Review

Cure of experimental botulism and antibotulismic effect of toosendanin[1]

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ABSTRACT

Botulinum neurotoxins (BoNTs), a group of bacterial proteins that comprise a light chain disulfide linked a heavy chain, are the most lethal biotoxins known to mankind. By inhibiting neurotransmitter release, BoNTs cause severe neuroparalytic disease, botulism. A series of important findings in the past 10 years which displayed the molecular targets of BoNTs and hence proposed a four-step action mechanism to explain BoNT intoxication greatly advanced the study of antibotulismic drug. In this article, we reviewed these progresses and anti-botulismic compounds found in recent years. These compounds function due to their facilitation on neurotransmitter release or to their interference on the binding, internalization, translocation, and endopeptidase activity of the toxins. Toosendanin is a triterpenoid derivative extracted from a digestive tract-parasiticide in Chinese traditional medicine. Chinese scientists have found that the compound is a selective prejunctional blocker. In spite of sharing some similar action with BoNT, toosendanin can protect botulism animals that have been administrated with lethal doses of BoNT/A or BoNT/B for several hours from death and make them restore normal activity. The neuromuscular junction preparations isolated from the rats that have been injected with toosendanin tolerate BoNT/A challenge. Toosendanin seems to have no effect on endopeptidase activity of BoNT, but blocks the toxin approach to its enzymatic substrate.

INTRODUCTION

The botulinum neurotoxins (BoNTs) synthesized by strains of the anaerobic bacteria, *Clostridium botulinum*, are the most lethal biotoxins known to mankind. BoNTs comprise a family of seven immunologically distinct neurotoxic proteins (BoNT/A-/G). These toxins act on nerve terminals to block neurotransmitter release[1]. BoNT poisoning results in inhibition of synaptic transmission at the skeletal neuromuscular junction and subsequent respiratory failure[2]. Although the incidence of botulism from foodborne sources has decreased considerably due to improvement in standard of living and public health protection, sporadic outbreaks are still reported every year in the world. Moreover, owing of their extremal potency and ease of production, the BoNTs are considered to be formidable threat agents

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and botulism is also a significant public health problem.

Since 1890s, numerous attempts have been made to develop approved pharmacological treatments for BoNT intoxication, although these efforts have achieved a few successes. Currently, the only approved treatment for botulinum intoxication is infusion of equine antitoxin. However, the antitoxin is effective in the early stages of BoNT exposure, becoming refractory once symptoms appear. And no antibody can recognize all toxin serotypes and produce universal neutralization. Clearly, a safe and reliable agent, especially one that would be effective after BoNT poisoning, would be desirable. In China, the scientists found that toosendanin (TSN), a plant-derived triterpenoid derivative extracted from Chinese traditional medicine, is an effective antibotulismic compound[3]. In this article, after a simple description on the molecular mechanisms of BoNT blocking neurotransmitter release we will review the studies on cure of experimental botulism and that to demonstrate antibotulismic effect of TSN.

MOLECULAR MECHANISMS OF BoNT BLOCKING NEUROTRANSMITTER RELEASE

The bacteria produce BoNT as single-chain polypeptide of ~150 kDa. When proteolytically activated by digestive enzymes the polypeptide chain is cleaved to generate a ~100 kDa heavy chain and a ~50 kDa light chain, coupled by a single disulfide bond and non-covalent interactions. According to the currently accepted view, ie, the four-step action hypothesis[4-6], the heavy chain of the neurotoxin binds selectively to ectoreceptors on the nerve terminal (binding). Once binding to the ectoreceptors, BoNTs cross the plasm membrane by a process of receptor-mediated endocytosis, resulting in entrapment of toxin in an endosomal structure (internalization). Then through an ATP-dependent process the intra-endosomal pH lowers to ~4.5, hence leading to a conformational change in the toxin molecule and separation of two chains. Subsequently, the hydrophobic domains of the N-terminal region of the heavy chain insert into the endosomal membrane, forming a channel for the translation of light chain into the cytosol (translocation). The light chain as proteolysisase to cleave its substrate in cytosol, one of SNARE proteins, is the last step, the intracellular action of the toxin.

During the past 10 years, a series of important findings displayed the molecular action mechanism of BoNT. These findings have greatly advanced our understanding of the cellular biology of exocytosis. Now, the clostridial neurotoxins are used as tools to investigate the molecular events of neurotransmitter release. Sequence comparison has revealed that the light chain of all BoNT serotypes has a highly conserved 20-residue-long segment, located in the middle of the peptide, containing the His-Glu-Xaa-Xaa-His zinc-binding motif of zinc-endopeptidases[7]. Each of the seven serotypes of BoNT as a zinc-dependent protease cleaves one of the three SNARE proteins which are necessary for the vesicle fusion in transmitter release: synaptobrevin (vesicle-associated membrane protein, VAMP), SNAP-25 (synaptosomal associated protein of 25 kDa) and syntaxin[6]. It is now established that the VAMP is the target for BoNT/B[8], BoNT/D[9], BoNT/F[10], and BoNT/G[11]. The specific target for cleavage by BoNT/A or BoNT/E is SNAP-25[12,13]. The target protein for BoNT/C is syntaxin[14]. More precisely, the cleavage sites of the various toxins for the three SNARE proteins are confirmed. BoNT/A cleaves SNAP-25 at the Gln197-Arg198 peptide bound but not hydrolyzes the Gln15-Arg16 in the same protein. BoNT/B cleaves VAMP at the same peptide bound (Gln76-Phe77). BoNT/C cleaves syntaxin Ia at the Lys253-Ala254 peptide bound but dose not affect the Lys260-Ala261 bound of syntaxin, moreover, BoNT/C also cleaves SNAP-25 at the Arg198-Ala199 peptide bound and not at the Arg17-Ala18 bound. BoNT/D cleaves VAMP at the Lys59-

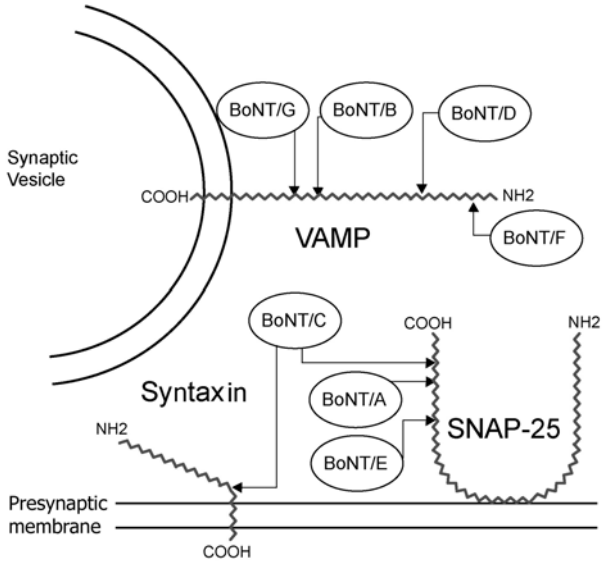

Fig 1. Molecular targets of botulinum neurotoxins. The three synaptic proteins, syntaxin, SNAP-25, and VAMP, cleaved by toxin light chains are shown. The cleavage sites are indicated by arrows.

Leu60 peptide bound, but not the site of Lys83-Leu84 within the same molecule. BoNT/E cleaves SNAP-25 at the Arg180-Ile181 peptide bound, but not the bound between Arg59 and Ile60. BoNT/F cleaves VAMP at the Gln58-Lys59 peptide bond. BoNT/G cleaves VAMP at the Ala81-Ala82 peptide bound[6].

STUDES ON K^+ CHANNEL BLOCKER AS CURE OF BOTULISM

K^+ channel blockers have been examined in the treatment of human botulism outbreaks during past 20 years. Among the various K^+ channel blockers, 3,4-diaminopyridine (3,4-DAP) is the least toxic of the available K^+ blockers and is highly effective in antagonizing muscle paralysis following BoNT/A exposure *in vitro* [15-17].

In rat diaphragm muscle, after the muscle was paralyzed by BoNT/A exposure, 3,4-DAP induced a rapid and pronounced increase of twitch tensions. The beneficial effect of 3,4-DAP was well maintained and underwent little or no decrement relative to control for at least 8 h after addition[17]. Combining of *in vivo* and *in vitro* recording techniques, Adler *et al*[18] studied the actions of 3,4-DAP in the rat extensor digitorum longus (EDL) muscle following local inhibition of neuromuscular transmission by BoNT. The results showed that 3,4-DAP iv potentiated twitch tensions markedly in BoNT/A intoxicated muscle. The sensitivity of the EDL muscle to 3,4-DAP did not diminish with time or with repeated application. Since a major barrier to the clinical use of 3,4-DAP is its brief duration, Adler *et al* attempted to deliver the drug via osmotic minipump infusion. It was found sustained delivery of the drug would be required for the entire period of BoNT intoxication to maintain muscle function [19].

In spite of the numerous advantages of 3,4-DAP, there are also a number of problems associated with its use in cure of botulism. First, the efficacy of 3,4-DAP is limited primarily to BoNT/A. Second, in human cases of botulism, the aminopyridines have been reported to produce marked increase in the strength of limb muscle, but only minimal increases in respiratory muscles and no return of spontaneous ventilation[17]. Third, clinical use of aminopyridines has occasionally elicited seizures[20]. The first limitation is inherent in the differences among the mechanisms of action of the toxins and can not be readily altered. The second limitation results presumably from the inability to attain an adequate plasma concentration of 3,4-DAP due to the severity of the toxic side effects of high doses of aminopyridines. The third limitation results from the slow but finite penetration of 3,4-DAP across the blood-brain barrier[21].

In order to overcome these difficulties, various attempts were executed. One strategy is to selectively enhance the postsynaptic effects of acetylcholine by use of an anticholinesterase agent. However, the results showed that the cholinesterase inhibitor neostigmine and pyridostigmine were without benefit to antagonizing the effect of BoNT/A when used on its own, and only of limited value when applied with 3,4-DAP in rat diaphragm muscle[18,19]. Furthermore, a more pronounced tetanic fade was observed when neostigmine was co-administered with 3,4-DAP[18]. Lowering the dose of 3,4-DAP would be expected to reduce the risk of seizures, but this procedure would also decrease the therapeutic effect of the compound. A potential solution is to use a combination of two K^+ channel blockers such as 3,4-DAP and tetraethylammonium (TEA). In rat diaphragm muscle, TEA produced an initial potentiation of twitch tensions that were previously depressed by incubation with BoNT/A. However, continuous exposure to TEA led to a progressive reduction of contractility. Similarly, addition of TEA to preparations where tensions were partially restored by addition of 3, 4-DAP led initially to a further potentiation of tension but ultimately the tensions were considerably lower than those recorded with 3,4-DAP in the absence of TEA. These results suggest that the postsynaptic inhibitory actions of TEA preclude its utility as an adjunct to 3,4-DAP therapy. Although TEA itself was unsuccessful, the concept of supplementing 3,4-DAP with a K^+ channel blocker that inhibits Ca^{2+}-activated K^+ channels and has fewer central nervous system side effects is valid [18].

The efficacy of 3,4-DAP in antagonizing the action of BoNT/A is generally attributed to its ability to enhance the influx of Ca^{2+} as a result of inhibiting voltage-dependent K^+ currents. Inhibition of K^+ channel results in a marked potentiation of transmitter release. So, 3,4-DAP would appear to be useful for increasing muscle tone and strength during cure of botulism. However, The limited contractile activity facilitated by 3,4-DAP did not lead to correction of the underlying impairments of transmitter release in BoNT/A-injected muscle. It appears that little improvement in the BoNT/A-impaired exocytotic apparatus occurred in spite of continues release of ACh during the presence of 3,4-DAP. Moreover, active toxin may be present in the

terminal during and even after 3,4-DAP treatment. If active toxin does indeed persist, blockade of this activity or elimination of the toxin in the nerve terminal may constitute the most critical problem for effective therapy.

CHEMICALS TO INTERFERE WITH BINDING OF THE TOXIN

In the past 2 years, Japanese scientists found that black tea extract, thearubigin fraction (TRB), protected against the effect of BoNTs. In mouse phrenic nerve-diaphragm preparations, BoNT/A mixed with TRB did not induce muscle paralysis. The protective effect of TRB extended to BoNT/B and BoNT/E. Further studies showed that the specific binding of [^{125}I]BoNT/A, /B, or /E to rat cerebrocortical synaptosomes was inhibited by mixing iodinated toxins with TRB. The elution profile of [^{125}I]BoNT/A, /B, or /E on Sephadex G-50 column chromatography was different from that of toxins mixed with TRB. The effects of TRB were dose-dependent. These results indicate that TRB protects against the action of BoNTs by binding with the toxins [22,23]. The structural elucidation of the compounds by means of NMR spectroscopy revealed that three flavonoids were found to have the major activity. These are keampfetrin (kaempfenol-3-O-[glc-(6-1)-rha- (3-1)-glc]), nicotiflorin (kaempferol-3-O-[glc-(6-1)-rha]), and quercetin glycoside[24].

Although it is unclear which molecule is responsible for BoNT binding in presynaptic membrane, the receptors possess at least one sialic acid residue since it was observed that gangliosides, which are sialic acid-containing glycosphingolipids, bound to toxins and caused loss of activity[25,26]. The fact that lectin antagonized the effect of BoNT also supports the hypothesis that sialic acid-containing molecules are receptors for BoNTs. Lectin from Limax flavus (LFL) and lectin from Triticum vulgaris (TVL), both of which have affinity for sialic acid, show significant inhibition of various serotypes of BoNT binding to brain membrane preparations. The lectins behaved as competitive antagonists[27]. Studies on isolated neuromuscular preparations showed that TVL did not affect neuromuscular transmission but produced a significant antagonism of the onset of BoNT/B-induced paralysis. The lectin was effective when incubated with tissues or with toxin, but it was ineffective when incubated with tissue that had already bound toxin. These experiments indicated that the lectin was acting at the level of the membrane receptor rather than some later step in the poisoning process to produce antagonism of the toxins[27]. Kalandakanond and Coffield also found that incubation of phrenic nerve hemidiaphragm preparations in TVL before the addition of BoNT/A antagonized action of the toxin as demonstrated by an increased latent period of muscle paralysis. The time to paralysis in TVL-treated tissues was approximately 5 fold longer than in toxin-treated tissues without TVL. Furthermore, the antagonism of toxin binding by TVL was correlated with significant antagonism of substrate cleavage in the same tissues. The decrease of SNAP-25 immunoreactivity in tissues treated with BoNT/A alone was significant greater than in tissues treated with TVL+BoNT/A[28].

ANTAGONISTS AT pH-DEPENDENT TRANSLOCATION STAGE OF THE TOXINS

After binding to receptors on the surface of nerve endings, the toxin is internalized by endocytosis. Then the toxin translocates from the endosome to the cytosol by a pH-dependent process. Some drugs act on this step to antagonize the effect of BoNTs. One of these compounds is amine. Simpson[29] reported that ammionium chloride and methylamine hydrochloride produce concentration- and time-dependent antagonism of the onset of neuromuscular blockade caused by BoNT/A, /B, and /C. The drugs exerted their effects only when they were added either before or within 10-20 min after toxin challenge. At concentrations that produce antagonism of onset of BoNT-induced paralysis, ammonium chloride and methtlamine hydrochloride did not inactivate toxin molecules, nor did they produce irreversible changes in tissue function. Furthermore, the drugs did not inhibit BoNT binding to receptor and did not reverse neuromuscular blockade, but acted solely to antagonize internalization of toxins.

Acidification of endosome depends on an endosomal H^+-ATPase that acts as a proton pump to accumulate H^+s from the cytoplasm into the lumen of an endosome. Application of H^+-permeant ionophore can deplete this pH gradient without acting on ATP hydrolysis[30,31]. Sheridan[32] found that two ionophores, nigericin and monensin, that increase membrane permeability to H^+ and K^+ or H^+, Na^+, and K^+, respectively, block endosomal acidification by acting as H^+ shunts to neutralize pH gradients. Nanomolar concentrations of nigericin or monensin delayed development of blockade in BoNT/A-or BoNT/B-treated muscles two to three

fold over onset times in unprotected muscles. However, higher concentration of ionophores directly blocked synapses. Thus, nigericin and monensin could delay onset of BoNT paralysis only over a narrow range of concentrations[32].

Previous investigation demonstrated that chloroquine, currently used clinically as antimalarial drug, was effective in delaying BoNT/A-induced block[33]. Further studies showed that among the aminoquinoline compounds tested, those having a 7-chloro-4-aminoquinoline configuration, similar to chloroquine (or the structurally similar 6-chloro-9-aminoquinoline group in quinacrine), were effective to prolong the time required for BoNT/A to block neuromuscular transmission[34,35]. The mechanism of action of these antimalarial agents is probably through the rising of endosomal pH. Some of these drugs could also act by inhibiting toxin-induced channel formation[34].

ZINC-DEPENDENT METALLOPROTEASE INHIBITOR

The light chains of BoNTs are zinc-dependent metalloproteases. Zn^{2+} is required for the intracellular activity of the toxins. Therefore, metallo-endopeptidase inhibitors and heavy metal chelators are considered to be effective to antagonize the inhibitory action of BoNTs.

Deshpande *et al*[36] examined the ability of three metalloprotease inhibitors to delay the onset or to prolong the time to 50 % block of nerve-elicited muscle twitch tension in the mouse phrenic-nerve diaphragm after BoNT/A or BoNT/B application. It was found that of three compounds tested only phosphoramidon, the clinically used angiotensin converting enzyme inhibitor, significantly delayed the onset of muscle paralysis by BoNT/B but showed no effect in prolonging block by BoNT/A. Two other metalloprotease inhibitors captopril and peptide hydroxamate failed to show any protection in either delay in onset or in prolongation of the time-course of paralysis by either serotype of BoNT. Although pretreatment or preincubation of BoNT with phosphoramidon demonstrated to be effective, the effect of phosphoramidon was limited. Several reasons could account for this limited effectiveness. It is likely that in spite of its high lipid solubility, adequate intracellular concentrations were not built up in order to offer more protection than seen in these experiments. Among the phosphorus-containing amino acids and peptides that have received extensive attention as inhibitors of metalloproteases, phosphoramidon may not be an ideal candidate for protection against various serotypes of BoNT.

IDC1578 (7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin), a phosphoramidon analog was assessed for its ability to inhibit the catalytic activity of BoNT/B light chain using a sensitive fluorescence assay. Pretreating the BoNT/B light chain with IDC 1578 produced a concentration-dependent inhibition of BoNT/B-mediated proteolysis with an $IC_{50}\approx27.6$ μmol/L. For comparison, captopril, a well-known zinc metalloprotease inhibitor, generated less than 10 % inhibition at a concentration of 5 μmol/L. ICD 1578 is the most potent antagonist of BoNT/B light chain thus far described[37].

N,N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN) is a heavy metal chelator. TPEN can markedly delay the time to block of elicited muscle tensions in isolated nerve-muscle preparations exposed to BoNT, and TPEN is effective on all serotypes of BoNT. The mechanism appears to be chelation of the catalytically important zinc at the active site of the BoNT light chain[38,39]. To determine the protective efficacy of TPEN against BoNTs *in vivo*, mice were injected ip with TPEN as a single bolus or as multiple injections 30 min before and 0, 2, 4, and 6 h following iv challenges with BoNT/A or B. TPEN treatment did not alter the lethality of BoNT/A or /B-injected mice but did produce a significant delay in the time to death, suggesting TPEN may be of potential benefit. The acute toxicity experiment showed that mice could tolerate low doses of TPEN (≤10 mg/kg), but higher doses of TPEN (≥30 mg/kg) produce acute toxicity leading to convulsions and death within 20 min. Since the therapeutic and toxic actions of TPEN are both related to zinc chelation, it is suggested that the use of TPEN would need to be restricted to low doses as part of a combination therapy[40]. When BoNT/A was pretreated with Ca-EDTA and TPEN, and the mixture of toxin and chelators was added to phrenic nerve hemidiaphragm preparations that had been pretreated with TPEN, the time to paralysis in chelator-treated tissues was approximately 2.5 fold longer than in toxin-treated tissues without chelator. Furthermore, the effect of chelator on paralysis time was correlated with a reduction in substrate cleavage. The decrease of SNAP-25 immunoreactivity in tissues treated with toxin plus chelator was significant less than in tissues treated with BoNT/A alone[28].

TOOSENDANIN, AN INGREDIENT FROM CHINESE TRADITIONAL MEDICINE

An effective antibotulismic agent both *in vitro* and *in vivo* TSN ($C_{30}H_{38}O_{11}$, FW=574), a triterpenoid derivative (Fig 2), is an active ingredient extracted from bark and fruit of the plant belonging to family *Melia*[41, 42], which had been used as digestive tract-parasiticide and agricultural insecticide in ancient China[3,43]. TSN has been demonstrated to be an effective antibotulismic compound both *in vivo* and *in vitro*, and a selective presynaptic blocker sharing some similar action with BoNT. The compound can prevent botulism animals (mouse, rat, and monkey) from death and make them restore normal activity. The neuromuscular junction preparations isolated from the rats several days after a single injection of TSN tolerate BoNT/A challenge.

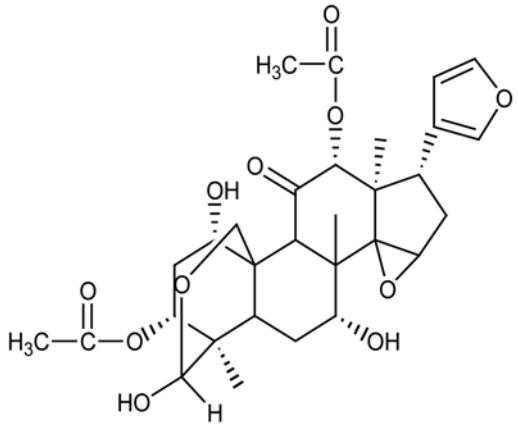

Fig 2. The chemical structure of toosendanin.

As shown in Tab 1, TSN-treatment (iv, sc, or po) saved the botulism mice from death[44]. The effect was dose-dependent. The optimum time for TSN therapy was 1-6 h after BoNT challenge. Increasing BoNT/A to 4.7 LD_{50}, the surviving rats still reached 25 % after TSN-treatment. In the experiments performed on botulism monkey, similar therapeutic effect of TSN was also observed[44,45]. Each of rhesus monkeys was given one MLD of BoNT/A subcutaneously, the TSN therapy (iv, 0.9-1.0 mg/kg) was initiated 24 h after BoNT/A challenge. In the TSN-treated group, 10 of 13 monkeys survived and restored to normal activity, while only 2 of 12 monkeys in the TSN-untreated control group. By the way, for the BoNT/B or /E-induced mouse botulism, TSN had similar therapeutic effect.

Experiments performed on rat phrenic nerve-diaphragm preparation showed that the preparations suffered a 30 min incubation in a TSN-containing (1.7×10^{-6} g/mL) solution, their contraction response to indirect stimulation had no any sign of decrease for 5 h. However, the TSN-incubated preparations had been entrusted an anti-BoNT/A ability, the paralysis onset of the preparations after applying same dosage of BoNT/A was significantly delayed[46]. Similar result was obtained from mouse diaphragm preparations. In that case, a 5-min preincubatim with TSN was enough to make the preparations a high tolerance to BoNT/A[47]. It was also found that the high tolerance to BoNT/A was observed

Tab 1. Therapeutic effect of toosendanin (TSN) on experimental mouse botulism (survived rate, %).(1)

	Dosage of TSN(2) (mouse LD_{50})				Administration time after BoNT/A (h)(3)					Dosage of BoNT/A(4) (mouse LD_{50})			
	0.1	0.2	0.3	0.4	1	6	12	18	24	1.7	2.7	3.7	4.7
i v	2.8	41.7	88.9	91.2	86.7	100.0	73.3	6.7	0.0	95.0	90.0	40.0	25.0
sc	6.7	30.0	86.9	93.3	96.7	93.3	73.3	13.3	3.3	100.0	95.0	20.0	15.0
po	36.6	73.3	70.0	53.3	62.5	70.0	30.0	10.0	0.0	-	-	-	-

(1) Data are from Li PZ *et al* (ref 44).

(2) Mice more than 30 in each group received a subcutaneous injection of mouse MLD of BoNT/A, 1 h late TSN was administrated to the mice in the therapeutic group. All mice in control groups died within 4 d after the toxin challenge. Mouse MLD of TSN: 14.6 mg/kg (iv), 15.2 mg/kg (sc), 550 mg/kg (po).

(3) Mice more than 30 in each group received a subcutaneous injection of mouse MLD of BoNT/A, various time after the toxin challenge, TSN (9 mg/kg for iv and sc, 220 mg/kg for po) was administrated to the mouse in therapeutic groups. All mice in control groups died within 4 d after the toxin challenge.

(4) Twenty mice in each group received a subcutaneous injection of mouse MLD of BoNT/A, and 1 h later, TSN (9 mg/kg) was injected intravenously to the mice in therapeutic groups. All mice in control groups died within 4 d after BoNT/A challenge.

in the neuromuscular preparations isolated from the rats that were preinjected with TSN (see Tab 2 also).

Tab 2. Toosendanin (TSN) delayed the neuromuscular block induced by BoNT/A.

No experiment	Origin and pretreatment of preparation	Blocking time by BoNT/A(min)	N
One	Isolated from normal rats		
	Non-pretreatment	52±3.6	4
	30 min preincubation with TSN	213±26.3	4
Two	From non-TSN injected rats	41.3±3.2	7
	From TSN-injected rats		
	6 h after TSN	121.0±12.0	9
	16 h after TSN	108.7±15.2	9

Data from Shi and Xu (ref 46)

TSN-evoked transmitter release facilitation By a series of studies, TSN has been demonstrated to be a selective presynaptic blocker acting on neurotransmitter release [48-56]. There are some similarities in the blocking action between TSN and BoNT/A, such as the dosage-effect relation, temperature-effect relation, Ca^{2+}- and nerve activity-dependent, irreversibility and always with a latent period of their blocking action, and completely blocking the quantal release of neurotransmitter at last by eliminating the Ca^{2+}-sensitive of release. However, an obvious difference between them is that TSN induced a two-phase change on transmitter release. Namely, there is a facilitatory phase before inhibition. In the phase, both the transmitter release and the Ca^{2+}-sensitivity of the release machinery are higher than those of control[3,51]. The two-phase effect restrict neither on neuromuscular junction nor cholinergic synapse[55,56].

The facilitatory phase in the neuromuscular preparations was related to the high anti-BoNT ability in time course[57]. In the experiments, the rats injected with (7 mg/kg, sc) or without TSN-containing solutions were sacrificed at various time (from 15 min to several days) after the injection, then two phrenic nerve-diaphragm preparations were isolated from each rat: one for intracellular recording of miniature end-plate potential (MEPP), another for determining paralysis time after applying BoNT/A. The results showed that the high BoNT tolerance of the preparation occurred within 2 h after TSN injection and lasted for several days. In the period, the frequency of MEPP was higher than control. (Fig 3).

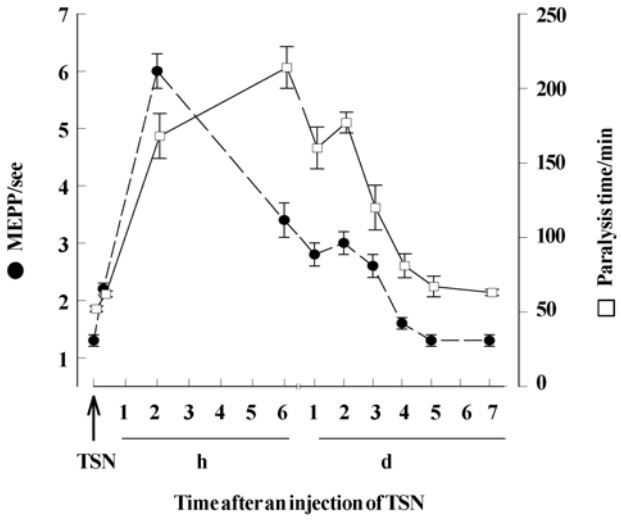

Fig 3. Changes of MEPP frequency (●) and paralysis time after applying BoNT/A (□) of phrenic nerve-diaphragam preparations isolated at different time from the rats after an injection of TSN. Data are from ref 57.

Transmitter release is regulated by membrane ion-channels and closely related to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) change. It was reported that TSN inhibited several kinds of neural K^+ channels, such as the perineurial fast K^+-currents at mouse motor nerve terminals[58], the whole-cell delayed rectifier K^+ current in NG108-15 cells[59], as well as the large- and small-conductance Ca^{2+}-activated K^+-currents[60,61] and inward rectifier K^+ current[62] recorded by single-channel patch-clamping from CA1 pyramidal neurons of rat hippocampus. An inhibition of inward rectifier K^+ current in guinea pig papillary muscle by TSN was also observed[63].

TSN seems to have different actions on various Ca^{2+}-channels. It was reported that in mouse motor nerve terminals, TSN inhibited the slow Ca^{2+}-current, while had no effect on the fast Ca^{2+}-current[64]. The slow and fast Ca^{2+} current are presumed to be mediated by P- and Q-type channels respectively. At frog nerve terminals, most of I_{Ca} corresponds to the N-type I_{Ca}, TSN had no inhibitory effect on it, while selectively increased a nifedipine-sensitive composition of I_{Ca}[64]. In NG108-15 cell, TSN increased Ca^{2+}conductance of membrane[65] and high-voltage activated ω-conotoxin MVIIC-resistent and nifedipine-sensitive I_{Ca}, while had

no effect on T-, N- and Q-type I_{Ca}[66]. An increase in $[Ca^{2+}]_i$ which was mediated by Ca^{2+}-influx via L-type channels in NG108-15 cells was also detected by laser confocal microscopic imaging[67]. These data indicate that TSN is a selective agonist of L-type Ca^{2+} channels.

Protecting SNARE protein from being cleaved by BoNT/A The TSN-induced inhibition on K^+-channels and elevation in $[Ca^{2+}]_i$ will facilitate neurotransmitter release and could be related to antibotulismic effect of TSN. However, the molecular mechanisms of anti-BoNT of the compound need to be elucidated. Our previous study showed that the binding site of ^{3}H-TSN existed on the rat cerebral synaptosome[68]. Recently, by means of western blotting, we observed that TSN-incubation did not change the electrophoresis pattern and the amounts of SNAP-25, syntaxin and synaptobrevin/VAMP in rat cerebral synaptosomes, but made the synaptosomes resistant to BoNT/A-mediated cleavage of SNAP-25. The antagonizing effect did not result from an inhibition of endopeptidase activity of light chain in BoNT/A by TSN, since TSN-incubated synaptosomce membrane fraction did not resistant to the cleavage of SNAP-25 by the light chain. We suggested that to block the approach of the light chain of the toxin as proteolysis enzyme with its substrate in some way was responsible for the TSN-induced antibotnlismic effect[69].

In conclusion, a series of findings in the past 10 years which revealed the molecular targets of BoNTs and hence proposed the four-step mechanism to explain BoNT intoxication greatly advanced the studies of antibotulismic drugs. Up to now, several compounds have been demonstrated to antagonize experimental botulism through interfering a certain step of action of BoNTs. Among them, TSN, a triterpenoid derivative from Chinese traditional medicine, is the most effective and is worthy to further investigate.

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