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Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism

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ABSTRACT

Botulinum neurotoxins (BoNTs) form a growing family of metalloproteases with a unique specificity either for VAMP, SNAP25 or syntaxin. The BoNTs are grouped in seven different serotypes indicated by letters from A to G. These neurotoxins enter the cytosol of nerve terminals *via* a 100 kDa chain which binds to the presynaptic membrane and assists the translocation of a 50 kDa metalloprotease chain. These two chains are linked by a single disulfide bridge which plays an essential role during the entry of the metalloprotease chain in the cytosol, but thereafter it has to be reduced to free the proteolytic activity. Its reduction is mediated by thioredoxin which is continuously regenerated by its reductase. Here we show that inhibitors of thioredoxin reductase or of thioredoxin prevent the specific proteolysis of VAMP by the four VAMP-specific BoNTs: type B, D, F and G. These compounds are effective not only in primary cultures of neurons, but also in preventing the *in vivo* mouse limb neuroparalysis. In addition, one of these inhibitors, Ebselen, largely protects mice from the death caused by a systemic injection. Together with recent results obtained with BoNTs specific for SNAP25 and syntaxin, the present data demonstrate the essential role of the thioredoxin–thioredoxin reductase system in reducing the interchain disulfide during the nerve intoxication mechanism of all serotypes. Therefore its inhibitors should be considered for a possible use to prevent botulism and for treating infant botulism.

1. Introduction

Several species of anaerobic bacteria of the genus *Clostridium* produce botulinum neurotoxins which belong to seven different serotypes (BoNT/A-/G) [1,2]. Their number is rapidly growing and many different sub-serotypes are presently known. The biological and toxicological properties of these novel BoNTs are poorly understood, but the limited amount of experimental data indicate that they act predominantly at peripheral cholinergic nerve terminals, causing a long lasting blockade of acetylcholine release with ensuing paralysis of skeletal and autonomic nerve terminals, characteristic of botulism [3]. Apart from BoNT/D [4–6], BoNTs are the most toxic poisons for humans and are classified as potential bioterrorist weapons [7,8]. This extremely high toxicity results from their neurospecificity and from their catalytic activity, which leads to knock-out of proteins essential to the neurotransmitter release apparatus [2,9]. All BoNTs consist of a metalloprotease light chain (L, 50 kDa) and a heavy chain (H, 100 kDa) linked by a strictly conserved interchain disulfide bond. This molecular structure has been shaped during evolution in order to exploit essential physiological features of the vertebrate nervous system. Indeed BoNTs bind specifically to peripheral nerve terminals presynaptic membrane [10] *via* the C-terminus of the H chain which interacts with polysialogangliosides leading to toxin accumulation. The subsequent binding to a protein receptor, transiently exposed on the membrane, is harnessed for their endocytosis [11,12]. In the case of BoNT/A the endocytic organelles were identified as synaptic vesicles [13,14]. Similar data are not available for the other BoNTs, but several experiments performed with vacuolar ATPase proton pump inhibitors clearly indicate that all these neurotoxins enter the lumen of an acidic compartment [15,16]. Indeed it is established that all serotypes have to undergo a low-pH driven

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membrane translocation of the L chain, mediated by the N-terminal part of H chain [9,17,18]. Once on the cytosolic side, the L metalloprotease remains attached to the H chain *via* the interchain disulfide bridge. This bond is strictly conserved among serotypes, sub-serotypes and also tetanus neurotoxin, which is structurally and functionally related to BoNTs. Remarkably, the premature reduction of this disulfide completely abrogates the toxicity of all clostridial neurotoxins, underscoring its fundamental role in the intoxication process [19–23]. The reduction of this bond is essential to release the catalytic activity of the L metalloprotease within the cytosol versus the three SNARE proteins [20]. Indeed, also in the test-tube BoNTs cannot cleave their recombinant substrates unless this linkage is reduced [24,25]. Once enabled through reduction, the L chain of BoNT/B, /D, /F and /G cleave VAMP at different peptide bonds, BoNT/A and /E cleave SNAP25, while BoNT/C is particular because it is the only one capable to cleave two substrates, SNAP25 and syntaxin [26,27].

We recently reported that the thioredoxin reductase (TrxR)–thioredoxin (Trx) redox system is present on the cytosolic surface of synaptic vesicles and that its inhibition with specific drugs very effectively prevented the neuroparalysis induced by the three SNAP25 specific BoNTs (A, C and E) [28,29]. Here, we extended the study to the four VAMP-specific BoNTs (B, D, F and G) [16,30] using the four chemicals whose structures are shown in Fig. 1 and which are well characterized inhibitors of TrxR–Trx system. Myricetin is a flavonoid which reacts with the selenium atom present in the active site of the reduced TrxR, providing its irreversible inhibition [31]. Curcumin is a polyphenol of vegetal origin that irreversibly inhibits TrxR forming a 1:2 adduct [32]. In both cases, the direct consequence of inhibition is the loss of Trx reducing potential. PX12 acts mainly on thioredoxin by alkylating a non-catalytic cysteine residue, generating a steric hindrance that prevents the interaction with its reductase. As a result, Trx remains permanently in the oxidized, inactive, form [33,34]. Ebselen acts on both members of the redox couple, as it is an excellent substrate for the mammalian TrxR and a highly efficient oxidant of reduced Trx. Thus, Ebselen prevents the normal function of both enzymes [35].

Together with our previous reports [28,36], the present results provide a strong indication that the reduction of the single interchain disulfide bond is a newly identified key event in nerve intoxication of all BoNTs. We therefore propose that TrxR–Trx inhibitors can be considered as a novel and general class of anti-BoNTs drugs and discuss their possible use in humans.

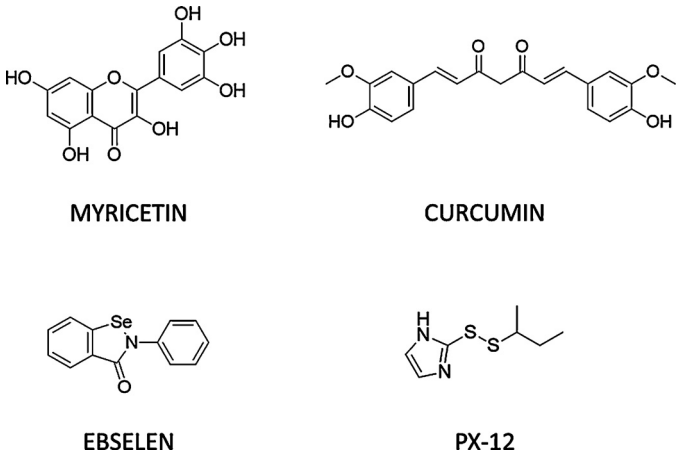

Fig. 1. Thioredoxin–thioredoxin reductase inhibitors used in this study. 3,3′,4′,5,5′,7-Hexahydroxyflavone (Myricetin) and (E,E)-1,7-bis(4-hydroxy-3 methoxyphenyl)-1,6 heptadiene-3,5-dione (Curcumin) preferentially inhibit thioredoxin reductase, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) both thioredoxin and thioredoxin reductase and 2-[(1-methylpropyl) dithio]-1H-imidazole (PX12) inhibits thioredoxin.

2. Materials and methods

2.1. Reagents

3,3′,4′,5,5′,7-Hexahydroxyflavone (Myricetin), (E,E)-1,7-bis(4-hydroxy-3 methoxyphenyl)-1,6 heptadiene-3,5-dione (Curcumin), cytosine β-D-arabinoside, DNAse I and poly-L-lysine were purchased from Sigma– Aldrich. 2-[(1-Methylpropyl) dithio]-1H-imidazole (PX12) was purchased from Santa Cruz Biotechnology and 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) was purchased from Cayman Chemical. Antibodies: VAMP2 (104 211) and Syntaxin-1A (110 111) were from Synaptic System, SNAP25 (SMI81, ab24737) was from Abcam. Botulinum neurotoxins B, D and G were produced in *Escherichia coli via* recombinant methods [37–39] whilst BoNT/F was purified as previously described [40].

2.2. Neuronal cultures

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 6- to 8-days-old rats as previously described [41]. Briefly, cerebella were isolated, mechanically disrupted and then trypsinized in the presence of DNase I. Cells were then collected and plated into 24 well plates, pre-coated with poly-L-lysine (50 μg/ml), at a cell density of 4×10^5 cells per well. Cultures were maintained at 37 °C, 5% CO_2, 95% humidity in BME supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine and 50 μg/ml gentamicin (hereafter indicated as complete culture medium). To arrest growth of non-neuronal cells, cytosine arabinoside (10 μM) was added to the medium 18–24 h after plating.

2.3. Botulinum neurotoxins inhibition assay on CGNs

CGNs at 6–8 days *in vitro* (DIV) were incubated with increasing concentrations of the indicated inhibitor in complete culture medium for 30 min at 37 °C. Thereafter, the indicated toxin was diluted in complete culture medium and added to CGNs in order to obtain the following final concentrations: BoNT/B (2 nM) or BoNT/F (4 nM) or BoNT/G (4 nM). Incubation was prolonged for 12 h at 37 °C. In the case of BoNT/D, owing to its potency, the toxin was added at a final concentration of 0.025 nM and incubated for 15 min at 37 °C. The neuronal culture was then washed and the culture medium with the same concentration of inhibitor was restored for 12 h. Toxicity was evaluated following the specific proteolytic activity of BoNTs *via* immunoblotting with antibodies specific for VAMP2, SNAP25 and syntaxin. All inhibitors were dissolved in DMSO and stored at −80 °C.

2.4. Immunoblotting

Cells were directly lysed with Laemmli sample buffer containing protease inhibitors (complete Mini EDTA-free, Roche). Cell lysates were loaded onto a 4–12% NuPage gel (Life technologies) and separated by electrophoresis in 1X MES buffer (Life technologies). Proteins were transferred onto Protran nitrocellulose membranes (Whatman) and saturated for 1 h in PBST (PBS, 0.1% Tween 20) supplemented with 5% non-fatty milk. Incubation with primary antibodies was performed overnight at 4 °C. The membranes were then washed three times with PBST and incubated with secondary HRP-conjugated antibodies for 1 h. Finally, membranes were washed twice with PBST and once with PBS; visualization was carried out using Luminata Crescendo (Merck Millipore).

2.5. Immunocytochemistry

Neurons were seeded onto 13 mm round glasses in 24-well plates at a cell density of 4×10^5 cells per well. CGNs at 6–8 DIV were pre-incubated for 30 min with the indicated concentration of inhibitor in complete culture medium at 37 °C and 5% CO_2. BoNT/D was added to reach a final concentration of 0.2 nM and the incubation carried out for 10 min at 4 °C. Neurons were washed and the incubation with the indicated inhibitor in the same medium was prolonged for 90 min at 37 °C and 5% CO_2. After treatments, isolated CGNs were fixed for 10 min with 4% paraformaldehyde in PBS and processed for immunocytochemistry. Coverslips were mounted using Fluorescent Mounting Medium (Dako) and examined by epifluorescence (Leica DMIRE2) microscopy. BoNT/D activity was evaluated following the decrease of VAMP2 staining, detected with an antibody specific for the intact form of the protein.

2.6. Digit abduction score assay

Swiss-Webster adult male CD1 mice weighing 26–28 g were housed under controlled light/dark conditions, and food and water were provided *ad libitum*. All experiments were performed in accordance with the European Communities Council Directive n° 2010/63/UE and approved by the Italian Ministry of Health. Curcumin and PX12 were dissolved in ethanol to make stock solutions (10 mg/ml and 5 mg/ml, respectively and stored at −20 °C), whilst Ebselen was dissolved in DMSO (10 mg/ml and stored at −20 °C). A freshly opened aliquot of Ebselen must be used. Hind limb skeletal muscles were injected (total injection volume 25 μl) with 0.02 mg of Curcumin or PX12 or Ebselen or vehicle alone (8% ethanol or DMSO in 0.9% NaCl with 0.2% gelatin). After 30 min, muscles were further injected with BoNT/B (0.5 pg/g) or BoNT/D (0.02 pg/g) or BoNT/F (2 pg/g) or BoNT/G (5 pg/g) or vehicle alone (0.9% NaCl with 0.2% gelatin). Hind limbs paralysis was evaluated at least once per day using the Digit Abduction Score (DAS) assay, performed as previously reported [42,43].

2.7. Lethality assay

Swiss-Webster adult male CD1 mice weighing 24–26 g were housed under controlled light/dark conditions, and food and water were provided *ad libitum*. All experiments were performed in accordance with the European Communities Council Directive n° 2010/63/UE and approved by the Italian Ministry of Health. A stock solution of Ebselen in DMSO was prepared (7.5 mg/ml). Mice were conditioned for 3 days with intraperitoneal (i.p.) injections of Ebselen at a dose of 7.5 mg/kg or with vehicle (DMSO) every 12 h. Each experiment was conducted with a freshly opened aliquot of Ebselen. The third day, BoNT/B or BoNT/D or BoNT/F was prepared as a stock solution (BoNT/B 0.9 pg/μl, BoNT/D 0.04 pg/μl and BoNT/F 2.5 pg/μl in 0.9% NaCl with 0.2% gelatin), and 30 min after the injection of the last inhibitor dose, mice were weighted and i.p. injected with 1 μl/g body weight, roughly corresponding to a 2 fold MLD_{50} for each toxin. The respective MLD_{50} have been determined through preliminary experiments: BoNT/B 0.45 ng/kg, BoNT/D 0.02 ng/kg and BoNT/F 1.25 ng/kg). Mice were monitored every 4 h for 96 h, at which the experiment was considered ended.

3. Results

3.1. Inhibitors of thioredoxin reductase and thioredoxin prevent cleavage of VAMP by botulinum neurotoxins type B, D, F and G in cultured neurons

The most convenient and rapid way to screen the ability of TrxR–Trx inhibitors in blocking the VAMP-specific BoNTs toxicity is the use of sensitive neuronal cultures. Fig. 2 shows that, upon overnight incubation of primary cultures of cerebellar granular neurons, 2 nM BoNT/B cleaves its substrate, as evaluated by

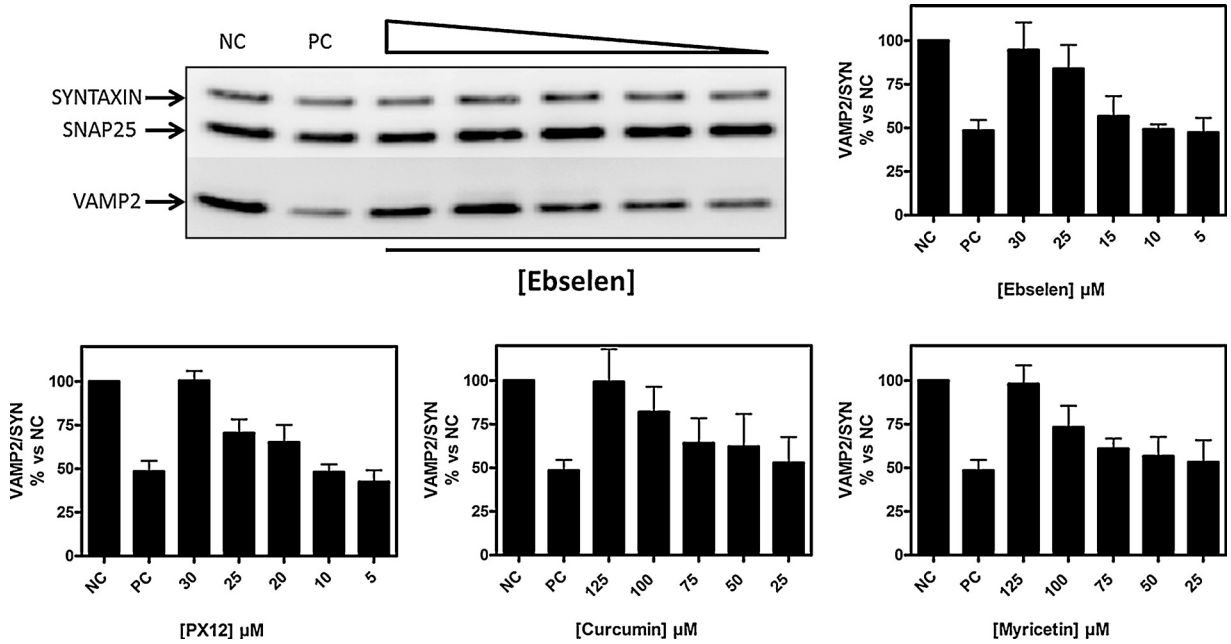

Fig. 2. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/B-induced cleavage of VAMP2 in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitors at 37 °C for 30 min. 2 nM BoNT/B was then added and incubation prolonged for 12 h at 37 °C; cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

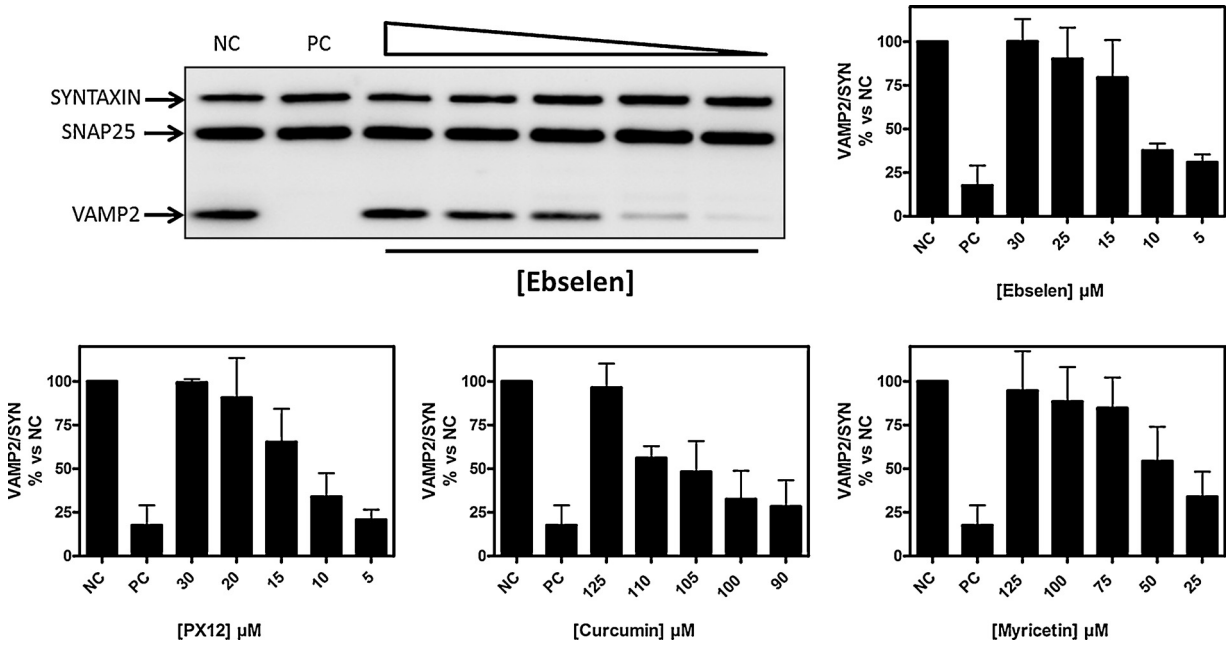

Fig. 3. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/D-induced cleavage of VAMP2 in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitor at 37 °C for 30 min. BoNT/D 0.025 nM was added for 15 min, cells were washed, and culture medium with the same concentration of inhibitor was restored and incubation prolonged for 12 h at 37 °C. Cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

western blotting with an antibody specific for the intact form of the protein; a residual portion of VAMP2 not accessible to the metalloprotease L chain of BoNT/B is a common finding [19,44,45]. At the same time, Ebselen, PX12, Curcumin and Myricetin prevent the cleavage of VAMP2 in a dose dependent mode. Importantly, at the doses used here, these drugs do not affect cell viability, nor do they inhibit the cleavage of a recombinant VAMP2 (not shown).

Fig. 3 shows that BoNT/D is particularly active in CGNs and a concentration as low as 0.025 nM cleaves nearly all VAMP2. Nevertheless, the four compounds are effective in blocking such an activity with a profile of concentration dependence similar to that found with BoNT/B (Fig. 2), as determined by immunoblotting. This is even more evident in Fig. 4, which documents their effect using an immunofluorescence analysis. Importantly, the two assays provide fully consistent results even though different toxin concentrations, binding and incubation times were used. This reinforces the conclusion about the preventing activity of the four drugs used here.

We have no a direct explanation for the much higher potency of BoNT/D in VAMP2 cleavage with respect to BoNT/B, but it could be ascribed to a faster entry into the cytosol of BoNT/D than BoNT/B, which was reported to be particularly slow in cell cultures [44]. This may also explain why, when TrxR is not completely inhibited as in the case of lower concentrations of Curcumin, BoNT/D activity appears to be less blocked as compared to BoNT/B. Nevertheless, the comparable inhibition profile of these two BoNTs strongly indicates that the reduction of the interchain bond, catalyzed by the TrxR–Trx system, is of similar and essential importance to enable their intraneuronal catalytic activity. This conclusion is reinforced by the similar data obtained using BoNT/F and BoNT/G, whose inhibition profiles are reported in Fig. 5 and Fig. 6, respectively. Importantly, we achieved this result by using four different compounds, which belong to different chemical classes, have different molecular structures as well as different mechanisms to inhibit TrxR–Trx system.

3.2. Inhibitors of thioredoxin reductase and thioredoxin effectively lower the reversible flaccid paralysis induced by botulinum neurotoxins type B, D, F and G in mice

One remarkable aspect of BoNTs action *in vivo* is that they induce a reversible peripheral neuroparalysis. This property is clearly documented by the black traces in the panels of Fig. 7 which shows the recovery time course of mouse limb muscles function after the paralysis induced by a single local injection of BoNT/B, /D, /F and /G in sub-lethal doses. The neuromuscular paralytic effect was evaluated over time with the well-established DAS assay [42,43], which assigns a score to the severity of muscles paralysis according to the capability of the mouse to move the hind limb fingers, upon injection of BoNTs close to the EDL (*Extensor Digitoris Longus*) muscle. This score ranges from 0 (no paralysis) up to 4 (all fingers are paralyzed). Even though the paralytic effect exerted by the four BoNTs has different durations, it is worth noting that the local injection of the TrxR–Trx inhibitors effectively reduced both the severity and the duration of the paralysis. Owing to the very large number of sampling required by this type of analysis, we used three different inhibitors to dissect the entire TrxR–Trx system: Curcumin (inhibitor of TrxR), PX12 (inhibitor of Trx), and Ebselen (inhibitor of TrxR and Trx). All of them very effectively prevented the peripheral neuroparalysis induced by the four different BoNTs, with Ebselen being slightly more potent (Fig. 7).

3.3. Ebselen effectively prevents mice death caused by botulinum neurotoxins type B, D and F

On the basis of the results reported above, it became very relevant to test the capacity of the TrxR–Trx inhibitors in preventing the development of botulism upon systemic delivery of the four neurotoxins. Since such tests would have required a very large number of animals, we confined the experiments to one of the drugs. Ebselen was chosen because it best protected against paralysis in the DAS assay and because it has been used in clinical

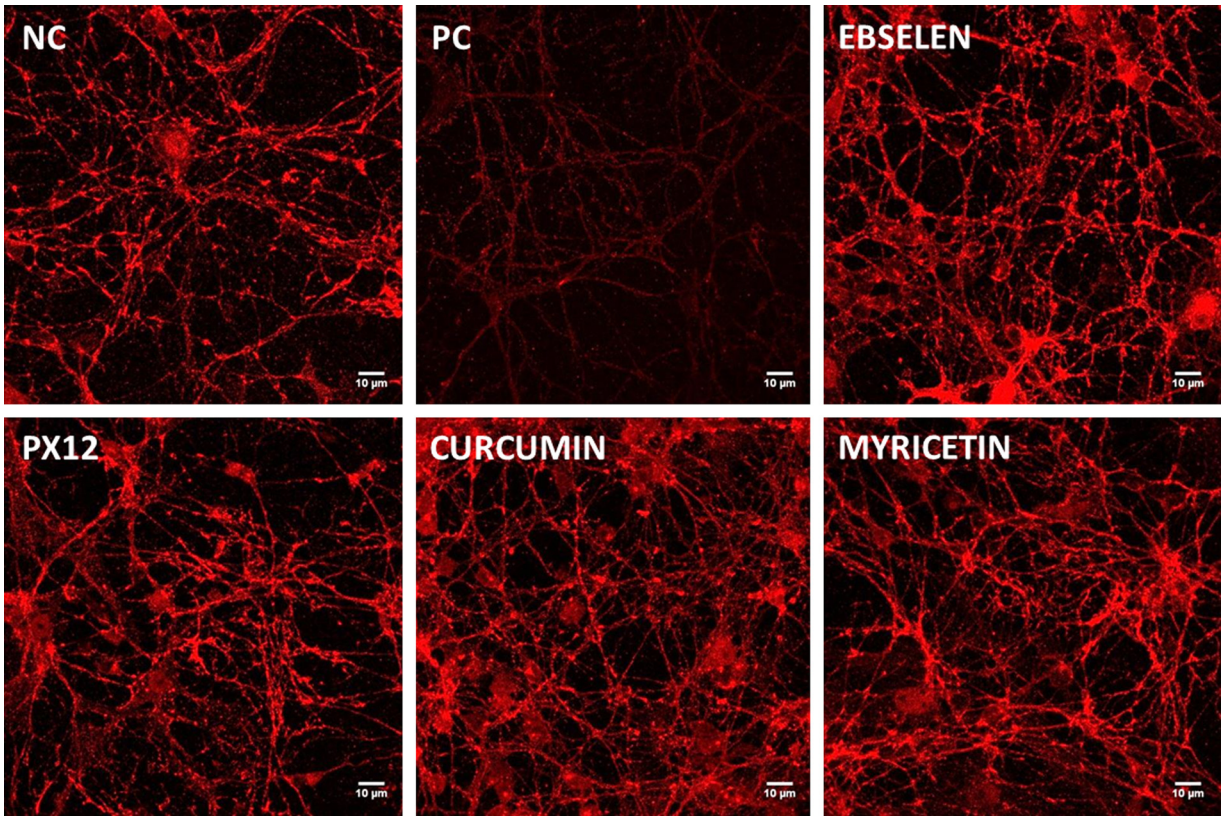

Fig. 4. Immunocytochemical evaluation of BoNT/D activity blockage by Inhibitors of thioredoxin–thioredoxin reductase system. CGNs were treated with Trx–TrxR inhibitors (Curcumin 100 μM, Ebselen 30 μM, Myricetin 100 μM or PX12 25 μM) or vehicle (NC, no toxin added; PC, only toxin added) at 37 °C. After 30 min, BoNT/D 0.2 nM was added for 10 min at 4 °C after which neurons were washed and incubated with the same concentration of inhibitors for further 90 min at 37 °C. Thereafter neurons were fixed and VAMP2 cleavage was assessed using a specific antibody. The images are representative of three independent sets of experiments (scale bar 10 μm).

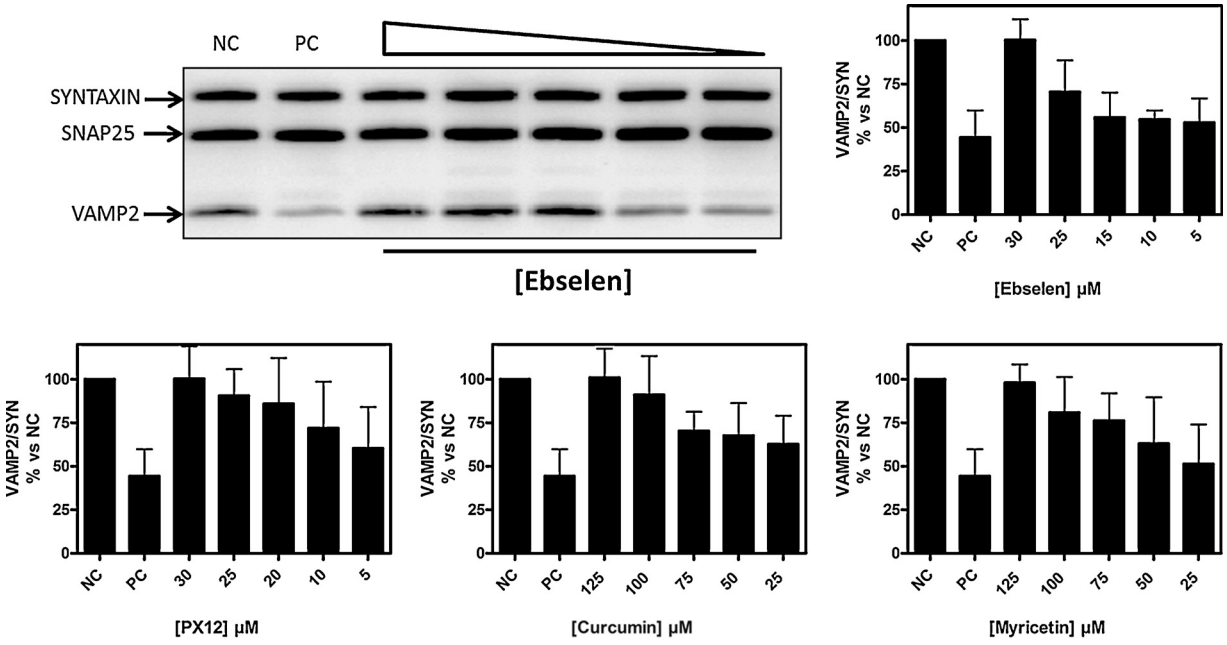

Fig. 5. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/F-induced cleavage of VAMP2 in in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitor at 37 °C for 30 min. 4 nM BoNT/F was then added and incubation prolonged for 12 h at 37 °C, cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

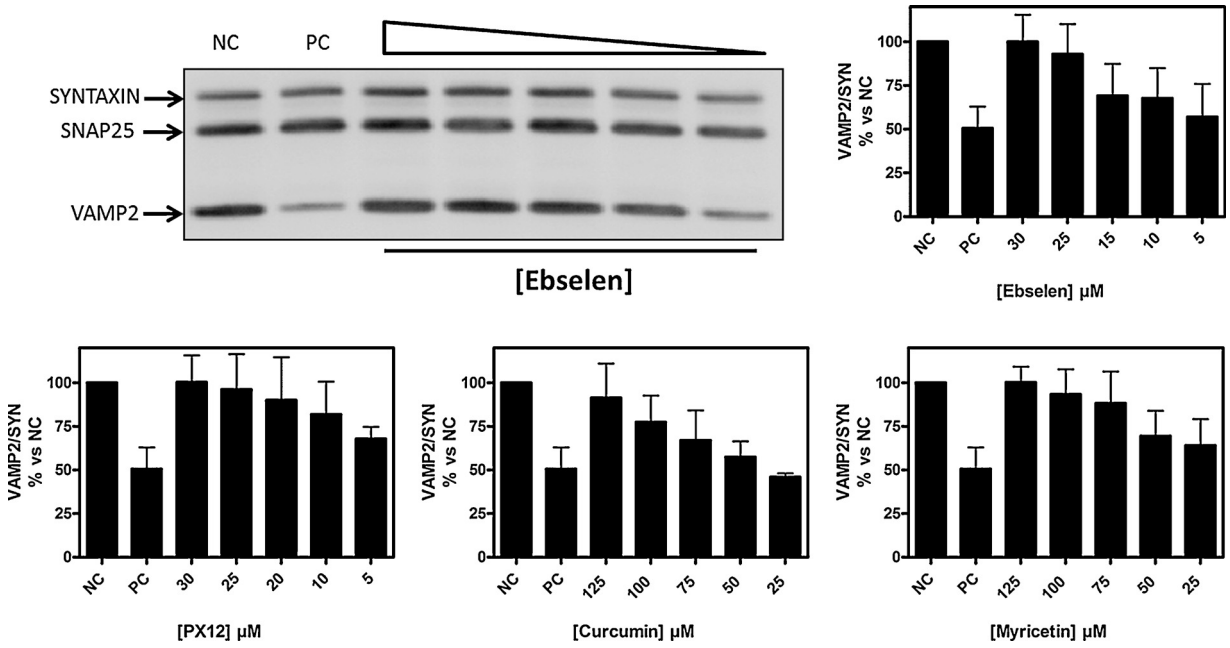

Fig. 6. Thioredoxin–thioredoxin reductase inhibitors prevent the BoNT/G-induced cleavage of VAMP2 in neuronal culture. CGNs were incubated with the indicated concentrations of inhibitor at 37 °C for 30 min. 4 nM BoNT/G was then added and incubation prolonged for 12 h at 37 °C, cells were then lysed and the VAMP2 content was estimated with an antibody recognizing the intact form of VAMP2. Syntaxin and SNAP25 staining was used as loading control. Upper left panel shows a representative immunoblot, obtained in experiments with Ebselen (NC, no toxin added; PC, only toxin added). Graphs show the quantification, of the experiments performed with the reported inhibitors, for VAMP2 determined as a ratio to Syntaxin staining, taking the value of non-treated cells as 100%. SD values derive from at least three independent experiments.

trials in humans for other diseases [46,47]. In addition, we focused our attention on BoNT/B and BoNT/F, because these serotypes are involved in human botulism and BoNT/D because it is often associated to animal botulism [2,3]. Type G was not tested because

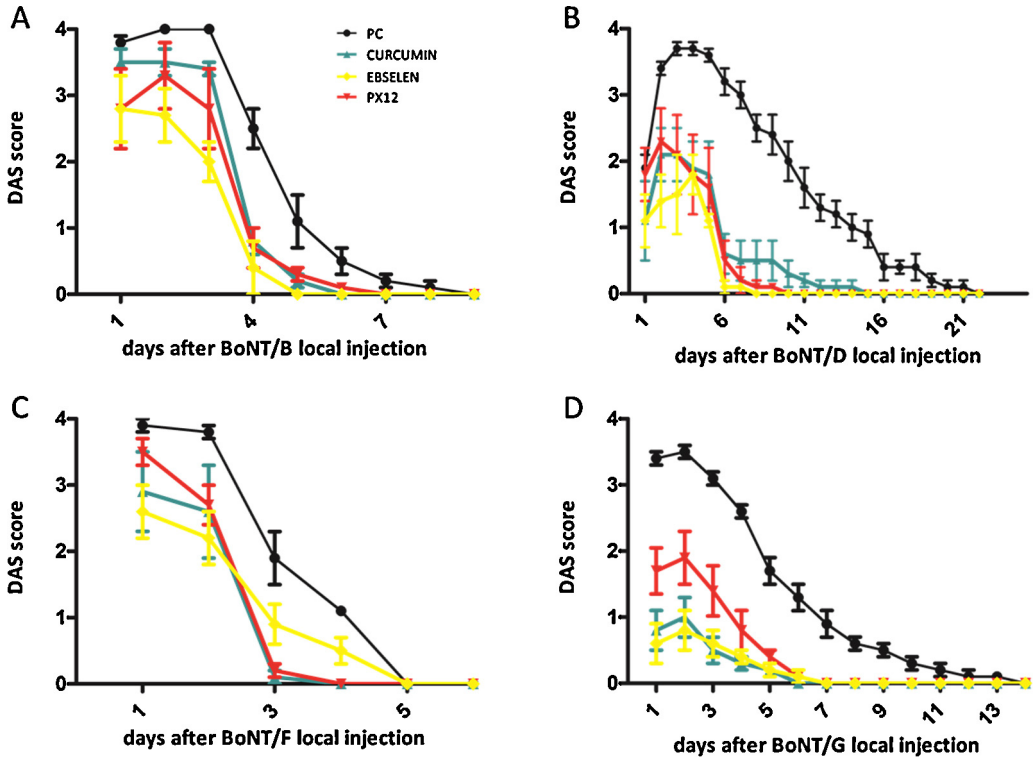

Fig. 7. Thioredoxin–thioredoxin reductase inhibitors decrease the local paralysis induced by BoNT/B, /D, /F and G. 0.02 mg of Curcumin (green traces) or PX12 (red traces) or Ebselen (yellow traces) or vehicle (PC, black traces) were injected in the hind limb of adult male CD1 mice. After 30 min the same hind limbs were injected with 0.5 pg/g of BoNT/B (A) or 0.2 pg/g of BoNT/D (B) or 2 pg/g of BoNT/F (C) or 5 pg/g of BoNT/G (D) and the severity of local paralysis was evaluated and reported as DAS score (see Section 2). DAS score of animals treated with only inhibitors are not shown for clarity. DAS values are means ± SEM from three individual experiments of at least eight animals per condition.

it has not been reported to cause botulism in humans or animals. Fig. 8 shows that Ebselen substantially protects mice from intraperitoneally injected BoNT/B, or BoNT/D or BoNT/F. Noteworthy, all mice treated displayed the symptoms typical of botulism, such as a generalized flaccid paralysis of muscles, but Ebselen-treated animals displayed milder symptoms and in several cases they were not severe enough to cause the respiratory failure or to prevent drinking and feeding that generally contribute to death of injected mice in the animal cage. Importantly, survivors fully recovered from developed symptoms in few days, suggesting that large amounts of the injected toxin did not reached VAMP.

4. Discussion

The results presented here are very relevant in several respects. The first one is that they provide experimental evidence that thioredoxin reductase-thioredoxin disulfide reducing system cleaves the single interchain disulfide bond of the four BoNT serotypes whose L chain proteolyses VAMP: BoNT/B, /D, /F and /G. Together with our previous reports on the SNAP25 and syntaxin cleaving BoNTs [28,36], it can now be concluded that this molecular event, which takes place on the cytosolic face of an intracellular acidic compartment [20], is an essential step of the cellular mechanism of intoxication of all BoNT serotypes. So essential, that it is sufficient to inhibit the TrxR–Trx redox system to completely prevent the toxicity of these very powerful neurotoxins in cultured neurons. This conclusion is even more important if one considers the large number of novel BoNTs that are being discovered [48,49]. They can be classified as subtypes of the main seven serotypes, which have now all been analyzed with respect to disulfide reduction (the present paper and [28,36]). Therefore, it can be concluded that the release in the cytosol of the L chain metalloprotease activity of all clostridial neurotoxins requires reduction of the interchain disulfide bridge by the TrxR–Trx system.

An important feature of BoNTs is their reversibility of action. This remarkable property has been exploited to evaluate the respective potency and duration of the different serotypes *in vivo* [50,51] through the DAS assay. This test is based on the intramuscular injection of a limited amount of BoNTs which can induce the local paralysis of mice hind legs without causing their death [42,43]. This is facilitated by a very limited diffusion from the site of injection, a feature which becomes very relevant in the therapeutic use of BoNTs, particularly when small muscles are injected [52,53]. Given that the intracellular degradation of the L chain seems to be the main reason of BoNTs reversibility of action, the duration of paralysis primarily depends on the amount L chains which have entered the nerve terminals, beside the intrinsic properties of the different L chains may also play a significant role [27,54,55]. Therefore, the second valuable result described here is that inhibitors of the TrxR–Trx system are general inhibitors of all BoNTs and are capable of preventing to a large extent their local neuroparalytic action in mice by reducing the number of L chains which have entered the nerve terminals. Accordingly, this result indicates the possible employment of these inhibitors in accidental events of over dosage of a BoNT during its therapeutic use.

We also assayed the efficacy of TrxR–Trx inhibitors in protecting mice from a systemic injection of BoNTs. This assay better recapitulates botulism, i.e., a generalized peripheral neuroparalysis which generally develops following the toxin absorption from a large organ such as the intestine (alimentary botulism) [2]. Botulism is reversible, provided that the intoxicated patient is mechanically ventilated to prevent death by respiratory muscles paralysis. Because of local regulations on experimentation involving animals, we could not assay the efficacy of all the inhibitors tested in neuronal cultures and capable of preventing local neuroparalysis (DAS assay), in mice lethality tests. However, using Ebselen we have provided a proof of principle that inhibitors of the TrxR–Trx system can effectively prevent the development of the flaccid paralysis caused by BoNT and protect a sizeable fraction of animals from deadly effects. Notably, the survivors recovered completely. This result is very relevant as it suggests that Ebselen, and probably the other TrxR–Trx inhibitors, can be used to prevent botulism in humans and in animals. This is also valid in the case of BoNT/D which, in our hands, is the most toxic of all BoNTs in mice with a MLD_{50} of 0.02 ng/kg to be compared with the literature data of 0.4 ng/kg [56].

On the basis of the present knowledge about the BoNTs mechanism of neuron intoxication, it is clear that once the reduction of the interchain disulfide bond has released the L chain metalloprotease activity, the inhibitors tested here are no longer effective. In other words, these inhibitors cannot be considered for the use after the symptoms of botulism have developed. Therefore the drugs tested here are to be considered as prophylactic and the limitation of a prophylaxis has been discussed before [57]. Notwithstanding, if given soon after diagnosis, these inhibitors may lessen symptoms severity by preventing the entry of circulating BoNT, and therefore shorten the period of hospitalization which is associated with the high costs of intensive care. It is indeed known that in adult botulism caused by the ingestion of BoNT poisoned food, there is a long persistence of the toxin in the general circulation [58,59]. Moreover, having a good record of safety in humans, as deduced from previous trials [60–65], these drugs may have a great potential in the treatment of human botulism where a continuous release of freshly produced BoNT takes place, which is the case of infant botulism [66]. In this form of the disease, the BoNT producing Clostridia colonize the intestine,

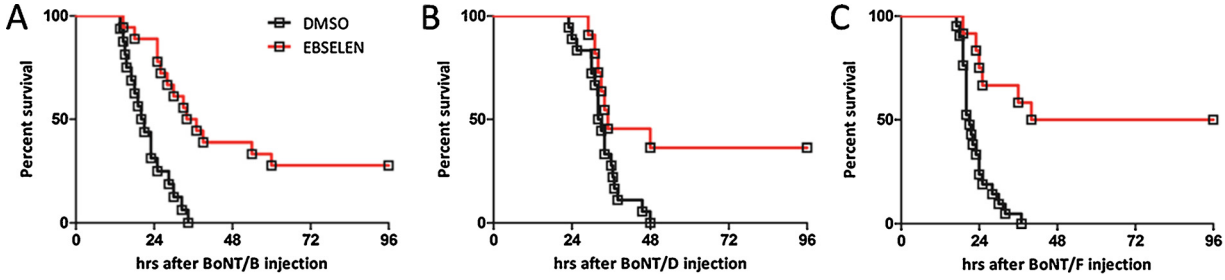

Fig. 8. Ebselen prevents the lethality of BoNT/B, /D and /F. Adult male CD1 mice were preconditioned with Ebselen 7.5 mg/kg ($n = 15$) or vehicle ($n = 15$) as described in Section 2. Thereafter, 2xMLD_{50} of BoNT/B (A) or BoNT/D (B) or BoNT/F (C) were injected i.p. Animals were monitored every 4 h for 96 h, after which the experiment was considered concluded. Graphs report the survival curves analyzed with the Log-rank (Mantel–Cox) test, and found to be significantly different (BoNT/B: $p < 0.0001$; BoNT/D: $p < 0.0058$; BoNT/F: $p < 0.0022$) from inhibitor free controls.

owing to the lack of competitive bacterial flora, and release the toxin in the general circulation for long periods of time [2,67].

There is growing interest in finding new mechanism-based antidotes against BoNTs, and some molecules were found to have beneficial potential (Fischer, Nakai et al., 2009). The main advantage of such molecules is that they act regardless of the serotypes causing envenomation. This is the more important in light of the large number of different BoNTs that are being discovered. These drugs can be administered without knowing the BoNT serotype and sub-type, thus saving the time required for toxin characterization. This is also relevant to those cases of botulism caused by Clostridia producing more than one BoNT (Barash and Arnon 2004, Barash and Arnon 2013, Dover, Barash et al., 2013, Maslanka, Lúquez et al., 2015).

Authors contributions

(1) Study conception and design: M.P. and C.M.
(2) Acquisition, analysis and/or interpretation of data: G.Z., D.A.T., M.P. and C.M.
(3) Drafting/revision of the work for intellectual content and context: M.P., G.Z., D.A.T., O.R., T.B. and C.M.
(4) Final approval and overall responsibility for the published work: G.Z., D.A.T., M.P., T.B., C.C.S., S.F., F.L., O.R. and C.M.

Conflict of interests

The authors declare that they have no conflict of interests.

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