Gantacurium and CW002 Do Not Potentiate Muscarinic Receptor-mediated Airway Smooth Muscle Constriction in Guinea Pigs

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ABSTRACT

Background: Neuromuscular blocking agents are an integral component of general anesthesia. In addition to their intended pharmacologic target on skeletal muscle nicotinic receptors, undesirable airway effects (*i.e.*, bronchoconstriction) can result from neuromuscular blocking agents' affinity for airway muscarinic receptors. We questioned whether two new members of a bisquaternary nondepolarizing muscle relaxant family, gantacurium and CW002, demonstrated detrimental effects of airway muscarinic receptors using an *in vivo* model in guinea pig airways.

Methods: Urethane-anesthetized male guinea pigs were ventilated through a tracheostomy with continuous digital recordings of pulmonary inflation pressure and heart rate. The dose for 95% twitch suppression for gantacurium, CW002, cisatracurium, and rapacuronium was defined in the guinea pig. Transient and reproducible changes in pulmonary inflation pressure and heart rate were recorded after vagal nerve stimulation or intravenous injection of acetylcholine before and after pretreatment with cumulatively increasing concentrations of gantacurium, CW002, cisatracurium or a single concentration of rapacuronium.

Results: The doses for 95% twitch suppression for gantacurium, CW002, cisatracurium, and rapacuronium were 0.064 ± 0.006, 0.012 ± 0.0006, 0.10 ± 0.003, and 0.31 ± 0.05 mg/kg, respectively. Gantacurium, CW002, and cisatracurium were without effects on baseline pulmonary inflation pressures and were devoid of significant interactions with M2 and M3 muscarinic receptors *in vivo*.

Conclusion: These findings suggest that gantacurium and CW002 are devoid of significant effects at airway muscarinic receptors

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particularly M3 receptors on bronchial smooth musculature at doses several fold higher than those required for functional muscle paralysis.

What We Already Know about This Topic

- ❖ Some neuromuscular blocking drugs can induce or exacerbate bronchoconstriction by interacting with muscarinic receptors to augment the effect of acetylcholine
- ❖ Gantacurium and CW002 may avoid this effect

What This Article Tells Us That Is New

- ❖ In anesthetized guinea pigs, gantacurium and CW002 were devoid of effects on airway muscarinic receptors at doses several fold higher than those required for muscle paralysis

NEUROMUSCULAR blocking agents are used to facilitate tracheal intubation and to maintain muscle relaxation during many surgical procedures. In addition to their intended target, the nicotinic receptor on skeletal muscle, muscle relaxants can initiate bronchoconstriction *via* histamine release,[1,2] interaction with airway muscarinic receptors,[3–7] or anaphylaxis.[8–10] We have previously demonstrated that muscle relaxants that antagonize prejunctional parasympathetic M2 muscarinic autoreceptors can potentiate vagal nerve-induced bronchoconstriction,[6] a common event accompanying irritation of the upper airway during intubation and suctioning. In addition, we have demonstrated that some muscle relaxants exhibit an additional detrimental effect on airway muscarinic receptors: potentiation of the effect of acetylcholine at airway smooth muscle M3 muscarinic receptors by a positive allosteric effect at this receptor.[4,5]

Histamine release is one of the known risk factor for bronchospasm and has been attributed to some neuromuscular blocking agents, such as curare,[11–13] atracurium,[13] and mivacurium.[13] Instrumentation of the well-innervated upper trachea initiates an irritant reflex that results in the release of acetylcholine from parasympathetic nerves that activate M2 and M3 muscarinic receptors in airway smooth muscle, resulting in bronchoconstriction.[14] Typically, ongoing acetylcholine release from parasympathetic nerves is terminated by acetylcholine acting on M2 muscarinic inhibitory auto-feed-

back receptors present in the presynaptic terminals of post-ganglionic parasympathetic nerves.[15,16] However, nondepolarizing muscle relaxants are known to have differential antagonistic affinities for M2 muscarinic receptors.[6,7] Agents that have a higher affinity for the M2 *versus* the M3 muscarinic receptor can lead to selective inhibition of M2 muscarinic autoreceptors on parasympathetic nerves during periods of parasympathetic nerve stimulation[17] (*e.g.*, intubation) and result in enhanced release of acetylcholine, which then acts on unopposed M3 muscarinic receptors in airway smooth muscle, facilitating bronchospasm. An additive detrimental effect of allosteric potentiation of the effect of acetylcholine on postjunctional M3 muscarinic receptors on airway smooth muscle was discovered with a recently discontinued muscle relaxant, rapacuronium.[4,5]

Studies of rapacuronium and muscarinic receptors were originally pursued to identify the mechanism by which rapacuronium caused bronchoconstriction, so that newly synthesized muscle relaxants intended for clinical use could be screened for these detrimental interactions with airway muscarinic receptors. A new family of bisquaternary nondepolarizing muscle relaxants that have advantageous clinical kinetic profiles (rapid onset, ultra-short and short duration, and immediate antagonism by cysteine) are currently in development.[18–20] Gantacurium (GW280430A) has not demonstrated detrimental airway effects in early clinical trials,[18] and animal studies suggest a lack of effect of gantacurium on cardiac M2 muscarinic receptors.[20] A second compound of intermediate duration in this family, CW002, has also demonstrated a limited cardiopulmonary side effect profile and rapid reversibility by cysteine in animal studies. However, neither of these previous studies was designed to detect with high sensitivity the potential of these new relaxants to interact with airway muscarinic receptors nor were these studies designed to directly compare gantacurium and CW002 alongside rapacuronium, the relaxant that is now the standard for detrimental interactions with airway muscarinic receptors. We sought to evaluate the potential interaction of gantacurium and CW002 with airway muscarinic receptors using a well-characterized *in vivo* guinea pig model of airway constriction. This type of airway safety evaluation is of key strategic importance in the development of new neuromuscular blocking drugs that may be used to facilitate intubation. A comparative lack of effect in these studies *versus* the recently established detrimental properties of rapacuronium would suggest an improved safety profile in reducing the possibility of reactive bronchospasm during airway instrumentation.

Materials and Methods

Male Hartley guinea pigs (approximately 400 g) were used. Guinea pigs were handled in accordance with the standards established by the U.S. Animal Welfare Acts set forth in the National Institutes of Health guidelines, and all protocols were approved by the Columbia University Animal Care and Use Committee (New York, NY).

Reagents

Powdered gantacurium (AV430A) was supplied by Avera Pharmaceuticals (San Diego, CA), and CW002 was supplied by Weill Cornell Medical College. The clinical formulation of rapacuronium (Organon, Roseland, NJ) and cisatracurium (Abbott Laboratories, Abbott Park, IL) was used in these studies. All drugs were serially diluted in physiologic saline for *in vivo* studies. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Animal Model

Protocols were performed as previously described.[5] In brief, Hartley male guinea pigs (approximately 400 g) were anesthetized with 1.7 g/kg intraperitoneal urethane. Depth of anesthesia was monitored by changes in respiratory rate and response to foot pinch before paralysis. Surgical intervention did not begin until there was an absence of response to foot pinch. A 20% increase in respiration rate or a withdrawal response to foot pinch before paralysis was taken as a potential indication of inadequate anesthesia at which time incremental doses of urethane (0.2 g/kg intraperitoneal) were given until respiratory rate or foot withdrawal was unresponsive to stimulation. The trachea was cannulated with a 1-inch and 16-gauge intravenous catheter, and the animal was ventilated by a positive pressure, constant volume animal ventilator (3 ml tidal volume at 60 breaths/min) (model 683; Harvard Apparatus Co., South Natick, MA). Peak pulmonary inflation pressure (Ppi) was measured just proximal to the cannula through a side port *via* rigid plastic extension tubing connected independently to two pressure modules with differing ranges of sensitivity (TSD160B 0–12.5 cm H_2O and TSD160C 0–25 cm H_2O; Biopac Systems Inc., Goleta, CA). Both jugular veins were cannulated with PE-50 tubing for the administration of drugs, at which time the animals were paralyzed with succinylcholine (170 $\mu g \cdot kg^{-1} \cdot min^{-1}$). The carotid artery was cannulated with PE-50 tubing and connected to a pressure transducer inline with a Biopac TSD104A module for monitoring heart rate and blood pressure. Throughout the experiments, sympathetically mediated changes in hemodynamics (hypertension and tachycardia) were monitored as an indicator of the depth of anesthesia after muscle paralysis. A 20% increase in heart rate or systolic blood pressure was treated with incremental urethane (0.2 g/kg intraperitoneal). Both vagus nerves were tightly tied, but not cut, and the distal ends were placed on shielded electrodes. In a separate group of animals, ED_{95} for twitch suppression of sciatic nerve stimulation of gastrocnemius muscle response was measured for gantacurium, CW002, cisatracurium, and rapacuronium. Animals were instrumented as earlier, except that succinylcholine was omitted and the vagus nerves were not isolated. The tendon of the gastrocnemius muscle was surgically exposed and attached with a silk suture to an FT03 force transducer, and preload was set at 50 g. Two 22-gauge needles were percutaneously placed near the sciatic nerve to which electrodes were connected. Continuous DC square wave impulses (0.15 Hz,

2–10 V, 0.2 ms pulse width) were delivered from a Grass-Telefactor S88 stimulator to achieve a maximal twitch response in each animal. All pressure transducers were connected to a Biopac MP100A acquisition system, and data were continuously captured using Acknowledge software, version 3.7.3 (Biopac Systems).

Determination of ED_{95} for Gantacurium, CW002, Cisatracurium, and Rapacuronium in the Guinea Pig

After the establishment of the magnitude of the gastrocnemius twitch response, the potency of muscle paralysis of each muscle relaxant was tested in individual animals. CW002 and cisatracurium were given as cumulatively increasing concentrations during continuous stimulation and recording of twitch responses. The subsequent dose of each muscle relaxant was given when the diminution of the twitch from the previous dose had achieved a stable plateau (typically 5–10 min for CW002 and cisatracurium). Because of their relatively shorter duration of action, incrementally increasing doses of gantacurium and rapacuronium were given after the twitch response from the previous dose had totally returned to baseline values for 60 min. Dose response curves for twitch inhibition were calculated from four independent experiments from which 12–16 points were plotted. The percentage of twitch suppression was converted to logits, and the ED_{95} for twitch blockade was calculated from the regression line.

Effects of Muscle Relaxants on Vagal Nerve-stimulated or Intravenous Acetylcholine-stimulated Increases in Pulmonary Inflation Pressure and Heart Rate

The effects of each dose of each muscle relaxant on airway and cardiac M2 muscarinic receptor function were assessed by the effect on the magnitude of the vagally induced increase in pulmonary inflation pressure and the inhibition of bradycardia, respectively. The effect of each dose of each muscle relaxant on postjunctional M3 muscarinic receptors (*i.e.*, M3 muscarinic receptors on airway smooth muscle) was assessed by the effect on the magnitude of intravenous acetylcholine-induced increase in pulmonary inflation pressure. The potential ability of each dose of each muscle relaxant to release histamine was determined by assessing the effects of each dose of each muscle relaxant on baseline pulmonary inflation pressure[3,5] in the absence of vagal nerve or intravenous acetylcholine stimuli.

Electrical stimulation of both vagus nerves (10–20 Hz, 10–20 V, 0.2 ms pulse duration, 10–12 s train duration) produced transient increases in Ppi and bradycardia. A single voltage was selected for each animal within a range of 10–20 V to yield similar increases in airway pressure between animals. The nerves were stimulated at approximately 10-min intervals alternating with injections of intravenous acetylcholine (5–12 μg/kg in a volume of 0.15 ml physiologic saline) approximately every 10 min to directly stimulate postjunctional muscarinic receptors on the heart and airway smooth muscle. The dose of intravenous acetylcholine was chosen to yield similar increases in airway pressure between

animals and similarly to the vagal nerve stimulation (VNS)-induced increase in Ppi.

At least three repetitive cycles of alternating VNS followed 5 min later by intravenous acetylcholine were given to establish stable baseline responses of Ppi and heart rate. Subsequently, cumulative doses of muscle relaxants (each 0.15 ml) were administered intravenously, with each dose followed approximately 3 min later by a VNS and then 5 min later by an intravenous acetylcholine stimulus. Independent experiments were performed for each muscle relaxant in six animals. Cumulative doses of CW002 (0.03–0.6 mg/kg), gantacurium (0.075–1.5 mg/kg), or cisatracurium (0.025–0.5 mg/kg) were administered. Rapacuronium (8 mg/kg) was given after the last dose of each muscle relaxant in every animal (approximately 10 times the ED_{95} for twitch suppression in humans). At the end of the experiment, 0.5 mg/kg atropine was given to confirm that VNS- and intravenous acetylcholine-induced increase in Ppi and bradycardia was mediated *via* muscarinic receptors.

The effects of each dose of each muscle relaxant on airway and cardiac M2 muscarinic receptor function were assessed by augmentation of the magnitude of VNS-induced increases in Ppi and the inhibition of bradycardia, respectively. The effect of each dose of each muscle relaxant on postjunctional muscarinic receptors (*i.e.*, M3 muscarinic receptors on airway smooth muscle) was assessed by the effect on the magnitude of intravenous acetylcholine-induced increases in Ppi. Any direct effect of each dose of each muscle relaxant on baseline airway tone was also assessed.

Data Analysis

All values are given as mean $\pm$ SEM, where n represents the number of individual animals studied *in vivo*. The changes in Ppi or heart rate after the addition of muscle relaxants were compared with the initial change in Ppi or heart rate induced by VNS or intravenous acetylcholine using two-way analysis of variance with a *post hoc* Student *t* test with Bonferroni correction for multiple comparisons. Instat software (GraphPad Software Inc., San Diego, CA) was used with statistical significance set at $P < 0.05$.

Results

Determination of ED_{95} for Twitch Suppression

The potency for neuromuscular blockade (ED_{95}) for gantacurium, CW002, cisatracurium, and rapacuronium in the guinea pig was 0.064 ± 0.006, 0.012 ± 0.0006, 0.10 ± 0.003, and 0.31 ± 0.05 mg/kg, respectively (table 1). The potency ratio of each compound relative to rapacuronium and the dose ratio of the highest dose tested in the airway response study compared with the twitch response are also presented in table 1.

In Vivo *Studies of Muscle Relaxant Interaction with M2 or M3 Muscarinic Receptors*

The effects of intravenously administered muscle relaxants (cumulatively increasing doses up to 50 times the ED_{95} for

Table 1. Muscle Relaxant ED$_{95}$ for Twitch Suppression and Comparable Potencies

Compound	ED$_{95}$ for Twitch Suppression (mg/kg)	Potency Ratio of Each Compound to Rapacuronium (ED$_{95}$ of Rapacuronium/ED$_{95}$ of Each Compound)	Highest Dose Used in Airway Study (mg/kg)	Dose Ratio of Highest Dose Used in Airway Study to ED$_{95}$ for Twitch Suppression (Highest Dose of Airway Study/ED$_{95}$ of Each Compound)
CW002	0.012 ± 0.0006	25.8	0.6	50.0
Gantacurium	0.064 ± 0.006	4.8	1.5	23.4
Cisatracurium	0.10 ± 0.003	3.1	0.5	5.0
Rapacuronium	0.31 ± 0.05	1.0	8	25.8

twitch suppression in guinea pigs) were assessed on pulmonary inflation pressures and heart rate in the absence or presence of VNS or intravenously administered acetylcholine. Baseline pulmonary inflation pressure was 8.76 ± 0.18 cm H$_2$O before VNS and increased to 15.7 ± 0.62 cm H$_2$O after VNS, with an average VNS-induced increase of 79.4 ± 6.4% in pulmonary inflation pressure. Baseline pulmonary inflation pressure was 8.91 ± 0.20 cm H$_2$O before intravenous acetylcholine and increased to 16.5 ± 0.86 cm H$_2$O after intravenous acetylcholine, with an average acetylcholine-induced increase of 85.2 ± 9.6% in pulmonary inflation pressure. There was no significant difference in baseline pulmonary inflation pressure or in the magnitude of the induced increase between VNS and intravenous acetylcholine.

Acetylcholine released by VNS activates cardiac M2 muscarinic receptors inducing a transient bradycardia. The magnitude of the VNS-induced bradycardia before the administration of muscle relaxants is defined as 100%. Antagonism of M2 muscarinic receptors by cumulatively increasing concentrations of muscle relaxants reduces this induced bradycardia by VNS and is expressed as the percent of initial bradycardia. Resting heart rate was 234.3 ± 9.2 bpm and transiently decreased by 81.5 ± 2.5% with VNS.

CW002 (0.03–0.6 mg/kg; n = 6) alone did not affect baseline pulmonary inflation pressure or heart rate (data not shown). CW002 had no significant effect on either VNS-induced (fig. 1) or intravenous acetylcholine-induced increases (fig. 2) in pulmonary inflation pressure within and above clinically relevant concentrations. A high dose of CW002 (0.6 mg/kg; 50 times the ED$_{95}$ for twitch suppression in guinea pigs) only slightly (20%) attenuated VNS-induced bradycardia (fig. 3).

Gantacurium (0.075–1.5 mg/kg; n = 6) alone did not affect baseline pulmonary inflation pressure or heart rate (data not shown). Gantacurium had no significant effect on either VNS-induced (fig. 1B) or intravenous acetylcholine-induced increases (fig. 2B) in pulmonary inflation pressure within and above clinically relevant concentrations. A high dose of gantacurium (1.5 mg/kg; 23 times the ED$_{95}$ for twitch suppression in guinea pigs) attenuated VNS-induced bradycardia (fig. 3B).

Cisatracurium (n = 6), which shares some similarity in molecular structure to the new bisquaternary muscle relaxants and which has been shown previously to have no effect on airway muscarinic receptors,[6] was used as a negative control in the current studies. Cisatracurium (0.025–0.5 mg/kg) alone did not affect baseline pulmonary inflation pressure or heart rate (data not shown). Cisatracurium had no significant effect on either VNS- (fig. 1C) or intravenous acetylcholine-induced (fig. 2C) increases in pulmonary inflation pressures. Higher concentrations of cisatracurium dose dependently attenuated VNS-induced bradycardia (fig. 3C). Cisatracurium administration alone had no effect on airway pressures.

Rapacuronium (n = 18), which has been previously shown in this model to potentiate both VNS- and intravenous acetylcholine-induced increases in pulmonary inflation pressure,[5] was used as a positive control. Rapacuronium (8 mg/kg; 26 times the ED$_{95}$ for twitch suppression in guinea pigs), given after the last dose of each muscle relaxant in every animal, had no effect on baseline pulmonary inflation pressure but potentiated both VNS- (fig. 1) and intravenous acetylcholine-induced (fig. 2) increases in pulmonary inflation pressure and prevented VNS-induced bradycardia (fig. 3). After a single dose of atropine (0.5 mg/kg; n = 18), rapacuronium-induced increases in pulmonary inflation pressure or bradycardia after VNS or intravenous acetylcholine were completely blocked confirming the muscarinic receptor mediation of these responses.

Discussion

The primary finding of this study is that gantacurium and CW002 at up to 23 and 50 times the ED$_{95}$ of twitch suppression in guinea pigs, respectively, had no significant effect on either vagal nerve stimulated- or intravenous acetylcholine-induced increases in pulmonary inflation pressures *in vivo* in guinea pigs. In contrast, rapacuronium, a muscle relaxant previously shown in this model to have detrimental interactions with airway muscarinic receptors, potentiated both nerve- and acetylcholine-induced increases in pulmonary inflation pressures *in vivo*. The ED$_{95}$ for twitch suppression by gantacurium in the guinea pig (0.06 ± 0.006 mg/kg) is identical to that previously determined in monkeys[20] and three times more potent than that found in humans.[18]
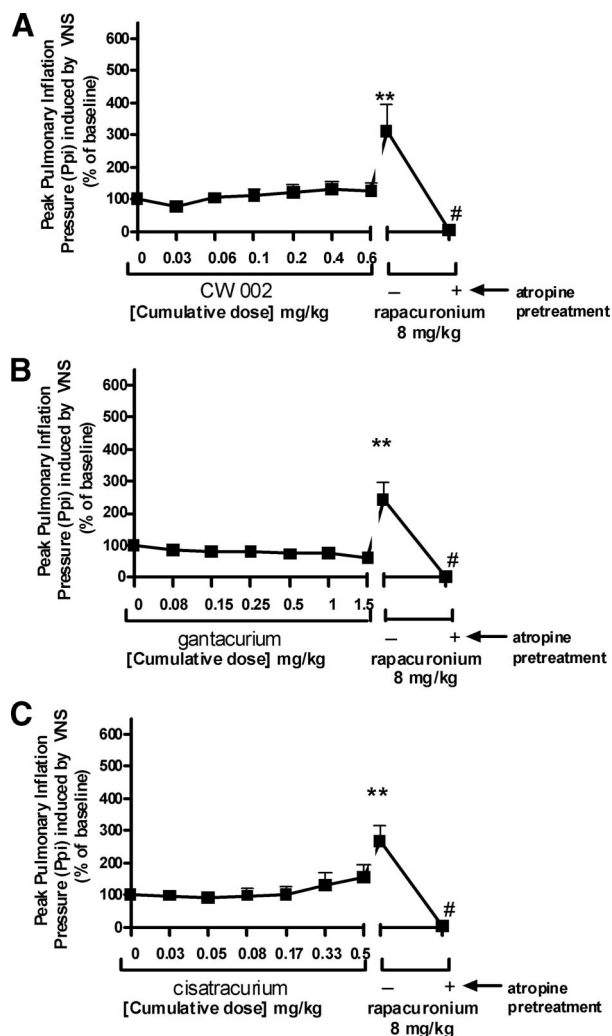
Fig. 1. *In vivo* effects of muscle relaxants on vagal nerve–induced peak pulmonary inflation pressures in guinea pigs to measure antagonism of airway M2 muscarinic receptor. Peak pulmonary inflation pressure expressed as a percent of baseline effect of vagal nerve stimulation (VNS) in the presence of cumulatively increasing concentrations of intravenous muscle relaxants (n = 6). After each dose response, each animal received a single intravenous dose of rapacuronium as a positive control. Subsequently, each animal received atropine to confirm the muscarinic origin of measured responses. CW002, gantacurium, and cisatracurium (negative control) were without significant effects on increases in pulmonary inflation pressure induced by vagal nerve stimulation. Rapacuronium (positive control) caused 200–400% increases in pulmonary inflation pressure (indicative of M2 muscarinic receptor antagonism), which was blocked after atropine administration. The rapacuronium effect was clearly demonstrated in each animal after the lack of such property had been shown for one of the comparative compounds. ** $P < 0.01$ compared with initial baseline, # $P < 0.05$ compared with rapacuronium in absence of atropine.

Guinea pig airways and heart rate responses have been widely used models to characterize muscle relaxants interactions with M2 and M3 muscarinic receptors.[3,5,17,21] Stimulation of parasympathetic nerves in the airway either by direct VNS *in vivo* or by electrical field stimulation *in vitro* releases acetylcholine that acts on M3 muscarinic receptors on airway smooth muscle to cause smooth muscle contrac-
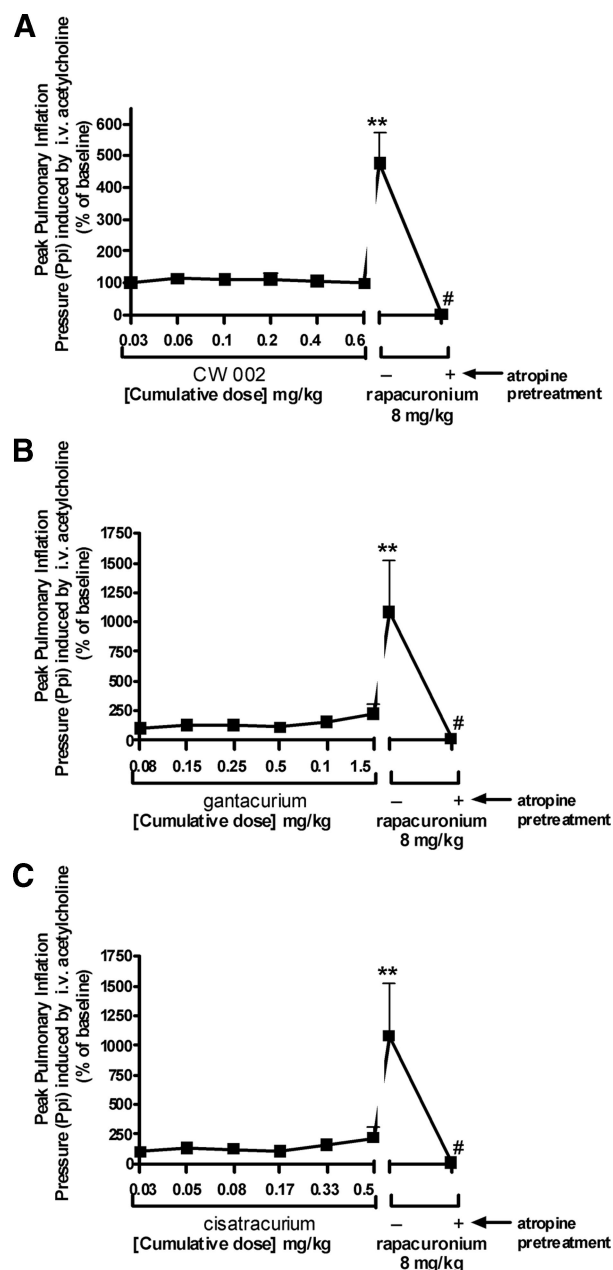

Fig. 2. *In vivo* effects of muscle relaxants on intravenous acetylcholine-induced peak pulmonary inflation pressures in guinea pigs to measure allosteric potentiation of airway smooth muscle M3 muscarinic receptor. Peak pulmonary inflation pressure expressed as a percent of baseline effect of intravenous acetylcholine in the presence of cumulatively increasing concentrations of intravenous muscle relaxants (n = 6). After each dose response, each animal received a single intravenous dose of rapacuronium as a positive control. Subsequently, each animal received atropine to confirm the muscarinic origin of measured responses. CW002, gantacurium, and cisatracurium (negative control) were without significant effects on increases in pulmonary inflation pressure induced by intravenous acetylcholine. Rapacuronium (positive control) significantly increased pulmonary inflation pressure (indicative of positive allosteric effects with acetylcholine at the M3 muscarinic receptor) in every animal. This response was blocked after atropine administration. ** $P < 0.01$ compared with initial baseline, # $P < 0.05$ compared with rapacuronium in absence of atropine.
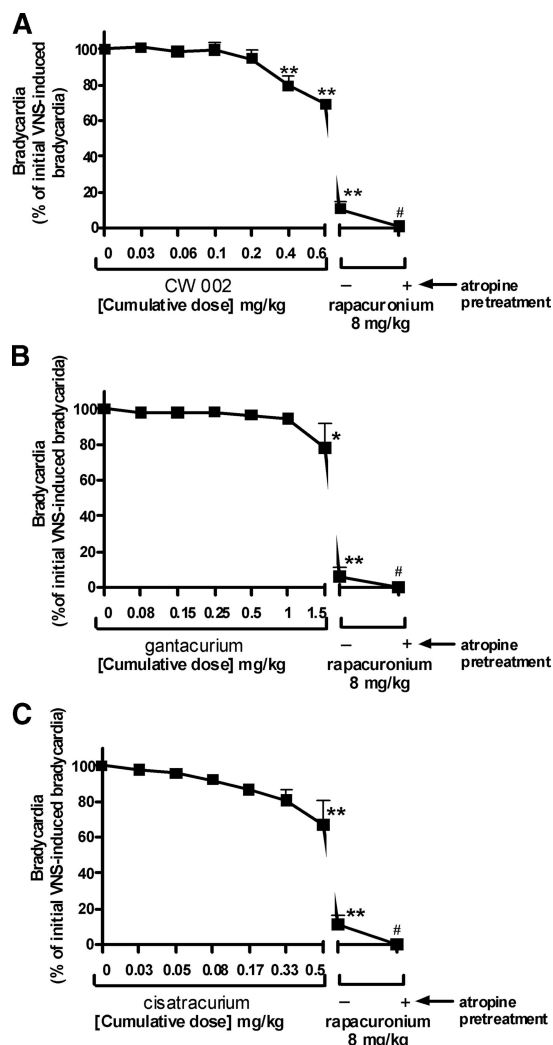
Fig. 3. *In vivo* effects of muscle relaxants on vagal nerve–induced bradycardia in guinea pigs to measure antagonism of cardiac M2 muscarinic receptor. The magnitude of vagal nerve stimulation (VNS)-induced bradycardia before muscle relaxants was expressed as 100%. Antagonism of cardiac M2 muscarinic receptors by muscle relaxants resulted in reduced bradycardia during subsequent VNS challenges, which is expressed as a percent of the initial baseline VNS-induced bradycardia. Cumulatively increasing concentrations of intravenous muscle relaxants were administered with measurements of VNS-induced bradycardia between each dose (n = 6). After each completed dose response, each animal received a single intravenous dose of rapacuronium as a positive control. Subsequently, each animal received atropine to confirm the muscarinic origin of measured responses. CW002 did not block vagal nerve–induced bradycardia until a dose of 0.4 mg/kg, 33 times the ED_{95} for twitch suppression in guinea pigs, which showed a weak response (20% block). Gantacurium did not block vagal nerve–induced bradycardia until a dose of 1.5 mg/kg (23 times the ED_{95} for twitch suppression in guinea pigs) produced a weak response (20% block). Cisatracurium did not block vagal nerve-induced bradycardia until a dose of 0.5 mg/kg (five times the ED_{95} for twitch suppression in guinea pigs) produced a weak response (30% block). Rapacuronium (8 mg/kg; 26 times ED_{95} for twitch suppression in guinea pigs) profoundly blocked VNS-induced bradycardia (90–95% block) in all animals (n = 18) indicative of cardiac M2 muscarinic receptor antagonism. * $P < 0.05$ compared with initial baseline, ** $P < 0.01$ compared with initial baseline, # $P < 0.05$ compared with rapacuronium in absence of atropine.

tion measured by an increase in pulmonary inflation pressure *in vivo* or direct smooth muscle contraction of an isolated airway ring *in vitro*. Typically, the released acetylcholine acts on inhibitory auto-feedback M2 muscarinic receptors on airway postganglionic parasympathetic nerves to inhibit further acetylcholine release.[21] In the heart, the stimulation of vagal nerves *in vivo* releases acetylcholine to act on cardiac M2 muscarinic receptors to induce bradycardia. Thus, the measurements of pulmonary inflation pressure or heart rate *in vivo* during VNS allows for the evaluation of M2 muscarinic receptor function. An antagonist or negative allosteric modulator at M2 muscarinic receptors (including some muscle relaxants)[17] would block the airway parasympathetic nerve inhibitory M2 muscarinic receptor, leading to enhanced acetylcholine release and increased smooth muscle contraction, and would block parasympathetic nerve induced M2 muscarinic receptor-mediated bradycardia. This model also allows for the study of intravenously administered acetylcholine acting directly on the airway smooth muscle M3 muscarinic receptor inducing airway contraction or the cardiac M2 muscarinic receptor inducing bradycardia.

These models of whole animal airway responses and isolated airway contractile responses combined with *in vitro* receptor pharmacology were instrumental in elucidating the detrimental effect of rapacuronium on airway smooth muscle constriction that led to its removal from clinical practice.[22,23] Although rapacuronium antagonized the M2 muscarinic receptor similar to other muscle relaxants,[6,17,24] its unique and central detrimental effect was its potentiation of the action of acetylcholine at airway smooth muscle M3 muscarinic receptors.[4,5] This allosteric potentiation by rapacuronium at the M3 muscarinic receptor was demonstrated in both isolated guinea pig airway rings *in vitro*[4] and intact whole guinea pig airways *in vivo*,[5] and this same *in vivo* model was therefore used in the current study for the evaluation of the new bisquaternary nondepolarizing muscle relaxants gantacurium and CW002 studied in parallel with rapacuronium.

In vivo, with doses of CW002 up to 50 times the determined ED_{95} for twitch suppression in guinea pigs (0.6 mg/kg), there was no evidence of potentiation of vagal nerve-induced increases in pulmonary inflation pressure. There was evidence of M2 muscarinic receptor antagonism of the M2 muscarinic receptors on the heart illustrated by the blockade of vagal nerve-induced bradycardia, but this effect only occurred at doses that are likely to be supratherapeutic ($\geq$0.4 mg/kg). As demonstrated before,[5] rapacuronium (8 mg/kg; 26 times the ED_{95} for twitch suppression in guinea pigs) caused a nearly twofold increase in pulmonary inflation pressures and complete blockade of bradycardia after VNS in these same animals. These results suggest that *in vivo* attenuation of bradycardia may be a more sensitive indicator of M2 muscarinic receptor antagonism than potentiation of vagal nerve–induced increases in pulmonary inflation pressure or that stimulating the vagus nerve activates other fiber subtypes to the airways (*e.g.*, nonadrenergic, noncholinergic)

in addition to parasympathetic cholinergic fibers. Nonetheless, the central result is that CW002 does not have significant effects on M2 muscarinic receptor function *in vivo* within clinically relevant doses.

Similarly, CW002, at clinically relevant concentrations, did not exhibit significant potentiation of acetylcholine effects at M3 muscarinic receptors *in vivo*. At concentrations up to 50 times the determined ED_{95} for twitch suppression in guinea pigs, CW002 demonstrated no potentiation of intravenous acetylcholine-induced increases in pulmonary inflation pressure, indicating a lack of effect of CW002 at postjunctional M3 muscarinic receptors. As demonstrated before, rapacuronium caused a nearly fourfold increase in pulmonary inflation pressures after intravenous acetylcholine in these same animals.

Only the highest *in vivo* dose of gantacurium tested (1.5 mg/kg = 23 times the ED_{95} for twitch suppression in guinea pigs) showed a small but significant blockade of vagal nerve-induced bradycardia. Gantacurium demonstrated no interaction with M3 muscarinic receptors *in vivo*.

Cisatracurium served as a negative control in the current study because of the previous demonstration that it has no detrimental interactions with muscarinic receptors at clinically relevant concentrations. Interestingly, we were able to detect a small but significant attenuation of vagal nerve-induced bradycardia at the highest dose tested (0.5 mg/kg = 5 times ED_{95} for twitch suppression in guinea pigs), suggesting weak M2 muscarinic receptor antagonism at this high dose. There was no effect of cisatracurium on vagal nerve-induced increases in pulmonary inflation pressures even at this extreme dose, again supporting that in this *in vivo* model, blockade of vagal nerve–induced bradycardia is a more sensitive measure of M2 muscarinic receptor antagonism than is potentiation of vagal nerve–induced bronchoconstriction. Our previous *in vivo* study demonstrated that 1.5 mg/kg rapacuronium, which is 4.8 times the ED_{95} for twitch suppression in guinea pig, was sufficient for potentiating intravenous acetylcholine-induced bronchoconstriction.[5] This suggests that the concentration of cisatracurium used in the current study (0.5 mg/kg or 5 times the ED_{95} for twitch suppression) was sufficiently high to serve as an appropriate negative control in these studies.

Although both antagonism of the prejunctional neural M2 muscarinic receptor and allosteric potentiation of acetylcholine at the postjunctional muscle M3 muscarinic receptor could theoretically potentiate airway smooth muscle constriction, clinical experience with muscle relaxants would suggest that potentiation at the M3 muscarinic receptor is far more detrimental to airway tone.[4,5,23] Pancuronium and gallamine have long been known to be potent M2 muscarinic receptor antagonists,[17] yet clinical experience suggests that these muscle relaxants did not cause bronchoconstriction even when administered during periods of heightened parasympathetic nerve activity (*e.g.*, intubation). This is likely because of pancuronium's antagonism of postjunctional M3 muscarinic receptors and gallamine's lack of significant affinity for the M3 muscarinic receptor.[7] Conversely, rapacuronium was found to potentiate acetylcholine's action at M3 muscarinic receptor by a positive allosteric mechanism.[4] In the current study, neither CW002 nor gantacurium was found to have significant potentiation of acetylcholine action at postjunctional M3 muscarinic receptors in intact airways of the guinea pig.

In addition to interactions with muscarinic receptors, additional mechanisms exist by which muscle relaxants can potentiate bronchoconstriction, including the release of histamine. The *in vivo* study of pulmonary inflation pressures is an ideal model to detect potential airway tone effects of histamine release,[3] and we have previously shown in this model that mivacurium-induced increases in pulmonary inflation pressure are blocked by the histamine receptor antagonist pyrilamine.[5] The structural group to which CW002 and gantacurium belong has the tendency to release histamine. Gantacurium produced clinically relevant histamine release at 0.54–0.72 mg/kg ($\sim$1–1.5 times intubation dose) in humans.[18] Despite the increase in plasma histamine, there was no evidence of bronchoconstriction up to 50 times the ED_{95} for twitch suppression (3.2 mg/kg) in dogs.[19] In the current study, there was no effect on baseline pulmonary inflation pressure of the highest doses of CW002 (0.6 mg/kg) or gantacurium (1.5 mg/kg) (50 and 23 times the ED_{95} for twitch suppression in guinea pigs, respectively), indicating that these drugs do not release amounts of histamine that might increase pulmonary inflation pressures in the guinea pig airway.

In summary, using a well-defined *in vivo* guinea pig model previously used to define the mechanism of rapacuronium's detrimental interaction with airway muscarinic receptors, we show that CW002 and gantacurium do not demonstrate interactions with airway M2 and M3 muscarinic receptors at concentrations up to 50 times their ED_{95} concentrations for muscle relaxation.

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