

Comparative Pharmacodynamics Study of 3 Different Botulinum Toxin Type A Preparations in Mice

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BACKGROUND A new complexing protein-free botulinum toxin Type A (CBoNT) with the same mechanism of action as the botulinum toxin complex onabotulinumtoxinA (OBoNT) and complexing protein-free inco-botulinumtoxinA (IBoNT) was recently developed.

OBJECTIVE To compare the local paresis and chemodeneration efficacy of 3 different botulinum toxin Type A preparations in mice.

MATERIALS AND METHODS Efficacy and duration of action of CBoNT, OBoNT, and IBoNT after a single intramuscular injection to the right gastrocnemius was evaluated by digit abduction score (DAS) and compound muscle action potential (CMAP) assays.

RESULTS Mouse DAS and CMAP responses were comparable between CBoNT and OBoNT, indicating similar paresis and chemodeneration efficacy, as well as duration of action. Both botulinum toxins showed significantly higher efficacy and longer duration of action than IBoNT. Similarly, mean DAS potency of CBoNT (ED_{50} : 3.85 ± 0.34 U/kg) and OBoNT (ED_{50} : 4.13 ± 0.07 U/kg) were significantly higher compared with IBoNT (ED_{50} : 6.70 ± 0.83 U/kg).

CONCLUSION CBoNT displays the same efficacy as OBoNT as shown by their comparable chemodeneration and local paretic effects, and demonstrates superior efficacy and duration of action compared with IBoNT. Likewise, CBoNT has comparable DAS potency to OBoNT and is superior to IBoNT.

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Botulinum neurotoxins (BoNT) are produced by anaerobic bacterium *Clostridium botulinum* and related species, and are the most potent protein neurotoxins known so far.¹ Botulinum neurotoxins causes botulism, a rare but potentially fatal illness characterized by progressive flaccid paralysis.² There are 7 antigenically distinct BoNT serotypes (A to G). Botulinum neurotoxins Types A, B, D, and F have been associated with human botulism.³ Botulinum neurotoxins disrupt the fusion of synaptic vesicles and plasma membrane in peripheral cholinergic nerve terminals by cleaving

SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors) and prevent the release of acetylcholine (ACh) neurotransmitters at neuromuscular junctions. This mechanism of action has made BoNT a highly suitable therapeutic agent for spasticity-related muscle disorders.^{4,5}

Botulinum toxin Type A (BoNT/A) is the first and most widely used BoNT serotype approved for clinical and cosmetic purposes.⁵ The first reported clinical application of BoNT/A was for treatment of strabismus as an alternative to surgical correction.⁶ Since then, its

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therapeutic application has expanded to various indications related to muscle spasticity, such as blepharospasm, hemifacial spasm, cervical dystonia, chronic migraine, acquired equinus deformity, sialorrhea, and urinary incontinence. BoNT/A is also used to temporarily correct moderate-to-severe glabellar, lateral canthal, and forehead lines, as well as auxiliary hyperhidrosis.^{7,8} Unit potency of different BoNT/A products are generally not interchangeable because of underlying differences in the manufacturing process, formulation, and testing method used.⁹

The 3 leading BoNT/A products—onabotulinumtoxinA (OBoNT, BOTOX/BOTOX Cosmetic; Allergan Inc., Irvine, CA), abobotulinumtoxinA (ABoNT; Dysport/Azzalure; Ipsen Ltd., Wrexham, United Kingdom), and incobotulinumtoxinA (IBoNT; Xeomin/Bocouture; Merz Pharmaceuticals GmbH, Frankfurt, Germany) are manufactured using *C. botulinum* Type A strain. OBoNT and ABoNT contain neurotoxin complexes, whereas IBoNT contains complexing-protein-free neurotoxin.¹⁰ Another complexing protein-free BoNT/A formulation marketed under the name Coretox (CBoNT; Medytox Inc.) was recently approved by South Korea's Ministry of Food and Drug Safety for the temporary improvement of glabellar wrinkles after completing the Phase 3 clinical trial (NCT03908008) in 2014.

The present study compares the pharmacodynamics of CBoNT, OBoNT, and IBoNT using mouse models that evaluate chemodenervation and local paresis. This investigation was conducted to establish performance comparability between CBoNT and the other 2 BoNT/A formulations.

Materials and Methods

Animals

Six to 7-week-old female ICR/CD-1 mice were purchased from Orient Bio, Inc. (Sandaewon-dong, Seongnam-si, South Korea), housed in groups of 5 in a 12-hour light/dark cycle, and given free access to food and water. All protocols were approved by the Institutional Animal Care and Use Committee of Medytox Inc.

Toxin Preparation and Administration

OBoNT, IBoNT, and CBoNT were prepared and administered following the method described previously.¹¹ Each unexpired 100 U vial of the BoNT/A formulations was reconstituted to 200 U/mL, then serially diluted to arrive at doses equivalent to 1.2, 3.3, 4.0, 9.8, 12, and 40 U/kg body weight using preservative-free normal saline vehicle.

Before BoNT/A injection, mice were anesthetized with an intraperitoneal (IP) dose of 60 mg/kg ketamine hydrochloride and 12 mg/kg xylazine, and the right hindlimb was shaved. Each BoNT/A preparation or vehicle was administered into the right gastrocnemius at 0.2 mL/kg using a 25- μ L Hamilton syringe. Groups of 5 mice for OBoNT and IBoNT, and 15 mice for CBoNT were each given a single intramuscular (IM) dose of 3.3 or 9.8 U/kg of the BoNT/A formulations. Another 5 mice were given placebo as a negative control. For determining the median IM effective dose (ED₅₀), a single IM injection of 1.2, 4.0, 12, or 40 U/kg of CBoNT, OBoNT, or IBoNT was each administered to groups of 6 mice, with the injections performed sequentially from low to high dosage. The average ED₅₀ was obtained from 3 independent experiments.

Digit Abduction Score Assay

A modified digit abduction score (DAS) assay was used to evaluate local paresis on the injected leg.^{11,12} Briefly, mouse was suspended by the tail to elicit the characteristic startled response manifested by extension of the hindlimb followed by abduction of the hind digits. Digit abduction score of the right hindlimb was determined by 2 blinded observers using a 5-point scale from 0 (full digit abduction) to 4 (complete absence of digit abduction and leg extension). Digit abduction score was assessed before BoNT/A injection and daily for the first 3 days, then at various intervals thereafter up to 48 days.

Compound Muscle Action Potential Measurement

Change in compound muscle action potential (CMAP) was used to evaluate chemodenervation on the injected leg. Compound muscle action potential amplitude

on the right hindlimb was measured using Nicolet VikingQuest (Viasys Healthcare, Madison, WI) following the method described previously.¹³ Briefly, mice were anesthetized, followed by electrical stimulation with 10 to 20 mA for 0.1 ms to the target muscle on the injected leg and recording of the CMAP amplitude. Compound muscle action potential measurement was performed before BoNT/A injection to establish the baseline CMAP amplitude ($CMAP_{baseline}$), then daily for the first 3 days, and at various intervals thereafter up to 132 days.

Data and Statistical Analyses

Mean and SD of DAS and CMAP amplitudes were obtained. Area under the curves for DAS (DAS_{AUC}) and CMAP ($CMAP_{AUC}$) were calculated from DAS and CMAP response versus time curves, respectively. Area above the curve for CMAP ($CMAP_{AAC}$) was calculated using the formula $CMAP_{baseline} AUC - CMAP_{AUC}$, where $CMAP_{baseline} AUC$ is the estimated $CMAP_{AUC}$ in the absence of BoNT/A treatment, extrapolated from the $CMAP_{baseline} \cdot ED_{50}$, which represents DAS potency, was calculated from the maximum DAS dose-response curves fitted using three-parameter logistic equation.¹⁴ Maximum DAS response (DAS_{max}), lowest CMAP amplitude ($CMAP_{min}$), DAS_{AUC} , $CMAP_{AAC}$ and ED_{50} were compared using one-way analysis of variance (ANOVA) followed by post-hoc *t*-tests. Duration of action was compared using two-way ANOVA, followed by one-way ANOVA and *t*-test of the DAS and CMAP responses at different time points. A $p < .05$ was considered statistically significant.

Results

Comparing Local Paretic Effects

Local paresis in mice induced by CBoNT, OBoNT, and IBoNT was evaluated by DAS. Mice injected with placebo displayed full digit abduction ($DAS = 0$) throughout the observation period (data not shown). In contrast, all 3 BoNT/A formulations displayed $DAS_{max} 2 \sim 3$ days after administration in both the 3.3 and 9.8 U/kg dose (Figure 1). As shown in Table 1, the 3-fold higher dosage of CBoNT, OBoNT, and IBoNT

significantly increased DAS_{max} ($p < .01$). At the 3.3 U/kg dose, there were no significant differences in DAS_{max} , duration of action, and DAS_{AUC} among the 3 BoNT/A formulations. At the 9.8 U/kg dose, CBoNT and OBoNT showed comparable DAS_{max} and duration of action, whereas IBoNT elicited a lower DAS_{max} and shorter duration of action than the other 2 BoNT/A formulations. Similarly, the DAS_{AUC} of CBoNT (27.9 ± 15.5 DAS-day) was also comparable with OBoNT (27.2 ± 22.1 DAS-day) at this higher dosage, and was significantly higher than IBoNT (11.7 ± 5.3 DAS-day).

Comparing Level and Duration of Chemodenervation

The same groups of animals administered with 3.3 and 9.8 U/kg of the BoNT/A formulations were evaluated by CMAP assay. In the placebo group, the average

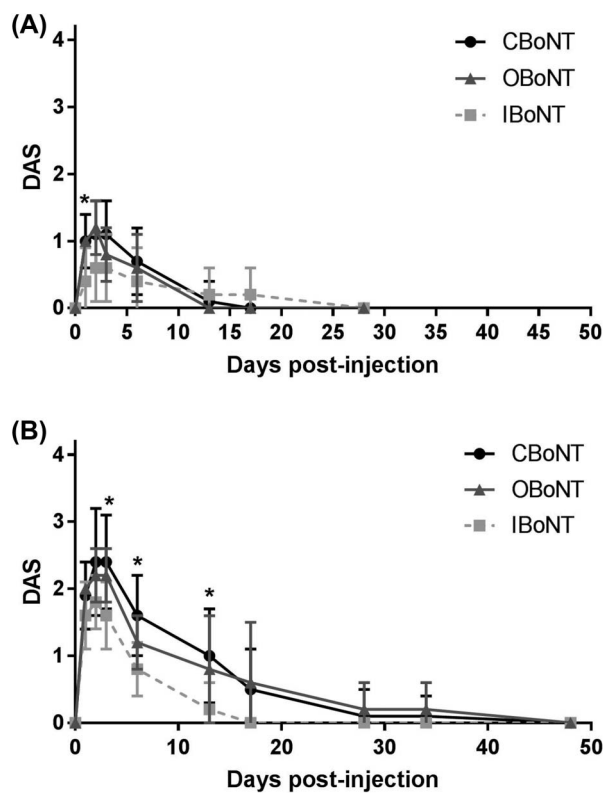


Figure 1. Level and duration of local paresis on the injected leg assessed by digit abduction score (DAS) assay. CBoNT, OBoNT, or IBoNT injected via intramuscular route to the right gastrocnemius at a dose of 3.3 U/kg (A) or 9.8 U/kg (B). All data points represent mean \pm SD. Asterisks indicate significant differences between CBoNT and IBoNT ($p < .01$).

TABLE 1. Digit Abduction Score (DAS) Response Observed on the Injected Leg After Intramuscular Administration of Different BoNT/A Preparations

BoNT/A	Dose (U/kg)	No. of Animals (Responsive/Total)	DAS _{max}	Duration of Action (d)	DAS _{AUC} (DAS·d)
CBoNT	3.3	14/15	1.1 ± 0.5	10.5 ± 4.5	7.9 ± 4.2
	9.8	15/15	2.5 ± 0.7	22.3 ± 9.8*	27.9 ± 15.5*
OBoNT	3.3	5/5	1.2 ± 0.4	9.6 ± 4.8	6.8 ± 3.6
	9.8	5/5	2.2 ± 0.4	23.8 ± 14.9	27.2 ± 22.1
IBoNT	3.3	3/5	0.6 ± 0.5	9.4 ± 11.7	6.8 ± 9.3
	9.8	5/5	1.8 ± 0.4	12.4 ± 4.0	11.7 ± 5.3

*Data expressed as mean ± SD ($p < .05$ using 2-tailed t -test vs IBoNT).

DAS_{max}, highest digit abduction score; DAS_{AUC}, total DAS response versus time.

CMAP amplitude on the injected leg ranged between 47.58 and 48.94 millivolts (mV) throughout the observation period, with one of the animal showing the highest coefficient of variation of 4.7% (data not shown). This suggests that the impact of the procedure on the reproducibility of CMAP measurement is negligible, and that a full recovery from chemodeneration effects can be considered once the CMAP amplitude on the injected leg reaches 95% of the CMAP_{baseline}. The CMAP_{baseline} observed in the treatment groups averaged between 47.94 and 48.40 mV, which was within the normal range (data not shown). CBoNT, OBoNT, and IBoNT caused significant chemodeneration for both doses tested, with CMAP_{min} recorded within 3 days after administration (Figure 2). Similar to the DAS response, the higher dosage elicited a more significant decrease in CMAP_{min} ($p < .01$), as shown in Table 2. At the 9.8 U/kg dose, CBoNT and OBoNT showed comparable levels of chemodeneration with CMAP_{min} of 0.27 ± 0.09 mV and 0.24 ± 0.09 mV, respectively, whereas IBoNT (CMAP_{min}: 0.79 ± 0.19 mV) was significantly lower ($p < .01$). In addition, the duration of action of CBoNT (120.4 ± 12.5 days) was longer compared with OBoNT (116.2 ± 17.0 days) and IBoNT (104.2 ± 12.0 days). Moreover, the difference in the duration of action between CBoNT and IBoNT was statistically significant ($p < .05$). Based on their calculated CMAP_{AAC}, the chemodeneration effects of both CBoNT ($2,713.61 \pm 336.70$ mV·day) and OBoNT ($2,657.01 \pm 421.81$ mV·day) was significantly higher than IBoNT (1854.75 ± 135.63 mV·day) at this higher dosage ($p < .01$).

Comparing Digit Abduction Score Potency

DAS potency of the 3 BoNT/A formulations was determined by the average ED₅₀ of the DAS response from 3 independent experiments. The maximum DAS response for each dose of CBoNT, OBoNT, and IBoNT, ranging from 1.2 to 40 U/kg, was plotted (Figure 3) and used to calculate ED₅₀. CBoNT and OBoNT showed comparable DAS potency, with an ED₅₀ of 3.85 ± 0.34 U/kg and 4.13 ± 0.07 U/kg, respectively. By comparison, the ED₅₀ of IBoNT (6.70 ± 0.83 U/kg) was significantly higher than CBoNT ($p < .01$) and OBoNT ($p < .05$), indicating that IBoNT has a lower DAS potency compared with the other 2 BoNT/A formulations.

Discussion

The active substance in all BoNT/A formulations is the 150 kDa BoNT/A holotoxin. This holotoxin is synthesized as a single polypeptide chain post-translationally nicked to yield the 100 kDa Heavy Chain (H_C) and 50 kDa Light Chain (L_C) segments, covalently bound by a disulfide bridge. During synthesis, the holotoxin forms multi-protein complexes with nontoxic nonhemagglutinin protein and hemagglutinin components HA-70, HA-33, and HA-17 to generate different molecular size complex, the largest of which is the 900 kDa 19 S LL-PTC (progenitor toxin complex).¹⁵⁻¹⁷

The manufacturing process for all commercial BoNT/A differs among manufacturers, this includes the strain of *C. botulinum* Type A used, purification

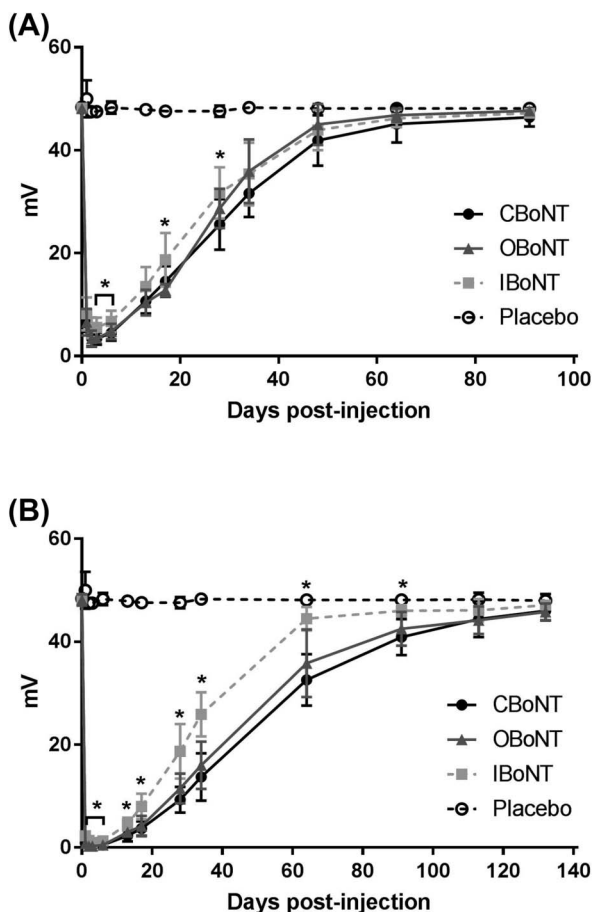


Figure 2. Level and duration of chemodenervation assessed by changes in compound muscle action potential (CMAP) amplitude of the injected leg muscle. CBoNT, OBoNT, or IBoNT injected via intramuscular route to the right gastrocnemius at a dose of 3.3 U/kg (A) or 9.8 U/kg (B). All data points represent mean \pm SD. Asterisks indicate significant differences between CBoNT and IBoNT ($p < .05$).

method and choice of excipients, resulting in different formulations.^{9,18,19} The formulation of OBoNT contains the 900 kDa neurotoxin complex, whereas

IBoNT and CBoNT formulations contain only the purified 150 kDa botulinum neurotoxin protein.^{20–23} The mouse LD₅₀ assay is the standard method used for determining BoNT/A potency based on their intraperitoneal median lethal dose. However, the conditions for this assay that could significantly affect potency such as the strain, sex and age of mice, composition of the reconstitution buffer and diluent, and injection volume have not been harmonized.^{24–26} Thus, the dose units indicated on the label of different BoNT/A products are generally not interchangeable, and that any BoNT/A formulation should be used according to the guidelines of their respective manufacturer.^{9,19,22}

Several clinical studies comparing BoNT/A dose potencies have generally agreed on the noninferiority of IBoNT to OBoNT in therapeutic performance and patient satisfaction.^{21,27–30} Others, however, have reported either lower clinical efficacy or shorter duration of action for IBoNT compared with OBoNT.^{31–33} Similarly, nonclinical studies comparing the potency or efficacy of OBoNT and IBoNT have disagreed on their dose equivalence. Some have demonstrated comparable dose potencies between the 2 BoNT/A products using the mouse LD₅₀ or CMAP assays,^{34,35} whereas others have reported lower potencies for IBoNT compared to OBoNT when the same dose units are tested using the mouse LD₅₀ assay.^{36,37} Interestingly, nonclinical studies that evaluated the paralytic effects of BoNT/A in mice have reported lower dose efficacy of IBoNT compared with OBoNT, including the study that asserted comparable dose potencies between the

TABLE 2. Compound Muscle Action Potential Evaluation on the Right Leg After Intramuscular Injection With Different BoNT/A Preparations

BoNT/A	Dose (U/kg)	No. of Animals (Responsive/Total)	CMAP _{min} (mV)	Duration of Action (d)	CMAP _{AAC} (mV·d)
CBoNT	3.3	15/15	2.59 \pm 0.57*	65.1 \pm 15.1	1,379.24 \pm 155.08*
	9.8	15/15	0.27 \pm 0.09†	120.4 \pm 12.5*	2,713.61 \pm 336.70†
OBoNT	3.3	5/5	2.80 \pm 1.07	54.4 \pm 8.8	1,228.05 \pm 168.37
	9.8	5/5	0.24 \pm 0.09†	116.2 \pm 17.0	2,657.01 \pm 421.81†
IBoNT	3.3	5/5	3.96 \pm 1.80	63.0 \pm 17.6	1,174.36 \pm 120.94
	9.8	5/5	0.79 \pm 0.19	104.2 \pm 12.0	1854.75 \pm 135.63

*Data expressed as mean \pm SD ($p < .05$, using 2-tailed *t*-test vs IBoNT).

†Data expressed as mean \pm SD ($p < .01$ using 2-tailed *t*-test vs IBoNT).

CMAP_{min}, lowest CMAP amplitude; CMAP_{AAC}, Total CMAP reduction versus time.

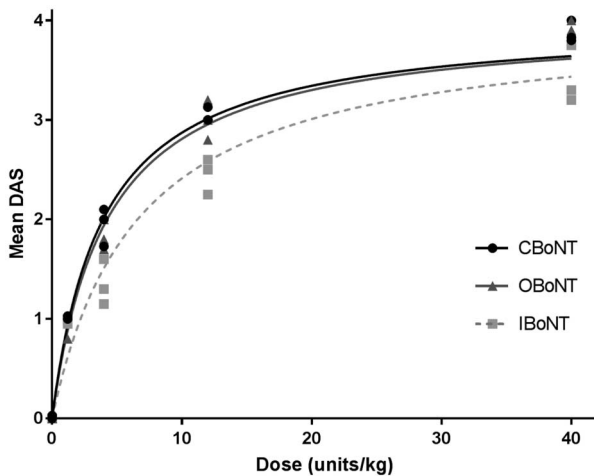


Figure 3. Dose-maximal digit abduction score (DAS) response curve. Dose is expressed in U/kg of body weight. All lines represent best-fit three-parameter logistic regression curves and mean values from 3 independent experiments. Digit abduction score potency based on the median effective dose (ED_{50}) was derived from this graph.

2 BoNT/A formulations based on CMAP.^{35,37,38} The present study similarly observed lower efficacy for IBoNT compared with OBoNT based on DAS response and DAS potency, whereas their relative CMAP reduction were comparable to the results for similar doses tested in a previous study.³⁵

A clinical study comparing the chemodenervation effects of CBoNT and OBoNT on the extensor digitorum brevis in healthy volunteers reported comparable efficacies between these 2 BoNT/A formulations.²³ Similarly, the present nonclinical study, which evaluated DAS and CMAP responses of mice after BoNT/A injection, have shown comparable efficacy and duration of action between CBoNT and OBoNT, suggesting that these 2 BoNT/A formulations are equi-efficacious. In contrast, CBoNT displayed more effective chemodenervation and local paresis than IBoNT, with a longer duration of action. In addition, CBoNT and OBoNT showed comparable DAS potency, whereas IBoNT displayed lower DAS potency than the other 2 BoNT/A formulations. Based on the DAS potency test, a dose conversion ratio for the nonclinical efficacy between OBoNT, CBoNT, and IBoNT is estimated to be 1.0:0.93:1.62.

Complexing proteins play an important role in protecting the core neurotoxin from the harmful acidic

environment of the gut and ensure successful translocation of BoNT/A across the intestinal mucosa.^{15,39,40} However, they are not involved in the mode of action of BoNT/A, whereas their role in maintaining the stability of the manufactured holotoxin is debatable.⁴¹ Conversely, the presence of complexing proteins in BoNT/A formulations has been associated with an increased risk of secondary nonresponse, defined as the diminishing or complete loss of response to subsequent BoNT/A injections. Secondary nonresponse can be attributed to many factors, one of which is the formation of neutralizing antibodies (NAbs) that bind to the BoNT/A holotoxin and block its mode of action.⁴² Although there is no direct evidence linking the complexing proteins with NAbs formation, one study had shown that patients who had developed NAbs-related secondary nonresponse from OBoNT or ABoNT that eventually waned before injection with IBoNT did not show formation of NAbs as a response to the subsequent injection.⁴³ Because of this, BoNT/A formulations devoid of complexing proteins has been suggested to offer a therapeutic advantage over BoNT/A complexes.^{10,41,44}

Other factors may contribute to differences in potency and efficacy of BoNT/A formulations. The comparative pharmacodynamics study described here was performed using only 2 different batches of the BoNT/A formulations and thus recommends testing other batches to establish their relative potency. Also, the dose conversion ratio reported in this study is based on the relative DAS potency of the BoNT/A formulations in mice, and may not necessarily reflect the equivalent therapeutic efficacy in human patients.

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