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Use of botulinum toxins in cancer therapy

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A recent study has demonstrated for the first time that botulinum neurotoxin (BoNT) briefly opens tumour vessels, allowing more effective destruction of cancer cells by radiotherapy and chemotherapy. This review discusses the implications of BoNTs in cancer treatment. After briefly reviewing the different BoNT serotypes, their pharmacological activities and their general use in medicine, the authors focus on their possible application in cancer and describe how BoNTs have been used so far to treat spasm related to tumour or to therapies. By dissecting the mechanisms of action leading to a potentiation of anticancer therapy, it can be seen that BoNTs act by an effect on the tumour microenvironment rather than by a direct cytotoxic effect on tumour cells.

Keywords: chemotherapy, oxygenation, perfusion, radiotherapy, tumour microenvironment

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1. Introduction

A recent study by Ansiaux *et al.* [1], published in *Clinical Cancer Research* in 2006, demonstrated that botulinum neurotoxin (BoNT) briefly opens tumour vessels, providing an opportunity for more effective destruction of cancer cells by radiotherapy and chemotherapy. This paper describes, for the first time, how BoNT is helpful in targeting resistant tumours for treatment, and re-examines the significance of BoNTs in cancer treatment. After reviewing the different BoNT serotypes, their pharmacological activities and their general use in medicine, this review focuses on their possible application in cancer. How have they been used so far to treat spasms related to tumours or therapies? By dissecting the mechanisms of action leading to a potentiation of cancer therapy, is it possible to demonstrate a direct cytotoxic effect of BoNTs? Or should we rather look at their effect on the tumour microenvironment? These are the questions that this paper addresses and that the authors try to answer in the present review.

2. Types and structures of botulinum toxins

BoNTs are neurotoxins produced by an anaerobic Gram-positive sporulating organism called *Clostridium botulinum*. There are seven serotypes of BoNT (indicated with the letters A – G) that are produced by distinct *Clostridia*, differing in antigenicity and biochemical activity [2-7]. All of these serotypes share the same pharmacological activity: they all inhibit acetylcholine release from nerve terminals. Toxin types A, B, E and F are well-established causes of human botulism, whereas types C and D cause illness in other animals. Type G has not been established as a cause of either human or animal disease.

The toxin is synthesised in the bacterial cytosol as an inactive single-chain protein that is ~ 150 kDa and 1200 – 1300 amino acids [7,8]. The organisms that manufacture the BoNT-A and -B that are used in the clinic possess a protease that

nicks (cleaves) the molecule to create an active di-chain molecule in which the ~ 50-kDa light chain (L) and the ~ 100-kDa heavy chain (H; **Figure 1**) remain linked via a disulfide bond and noncovalent interactions [4-8]. The proportion of single to di-chain toxin is dependent on the toxin's serotype and whether or not the bacterial strain expresses the appropriate protease [9]; for example, BoNT-A is recovered from cultures that are > 95% nicked [10]. For type B, only a portion of neurotoxin molecules are nicked [11]. In contrast, certain strains of *Clostridia* (such as type E) are completely un-nicked (a single chain released) but this does not necessarily diminish the potential for disease because this single chain is then cleaved by exogenous (host) proteases [12-14]. To date, only the crystallographic structures of BoNT-A and B have been determined [15,16], revealing that the H chain (amino acids 449 – 1296) is composed of 2 domains (each ~ 50 kDa; **Figure 1**). The ~ 50-kDa receptor binding domain, termed H_C, consists of two subdomains. The N-terminal β-barrel part of H_C (HcN) consists of an amino acid sequence that is highly conserved among BoNTs, suggesting a closely similar three-dimensional structure. In contrast, the sequence of the C-terminal β-trefoil part of H_C (HcC) is poorly conserved and seems to play an important role in neurospecific binding. Those two subdomains are linked by an α-helix and create a cleft at the subdomain interface. The H_N domain presents two antiparallel α-helices similar to those of the membrane-interacting proteins. The H_N domain of BoNTs is highly homologous and is implicated in the transmembrane translocation of the L chain into the cytosol (by forming channels in cell membranes). The L chain of BoNTs (amino acids 1 – 448) is an endopeptidase with the same active site zinc-binding motif (His-Glu-X-X-His; **Figure 1**), which exerts its action in the cytosol of the contaminated nerve terminal, and cleaves proteins of the soluble NSF (*N*-ethyl maleimide-sensitive factor) attachment protein receptor (SNARE) complex [17-20]. Synaptobrevin, SNAP-25 and syntaxin form this SNARE heterotrimeric coil-coiled complex, which induces the juxtaposition of vesicles to the target membrane complex and is involved in their fusion and subsequently in the exocytosis of acetylcholine. BoNT serotypes are specific proteases that recognise and cleave specific proteins of the SNARE complex and subsequently cause distinct patterns of neuromuscular paralysis [6,12,21]. BoNT-B, D and F and G cleave a vesicle-associated membrane protein (also called synaptobrevin) at different single peptide bonds. BoNT-A and E cleave a synaptosomal-associated protein of 25 kDa (SNAP-25) at different sites within the C terminus, whereas BoNT-C cleaves both syntaxin and SNAP-25. Note that the molecular basis of the specificity of metalloprotease activity for the three SNAREs is only partially known. At present, it seems that a nine-residue motif (the SNARE motif, which is composed of three carboxylate residues alternated with hydrophobic and hydrophilic residues and that are present in SNARE proteins) is involved in the specificity of action of BoNTs [2,3,5,7].

For all serotypes, the released toxin is a complex composed of the toxin itself (holotoxin) with non-toxic neurotoxin-associated proteins (NAPs). Depending on the bacterial strain or on the preparation, the size of the toxin complex changes in function of the NAPs. For example, BoNT-A is released as 300-, 500- or 900-kDa complexes [3]; BoNT-B and C as 500- and 700-kDa complexes; BoNT-D as 300- and 500-kDa complexes; and BoNT-E and F as ~ 300-kDa complexes. The complexes (i.e., MW > 150 kDa) are believed to contain a non-toxin haemagglutinin protein and a non-toxin non-haemagglutinin protein [14]. Neither of these proteins plays a role in toxin-induced blockade of acetylcholine-mediated transmission. Indeed, it is unlikely that the intact multimeric complex even reaches nerve endings. Although auxiliary proteins play no meaningful role at the site of toxin action, this does not mean that they are unimportant. On the contrary, these two non-toxin proteins provide stability against denaturation of the BoNT molecule and protection against digestive acids and proteolytic enzymes when the toxin is ingested. Most of the interactions between the holotoxin and the NAPs occur through the binding domain [3,22].

Among the serotypes, BoNT-A has been the most widely studied for therapeutic purposes. Recently, BoNT-B has also become commercially available. The different toxin preparations are listed in **Table 1**.

3. Pharmacological activity of botulinum toxins

Most of the studies have focused on the cholinergic activity of BoNTs. In physiological conditions, nerve stimulation induces a depolarisation of the axon terminal that results in the release of acetylcholine into the synaptic cleft and subsequently muscle contraction. When BoNTs are present, this phenomenon is blocked by the following described mechanism, which is similar whichever toxin serotype is used. BoNTs enter the organism by several ways. The two main ones are by contaminated foods or wound infection, either by therapeutic or cosmetic injection. As well as these routes of entry, inhalation, accidental trauma and surgery are other ways that the toxins can enter. Once the toxin enters the organism, it must – in the case of an orally or inhalation entrance – cross the membrane barriers by transcytosis to reach the general circulation. To access the extracellular space in the vicinity of its target organs (which are cholinergic nerve cells), the botulinum toxin exits the vasculature by a mechanism that is still unclear. Just after, when the toxin reaches peripheral cholinergic nerve endings, there is once again a sequence of membrane-penetrating events [14]. Only the injection route is of interest for cancer therapy. From the site of injection, BoNTs diffuse to the presynaptic membrane at a neuromuscular junction. Neuron intoxication then begins in four steps [2,4,7,12,14]. First, the toxin-binding domain (H_C) mediates interaction

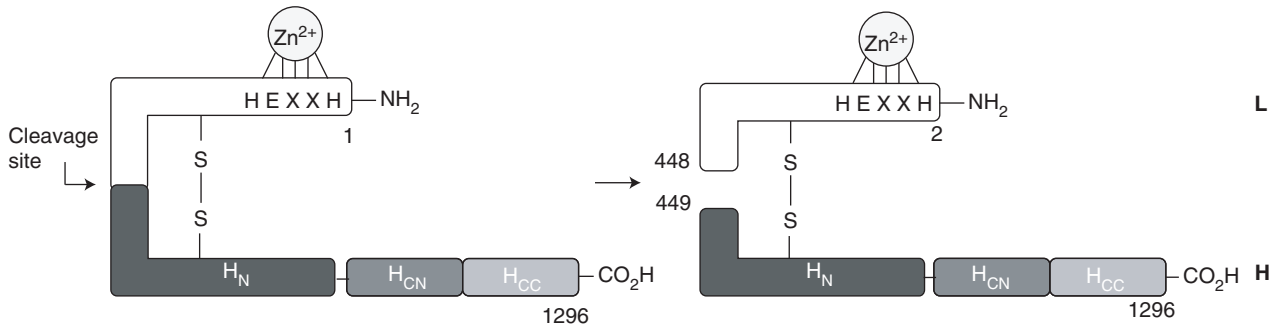

Figure 1. Schematic representation of BoNT-A. BoNT-A is synthesised as a single inactive protein (**A**), which is activated by proteolytic cleavage (arrow) to give a di-chain held together by a disulfide bond (**B**). The H chain (grey) is divided into the H_N and H_C fragments. The H_C chain consists of two subdomains: H_{CN} and H_{CC}. The H_{CC} subdomain is involved in neurospecific binding. The H_N subdomain is implicated in the translocation of the L chain. The L chain (white) is the endopeptidase responsible for the toxic effect, with a zinc-binding motif. Numbers indicate the amino acids.
BoNT: Botulinum neurotoxin; H: Heavy chain; H_N: N-terminal fragment; H_C: C-terminal fragment; L: Light chain.

Table 1. Preparations of botulinum neurotoxins.

Toxin serotype	Trade name	Pharmaceutical company
A	Botox®*	Allergan, Inc. (USA and Europe)
	Botox® Cosmetic-Vistabel®*	Allergan, Inc. (USA and Europe)
	Dysport®*	Ipsen, Inc. (Europe)
	Reloxin®*	Ipsen, Inc. (USA)
	Linurase®§	Prollenium Medical Technologies (Canada)
	BTX-A®‡	Lanzhou Biological Products Institute (China)
	Neuronox®‡	Medy-Tox (South Korea)
	Puretox®‡§	Mentor Corp. (USA and Europe)
	Xeomin®‡§	Merz Pharma (Germany)
B	Myobloc®*	Elan Pharmaceuticals (USA)
	NeuroBloc®*	Elan Pharmaceuticals (Europe)

*Commercially available. ‡Not licensed for worldwide therapeutic use at this time. §Highly purified (free of complexing proteins).

between BoNT and the presynaptic nerve membrane via the ganglioside and protein receptors found specifically on cholinergic nerve endings [23-30]. Second, this is followed by internalisation of the toxin by endocytosis. Third, the acidity of the endosome changes the conformation of the BoNT translocation domain, which inserts into the lipid bilayer of the vesicle membrane to form a pore, providing passage for the catalytic domain into the cytosol [31,32]. The fourth step is proteolytic cleavage [12] of one of the three SNARE proteins (see Section 2). This activity is characterised by dissociation of the catalytic domain from the translocation domain by cleavage of the disulfide bond. The end result is that the neurotransmitter vesicles cannot fuse with the neuronal membrane and then cannot release neurotransmitter into the synapse, which, in striated muscles, results in flaccid paralysis. BoNTs have also been successfully used to treat spastic disorders of smooth muscle and dysfunction of glandular secretion.

When injected into striated muscle, paralysis generally occurs within 1 – 5 days, peaks at 2 – 3 weeks, and lasts for a maximum of 3 or 4 months [5,6]. The duration of induced neuromuscular paralysis varies as a function of the BoNT used. An experiment in mice measuring the loss of toe-spread reflex (which indicates paralysis) into the hind leg demonstrated that the rank order of duration of paresis was BoNT-A > C1 > > B > F > E [33,34]. This is consistent with results found in humans. The recovery of functional contraction is attributed to axonal sprouting, formation of new neuromuscular junctions and to SNARE protein complex turnover [5,8,21,35]. In the case of BoNT-A, animal experiments show that extensive nerve terminal remodelling occurs with axonal sprouts appearing by days 3 – 5 [36]. These nerve

outgrowths continued to grow to reach a length of ~ 150 µm at day 42, and acquire endo- and exocytic activity. At day 28, nerve-evoked muscle twitch could be detected as vesicle recycling occurred in the sprouts (not in the parent terminals) and nicotinic acetylcholine receptors were progressively found on the muscle membrane adjacent to the sprout contacts. This resulted in a progressive switch in neural activity from the original nerve endings to their sprouts. Meanwhile, other alterations took place. Nerve sprouting continued to increase until day 42 but was then followed by a slight reduction in sprouting for a 3-week period (from day 42 to 63). There followed a recession in vesicle turnover in the outgrowths followed by total sprout retraction concomitant with a return of synaptic activity to the original endplates [36]. A return to normal nerve muscle transmission – as existed before BoNT-A poisoning – was thus achieved after ~ 90 days [36]. Hence the effect of BoNTs is fully reversible. In the case of multiple injections, a more complicated pattern of remodelling occurs, resulting in a cumulative effect and it is then possible to detect sprouts for months after the injections. Note that nerve sprouting and the time course depends on the serotype injected; for example, BoNT-E does not induce sprouts and synaptic activity recovers by day 3. The half-lifes of the toxins' inhibitory activities have been calculated from cerebellar granule neurons and range from < 24 h for BoNT-E, to 2 and 10 days for BoNT-F and B, respectively. Values for BoNT-A and C exceeded the length of the experiment and are > > 31 days and > > 18 days, respectively [33].

For all BoNT preparations, doses are expressed in 'mouse units': a unit is the amount of the neurotoxin complex protein that is lethal in 50% of female Swiss-Webster mice following an intraperitoneal injection (IP LD_{50}) [6,37]. Note that toxin units are not clinically equivalent or interchangeable among toxin serotypes and also differ for different formulations of the same serotype; for example, 1 unit of Botox® (Allergan, Inc.) is approximately equivalent to 2 – 5 units of Dysport® (Ipsen, Inc.) [38-40].

There are correlations between the BoNT dose and the extent of paresis and between the BoNT dose and the duration of action. The higher the BoNT dose is, the higher the degree and duration of action; however, a saturation phenomenon occurs with high doses that explains the maximal action duration of 3 months [5]. Importantly, it can be demonstrated using radiolabelled BoNT-A in animal models that the toxin remains in the injected tissue, only diffusing over a distance of 7 mm from the injection site [41]. This diffusion is larger in humans when using larger volumes for the injection [5,42]. It is important to note that antibodies against BoNTs can be formed after a few injections and the duration and extent of the therapeutic effect can then be reduced and sometimes suppressed. The duration of action depends on the patient and on the pathology being treated. In practice, a patient is treated with the lowest effective dose and frequent repeat injections are avoided to minimise the immune response. As a result, the duration of action is usually stable. What is known about the action of BoNT on striated muscle also seems to apply to the autonomic nervous system [5,6].

4. Clinical uses of botulinum toxins

As mentioned in Section 3, serotype A is the most powerful toxin and was the first to be developed for clinical therapeutics. The first use of BoNT-A for injectable selective muscle weakening was in the treatment of strabismus in 1980 by Scott [43]. Of the other serotypes, type B has recently become commercially available. At present, only BoNT-A and B are considered as clinically relevant therapeutic agents and are used in a wide range of medical conditions that are characterised by local, involuntary muscle hyperactivity (**Box 1**). These include dystonias, eye movement disorders, spasticity, smooth muscle disorders and exocrine gland hyperactivity as well as pain syndromes [6,8,44-46]. BoNT-A is also very popular for its aesthetic effect to rejuvenate the ageing face [47]. New indications continue to appear, such as BoNT use in the preoperative setting to allow surgical positioning and access [48].

5. Use of botulinum toxins in cancer therapy

A review of the literature about BoNT use in cancer has provided very few results. The authors group the results into four categories: BoNTs used as adjuvants to relieve spasms due to cancer or to therapies; BoNTs proposed to possess a cytotoxic effect; BoNTs used to open the tumour vascular bed; and the use of *Clostridium* spores in anticancer therapies.

5.1 Botulinum toxins as adjuvants to relieve spasms due to cancer or to therapies

Most of the retrieved articles concerned injections of BoNTs to treat symptoms associated with the cancer itself or those resulting from side effects after chemotherapy and/or radiotherapy. It is well known that radiotherapy and chemotherapy can induce complications such as spastic contractures and painful muscle spasms. In such cases, treatment with BoNT-A injections can be helpful. Radiation-induced trismus due to secondary myokymia of the masseter muscle after treatment for palatal adenocarcinoma has been treated effectively with BoNT-A in the past [49]. Only one report in the literature describes the painful post-irradiation muscle spasms of the head and neck musculature (specifically of the sternocleidomastoid muscle) and discusses the use of BoNT-A to help manage the disorder [50]. An increasing use of radiation therapy in pelvic malignancy has led to a rise in the incidence of chronic radiation proctitis, which can vary from milder to severe forms, potentially affecting a patient's quality of life. A first case of severe radiation-induced proctitis successfully treated with BoNT-A (when all other treatments were ineffective) was reported in 2003 [51]. Concurrence of acute lymphoblastic leukaemia and neurological disorders in

Box 1. Clinical uses of botulinum neurotoxins.

Focal dystonias
Cervical dystonia (torticollis)
Blepharospasm (eyelid closure)
Laryngeal dystonia (spasmodic dysphonia)
Oromandibular–facial–lingual dystonia
Task-specific dystonia (occupational cramps)
Limb and axial dystonia
Other focal dystonias (idiopathic and secondary)

Eye movement disorders
Strabismus (disorder of conjugate eye movement)
Nystagmus
Chronic sixth nerve palsy

Spacticity
Stroke
Cerebral palsy
Traumatic brain injury
Multiple sclerosis
Spinal cord injury

Disorders of localised muscle spasms and pain
Headache (tension, migraine and cervogenic)
Chronic low back pain
Myofacial pain syndrome
Fibromyalgia
Piriformis syndrome
Radiculopathy

Smooth muscle hyperactive disorders
Sphincter-detrusor dyssynergia
Detrusor hyper-reflexia
Achalasia cardia
Hirschsprung disease
Sphincter of Oddi dysfunctions
Chronic anal fissures

Exocrine gland hyperactivity
Focal hyperhidrosis (axillary and palmar)
Sialorrhoea
Frey's syndrome
Crocodile tears syndrome

Other involuntary movements
Haemifacial spasm
Tics
Tremors (voice, head and limb)
Myokymia and synkinesis
Palatal myoclonus
Hereditary muscle cramps

Cosmetic use
Hyperkinetic facial lines (glabellar frown lines, crow's feet and wrinkles)
Hypertrophic platysma muscle bands

Miscellaneous
Stuttering
Bruxism
Freezing of gait
Sixth nerve disorders

children was described. Spastic leg contractures, to which a peripheral neuropathy associated with vincristine administration was added, was successfully treated by botulinum toxin therapy in a 2-year-old boy [52]. BoNT-A was also used to reduce ocular motility disturbances such as diplopia induced by plaque brachytherapy in the treatment of uveal melanoma [53]. BoNT-A was also able to compensate diplopia-associated tumour (nasopharyngeal carcinoma) resulting from sixth nerve palsy [54]. Frey's syndrome (gustatory sweating) is well recognised as a postoperative complication of parotidectomy. The most prevalent theory is the aberrant regeneration of secretomotor parasympathetic neurons of the auriculotemporal nerve that has lost its target organ. Intracutaneous injection of BoNT-A was reported to block the cholinergic autonomic fibres responsible and the subsequent signal transduction of nerve fibres stimulating sweat glands [55,56]. In all of these cases, BoNT is used to induce prolonged inhibition of muscle spasm and/or pain. More research is needed to clarify the aetiology, incidence, predisposing factors, time course of spasm development and association with combined chemotherapy and radiation dose dependency.

5.2 Botulinum toxins: do they possess a cytotoxic effect?

The release of neurotransmitters by spinal cord neurons in culture, including primary motorneurons, is very sensitive to clostridial neurotoxins [12]. In these cells, BoNT-A was found to cause no detectable morphological changes, whereas BoNT-C causes rapid swelling of synaptic terminals followed by degeneration of axons, dendrites and cell bodies [57] and the collapse of growth cones in chick dorsal root ganglia [58]. As a consequence of these cellular alterations, BoNT-C (uniquely among clostridial neurotoxins) causes the death of spinal cord neurons in culture but not of other CNS neurons *in vitro*, and BoNT-C causes the loss of motor neurons in humans [12].

Regarding tumour cells, there is only one report that suggests a direct cytotoxic effect of BoNT-A. This report is not published in a peer-reviewed journal but is part of a patent [101]. The experiment reported the *in vitro* effect of Botox on several cancer lines using the Oncotech Extreme Drug Resistance Assay (EDR®) assay (incorporation of ^{3}H-thymidine as a measure of cell division). Tumour cells were evaluated in duplicate for viability in the presence of Botox using concentrations varying from 0.001 U/ml to 20 U/ml. Untreated cells were used as negative controls, whereas cells treated with cisplatin were used as positive controls. The levels of inhibition were in the range of 0 – 28% and incorporated ^{3}H-thymidine compared with control. The dose–effect relationship effect possessed a rather strange feature, with a maximal value of Botox of ~ 0.1 U/ml. Lower concentrations and, more surprisingly, higher concentrations were less active or not active at all. No *in vivo* evidence for direct cytotoxicity against tumour cells has been reported so far.

5.3 Botulinum toxins as a way to open the tumour vascular bed to enhance cytotoxic treatments

The recent publication by Ansiaux *et al.* [1] demonstrated a novel effect of BoNTs that could be very useful in the treatment of cancers. The starting point of the study simply considered the main factors underlying the resistance of tumours to cytotoxic treatments. Tumour hypoxia is well known to be a key factor in the response to irradiation, whereas adequate perfusion is mandatory for the delivery of chemotherapeutic drugs; therefore, radiotherapy and chemotherapy could benefit from transient reoxygenation and reperfusion of the tumour just before the application of the cytotoxic treatment. The starting paradigm of these authors was that BoNTs could interfere with neurotransmitter release at the perivascular sympathetic varicosities, leading to inhibition of the neurogenic contraction of tumour vessels, therefore, improving tumour perfusion and oxygenation. To test this hypothesis, myograph assays [59] were used to characterise the effect of BoNT-A on tumour vessels. These experiments confirmed that BoNT-A could interfere with the release of neurotransmitters and with neurogenic vasoconstriction. The *in vivo* application of this concept allowed us to demonstrate significant reoxygenation and reperfusion of tumours after local intra-tumour administration of BoNT-A by recently developed techniques (electron paramagnetic resonance oximetry [60,61] and dynamic contrast-enhanced MRI [62]). A dramatic increase in the efficacy of X-ray radiotherapy and cyclophosphamide therapy was observed when these treatments were given at the time of maximal reoxygenation and reperfusion (**Figure 2**) [1]. Recently, Cron *et al.* also provided evidence for an increase in the delivery of gemcitabine into tumours following treatment with BoNT-A, results that were consistent with an increase in the therapeutic efficacy of this combination of treatments [63]. Another very important finding of a study by authors' laboratory group [1] was that the benefit from BoNT was directly related to a change in the tumour microenvironment and not to a direct cytotoxic or radiosensitising effect. Indeed, when applied *in vitro*, BoNT did not alter apoptosis in tumour cells or induce any radiosensitising effect. Moreover, no change in tumour growth was observed *in vivo* when using BoNT alone. The authors' laboratory group observed a beneficial effect only when cytotoxic treatment was applied after the reoxygenation and reperfusion induced by the local administration of BoNT. Interestingly, because BoNTs are already widely used clinically, with established absence of long-term toxicity (absence of systemic effects) when used appropriately [64], it is easy to envisage clinical trials with local administration of BoNT in easily accessible tumours. The authors' laboratory group do not anticipate any major concerns with the use of BoNT. Local administration is associated with limited diffusion into surrounding tissues. Moreover, as the therapeutic benefit is mediated by an oxygen effect, a radiosensitising effect on normal tissues is unlikely: the radiosensitising effect is likely to be higher for hypoxic tumour regions than for well-oxygenated tissues.

5.4 Use of *Clostridium* spores in anticancer therapies

Species of anaerobic bacteria, particularly *Clostridia*, are well known to exclusively localise and germinate in hypoxic regions that are common to solid tumours. Indeed, intravenous administration of toxin-free *Clostridium* spores that did not cause noticeable systemic toxicity revealed that bacterial growth was restricted to tumours and was not observed in the liver, spleen, kidneys, lungs or brain [65]. The targeting of clostridial spores to tumours is now considered as a potent new therapeutic way for tumour destruction. Indeed, administration of clostridial spores (intra-tumourally and intravenously) such as *C. butyricum M-55* to cancer mice and patients induced a bacteriolytic breakdown of large parts of the tumour [66]. This oncolysis (tumour destruction) was explained by the stimulation of the host immune system and by the bacterial products that are released into the tumour microenvironment [65]. Unfortunately, this single treatment with oncolytic clostridia did not always succeed in tumour control and sometimes induced toxicity; therefore, new strategies must be developed to find more efficient alternatives. Thanks to the evolution of the genetic-engineering techniques, Brown and colleagues developed the idea of endowing harmless clostridia with antitumour properties [66]. The clostridial-directed enzyme prodrug therapy was subsequently introduced. The aim is to produce an enzyme within the tumour capable of metabolising a systemically introduced, non-toxic prodrug into toxic metabolite. The principle is that the *Clostridium* is endowed with a gene that encodes the prodrug-converting enzyme. An innocuous prodrug is subsequently delivered to the tumour and is converted to a highly cytotoxic compound [65]. *In vitro* and intra-tumoural administration studies revealed prodrug-converting activity; however, intravenously administration of recombinant spores with the prodrug revealed no or few therapeutic effect [65]. This was probably due to a limited colonisation and consequently insufficient levels of the prodrug-converting enzyme. The choice of clostridial host to use in clostridial-directed enzyme prodrug therapy is largely dictated by the availability of genetic systems. To date, only relatively few clostridial species have proven to be amenable to genetic manipulation such as *C. perfingens*, *C. acetobutylicum* and *C. sporogenes*. An alternative approach is to genetically engineer clostridia to allow them to secrete a biologically active protein such as TNF-α.

6. Expert opinion

The medical indications for BoNTs are large; however, the application of BoNTs in cancer has so far been limited to the treatment of spasm symptoms associated with the disease itself or with its cytotoxic therapy. The suggestion that BoNTs may be used to treat cancer directly is novel. As discussed in this review, claims that this therapy may have a direct cytotoxic effect on cancer cells [101] are rather debatable as the level of toxicity reached was low and without any sensible dose–effect relationship. In the study by Ansiaux *et al.* [1], no direct

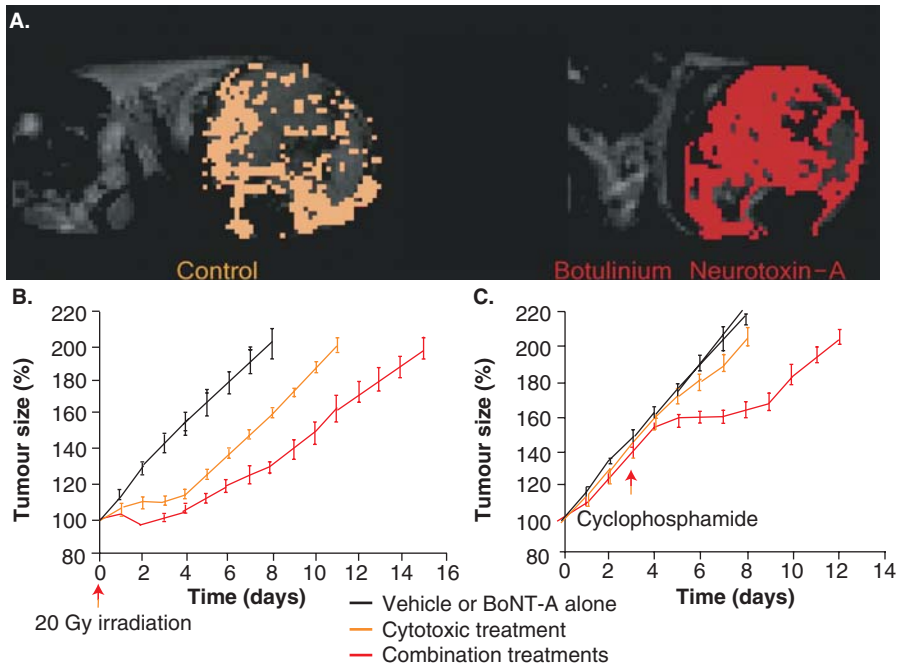

Figure 2. Effect of local administration of BoNT-A on tumour perfusion and treatment. A. Typical MRI images of FSAII tumours showing the perfused pixels 3 days after a single injection of BoNT-A 29 U/kg (red) or vehicle (orange). **B** and **C.** Effect of the combination of BoNT-A with cytotoxic treatments. **B.** Treatment with 20 Gy radiotherapy. **C.** Treatment with cyclophosphamide 50 mg/kg. Note that BoNT-A alone does not exert any cytotoxic effect and that the combination potentiates the cytotoxic treatment.
BoNT-A: Botulinum neurotoxin A; FSAII: Fibrosarcoma.

cytotoxic effect or promoting effect of cytotoxic treatment was observed when BoNT was incubated directly with tumour cells; therefore, it is unlikely that the inhibition of cell division will be large enough for BoNT to be used alone to treat cancer. Instead of considering the possible toxicity of this therapy, it seems more promising to focus on the profound effect that BoNTs induce on the tumour microenvironment. The inhibition of neurogenic contractions of tumour vessels is particularly interesting for potentiating cytotoxic therapies. The delivery of cytotoxic drugs is increased as tumour perfusion is increased and, at the same time (with an increased number of oxygen carrying red blood cells inside the tumour), there is reoxygenation of the tumour, thus potentiating radiation therapy. Therefore, the effect of BoNTs on tumours should really be interpreted in the context of the attempts to manipulate the tumour microenvironment to increase the efficacy of anticancer therapeutics. Mechanistically, tumour hypoxia results from an imbalance between oxygen delivery and oxygen consumption, either of which may be potentially targeted by therapeutic interventions. Oxygen delivery may be increased by increasing tumour perfusion with drugs acting on vascular tone [59,60,67] or during the early normalisation phase at the start of antiangiogenic treatments [68,69], by breathing oxygen-enriched gases [70,71], by changing the haemoglobin saturation [72,73] or during the course of radiotherapy [74-76]. Tumour hypoxia can also be alleviated by decreasing oxygen consumption: several drugs that inhibit mitochondrial respiration (such as meta-iodobenzylguanidine [77], insulin [78] and COX-2 inhibitors [79]) have the potential to increase tumour oxygenation and thereby enhance radiosensitivity.

Where do BoNTs fit in among these strategies? BoNTs have several advantages that can be emphasised: BoNT acts on both perfusion and oxygenation, increasing the efficacy of both radiotherapy and chemotherapy; BoNT has a long-term action, allowing potentiation of radiotherapy in fractionated regimens; BoNT has no systemic side effects; and BoNT is already used in many clinical situations, allowing a direct transfer from preclinical studies to clinical trials. In addition, the proof-of-concept that was demonstrated with BoNT-A can be extended to other BoNTs as clostridial neurotoxins possess multi-domain structures. Advances in exploring structure–function relationships could facilitate in determining new therapeutics (active parts of the toxin or peptidomimetic drugs) [80]. Finally, it should be emphasised that the transfer of these strategies to humans clearly benefits from the development of new imaging modalities that allow characterisation of the evolution of the tumour microenvironment during therapies.

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•• **A review of the recent advances in the roles of the individual domains present in BoNTs and their potential pharmacological activities.**

Patent

101. ALLERGAN, INC: US0031648 (2005).

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