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The thioredoxin reductase – Thioredoxin redox system cleaves the interchain disulphide bond of botulinum neurotoxins on the cytosolic surface of synaptic vesicles

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ABSTRACT

Botulinum neurotoxins (BoNTs) are Janus toxins, as they are at the same time the most deadly substances known and one of the safest drugs used in human therapy. They specifically block neurotransmission at peripheral nerves through the proteolysis of SNARE proteins, i.e. the essential proteins which are the core of the neuroexocytosis machinery. Even if BoNTs are traditionally known as seven main serotypes, their actual number is much higher as each serotype exists in many different subtypes, with individual biological properties and little antigenic relations. Since BoNTs can be used as biological weapons, and the only currently available therapy is based on immunological approaches, the existence of so many different subtypes is a major safety problem. Nevertheless, all BoNT isoforms are structurally similar and intoxicate peripheral nerve endings via a conserved mechanism. They consist of two chains linked by a unique disulphide bond which must be reduced to enable their toxicity. We found that thioredoxin 1 and its reductase compose the cell redox system responsible for this reduction, and its inhibition via specific chemicals significantly reduces BoNTs activity, *in vitro* as well as *in vivo*. Such molecules can be considered as lead compounds for the development of pan-inhibitors.

1. Introduction

Botulinum neurotoxins, together with Tetanus neurotoxin (TeNT), form the family of clostridial neurotoxins (CNTs). They are the most lethal bacterial toxins, so toxic that the lethal dose for humans is estimated to be around 1 ng per kg of body weight (Gill, 1982). Such potency can be ascribed to their specific inhibition of a fundamental physiologic event, that of neurotransmission. BoNTs specifically bind and intoxicate the neuromuscular junction, where they block neurotransmitter release, resulting in a progressive flaccid paralysis (Johnson and Montecucco, 2008; Rossetto et al., 2014). In contrast, TeNT binds to peripheral nerves, is retrogradelly transported inside motor axons and taken up within inhibitory interneurons in the spinal cord. Here, it blocks neurotransmitter release with a molecular mechanism identical to BoNTs, but causing a spastic paralysis (Schiavo et al., 2000). Notably, CNTs do not cause the degeneration of poisoned nerve, and, if mechanical ventilation is timely performed, affected individuals survive and fully recover. Exploiting this feature, BoNTs have become extraordinary therapeutics for the treatment of many pathological conditions caused by the hyperactivity of cholinergic terminals, and one of the safest and most versatile drug available on the market (Hallett et al., 2013; Naumann et al., 2013; Rossetto et al., 2001). In addition, thanks to the comprehension of their mechanism of action, CNTs have become useful and sophisticated tools for the study of neuronal physiology (Pantano and Montecucco, 2013).

CNTs are produced by different species of the genus Clostridium (Rossetto et al., 2014). There is one single tetanus neurotoxin but many different botulinum neurotoxins. They have been

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traditionally classified as seven main distinct toxins (BoNT/A-G), distinguished on the basis of their immunological properties (serotypes). Recently, the development of next generation sequencing has permitted the analysis of clinical cases of botulism accumulated over time. As a result, it has become rapidly clear that neurotoxigenic Clostridia have considerable genetic heterogeneity in terms of genome organization, toxin gene clusters, and most importantly, toxin sequences variability (Hill and Smith, 2013). Accordingly, the number of BoNT subtypes has dramatically grown, reaching more than forty molecules in a few years, and this number is continuously increasing (Montecucco and Rasotto, 2015). They have been categorized as subserotypes, i.e. toxins immunogenically related to the parental serotypes but with an aminoacidic composition difference higher than 2.6% (indicated as BoNT/A1, BoNT/A2, etc.) (Hill and Smith, 2013; Rossetto et al., 2014; Smith et al., 2007). At variance, some others are composed by the recombination of different serotypes: accordingly, they have been classified as mosaic toxins and indicated as BoNT/CD and/DC (Moriishi et al., 1996a, 1996b). Recently, a toxin isolated from a case of infant botulism was proposed to be a new serotype, but later analysis showed it to be a chimera of A1 and F5 (Kalb et al., 2015). Remarkably, this finding raised the issue of serotype definition, but, most importantly, this concretely embodies the limitation of using antisera for the treatment of botulism, and questions the possibility of developing a universal vaccine covering all the BoNTs. This is even more relevant as BoNTs can be employed as bioweapons (Centers for Disease Control and Prevention, 2012). This calls for implementing more studies aiming at the discovery of new inhibitors capable of blocking BoNTs regardless of their antigenic differences.

2. BoNTs mechanism of action as target for new inhibitors

The available crystallographic structures of BoNTs (Kumaran et al., 2009; Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000) show they share an overall highly conserved molecular architecture, which is functional to their mechanism of action. The structure, which is similar for TeNT too, is constituted by two main chains: L, 50 kDa and H, 100 kDa, held together by a single interchain disulphide bond and non-covalent interactions. The C-terminal part of H (HC) mediates the neurospecific binding and the internalization of the toxin into peripheral nerve terminals via a double receptor mechanism (Montecucco, 1986; Rummel, 2013). Firstly, BoNTs accumulate on the plasma membrane via the recognition of polysialogangliosides, molecules highly enriched in the neuronal plasma membrane (Binz and Rummel, 2009). Thereafter, BoNTs enter the nerve as a result of the interaction with the luminal domain of a synaptic vesicle protein, which may differ for the different BoNTs (Benoit et al., 2014; Dong et al., 2008, 2003, 2007, 2006; Mahrhold et al., 2006; Nishiki et al., 1994; Peng et al., 2012; Rummel et al., 2004). At variance, nidogen 1 and 2, two extracellular matrix proteins, have been suggested to be the peripheral receptor of TeNT (Bercsenyi et al., 2014), mediating its entry into vesicles undergoing retrograde axonal transport up to the spinal cord (Deinhardt et al., 2006). TeNT is then delivered to inhibitory interneurons where is internalized inside SV (Matteoli et al., 1996), as BoNTs do. Here, the HN domain of TeNT and BoNTs assist the membrane translocation of the L chain in a process driven by the luminal acidification (Fischer and Montal, 2007b; Montal, 2010). The L chain is a Zn^{2+} dependent metalloprotease that targets specifically the three SNARE proteins: BoNT/B,/D,/F,/G and TeNT cleave VAMP1/2 at different positions (Schiavo et al., 1992, 1993a, 1993c), BoNT/A and/E cleave two different peptide bonds away from the C-terminus of SNAP25 (Blasi et al., 1993; Schiavo et al., 1993b), BoNT/C is unique as it cleaves both SNAP25 and Syntaxin1A-1B (Pantano and Montecucco, 2013). SNAREs form a heterotrimeric complex (Sutton et al., 1998) that constitutes the core of the neuroexocytosis nanomachine and their proteolysis impairs its assembly and/or function (Pantano and Montecucco, 2013).

Remarkably, each molecule may exert the same toxic mechanism with its own specific features, i.e. different binding partners, different trafficking, different substrates and exclusive enzyme–substrate interactions. At the same time, it is even more surprising that despite of a great variability within their primary structure, CNTs have evolved to have a high similar mechanism to exploit nerve terminals: this offers the possibility to rationally design new molecules capable of inhibiting BoNTs, independently from their antigenic properties.

3. The interchain disulphide bond reduction as a rational target

Besides the structural role, the interchain disulphide bond plays a fundamental role in BoNTs toxicity. The first evidence was provided by the lack of toxicity *in vivo* of a previously reduced tetanus neurotoxin (Schiavo et al., 1990). Later, an elegant study of BoNT dynamics at single molecule resolution (Fisher and Montal, 2006) demonstrated that an intact disulphide linkage is an essential prerequisite for productive translocation across the channel arranged by HN, and that reduction occurs on the cytosolic side (Fischer and Montal, 2007a). Accordingly, it was later proposed that the positioning, the size and the high hydrophobicity of the two sulfur atoms, together with a group of conserved acidic and protonable aminoacids within the same surface of a BoNT molecule, are pivotal in initiating the translocation event (Pirazzini et al., 2011; Rossetto et al., 2014). Although the role of the disulfide bond during translocation is known in molecular details only for BoNT/A, none of the different serotypes display the protease activity unless the interchain disulphide is reduced (Schiavo et al., 1993b). Accordingly, the aforementioned mechanism for BoNT/A can be safely extended to all other toxin isoforms, leading to the more general deduction that the reduction of the interchain disulphide bond within nerve terminal cytosol is a "*conditio sine qua non*" to free the metalloprotease activity of botulinum neurotoxins, and therefore represents a rationale for the development of mechanism-based antitoxins.

4. Thioredoxin–thioredoxin reductase inhibitors as a new class of antitoxins

Cells possess many different redox systems and their presence within key compartments (nucleus, mitochondria, endoplasmic reticulum and cytosol) explains their cardinal role in managing redox reactions (Arner and Holmgren, 2000; Hanschmann et al., 2013; Holmgren, 1985; Powis and Kirkpatrick, 2007). Notably, the understanding of redox biochemistry has rapidly and radically evolved over the last few years. The initial picture of an overall redox balance that must be simply maintained to avoid pathological conditions, has turned into a complex network of specific, compartmentalized and reversible redox reactions regulating the activity of key proteins involved in many intracellular, as well as extracellular, physiologic events (Hanschmann et al., 2013). The reducing system responsible for the disulphide reduction of BoNTs was shown to act on the cytosolic side (Fischer and Montal, 2007a). The thioredoxin 1-thioredoxin reductase 1 (TrxR-Trx) and the glutathione–glutathione reductase systems are important redox systems in the cytosol and those involved in controlling protein disulphides. They act via the so called "dithiol mechanism" through which an electron flow is shuttled from NADPH to thioredoxin or