The role of the single interchains disulfide bond in tetanus and botulinum neurotoxins and the development of antitetanus and antibotulism drugs

Ornella Rossetto[1] | Marco Pirazzini[1] | Florigio Lista[2] | Cesare Montecucco[1,3]

[1] Dipartimento di Scienze Biomediche, Università di Padova, Padua, Italy

[2] Sezione di Istologia e Biologia Molecolare, Centro di ricerca Medica e Veterinaria del Ministero della Difesa, Rome, Italy

[3] Istituto Neuroscienze del CNR, Università di Padova, Padua, Italy

Correspondence
Cesare Montecucco, Istituto Neuroscienze del CNR, Università di Padova, Via Ugo Bassi 58/B, Padua 35121, Italy.
Email: cesare.montecucco@unipd.it

Abstract

A large number of bacterial toxins consist of active and cell binding protomers linked by an interchain disulfide bridge. The largest family of such disulfide-bridged exotoxins is that of the clostridial neurotoxins that consist of two chains and comprise the tetanus neurotoxins causing tetanus and the botulinum neurotoxins causing botulism. Reduction of the interchain disulfide abolishes toxicity, and we discuss the experiments that revealed the role of this structural element in neuronal intoxication. The redox couple thioredoxin reductase–thioredoxin (TrxR-Trx) was identified as the responsible for reduction of this disulfide occurring on the cytosolic surface of synaptic vesicles. We then discuss the very relevant finding that drugs that inhibit TrxR-Trx also prevent botulism. On this basis, we propose that ebselen and PX-12, two TrxR-Trx specific drugs previously used in clinical trials in humans, satisfy all the requirements for clinical tests aiming at evaluating their capacity to effectively counteract human and animal botulism arising from intestinal toxaemias such as infant botulism.

KEYWORDS

botulinum toxins, clostridial neurotoxins, inhibitors, tetanus toxin, thioredoxin

1 | INTRODUCTION

Bacteria produce thousands of toxins that are released extracellularly and affect key physiological functions of plants and animals (host). Thus, they play a major role in the biological arena of host–pathogen interactions, which lays at the foundation of the evolution of the host. After the proposals of Haldane (1949), Van Valen (1974), and Ebert and Hamilton (1996), it is now well established that pathogens are a major driving force of animal and plant evolution.

In general, bacterial protein toxins are produced to harness the host defence in such a way as to promote the growth and diffusion in the environment of bacteria, which spend at least part of their existence in or on animals or plants.

Therefore, the study of the mechanism of action of bacterial toxins leads to the following:

a. A better understanding of the pathogenesis of the disease caused by the microorganism;

b. To learn about specific physiological functions of the host, including the immune reactions;

c. The development of novel therapeutics, including vaccines, inhibitors, and so forth;

d. The use of the toxins themselves as therapeutics, which is exemplified by the almost incredible case of botulinum neurotoxin (BoNT), which is, at the same time, the most poisonous poison and a most valuable therapeutic used for all conditions that are originated in humans and other animals by the hyperfunction of cholinergic peripheral nerve terminals (Pirazzini, Rossetto, Eleopra, & Montecucco, 2017).

At the same time, an analysis of the evolution and lifestyle of the toxigenic pathogen may contribute to understand the mechanism of action of the toxin and to put it in the context of the disease caused in the host.

2 | A-B BACTERIAL PROTEIN TOXINS

Many bacterial proteins consist of two protomers: an active (A) part and a host cell binding (B) protomer, which are linked by a single interprotomeric disulfide bond, which is essential for their biological activity. The enzymatically active subunit is present in a single copy, and it is disulfide linked to the B protomer, which may consist of one subunit, such as in diphtheria toxin (DT), or several subunits such as in cholera toxin. We will focus in this review on tetanus neurotoxin (TeNT) and BoNT and on their interchain SS bond, which bridges the L (light, 50 kD) and the H (heavy, 100 kDa) chains. For more complete reviews on these neurotoxins, the reader is referred to Binz (2013), Dong, Masuyer, and Stenmark (2018), Montal (2010), Pirazzini et al. (2017), Rossetto, Pirazzini, and Montecucco (2014), and Rummel (2016).

TeNT and BoNT are also commonly indicated as clostridial neurotoxins, because until few years ago, they were known to be produced only by bacteria of the genus *Clostridium*. This indication is no longer correct as toxins with very similar structure and enzymatic target were recently discovered using bioinformatic methods in non-clostridial bacterial species (Brunt, Carter, Stringer, & Peck, 2018; Doxey, Mansfield, & Montecucco, 2018; Mansfield, Adams, & Doxey, 2015; Mansfield & Doxey, 2018; Mansfield et al., 2019; Zhang et al., 2018; Zornetta et al., 2016). However, these "bioinformatics" toxins have not been yet associated to diseases nor it is known if they are produced at all in the natural environment (Doxey et al., 2018). On the other hand, TeNT and BoNT do cause tetanus and botulism, respectively, and the molecular mechanism of pathogenesis of these deadly diseases is known in some details.

3 | TETANUS

Tetanus is an acute and often fatal disease characterized by a spastic paralysis. Almost in all cases, it is caused by the contaminations of wound with the ubiquitous spores of *Clostridium tetani*. If the strain harbours the plasmid encoding for TeNT and if the wound is at least partially necrotic and anaerobic, the bacterium germinates and produces the toxin that enters the general circulation and reaches, by retroaxonal transport inside motor axons and transynaptic movement, second-order neurons in the spinal cord (Bercsenyi et al., 2014;

Brooks, Curtis, & Eccles, 1955, 1957; Schwab, Suda, & Thoenen, 1979). Therein, it blocks neurotransmitter release and function of inhibitory interneurons (Bercsenyi et al., 2014; Brooks et al., 1955, 1957; Schwab et al., 1979). As a consequence, the balanced contraction of opposing skeletal muscles, which is at the basis of physiological movements, is lost, and muscles are fully contracted and work one against the other, leading to muscle spasms and seizures. *Generalised tetanus* is the most common form of the disease, and it usually begins with a characteristic facial trismus (lockjaw; Weinstein, 1973). Subsequently, a neck stiffness develops and later spreads to affect the muscles of the spinal cord, of the abdomen and of the legs. Tetanic seizure is very painful and is characterized by a sudden burst of tonic contraction of muscle groups causing *opisthotonos*, flexion and adduction of the arms, and extension of the lower extremities. Spasms can be so intense to cause bone fracture or myofibre damage. Pharyngeal and/or thoracic muscle spasms may lead to respiratory compromise, frequently requiring endotracheal intubation. Later, autonomic symptoms develop with alterations of blood pressure and of the cardiac rhythm and sweating. Glottal and laryngeal spasms may develop and cause cyanosis and asphyxia if not promptly relieved by medical or surgical means. Dysuria or urinary retention may also supervene. One dramatic aspect of tetanus is that the patient is fully conscious during such episodes and experiences intense pain.

A milder form of the disease is *local tetanus*, characterized by rigidity of the group of muscles close to the site of injury, which may persist for a considerable period of time without further developments or it may proceed to generalised tetanus. Tetanus has almost disappeared in those countries where vaccination with tetanus toxoid is enforced. Yet, in those areas of the world where vaccination programs are inadequate, it is still a major killer. In this respect, a horrible though frequent form of tetanus, usually fatal, is *tetanus neonatorum*, which is acquired by newborns, whose umbilical cord was cut using nonsterile tools and whose mothers were not immunised against TeNT (Galazka & Gasse, 1995; Vouking, Tadenfok, & Ekani, 2017).

No specific drugs to block tetanus once symptoms become evident are available, whereas the disease is completely prevented by anti-TeNT-specific antibodies.

4 | BOTULISM

Botulism is a rare (incidence of one to five cases per 10 million) but severe and potentially lethal disease (Fleck-Derderian et al., 2017). Contrary to tetanus, botulism is characterized by a flaccid paralysis mainly of cholinergic nerve terminals of the skeletal and autonomic peripheral nervous systems (Chatham-Stephens et al., 2017; Erbguth, 2008; Johnson & Montecucco, 2008). According to the portal of entry of BoNT into the body, six forms of botulism can be distinguished (Rossetto et al., 2014): (a) *Food-borne botulism*, which occurs after the ingestion of preformed BoNT in food; (b) *infant botulism* and (c) *adult intestinal botulism*, which are toxoinfections associated with the entry of spores in the intestine, their germination into toxigenic strains of Clostridia, and the local and prolonged production of BoNTs; (d)

wound botulism is similar to the initial phase of tetanus with spore contamination of wounds, bacterial germination, and production of BoNT that then diffuses away from the site of production; (e) *iatrogenic botulism* usually follows the therapeutic or cosmetic use of BoNTs, when an excessive amount of toxin is injected and diffuses to nearby areas causing paralysis; and (f) *inhalational botulism* resulting from accidental or deliberate release of aerosolised toxins (Holzer, 1962).

The major symptoms of botulism are abdominal pain and constipation, respiratory deficits or distress, and a progressive paralysis that begins with the small facial muscles of the eyes (diplopia, blurring vision, and ptosis), of swallowing (dysphagia) followed by descending flaccid and symmetric paralysis of skeletal and autonomic cholinergic nerve terminals, which include nausea, vomiting, and dry mouth. The disease may progress to compromise respiration. As in tetanus, patients are fully conscious but cannot control any body function nor communicate. Botulism patients can be saved by mechanical ventilation as the BoNT-induced neuroparalysis is reversible with the decay of the metalloprotease L chain of the toxin inside the intoxicated neurons. This is an important feature of both TeNT and BoNTs: They intoxicate and block neurotransmitter release from nerve terminals, but do not kill the neurons. Hence, with time, TeNT and BoNTs are degraded, and neuroexocytosis impairment is reinstated. Seven serotypes of BoNT are known, and the time of recovery depends on the dose and on the serotype of BoNT with the following order: BoNT/A ~ BoNT/C > BoNT/B ~ BoNT/D ~ BoNT/F ~ BoNT/G > BoNT/E (Pirazzini & Rossetto, 2017).

5 | TETANUS NEUROTOXINS

TeNT is always mentioned and discussed as a single toxin, at variance from the many BoNTs. But this is no longer appropriate as novel TeNT isoforms with slight, but significant, amino acid sequence changes are reported (Alam, Dixit, Tomar, & Singh, 2010; Bruggemann et al., 2015; Cohen, Wang, Shen, Wu, & Keller, 2017; Dixit, Alam, & Singh, 2006), and surely many more will be found as the sequence of more *C. tetani* TeNT encoding plasmids become available.

The structure of TeNT has been resolved only recently, and it shows the typical three domains BoNT structure (Figure 1) consisting of the 50-kDa L domain endowed with a Zn^{2+}-dependent metalloprotease activity, the HN domain (50 kDa, N-terminal half of the H chain), and the HC domain (50 kDa, C-terminal half of the H chain; Masuyer et al., 2017). TeNT is produced as a single polypeptide chain of 150 kDa with two disulfide bonds: one, conserved in BoNTs, links Cys439 to Cys467 and connects the L and HN domains; the other one joins Cys869 and Cys1093 and forms an intrachain disulfide bridge internal to the HC domain, which is unique of TeNT (Masuyer et al., 2017).

The peptide loop subtended by the Cys439–Cys467 disulfide bond includes several protease cleavage sites, and therefore, TeNT isolated from *C. tetani* supernatants is mostly in the disulfide-bridged di-chain form (Krieglstein, Henschen, Weller, & Habermann, 1991).

The three domains are functional to the process of entry of TeNT into neurons. The HC domain interacts at the NMJ with nidogen, a protein of the basal lamina facing the presynaptic membrane of the motor neuron (Bercsenyi et al., 2014). TeNT then binds to polysialogangliosides of the presynaptic membrane and enters the lumen of signalling endosomes, whose function is controlled by rab5 and rab7 and moves retroaxonally to the cell body located in the spinal cord (Deinhardt et al., 2006). Therefrom, TeNT is released in the intersynaptic space enclosed between the motoneuron and the inhibitory interneurons that ensure the balanced contraction of opposing skeletal muscles (Brooks et al., 1955; Montecucco & Schiavo, 1995), and it enters inside synaptic vesicle and translocates its L chain in the cytosol (see below). Here, it cleaves specifically the vesicle protein VAMP (vesicle-associated membrane protein; Schiavo et al., 1992) and blocks the release of the vesicular neurotransmitter glycine. This impairs the regulatory activity of the spinal cord inhibitory interneurons causing a spastic paralysis (Brooks et al., 1955, 1957).

6 | BOTULINUM NEUROTOXINS

These neurotoxins are structurally closely similar to TeNT and are produced by different bacteria, mainly Clostridia (*botulinum, butyrricum,*
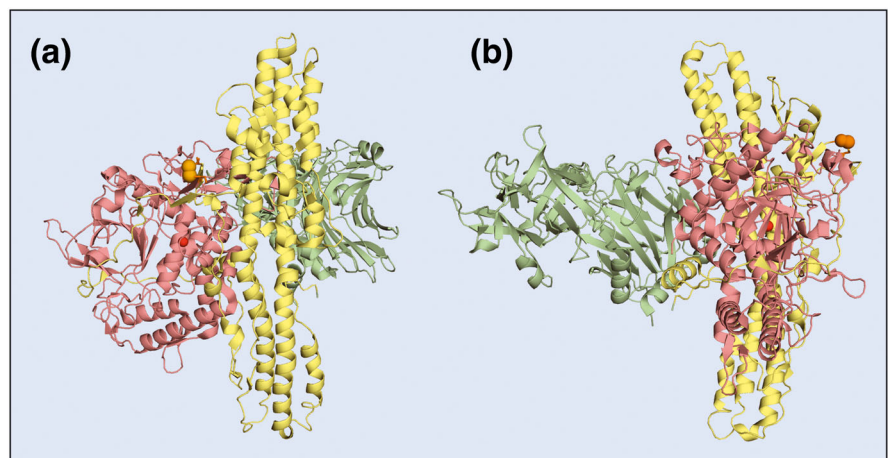

FIGURE 1 Crystallographic structure of tetanus neurotoxins. The picture is modified from Masuyer, Conrad, and Stenmark (2017). The L domain is in pink, the HN domain in yellow, and the HC domain in green. The two sulfur atoms of the interchain disulfide bond is in orange. The red sphere represents the zinc atom present in the metalloprotease active site of the L domain. (a and b) Two different orientations of the molecules to appreciate the relative orientations of the three domains

barati, and *argentinensis*; Peck et al., 2017; Williamson et al., 2016). They are synthesised as single 150-kDa chain in the bacterial cytosol where they associates with a non-toxic nonhaemagglutinin protein (NTNH) which is very similar to BoNT. The BoNT/NTNH dimer associates with additional non-toxic proteins to form high molecular weight complexes that are believed to protect the toxin during its passage through the GI tract and, possibly, assists BoNT during its intestinal adsorption (Bonventre, 1979; Fujinaga & Popoff, 2018; K.-H. Lam & Jin, 2015). The BoNTs are then released by bacterial autolysis and nicked by proteases at the peptide loop connecting the L and H chains, which remain associated via noncovalent forces, by a belt that encircles the L chain and by the highly conserved interchain disulfide bond. Serotyping has characterized seven antigenically distinct types of BoNT: A, B, C, D, E, F, and G, plus some chimeric toxins (BoNTC/D, D/C and F5A; Dong et al., 2018; Rossetto et al., 2014; Smith & Sugiyama, 1988; Tehran & Pirazzini, 2018). In recent years, many dozens of novel BoNT sequences have been determined. Nearly all the "novel" BoNTs belong to one serotype or another, and therefore, they are defined as subtypes and abbreviated as BoNTA1, BoNT/A2, and so forth (Montecucco & Rasotto, 2015; Peck et al., 2017).

Very recently, four *bont* genes were identified by bioinformatic methods. When expressed in *Escherichia coli*, the corresponding proteins were not recognised by available standard anti-BoNT antisera. They were identified in *Weissella oryzae* (Mansfield et al., 2015; Zornetta et al., 2016), in strain 111 of *C. botulinum* (Sicai Zhang et al., 2017), in the genome of a strain IDI0629 of *Enterococcus faecium* (Brunt et al., 2018; S. Zhang et al., 2018), and in *Cryseobacterium piperi* (Mansfield et al., 2019). Most notably, both the *Weissella* and the *Cryseobacterium* sequences lack the SS bond connecting the L and HN domain.

7 | MECHANISM OF PARALYSIS OF NERVE TERMINALS BY TeNT AND BoNTs

These neurotoxins act inside nerve terminal and reach their cytosolic targets in a multistep process that exploits cell trafficking events, similarly in general terms, to that used by many other disulfide-bridged toxins acting inside cells consisting of four steps.

BoNTs bind to the presynaptic membrane by a peculiar double-receptor mechanism. In fact, only after binding a polysialoganglioside molecule and then the lumenal domain of a synaptic vesicle protein, BoNT binding becomes productive and is followed by endocytosis. Polysialogangliosides are highly enriched on the presynaptic membrane and project out of the membrane surface their negatively charged oligosaccharide portion acting as sort of capturing antennae (Montecucco, 1986). On the other hand, BoNT is dipoles with the positive pole located close to the conserved ganglioside-binding site. This feature leads to a reorientation of the tumbling BoNT molecule while approaching to the membrane in such a way that almost any hit with the ganglioside results in toxin binding (Fogolari, Tosatto, Muraro, & Montecucco, 2009). BoNT/A and BoNT/E bind to SV2, their protein receptor, whereas BoNT/B, BoNT/G, and BoNT/DC bind to the synaptic vesicle protein synaptotagmin I/II (Binz & Rummel, 2009; Dong et al., 2018). Such a binding is followed by endocytosis inside synaptic vesicle. This has been demonstrated for TeNT in CNS neurons (Matteoli et al., 1996) and for BoNT/A in neurons in culture (Harper et al., 2011; Harper et al., 2016) and at the mouse neuromuscular junction (Colasante et al., 2013). The intracellular trafficking of other serotypes is not yet known, though in most cases, endocytosis within synaptic vesicle is the first step of entry. There is solid evidence that all BoNTs translocate the L chain across the membrane driven by a structural change induced by the acidic pH of the intracellular compartment (synaptic vesicle or endosome; Pirazzini et al., 2016). In the translocation process, a transmembrane ion channel is formed by the H chain (Fischer & Montal, 2013; Montal, 2010), though it has yet to be shown that the L chain crosses the membrane inside this channel.

8 | ON THE POSSIBLE ROLE OF THE SINGLE DISULFIDE BOND BRIDGING THE L AND HN DOMAIN OF TeNT AND BoNT

The interchain disulfide bond is highly conserved among neurotoxic TeNTs and BoNTs. The first evidence that this disulfide bond of TeNT is essential for neurotoxicity was obtained by Schiavo, Papini, Genna, and Montecucco (1990) using the thioredoxin reductase–thioredoxin redox couple (TrxR-Trx), discovered by Holmgren (1985) to reduce it specifically and iodoacetamide to block the SH groups; the intrachain SS bond present within the HC domain remains intact. The interchain SS-reduced TeNT was almost non-toxic (Schiavo et al., 1990). Kistner and Habermann (1992) showed that this reduction could be performed in TeNT and in BoNT by a TrxR-Trx purified from brain cortex (Kistner & Habermann, 1992). It was then demonstrated that the interchain SS bond is essential for the entry of the toxin into the nerve terminal (de Paiva et al., 1993). Simpson, Maksymowych, Park, and Bora (2004) showed that this disulfide bond is essential for the activity of all BoNT serotypes and that its reduction causes a change in the respective orientation of the two chains with a relocation of the belt that encircles the L domain (Simpson et al., 2004).

A major step forward in the understanding of the precise intracellular site and activity of the interchain SS bond involvement was made using the patch clamp technique in Neuro2A cells (Fischer & Montal, 2007). They found that the SS bond had to be intact for the BoNT molecule to translocate from the lumenal vesicle side to the cytosolic side. Reduction on the cytosolic side then led to full opening of the ion channel made by H chain across the membrane at acidic pH values. The same conclusion was reached using a protocol that promotes the low pH-induced translocation of TeNT and BoNT from the cell surface into the cytosol (Pirazzini, Rossetto, Bolognese, Shone, & Montecucco, 2011). Using a reporter bound to the L chain, it was found that an intact interchain SS bond is necessary for membrane translocation of the L chain (Zuverink, Chen, Przedpelski, Blum, & Barbieri, 2015). This sequence of events is very similar to that followed by the prototype of three-domain disulfide-bridged protein

toxins, that is, DT, which also forms transmembrane ion channel at low pH (Collier, 2001; Oh, Senzel, Collier, & Finkelstein, 1999).

On the basis of similarities among DT, TeNT, and BoNT with respect to low pH-driven membrane translocation, of experiments of hydrophobic membrane photolabelling with photoactivatable phospholipids of these toxins under different conditions (Montecucco et al., 1986; Montecucco, Schiavo, & Dasgupta, 1989; Papini et al., 1987), of the effect of cargoes attached to the N-terminus of these toxins (Bade et al., 2004; Madshus, Olsnes, & Stenmark, 1992; Zuverink et al., 2015), and of the physico-chemical properties of sulfur atoms (Creighton, 1992), we suggest that the first part of TeNT and BoNT molecules that penetrates the membrane is the interchain SS bond (as depicted in Figure 2) implying that the membrane insertion at low pH begins with the C-terminus of the L chain and the N terminus of HN. Available experimental evidence support this proposal (Bade et al., 2004; Madshus et al., 1992; Zuverink et al., 2015). It should also be considered that sulfur atoms are large,

FIGURE 2 Membrane interaction of botulinum neurotoxins at low pH. The interchain disulfide resides on the surface of the BoNT that has been identified to be the one that interacts at low pH with the membrane plane (Lam et al., 2018; Pirazzini et al., 2011; Pirazzini et al., 2013). (a) The SS bond is surrounded by hydrophobic residues (in black), which are likely to mediate the interaction with the hydrophobic core of the lipid bilayer. In the same surface, a set of conserved high pKa carboxylates are present, which will lose their charge at the pH values reached inside synaptic vesicles (Pirazzini et al., 2013). (b) A possible orientation of the SS containing surface at low pH with respect to the membrane represented by grey rectangle

deformable, and hydrophobic and that around the protein surface including the disulfide bond, and within the fork subtended by the SS bond, there are several hydrophobic residues (depicted in black in Figure 2). In addition, right below the SS bond, there are buried α-helices that switch into a surface-exposed hydrophobic β-hairpin at low pH, acting as a molecular appendix that flips out and penetrates the membrane (Lam et al., 2018). It is possible that the disulfide-linked fork is among the first parts of the L domain that emerge on the cytosolic side.

9 | THE INTERCHAIN DISULFIDE BOND OF TeNT AND BoNTs IS SPECIFICALLY REDUCED BY TrxR-Trx REDOX SYSTEM

Previous work had established that the TrxR-Trx is capable of reducing specifically the interchain disulfide bond of both TeNT and BoNT and that this reduction is necessary to free the metalloprotease activity of the L chain (de Paiva et al., 1993; Kistner & Habermann, 1992; Schiavo et al., 1990; Schiavo et al., 1992). However, several redox systems are operating in cells, and some of them are compartmentalised. In addition, no redox enzyme was reported to be associated with the synaptic vesicle in careful mass spectrometry studies of the chemical compositions of synaptic vesicles (Boyken et al., 2013; Morciano et al., 2005; Morciano, Beckhaus, Karas, Zimmermann, & Volknandt, 2009; Takamori et al., 2006). Using a pharmacological approach on different primary neurons in culture, we obtained evidence that, among the various protein reducing systems, TrxR-Trx is the one responsible for the reduction of the interchain disulphide bond of TeNT and of BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F and BoNT/G (Pirazzini et al., 2013;Pirazzini et al., 2014 ; Zanetti et al., 2015). Preparations of synaptic vesicles, not treated with carbonate buffers that strip extrinsic membrane associate proteins, as usually done before the mass spectrometry analysis, revealed the presence of both TrxR-Trx on the cytosolic surface of synaptic vesicles, in the right position to reduce the SS bond as the L chain emerges from the membrane.

10 | INHIBITORS OF THE TrxR-Trx REDOX SYSTEM PREVENT BOTULISM AND THE PERIPHERAL NEUROPARALYTIC ACITIVITY OF BoNTs

There is evidence that the activity of TrxR-Trx is altered in several human pathologies including rheumatoid arthritis, ischaemia, some forms of cancer (Arner & Holmgren, 2000; Hanschmann, Godoy, Berndt, Hudemann, & Lillig, 2013; Holmgren & Lu, 2010). Therefore, it is not surprising that several inhibitors of thioredoxin reductase or thioredoxin or both have been discovered, and some of them have been tested in clinical trials in humans (Mahmood, Abderrazak, El Hadri, Simmet, & Rouis, 2013; Tonissen & Di Trapani, 2009). We have tested available TrxR-Trx-specific drugs and have shown that they prevent the cleavage of the substrates of TeNT and of all BoNT serotypes. Remarkably, ebselen and PX-12 are very effective in
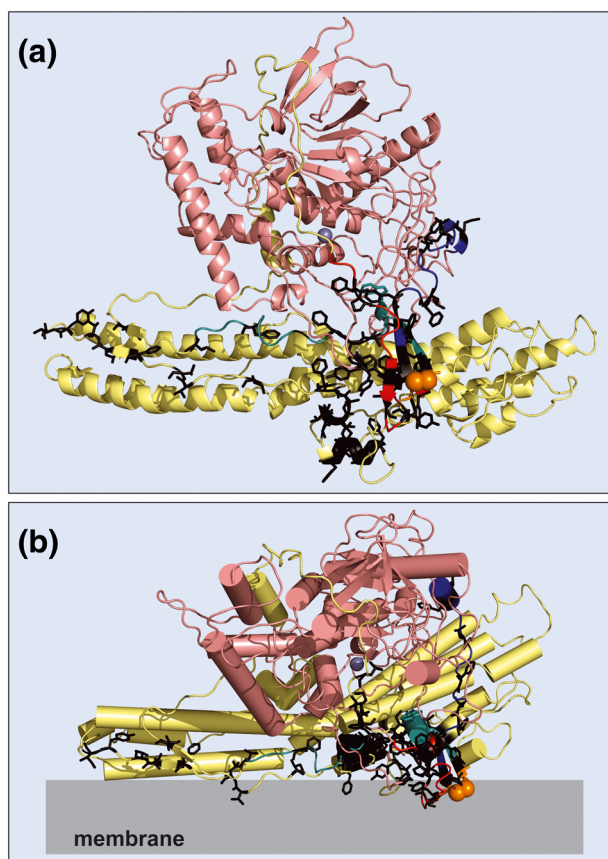
preventing animal paralysis, and ebselen is capable of reducing the number of mice death caused by different BoNTs (Pirazzini et al., 2014; Zanetti et al., 2015).

It should be underlined that the inhibitory activity of these TrxR-Trx-specific drugs defines the reduction of the single interchain SS bridge of clostridial neurotoxins as an essential step in the neuroparalysis induced by TeNT and BoNTs. Therefore, we propose this process consists of five steps: (a) binding, (b) internalisation, (c) membrane translocation, (d) interchain disulfide reduction, and (e) target modification. This appears to be valid also for DT. Indeed, thioredoxin was present in a complex isolated from endosomes and shown to be involved in the delivery of the A subunit of DT in the cytosol (Murphy, 2011; Ratts et al., 2003). Moreover, reduction of the interchain disulfide bond was demonstrated to be the rate limiting step of the process of translocation of DT into the cytosol (Papini, Rappuoli, Murgia, & Montecucco, 1993). In addition, both DT and the clostridial neurotoxins appear to require the assistance of Hsp90 and possibly other cytosolic chaperones to refold their enzymatic subunits in the cytosol (Azarnia Tehran et al., 2017; Murphy, 2011; Ratts et al., 2003; Schuster et al., 2017).

The fact that reduction of the interchain SS bond by TrxR-Trx is a common essential step in neuroparalysis by all serotypes of BoNT and TeNT makes TrxR-Trx-specific drugs effective pan-inhibitors of the clostridial neurotoxins not depending on their serotypes, as antibodies are (Pirazzini & Rossetto, 2017). Clearly, such drugs have the limitation that they do not block the metalloprotease activity once the L domain is in the cytosol and hence are unlikely to provide benefit in the case of a bioterrorist attack employing BoNT, but they are fully adequate to prevent botulism in personnel operating in a domain where the BoNTs could be used as bioweapons. On the other hand, TrxR-Trx-inhibiting drug are very likely to be useful in the case of botulism caused by intestinal toxaemia.

11 | TrxR-Trx INHIBITORS IN THE TREATMENT OF INFANT BOTULISM AND ADULT INTESTINAL BOTULISM

Infant botulism was a late discovery because its symptoms are difficult to be identified in newborns (Midura & Arnon, 1976; Pickett, Berg, Chaplin, & Brunstetter-Shafer, 1976). Notably, this disease may develop only in the first period of life (1/2 to 11 months of age) following the ingestion of clostridial spores that germinate in the infant gut whose microbiome is not capable to outcompete neurotoxigenic Clostridia as it would normally occur in the adult (Arnon, 1986). This rationale is fully supported by experiments with germ-free mice and with antibiotic-treated mice (Moberg & Sugiyama, 1979; Burr & Sugiyama, 1982). Following the definition of infant botulism, an intestinal toxaemia in adults was described (Chia, Clark, Ryan, & Pollack, 1986). These two forms of botulism are now referred to as "intestinal toxaemia botulism." As it has been well discussed by Arnon (1995), predisposing causes of this disease are altered intestinal microflora either due to the young age or to broad spectrum antibiotics, or

altered intestinal tract physiology or anatomy. In any case, the neurotoxigenic Clostridia in the intestine produce BoNT. The site(s) of absorption in human has not been well defined. In rabbits and rats, it occurs in the upper small intestine and in the ileum, whereas in chicken, the caecum is the main site involved. The toxin appears first in the lymph and then in the blood in the 150-KDa BoNT form, which is then distributed in the entire body and binds to peripheral cholinergic nerve terminals (Heckly, Hildebrand, & Lamanna, 1960; May & Whaler, 1958; L. Simpson, 2013).

The human and hospital costs of intestinal toxaemia botulism are very high because of the frequent need of the intensive care unit (Arnon, 1995). The only available specific treatment is a human botulism immune globulin (BIG-IV) derived from the immune plasma of donors immunised with pentavalent (A to E) toxoid. BIG-IV was generated thanks to the involvement of the California Department of Health Services and of the "heroic" effort of Dr. S.S. Arnon (Arnon, 2007). Treatment with BIG-IV resulted to be very successful as it shortened the mean hospital stay of infant botulism patients from 5.7 to 2.6 weeks, and it reduced the duration of intensive care by 3.2 weeks and of mechanical ventilation by 2.6 weeks; at the same time, no serious adverse effects attributable to this treatment was recorded (Arnon, 2007).

There is no evidence that an antibody molecule can reach the cytosol and block the toxin enzymatic domain, and therefore, BIG-IV can only act on the extracellular toxin, which is continuously produced. Drugs such as ebselen and PX-12 act further down in the chain of events leading to intoxication because they can penetrate cells and act on TrxR-Trx. For this reason, in principle, they should have an effect comparable or superior with that of BIG-IV. But the major advantages with respect to the use of specific anti-BoNTs immunoglobulins are that (a) these chemical inhibitors act irrespectively of the BoNT serotype responsible for the toxaemia, and therefore, they can be given before knowing the BoNT serotype involved; (b) they have much lower cost of production; (c) they have much longer shelf life; and (d) they are safe at the doses used in the clinical trial so far performed in humans.

Their chemical synthesis is well established and is not expensive. Moreover, ebselen has been already used in clinical trials, including acute ischaemic stroke, aneurysmal subarachnoid haemorrhage, cerebral ischaemia, bipolar disorders and hearing loss found to be non-toxic (Kil et al., 2017; Masaki et al., 2016; Ogawa et al., 1999; M. Parnham & Sies, 2000; M. J. Parnham & Sies, 2013; Saito et al., 1998; Yamaguchi et al., 1998). In these studies, ebselen was used at doses comparable to those used in mice (Pirazzini et al., 2014; Zanetti et al., 2015). In fact, in clinical trials, a range of regimens was used from the initial studies employing 150 mg of ebselen per person per day per 2 weeks to 200 mg up to 600 mg twice per day per person per 6 days to 3600 mg once per day. In mice experiments, we used 7.5 mg/kg twice per day for 4 days.

Also, PX-12 has been already tested in several clinical trials for efficacy against human solid tumours of different origins (Baker et al., 2013; Baker et al., 2006; Galmarini, 2006; Ramesh K Ramanathan et al., 2011; Ramanathan et al., 2007; Ramanathan et al., 2012). Also,

in the case of PX-12, we used in mice drug doses non-toxic in humans. In mice, we found that both ebselen and PX-12 are very effective in preventing the paralysis caused by the different BoNT serotypes. They act as general inhibitors of all BoNT serotypes because they block a common step of the mechanism of entry of these neurotoxins in the cytosol of nerve terminals. Therefore, the proposal of performing trials of the use of ebselen and PX-12 in the treatment of patient suffering from intestinal toxaemia botulism, where there is a continuous production and release of BoNT in the intestinal lumen, is well grounded.

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ORCID

Cesare Montecucco https://orcid.org/0000-0001-7361-5688

REFERENCES

Alam, S. I., Dixit, A., Tomar, A., & Singh, L. (2010). Comparative genomic analysis of a neurotoxigenic *Clostridium* species using partial genome sequence: Phylogenetic analysis of a few conserved proteins involved in cellular processes and metabolism. *Anaerobe*, 16(2), 147–154. https://doi.org/10.1016/j.anaerobe.2009.06.001

Arner, E. S. J., & Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry*, 267(20), 6102–6109. https://doi.org/10.1046/j.1432-1327.2000.01701.x

Arnon, S. S. (2007). Creation and development of the public service orphan drug Human Botulism Immune Globulin. *Pediatrics*, 119(4), 785–789. https://doi.org/10.1542/peds.2006-0646

Arnon, S. S. (1986). Infant Botulism: Anticipating the second decade. *J Infect Dis*, 154 (2), 201–206.

Arnon, S. S. (1995). Botulism as an intestinal toxemia. In Blaser, M. et al. (Eds.), *Infections and Gastrointestinal Tract* (pp. 257–271). N.Y.: Raven press.

Azarnia Tehran, D., Pirazzini, M., Leka, O., Mattarei, A., Lista, F., Binz, T., ... Montecucco, C. (2017). Hsp90 is involved in the entry of clostridial neurotoxins into the cytosol of nerve terminals. *Cellular Microbiology*, 19(2). https://doi.org/10.1111/cmi.12647

Bade, S., Rummel, A., Reisinger, C., Karnath, T., Ahnert-Hilger, G., Bigalke, H., & Binz, T. (2004). Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. *Journal of Neurochemistry*, 91(6), 1461–1472. https://doi.org/10.1111/j.1471-4159.2004.02844.x

Baker, A. F., Adab, K. N., Raghunand, N., Chow, H. H. S., Stratton, S. P., Squire, S. W., ... Dragovich, T. (2013). A phase IB trial of 24-hour intravenous PX-12, a thioredoxin-1 inhibitor, in patients with advanced gastrointestinal cancers. *Investigational New Drugs*, 31(3), 631–641. https://doi.org/10.1007/s10637-012-9846-2

Baker, A. F., Dragovich, T., Tate, W. R., Ramanathan, R. K., Roe, D., Hsu, C. H., ... Powis, G. (2006). The antitumor thioredoxin-1 inhibitor PX-12 (1-methylpropyl 2-imidazolyl disulfide) decreases thioredoxin-1 and VEGF levels in cancer patient plasma. *The Journal of Laboratory and Clinical Medicine*, 147(2), 83–90. https://doi.org/10.1016/j.lab.2005.09.001

Bercsenyi, K., Schmieg, N., Bryson, J. B., Wallace, M., Caccin, P., Golding, M., ... Schiavo, G. (2014). Nidogens are therapeutic targets for the prevention of tetanus. *Science*, 346(6213), 1118–1123. https://doi.org/10.1126/science.1258138

Binz, T. (2013). Clostridial neurotoxin light chains: Devices for SNARE cleavage mediated blockade of neurotransmission. *Current Topics in Microbiology and Immunology*, 364, 139–157.

Binz, T., & Rummel, A. (2009). Cell entry strategy of clostridial neurotoxins. *Journal of Neurochemistry*, 109(6), 1584–1595. Epub 2009 Apr 1528. https://doi.org/10.1111/j.1471-4159.2009.06093.x

Bonventre, P. F. (1979). Absorption of botulinal toxin from the gastrointestinal tract. *Reviews of Infectious Diseases*, 1(4), 663–667. https://doi.org/10.1093/clinids/1.4.663

Boyken, J., Gronborg, M., Riedel, D., Urlaub, H., Jahn, R., & Chua, J. J. (2013). Molecular profiling of synaptic vesicle docking sites reveals novel proteins but few differences between glutamatergic and GABAergic synapses. *Neuron*, 78(2), 285–297. https://doi.org/10.1016/j.neuron.2013.02.027

Burr, D. H., & Sugiyama, H. (1982). Susceptibility to enteric botulinum colonization of antibiotic-treated adult mice. *Infect Immun*, 36(1), 103–106.

Brooks, V. B., Curtis, D. R., & Eccles, J. C. (1955). Mode of action of tetanus toxin. *Nature*, 175(4446), 120–121. https://doi.org/10.1038/175120b0

Brooks, V. B., Curtis, D. R., & Eccles, J. C. (1957). The action of tetanus toxin on the inhibition of motoneurones. *The Journal of Physiology*, 135(3), 655–672. https://doi.org/10.1113/jphysiol.1957.sp005737

Bruggemann, H., Brzuszkiewicz, E., Chapeton-Montes, D., Plourde, L., Speck, D., & Popoff, M. R. (2015). Genomics of *Clostridium tetani*. *Research in Microbiology*, 166(4), 326–331. https://doi.org/10.1016/j.resmic.2015.01.002

Brunt, J., Carter, A. T., Stringer, S. C., & Peck, M. W. (2018). Identification of a novel botulinum neurotoxin gene cluster in *Enterococcus*. *FEBS Letters*, 592(3), 310–317. https://doi.org/10.1002/1873-3468.12969

Chatham-Stephens, K., Fleck-Derderian, S., Johnson, S. D., Sobel, J., Rao, A. K., & Meaney-Delman, D. (2017). Clinical features of foodborne and wound botulism: A systematic review of the literature, 1932-2015. *Clinical Infectious Diseases*, 66(suppl_1), S11–s16.

Chia, J. K., Clark, J. B., Ryan, C. A., & Pollack, M. (1986). Botulism in an adult associated with food-borne intestinal infection with *Clostridium botulinum*. *The New England Journal of Medicine*, 315(4), 239–241. https://doi.org/10.1056/NEJM198607243150407

Cohen, J. E., Wang, R., Shen, R. F., Wu, W. W., & Keller, J. E. (2017). Comparative pathogenomics of *Clostridium tetani*. *PLoS ONE*, 12(8) e0182909). https://doi.org/10.1371/journal.pone.0182909

Colasante, C., Rossetto, O., Morbiato, L., Pirazzini, M., Molgo, J., & Montecucco, C. (2013). Botulinum neurotoxin type A is internalized and translocated from small synaptic vesicles at the neuromuscular junction. *Molecular Neurobiology*, 48(1), 120–127. https://doi.org/10.1007/s12035-013-8423-9

Collier, R. J. (2001). Understanding the mode of action of diphtheria toxin: A perspective on progress during the 20th century. *Toxicon*, 39(11), 1793–1803. https://doi.org/10.1016/S0041-0101(01)00165-9

Creighton, T. E. (1992). *Proteins: Structures and molecular properties*. Oxford: Freeman & Co.

de Paiva, A., Poulain, B., Lawrence, G. W., Shone, C. C., Tauc, L., & Dolly, J. O. (1993). A role for the interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly. *The Journal of Biological Chemistry*, 268(28), 20838–20844.

Deinhardt, K., Salinas, S., Verastegui, C., Watson, R., Worth, D., Hanrahan, S., ... Schiavo, G. (2006). Rab5 and Rab7 control endocytic sorting along

the axonal retrograde transport pathway. *Neuron*, *52*(2), 293–305. https://doi.org/10.1016/j.neuron.2006.08.018

Dixit, A., Alam, S. I., & Singh, L. (2006). Sequencing and phylogenetic analysis of neurotoxin gene from an environmental isolate of *Clostridium* sp.: Comparison with other clostridial neurotoxins. *Archives of Toxicology*, *80*(7), 399–404. https://doi.org/10.1007/s00204-006-0062-8

Dong, M., Masuyer, G., & Stenmark, P. (2018). Botulinum and Tetanus Neurotoxins. *Annu Rev Biochem*, *88*, 2.1–2.27. https://doi.org/10.1146/annurev-biochem-013118-111654

Doxey, A. C., Mansfield, M. J., & Montecucco, C. (2018). Discovery of novel bacterial toxins by genomics and computational biology. *Toxicon*, *147*, 2–12. https://doi.org/10.1016/j.toxicon.2018.02.002

Ebert, D., & Hamilton, W. D. (1996). Sex against virulence: The coevolution of parasitic diseases. *Trends in Ecology & Evolution*, *11*, 79–82. https://doi.org/10.1016/0169-5347(96)81047-0

Erbguth, F. J. (2008). From poison to remedy: The chequered history of botulinum toxin. *Journal of Neural Transmission (Vienna)*, *115*(4), 559–565. https://doi.org/10.1007/s00702-007-0728-2

Fischer, A., & Montal, M. (2007). Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. *The Journal of Biological Chemistry*, *282*(40), 29604–29611. https://doi.org/10.1074/jbc.M703619200

Fischer, A., & Montal, M. (2013). Molecular dissection of botulinum neurotoxin reveals interdomain chaperone function. *Toxicon*, *75*, 101–107. https://doi.org/10.1016/j.toxicon.2013.01.007

Fleck-Derderian, S., Shankar, M., Rao, A. K., Chatham-Stephens, K., Adjei, S., Sobel, J., … Pillai, S. K. (2017). The epidemiology of foodborne botulism outbreaks: A systematic review. *Clinical Infectious Diseases*, *66*(suppl_1), S73–S81. https://doi.org/10.1093/cid/cix846

Fogolari, F., Tosatto, S. C. E., Muraro, L., & Montecucco, C. (2009). Electric dipole reorientation in the interaction of botulinum neurotoxins with neuronal membranes. *FEBS Letters*, *583*(14), 2321–2325. https://doi.org/10.1016/j.febslet.2009.06.046

Fujinaga, Y., & Popoff, M. R. (2018). Translocation and dissemination of botulinum neurotoxin from the intestinal tract. *Toxicon*, *147*, 13–18. https://doi.org/10.1016/j.toxicon.2017.10.020

Galazka, A., & Gasse, F. (1995). The present status of tetanus and tetanus vaccination. *Current Topics in Microbiology and Immunology*, *195*, 31–53.

Galmarini, C. M. (2006). Drug evaluation: The thioredoxin inhibitor PX-12 in the treatment of cancer. *Current Opinion in Investigational Drugs*, *7*(12), 1108–1115.

Haldane, J. B. S. (1949). Disease and Evolution. *La Ricerca Scientifica* (suppl. 19), 68–75.

Hanschmann, E. M., Godoy, J. R., Berndt, C., Hudemann, C., & Lillig, C. H. (2013). Thioredoxins, glutaredoxins, and peroxiredoxins-molecular mechanisms and health significance: From cofactors to antioxidants to redox signaling. *Antioxidants & Redox Signaling*, *19*(13), 1539–1605. https://doi.org/10.1089/ars.2012.4599

Harper, C. B., Martin, S., Nguyen, T. H., Daniels, S. J., Lavidis, N. A., Popoff, M. R., … Meunier, F. A. (2011). Dynamin inhibition blocks botulinum neurotoxin type A endocytosis in neurons and delays botulism. *The Journal of Biological Chemistry*, *286*(41), 35966–35976. https://doi.org/10.1074/jbc.M111.283879

Harper, C. B., Papadopulos, A., Martin, S., Matthews, D. R., Morgan, G. P., Nguyen, T. H., … Meunier, F. A. (2016). Botulinum neurotoxin type-A enters a non-recycling pool of synaptic vesicles. *Scientific Reports*, *6*, 19654. https://doi.org/10.1038/srep19654

Heckly, R. J., Hildebrand, G. J., & Lamanna, C. (1960). On the size of the toxic particle passing the intestinal barrier in botulism. *The Journal of Experimental Medicine*, *111*, 745–759. https://doi.org/10.1084/jem.111.6.745

Holmgren, A. (1985). Thioredoxin. *Annu Rev Biochem*, *54*, 237–271.

Holmgren, A., & Lu, J. (2010). Thioredoxin and thioredoxin reductase: Current research with special reference to human disease. *Biochemical and Biophysical Research Communications*, *396*(1), 120–124. https://doi.org/10.1016/j.bbrc.2010.03.083

Holzer, E. (1962). Botulism caused by inhalation. *Medizinische Klinik*, *57*, 1735–1738.

Johnson, E. A., & Montecucco, C. (2008). Botulism. In *Handbook of clinical neurology* (Vol. 91) (pp. 333–368). Amsterdam: Elsevier BV. https://doi.org/10.1016/S0072-9752(07)01511-4

Kil, J., Lobarinas, E., Spankovich, C., Griffiths, S. K., Antonelli, P. J., Lynch, E. D., & Le Prell, C. G. (2017). Safety and efficacy of ebselen for the prevention of noise-induced hearing loss: A randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet*, *390*(10098), 969–979. https://doi.org/10.1016/S0140-6736(17)31791-9

Kistner, A., & Habermann, E. (1992). Reductive cleavage of tetanus toxin and botulinum neurotoxin A by the thioredoxin system from brain. Evidence for two redox isomers of tetanus toxin. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *345*(2), 227–234. https://doi.org/10.1007/BF00165741

Krieglstein, K. G., Henschen, A. H., Weller, U., & Habermann, E. (1991). Limited proteolysis of tetanus toxin. Relation to activity and identification of cleavage sites. *European Journal of Biochemistry*, *202*(1), 41–51. https://doi.org/10.1111/j.1432-1033.1991.tb16342.x

Lam, K. H., Guo, Z., Krez, N., Matsui, T., Perry, K., Weisemann, J., … Jin, R. (2018). A viral-fusion-peptide-like molecular switch drives membrane insertion of botulinum neurotoxin A1. *Nature Communications*, *9*(1), 5367. https://doi.org/10.1038/s41467-018-07789-4

Lam, K.-H., & Jin, R. (2015). Architecture of the botulinum neurotoxin complex: A molecular machine for protection and delivery. *Current Opinion in Structural Biology*, *31*, 89–95. https://doi.org/10.1016/j.sbi.2015.03.013

Madshus, I. H., Olsnes, S., & Stenmark, H. (1992). Membrane translocation of diphtheria toxin carrying passenger protein domains. *Infection and Immunity*, *60*(8), 3296–3302.

Mahmood, D. F., Abderrazak, A., El Hadri, K., Simmet, T., & Rouis, M. (2013). The thioredoxin system as a therapeutic target in human health and disease. *Antioxidants & Redox Signaling*, *19*(11), 1266–1303. https://doi.org/10.1089/ars.2012.4757

Mansfield, M. J., Adams, J. B., & Doxey, A. C. (2015). Botulinum neurotoxin homologs in non-*Clostridium* species. *FEBS Letters*, *589*(3), 342–348. https://doi.org/10.1016/j.febslet.2014.12.018

Mansfield, M. J., & Doxey, A. C. (2018). Genomic insights into the evolution and ecology of botulinum neurotoxins. *Pathogens and Disease*, *76*(4). https://doi.org/10.1093/femspd/fty040

Mansfield, M. J., Wentz, T. G., Zhang, S., Lee, E. J., Dong, M., Sharma, S. K., & Doxey, A. C. (2019). Bioinformatic discovery of a toxin family in *Chryseobacterium piperi* with sequence similarity to botulinum neurotoxins. *Scientific Reports*, *9*(1), 1634. https://doi.org/10.1038/s41598-018-37647-8

Masaki, C., Sharpley, A. L., Cooper, C. M., Godlewska, B. R., Singh, N., Vasudevan, S. R., … Cowen, P. J. (2016). Effects of the potential lithium-mimetic, ebselen, on impulsivity and emotional processing. *Psychopharmacology*, *233*(14), 2655–2661. https://doi.org/10.1007/s00213-016-4319-5

Masuyer, G., Conrad, J., & Stenmark, P. (2017). The structure of the tetanus toxin reveals pH-mediated domain dynamics. *EMBO Reports*, *18*(8), 1306–1317. https://doi.org/10.15252/embr.201744198

Matteoli, M., Verderio, C., Rossetto, O., Iezzi, N., Coco, S., Schiavo, G., & Montecucco, C. (1996). Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(23), 13310–13315. https://doi.org/10.1073/pnas.93.23.13310

May, A. J., & Whaler, B. C. (1958). The absorption of *Clostridium botulinum* type A toxin from the alimentary canal. *British Journal of Experimental Pathology*, *39*(3), 307–316.

Midura, T. F., & Arnon, S. S. (1976). Infant botulism. Identification of *Clostridium botulinum* and its toxins in faeces. *Lancet*, *2*(7992), 934–936.

Moberg, L. J., & Sugiyama, H. (1979). Microbial ecological basis of infant botulism as studied with germfree mice. *Infection and Immunity*, *25*(2), 653–657.

Montal, M. (2010). Botulinum neurotoxin: A marvel of protein design. *Annual Review of Biochemistry*, *79*, 591–617. https://doi.org/10.1146/annurev.biochem.051908.125345

Montecucco, C. (1986). How do tetanus and botulinum toxins bind to neuronal membranes. *Trends in Biochemical Sciences*, *11*(8), 314–317. https://doi.org/10.1016/0968-0004(86)90282-3

Montecucco, C., & Rasotto, M. B. (2015). On botulinum neurotoxin variability. *MBio*, *6*(1). https://doi.org/10.1128/mBio.02131-14

Montecucco, C., & Schiavo, G. (1995). Structure and function of tetanus and botulinum neurotoxins. *Quarterly Reviews of Biophysics*, *28*(04), 423–472. https://doi.org/10.1017/S0033583500003292

Montecucco, C., Schiavo, G., Brunner, J., Duflot, E., Boquet, P., & Roa, M. (1986). Tetanus toxin is labeled with photoactivatable phospholipids at low pH. *Biochemistry*, *25*(4), 919–924. https://doi.org/10.1021/bi00352a027

Montecucco, C., Schiavo, G., & Dasgupta, B. R. (1989). Effect of pH on the interaction of botulinum neurotoxins A, B and E with liposomes. *The Biochemical Journal*, *259*(1), 47–53. https://doi.org/10.1042/bj2590047

Morciano, M., Beckhaus, T., Karas, M., Zimmermann, H., & Volknandt, W. (2009). The proteome of the presynaptic active zone: From docked synaptic vesicles to adhesion molecules and maxi-channels. *Journal of Neurochemistry*, *108*(3), 662–675. https://doi.org/10.1111/j.1471-4159.2008.05824.x

Morciano, M., Burré, J., Corvey, C., Karas, M., Zimmermann, H., & Volknandt, W. (2005). Immunoisolation of two synaptic vesicle pools from synaptosomes: A proteomics analysis. *Journal of Neurochemistry*, *95*(6), 1732–1745. https://doi.org/10.1111/j.1471-4159.2005.03506.x

Murphy, J. R. (2011). Mechanism of diphtheria toxin catalytic domain delivery to the eukaryotic cell cytosol and the cellular factors that directly participate in the process. *Toxins (Basel)*, *3*(3), 294–308. https://doi.org/10.3390/toxins3030294

Ogawa, A., Yoshimoto, T., Kikuchi, H., Sano, K., Saito, I., Yamaguchi, T., & Yasuhara, H. (1999). Ebselen in acute middle cerebral artery occlusion: A placebo-controlled, double-blind clinical trial. *Cerebrovascular Diseases*, *9*(2), 112–118. https://doi.org/10.1159/000015908

Oh, K. J., Senzel, L., Collier, R. J., & Finkelstein, A. (1999). Translocation of the catalytic domain of diphtheria toxin across planar phospholipid bilayers by its own T domain. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(15), 8467–8470. https://doi.org/10.1073/pnas.96.15.8467

Papini, E., Rappuoli, R., Murgia, M., & Montecucco, C. (1993). Cell penetration of diphtheria toxin. Reduction of the interchain disulfide bridge is the rate-limiting step of translocation in the cytosol. *The Journal of Biological Chemistry*, *268*(3), 1567–1574.

Papini, E., Schiavo, G., Tomasi, M., Colombatti, M., Rappuoli, R., & Montecucco, C. (1987). Lipid interaction of diphtheria toxin and mutants with altered fragment B. 2. Hydrophobic photolabelling and cell intoxication. *European Journal of Biochemistry*, *169*(3), 637–644. https://doi.org/10.1111/j.1432-1033.1987.tb13655.x

Parnham, M., & Sies, H. (2000). Ebselen: Prospective therapy for cerebral ischaemia. *Expert Opinion on Investigational Drugs*, *9*(3), 607–619. https://doi.org/10.1517/13543784.9.3.607

Parnham, M. J., & Sies, H. (2013). The early research and development of ebselen. *Biochemical Pharmacology*, *86*(9), 1248–1253. https://doi.org/10.1016/j.bcp.2013.08.028

Peck, M. W., Smith, T. J., Anniballi, F., Austin, J. W., Bano, L., Bradshaw, M., ... Stringer, S. C. (2017). Historical perspectives and guidelines for botulinum neurotoxin subtype nomenclature. *Toxins (Basel)*, *9*(1). https://doi.org/10.3390/toxins9010038

Pickett, J., Berg, B., Chaplin, E., & Brunstetter-Shafer, M. A. (1976). Syndrome of botulism in infancy: Clinical and electrophysiologic study. *The New England Journal of Medicine*, *295*(14), 770–772. https://doi.org/10.1056/NEJM197609302951407

Pirazzini, M., Azarnia Tehran, D., Leka, O., Zanetti, G., Rossetto, O., & Montecucco, C. (2016). On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. *Biochimica et Biophysica Acta*, *1858*(3), 467–474. https://doi.org/10.1016/j.bbamem.2015.08.014

Pirazzini, M., Azarnia Tehran, D., Zanetti, G., Megighian, A., Scorzeto, M., Fillo, S., ... Montecucco, C. (2014). Thioredoxin and its reductase are present on synaptic vesicles, and their inhibition prevents the paralysis induced by botulinum neurotoxins. *Cell Reports*, *8*(6), 1870–1878. https://doi.org/10.1016/j.celrep.2014.08.017

Pirazzini, M., Bordin, F., Rossetto, O., Shone, C. C., Binz, T., & Montecucco, C. (2013). The thioredoxin reductase-thioredoxin system is involved in the entry of tetanus and botulinum neurotoxins in the cytosol of nerve terminals. *FEBS Letters*, *587*(2), 150–155. https://doi.org/10.1016/j.febslet.2012.11.007

Pirazzini, M., & Rossetto, O. (2017). Challenges in searching for therapeutics against botulinum neurotoxins. *Expert Opinion on Drug Discovery*, *12*, 1–14.

Pirazzini, M., Rossetto, O., Bolognese, P., Shone, C. C., & Montecucco, C. (2011). Double anchorage to the membrane and intact inter-chain disulfide bond are required for the low pH induced entry of tetanus and botulinum neurotoxins into neurons. *Cellular Microbiology*, *13*(11), 1731–1743. https://doi.org/10.1111/j.1462-5822.2011.01654.x

Pirazzini, M., Rossetto, O., Eleopra, R., & Montecucco, C. (2017). Botulinum neurotoxins: Biology, pharmacology, and toxicology. *Pharmacological Reviews*, *69*(2), 200–235. https://doi.org/10.1124/pr.116.012658

Ramanathan, R. K., Abbruzzese, J., Dragovich, T., Kirkpatrick, L., Guillen, J. M., Baker, A. F., ... Von Hoff, D. D. (2011). A randomized phase II study of PX-12, an inhibitor of thioredoxin in patients with advanced cancer of the pancreas following progression after a gemcitabine-containing combination. *Cancer Chemotherapy and Pharmacology*, *67*(3), 503–509. https://doi.org/10.1007/s00280-010-1343-8

Ramanathan, R. K., Kirkpatrick, D. L., Belani, C. P., Friedland, D., Green, S. B., Chow, H. H., ... Dragovich, T. (2007). A phase I pharmacokinetic and pharmacodynamic study of PX-12, a novel inhibitor of thioredoxin-1, in patients with advanced solid tumors. *Clinical Cancer Research*, *13*(7), 2109–2114. https://doi.org/10.1158/1078-0432.CCR-06-2250

Ramanathan, R. K., Stephenson, J. J., Weiss, G. J., Pestano, L. A., Lowe, A., Hiscox, A., & Richards, D. A. (2012). A phase I trial of PX-12, a small-molecule inhibitor of thioredoxin-1, administered as a 72-hour infusion every 21 days in patients with advanced cancers refractory to standard therapy. *Investigational New Drugs*, *30*(4), 1591–1596. https://doi.org/10.1007/s10637-011-9739-9

Ratts, R., Zeng, H., Berg, E. A., Blue, C., McComb, M. E., Costello, C. E., ... Murphy, J. R. (2003). The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *The Journal of Cell Biology*, *160*(7), 1139–1150. https://doi.org/10.1083/jcb.200210028

Rossetto, O., Pirazzini, M., & Montecucco, C. (2014). Botulinum neurotoxins: Genetic, structural and mechanistic insights. *Nature Reviews. Microbiology*, *12*(8), 535–549. https://doi.org/10.1038/nrmicro3295

Rummel, A. (2016). Two feet on the membrane: Uptake of clostridial neurotoxins. *Current Topics in Microbiology and Immunology*, *406*, 1–37.

Saito, I., Asano, T., Sano, K., Takakura, K., Abe, H., Yoshimoto, T., ... Ishibashi, S. (1998). Neuroprotective effect of an antioxidant, ebselen, in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage. *Neurosurgery*, *42*(2), 269–277. https://doi.org/10.1097/00006123-199802000-00038

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. R., & Montecucco, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*, *359*(6398), 832–835. https://doi.org/10.1038/359832a0

Schiavo, G., Papini, E., Genna, G., & Montecucco, C. (1990). An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. *Infection and Immunity*, *58*(12), 4136–4141.

Schuster, M., Schnellm, L., Feiglm, P., Birkhoferm, C., Mohrm, K., Roeder, M., ... Barth, H. (2017). The Hsp90 machinery facilitates the transport of diphtheria toxin into human cells. *Scientific Reports*, *7*(1), 613. https://doi.org/10.1038/s41598-017-00780-x

Schwab, M. E., Suda, K., & Thoenen, H. (1979). Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. *The Journal of Cell Biology*, *82*(3), 798–810. https://doi.org/10.1083/jcb.82.3.798

Simpson, L. (2013). The life history of a botulinum toxin molecule. *Toxicon*, *68*, 40–59. https://doi.org/10.1016/j.toxicon.2013.02.014

Simpson, L. L., Maksymowych, A. B., Park, J. B., & Bora, R. S. (2004). The role of the interchain disulfide bond in governing the pharmacological actions of botulinum toxin. *The Journal of Pharmacology and Experimental Therapeutics*, *308*(3), 857–864. https://doi.org/10.1124/jpet.103.058149

Smith, L. D. S., & Sugiyama, H. (1988). *Botulism: The organism, its toxins, the disease*. Springfield, Illinois: Charles C. Thomas Publisher, Limited.

Takamori, S., Holt, M., Stenius, K., Lemke, E. A., Gronborg, M., Riedel, D., ... Jahn, R. (2006). Molecular anatomy of a trafficking organelle. *Cell*, *127*(4), 831–846. https://doi.org/10.1016/j.cell.2006.10.030

Tehran, D., & Pirazzini, M. (2018). Novel botulinum neurotoxins: Exploring underneath the iceberg tip. *Toxins (Basel)*, *10*(5). https://doi.org/10.3390/toxins10050190

Tonissen, K. F., & Di Trapani, G. (2009). Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy. *Molecular Nutrition & Food Research*, *53*(1), 87–103. https://doi.org/10.1002/mnfr.200700492

Van Valen, L. (1974). Two modes of evolution. *Nature*, *252*(5481), 298–300.

Vouking, M. Z., Tadenfok, C. N., & Ekani, J. M. E. (2017). Strategies to increase immunization coverage of tetanus vaccine among women in sub Saharan Africa: A systematic review. *The Pan African Medical Journal*, *27*(Suppl 3), 25–25.

Weinstein, L. (1973). Tetanus. *The New England Journal of Medicine*, *289*(24), 1293–1296. https://doi.org/10.1056/NEJM197312132892408

Williamson, C. H., Sahl, J. W., Smith, T. J., Xie, G., Foley, B. T., Smith, L. A., ... Hill, K. (2016). Comparative genomic analyses reveal broad diversity in botulinum-toxin-producing *Clostridia*. *BMC Genomics*, *17*, 180. https://doi.org/10.1186/s12864-016-2502-z

Yamaguchi, T., Sano, K., Takakura, K., Saito, I., Shinohara, Y., Asano, T., & Yasuhara, H. (1998). Ebselen in acute ischemic stroke: A placebo-controlled, double-blind clinical trial. Ebselen Study Group. *Stroke*, *29*(1), 12–17.

Zanetti, G., Azarnia Tehran, D., Pirazzini, M., Binz, T., Shone, C. C., Fillo, S., ... Montecucco, C. (2015). Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism. *Biochemical Pharmacology*, *98*(3), 522–530. https://doi.org/10.1016/j.bcp.2015.09.023

Zhang, S., Lebreton, F., Mansfield, M. J., Miyashita, S. I., Zhang, J., Schwartzman, J. A., & Dong, M. (2018). Identification of a botulinum neurotoxin-like toxin in a commensal strain of *Enterococcus faecium*. *Cell Host & Microbe*, *23*(2), 169–176.e166. https://doi.org/10.1016/j.chom.2017.12.018

Zhang, S., Masuyer, G., Zhang, J., Shen, Y., Lundin, D., Henriksson, L., Stenmark, P. (2017). Identification and characterization of a novel botulinum neurotoxin. 8, 14130.

Zornetta, I., Azarnia Tehran, D., Arrigoni, G., Anniballi, F., Bano, L., Leka, O., ... Montecucco, C. (2016). The first non *Clostridial* botulinum-like toxin cleaves VAMP within the juxtamembrane domain. *Scientific Reports*, *6*, 30257. https://doi.org/10.1038/srep30257

Zuverink, M., Chen, C., Przedpelski, A., Blum, F. C., & Barbieri, J. T. (2015). A heterologous reporter defines the role of the tetanus toxin interchain disulfide in light-chain translocation. *Infection and Immunity*, *83*(7), 2714–2724. https://doi.org/10.1128/IAI.00477-15