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A saxitoxin-binding aptamer with higher affinity and inhibitory activity optimized by rational site-directed mutagenesis and truncation

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ABSTRACT

Saxitoxin (STX), a member of the family of paralytic shellfish poisoning toxins, poses toxicological and ecotoxicological risks. To develop an analytical recognition element for STX, a DNA aptamer (APT^{STX1}) was previously discovered via an iterative process known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) by Handy et al. Our study focused on generating an improved aptamer based on APT^{STX1} through rational site-directed mutation and truncation. In this study, we generated the aptamer, M-30f, with a 30-fold higher affinity for STX compared with APT^{STX1}. The Kd value for M-30f was 133 nM, which was calculated by Bio-Layer Interferometry. After optimization, we detected and compared the interaction of STX with aptamers (APT^{STX1} or M-30f) through several techniques (ELISA, cell bioassay, and mouse bioassay). Both aptamers' STX-binding ability was demonstrated in all three methods. Moreover, M-30f performs better than its parent sequence with higher suppressive activity against STX. As a molecular recognition element, M-30f has good prospects for practical application.

1. Introduction

Saxitoxin (STX), with the molecular formula $C_{10}H_{17}N_7O_4$ (Molecular Weight = 299), is one of the most potent natural neurotoxins known; it is highly polar due to the presence of two guanidinium groups (Fig. 1, Wiese et al., 2010). Along with dozens of analogues, STX makes up a family of neurotoxins, which are classified as paralytic shellfish toxins (PSTs), and may cause paralytic shellfish poisoning (PSP; Etheridge, 2010). As reported, STX can be synthesized by multiple species of cyanobacteria and dinoflagellates (Perreault et al., 2011). *In vivo* accumulation of STX by filter-feeding bivalves and fish, and the subsequent transfer through the food chain, can result in human illnesses or even death (Cusick and Sayler, 2013). STX is toxic mainly because of its effect on the nervous system, where it blocks voltage-gated sodium channel in excitable cells (Tian et al., 2014). Its LD_{50} in mice is 8–10 μg kg^{-1} *i.p.*, 3.4 μg kg^{-1} *i.v.* and 260 μg kg^{-1} by oral administration (Falconer, 2008). These data demonstrates the importance of monitoring programs for STX. In many countries, the regulatory limit for PSP toxins in shellfish has been established as 800 μg of STX equivalents/kg of shellfish meat or 4 mouse unit (MU) of PSP toxins/g of shellfish meat (Kawatsu et al., 2014).

The longstanding regulatory method for validating biological threats is the internationally accredited AOAC biological method (Mouse bioassay 959.08), with only the United Kingdom using the relatively newly accredited AOAC Official HPLC Method 2005.06 as an alternative (Campbell et al., 2009). However, a rapid, high throughput screening assay for effective monitoring is still needed, which could also reduce animal usage. The most important part of a screening assay is its recognition element, e.g., the antibodies and receptors that are usually adopted (Handy et al., 2013). However, many challenges remain, e.g., the limited availability of antibodies and the use of animals. Handy et al. (2013) reported the discovery of a DNA aptamer named APT^{STX1} that targets STX, which could serve as an alternative analytical recognition element in diagnostic assays for STX (Fig. 2; Handy et al., 2013).

Aptamers are short strands of DNA or RNA that can fold into unique three-dimensional structures and bind with high affinities and specificities to targets such as ions, proteins, low molecular weight metabolites, sugar moieties, lipids, and even whole cells (Kong and Byun, 2013). They are typically screened out from a

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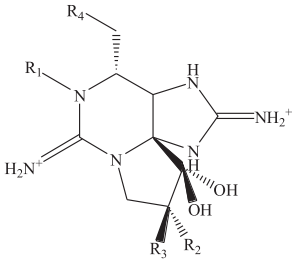

Fig. 1. Structure of STX.

random library comprising 10^{12}–10^{15} oligonucleotide sequences that are synthesized in vitro by a SELEX process (Systematic Evolution of Ligands by Exponential Enrichment) (Radom et al., 2013; Shigdar et al., 2013). Because of their numerous advantages compared to antibodies, e.g., stability and non-immunogenicity, aptamers are promising for detection and clinical applications (Wang et al., 2011). To date, several aptamers have entered clinical trials as antagonists, agonists or targeting molecules in drug delivery; the most successful one, pegaptanib, has been approved by the FDA for the treatment of vascular ocular disease (Radom et al., 2013). There has also been considerable progress towards applications of aptamers for laboratory techniques and medical diagnostics (Radom et al., 2013).

However, primary aptamers obtained by SELEX are not suitable for direct clinical or laboratory application. Optimization is needed to improve and regulate their functions (Wang et al., 2011). In recent years, a number of attempts have been made to optimize present aptamers through several common strategies, e.g., truncation, chemical modification, LNA replacement, mutation and 3' or 5' capping (Wang et al., 2011; Yang et al., 2011; Nonaka et al., 2013; Bullock et al., 2000; Pasternak et al., 2011). In this study, we adopted rational site-directed mutagenesis and truncation to optimize APT^{STX1}. First, we introduced mutations to improve its conformational stability and strengthen its interaction with STX. Although a number of aptamers have been identified by SELEX, it sometimes fails to identify aptamers that bind with their target with high affinity, because of amplification bias of PCR and reduced library diversity caused by experimental manipulation (Nonaka et al., 2013). Different groups have engineered mutated aptamers that bind to targets with improved affinity compared to the wild type (Nonaka et al., 2013; Bullock et al., 2000). Similarly, by introducing site-directed mutations on the basis of secondary structure prediction, we attempted to get an improved underlying aptamer with higher affinity. It is known that G-quadruplexes are nucleic acids structures, characterized by unique highly ordered architecture and high stability (Ruttkay-Nedecky et al., 2013). Some sequences remain folded under physiological conditions and at temperatures above 90 °C (Ruttkay-Nedecky et al., 2013). Therefore, we presume that if an aptamer can form a G-quadruplex after slight mutation, its affinity could be improved based on its strengthened structural stability. We designed experiments to verify our hypothesis. As expected, we obtained a mutated quadruplex-forming aptamer with higher affinity to STX compared to the parent aptamer. Subsequently, we adopted truncation to reduce the cost of the molecule and find the key binding structure. Generally, not all nucleotides of a certain aptamer are necessary for direct interaction with the target or for folding into the structure that facilitates target binding. Moreover, longer sequences result in lower yield and higher cost of synthesis (Shangguan et al., 2007). Thus, truncation is necessary during the optimization process. At the same time, it aids in exploring the relationship between the aptamer structure and function and in characterizing the target binding motif.

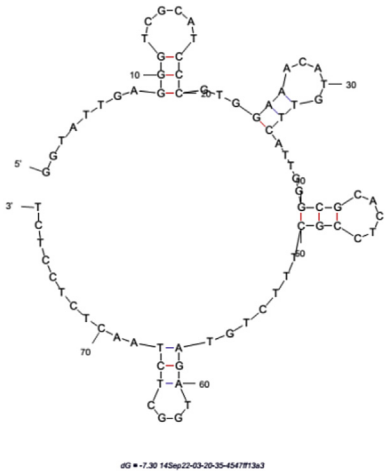

Fig. 2. Structure of APT^{STX1} generated by the mfold web server.

After optimization of aptamer APT^{STX1}, we investigated the interaction of the pre- and post-optimized aptamers with STX through different methods: ELISA, a cell bioassay and mouse bioassay. By these methods, STX was detected as a control following standard procedures. After the introduction of aptamers in these detection systems, differences could be observed compared to controls, which verified aptamers' ability of binding with STX. Besides, we compared the performances of different aptamers in these assays. Through these three methods, we obtained an overall understanding of their STX-binding ability and function.

2. Experimental

2.1. Materials and reagents

All synthetic oligonucleotides were synthesized by Sangon Biotech (Shanghai, China). STX diacetate was purchased from Taiwan Algal Science Inc (Taiwan). Ultrafiltration tubes used were Amicon Ultra-0.5 mL and Ultra-4 mL centrifugal desalting filters with a 3 KD molecular cutoff, obtained from EMD Millipore (Alberta, Canada). Ouabain(10 mM, aqueous) and Veratridine (1 mM, in 0.01 M HCl) were purchased from Sigma–Aldrich Co. LLC(mainland, China). Nuclease free water (IDT) was used in the preparation of all reagents used for experiments. PBS-T used was 10 mM phosphate buffer, 2.7 mM KCl, 140 mM NaCl, 0.05% Tween-20, pH 7.4 (Sigma, St. Louis, MO). All other chemical reagents used were analytical grade.

2.2. Evaluation of STX-aptamers binding using Bio-Layer Interferometry sensors

STX-aptamers binding was evaluated using Bio-Layer Interferometry sensors in an OctetRED 96 system (ForteBio, Shanghai). The principle and analysis procedures used herein were as detailed in Concepcion et al. (2009). Briefly, aptamer immobilization was performed using the streptavidin-biotin coupling method. SSA(enhanced streptavidin) sensors (ForteBio) were prepared by the procedure of equilibration (1 min), biotinylated aptamer coupling(1 μM, 5 min), dissociation(5 min), and equilibration (2 min); One reference channel was also prepared via equilibration. The running buffer used for immobilization and sample analysis was PBS-T at room temperature. Lyophilized aptamers were reconstituted in PBS-T to a concentration of 100 μM, then diluted in PBS-T to 10 μM and 1 μM, heated at 95 °C for 5 min, and cooled on ice for 10 min prior to use. All aptamers used for experiments herein were required to undergo the heating and cooling procedure