jiang et al. 2019). In our study, CHAT and ACHE were identified as HUBs, linked to the biological processes we selected as relevant for the action of STX. Thus, the overexpression of CHAT along with the blockade of $Na^+$ channels could prevent the release of ACH, leading to ACH intracellular accumulation and triggering another process mediated by ACHE, that results in apoptosis through mitochondrial and caspase pathways. On the other hand, the ACH accumulation increases the levels of ROS, which implies in the induction of chromosomal instability (Xi et al. 2015), as observed in the *in vitro* CBMN assay.

The damage on the genetic material triggers repair mechanisms, which we identified with the HUB node represented by the ERCC protein. ERCC2 is a protein that actively works on the nucleotide excision repair (NER) (Li et al. 2019). The accumulation of damage, above the repair capacity by NER, triggers another pathway of action by phosphorylation of AKT1 (another HUB node in our analysis). AKT1 modulates several pathways, including TP53 and P21, both HUB nodes, which act in the forwarding of the apoptotic mechanism and in the cell cycle progression, respectively (Ersahin et al. 2015). The effect of STX on the cell cycle might also occurs through the direct inhibition of CDK5 and CDK8, two HUB nodes observed in our *in silico* model. Inhibition of CDK5 prevents cell cycle progression (cytostasis), along with the activation of apoptotic pathway by TP53.

Based on the results obtained from the *in vitro* and *in silico* analysis, we suggested a mechanism of action that allows us to observe the possible effect of STX on SH-SY5Y cell line (Figure 4). The overexpression of CHAT in the SH-SY5Y cell line, and the blockade that STX exerts on

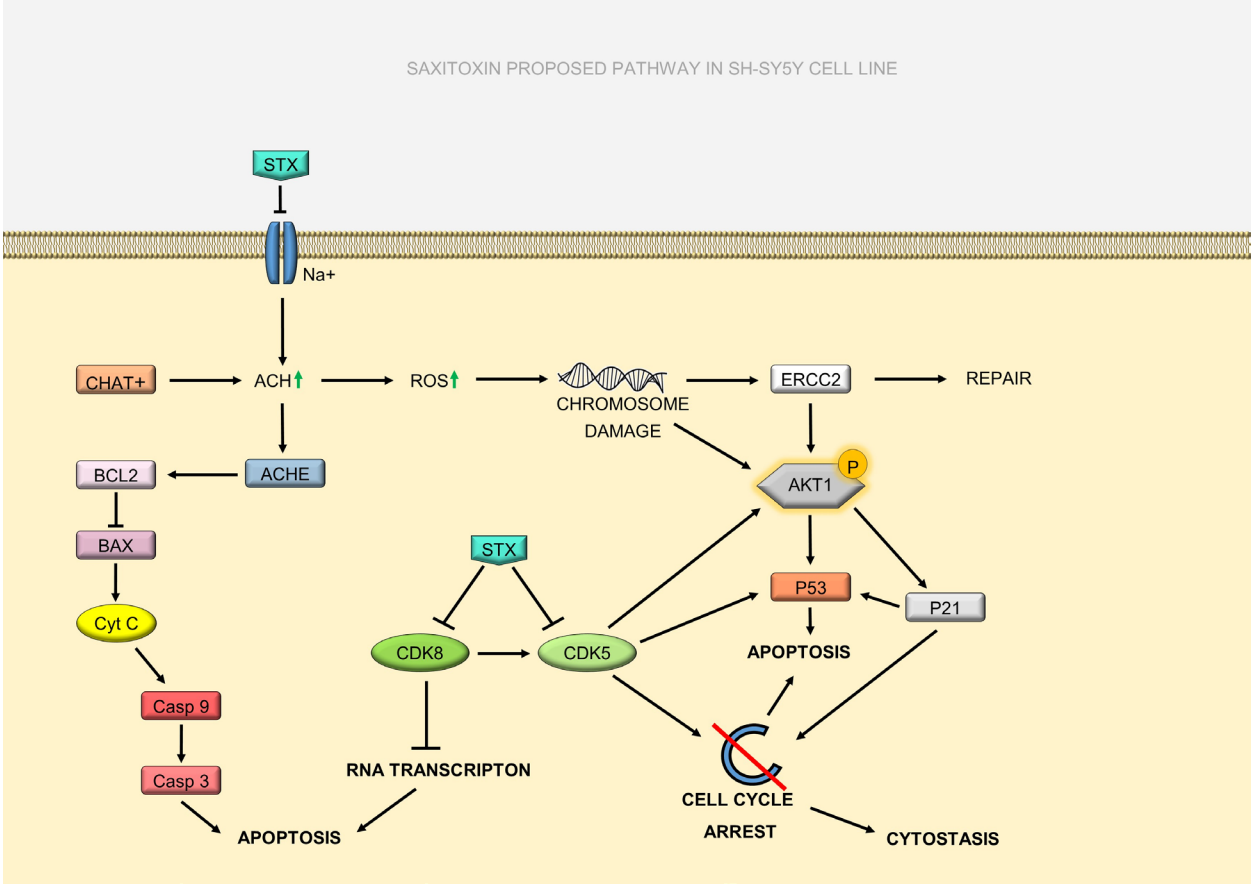


**Figure 4. Mechanism of action of STX in SH-SY5Y cell line. STX acts by directly blocking $Na^+$ channels as well as inhibiting cyclin-dependent kinases 5 and 8 (CDK5 and CDK8). The overexpression of CHAT, the protein responsible for the synthesis of ACH, and the blockade of $NA^+$ channels result in the accumulation of cytosolic ACH. Difficulty in degradation leads to activation of apoptosis via activation of the caspase cascade. The accumulation of ACH leads to the generation of ROS, which induce chromosomal mutations and genomic instability, triggering the apoptotic pathway by TP53 and/or cell cycle arrest (cytostatic effect) by P21.**

the functional capacity of $Na^+$ channels, results in ACH cytosolic accumulation, which will reduce ACHE degradation capacity, leading to apoptosis via BCL2 activation, inactivating BAX and triggering caspase pathway. The accumulation of ACH favors the increase of ROS, which, more directly, result in genotoxic damage. Thus, NER mediated ERCC2 results in cell cycle blockade by P21, activating AKT1. Activated AKT1 modulates several central processes for cell maintenance, including apoptosis by TP53.

The blockage of cell cycle progression could be explained along with the P21-mediated mechanism. STX works directly blocking the action of CDK5 and CDK8, both of which are closely associated with cell cycle progression and control. CDK5 inhibition, besides cell cycle arresting, can activate TP53 and trigger cell death. Similarly, CDK8 blockade arrests cell cycle as well as indirectly mediates the progression of caspase cascade.

It is worth mentioning that according to the Brazilian Ministry of Health (Regulation number 2914/2011) the analysis of saxitoxin in drinking water is mandatory, with the maximum acceptable limit of 3 µg/L (Fitzgerald et al. 1999,

Brazilian Ministry of Health 2011). Based on this study, STX induced cytostasis and chromosome instability in concentrations of 2.5 – 10 µg/L in a dose-response relationship and apoptosis in the concentration of 10 µg/L in a human-derived neural cell line. Our data along with other studies available in scientific literature should be taken into consideration when setting up guidelines for the maximum STX concentrations in water. Finally, the possibility of associating data obtained through genetic toxicology with *in silico* tools, opens a new perspective for understanding the mechanisms and responses at the level of DNA and proteins induced by cyanotoxins.

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## SUPPLEMENTARY MATERIAL

### Figure S1

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Juliany Constante performed the experiments and analyzed the data. Juliana Al Khateeb performed the experiments and analyzed the data. Ana Paula de Souza performed the experiments, analyzed the data and wrote the paper. Felipe Umpierre Conter performed the system biology analysis. Maurício Lehmann contributed materials and wrote the paper. João Sarkis Yunes performed saxitoxin extraction and revised the paper. Rafael Rodrigues Dihl designed the experiments, contributed reagents and materials, analyzed the data and wrote the paper.