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Comparative effectiveness of Calabadiion and sugammadex to reverse non-depolarizing neuromuscular blocking agents

Friederike Haerter^{a,*} [cand.med.], Jeroen Cedric Peter Simons^{a,*} [cand.med.], Urs Foerster, M.D.^{b,*}, Ingrid Moreno Duarte, M.D.^a, Daniel Diaz-Gil^a [cand.med.], Shweta Ganapati, B.S.^c, Katharina Eikermann-Haerter, M.D.^d, Cenk Ayata, M.D.^d, Ben Zhang, Ph.D.^c, Manfred Blobner, M.D.^b, Lyle Isaacs, Ph.D.^c, and Matthias Eikermann, M.D., Ph.D.^{a,e}

Manfred Blobner: blobner@lrz.tum.de; Lyle Isaacs: lisaacs@umd.edu

^aDepartment of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, 55 Fruit Street, Boston, Massachusetts 02114

^bClinic of Anesthesiology, Klinikum rechts der Isar, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany

^cDepartment of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742-4454

^dDepartment of Radiology, Massachusetts General Hospital and Harvard Medical School, 55 Fruit Street, Boston, Massachusetts 02114

^eDepartment of Anesthesia and Critical Care Medicine, University Hospital Essen, University Duisburg-Essen, Hufelandstrasse 55, 45147 Essen, Germany

Abstract

Background—We evaluated the comparative effectiveness of calabadiion 2 to reverse non-depolarizing neuromuscular blocking agents (NMBAs) by binding and inactivation.

Methods—The dose-response relationship of drugs to reverse vecuronium, rocuronium, and cisatracurium-induced neuromuscular block (NMB) was evaluated *in vitro* (competition binding assays and urine analysis), *ex vivo* (n=34; phrenic nerve hemidiaphragm preparation) and *in vivo* (n=108; quadriceps femoris muscle of the rat). Cumulative dose-response curves of calabadiions, neostigmine, or sugammadex were created *ex vivo* at steady-state deep NMB. In living rats, we studied the dose-response relationship of the test drugs to reverse deep block under physiological conditions and we measured the amount of calabadiion 2 excreted in the urine.

Results—*In vitro* experiments showed that calabadiion 2 binds rocuronium with 89 times the affinity of sugammadex ($K_a = 3.4 \times 10^9 \text{ M}^{-1}$ and $K_a = 3.8 \times 10^7 \text{ M}^{-1}$). Urine analysis (proton

Corresponding author: Matthias Eikermann, MD, PhD, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, 55 Fruit Street, Boston, MA02114, Phone: +1-617-643-2399, Fax: +1-617-726-5985, meikermann@partners.org.

*These authors made equal contributions to this manuscript.

Conflicts of interest disclosure

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nuclear magnetic resonance), competition binding assays *and ex vivo* study results obtained in the absence of metabolic deactivation are in accordance with an 1:1 binding ratio of sugammadex and calabadiion 2 toward rocuronium. In living rats, calabadiion 2 dose-dependently and rapidly reversed all NMBAs tested. The molar potency of calabadiion 2 to reverse vecuronium and rocuronium was higher compared to sugammadex. Calabadiion 2 was eliminated renally, and did not affect blood pressure or heart rate.

Conclusion—Calabadiion 2 reverses NMB-induced by benzyliisoquinolines and steroidal NMBAs in rats more effectively, i.e. faster, than sugammadex. Calabadiion 2 is eliminated in the urine and well tolerated in rats.

Keywords

Reversal of neuromuscular blockade; encapsulation; safety

Introduction

Reversal of neuromuscular blocking agents (NMBAs) is an important strategy for accelerating recovery from neuromuscular blockade (NMB).^{1–4} Acetylcholinesterase (AChE) inhibitors, typically neostigmine, are the only drugs to reverse NMB approved by the US FDA. AChE inhibitors cannot reverse deep NMB^{5–7} and may even induce muscle weakness when administered in large doses following recovery from NMBAs.⁸

Sugammadex, an anionic γ -cyclodextrin derivative, reverses NMB by binding and thereby inactivating steroidal NMBAs.⁹ Hypersensitivity to sugammadex as well as dose-dependent sugammadex-induced anti-coagulation (increases activated partial thromboplastin time and prothrombin time) were reported.^{10–12} While sugammadex is used outside the USA the FDA has declined it twice based on the appearance of these side effects.¹³

Over the past decade, the preparation and molecular recognition properties of a new class known as cucurbiturils have been thoroughly explored.^{14,15} Calabadiion 1 – a cucurbituril derivative – reverses the steroidal non-depolarizing NMBAs rocuronium and vecuronium as well the benzyliisoquinoline cisatracurium by encapsulation.¹⁶ However, the *in vitro* binding affinity of calabadiion 1 toward rocuronium is slightly less than the reported binding affinity of sugammadex.¹⁷ Calabadiion 2 (fig. 1) is a major leap forward in selectivity and binding affinity towards NMBAs. The selectivity of calabadiion 2 for rocuronium is 18900 times that of acetylcholine, whereas calabadiion 1's selectivity to rocuronium was only 350 times higher than to acetylcholine.

A major goal of this study was to conduct a head-to-head comparison of the binding affinity to steroidal NMBAs between calabadiion 2 and sugammadex *in vitro*, *ex vivo* (in the absence of drug metabolism) and *in vivo*. In addition, we evaluated the effectiveness of calabadiion 2 to reverse cisatracurium-induced NMB.

Methods

Calabadiion 1 and 2 were synthesized in the Isaacs laboratory (University of Maryland, College Park, Maryland, USA) according to the published procedures.¹⁸

In vitro

To determine the relative binding affinity of calabadion 2 and sugammadex toward rocuronium, we allowed calabadion 2 and sugammadex to compete for a limited amount of rocuronium in one solution (pH = 7.4; 25°C). We determined the concentrations of calabadion 2•rocuronium and sugammadex•rocuronium by ^1H NMR spectroscopy by integration of the diagnostic resonances for the CH_3 -groups of rocuronium in the calabadion 2•rocuronium complex which appear between 0.3 and -0.3 parts per million (ppm) relative to an internal standard of known concentration. Substitution of the concentrations into the usual equilibrium expressions along with mass balance considerations allowed us to calculate the difference in binding affinity between calabadion 2 and sugammadex towards rocuronium.

To determine the concentrations of calabadion 2 and rocuronium in the urine samples from the rats, we used a different ^1H NMR assay. For this assay, we evaporate the urine samples and then redissolve them in buffered D_2O to allow for ^1H NMR analysis. We determine the concentrations of calabadion 2 and rocuronium by integration of the diagnostic resonances for the methyl-groups of rocuronium in the calabadion 2•rocuronium complex which appear between 0.3 and -0.3 ppm relative to an internal standard of known concentration.

To monitor the transformation of cisatracurium over time in the Krebs-Henseleit buffer to laudanosin and pentamethylenediacrylate ($\text{H}_2\text{C}=\text{CHCO}_2(\text{CH}_2)_5\text{O}_2\text{CCH}=\text{CH}_2$) by Hoffmann degradation we performed a third different ^1H NMR assay. In this assay, we monitored the formation of pentamethylenediacrylate by the appearance of the diagnostic resonances for the terminal $\text{HC}=\text{CH}_2$ groups. We integrate these diagnostic resonances normalized with respect to the initial concentration of cisatracurium reflected by the integral for the resonances of the central $(\text{CH}_2)_3$ group of cisatracurium and pentamethylenediacrylate to determine the concentration of pentamethylenediacrylate as a function of time.

To determine the binding constant for the calabadion 2•succinylcholine complex we performed a UV/Vis competition assay as published previously.¹⁷ In brief, we prepare a solution of calabadion 2•Rhodamine 6G which has a known K_a ($4.8 \pm 0.1 \times 10^5 \text{ M}^{-1}$) and which displays a different UV/Vis spectrum than Rhodamine 6G alone. Upon addition of increasing concentrations of succinylcholine we observe that the UV/Vis spectrum reverts toward that of free Rhodamine 6G which reflects the competitive binding. We monitor the change in UV/Vis absorbance and fit it to a standard competition binding model as published previously to determine the K_a value for calabadion 2•succinylcholine.¹⁷

Ex vivo

Animals - Ex vivo—A total of 34 male Sprague–Dawley rats whose weights ranged from 340–406 g were used. Institutional guidelines for animal care and usage for research purposes were strictly followed. All procedures involving animals were approved by the Regierung von Oberbayern (AZ: 55.2-1-54-2532.3-88-13) and experiments were conducted at the research laboratories of Klinikum Rechts der Isar in Munich, Germany.

Experimental Procedures – Ex vivo—Rats were anesthetized with CO₂ for decapitation, in order to dissect two hemi-diaphragm preparations per rat with intact phrenic nerve.

Each hemi-diaphragm was secured to a phrenic nerve-diaphragm tissue holder and mounted in a 65 ml tissue bath (ISO-07-TSZ2D, Experimetria Ltd., Hungary) with a Krebs-Henseleit buffer solution (KH₂PO₄ 1.0 mM, MgSO₄·7H₂O 0.6 mM, KCl 5.0 mM, NaHCO₃ 30.0 mM, α-D-Glucose 20.0 mM, NaCl 118 mM, CaCl₂ 2.5 mM) maintained at 37°C (AMP-09 Temperature controller, Experimetria Ltd., Hungary) and 95% O₂ and 5% CO₂ (Vol %) insufflation.

The hemi-diaphragms were attached to an isometric force-displacement transducer (FSG-01/200 Force Transducer, Experimetria Ltd., Hungary) at the centrum tendineum using a crocodile clip (AGF1 Crocodileclip, SKS Hirschmann, Germany). Measurements were amplified by AMP-01-SG Classic bridge amplifier, and recorded with the S.P.E.L. Advanced Isosys software (Experimetria Ltd., Hungary). A resting force of 10 mN was applied for 20 min followed by 50 mN to assess a resting and peak force during the experiment. The phrenic nerve was stimulated with a 2 Hz train-of-four (TOF) stimulus every 15 s (rectangular pulses of 0.2 ms duration with a supra-maximal voltage) using a square wave stimulator (ST-03-O4, Experimetria Ltd., Hungary). To ensure a steady-state, stimulation was continued for 15 min before further measurements were taken.

The NMB-induced by rocuronium and cisatracurium was quantified as the depression of the force amplitude of the first twitch response (T1) to the TOF-stimulus. In order to decrease the interindividual variability when making comparison of calabadiol 2's effects to reverse benzyloquinolines and steroids, one hemi-diaphragm preparation of each rat was treated with rocuronium, and the other with cisatracurium.

Rocuronium and cisatracurium were added in steps of 150 µg and 100 µg, respectively, until the T1 value was decreased by more than 10% compared to the control value. Following each NMBA increment a stable T1 value, i.e. three consecutive T1 with less than 3% deviation, was achieved after 5 to 8 min. The NMBA boluses were therefore added every consecutive 10 min. Based on these measurements, we then subsequently applied a 1.5 times the EC₉₉ of rocuronium (62µM) and cisatracurium (16µM) to evaluate effectiveness of the reversal agents. Neuromuscular transmission recovery was assessed by recovery of T1 and TOF ratio (ratio of the twitch response of the fourth to the first stimulus of each TOF stimulation).

In order to evaluate their effectiveness, the reversal agents were administered every 10 min until recovery of the TOF ratio to 0.9.

To evaluate rocuronium reversal, we administered an initial bolus of sugammadex or calabadiol 2, each 5 mg, followed by repetitive doses of 0.4 mg. For cisatracurium reversal, an initial dose of 8 mg calabadiol 2 was added to the tissue bath, followed by repetitive doses of 1.5 mg. In order to address the impact of the Hoffmann degradation on the concentration of cisatracurium when cisatracurium was used as NMBA, 10 additional hemi-diaphragm preparations were incubated in 6 µM cisatracurium bath (EC₉₀) to evaluate

spontaneous recovery. In these preparations the twitch response was measured every 10 min, and the expected spontaneous sigmoidal recovery course was observed. Linearization by logit transformation revealed a mean increase of twitch responses by 0.26 each 10 min. The twitch response values of the reversal studies were corrected accordingly, before the individual concentration response relations were calculated. In order to account for the time dependent degradation of cisatracurium into laudanosin and pentamethylenediacrylate (Hoffmann degradation) we calculated the increase in the concentration of pentamethylenediacrylate. The concentration of cisatracurium in Krebs-Henseleit buffer (initial concentration: 3.5 mM, pH = 7.4, 37.5°C, ingredients as described above in this section) was repeatedly measured by ^1H NMR every 10 min for 1 hour, and then hourly for 5 h. The concentration of pentametyldiacrylate was measured. The ^1H NMR resonances for the characteristic terminally located alkene protons increased over time and were quantified. Under these conditions the magnitude of decrease in cisatracurium concentration amounted to $18\% \ln(t[h]) + 41\%$, i.e. 8.3% every 10 min. The cisatracurium concentrations used to calculate the affinity to calabadion 2 were corrected based on measurements of Hoffman degradation by ^1H NMR obtained over one hour.

In vivo

Animals—A total of 108 male Sprague–Dawley rats (227 – 356 g) were used.

Institutional guidelines for animal care and usage for research purposes were strictly followed. All procedures involving animals were approved by the Institutional Animal Care and Use Committees at Harvard Medical School, and experiments were conducted at the research laboratories of the Massachusetts General Hospital, Boston, Massachusetts, USA.

General Surgical Procedures—Rats were anesthetized (5% Isoflurane, maintained to 1.5%) before cannulation of their left femoral vein and artery for drug administration, blood pressure analyses, and blood gas analyses. The animals were tracheotomized to maintain normal breathing throughout the surgery. The body temperature was monitored with a rectal temperature probe (regulation to 37 ± 1 °C). Blood pressure was recorded continuously throughout the experiment and blood gas analyses were performed 1 min before, and 10 min and 20 min after administration of the relaxant.

Total urine volume was collected in 8 rats at the end of 60 min after the injection of calabadion 2 by a single puncture in the bladder, after which the animal was euthanized.

Assessment of Neuromuscular Transmission—For evaluation of neuromuscular transmission, the femoral nerve was stimulated with two subcutaneously inserted needle electrodes on a supra-maximal level. The evoked response of the quadriceps femoris muscle was detected via accelerometry with a TOF-Watch SX Monitor (Organon Ireland Ltd, a part of Schering-Plough Corporation, Dublin, Ireland), as has been published before.^{16,19} The transducer was inserted subcutaneously ventromedially at the proximal end of the thigh, next to the tuberosity of tibia. After determination of the supramaximal stimulation current, the femoral nerve was continuously stimulated at 1 Hz until twitch height reached a stable plateau, which was defined as TOF 100%. Stimulation was applied for at least 10 min before any drug injection. We prepared envelopes for a block-randomization in groups of 12 rats at

a time. After surgical preparation of the rat had been finished, a card was blindly drawn to define the reversal drug and dose to be administered. The person who conducted the experiment was blinded throughout the experiment. After twitch recovery to 50% of baseline the single twitch stimulation mode was changed to the TOF stimulation mode with a 12 s-interval in order to capture shallow levels of NMB. TOF monitoring was continued until 20 min after injection of the reversal agent.

Experimental Procedures

Drug administration

In order to compare the dose-response relationship of NMB reversibility between calabadiol 2, calabadiol 1, neostigmine, and sugammadex we measured time to recovery of neuromuscular transmission following intravenous injection of the two-fold ED₉₀ of rocuronium (3.5 mg/kg), vecuronium (0.7 mg/kg), or cisatracurium (0.6 mg/kg), (table 1).^{16,20} Previous experiments indicated a group size of 4 to be sufficient.¹⁶ At the onset of apnea, mechanical ventilation was started and the test-drug (reversal agent or saline) was injected 30 sec later. Mechanical ventilation was terminated when animals started sufficient breathing. The rats were observed for at least 30 min, rats with bladder puncture for at least 60 min after administration of the relaxant.

In order to evaluate the effects of calabadiols on the subsequent administration of succinylcholine (calabadiol 2•succinylcholine $K_a = (2.8 \pm 0.1) \times 10^6 \text{ M}^{-1}$), the depolarizing NMBA was administered 60 sec after calabadiol 1/2-assisted recovery from vecuronium-induced relaxation. We compared the onset of NMB after succinylcholine-induced relaxation between the conditions pretreatment (with calabadiol 1 or calabadiol 2) or no pretreatment (placebo).

Drugs

Rocuronium 3.5 mg/kg (2 times ED₉₀), vecuronium 0.7 mg/kg (2 times ED₉₀), cisatracurium 0.6 mg/kg (2 times ED₉₀), succinylcholine 0.9 mg/kg (2 times ED₉₀), neostigmine/glycopyrrolate 0.06/0.012 mg/kg, and sugammadex were diluted in 0.5 ml water. Calabadiol 1 and 2 were diluted in 0.5 ml H₂O. All drugs were administered over 5 sec. Isoflurane (Flurane; Baxter Healthcare Corporation, Deerfield, IL), cisatracurium (Abbott Laboratories, Abbott Park, IL), rocuronium (Zemuron, MERCK, NJ), vecuronium (Novaplus, Irving, TX), succinylcholine (Hospira, Inc., Lake Forest, IL), and neostigmine/glycopyrrolate (West-Ward, Eatontown, NJ) were obtained from clinical supplies. Sugammadex (Merck, WhiteHouse Station, NJ) was imported.

Quantification of Urinary Excretion of Calabadiol 2 and NMBAs by ¹H NMR Spectroscopy

From the urine samples of rats injected with rocuronium or cisatracurium, we took 0.1 ml aliquots of the urine and evaporated them under high vacuum. The residue was dissolved in a solution containing a known concentration of 2,2,3,3-d(4)-3-(trimethylsilyl)propanoic acid sodium salt (TMSP) as internal standard and the sample was diluted to a total volume of 0.6 ml with 20 mM phosphate buffered D₂O (pH = 7.4). The concentrations of the calabadiol 2•rocuronium complexes were determined by ¹H NMR spectroscopy (fig. 2) by comparing

the integral for diagnostic peaks of the complex (CH_3 groups at 0.23 ppm and -0.32 ppm, 3H each) relative to the internal standard (-0.02 ppm, 9H). Concentrations of free rocuronium were measured by adding a two-fold molar excess of calabadion 2 to the above solution. Addition of excess calabadion 2 bound any free rocuronium as the calabadion 2-rocuronium complex. Conversely, addition of excess rocuronium allowed the measurement of calabadion 2 not originally bound to rocuronium. We measured only total concentrations of calabadion 2 for the cisatracurium samples.

Statistical Analysis

We conducted a pre-clinical hypothesis-driven drug development study and tested calabadion 2 for one indication, reversal of non-depolarizing NMB. We tested the research hypotheses that calabadion 2 increases speed of recovery from NMB compared with available comparators. The primary aim of the *in vivo* experiments was to compare the effect of calabadion 2 in reversing benzylisoquinolines and steroidal NMBA relative to neostigmine, placebo, and sugammadex (the latter comparison was made for steroids only). The primary outcome variable was time to recovery from NMB expressed as time to onset of breathing and time to recovery of TOF-ratio to 90%.

In order to address the primary aim, we tested the hypotheses that calabadion 2 increases speed of recovery from benzylisoquinoline and steroidal NMBA-induced NMB compared to neostigmine, calabadion 1 and placebo in a dose dependent fashion. We included all measurements of time to recovery across muscle function types, using a mixed linear model (compound symmetry repeated covariance type).

To evaluate effects on time to recovery from NMB, we included relaxant (vecuronium, rocuronium or cisatracurium), reversal agent (calabadion 2, calabadion 1, neostigmine or placebo), reversal agent dose in mg/kg and observed muscle function type (recovery of spontaneous breathing vs. extremity TOF-ratio) as repeated independent variables and time to recovery as the dependent variable. We tested for an interaction between the administered reversal agent and the slope of the reversal agent dose.

In order to evaluate the secondary aim, we hypothesized that calabadion 2 reverses steroidal NMBA-induced NMB with a higher potency than sugammadex. As for the primary aim we included all measurements of time to recovery across muscle function types, using a mixed linear model (compound symmetry repeated covariance type) and evaluated the effects of both reversal agents on the dependent variable time to recovery from NMB including relaxant (vecuronium or rocuronium), reversal agent (calabadion 2 or sugammadex), reversal agent dose (adjusted for molecular weight (1641 g/mol for calabadion 2 vs. 2178 g/mol for sugammadex)) and observed muscle function type as repeated independent variables. We tested for an interaction between the administered reversal agent and the slope of the reversal agent dose.

In the ex-vivo experiments, twitch response was defined as the difference of resting force and peak force. Assuming that the relationship between NMBA/reversal agent concentrations with the TOF ratio are governed by the Hill equation, linear regressions of the TOF ratio in a logit scale and the concentrations in natural log scale were created for

each preparation.²¹ The transformed variables of the individual linear regressions, i.e. the individual slopes and intercepts, were statistically evaluated by an analysis of covariance for repeated measurements using either NMBA or reversal agent (between groups) or regression variable (within group) as independent factors. The concentration effect relation is described by the slope and the intercept and by the effective concentration for a 50 % effect (EC₅₀).

Data is presented as mean $\pm$ SD unless otherwise specified. $P < 0.05$ was considered to be the minimum criterion for statistical significance and no attempts were made to adjust for multiple comparisons. Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL).

Results

In vitro

Figure 3 shows the ¹H NMR spectra recorded for rocuronium, calabadiion 2•rocuronium, sugammadex•rocuronium, and a mixture of rocuronium (199.8 μ M), calabadiion 2 (186.8 μ M), and sugammadex (2399.7 μ M) in 20 mM phosphate buffered D₂O at pH 7.4. As can be seen in Figures 3B and 3C, the resonances for the axial steroidal CH₃ groups appear at different chemical shifts in the calabadiion 2•rocuronium (≈ 0.3 and -0.3 ppm) and sugammadex•rocuronium (≈ 1.0 ppm) complexes. Figure 3D shows that these axial steroidal CH₃ groups appear as separate resonances even when calabadiion 2 and sugammadex are competing to bind to rocuronium. This advantageously allows us to integrate these resonances to determine the concentrations of calabadiion 2•rocuronium and sugammadex•rocuronium in the solution at equilibrium. Using the usual equilibrium and mass balance expressions as detailed below in this section, we are able to calculate that calabadiion 2 binds 89 times more tightly to rocuronium than sugammadex does. When combined with the known K_a value for the calabadiion 2•rocuronium ($K_a = 3.4 \times 10^9 \text{ M}^{-1}$)¹⁷, allows us to determine the K_a value for sugammadex•rocuronium ($K_a = 3.8 \times 10^7 \text{ M}^{-1}$) in the 20 mM phosphate buffer employed herein. When comparing the K_a value for calabadiion 2•rocuronium, with the binding affinity of sugammadex•rocuronium, by using the same method that is a competition ¹H NMR assay, calabadiion 2 has a substantially higher binding affinity to rocuronium (K_a value for calabadiion 2•rocuronium = $3.4 \times 10^9 \text{ M}^{-1}$) versus $3.8 \times 10^7 \text{ M}^{-1}$, respectively.

The mass balance expression ($[\text{rocuronium}]_{\text{total}} = 199.8 \text{ } \mu\text{M} = [\text{calabadiion 2} \cdot \text{rocuronium}] + [\text{sugammadex} \cdot \text{rocuronium}]$) allowed us to calculate the concentration of the complex calabadiion 2•rocuronium (=136.1 μ M), and sugammadex•rocuronium (= 63.7 μ M). Assuming a closed system we calculated the concentration of free calabadiion 2 (=50.7 μ M) and sugammadex (=2336 μ M) based on the spectral analysis (fig. 3) of their complexes with rocuronium. The relative binding constant ($K_{\text{rel}} = ([\text{calabadiion 2} \cdot \text{rocuronium}] [\text{sugammadex}]_{\text{free}}) / ([\text{sugammadex} \cdot \text{rocuronium}] [\text{calabadiion 2}]_{\text{free}})$) was obtained as $K_{\text{rel}} = 98.4$. In a solution containing 186.8 μ M calabadiion 2, 199.8 μ M rocuronium, and 4.7993 mM sugammadex the relative binding constant was comparable ($K_{\text{rel}} = 81.45$). The value of K_a for calabadiion 2•rocuronium of $3.4 \times 10^9 \text{ M}^{-1}$ and the K_a of sugammadex•rocuronium of $3.8 \times 10^7 \text{ M}^{-1}$ allows us to conclude that the binding affinity of calabadiion 2•rocuronium compared with sugammadex•rocuronium is 89 times higher. We determined the K_a value for

calabadiol 2•succinylcholine as $2.8 \times 10^6 \text{ M}^{-1}$ by monitoring the change in UV/Vis absorbance and fit it to a standard competition binding model as published previously.¹⁷

Ex vivo

Rocuronium and cisatracurium-induced a concentration-dependent decrease in twitch height of the rat hemi-diaphragm with an EC₅₀ of 12 μM and 3 μM , respectively (table 2).

The EC₅₀ of calabadiol 2 and sugammadex in reversing rocuronium (1.5xEC₉₉) were within the same range (assessed by TOF, 61 μM vs. 49 μM and assessed by T1, 53 μM vs. 43 μM , respectively, fig. 4) suggesting that calabadiol 2 like sugammadex binds rocuronium with a 1:1 binding ratio. For the reversal of cisatracurium we identified an EC₅₀ of calabadiol 2 of 80 μM when assessed by T1 and of 100 μM when assessed by TOF. We did not identify any reversal of cisatracurium with sugammadex at concentrations up to 1000 μM .

In vivo experiments

Calabadiol 2 reversed vecuronium ($p < 0.01$ for main effect of reversal agent dose, fig. 5)-, rocuronium ($p < 0.001$, fig. 6)-, and cisatracurium-induced ($p < 0.01$, fig. 7) NMB dose dependently. At the highest doses, the time to recovery of NMB amounted to $16.6 \pm 7.9 \text{ sec}$, $13.6 \pm 9.2 \text{ sec}$ and $15.8 \pm 5.6 \text{ sec}$ after reversal administration respectively, with significantly faster recovery of spontaneous breathing compared to TOF-recovery ($p < 0.001$ for interaction effect of reversal agent and muscle group).

Time to recovery from NMB was dose dependently significantly faster across all three relaxants when reversed with calabadiol 2 compared to neostigmine and placebo ($p < 0.001$ for interaction effect of reversal agent and reversal agent dose on recovery time).

Reversal of steroidal NMBAs

Calabadiol 2 reversed vecuronium ($p < 0.01$ for main effect of reversal agent dose), and rocuronium-induced ($p < 0.001$) NMB dose dependently. Time to recovery from vecuronium-induced NMB to onset of breathing was significantly faster following calabadiol 2 compared with sugammadex, calabadiol 1, neostigmine, and placebo, (fig. 5, 6).

The molar potency (interaction between number of molecules of reversal agent administered and time to recovery of spontaneous breathing following administration of steroidal NMB) of calabadiol 2 to reverse the effects of vecuronium was greater compared with sugammadex ($p < 0.05$) (fig. 7).

Reversal of benzyloquinolines

Calabadiol 2 reversed cisatracurium-induced NMB dose dependently ($p < 0.01$ for main effect of reversal agent dose). Time to recovery of muscle function (expressed as TOF 0.9 and spontaneous breathing recovery) following cisatracurium-induced NMB was significantly shorter with calabadiol 2 compared to placebo and neostigmine/glycopyrrolate (fig. 8).

Effects of previous Calabadion reversal of non-depolarizing NMBAs on succinylcholine-induced NMB

Onset of action and peak NMB of succinylcholine did not differ in animals pretreated with calabadion 1 and calabadion 2 to reverse vecuronium-induced NMB compared with those who received placebo (fig. 9).

Adverse Effects

We did not observe any adverse effects of calabadion 2 on heart rate, blood pressure, or arterial blood gas parameters. No signs of residual blockade or recurarization were detected.

Urinary Excretion of Calabadion 2

One hour after i.v. administration of calabadion 2 (40 – 80 mg/kg), $49 \pm 31\%$ (mean $\pm$ SD) of the drug was detected in the urine samples. When administered in low dosage (5–10 mg/kg) $62 \pm 17\%$ of calabadion 2 was detected in urine.

Discussion

Calabadion 2 is a broad-spectrum agent to rapidly reverse deep vecuronium-, rocuronium, and cisatracurium-induced NMB in a dose-dependent manner. Calabadion 2, like sugammadex, reverses steroidal NMBAs with a 1:1 binding ratio, has a higher binding affinity *in vitro*, and provides a higher molar potency to reverse steroidal-induced NMB *in vivo*. Calabadion 2 was well tolerated in the rat and a substantial amount of calabadion 2 was eliminated by the kidney within 1 h.

The 1:1 binding between calabadion 2 and NMBAs was previously established by ^1H NMR integration of resonances for calabadion 2 relative to NMBA and also by Job plots.¹⁷ In this study, the ^1H NMR experiments used for head-to-head binding assay and the urine analysis reconfirmed the 1:1 binding.

In combination, our current head-to-head competition experiment between calabadion 2 and sugammadex for rocuronium (calabadion 2 binds 89 times stronger than sugammadex) and our previous determination that calabadion 2 binds rocuronium stronger than calabadion 1, establishes that calabadion 2 has superior binding affinity.¹⁷ We attribute the superior binding affinity of calabadion 2 toward rocuronium to several factors. First, the naphthalene walls of calabadion 2 define a hydrophobic box that is complementary to the hydrophobic steroidal skeleton of rocuronium. Second, and more importantly, the ureidyl C=O portals of calabadion 2 exhibit strong ion-dipole interactions towards the cationic N-atoms of rocuronium that are not possible with sugammadex.

The higher *in vitro* binding affinity to steroidal NMBAs of calabadion 2 compared to sugammadex has the potential to translate to a high *in vivo* molar potency in the rat. The high binding affinity is an encouraging pharmacological observation. Reversing drugs by encapsulating carries the risk of non-specific binding to biologically relevant plasma molecules or medications the patients may also have taken. The experience with sugammadex shows its side effects (inhibiting effects on coagulation) are dose-dependent.²² While we have so far not observed inhibiting effects of calabadion 2 on coagulation, it will

be important to use the lowest effective dose of a drug that inactivates NMBAs by binding. Our data showing the higher *in vitro* and *in vivo* (fig. 5–8) binding affinity of calabadion 2 compared with sugammadex suggest calabadion 2 may have a slightly wider therapeutic range. Further experiments to evaluate the potential of calabadion 2 to bind to alternate targets are currently being performed.

Sugammadex, calabadion 1 and calabadion 2, as been shown in this study, are renally eliminated within a short time.¹⁶ This pharmacokinetic profile helps to prevent long-term effects of the containers on medications administered during the postoperative course.

In our *ex vivo* experiments we observed similar values of the EC₅₀ of calabadion 2 and sugammadex (TOF 0.5 reversal after calabadion 2 injection: 61 μ M (95 CI: 41 – 88), and after sugammadex: 54 μ M (32 – 73) as well as slope of the recovery curves (fig. 4) (TOF 0.5 reversal after calabadion 2 injection: 22 – 27 and after sugammadex 20 – 24) in reversing rocuronium (Table 2), indicating that calabadion 2, like sugammadex, binds to steroidal NMBAs in a 1:1 ratio. Of note, under the conditions of the steady-state *ex vivo* preparation, differences in binding affinity between sugammadex and calabadion 2 did not and are not expected to translate into effects on EC₅₀ of these compounds.

Within the rat hemi-diaphragm preparation we created an environment of steady-state NMB that does not contain plasma proteins. Therefore, high binding affinity/selectivity is not expected to translate into increased speed of recovery from neuromuscular blockade under these conditions. Since the *ex vivo* diffusion distance is relatively long compared to a perfused muscle or an *in vitro* solution, rocuronium's ability to pass through the tissue is limited, even with a maximum concentration gradient created by calabadion 2 or sugammadex encapsulation. Accordingly, due to the initial excess of rocuronium molecules at post-junctional receptors, their removal does not result in an instant recovery of twitch height. However, as soon as we achieve a reduction to 70% receptor occupation, we will observe a complete recovery "reversal" of the twitch height.²³

Sugammadex reverses rocuronium and vecuronium-induced NMB by encapsulation into its lipophilic cavity, but does not bind to benzylisoquinolines, e.g. cisatracurium, that account for about one third of the market volume of NMBAs.^{24–26}

Not all mammalian species react in the same way to neuromuscular blocking substances.²⁷ Rats are more resistant to non-depolarizing NMBAs and more sensitive to depolarizing NMBAs than humans. The dose–response curve of calabadion and sugammadex to reverse the effects of non-depolarizing NMBAs should be shifted to the left in humans compared with this study in rats. Accordingly, lower doses of an encapsulating agent should be required in humans compared to those required to reverse rocuronium in the rat, which is also supported by published preclinical sugammadex data.^{28,29} Ultimately, dose – response studies in humans are required to define the risk – benefit ratio of calabadion 2 when given for reversal of non-depolarizing NMBAs.¹⁸

Calabadion 1 was the first reversal agent that reversed deep cisatracurium-induced NMB by binding and encapsulation, an unmet need since no alternative medication is available to reverse deep cisatracurium-induced NMB. Calabadion 2 has an almost 5 times higher

binding affinity towards cisatracurium compared to calabadion 1, and substantially lower doses are required to reverse cisatracurium *in vivo*.¹⁷ The affinity of calabadions to cisatracurium is lower than their affinity toward steroidal NMBAs. In contrast, sugammadex does not bind to cisatracurium and the binding affinity of calabadion 2•cisatracurium $K_a = 4.8 \times 10^6 \text{ M}^{-1}$ is one-eighth that of lower than sugammadex•rocuronium ($K_a = 3.8 \times 10^7 \text{ M}^{-1}$). We demonstrated a stable and comparable clinical effect, when the doses had been adapted appropriately. Importantly, reappearance of breathing after reversal of cisatracurium with calabadion 2 was sufficiently fast ($17.5 \pm 6.5 \text{ sec}$ for calabadion 2 – 60 mg/kg - compared to $465 \pm 196 \text{ sec}$ for placebo and $291 \pm 99 \text{ sec}$ for neostigmine and compared to $47 \pm 13 \text{ sec}$ for the previously published calabadion 1 – 150 mg/kg).¹⁶

We attribute the enhanced binding affinity of calabadion 2 (fig. 5–8) for its targets to its larger hydrophobic cavity which is shaped by two naphthalene walls as opposed to calabadion 1 which features two benzene walls.³⁰ Calabadion 2 is currently developed as a new broad-spectrum NMBA reversal agent. Unlike sugammadex, calabadion 2 also reverses cisatracurium-induced NMB. We were able to show this effect at each level of our experiments, i.e. *in vitro*, *ex vivo*, as well as *in vivo*.

In clinical practice, surgical complications may occur that require a second (emergency) surgical procedure under anesthesia, shortly after the reversal of the NMBA with an agent that inactivates NMBAs, such as sugammadex or calabadion. It is possible to administer a higher dose of the non-depolarizing NMBA injected before the reversal. Cammu et al. found an inverse relationship between the onset time and the time interval between sugammadex and the repeat administration of rocuronium, and a direct relationship between the duration of NMB and the time interval between sugammadex and the repeat administration of rocuronium.³¹ However, it is currently recommended that after initial reversal of NMB with sugammadex, 24 h should be allowed before rocuronium or vecuronium can be re-administered. Based on European Medicines Agency recommendations, succinylcholine can be given.³² Given the substantial *in-vitro* affinity of calabadion 2 to succinylcholine, we conducted an additional set of experiments to evaluate whether succinylcholine can be used following calabadion reversal of non-depolarizing NMBA. Our data (fig. 9) show that succinylcholine can be used safely and effectively directly after administration of calabadion 2 to reverse a non-depolarizing NMBA.

The concept behind our study brings important and new input in the field of drug development. The model of drug inactivation by encapsulation will become even more important in the future since the clinically meaningful problems such as of adverse and lingering effects of anesthetics and NMBAs or cocaine intoxication have not yet been solved.³³ Using the principle of encapsulation the relationship between binding affinity to the target drug to be reversed and the binding affinity to other chemically similar compounds that are not to be inactivated (that is binding selectivity) has to be considered.

New encapsulating drugs that affect neuromuscular transmission have to be tested *in vitro*, *ex vivo* and *in vivo* for measurements of binding affinity and selectivity. The clinician scientist should consider that differences in binding affinity and selectivity may translate to effects on speed of reversal *in vivo*, as been demonstrated in our study. In contrast, in a

steady-state ex vivo model (such that the phrenic nerve hemidiaphragm model) that does not contain plasma proteins, high binding affinity/selectivity does not translate to increased drug potency.

In summary, we show that calabadion 2 is a broad-spectrum reversal agent to rapidly reverse deep vecuronium-, rocuronium-, and cisatracurium-induced NMB in a dose-dependent fashion. Calabadion 2, like sugammadex, reverses steroidal NMBAs with a 1:1 binding ratio, has a higher binding affinity *in vitro*, and provides a higher molar potency to reverse steroidal-induced NMB *in vivo*. Calabadion 2 was well tolerated in the rat and substantial amount of calabadion 2 was eliminated unchanged by the kidney within 1 h.

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Fig. 1.

Calabadiion 2 (**A**), the second-generation cucurbituril receptor, features a cavity with naphthalene walls and binds with high affinity toward steroidal (**B**) ($K_a = 0.53\text{--}3.4 \times 10^9 \text{ M}^{-1}$) and benzyloquinoline ($K_a = 4.8 \times 10^6 \text{ M}^{-1}$) neuromuscular blocking agents.

Fig. 2. ^1H NMR Spectral analysis

(600 MHz, room temperature). The urine samples were evaporated and then diluted with deuterated 20 mM sodium phosphate buffer at pH = 7.4 prior to ^1H NMR analysis.

Chemical shift (in parts per million, ppm) is shown on the x-axes and signal intensity on the y-axes. Evaluation of rocuronium and calabadiol in the urine of 8 rats.

(A) Free rocuronium (2 mM) shows two resonances (a and b) for the steroidal CH_3 -groups.

(B) An equimolar mixture of calabadiol 2 and rocuronium (2 mM), a' and b' are resonances for the bound CH_3 -groups of calabadiol 2•rocuronium,

(C) Urine sample from a rat taken 2 hours after i.v. administration of rocuronium (3.5 mg/kg) followed by calabadiol 2 (10 mg/kg). a' and b' are resonances for the bound CH_3 -groups of calabadiol 2•rocuronium.

Fig. 3. ^1H NMR Spectral analysis

(600 MHz, room temperature). Head-to-head competition between sugammadex and calabadion 2 to assess their relative binding affinity toward rocuronium. Chemical shift (in parts per million, ppm) is shown on the x-axes and signal intensity on the y-axes.

(A) Rocuronium 2 mM, a and b are the resonances for the CH_3 -groups of free rocuronium,

(B) An equimolar solution of calabadion 2 and rocuronium (2 mM). a' and b' are resonances for the bound CH_3 -groups of calabadion 2•rocuronium.

(C) An equimolar solution of sugammadex and rocuronium (2 mM), a'' and b'' are resonances for the bound CH_3 -groups of sugammadex•rocuronium.

(D) A solution containing 186.8 μM calabadion 2, 199.8 μM rocuronium, and 2399.7 μM sugammadex.

Fig. 4.

Steady-state binding study in phrenic nerve hemidiaphragm preparations in a tissue bath (n=85). Mean and 95% confidence intervals are provided. The EC₅₀ (the median effective concentration) of calabadiol 2 and sugammadex in reversing rocuronium were within the same range (assessed by TOF, 61 μ M vs. 49 μ M and assessed by T1, 53 μ M vs. 43 μ M, respectively).

(A) Concentration effect of rocuronium and cisatracurium on T1 depression.

(B) Reversal of rocuronium and cisatracurium-induced neuromuscular blockade by calabadiol 2 and **(C)** reversal of rocuronium by sugammadex.

Fig. 5.

Recovery of onset of breathing efforts (A) and train-of-four ratio to 0.9 (B) following administration of the 2 times ED 90 of vecuronium (0.7mg/kg). Means and SD from 44 rats.

(A) Breathing onset. Calabation 2 accelerates recovery time significantly compared to placebo (* $p < 0.001$), neostigmine (# $p < 0.001$), calabation 1 (♦ $p < 0.01$) and sugammadex (@ $p < 0.01$).

(B) TOF 0.9 recovery. Calabation 2 decreases time to recovery significantly compared to placebo (* $p < 0.001$), neostigmine (# $p < 0.001$), and calabation 1 (♦ $p < 0.001$).

Fig. 6.

Recovery of onset of breathing efforts and train-of-four ratio to 0.9 following administration of the 2 times ED 90 of rocuronium (3.5 mg/kg). Means and SD from 32 rats.

(A) Breathing onset. Calabadiol 2 accelerates recovery time significantly compared to placebo (* $p < 0.001$), neostigmine (# $p < 0.001$) and sugammadex (@ $p < 0.001$).

(B) TOF 0.9 recovery. Calabadiol 2 decreases time to recovery significantly compared to placebo (* $p < 0.001$), neostigmine (# $p < 0.001$) and sugammadex (@ $p < 0.001$).

Fig. 7.

Recovery of onset of breathing efforts (A) and train-of-four ratio to 0.9 (B) following administration of the 2 times ED 90 of vecuronium. Means and SD from 28 rats.; the x-axis was normalized to account for the differences in molecular weight of sugammadex and calabadiol 2.

(A) Effects of the molecular concentration of calabadiol 2 and sugammadex given at peak neuromuscular block on time to **recovery of breathing** (* $p < 0.05$) and (B) **train of four 0.9 recovery**.

Fig. 8.

Recovery of onset of breathing efforts and train-of-four ratio to 0.9 following administration of the 2 times ED 90 of cisatracurium (0.6 mg/kg). Means and SD from 20 rats.

(A) Breathing onset. Calabadiion 2 accelerates recovery time significantly compared to placebo (* $p < 0.001$) and neostigmine (# $p < 0.001$).

(B) TOF 0.9 recovery. Calabadiion 2 decreases time to recovery significantly compared to placebo (* $p < 0.05$) and neostigmine (# $p < 0.05$).

Fig. 9.

Succinylcholine (0.9 mg/kg; 2 times ED₉₀)-induced neuromuscular block in the absence of calabadiion 2 pretreatment and following reversal of vecuronium (0.7 mg/kg)-induced neuromuscular block with calabadiion 2. Means and SD from 12 rats.

(A) Onset of twitch depression. Previous reversal with calabadiion 2 (10 mg/kg) did not influence time until reaching a second relaxation indicated by twitch depression < 0.5 significantly.

(B) Onset of apnea. Previous reversal with calabadiion 2 (10 mg/kg) did not influence the time until reaching a second relaxation indicated by time to apnea significantly.

Table 1

Drug administration scheme

Neuromuscular blocking agent	Calabadiion 1	Calabadiion 2	Sugammadex	Neostigmine/glycopyrrolate	Placebo
Rocuronium 3.5 mg/kg	---	5, 10, 25 mg/kg	5, 10, 25 mg/kg	0.06/0.012 mg/kg	0.5 ml
Vecuronium 0.7 mg/kg	10, 20, 30 mg/kg	2, 5, 10, mg/kg	5, 10, 25 mg/kg	0.06/0.012 mg/kg	0.5 ml
Cisatracurium 0.6 mg/kg	---	40, 60, 80 mg/kg	---	0.06/0.012 mg/kg	0.5 ml
Succinylcholine 0.9mg/kg following Vecuronium	40 mg/kg	10 mg/kg	---	---	0 ml

Table 2

Ex vivo analysis

Neuromuscular blocking agent	EC50 [μ M] mean	95%CI	Slope mean	95%CI	Intercept mean	95%CI	N
Concentration response relation							
Rocuronium	12	[9 to 15]	3.6	[3.3 to 4.0]	41	[37 to 45]	10
Cisatracurium	3.0	[1.8 to 5.0] *	3.7	[3.2 to 4.1]	47	[41 to 52] *	10
Sugammadex concentration response for T1 reversal of 1.5 x EC99 with...							
Rocuronium	43	[28 to 65]	-22	-24 to -20]	-219	-241 to -197]	10
Cisatracurium	> 1000		n.a.		n.a.		3
Sugammadex concentration response for TOF reversal of 1.5 x EC99 with...							
Rocuronium	49	[32 to 73]	22	[20 to 24]	219	[198 to 240]	11
Cisatracurium	> 1000		n.a.		n.a.		3
Calabadiion 2 concentration response for T1 reversal of 1.5 x EC99 with...							
Rocuronium	53	[30 to 88]	-20	-22 to -18]	-199	-221 to -178]	10
Cisatracurium	80	[54 to 179] *	-8.9	-10.3 to -7.5] *	-84	-97 to -71] *	9
Calabadiion 2 concentration response for TOF reversal of 1.5 x EC99 with...							
Rocuronium	61	[41 to 88]	24	[22 to 27]	237	[216 to 257]	10
Cisatracurium	100	[54 to 179] *	4.0	[3.5 to 4.5] *	37	[32 to 42] *	9

*The results take into account the spontaneous Hoffman degradation of cisatracurium (details are given in the methods section) n.a., not applicable since sugammadex does not bind to cisatracurium. Slope and intercept are calculated by linear regressions of the Train of four ratio (TOF) in a logit scale and the concentrations in natural log scale.