Title: "Pillar[6]MaxQ and sugammadex enhance recovery from rocuronium- and vecuronium-mediated neuromuscular blockade with similar effects in isoflurane-anesthetized rats.**"**

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METHODS

Animals. Male Sprague Dawley rats (starting weight 331±7 g; n = 29) were used (Charles River Laboratories, Wilmington, MA) under an approved MGH IACUC protocol. Overt physical or behavioral signs of disease or pathology were grounds for active study exclusion and no animals met this criteria. Four animals died during the course of the study from complications related to anesthesia or airway instrumentation. There were no deaths or overt toxicities attributable to sugammadex or P6AS.

Data acquisition. All data were acquired (1024 Hz) and instrumentation controlled using LabView software (National Instruments, Austin, TX) run on a Macintosh computer (Apple, Cupertino, CA) interfaced with multiple USB-6009 data acquisition boards (National Instruments).

Anesthesia and instrumentation. Rats were anesthetized with isoflurane (5% induction, 1.5% maintenance; Covetrus, Portland, ME) using an IsoTec3 variable bypass vaporizer and a routinely-calibrated Capnomac Ultima medical gas analyzer (GE Healthcare, Buckinghamshire, United Kingdom), instrumented with a 24G tail vein angiocatheter and a 14G orotracheal angiocatheter, and positioned supine. The orotracheal 14G angiocatheter was attached to a Luer T-piece through which a stable air/ anesthetic gas flow (1 LPM) was provided by mass flow controller (model GE50A with Type 247 power supply; MKS Instruments Inc., Andover, MA). Positive pressure mechanical ventilation (15/2.5 cm-H_2O at 30 BPM by cycling the gas flow) was initiated 60 sec prior to muscle relaxant administration, continuously monitored using an analog pressure gauge, and discontinued upon return of baseline accelerometer signal amplitude and strong breathing efforts. Rectal temperature was maintained at 37°C by a thermistor-controlled, automated heat lamp via Labview. Stainless steel needles (14 x 0.38 mm; Natus Medical, Inc., Middleton, WI) were inserted, two in the genioglossus muscle, and a third subcutaneously in the neck, for qualitative breathing assessment by electromyography (EMG) phasic bursting activity. The EMG signal was amplified (10,000-fold) and band-pass filtered (30-3,000 Hz) with an AC amplifier (QP511; Grass Instruments, West Warwick, RI).

Train-of-four and breathing monitoring. Two woundsclips (9 mm Autoclips, Braintree Scientific, Braintree, MA) were applied to the shaved groin skin over the left femoral nerve and were grasped each with an alligator clip lead. A nerve stimulator (TOF-Watch SX; Organon, New Jersey, NJ) provided repeated, supramaximal 2 Hz train-of-four (TOF) stimuli (T1, T2, T3, and T4) through the two alligator clip leads every 15 sec. An accelerometer (ADXL335, ± 3g; Analog Devices, Wilmington, MA) was affixed to the left hind paw and was used to quantify limb movement in response to femoral nerve stimulation. The accelerometer-affixed limb was tensioned with a rubber band (#19, 3.5"x1/16"; Staples, Framingham, MA) taped to the study platform to prevent migration. The right lower leg and tail base

were taped to the study platform for stability. Supramaximal stimulation (9.2±0.2 mA, n =117) was determined for each animal by increasing stimulation intensity from a minimum until the accelerometer signal amplitude plateaued. T4/T1 amplitude ratio (TOF ratio) and, more specifically, time to 90% recovery in TOF ratio (TOF_{90}) were calculated by *post hoc* signal analysis with a Labview peak detection virtual instrument algorithm. TOF-Watch SX generated TOF ratio values using the built-in accelerometer were erratic and unreliable. Because the animal would resume breathing in synchrony with the ventilator, it was difficult to gauge onset. Therefore, overt recurrence of regular, phasic genioglossus muscle EMG bursting activity was used to indicate breathing onset, more objectively, upon relaxant clearance or reversal.

Study drugs. Drugs were administered in normal (0.9%) saline 2 mls/kg IV final volume over 5 sec and were flushed in with 1 ml saline. P6AS (provided by Reversal Therapeutics, National Harbor, Maryland) was dissolved in saline (50 mg/ml), and prepared fresh each day or frozen for use the following day. Vecuronium (Hospira), rocuronium (Auromedics), and sugammadex (Bridion® /Merck) (MGH operating room pharmacy) were used. Vecuronium was dissolved in saline (1 mg/ml), aliquoted, frozen, and thawed prior to each study.

Study design and protocol. Rats were randomly assigned to one of four unblinded study groups by arbitrary cage selection: 1) vecuronium followed by saline or P6AS (8 rats), 2) vecuronium followed by saline or sugammadex (8 rats), 3) rocuronium followed by saline or P6AS (7 rats), and 4) rocuronium followed by saline or sugammadex (6 rats). Power analysis using values from prior published rat TOF_{90} data suggested a sample size of 6 would be sufficient to detect a 30% difference in mean values (P = 0.05 and Power = 80%).[1] Rats in each group received muscle relaxant (vecuronium, 0.7 mg/kg IV or rocuronium, 3.5 mg/kg IV both at 2 x ED_{90} dose[1]) followed by saline or reversal agent — a single dose each study session — in ascending doses. Rats recovered at least three days between study sessions.

Following instrumentation and positioning, supramaximal femoral nerve stimulation was applied repeatedly for 45 mins (TOF at 2 Hz every 15 sec). Saline, vecuronium, or rocuronium were administered at 15 min and saline, sugammadex or P6AS were administered at 15 min 30 sec. EMG and accelerometer signals were continuously monitored.

Data analysis and statistics. Since accelerometry yields a TOF ratio value slightly greater than 100% (i.e., T4 amplitude is slightly greater than T1), values were normalized to 100%, as others have done, using the average value over 5 min collected prior to muscle relaxant administration.[2] Data in Figure 1C and 1D reflect time from administration of reversal agent or saline at 15 min 30 sec. All data are reported as mean ± SEM and were compared by one-way ANOVA followed by a Sidak's *post hoc* test (Prism Software, San Diego, CA); a P-value less than 0.05 was significant. Time to recovery following saline treatment only (i.e., no reversal agent) within each study group was used as the negative control referenced in multiple comparisons to determine if the reversal agent, sugammadex or P6AS, shortened recovery times.

SUPPLEMENTAL FILE REFERENCES:

1. Hoffmann U, Grosse-Sundrup M, Eikermann-Haerter K, et al. Calabadion: A new agent to reverse the effects of benzylisoquinoline and steroidal neuromuscular-blocking agents. *Anesthesiology*. Aug 2013;119(2):317-25. doi:10.1097/ALN.0b013e3182910213

2. Suzuki T, Fukano N, Kitajima O, Saeki S, Ogawa S. Normalization of acceleromyographic train-of-four ratio by baseline value for detecting residual neuromuscular block. *Br J Anaesth*. Jan 2006;96(1):44-7. doi:10.1093/bja/aei273