A Novel Strategy to Reverse General Anesthesia by Scavenging with the Acyclic Cucurbit[n]uril-type Molecular Container Calabadion 2

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ABSTRACT

Background: Calabadion 2 is a new drug-encapsulating agent. In this study, the authors aim to assess its utility as an agent to reverse general anesthesia with etomidate and ketamine and facilitate recovery.

Methods: To evaluate the effect of calabadion 2 on anesthesia recovery, the authors studied the response of rats to calabadion 2 after continuous and bolus intravenous etomidate or ketamine and bolus intramuscular ketamine administration. The authors measured electroencephalographic predictors of depth of anesthesia (burst suppression ratio and total electroencephalographic power), functional mobility impairment, blood pressure, and toxicity.

Results: Calabadion 2 dose-dependently reverses the effects of ketamine and etomidate on electroencephalographic predictors of depth of anesthesia, as well as drug-induced hypotension, and shortens the time to recovery of righting reflex and functional mobility. Calabadion 2 displayed low cytotoxicity in MTS-3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium–based cell viability and adenylate kinase release cell necrosis assays, did not inhibit the human ether-à-go-go-related channel, and was not mutagenic (Ames test). On the basis of maximum tolerable dose and acceleration of righting reflex recovery, the authors calculated the therapeutic index of calabadion 2 in recovery as 16:1 (95% CI, 10 to 26:1) for the reversal of ketamine and 3:1 (95% CI, 2 to 5:1) for the reversal of etomidate.

Conclusions: Calabadion 2 reverses etomidate and ketamine anesthesia in rats by chemical encapsulation at nontoxic concentrations. (ANESTHESIOLOGY 2016; 125:333-45)

CURRENTLY used intravenous anesthetics such as ketamine and etomidate are clinically employed in a variety of settings. Ketamine is used to induce anesthesia,[1] to achieve sedation and analgesia during mechanical ventilation, and to treat patients with chronic pain or psychiatric problems, including the estimated 10 to 30% of patients with major treatment-resistant depression.[2] Etomidate, a rapid acting and cardiovascular safe anesthetic, is frequently used in emergency cases,[3] for procedural sedation, and for anesthesia induction.[4] Up to this point, these intravenous anesthetics have no mechanism of pharmacologic reversal.

Attempts to achieve faster emergence from general anesthesia have been directed toward counteracting specific physiologic sedating effects by stimulating opposing systems, for example, activating the arousal systems.[5] In addition, other researchers develop short-acting ketamine and etomidate that achieve faster recovery.[6–8]

What We Already Know about This Topic

- Termination of the effect of anesthetic agents is generally a passive process governed by their pharmacokinetics
- The γ-cyclodextrin sugammadex reverses the neuromuscular blocking effects of rocuronium by encapsulation, as a result of which the drug is unable to bind to the acetylcholine receptor

What This Article Tells Us That Is New

- The acyclic cucurbit[n]uril molecular container calabadion 2 dose-dependently decreased effects of ketamine and etomidate on electroencephalographic predictors of depth of anesthesia by encapsulation at nontoxic concentrations in rats
- At doses sufficient to reverse neuromuscular blockade, calabadion 2 had minimal effects on anesthetic depth or duration
- The effects of propofol and isoflurane were not reversed by calabadion 2

An exciting opportunity to overcome the limitations of reanimation by accomplishing an actual reduction of

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anesthetic agents has emerged with the characterization of the cucurbit[n]urils (CB[n]) molecular containers, which bind tightly and selectively to a variety of cations.[9,10] A particularly promising new subgroup of the acyclic CB[n] is the calabadions.[11,12] The development of narrow-spectrum high-affinity macromolecular binders as antidotes has been focused mainly on neuromuscular blockers and anticoagulants,[13] and previous studies[14] have demonstrated the effectiveness of molecular containers in scavenging excess neuromuscular blockers to speed postsurgical recovery from paralysis.

In this article, we explore the potential use of calabadion 2 as a lead drug to inactivate ketamine and etomidate. We tested the proof of concept that acyclic CB[n] may function as true anesthesia reversal agents by reducing levels of etomidate and ketamine in rats through encapsulation followed by renal excretion. Calabadions might have the potential to reduce operating room time and costs, to reduce the risk of postoperative complications, and to counteract accidental overdose in both clinical and recreational settings.

Materials and Methods

Chemistry

Calabadion 2 was synthesized according to the published procedure.[15] The binding constants (K_D) for the calabadion 2•ketamine and calabadion 2•etomidate complexes were determined by changes in UV/Vis competition assays,[16] with the calabadion 2•Rhodamine 6G complex ($K_a = 2.3 \pm 0.2 \times 10^6$/M), fitted to a competitive binding model as described previously.[11,12,17]

To establish the 1:1 stoichiometry between calabadion 2 and ketamine, we used Job's method of continuous variation.[18] We maintained the total molar concentration of the ketamine and calabadion 2 constant (1 mM), but varied their mole fractions. The 1H NMR (400 MHz, 20 mM sodium phosphate–buffered D_2O at pD = 7.4) resonance for calabadion 2 at 7.73 ppm was monitored. The change in chemical shift is proportional to the amount of complex formed.

Animals

All studies on rats (60 adult male *Sprague-Dawley* rats, strain code 400; mean ± SD, 294 ± 61 g) and mice (35 adult female *Swiss Webster* mice, strain code 551; mean ± SD, 22.5 ± 1.3 g) were conducted in accordance with the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, Massachusetts (Protocol 2011N00181) and the Subcommittee on Research Animal Care at the University of Maryland, College Park, Maryland (Protocol R-14-02), respectively.

Instrumentation of Sprague-Dawley *Rats*

For placement of intravenous lines, animals were anesthetized with 1.5% isoflurane. Temperature was controlled rectally and maintained at 37° ± 1°C with a thermostat-controlled heating pad. A total of 60 rats were used in this study, of which 32 were instrumented with two intravenous lines, an arterial line and a tracheal tube. Of the remaining 28 animals, 21 rats were only instrumented with a tail vein intravenous catheter (24 gauge 19 mm), while the other 7 did not undergo any instrumentation.

Effects of Calabadion 2 on Electrographic Metrics of Unconsciousness during Constant Anesthetic Infusion

The effects of calabadion 2 on etomidate- and ketamine-evoked unconsciousness were investigated by quantified changes in electrical brain activity, measured with an epidural electroencephalogram (EEG) electrode in 26 chronically instrumented rats.

Methods described by Vijn and Sneyd[19] and by Cotten *et al.*[20] were used in a group of 13 rats to continuously estimate the burst suppression ratio (BSR), the proportion of time the EEG signal spent in suppression during each 10-s epoch for the evaluation of reversal from etomidate-evoked unconsciousness. All studies were performed in a background of inhaled 1% isoflurane.

After an initial bolus administered over 40 s to achieve a BSR of approximately 70%, the infusion rate was decreased to a value between 0.1 and 0.3 mg kg^{-1} min^{-1} (average dose of 183.9 ± 28.4 μg kg^{-1} min^{-1}, mean ± SD) to derive at a steady-state BSR higher than 40% for at least 20 min before test drug administration. Animals were premedicated with 5 mg/kg dexamethasone to avoid symptoms of etomidate-induced adrenal suppression. After steady-state recordings, either a stepwise increasing calabadion 2 infusion of 40, 60, 80, and 100 mg kg^{-1} min^{-1} over 5 min each (n = 10) or a 20-min saline infusion of equivalent total fluid volume (n = 3) was administered in order to reverse the effects of the constantly maintained etomidate infusion on the BSR. Additionally, the blood pressure was monitored throughout the experiment for evaluation of the reversal of effects on the cardiovascular system.

In 13 rats used for the evaluation of reversal of ketamine anesthesia, we quantified the total EEG power during a continuous ketamine infusion titrated to abolishment of response to tail clamping. After all surgical procedures were completed, the dose of isoflurane was stepwise reduced and discontinued while a 0.67-mg kg^{-1} min^{-1} ketamine infusion was started. After 10 min of a sole ketamine infusion,

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we applied intermittent standardized tail clamping (25N) every 2 min to identify depth of anesthesia. Depending on response, the infusion rate was increased or decreased by 0.33 mg kg^{-1} min^{-1}, until a constant infusion of ketamine, during which we observed no response to 6 consecutive tail clamps, was achieved.[21]

After steady-state recordings, we administered an escalating calabadion 2 infusion with 20, 40, 60, and 80 mg kg^{-1} min^{-1} over a period of 5 min each with 40-s breaks in between (n = 10) or a saline infusion of equivalent volume and timing (n = 3). EEG and arterial blood pressure were continuously measured throughout the experiment.

EEG recordings were analog filtered between 0.3 and 300 Hz and digitized with a band–pass filter between 0.5 and 55 Hz. The spectrum of visually identified artifact-free episodes was then calculated using a fast Fourier transformation with a 1,024-bit Hann (cosine-bell) window.

Changes in total EEG power and mean arterial blood pressure (MAP) were quantified in response to test drug injection in comparison to steady-state ketamine.

To ensure that the observed effects were not caused by an interaction of calabadion 2 with isoflurane, we administered increasing amounts of calabadion 2 (20, 40, 60, and 80 mg kg^{-1} min^{-1} for 5 min each) in three rats anesthetized with a constant isoflurane anesthesia titrated to the abolishment of tail clamping and quantified EEG power, MAP, and heart rate.

Additionally, we administered an escalating phenylephrine infusion (4 to 10 μg kg^{-1} min^{-1}) in three rats anesthetized with a continuous ketamine infusion, to ensure that our changes in EEG can be interpreted as a result of shallower anesthesia, rather than nonspecific hemodynamic reactions.

Effects of Calabadion 2 on Time to Regain Righting Reflex after Single-bolus Anesthesia

We examined the effects of calabadion 2 on time to recovery from loss of righting reflex (LORR) after a single intravenous bolus of etomidate or ketamine in 14 adult male *Sprague-Dawley* rats. After instrumentation, animals were randomized to receive either an intravenous etomidate bolus (4 mg/kg) over 10 s or a 1-min infusion of ketamine (30 mg/kg). Once placed in the supine position, animals were randomized to receive either an intravenous infusion of calabadion 2 (80 mg kg^{-1} min^{-1} dissolved in distilled water) or saline, beginning 3 min after the anesthetic injection. Recovery from LORR was taken as the moment when the rat regained a standing or sternally recumbent position.[22]

Additionally, we tested in crossover experiments the effect of calabadion 2 on propofol anesthesia in five adult *Sprague-Dawley* rats randomized to receive an intravenous infusion of calabadion 2 (80 mg kg^{-1} min^{-1} dissolved in distilled water) and saline beginning 3 min after the intravenous injection of 20 mg/kg propofol. This was performed in two study days with 48-h recovery time between the experiments.

Effects of Calabadion 2 on Functional Mobility after Ketamine and Etomidate Anesthesia on the Balance Beam

Recovery of functional mobility impairment was quantified as previously described[23] using the balance beam test, a common method to assess motor coordination and balance of animals,[24] used as a predictor for pharmacologic impact on recovery.[25] The time rats remained on a wooden rod (diameter, 2.5 cm) was measured to evaluate balance and body strength. After recovery from LORR, animals were placed on the beam every 4 min starting at the intravenous anesthetic agent injection, or every 10 min starting at the intramuscular ketamine injection. Test performance was scored by a team member blinded to the study treatment as unable to maintain grip or balance on the beam (0 points); able to remain on the beam for up to 10 s (1 point), 11 to 20 s (2 points), or 21 to 30 s; or reaches support (3 points).[24]

Toxicology

We analyzed the effect of calabadion 2 on human leukocytes (THP-1), liver cells (HepG2), and kidney cells (HEK293).

The cell viability was measured using a MTS-3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium–based assay (CellTiter 96® AQueous Kit assay from Promega G3580, USA), and cell necrosis was determined *via* the quantification of the release of cytosolic adenylate kinase enzyme (Toxilight® BioAssay from Lonza LT07-117, Switzerland). These cells were exposed to 0.16, 0.4, 1, and 2.5 mM calabadion 2; hydroxypropyl-β-cyclodextrin; or erythromycin, as a point of comparison for a Food and Drug Administration–approved drug. In each cell type, the cell viability was normalized to the average values obtained from untreated cells. The cell lysis, on the other hand, was normalized to the values obtained from the incubation of the cells with distilled water, which induces cells lysis *via* osmotic shock.

In order to test the effect of calabadion 2 on human ether-à-go-go-related gene (hERG) currents, we used a Chinese Hamster Ovary cell line transfected with the hERG ion channel. The potassium flow was analyzed with patch clamp technology. The activity of the hERG channel of untreated cells was used to normalize the effect of increasing doses of calabadion 2 or the hERG inhibitor quinidine, both up to a dose of 25 μM.

In order to determine the mutagenic properties of calabadion 2, we used the bacteria reverse mutation assay (Ames Test MOLTOX® 31–100.2, histidine auxotroph strains of *Salmonella typhimurium*—not able to grow on histidine-deficient agar without a mutation). The mutagenicity of a compound was addressed by the ratio of the number of colonies growing after treatment with the test compound relative to untreated bacteria. Compounds that give ratios greater than 2.0, of 1.6 to 1.9, or of less than 1.6 are considered mutagenic, potentially mutagenic, or not mutagenic, respectively. In addition, the potential of calabadion 2 to be metabolized by the liver into a more toxic metabolite was assessed by incubation with rat liver extract (+S9) before treatment of bacteria. We used four different bacterial test strains to assess the mutagenicity of the compound (TA1535, TA 1537, TA 98, and TA 100) and administered 0.012, 0.037, 0.11, 0.33, or 1 mg calabadion 2 per plate plus 1.5 μg sodium azide, 6 μg daunomycin, 1 μg CR 191 acridine, or 10 μg 2-aminoanthracene per plate as control.

We analyzed the toxicity of calabadion 2 in 35 *Swiss Webster* mice by performing a dose escalation study. Groups (n = 7) of 4- to 6-week-old female mice were injected intraperitoneally daily with 29, 87, 145, and 203 mg/kg of calabadion 2 or not injected (untreated) for 14 consecutive days. The weight of each mouse was determined over a period of 28 days.

We further analyzed the toxicity of calabadion 2 in rats (n = 10) by performing a maximal tolerated dose escalation study. Adult male *Sprague-Dawley* rats (n = 6) were injected with escalating doses of calabadion 2 by intravenous injection for 5 consecutive days until the lethal dose was reached (100, 500, 1,000, 1,500, and 2,000 mg/kg). In the four remaining rats, we administered a nonlethal cumulative dose of 1.6 g/kg on 3 consecutive days (100, 500, and 1,000 mg/kg).

Based on the ratio of median lethal dose (LD_{50}) and median dose of calabadion 2 required to achieve an accelerated recovery from LORR with a 50% probability (ED_{50}), we calculated the therapeutic index of calabadion 2 in reversing etomidate and ketamine anesthesia.

The heart, lungs, liver, kidneys, and spleen of all 10 animals were harvested and fixated in 10% neutral buffered formalin. Samples were stained with hematoxylin and eosin and embedded in paraffin slides, and the organ tissue toxicity of calabadion 2 was evaluated by an independent pathologist.

Statistical Analysis

All data are reported as means ± SD unless otherwise specified. Statistical analysis was performed using SPSS 22.0 (SPSS, Inc., USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., USA). Descriptive analytics and visual inspection of the distribution including histogram, density plots, and Q-Q plots were applied. Normality was tested for using the Shapiro-Wilk test, and data were considered normally distributed when $P \geq 0.05$.

To assess the effects of calabadion 2 on EEG/BSR and blood pressure during a continuous infusion of etomidate or ketamine, we used a mixed linear model with an identity link function for normally distributed probability. Our mixed model included main effects of reversal agent (calabadion 2 *vs.* placebo) and dose, and the interaction term reversal of agent and dose as fixed effects while allowing intercepts to vary (random intercepts model). Goodness of fit was established using the likelihood ratio test (LRT) to compare the fit of the final model to the intercept-only model. The same model was used to evaluate the effects on blood pressure.

We used a paired Student's *t* test to assess differences in recovery time from LORR after etomidate, ketamine, and propofol anesthesia when administering calabadion 2 compared to saline in cross-over experiments in the same animals at different study days.

To evaluate the effect on postanesthetic etomidate- and ketamine-induced balance and coordination impairment, we used a mixed linear model with an identity link function for normally distributed probability. We tested for a fixed main effect of the reversal agent on the time needed to reach recovery milestones (score of 1, 2, and 3 after Combs and D'Alecy[24]), while allowing a subject specific intercept to vary as random effects.

All model assumptions were examined through model diagnostic plots, including residual plots and Q-Q plots. We examine whether the variance estimate was indistinguishable from 0 ($P > 0.05$). If so, fixed effects model is applied instead of the mixed model. Model comparison, if applied, was presented and conducted using ANOVA and comparing Bayesian information criterion values.

Inhibition of the hERG channel was analyzed using GraphPad Prism 6 to calculate statistical significance and IC_{50} values *via* nonlinear regression analysis, using a least squares (ordinary) fit. Goodness of fit was assessed using the R^2 value for the nonlinear regression and the SD of residuals (sy.x). Medium convergence criteria were used. That is, regression concluded when five consecutive iterations altered the sum of squares by less than 0.0001%. Model normality assumptions were examined and visualized through model diagnostic plots including residual plots and Q-Q plots. Additionally, normality of residuals was tested for using the Shapiro-Wilk test, and residuals were considered normally distributed when $P \geq 0.05$. The nonlinear regression used for quinidine passed the Shapiro-Wilk normality test in GraphPad Prism.

A Student's *t* test was used to detect differences between treatment conditions and untreated or distilled water–treated cells for the cell death and cell viability assay, respectively. The maximum tolerated dose study data were plotted as the average change in weight for each group plus ±1 SD. A Student's unpaired *t* test was performed to compare each dosage group to the untreated mice. A *P* value less than 0.05 was considered significant.

Results

Chemistry

The dissociation constants (K_D) of the calabadion 2•ketamine and calabadion 2•etomidate complexes were determined to be 5.1 ± 0.3 and 27.2 ± 5.0 μM, respectively (fig. 1; figs. S1 and S2, Supplemental Digital Content 1, http://links.lww.com/ALN/B291, showing the binding assays for both complexes).

The Job plots for the calabadion 2•ketamine and calabadion 2•etomidate complexes showed maxima at mole fractions of 0.5, which establishes the 1:1 nature of the calabadion 2•drug complexes (figs. S3 and S4, Supplemental Digital Content 1, http://links.lww.com/ALN/B291, establishing the stoichiometry of calabadion 2 and ketamine).

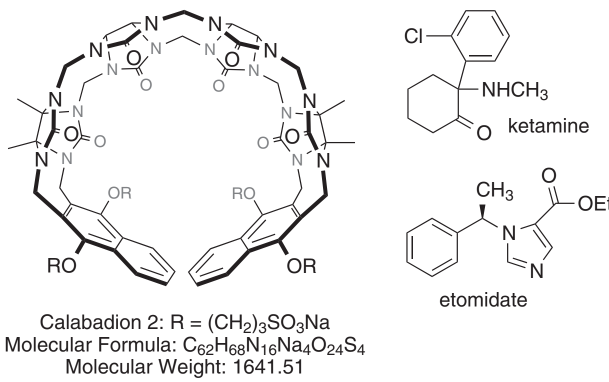

Fig. 1. Chemical structures of calabadion 2, ketamine, and etomidate.

Calabadion 2 Reverses Electrographic Metrics of Unconsciousness during Constant Anesthetic Infusion

Deepening anesthesia with etomidate is marked by lengthening of suppression periods in the EEG quantifiable as the BSR. An average dose of 183.9 ± 28.4 µg kg^{-1} min^{-1} was used to maintain the BSR at a stable rate of 63% (95% CI, 62 to 65%), deep enough such that a partial reversal could be achieved without awakening the animal. Calabadion 2, but not saline control, induced a dose-dependent decrease in BSR to 38% (95% CI, 24 to 51%; reversal agent × dose, $P = 0.001$, fig. 2A; n = 10; LRT $P < 0.001$; table S1, Supplemental Digital Content 1, http://links.lww.com/ALN/B291, displaying the effect sizes of fixed effects), while the MAP returned from 83% (95% CI, 80 to 86%) to 101% (95% CI, 96 to 105%) of preetomidate baseline (reversal agent × dose $P = 0.033$, fig. 2A; n = 10; LRT $P < 0.001$). These changes in brain function and blood pressure objectively demonstrate the ability of calabadion 2 to reverse the effects of etomidate.

Unlike etomidate, ascending levels of ketamine gradually increase EEG power. During continuous ketamine infusion titrated to abolish responses to a noxious stimulus (tail clamping), calabadion 2 induced a dose-dependent decrease in total EEG power to 63% (95% CI, 54 to 72%) of steady-state ketamine EEG power, indicating that calabadion 2 reversed the typical effects of ketamine in the EEG (reversal agent × dose $P < 0.001$, fig. 2B; n = 10; LRT $P < 0.001$, table S1, Supplemental Digital Content 1, http://links.lww.com/ALN/B291, displaying the effect sizes of fixed effects). During both calabadion 2 (n = 10) and saline (n = 3), all frequency bands behaved very similarly, without significant differences between individual bandwidths (fig. 3). In parallel, calabadion 2 injection resulted in a dose-dependent increase in MAP to almost 130% (95% CI, 117 to 142%) compared to preketamine baseline (96 mmHg) at the highest dose (n = 10), also indicating reversal of anesthesia (reversal agent × dose $P < 0.001$, fig. 2B; n = 10; LRT $P < 0.001$).

No significant changes in BSR (n = 3, $P = 0.22$), EEG power (n = 3, $P = 0.08$), or MAP (during etomidate, n = 3, $P = 0.939$; during ketamine, n = 3, $P = 0.697$) were observed during saline infusion (fig. 2, A and B).

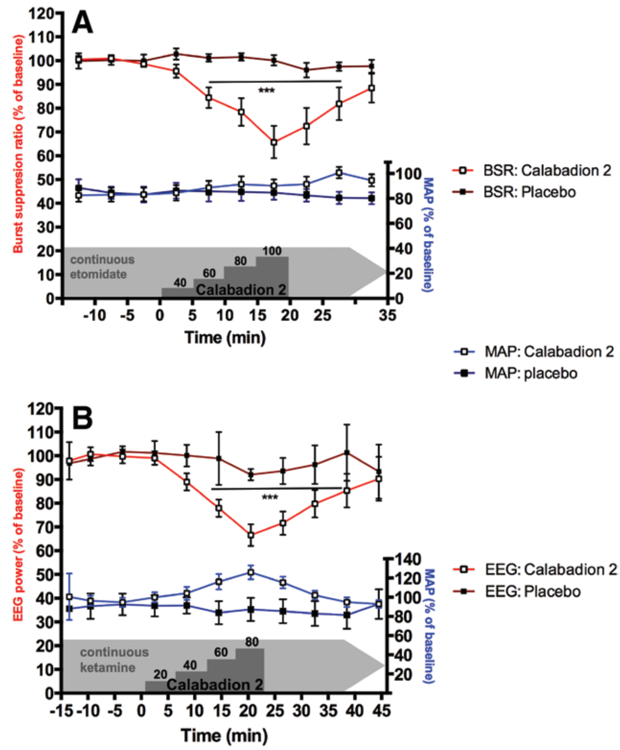

Fig. 2. Calabadion 2 decreases levels of unconsciousness during anesthesia with continuous administration of etomidate and ketamine. Effect of an escalating calabadion 2 intravenous infusion on (*A*) burst suppression ratio (BSR) and mean arterial blood pressure (MAP) during continuous etomidate intravenous infusion (titrated to an average dose of 184 µg kg^{-1} min^{-1}; n = 13) and on (*B*) electroencephalographic (EEG) power and MAP during continuous ketamine infusion (titrated to an average dose of 122 µg kg^{-1} min^{-1}; n = 13). Calabadion 2 decreased BSR during etomidate infusion and EEG power during ketamine infusion in a dose-dependent fashion and increased the MAP accordingly (***$P < 0.001$). BSR and EEG power are displayed as percent values from baseline (average value during continuous etomidate/ketamine infusion before test drug infusion). MAP is displayed as percent of mean MAP before start of etomidate/ketamine administration. *Error bars* represent the 95% CIs.

In contrast, continuous phenylephrine infusion during steady-state shallow ketamine anesthesia resulted in significant MAP increases without effects on EEG power (n = 3, $P = 0.024$). We did not observe any effects of calabadion 2 on EEG power, BSR, and MAP during and after the highest dose of the stepwise increasing calabadion 2 infusion when administered during constant isoflurane anesthesia.

Effects of Calabadion 2 on Time to Emergence from Anesthesia

Emergence from etomidate and ketamine anesthesia was assessed by measuring time to recovery from LORR, frequently used as a predictor for the level of anesthesia.[26,27] Relative to saline, calabadion 2 significantly decreased the time to recovery from LORR by almost 50% in etomidate-anesthetized rats (15.2 ± 1.4 *vs.* 26.9 ± 2.3 min, n = 7,

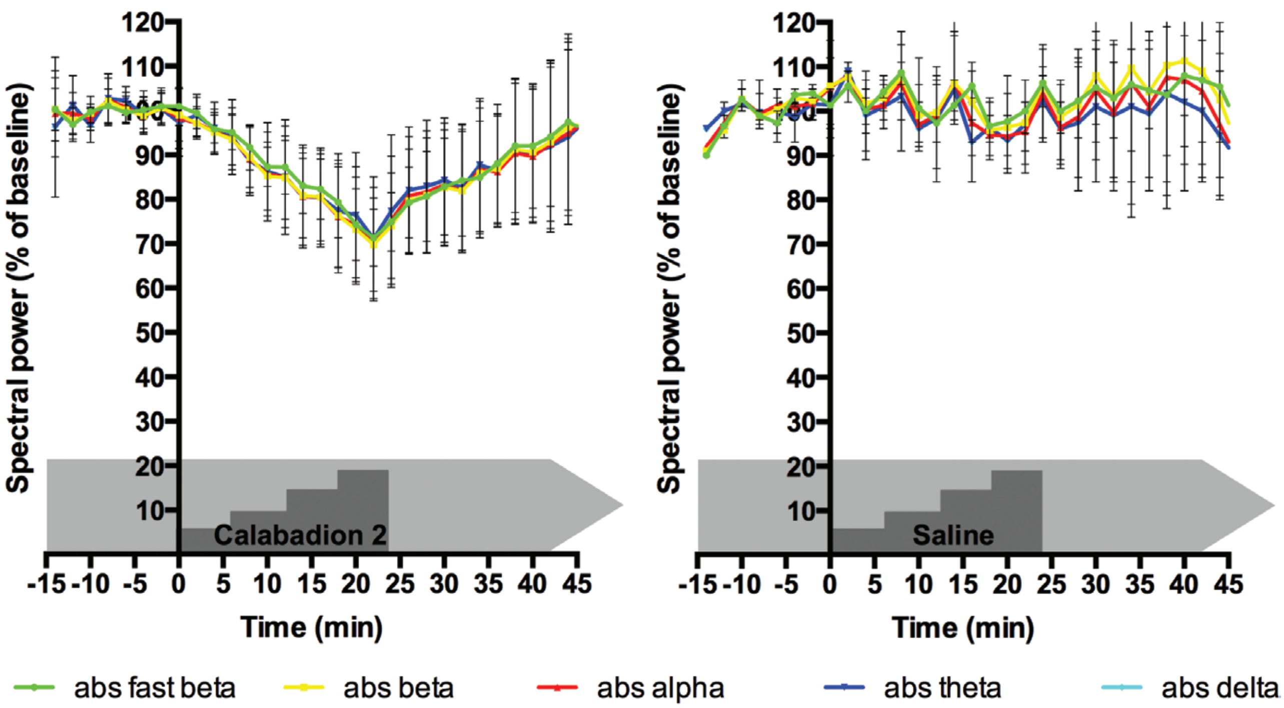

Fig. 3. Calabadion 2 effects on electroencephalographic frequency bands during anesthesia with continuous administration of ketamine. During both calabadion 2 (n = 10) and saline (n = 3), all frequency bands (δ, 1 to 4 Hz; θ, 4 to 8 Hz; α, 8 to 12 Hz; β, 12 to 25 Hz; and fast β, 25 to 30 Hz) behaved very similarly, without significant differences between individual bandwidths. *Error bar*s represent the 95% CIs. Abs = absolute.

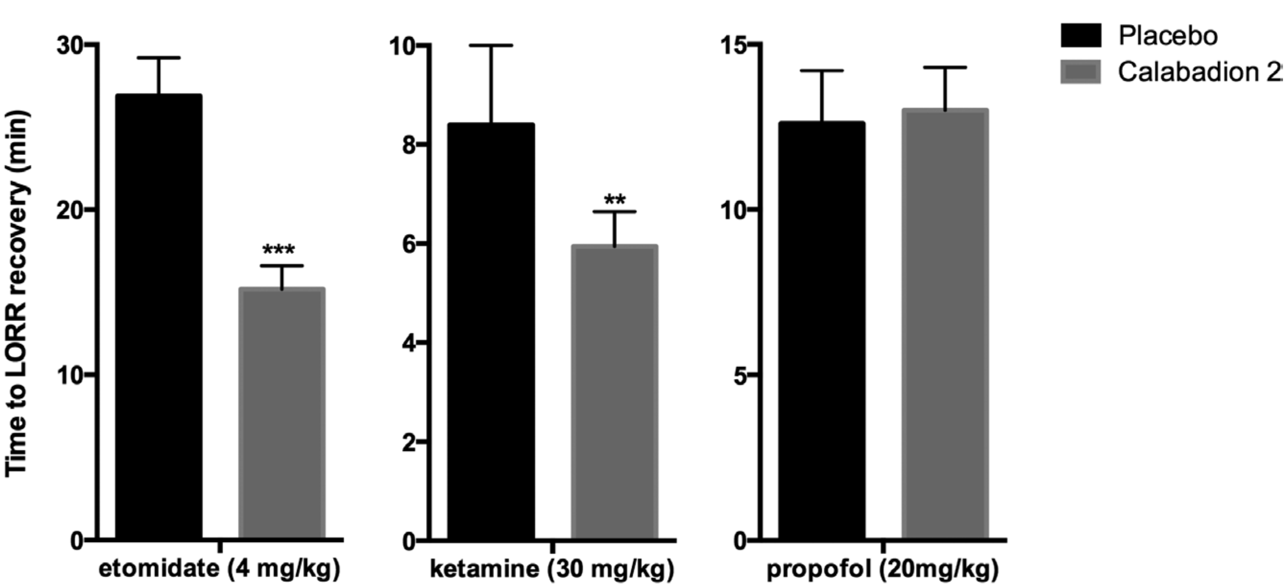

Fig. 4. Calabadion 2 accelerated recovery from single-bolus anesthesia with etomidate and ketamine and did not affect recovery from propofol anesthesia. Effect of calabadion 2 (80 mg kg^{-1} min^{-1}, intravenous) on time to recovery from loss of righting reflex (LORR) after administration of a single intravenous bolus of etomidate (4 mg/kg; n = 7), ketamine (30 mg/kg; n = 7), or propofol (30 mg/kg; n = 5). In the cases of etomidate and ketamine, recovery time was significantly shorter after calabadion 2 administration *versus* placebo (****P* < 0.001, ***P* = 0.023). Compared to saline, calabadion 2 did not affect the time to recovery from LORR after a propofol bolus (*P* = 0.672). Data are presented as ±SD.

P < 0.001, fig. 4) and by about 30% in ketamine anesthetized rats (6.0 ± 0.7 *vs.* 8.4 ± 1.6 min, n = 7, *P* = 0.023, fig. 4). The median dose of calabadion 2 required to achieve the described accelerated recovery from LORR with a 50% probability (ED_{50}) was 984 mg/kg (95% CI, 976 to 991 mg/kg) and 167 mg/kg (95% CI, 161 to 173 mg/kg) for the reversal of a 4 mg/kg intravenous etomidate bolus and a 30 mg/kg intravenous bolus of ketamine, respectively.

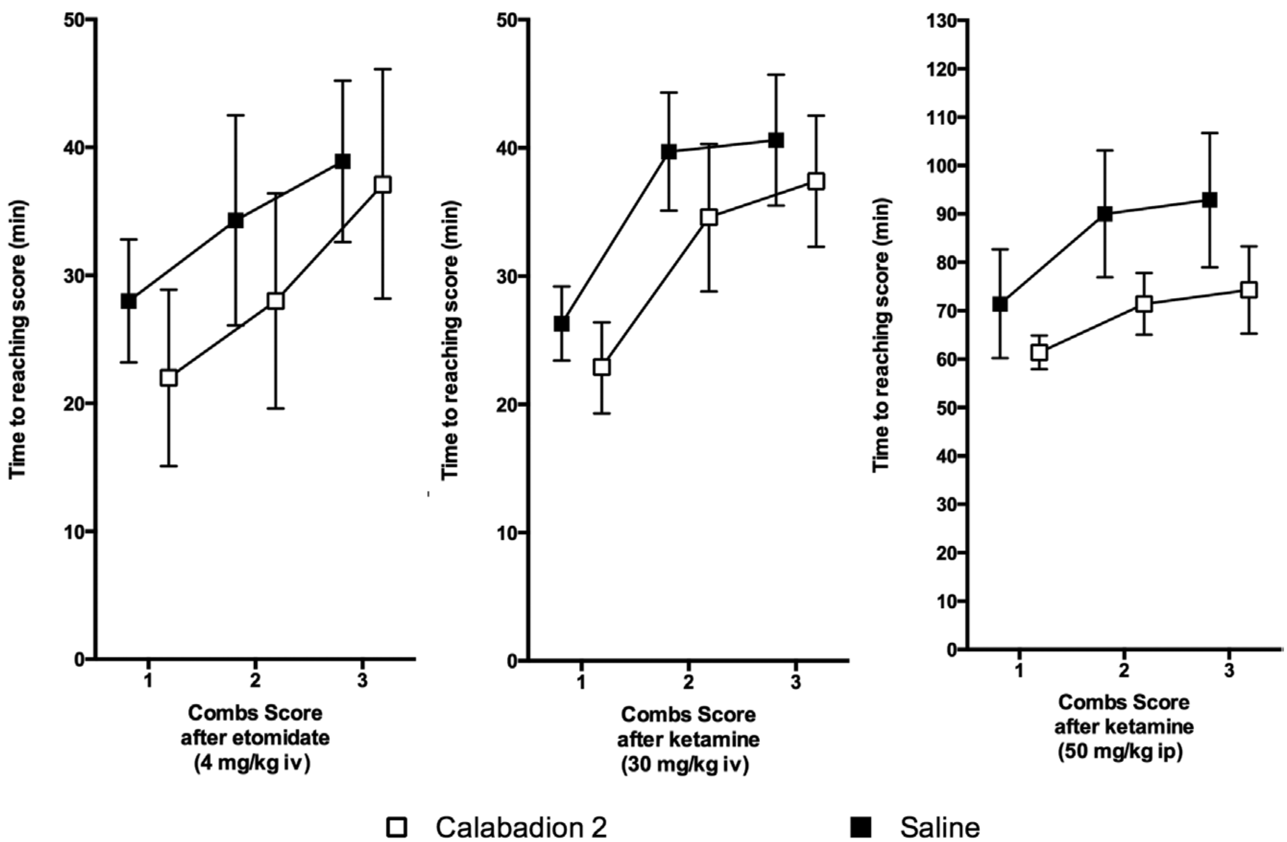

Fig. 5. Calabadion 2 accelerated recovery from postanesthetic functional mobility impairment. Effect of calabadion 2 on time to recovery of balance described by the Combs score after administration of a single bolus of etomidate (4 mg/kg intravenous [iv]; n = 7) or ketamine (30 mg/kg iv; n = 7; 50 mg/kg intramuscularly; n = 7) *versus* placebo. Test performance was scored as unable to maintain grip or balance on the beam (0 points); able to remain on the beam for up to 10 s (1 point), 11 to 20 s (2 points), or 21 to 30 s, or reaches support (3 points). Recovery time was significantly shorter after calabadion 2 *versus* placebo. After an intraperitoneal (ip) injection of calabadion 2, we observed the effect in accelerating recovery time to be significantly higher compared to iv administration (P < 0.001). *Error bars* represent the 95% CIs.

Calabadion 2 did not affect the time to recovery from LORR after a single bolus of propofol compared to saline (13.0 ± 1.3 *vs.* 12.6 ± 1.6 min, n = 5, P = 0.672, fig. 4).

Effects of Calabadion 2 on Postanesthesia Functional Mobility Impairment

We observed a significantly faster recovery of balance after anesthesia, when injecting calabadion compared to saline. Calabadion 2 significantly reduced the time slope of recovery by 4.9 min (95% CI, 1.1 to 8.6 min; P = 0.013; LRT P = 0.002) after administration of 4 mg/kg intravenous etomidate, by 3.9 min (95% CI, 1.5 to 6.3 min; P = 0.002; LRT P < 0.001) after 30 mg/kg intravenous ketamine, and by 15.7 min (95% CI, 9.4 to 22.0 min; P < 0.001; LRT P < 0.001) after 50 mg/kg intramuscular ketamine, as compared to saline (fig. 5 and table S2, Supplemental Digital Content 1, http://links.lww.com/ALN/B291, displaying the effect sizes of fixed effects). The faster recovery of balance may suggest a faster recovery of muscle strength and/or motor coordination after calabadion 2 injection for both anesthetics.

Calabadion 2 Is Not Toxic or Mutagenic

Calabadion 2 did not show any toxicity or mutagenic potential in a variety of tests (fig. 6). Even under stringent conditions (calabadion 2 up to 1 mM), we did not observe a significant reduction in cell viability of THP-1 and HepG2 cells, and only a slight dip on the HEK293 cells and no cell lysis (fig. 6, A and B). These results were very comparable to the toxicity observed after incubation of the same cell lines with the antibiotic erythromycin and the cyclodextrin, hydroxypropyl-β-cyclodextrin (fig. 7).

Treatment with calabadion 2 up to a concentration of 25 μM did not result in significant differences in the observed current at the hERG channel (IC_{50} more than 25 μM), indicating no inhibition of the hERG channel. In contrast, the positive control, quinidine, showed a distinct decrease from an average of 90 ± 4% to 1 ± 6% in the posttreatment current across the ion channel with increasing concentrations of the compound (IC_{50} = 1.66 μM, fig. 6C).

The ratio of the amount of colonies growing after treatment with calabadion 2 in the Ames test relative to untreated bacteria did not exceed 1.1 even at the highest dose (1 mg/ml),

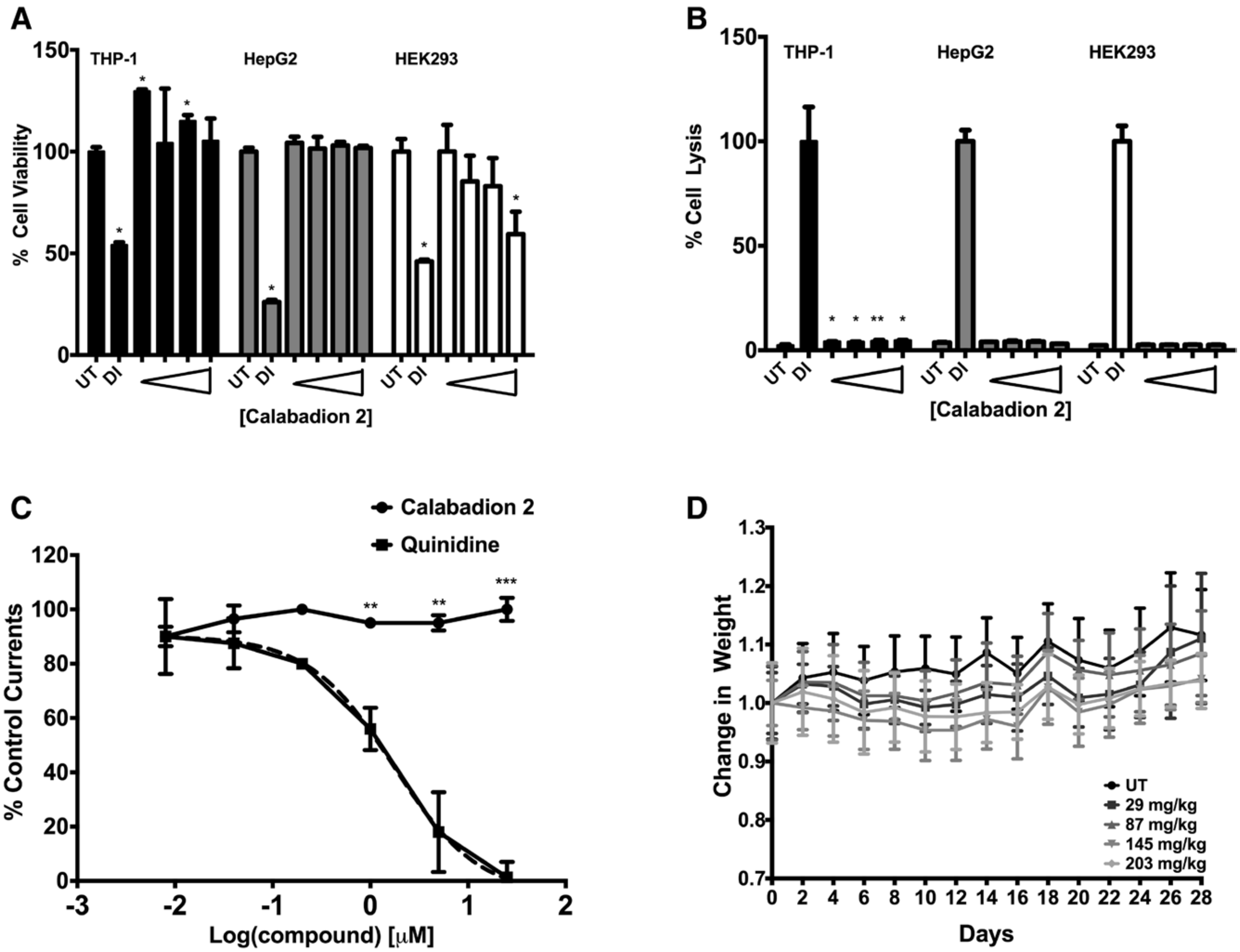

Fig. 6. Calabadion 2 does not induce acute cytotoxicity, inhibit the human ether-à-go-go-related (hERG) channel, or cause pathology in mice. The cell viability (*A*) or cell lysis (*B*) of human leukocytes (THP-1), liver cells (HepG2), and kidney cells (HEK293) after treatment with increasing doses (0.16 to 2.5 mM) of calabadion 2 is shown and compared to untreated (UT) and deionized water (DI). (*C*) The hERG assay was conducted using transfected Chinese Hamster Ovary (CHO)-hERG cells in an automated patch clamp study, with quinidine as a positive control (IC_{50} = 1.66 μM; best-fit, nonlinear line of regression, data are represented as ±SD). (*D*) *Swiss Webster* mice were injected intraperitoneally for 14 days either with increasing doses of calabadion 2 (milligram per kilogram of body weight) or left UT. The weight of each mouse was followed daily until 14 days posttreatment. The average and SD of the change in weight of the mice per treatment group (n = 7) is graphed. (*A–C*) The values are an average of at least three replicates with corresponding SD values (*P = 0.01 to 0.05; **P = 0.001 to 0.01; ***P < 0.001).

which indicates that calabadion 2 has no mutagenic potential (table 1).

Additionally, a maximum tolerated dose study in mice revealed a good tolerance of calabadion 2 without obvious side effects. The average weight change for mice in all groups did not fall below 95% after 28 days (fig. 6D).

Finally, a dose escalation study on 10 male *Sprague-Dawley* rats suggested a median lethal dose of 2.7 g/kg (LD_{50} = 2.7 g/kg [95% CI, 1.8 to 4.3]). Calabadion did not induce apparent toxic effects in efficacy experiments. The histopathologic evaluation of organs showed no significant lesions (*i.e.*, within normal limits) in the heart and spleen and mild to moderate vacuolation in the liver and kidney. In animals receiving lethal doses of calabadion 2 in escalating dose experiments, we observed mild cellular necrosis of parts of the lungs with fluid in the alveolar spaces, and occasional distension of the pulmonary alveolar capillaries with erythrocytes, which may be the consequence of pulmonary embolism when supratherapeutic, toxic doses are administered.

The therapeutic index of calabadion 2 in accelerating recovery of righting reflex was 16:1 (95% CI, 10 to 26:1) for the reversal of 30 mg/kg intravenous ketamine and 3:1 (95% CI, 2 to 5:1) for the reversal of 4 mg/kg intravenous etomidate. Calabadion 2 was well tolerated at effective doses. The detailed results of the histopathology studies are listed in table 2.

Discussion

The *in vitro* binding data show that calabadion 2 encapsulates etomidate and ketamine molecules. *In vivo* encapsulation translates to inactivation of clinical etomidate and

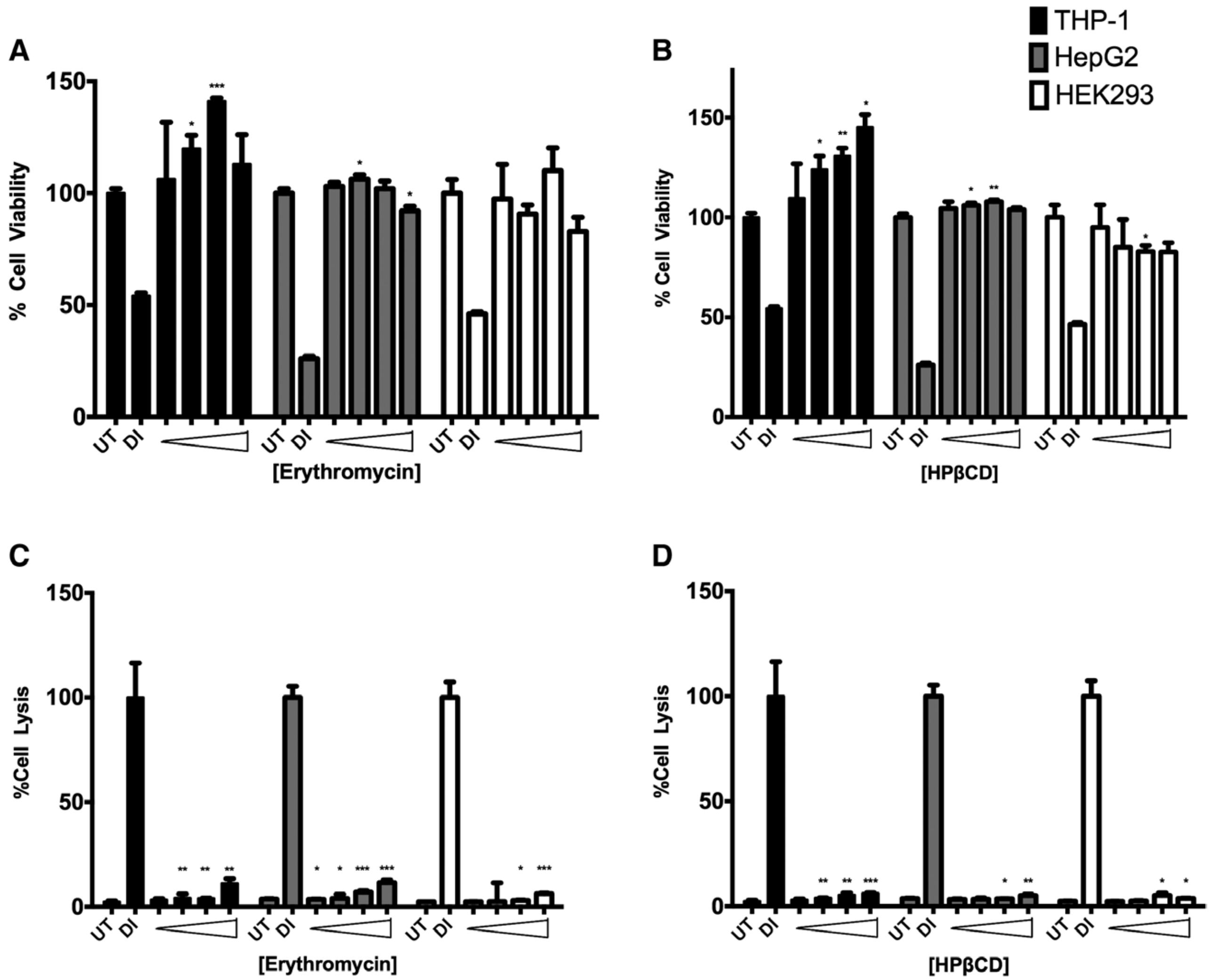

Fig. 7. Toxicity of erythromycin and HPβCD in *in vitro* cell assays. Monocytes (THP-1), liver (HepG2), and kidney (HEK293) cell lines were incubated with indicated doses (0.16 to 2.5 mM) of erythromycin (*A*, *C*) and cyclodextrin (HPβCD) (*B*, *D*). The untreated (UT) and cell death–inducing (DI) conditions are indicated as appropriate controls. The cell viability (*A*, *B*) and cell death (*C*, *D*) were analyzed, and results were normalized to UT groups or death induction controls, respectively. (*A–D*) The values are an average of at least three replicates with corresponding SD values (**P* = 0.01 to 0.05; ***P* = 0.001 to 0.01; ****P* < 0.001).

ketamine anesthesia. Our data indicate that calabadion 2 increases the level of consciousness during continuous anesthesia of etomidate and ketamine, decreases the time to emergence, and mitigates lingering effects on motor and cognitive function by sequestrating anesthetic agents so that they cannot act at the effect compartment. These reversal effects were dose-dependently achieved by nontoxic concentrations of calabadion 2. We provide the proof of concept that acyclic CB[n] can function as true anesthesia reversal agents by reducing levels of etomidate and ketamine in rats through encapsulation followed by renal excretion.

In clinical practice, emergence from general anesthesia is considered a passive process governed by anesthetic drug pharmacokinetics. Recently, Brown and coworkers[26] and Solt *et al.*[27] have described "reanimation" from general anesthesia: an active emergence with methylphenidate. Methylphenidate inhibits reuptake transporters for dopamine and norepinephrine in the brain,[28] and both neurotransmitters are known to promote arousal.[29] This was also observed after administration of a D1 dopamine receptor agonist[30] as well as electrical stimulation of the ventral tegmental area,[31] suggesting that dopamine release by ventral tegmental area neurons causes a profound arousal response sufficient to reverse the behavioral effects of general anesthesia.

While reanimation from general anesthesia aims to overpower the anesthetics at the receptor level by stimulation of this dopamine-mediated arousal pathway, calabadion 2 encapsulates the anesthetic agent without receptor interactions. This allows a reduction of anesthetic effects and potential side effects by decreasing the concentration of active molecules rather than stimulating other pathways. The encapsulation complex of calabadion 2 and molecules bound to it is excreted in the urine.

Table 1. Bacteria Reverse Mutation Assay (Ames Test) for Calabadion 2, in the Presence or Absence of Rat Liver S9 Fraction (–/+S9) in Order to Test for Metabolic Activation

Calabadion 2 concentration (mg)	TA 1535		TA 1537		TA 98		TA 100	
	–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
0	11 ± 2	8 ± 2	10 ± 5	7 ± 1	19 ± 2	27 ± 3	100 ± 27	109 ± 4
0.012	6 ± 1	10 ± 2	7 ± 2	2 ± 1	24 ± 4	24 ± 4	92 ± 11	114 ± 2
0.037	10 ± 5	6 ± 2	8 ± 2	4 ± 1	18 ± 8	27 ± 3	100 ± 6	112 ± 8
0.11	8 ± 3	7 ± 1	7 ± 2	6 ± 1	30 ± 10	23 ± 5	94 ± 4	91 ± 6
0.33	7 ± 4	10 ± 5	8 ± 1	6 ± 1	21 ± 5	24 ± 7	105 ± 9	101 ± 7
1	8 ± 3	7 ± 3	3 ± 2	5 ± 2	20 ± 5	29 ± 6	106 ± 6	97 ± 4
Ratio	0.72	0.87	0.3	0.71	1.05	1.07	1.06	0.88
Positive control	SA	2-AA	191-A	2-AA	D-myc	2-AA	SA	2-AA
	446	91	250	141	505	1,101	282	1,307

Displayed are numbers of colonies growing after treatment with the test compound (data are ±SD) and the ratio of the number of colonies growing after treatment with the test compound relative to untreated bacteria.

191-A = ICR 191 acridine; 2-AA = 2-aminoanthracene; D-myc = daunomycin; SA = sodium azide; TA 98–1535 = strain number of *Salmonella typhimurium*.

We defined emergence in rats as recovery of etomidate- and ketamine-specific EEG measures to levels reflecting higher consciousness, reversal of blood pressure effects of the anesthetic agents, and recovery of the righting reflex and of coordination. We found calabadion 2 encapsulation of ketamine and etomidate on EEG measures of brain function to be consistent with higher levels of consciousness. Both ketamine and etomidate disrupt frontal–parietal communication, leading to unconsciousness.[32] However, their neurophysiologic mechanisms of action are quite different, likely accounting for their different EEG effects and requiring different techniques for EEG quantification. Deep etomidate anesthesia is characterized by alternating periods of EEG suppression and activity, referred to as a burst suppression pattern, similarly observed with most γ-aminobutyric acid (GABA) types of anesthetics.[33] As opposed to anteriorization, the shift in occipital α activity to frontal α coherence also characteristic for GABA anesthetics, which develops rather abruptly as a function of anesthetic infusion,[34,35] the BSR progressively and continuously increases with deeper levels of anesthesia, reflecting a decrease in cerebral metabolic rate coupled with the stabilizing properties of adenosine triphosphate–gated potassium channels.[36,37] Unlike etomidate, sedation with ketamine does not produce a pattern of burst suppression.[21,33] Instead, ascending levels of ketamine gradually increase EEG power likely due to inhibition of *N*-methyl-D-aspartate–mediated glutamatergic inputs to GABAergic interneurons, leading to aberrant excitatory activity in the cortex, hippocampus, and limbic system.[38] Therefore, we quantified electrographic depth of ketamine by measuring total EEG power.[21] Calabadion 2 both dose-dependently decreased periods of suppression (BSR) during deep etomidate anesthesia and total EEG power in ketamine-anesthetized rats, showing a reversal of these anesthetics' EEG effects.

Because lingering postanesthetic effects may be caused by residual anesthetic molecules, we hypothesized that drug encapsulation with calabadion 2 would mitigate postemergence motor impairment. Toward this end, we evaluated the effects of calabadion 2 on functional mobility with the balance beam test, which has previously been used as a predictor for pharmacologic impact on the recovery process.[25] The balance beam test is indicative of subtle deficits in motor skills due to age, central nervous system lesions, and pharmacologic manipulations with a higher sensitivity for coordination impairment than other motor tests.[39] One group of experiments was conducted in order to analyze the encapsulation and reversal ability of calabadion 2 (intraperitoneally) even when not administered by the same route as the anesthetic (ketamine intramuscularly). This could be of high clinical importance in emergency situations, when intravenous injection is not possible (*e.g.*, after recreational ketamine overdose).

Calabadion 2 dose-dependently also reversed the etomidate-induced decrease in MAP, indicating a reversal of anesthesia depth-associated effects on the cardiovascular system. We also observed an increase in MAP when reversing ketamine. As opposed to our BSR-monitored experiments under deep etomidate anesthesia, we titrated a shallow ketamine anesthesia to achieve abolishment of response to tail clamping. As a consequence of further lowering anesthetic levels when reversing with calabadion 2, we observed an increase in MAP, further indicating awakening due to reversal.

To ensure the awakening reaction was not caused by nonspecific effects of calabadion 2 on the animal's hemodynamics, we applied a phenylephrine infusion in three rats anesthetized with an equally titrated ketamine infusion. This did not affect any ketamine-induced EEG patterns, indicating no reversal.

We did not observe any changes in BSR, EEG, or MAP during and after the highest doses (80 mg kg^{-1} min^{-1}) of calabadion 2 given during steady-state isoflurane anesthesia and no differences in recovery time during propofol anesthesia. This enforces our hypothesis that the observed effects are

Table 2. Effects of Lethal Doses of Calabadion 2 on Rat Organs

Organ	Calabadion Dose in Which the Pathology Finding Was Present	Pathology Finding
Heart	All doses	Within normal limits
Lung	Animals receiving 3.1–5.1 g/kg	Mild cellular necrosis, some apparent fluid in alveolar spaces and enlarged, foamy pulmonary alveolar macrophages Occasional distension of pulmonary alveolar capillaries with erythrocytes, and in a few animals evidence of possible pulmonary emboli Macrophages, especially in connective tissue or adipose tissue that were distended with a bluish, hematoxylin-positive material that appeared to be localized in the lysosomes
Liver	All animals that received 3.1–5.1 g/kg of calabadion One animal that received 1.6 g/kg	Mild to moderate vacuolation that is consistent with mild fat accumulation
Kidney	Five of 6 animals that received the 2 highest doses Not in the animals that received 1.6 g/kg dose	A mild vacuolation of the epithelium in the P1 and P2 segments of the proximal convoluted tubule
Spleen	All doses	Within normal limits

Pathologic evaluation of heart, lungs, liver, kidneys, and spleen of 10 rats after dose escalation study to determine the maximal tolerated dose with intravenous injection of calabadion 2 up to 5.1 g/kg. Organs were fixated in 4% formaldehyde, stored in 70% ethanol, stained with hematoxylin and eosin, and embedded in paraffin slides.

P1 = segment 1 of the proximal tubule; P2 = segment 2 of the proximal tubule.

caused by specific encapsulation and inactivation of etomidate and ketamine molecules.

The chemical structure of calabadion 2 features a glycoluril tetramer unit, which enables the compound to bind to hydrophobic and cationic species, the aromatic sidewalls impart affinity due to π-π interactions toward targets that contain aromatic rings in their structures, and finally the overall cavity size of calabadion 2 endows it with selectivity based on size. The preference for calabadion 2 toward ketamine and etomidate relative to other molecules like isoflurane or propofol reflects the absence of one or more of the structural-binding determinates in the latter compounds. The affinity of calabadion 2 for compounds that are neutral in water (*e.g.*, propofol, isoflurane) is typically less than 0.1% of its affinity for related cationic compounds.

The design of this study allows the conclusion that reversal of etomidate and ketamine with calabadion 2 is due to specific binding. Both anesthetics bind to calabadion 2 *in vitro* and reverse the drugs *in vivo*. The similar reduction in time to recovery from LORR is the consequence of high dose of calabadion 2 given to etomidate compared to ketamine-anesthetized rats—based on the different duration of action at a constant rate of calabadion 2 infusion. The therapeutic range of ketamine is pretty low in rodents, so we could only apply relatively small doses without cardiovascular compromise in rats. In contrast, at the recommended dose of etomidate used, duration of action was longer, and more calabadion 2 could be titrated to accelerate recovery from LORR.

Single boluses of both etomidate and ketamine are used during procedures of short duration, such as electroconvulsive therapy, or for emergency intubations,[40] and ketamine is often used as the anesthetic of choice in pediatric patients for minor surgical procedures, as well as in the developing world, where it is frequently used by nonanesthetists when monitoring equipment is poor or absent.[41] Use of higher dosages are associated with longer hospitalization, and typical complications described when used in pediatric patients include emesis, vomiting, rash, as well as recovery agitation and transient airway complications (*e.g.*, postsedative airway misalignment, or apnea).[42] Maybe even more importantly, ketamine is frequently abused.[43] The ability to directly reverse their effects would not only result in reduced complication rates and time to discharge, and a decreased costs of care in patients receiving etomidate and ketamine in such circumstances, but also provide an antidote in cases of abuse-associated intoxication.

Maintenance of sedation with ketamine and etomidate has been achieved in humans at plasma concentrations of 2 to 3 and 0.3 to 0.6 μg/ml,[44] respectively, and plasma concentrations in humans awakening from ketamine and etomidate were described at 1 and 0.28 μg/ml (*vs.* 5 and 0.44 μg/ml in rodents), respectively.[45–48] Even though we estimate one fifth and half dosages of calabadion 2 needed in humans to achieve an equivalent reversal of ketamine and etomidate, respectively, we expect a calabadion 2 excess concentration of 5.1 to 10.2 and of 2.5 to 34.6 μmol/l to achieve the 50 to 67% anesthesia concentration reduction needed to reverse ketamine anesthesia and the 6 to 53% reduction needed to reverse etomidate anesthesia in humans. The relatively high concentrations of excess calabadion 2 raise the question of its interaction with other endogenous molecules.

Given that plasma concentrations in rodents at recovery from LORR are in the micromolar range, while adrenal suppression is already achieved in the nanomolar range, we do not expect calabadion 2 to reverse etomidate-induced adrenal suppression at the doses used in this study to accelerate emergence from anesthesia.

Calabadion 2 reverses ketamine and etomidate with a narrow therapeutic index of 16:1 and 3:1, respectively, mainly explained by calabadion 2's design to reverse the neuromuscular blocking agents rocuronium, vecuronium, and cisatracurium, which is achieved at about one tenth of the doses used here.[14] At doses sufficient to reverse neuromuscular blockade, calabadion 2 has minimal effects on anesthetic depth or duration. The current studies demonstrate a proof of principle of etomidate reversal, similar to the proof of principle earlier published on the effectiveness of calabadion 1 to reverse cisatracurium,[49] where subsequent medicinal chemistry optimization allowed us to create a similar compound with higher affinity now used for drug development. Of note, the ED_{50} of calabadion 2 to reverse ketamine of 166 mg/kg is only about twice as high as the dose used to reverse cisatracurium, which might make a clinical use of calabadion 2 for the reversal of ketamine possible. Considering that lower dosages will be required to reverse anesthesia in humans and that we plan to explore potential changes in chemical structure to increase the affinity, we do not expect the narrow therapeutic range in this study to be a limitation for the reversal of anesthesia by encapsulation of active anesthetic molecules.

We are currently developing calabadions to be used for specific indications: to reverse neuromuscular blocking agents, to reverse intoxications with stimulants of abuse (ketamine, cocaine), and to reverse unwarranted side effects of ketamine and etomidate administered in clinical medicine. Each of the above indications will require generation of dose–response relationships, in order to define indications and contraindications, and in order to avoid side effects from displacement.

In conclusion, calabadion 2 accelerates emergence from etomidate and ketamine anesthesia and reverses evoked unconsciousness as well as lingering effects of these anesthetics that impair motor coordination in rats by chemical encapsulation at nontoxic concentrations.

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Competing Interests

L.I. and M.E. hold an equity stake in Calabash Bioscience, Inc. (College Park, Maryland), which develops Calabadions for biomedical applications. L.I., G.K.H., V.B., and M.E. are inventors on patents (WO2012/051413 A1) on topics related to the use of calabadions in biomedical applications. The other authors declare no competing interests.

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