Comparative Effectiveness of Calabadion and Sugammadex to Reverse Non-depolarizing Neuromuscular-blocking Agents


ABSTRACT

Background: The authors evaluated the comparative effectiveness of calabadion 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 calabadions, neostigmine, or sugammadex were created ex vivo at a steady-state deep NMB. In living rats, the authors studied the dose–response relationship of the test drugs to reverse deep block under physiologic conditions, and they measured the amount of calabadion 2 excreted in the urine.

Results: In vitro experiments showed that calabadion 2 binds rocuronium with 89 times the affinity of sugammadex (Kᵣ = 3.4 × 10⁻⁸ M⁻¹ and Kᵣ = 3.8 × 10⁻⁷ M⁻¹). The results of urine analysis (proton nuclear magnetic resonance), competition binding assays, and ex vivo study obtained in the absence of metabolic deactivation are in accordance with an 1:1 binding ratio of sugammadex and calabadion 2 toward rocuronium. In living rats, calabadion 2 dose-dependently and rapidly reversed all NMBAs tested. The molar potency of calabadion 2 to reverse vecuronium and rocuronium was higher compared with that of sugammadex. Calabadion 2 was eliminated renally and did not affect blood pressure or heart rate.

Conclusions: Calabadion 2 reverses NMB induced by benzylisoquinolines and steroidal NMBAs in rats more effectively, i.e., faster than sugammadex. Calabadion 2 is eliminated in the urine and well tolerated in rats. (Anesthesiology 2015; 123:1337-49)

REVERSAL of neuromuscular-blocking agents (NMBAs) is an important strategy for accelerating recovery from neuromuscular blockade (NMB).¹⁻⁴ Acetylcholinesterase inhibitors, typically neostigmine, are the only drugs to reverse NMB approved by the US Food and Drug Administration. Acetylcholinesterase inhibitors cannot reverse deep NMBs¹⁻² and may even induce muscle weakness when administered in large doses after recovery from NMBAs.⁸ Sugammadex, an anionic β-cyclodextrin derivative, reverses NMB by binding and thereby inactivating steroidal NMBAs.² Hypersensitivity to sugammadex and dose-dependent sugammadex-induced anticoagulation (increases activated partial thromboplastin time and prothrombin time) were reported.¹⁰⁻¹² Although sugammadex is used outside the United States, the Food and Drug Administration has declined it twice based on the observed side effects.¹³

What We Already Know about This Topic

- Sugammadex reverses neuromuscular blockade by binding and thus inactivating rocuronium and vecuronium.
- The cucurbituril derivative calabadion 1 reverses not only rocuronium and vecuronium but also cisatracurium. Its in vitro binding affinity for rocuronium is less than that of sugammadex.
- Calabadion 2’s affinity for rocuronium is nearly 20,000 times that for acetylcholine, whereas the affinity of calabadion 1 for rocuronium is only 350 times that for acetylcholine.

What This Article Tells Us That Is New

- Calabadion 2 rapidly reversed deep rocuronium-, vecuronium-, and cisatracurium-induced neuromuscular blockade in a dose-dependent manner.
- Calabadion 2 reversed rocuronium- and vecuronium-induced neuromuscular blockade with a 1:1 binding ratio, like sugammadex, but it had a higher in vitro binding affinity and a higher molar potency in vivo.

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Over the past decade, the preparation and molecular recognition properties of a new class known as cucurbiturils have been thoroughly explored. Calabadion 1—a cucurbituril derivative—reverses the steroidal nondepolarizing NMBAs rocuronium and vecuronium as well the benzylisoquinoline cisatracurium by encapsulation. However, the in vitro binding affinity of calabadion 1 toward rocuronium is slightly less than the reported binding affinity of sugammadex. Calabadion 2 (fig. 1) is a major leap forward in selectivity and binding affinity toward NMBAs. The affinity of calabadion 2 for rocuronium is 18,900 times that for acetylcholine, whereas the affinity of calabadion 1 for rocuronium is only 350 times higher than for acetylcholine.

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

Materials and Methods

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

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 CH3 groups of rocuronium in the calabadion 2•rocuronium complex that appear between 0.3 and −0.3 parts per million (ppm) relative to an internal standard of known concentration. Stimulation of the concentrations into the usual equilibrium expression along with mass balance considerations allowed us to calculate the difference in binding affinity between calabadion 2 and sugammadex toward 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 D2O 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 that appear between 0.3 and −0.3 ppm relative to an internal standard of known concentration.

To monitor the transformation of cisatracurium over time, we prepare a solution of calabadion 2•rhodamine 6G that has a known $K_a$ of $(4.8 \pm 0.1) \times 10^5$ M$^{-1}$ and which displays a different UV/Vis spectrum than rhodamine 6G alone. On addition of increasing concentrations of succinylcholine, we observe that the UV/Vis spectrum reverts toward that of free rhodamine 6G. We examined 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.

Ex Vivo

Animals. A total of 34 male Sprague–Dawley rats whose weights ranged from 340 to 406g were used. Institutional guidelines for animal care and usage for research purposes were followed. Institutional guidelines for animal care and usage for research purposes were followed. We determined the binding constant for the calabadion 2•succinylcholine complex, we performed a UV/Vis competition assay as published previously.

Fig. 1. Calabadion 2 (A), the second-generation cucurbituril receptor, features a cavity with naphthalene walls and binds with high affinity toward steroidal ($\beta$: $K_a = 0.53-3.4 \times 10^9$ M$^{-1}$) and benzylisoquinoline ($K_a = 4.8 \times 10^6$ M$^{-1}$) neuromuscular-blocking agents.
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, Munich, Germany.

Experimental Procedures. Rats were anesthetized with carbon dioxide for decapitation, to dissect two hemidiaphragm preparations per rat with intact phrenic nerve. Each hemidiaphragm was secured to a phrenic nerve diaphragm tissue holder and mounted in a 65 ml tissue bath (ISO-07-TSZ2D, Experimentia Ltd., Hungary) with a Krebs–Henseleit buffer solution (1.0 mM KH2PO4, 0.6 mM MgSO4·7H2O, 5.0 mM KCl, 30.0 mM NaHCO3, 20.0 mM α-D-glucose, 118 mM NaCl, 2.5 mM CaCl2) maintained at 37°C (AMP-09 Temperature controller, Experimentia Ltd.) and 95% O2 and 5% CO2 (Vol%) insufflations.

The hemidiaphragms were attached to an isometric force-displacement transducer (FSG-01/200 Force Transducer, Experimentia Ltd.) 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 (Experimentia Ltd.). A resting force of 10 mN was applied for 20 min followed by 50 mN to assess a resting and peak forces 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 supramaximal voltage) using a square wave stimulator (ST-03-O4, Experimentia Ltd.). 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. To decrease the interindividual variability when making comparison of calabadian 2’s effects to reverse benzylisoquinolines and steroids, one hemidiaphragm preparation of each rat was treated with rocuronium and the other was treated with cisatracurium.

Rocuronium and cisatracurium were added in steps of 150 and 100 µg, respectively, until the T1 value was decreased by more than 10% compared with the control value. After each NMBA increment, a stable T1 value, i.e., three consecutive T1 with less than 3% deviation was achieved after 5 to 8 min. Therefore, the NMBA boluses were added every consecutive 10 min. On the basis of these measurements, we then subsequently applied a 1.5 times the EC90 of rocuronium (62 µM) and cisatracurium (16 µM) to evaluate effectiveness of the reversal agents.

Neuromuscular transmission recovery was assessed by the recovery of T1 and TOF ratio (ratio of the twitch response of the fourth to the first stimulus of each TOF stimulation). 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 calabadian 2, each 5 mg, followed by repetitive doses of 0.4 mg. For cisatracurium reversal, an initial dose of 8 mg calabadian 2 was added to the tissue bath, followed by repetitive doses of 1.5 mg. To address the impact of the Hoffmann degradation on the concentration of cisatracurium when cisatracurium was used as NMBA, 10 additional hemidiaphragm 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.

The concentration of cisatracurium in Krebs–Henseleit buffer (initial concentration: 3.5 mM, pH = 7.4, 37.5°C, ingredients as described earlier in Experimental Procedures) was repeatedly measured by 1H NMR every 10 min for 1 h 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 calabadian 2 were corrected based on the measurements of Hoffman degradation by 1H NMR obtained over 1 h.

In Vivo Animals. A total of 108 male Sprague–Dawley rats (weight, 227 to 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.

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 and 20 min after administration of the relaxant. Total urine volume was collected in eight rats at the end of 60 min after the injection of calabadian 2 by a single puncture in the bladder, after which the animal was killed.

Assessment of Neuromuscular Transmission. For evaluation of neuromuscular transmission, the femoral nerve was
stimulated with two subcutaneously inserted needle electrodes on a supramaximal level. The evoked response of the quadriceps femoris muscle was detected using accelerometry with a TOF-Watch SX Monitor (Organon Ireland Ltd., a part of Schering-Plough Corporation, Ireland) as has been published previously. The transducer was inserted subcutaneously ventromedially at the proximal end of the thigh, next to the tuberosity of the 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 baseline. 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 to capture shallow levels of NMB. TOF monitoring was continued until 20 min after injection of the reversal agent.

**Experimental Procedures**

**Drug Administration**

To compare the dose–response relationship of NMB reversibility among calabadion 2, calabadion 1, neostigmine, and sugammadex, we measured time to recovery of neuromuscular transmission after intravenous injection of the twofold ED$_{90}$ of rocuronium (3.5 mg/kg), vecuronium (0.7 mg/kg), or cisatracurium (0.6 mg/kg; table 1). Previous experiments indicated a group size of four to be sufficient. At the onset of apnea, mechanical ventilation was started, and the test drug (reversal agent or saline) was injected 30 s later. Mechanical ventilation was terminated when animals started sufficient breathing. The rats were observed for at least 30 min, and rats with bladder puncture were observed for at least 60 min after administration of the relaxant.

To evaluate the effects of calabations on the subsequent administration of succinylcholine (calabadion 2•succinylcholine: \( K_c = (2.8 \pm 0.1) \times 10^6 \text{M}^{-1} \)), the depolarizing NMBA was administered 60 s after calabadion 1/2-assisted recovery from vecuronium-induced relaxation. We compared the onset of NMB after succinylcholine-induced relaxation between the conditions pretreatment (with calabadion 1 or calabadion 2) or no pretreatment (placebo).

**Drugs**

Rocuronium 3.5 mg/kg (two times ED$_{90}$), 0.7 mg/kg vecuronium (two times ED$_{90}$), 0.6 mg/kg cisatracurium (two times ED$_{90}$), 0.9 mg/kg succinylcholine (two times ED$_{90}$), 0.06/0.012 mg/kg neostigmine/glycopyrrolate, and sugammadex were diluted in 0.5 ml water. Calabation 1 and 2 were diluted in 0.5 ml H$_2$O. All drugs were administered over 5 s. Isoflurane (Flurane; Baxter Healthcare Corporation, USA), cisatracurium (Abbott Laboratories, USA), rocuronium (Zemuron, Merck, USA), vecuronium (Novaplus, USA), succinylcholine (Hospira, Inc., USA), and neostigmine/glycopyrrolate (West-Ward, USA) were obtained from clinical supplies. Sugammadex (Merck) was imported.

**Quantification of Urinary Excretion of Calabation 2 and NMBA s 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 D$_2$O (pH = 7.4). The concentrations of the calabation 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 and −0.32 ppm, 3H each) relative to the internal standard (−0.02 ppm, 9H). The concentrations of free rocuronium were measured by adding a twofold molar excess of calabadion 2 to the aforementioned solution. Addition of excess calabation 2 bound any free rocuronium as the calabation 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 preclinical hypothesis-driven drug development study and tested calabadion 2 for one indication, reversal of nondepolarizing 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 s relative to neostigmine, placebo, and

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**Table 1. Drug Administration Scheme**

<table>
<thead>
<tr>
<th>Neuromuscular-blocking Agent</th>
<th>Calabation 1 (mg/kg)</th>
<th>Calabation 2 (mg/kg)</th>
<th>Sugammadex (mg/kg)</th>
<th>Neostigmine/Glycopyrrolate (mg/kg)</th>
<th>Placebo (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 mg/kg rocuronium</td>
<td></td>
<td></td>
<td>5, 10, 25g</td>
<td>0.06/0.012</td>
<td>0.5</td>
</tr>
<tr>
<td>0.7 mg/kg vecuronium</td>
<td>10, 20, 30</td>
<td>2, 5, 10</td>
<td>5, 10, 25</td>
<td>0.06/0.012</td>
<td>0.5</td>
</tr>
<tr>
<td>0.6 mg/kg cisatracurium</td>
<td></td>
<td>40, 60, 80</td>
<td>—</td>
<td>0.06/0.012</td>
<td>0.5</td>
</tr>
<tr>
<td>0.9 mg/kg succinylcholine</td>
<td>40</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>

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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%.

To address the primary aim, we tested the hypotheses that calabadion 2 increases speed of recovery from benzylisoquinoline and steroidal NMBA–induced NMB compared with 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, and placebo), and reversal agent dose in milligrams per kilogram and observed muscle function type (recovery of spontaneous breathing vs. extremity TOF ratio) 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 and reversal agent concentrations with the TOF ratio is 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. 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 are presented as mean ± 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., USA).

Fig. 2. 1H NMR spectral analysis (600 MHz, room temperature). The urine samples were evaporated and then diluted with deuterated 20mM sodium phosphate buffer at pH = 7.4 before 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 calabadion in the urine of eight rats. (A) Free rocuronium (2 mM) shows two resonances (a and b) for the steroidal CH3 groups. (B) An equimolar mixture of calabadion 2 and rocuronium (2mM), a’ and b’ are resonances for the bound CH3 groups of calabadion 2•rocuronium. (C) Urine sample from a rat taken 2h after intravenous administration of rocuronium (3.5 mg/kg) followed by calabadion 2 (10 mg/kg). a’ and b’ are resonances for the bound CH3 groups of calabadion 2•rocuronium.
Results

In Vitro

Figure 3 shows the 1H NMR spectra recorded for rocuronium, calabadion 2•rocuronium, and sugammadex•rocuronium, and a mixture of rocuronium (199.8 μM), calabadion 2 (186.8 μM), and sugammadex (2,399.7 μM) in 20 mM phosphate-buffered D2O at pH 7.4. As shown in figure 3, B and C, the resonances for the axial steroidal CH3 groups appear at different chemical shifts in the calabadion 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 calabadion 2 and sugammadex are competing to bind to rocuronium. This advantageously allows us to integrate these resonances to determine the concentrations of calabadion 2•rocuronium and sugammadex•rocuronium in the solution at equilibrium. By using the usual equilibrium and mass balance expressions as detailed latter in this section, we are able to calculate that calabadion 2 binds 89 times more tightly to rocuronium than sugammadex does. When combined with the known K value for the calabadion 2•rocuronium (K = 3.4 × 10⁹ M⁻¹), we are able to determine the K value for sugammadex•rocuronium (K = 3.8 × 10⁷ M⁻¹) in the 20 mM phosphate buffer used herein. When comparing the K value for calabadion 2•rocuronium with the binding affinity of sugammadex•rocuronium, by using the same method that is a competition 1H NMR assay, calabadion 2 has a substantially higher binding affinity to rocuronium (K value: calabadion 2•rocuronium = 3.4 × 10⁹ M⁻¹ vs. sugammadex•rocuronium: 3.8 × 10⁷ M⁻¹, respectively).

The mass balance expression ([rocuronium]total = 199.8 μM = [calabadion 2•rocuronium] + [sugammadex•rocuronium]) allowed us to calculate the concentration of the complex calabadion 2•rocuronium (136.1 μM) and sugammadex•rocuronium (63.7 μM). Assuming a closed system, we calculated the concentration of free calabadion 2 (50.7 μM) and sugammadex (2336 μM) based on the spectral analysis (fig. 3) of their complexes with rocuronium. The relative binding constant (Krel = ([calabadion 2•rocuronium] [sugammadex]free)/([sugammadex•rocuronium] [calabadion 2]free)) was obtained as Krel = 98.4. In a solution containing 186.8 μM calabadion 2, 199.8 μM rocuronium, and 4.7993 mM sugammadex, the relative binding constant was comparable (Krel = 81.45). The value of K for calabadion 2•rocuronium of 3.4 × 10⁹ M⁻¹ and the K of sugammadex•rocuronium of 3.8 × 10⁷ M⁻¹ allows us to conclude that the binding affinity of calabadion 2•rocuronium compared with sugammadex•rocuronium is 89 times higher. We determined the K value for calabadion 2•succinylcholine as 2.8 × 10⁶ 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 hemidiaphragm with an EC50 of 12 and 3 μM, respectively (table 2). The EC50 of calabadion 2 and sugammadex in reversing rocuronium (1.5 × EC99) was within the same range (assessed by TOF, 61 vs. 49 μM and assessed by T1, 53 vs. 43 μM, respectively, fig. 4), suggesting that calabadion 2

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**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 CH3 groups of free rocuronium. (B) An equimolar solution of calabadion 2 and rocuronium (2 mM). a’ and b’ are resonances for the bound CH3 groups of calabadion 2•rocuronium. (C) An equimolar solution of sugammadex and rocuronium (2 mM), a” and b” are resonances for the bound CH3 groups of sugammadex•rocuronium. (D) A solution containing 186.8 μM calabadion 2, 199.8 μM rocuronium, and 2399.7 μM sugammadex.
bonds rocuronium with a 1:1 binding ratio similar to that of sugammadex. For the reversal of cisatracurium, we identified an EC$_{50}$ of calabadion 2 of 80 μM when assessed by T1 and 100 μM when assessed by TOF. We did not identify any reversal of cisatracurium with sugammadex at concentrations up to 1,000 μM.

**In Vivo Experiments**

Calabadion 2 reversed vecuronium-induced (P < 0.01 for main effect of reversal agent dose, fig. 5), rocuronium-induced (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 ± 7.9 s, 13.6 ± 9.2 s, and 15.8 ± 5.6 s after reversal administration, respectively, with significantly faster recovery of spontaneous breathing compared with 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 calabadion 2 compared with neostigmine and placebo (P < 0.001 for interaction effect of reversal agent and reversal agent dose on recovery time).

**Reversal of Steroidal NMBAs**

Calabadion 2 reversed vecuronium-induced (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 with calabadion 2 compared with sugammadex, calabadion 1, neostigmine, and placebo (figs. 5 and 6). The molar potency (interaction between number of molecules of reversal agent administered and time to recovery of spontaneous breathing after administration of steroidal NMBA) of calabadion 2 to reverse the effects of vecuronium was greater compared with sugammadex (P < 0.05; fig. 7).

**Reversal of Benzylisoquinolines**

Calabadion 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) after cisatracurium-induced NMB was significantly shorter with calabadion 2 compared with 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 2 to reverse vecuronium-induced NMB compared with those 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 intravenous administration of calabadion 2 (40 to 80 mg/kg), 62 ± 17% of calabadion 2 was detected in urine. When administered in low dosage (5 to 10 mg/kg), 62 ± 17% of calabadion 2 was detected in urine.

**Discussion**

Calabadion 2 is a broad-spectrum agent used 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

**Table 2. Ex Vivo Analysis**

<table>
<thead>
<tr>
<th>Concentration response relation</th>
<th>Mean EC$_{50}$ (μM)</th>
<th>95% CI</th>
<th>Mean Slope</th>
<th>95% CI</th>
<th>Mean Intercept</th>
<th>95% CI</th>
<th>N</th>
</tr>
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<tbody>
<tr>
<td>Rocuronium</td>
<td>12</td>
<td>9 to 15</td>
<td>3.6</td>
<td>3.3 to 4.0</td>
<td>41</td>
<td>37 to 45</td>
<td>10</td>
</tr>
<tr>
<td>Cisatracurium</td>
<td>3.0</td>
<td>1.8 to 5.0*</td>
<td>3.7</td>
<td>3.2 to 4.1</td>
<td>47</td>
<td>41 to 52*</td>
<td>10</td>
</tr>
<tr>
<td>Sugammadex concentration response for T1 reversal of 1.5 × EC$_{99}$ with ...</td>
<td>43</td>
<td>28 to 65</td>
<td>−22</td>
<td>−24 to −20</td>
<td>−219</td>
<td>−241 to −197</td>
<td>10</td>
</tr>
<tr>
<td>Cisatracurium</td>
<td>&gt;1,000</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Sugammadex concentration response for TOF reversal of 1.5 × EC$_{99}$ with ...</td>
<td>49</td>
<td>32 to 73</td>
<td>22</td>
<td>20 to 24</td>
<td>219</td>
<td>198 to 240</td>
<td>11</td>
</tr>
<tr>
<td>Cisatracurium</td>
<td>&gt;1,000</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Calabadion 2 concentration response for T1 reversal of 1.5 × EC$_{99}$ with ...</td>
<td>53</td>
<td>30 to 88</td>
<td>−20</td>
<td>−22 to −18</td>
<td>−199</td>
<td>−221 to −178</td>
<td>10</td>
</tr>
<tr>
<td>Cisatracurium</td>
<td>80</td>
<td>54 to 179*</td>
<td>−8.9</td>
<td>−10.3 to −7.5*</td>
<td>−84</td>
<td>−97 to −71*</td>
<td>9</td>
</tr>
<tr>
<td>Calabadion 2 concentration response for TOF reversal of 1.5 × EC$_{99}$ with ...</td>
<td>61</td>
<td>41 to 88</td>
<td>24</td>
<td>22 to 27</td>
<td>237</td>
<td>216 to 257</td>
<td>10</td>
</tr>
<tr>
<td>Cisatracurium</td>
<td>100</td>
<td>54 to 179*</td>
<td>4.0</td>
<td>3.5 to 4.5*</td>
<td>37</td>
<td>32 to 42*</td>
<td>9</td>
</tr>
</tbody>
</table>

Slope and intercept are calculated by linear regressions of the TOF in a logit scale and the concentrations in a natural log scale.

* The results take into account the spontaneous Hoffman degradation of cisatracurium (details are given in Materials and Methods).

NA = not applicable since sugammadex does not bind to cisatracurium; TOF = train-of-four.
Effectiveness of Calabadion and Sugammadex

provides a higher molar potency to reverse steroidal-induced NMB \textit{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 established previously by \textsuperscript{1}H NMR integration of resonances for calabadion 2 relative to NMBAs and also by Job plots.\textsuperscript{17} In this study, the \textsuperscript{1}H NMR experiments used for head-to-head

\textbf{Fig. 4.} Steady-state binding study in phrenic nerve hemidiaphragm preparations in a tissue bath (n = 85). Mean and 95\% CIs are provided. The EC\textsubscript{50} (the median effective concentration) of calabadion 2 and sugammadex in reversing rocuronium was within the same range (assessed by TOF, 61 vs. 49 \textmu M; assessed by T1, 53 vs. 43 \textmu M, respectively). (A) Concentration effect of rocuronium and cisatracurium on T1 depression. (B) Reversal of rocuronium- and cisatracurium-induced neuromuscular blockade by calabadion 2. (C) Reversal of rocuronium-induced neuromuscular blockade by sugammadex. TOF = train-of-four.
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 establish that calabadion 2 has superior binding affinity. 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 toward 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 with that of sugammadex has the potential to translate to a high in vivo molar potency in the rat. The high binding affinity is an encouraging pharmacologic observation. Reversing drugs by encapsulating carries the risk of nonspecific binding to biologically relevant plasma molecules or medications the patients may also have taken. The experience with sugammadex shows that its side effects (inhibiting effects on coagulation) are dose-dependent. Although we have so far not observed inhibiting effects of

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**Fig. 5.** Recovery of onset of breathing efforts (A) and train-of-four ratio to 0.9 (B) following administration of the two times ED_{50} of vecuronium (0.7 mg/kg). Means and SD from 44 rats. (A) Breathing onset. Calabadion 2 accelerates recovery time significantly compared with placebo (*P < 0.001), neostigmine (#P < 0.001), calabadion 1 (P < 0.01), and sugammadex (@P < 0.01). (B) TOF 0.9 recovery. Calabadion 2 decreases time to recovery significantly compared with placebo (*P < 0.001), neostigmine (#P < 0.001), and calabadion 1 (P < 0.001). TOF = train-of-four.

**Fig. 6.** Recovery of onset of breathing efforts and train-of-four ratio to 0.9 after administration of the two times ED 90 of rocuronium (3.5 mg/kg). Means and SD from 32 rats. (A) Breathing onset. Calabadion 2 accelerates recovery time significantly compared with placebo (*P < 0.001), neostigmine (#P < 0.001), and sugammadex (@P < 0.001). (B) TOF 0.9 recovery. Calabadion 2 decreases time to recovery significantly compared with placebo (*P < 0.001), neostigmine (#P < 0.001), and sugammadex (@P < 0.001). TOF = train-of-four.
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 to 8) binding affinity of calabadion 2 compared with sugammadex suggest that calabadion 2 may have a slightly wider therapeutic range. Currently, further experiments to evaluate the potential of calabadion 2 to bind to alternate targets are being performed.

Sugammadex, calabadion 1, and calabadion 2, as shown in this study, are renally eliminated within a short time. 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 EC$_{50}$ of calabadion 2 and sugammadex (TOF 0.5 reversal after calabadion 2 injection: 61 µM [95 CI, 41 to 88] and after sugammadex: 54 µM [32 to 73]) as well as slope of the recovery curves (fig. 4; TOF 0.5 reversal after calabadion 2 injection: 22 to 27 and after sugammadex: 20 to 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 EC$_{50}$ of these compounds.

Within the rat hemidiaphragm 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 NMB under these conditions. Because the ex vivo

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Fig. 7. Recovery of onset of breathing efforts (A) and TOF ratio to 0.9 (B) after administration of the two 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 calabadion 2. (A) Effects of the molecular concentration of calabadion 2 and sugammadex given at peak neuromuscular block on time to recovery of breathing (*P < 0.05) and (B) TOF 0.9 recovery. TOF = train-of-four.

Fig. 8. Recovery of onset of breathing efforts and TOF ratio to 0.9 after administration of the two times ED$_{90}$ of cisatracurium (0.6 mg/kg). Means and SD from 20 rats. (A) Breathing onset. Calabadion 2 accelerates recovery time significantly compared with placebo (*P < 0.001) and neostigmine (#P < 0.001). (B) TOF 0.9 recovery. Calabadion 2 decreases time to recovery significantly compared with placebo (*P < 0.05) and neostigmine (#P < 0.05). TOF = train-of-four.
diffusion distance is relatively long compared with a perfused muscle or an in vitro solution, the ability of rocuronium to pass through the tissue is limited, even with a maximum concentration gradient created by calabadion 2 or sugammadex encapsulation.

Accordingly, because of the initial excess of rocuronium molecules at postjunctional 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, which 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 nondepolarizing NMBAs and more sensitive to depolarizing NMBAs than humans. The dose–response curve of calabadi on and sugammadex to reverse the effects of nondepolarizing 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 with 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 nondepolarizing NMBAs.18

Calabadion 1 was the first reversal agent that reversed deep cisatracurium-induced NMB by binding and encapsulation, an unmet need because no alternative medication is available to reverse deep cisatracurium-induced NMB. Calabadion 2 has an almost five times higher binding affinity toward cisatracurium compared with calabadion 1, and substantially lower doses are required to reverse cisatracurium in vivo.17 The affinity of calabadion to cisatracurium is lower than their affinity toward steroidal NMBAs. In contrast, sugammadex does not bind to cisatracurium, and the binding affinity of calabadi on 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 ± 6.5 s for calabadion 2 to 60 mg/kg compared with 465 ± 196 s for placebo and 291 ± 99 s for neostigmine and compared with 47 ± 13 s for the previously published calabadion 1 to 150 mg/kg).16

We attribute the enhanced binding affinity of calabadion 2 (figs. 5 to 8) for its targets to its larger hydrophobic cavity that is shaped by two naphthalene walls as opposed to calabadion 1, which features two benzene walls.30 Currently, calabadion 2 is 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, and in vivo.

In clinical practice, surgical complications may occur that require a second (emergency) surgical procedure with anesthesia, shortly after the reversal of the NMBA with an agent that inactivates NMBAs, such as sugammadex or calabadi on. It is possible to administer a higher dose of the nondepolarizing NMBA injected before the reversal. Cammu et al.31 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.

However, currently, it is recommended that after initial reversal of NMB with sugammadex, 24 h should be allowed
before rocuronium or vecuronium can be readministered. Based on the 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 after calabadion reversal of nondepolarizing NMBA. Our data (fig. 9) show that succinylcholine can be used safely and effectively directly after administration of calabadion 2 to reverse a nondepolarizing 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 because the clinically meaningful problems such as of adverse and lingering effects of anesthetics and NMBA or cocaine intoxication have not yet been solved.33 By 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 has 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 NMBA 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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Competing Interests
Drs. Isaacs and Eikermann hold equity stakes in Calabash Biocience, Inc. (College Park, Maryland), which is developing calabadion 2 for biomedical application. The other authors declare no competing interests.

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