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

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Diaz-Gil, D., Mueller, N., Moreno-Duarte, I., Lin, H., Ayata, C., Cusin, C., Cotten, J. F., Eikermann, M. (2014). *Etomidate and Ketamine: Residual Motor and Adrenal Dysfunction that Persist beyond Recovery from Loss of Righting Reflex in Rats.* Pharmaceuticals (Basel) 8, 21-37.

Der experimentelle Teil dieser Arbeit wurde in den Zeitschriften „Pharmaceuticals (Basel)“ und „Anesthesiology“ publiziert. Der chemisch-experimentelle in vitro Anteil dieser Arbeit wurde durch das Team von PhD Lyle Isaacs, in Maryland durchgeführt. Die in vitro Toxikologie und pathologische Evaluation erfolgte durch das Team von Volker Briken, PhD, in Maryland. Principal Investigator der in vivo Versuche war Priv. Doz. Dr. Matthias Eikermann. Daniel Diaz Gil hat die in vivo Experimente unter Anleitung von Priv. Doz. Dr. Matthias Eikermann selbständig durchgeführt. Auf dem Boden der von Herrn Diaz Gil erhobenen in vivo Ergebnisse hat Herr Diaz Gil auch die von den Kooperationspartnern von Priv. Doz. Dr. Eikermann an den Universität in Maryland erhobenen Daten in seine Arbeit wissenschaftlich integriert. Die zum Thema publizierten Arbeiten wurden selbständig von Daniel Diaz Gil unter Anleitung von Priv. Doz. Dr. Matthias Eikermann verfasst.

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Introduction

General Anesthesia Recovery: an Unmet Medical Need

More than 21 million patients undergo general anesthesia in North America each year (Orser, 2008), and more than 18 million hours are collectively spent in recovery from anesthesia, generating a significant public health burden (Cullen et al., 2009). During this time patients are not only debilitated, but are also at risk for secondary injury related to residual impairment. All anesthetic regimens have powerful depressant effects on the central and peripheral nervous systems and it takes time for these systems to recover. An optimal anesthetic protocol would provide complete anesthesia, muscle relaxation and analgesia during the surgical procedure and permit complete recovery of consciousness, muscle strength and coordination at the end of the case. In practice, however, lingering effects of anesthetics and ancillary agents affect patients' safety and well-being. These effects delay recovery, necessitating prolonged monitoring in a dedicated postoperative anesthesia care unit (PACU), which increases the financial burden (estimated at over $4 billion per year) associated with the PACU (Habib et al., 2006; Simpson et al., 2013). Moreover, discharged patients may still be at risk of accidents due to impaired motor and cognitive functions (Whitlock et al., 2011). For example the American and Canadian Societies of Anesthesiologists recommend that patients do not drive, operate machines, or sign legal documents for at least 24 hours after any anesthetic is administered (Dobson et al., 2018). Accordingly, the development of methods to achieve faster emergence from general anesthesia and therefore alleviate lingering effects of the anesthetics outlined above, is viewed as an urgent unmet medical need (Avidan et al., 2014).

Ketamine and Etomidate

Currently used intravenous anesthetics such as ketamine and etomidate are clinically employed in a variety of settings. Ketamine is used to induce anesthesia (Haas et al., 1992), to achieve sedation and analgesia during mechanical ventilation, and to treat patients with chronic pain, or psychiatric problems including the estimated 10–30% of patients with major treatment resistant depression (Catena-Dell'Osso et al., 2013). Etomidate, a rapid acting and cardiovascular safe anesthetic, is frequently used in emergency cases (Godwin et al., 2005), for procedural sedation, and for anesthesia induction (Yang et al., 2015). Up to this point, these intravenous anesthetics have no mechanism of pharmacologic reversal.

Reversing Anesthesia

Attempts to achieve faster emergence from general anesthesia have been directed towards counteracting specific physiological sedating effects by stimulating opposing systems, for example by activating the arousal systems (McCarren et al., 2014). Although these sympathomimetic drugs may facilitate recovery of certain brain functions, the usefulness of stimulants may be limited by interpatient variability due to preexisting conditions and interaction with onboard drugs (e.g. antiepileptics), and most importantly, they do only reverse one component of anesthesia, as the anesthetic agents persist in the body. In addition, other researchers develop short-acting ketamine and etomidate derivates that achieve faster recovery (Harvey et al., 2015a; Harvey et al., 2015b; Pejo et al., 2012).

Calabadions

An exciting opportunity to overcome the limitations of reanimation by accomplishing an actual reduction of anesthetic agents has emerged with the characterization of the acyclic cucurbit[n]urils (CB[n]) molecular containers, which bind tightly and selectively to a variety

of cations (Cao et al., 2014; Liu et al., 2005). A particularly promising new subgroup of the acyclic cucurbit[n]urils is the Calabadions (Ma et al., 2012a; Shen et al., 2012). The development of narrow-spectrum high affinity macromolecular binders as antidotes has been focused mainly on neuromuscular blockers and anticoagulants (Forster et al., 2015), and previous studies (Haerter et al., 2015; Hoffmann et al., 2013; Staals et al., 2011a; Staals et al., 2011b) have demonstrated the effectiveness of molecular containers in scavenging excess neuromuscular blockers to speed post-surgical recovery from paralysis.

This research work is based on the idea that encapsulation of anesthetic agents may similarly speed recovery of function and is aimed to explore the potential use of calabadion 2 as a lead drug to inactivate effects of ketamine and etomidate, as well as to provide the proof of concept that acyclic cucurbit[n]urils 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, the risk of postoperative complications, and to counteract accidental overdose in both clinical and recreational settings (Diaz-Gil et al., 2016).

Hypotheses

The primary hypothesis of this study was that the effects of calabadion 2

(a) reverse ketamine and etomidate evoked unconsciousness and vegetative effects during a constant infusion of these two anesthetics,

(b) accelerate general emergence from etomidate and ketamine anesthesia,

(c) mitigate residual ketamine and etomidate induced functional mobility impairment after emergence, and

(d) reverse etomidate induced adrenal suppression.

Secondarily, it was hypothesized that this could be achieved at non-toxic doses of calabadion 2.

Materials and Methods

Chemistry

Calabadion 2 was synthesized according to the published procedure (Ma et al., 2012a). The binding constants (K_D) for the calabadion 2•ketamine and calabadion 2•etomidate complexes were determined by changes in ultraviolet-visible (UV/Vis) competition assays (Ma et al., 2012b), with the calabadion 2•Rhodamine 6G complex (K_a=2.3±0.2 x 10^6 M^{-1}), fitted to a competitive binding model as described previously (Ma et al., 2012a; Ma et al., 2012b; Shen et al., 2012).

To establish the 1:1 stoichiometry between calabadion 2 and ketamine Job's method of continuous variation was used (Huang, 1982). The total molar concentration of ketamine and calabadion 2 was maintained constant (1 mM), while their mole fractions were varied. The hydrogen-1 nuclear magnetic resonance (^{1}H NMR) (400 MHz, 20 mM sodium phosphate-buffered D_2O at pD = 7.4) 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 (93 adult male Sprague–Dawley rats, strain code 400; mean±SD, 315±78g) and mice (35 adult female *Swiss Webster* mice, strain code 551; mean±SD, 22.5±1.3g) were conducted in accordance with the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, MA (Protocol 2011N00181), and the Subcommittee on Research Animal Care at the University of Maryland, College Park, MD (Protocol R-14-02), respectively.

Instrumentation of Sprague-Dawley Rats

For the placement of intravenous (i.v.) lines animals were anesthetized with 1.5% isoflurane. Their body temperature was controlled rectally and maintained at 37±1 °C with a thermostat controlled heating pad. A total of 93 rats were used in this study, of which 32 were instrumented with two i.v. lines, an arterial line and a tracheal tube. Of the remaining 61 animals, 54 rats were only instrumented with a tail vein i.v. catheter (24 gauge 19mm), 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 electroencephalography (EEG)-electrode in 26 chronically instrumented rats (Diaz-Gil et al., 2016). Methods described by Vijn and Sneyd and by Cotten et al. 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 10s-epoch - for the evaluation of reversal of etomidate-evoked unconsciousness (Cotten et al., 2011; Vijn et al., 1998). To measure EEG signals the animals were placed in a stereotactic frame fitted with a nose cone, the skull was exposed and the periosteum removed. An epidural electrode was inserted 6 mm anterior of the lambda and 3 mm lateral of the midline to achieve temporal differentiation for the BSR measurements. This enhances higher frequency components while attenuating lower frequency components in the EEG signal, which eliminates the slow baseline drifts and delta activity while amplifying transient bursting activity, and therefore enhances sensitivity of BSR extraction (Cotten et al., 2011; Vijn et al., 1998). Time differentiation was performed digitally by taking the differences between two successive samples in the digitalized EEG, with the upper limit of burst suppresion detection (BSR=100%) equal to a 10s period without bursts.

Suppression was defined as an interval during which the time-differentiated EEG signal amplitude stays within an optimized suppression voltage window for at least 100 ms (Cotten et al., 2011). The suppression voltage window for each individual animal was empirically optimized by inducing brief EEG electrical silence with inhaled isoflurane, 4–5% delivered in 100% oxygen at 2 l/min from a calibrated agent-specific vaporizer into tracheostomy tube (Cotten et al., 2011). Consecutively the voltage window was reduced to the lowest value that produced a BSR measurement of more than 97% (typical value, approximately ± 0.5V) and the BSR was equilibrated at 1% isoflurane (Fig. 1 and 2).

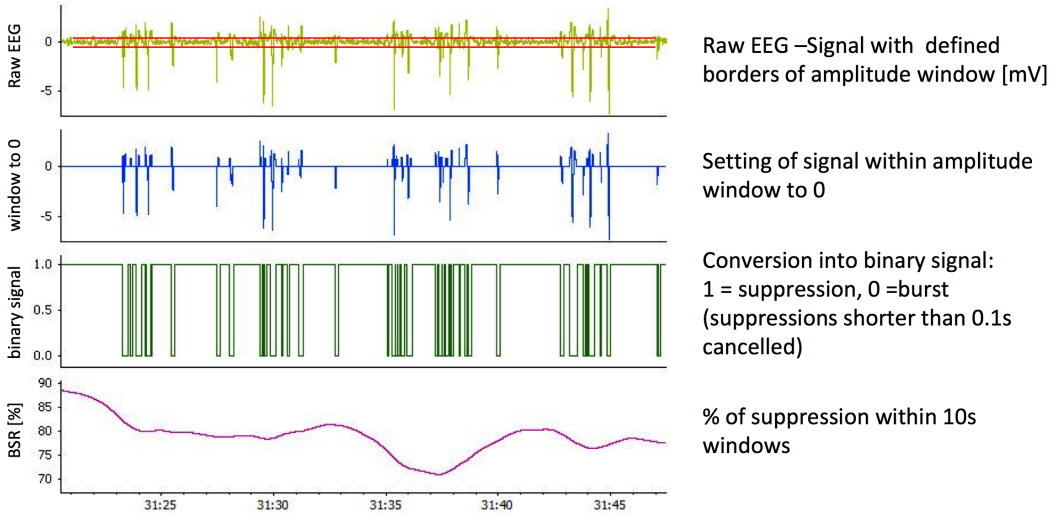


Figure 1. *Computerization of burst suppression ratio (BSR) modified after Vijn and Sneyd, BJA 1998 and Cotten el al., Anesthesiology 2011.*

Considering that, if successfully reversed etomidate would not consistently provide an adequate depth of anesthesia, all studies were performed in a background of inhaled isoflurane 1% to ensure an appropriate anesthesia of the instrumented animals during the experiment (Cotten et al., 2011).

After an initial bolus administered over 40s to achieve a BSR of approximately 70% (Fig. 2), the infusion rate was decreased to a value between 100 and 300 µg/kg/min (average dose of 183.9±28.4 µg/kg/min, mean±SD) to derive at a steady state BSR higher than 40% for at least 20 minutes before test drug administration. Animals were pre-medicated with 5mg/kg dexamethasone to avoid symptoms of etomidate-induced adrenal suppression.

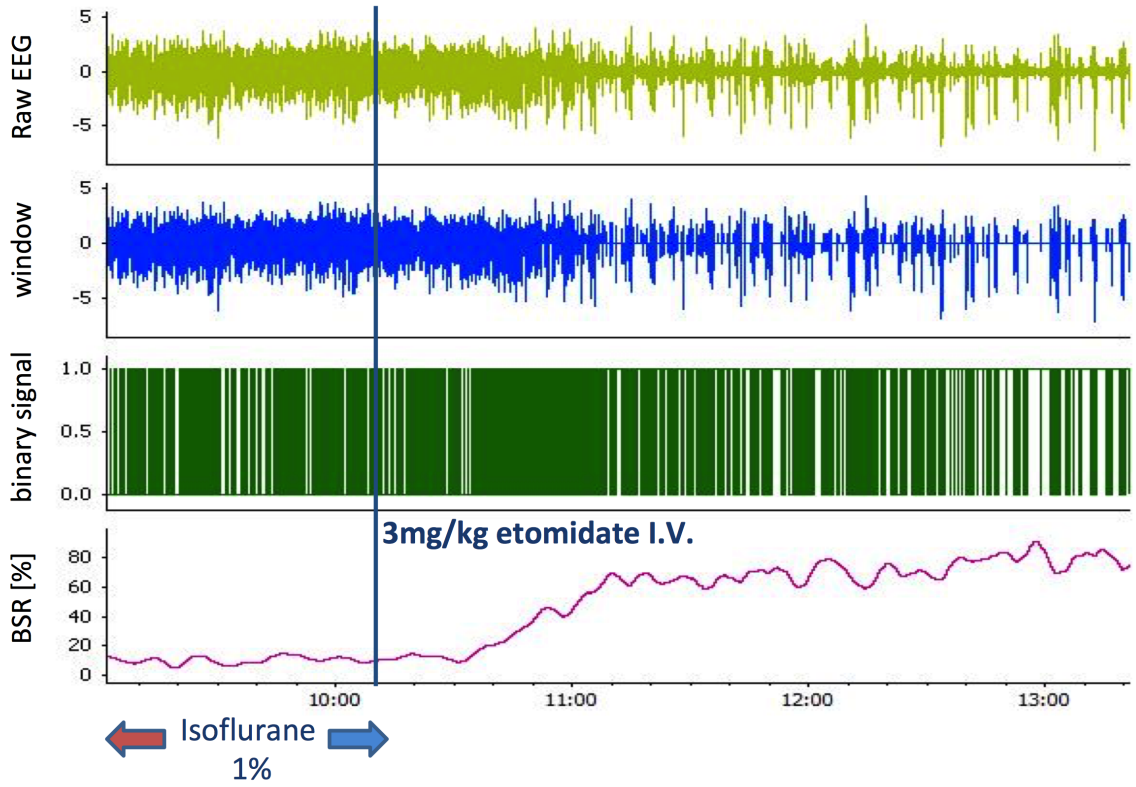


Figure 2. *Example of the changes in burst suppression ratio (BSR) after administration of 3mg/kg etomidate i.v. during 1% Isoflurane steady state.*

After steady state recordings either a stepwise increasing calabadion 2 infusion of 40, 60, 80 and 100 mg/kg/min 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. These animals were also placed in a stereotactic frame fitted with a nose cone, the skull was exposed and the periosteum removed. An epidural electrode 3 mm posterior to the bregma and approximately 1 mm lateral to the midline was inserted according to previous experiments performed in Dr. Eikermann's laboratory (Eikermann et al., 2012). After all surgical procedures were completed the dose of

isoflurane was stepwise reduced and discontinued while a 0.67 mg/kg/min ketamine infusion was started. Since ketamine concentrations at recovery of consciousness are still high enough to ensure analgesia, no baseline isoflurane was used during the ketamine experiments. After 10 min of a sole ketamine infusion, intermittent standardized tail-clamping (25 N) was applied every 2 min to identify depth of anesthesia. Depending on response, the infusion rate was increased or decreased by 0.33 mg/kg/min, until no response to 6 consecutive tail clamps during a constant dose of ketamine was observed (Eikermann et al., 2012).

After steady state recordings, calabadion 2 was infused with 20, 40, 60 and 80 mg/kg/min doses over a period of 5 min each with 40 sec 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 bandpass 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 1024 bit hann (cosine-bell) window, and the average EEG spectral power for the following frequency bands were calculated for every minute: total power 1-40 Hz, delta 1-4 Hz, theta 4-8 Hz, alpha 8-12 Hz, beta 2-25 Hz, high beta 25-30 Hz and gamma 30-40 Hz. Changes in total EEG power and MAP were quantified in response to the 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, increasing amounts of calabadion 2 (20, 40, 60 and 80mg/kg/min for 5min each) were administered in 3 rats anesthetized with a constant isoflurane anesthesia titrated to the abolishment of tail-clamping. In these rats, EEG power, mean arterial blood pressure and heart rate were quantified.

To ensure that the changes in EEG can be interpreted as a result of shallower anesthesia, rather than nonspecific hemodynamic reactions, additionally an escalating

phenylephrine infusion (4 to 10µg/kg/min) was administered in 3 rats anesthetized with a continuous ketamine infusion.

Effects of Calabadion 2 on Time to Regain Righting Reflex Following Single Bolus Anesthesia

The effects of calabadion 2 on the time to recovery from loss of righting reflex (LORR) were examined following a single i.v. bolus of etomidate or ketamine in 14 adult male *Sprague-Dawley* rats. After instrumentation, the animals were randomized to receive either an i.v. etomidate bolus (4 mg/kg) over 10 sec or a one minute infusion of ketamine (30 mg/kg). Once placed in the supine position, the animals were randomized to receive either an i.v. infusion of calabadion 2 (80 mg/kg/min dissolved in distilled water [H_2O]) or saline, beginning 3 min following the anesthetic injection. Recovery from LORR was taken as the moment when the rat regained a standing or sternally recumbent position (Franks, 2008).

Additionally, the effect of calabadion 2 on propofol anesthesia was tested in crossover experiments in 5 adult *Sprague-Dawley* rats randomized to receive an i.v. infusion of calabadion 2 (80 mg/kg/min dissolved in distilled H_2O) or saline beginning 3 min following the i.v. 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

Recovery of functional mobility impairment was quantified using the balance beam test, a common method to assess motor coordination and balance of animals (Combs et al., 1987; Diaz-Gil et al., 2016; Diaz-Gil et al., 2014), used as a predictor for pharmacologic impact on recovery (Goldstein et al., 1990). 14 adult male *Sprague-Dawley* rats randomized to receive either a single bolus of 4 mg/kg etomidate or 30 mg/kg ketamine i.v. followed by a continuous i.v. infusion of Calabadion 2 (80 mg/kg/min) or saline titrated

to recovery from LORR, and 7 rats receiving a single injection of 50 mg/kg ketamine intramuscular (i.m.) immediately followed by an intraperitoneal (i.p.) injection of 1000 mg/kg Calabadion 2 (3 ml) or saline were tested in a randomized crossover fashion. Following recovery from LORR, the animals were encouraged to cross over a narrow wooden beam (80 cm x 2.5 cm x 2.5 cm) into a darkened goal box at the opposite end, 60 cm over the ground (Chen et al., 2001; Dixon et al., 2003). As described by Dixon et al. noxious stimuli (bright light and high decibel white noise) were applied and terminated once the rat did not continue on the beam or entered the box. The animals were tested 12 times for a maximum of 60 sec (Dixon et al., 2003). All animals were previously trained in 3 independent training sessions one day before the first testing day and baseline measurements were conducted on the day of first testing. A Sony DCR-SX85 Handycam Camcorder recorded all testing. 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 i.v. anesthetic agent injection, or every 10 min starting at the i.m. 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 sec (1 point), 11 to 20 sec (2 points), or 21 to 30 sec or reaches support (3 points)(Combs et al., 1987)

Effects of Calabadion 2 on Adrenocortical Hormone Production after Etomidate Anesthesia

To evaluate the effects of calabadion 2 on etomidate-induced adrenal suppression, 18 rats were tested based on a protocol by Cotten et al. (etomidate + calabadion 2, n=6 vs. etomidate + placebo, n=6 vs. placebo + placebo, n=6) (Cotten et al., 2011). Each rat was given dexamethasone (0.2 mg/kg i.v.) at the beginning of each experiment to suppress the physiological stimulation of the adrenocortical hormone production by adrenocorticotropic hormone 1-24 (ACTH1-24). During the subsequent 2 h, the rats were allowed to rest in their cages, before they were re-anesthetized with isoflurane, as

previously described (Diaz-Gil et al., 2014). Before starting the experimental protocol, a blood sample was drawn to determine the un-stimulated baseline plasma corticosterone concentration. Subsequently, a second dose of dexamethasone was administered to keep the physiological ACTH production of the animal suppressed. High dose etomidate (4 mg/kg) or placebo was then administered as a bolus followed by a continuous Calabadion 2 infusion of 80 mg/kg/min or an equivalent volume of saline for 20 minutes. Adrenocortical function (i.e. responsiveness to ACTH1-24 administration) was assessed by repetitive administration of ACTH1-24 (25 µg/kg i.v.) and by measuring plasma corticosterone concentrations 30 min later. The first dose of ACTH1-24 was given directly before the test drug administration, and the plasma corticosterone concentration was measured 30 min later (blood sample 1 in figure 3). Immediately after drawing the blood sample 1, a second dose of ACTH1-24 was given and the plasma corticosterone concentration was measured 30 min later (blood sample 2 in Fig. 3). The same procedure was repeated a third time in order to obtain blood sample 3 (Fig. 3).

The volume of each blood sample was approximately 0.3 ml. The plasma corticosterone concentrations were determined as follows and previously described (Cotten et al., 2009; Diaz-Gil et al., 2014): Blood samples were centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant plasma was collected. The samples were frozen (-80°C) pending corticosterone measurement. After thawing and heat inactivation of corticosterone binding globulins (65°C for 20 min), and addition of 2.8 µmol/ml P-Xylenediamine (PDXA) to each sample in order to fully displace any molecules bound to calabadion 2, plasma baseline and ACTH1-24-stimulated corticosterone concentrations were quantified using and enzyme-linked immunosorbent assay (Cayman Chemicals, Ann Arbor, MI) and a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

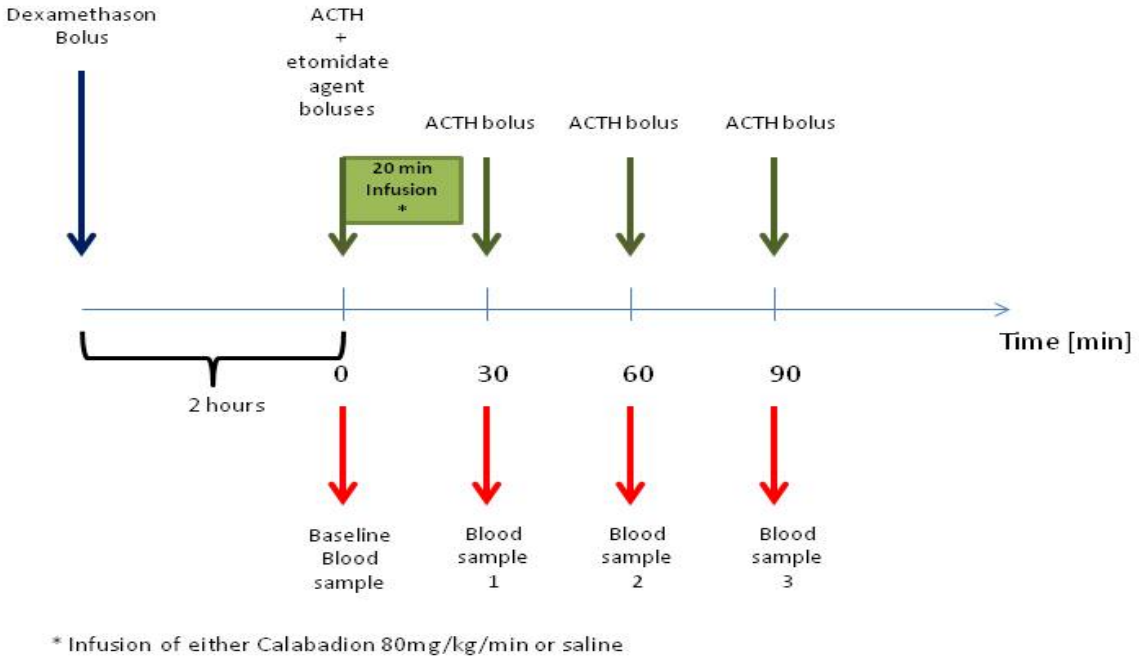


Figure 3. *Experiment flow chart to assess effects of Calabadion 2 on etomidate-induced adrenal suppression. Two hours following a dexamethasone bolus administration (0.2 mg/kg), a baseline blood sample was drawn prior to the administration of the first dose of ACTH 1-24 (25 µg/kg). Then a bolus of high dose etomidate (4 mg/kg) or saline was administered in a parallel group design followed by a 20 min infusion of either Calabadion 2 (80mg/kg/min) or saline. After 30-minute intervals, two further doses of ACTH were administered and blood samples were taken.*

Toxicology

The potential toxic effects of calabadion 2 were analyzed on human white blood cells (THP-1), liver cells (HepG2) and kidney cells (HEK293).

The cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) based assay (CellTiter 96® AQueous Kit assay from Promega G3580) and cell necrosis was determined via the quantification of the release of the cytosolic adenylate kinase enzyme (Toxilight® BioAssay from Lonza LT07-117). These cells were exposed to 0.16 mM, 0.4 mM, 1 mM, and 2.5 mM calabadion 2, hydroxypropyl-β-cyclodextrin (HP-β-CD) or erythromycin, as a point of comparison for a US Food and Drug Administration (FDA) 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 the ether-à-go-go-Related Gene (hERG) currents, a Chinese Hamster Ovary cell line transfected with the hERG ion channel was used. 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, a bacteria reverse mutation assay was used (Ames Test MOLTOX® 31-100.2, histidine auxotroph strains of S.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 1.6-1.9 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 the treatment of bacteria. Four different bacterial test strains were used to assess the mutagenicity of the compound (TA1535, TA 1537, TA 98 and TA 100). Calabadion 2 was added with a dose of 0.012, 0.037, 0.11, 0.33 or 1 mg per plate with an addition of 1.5 µg Sodium Azide, 6 µg Daunomycin, 1 µg CR 191 Acridine or 10 µg 2-Aminoanthracene per plate as control.

Additionally, the toxicity of calabadion 2 was analyzed in 35 Swiss Webster mice by performing a dose escalation study. Groups (n=7) of 4-6 week old female mice were injected i.p. daily with 29 mg/kg, 87 mg/kg, 145 mg/kg 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.

Further, the toxicity of calabadion 2 was analyzed 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 i.v. injection for 5 consecutive days until the lethal

dose was reached (100, 500, 1000, 1500 and 2000 mg/kg). In 4 additional rats a nonlethal cumulative dose of 1.6 g/kg was administered on 3 consecutive days (100, 500 and 1000 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}) the therapeutic index of calabadion 2 in reversing etomidate and ketamine anesthesia was calculated.

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 (H&E), 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 ± standard deviation (SD) unless otherwise specified. Statistical analysis was performed using SPSS 22.0 (SPSS Inc. Chicago, IL) and GraphPad Prism 6.0 (GraphPad Software, Inc. LaJolla, CA). Descriptive analytics and visual inspection of the distribution including histogram, density plots, quantile-quantile (Q-Q) plots were applied. Normality was tested for using the Shapiro-Wilk test and data was considered normally distributed when $P \geq 0.05$.

To assess the effects of calabadion 2 on EEG/BSR during a continuous infusion of etomidate or ketamine a mixed linear model with an identity link function for normally distributed probability was used. 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 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.

A paired t-test was used to assess the differences in time to recovery 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 post-anesthetic etomidate and ketamine induced balance- and coordination-impairment, a mixed linear model with an identity link function for normally distributed probability was used. It was 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 et al.)(Combs et al., 1987), while allowing a subject specific intercept to vary as random effects.

In order to assess the effects of calabadion 2 on etomidate-induced adrenal suppression, a mixed linear model with an identity link function for normally distributed probability was used. Our mixed model included main effects of reversal agent (calabadion 2 vs. placebo) and anesthetic agent (etomidate vs. placebo) as fixed effects while allowing intercepts to vary (random intercepts model).

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

Inhibition of the hERG channel was analyzed using GraphPad Prism 6 to calculate statistical significance and half maximal inhibitory concentration (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 standard deviation of residuals (sy.x). Medium convergence criteria were used. That is, regression concluded when five consecutive iterations altered the sum-of-squares by <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 was plotted as the average change in weight for each group plus or minus one standard deviation. A student's unpaired t test was performed to compare each dosage group to the untreated mice.

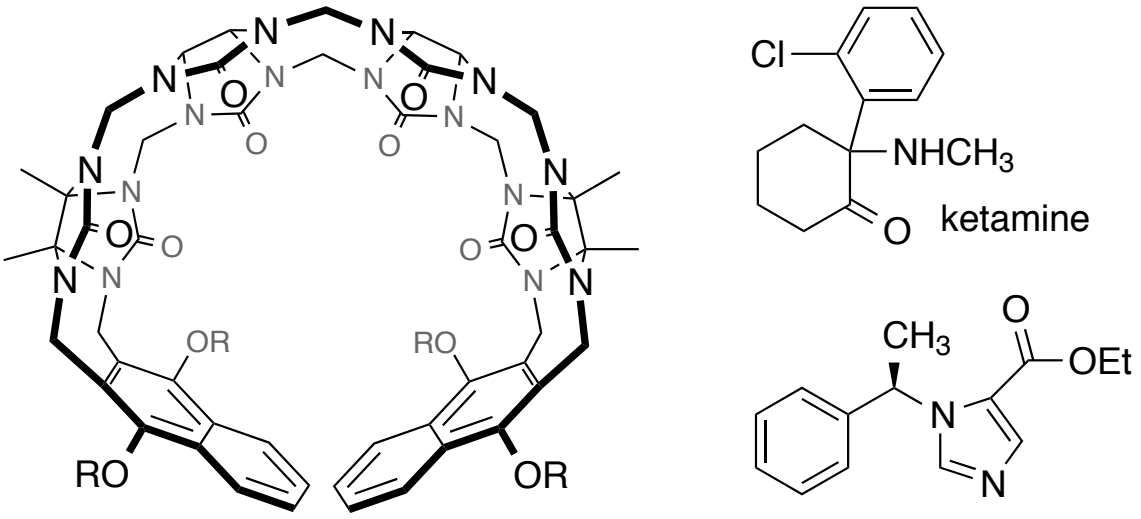
A P-Value < 0.05 was considered significant.

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 μM and 27.2 ± 5.0 μM, respectively (Fig. 4, 5 and 6).

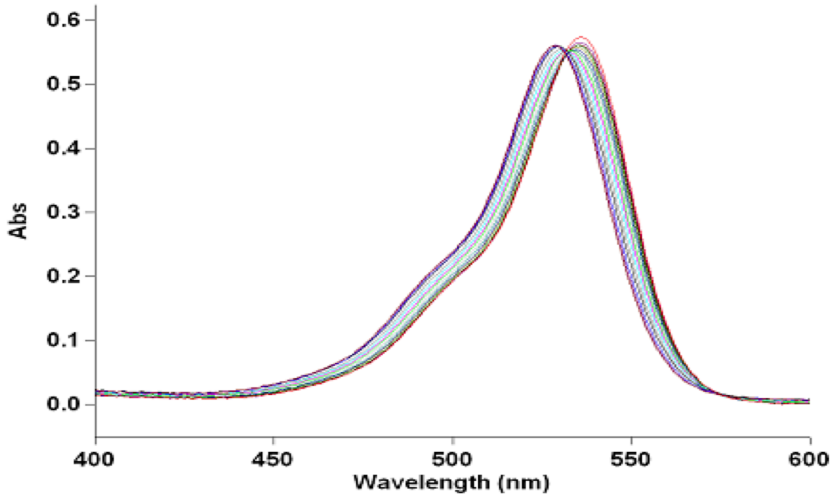
The Job plots for the calabadion 2•ketamine and calabadion 2•etomidate complexes, showed maxima at mole fractions of 0.5, which establishes the 1:1 nature of the calabadion 2•drug complexes (Fig. 7 and 8).



Calabadion 2: R = $(CH_2)_3SO_3Na$
Molecular Formula: $C_{62}H_{68}N_{16}Na_4O_{24}S_4$
Molecular Weight: 1641.51

Figure 4. *Chemical structures of calabadion 2, ketamine, and etomidate.*

a)



b)

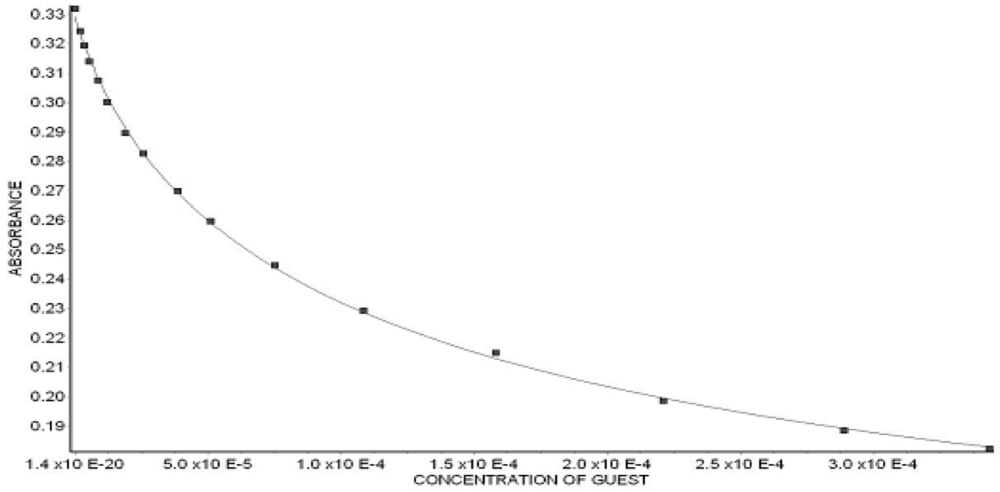
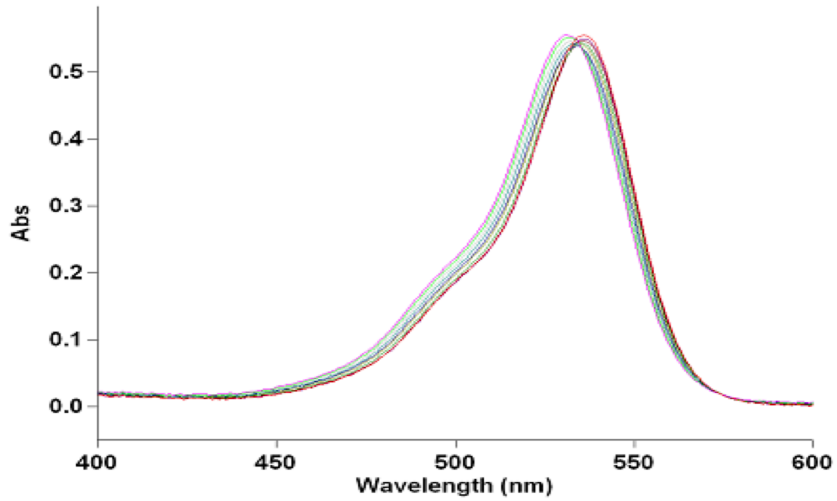


Figure 5. *Calabadion 2 binds to ketamine. a) UV/Vis spectra from the titration of calabadion 2 (9.18 µM) and rhodamine 6G (10.00 µM) with ketamine (0 – 0.35 mM) in 20 mM NaH_2PO_4 buffer (pH = 7.4); b) plot of the absorbance at a wavelength of 550 nm as a function of the concentration of ketamine. The solid line represents the best non-linear fit of the data to a competitive binding model (K_a = (1.8 ± 0.1) x 10^5 M^{-1}).*

a)



b)

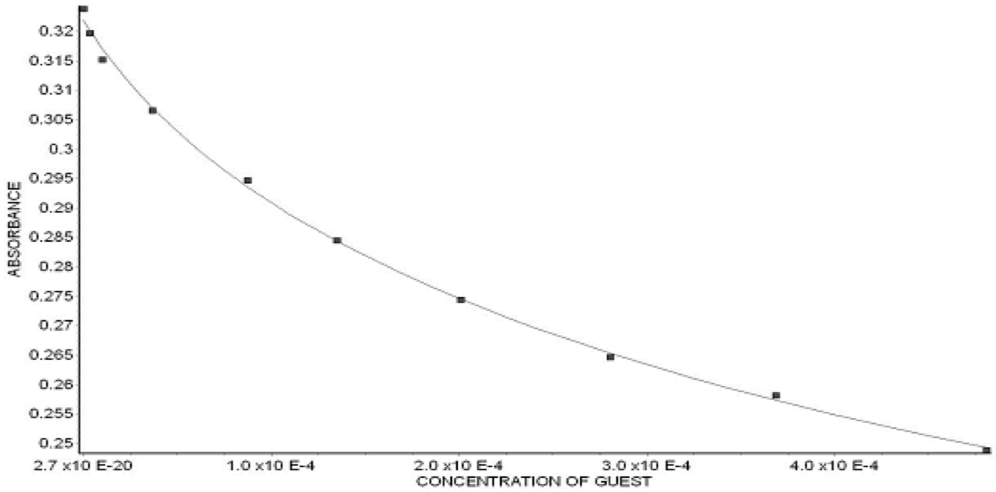


Figure 6. *Calabadion 2 binds to etomidate. a) UV/Vis spectra from the titration of Calabadion 2 (9.18 µM) and rhodamine 6G (10.00 µM) with etomidate (0 – 0.5 mM) in 20 mM NaH_2PO_4 buffer (pH = 7.4); b) plot of the absorbance at a wavelength of 550 nm as a function of the concentration of etomidate. The solid line represents the best non-linear fit of the data to a competitive binding model (K_a = (3.5 ± 0.5) x 10^4 M^{-1}).*

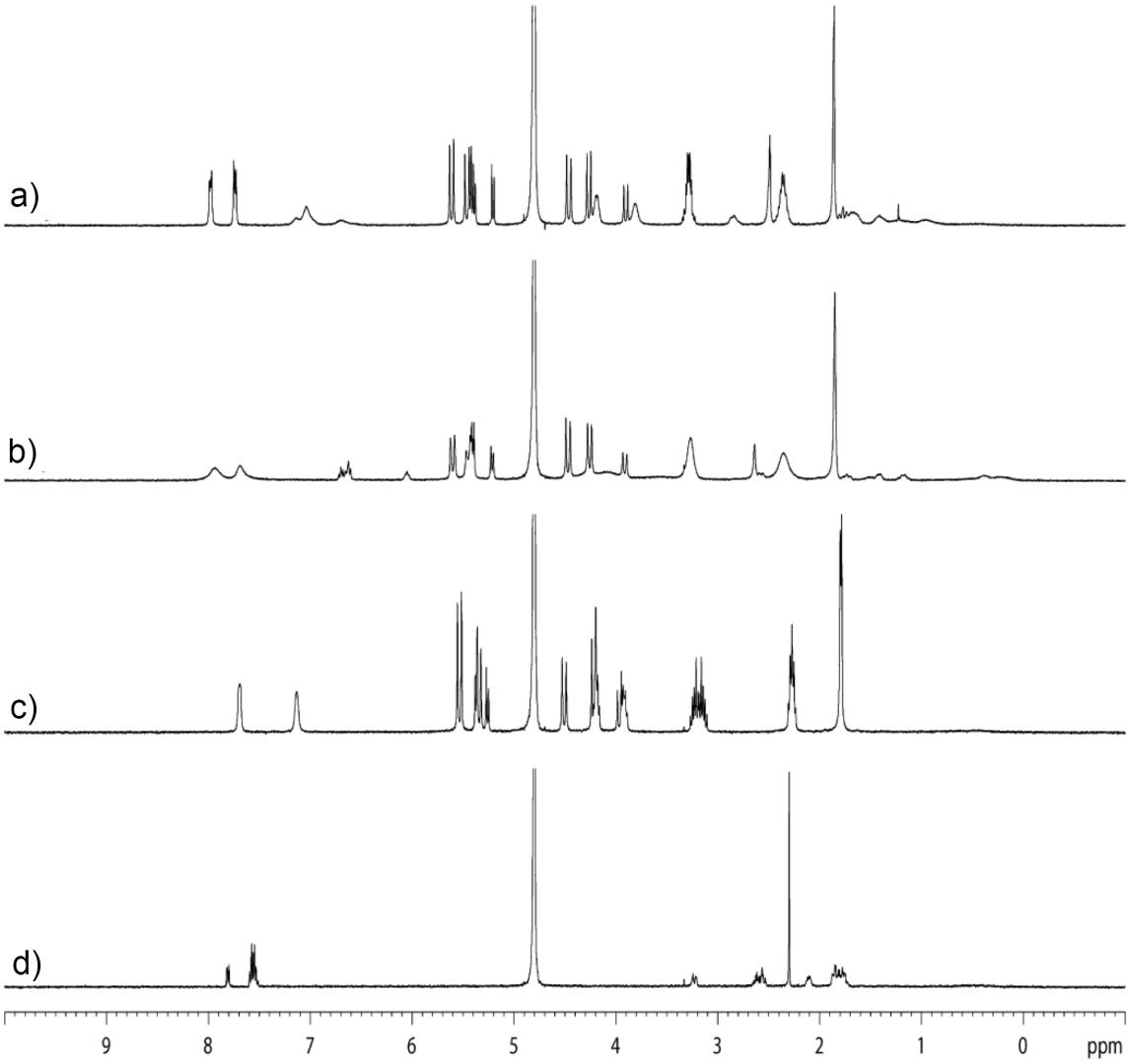


Figure 7. *Stoichiometry establishment of Calabadion 2 and ketamine.* ^{1}*H NMR spectra recorded (400 MHz, RT, deuterated sodium phosphate buffer at pD = 7.4) for a) a 2:1 mixture of ketamine (2 mM) and Calabadion 2 (1 mM), b) an equimolar mixture of ketamine and Calabadion 2 (2 mM), c) Calabadion 2 and d) ketamine.*

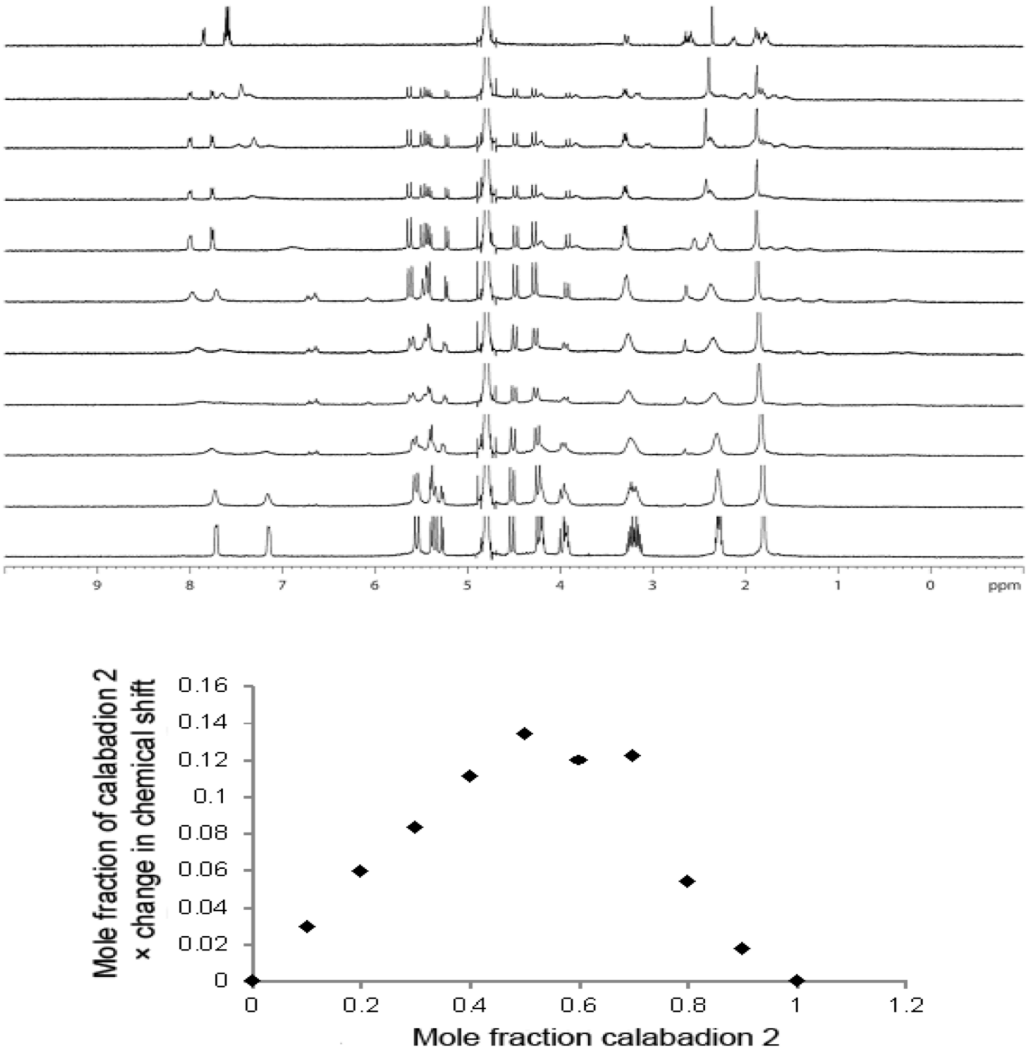


Figure 8. *Calabadion 2 and ketamine bind 1:1. Job plot establishing 1:1 binding of calabadion 2 (0-1 mM) with ketamine (0-1 mM) based on change in chemical shift at 7.73 ppm of calabadion 2 measured by* ^{1}*H NMR (400 MHz, deuterated sodium phosphate buffer at pD = 7.4)*

Calabadion 2 Reverses Electrographic Metrics of Unconsciousness during Constant Anesthetic Infusion

During anesthesia with etomidate the electroencephalogram (EEG) is characterized by periods of suppression alternating with periods of high amplitude activity, referred to as a burst-suppression pattern (Miller, 2010). Deepening anesthesia is marked by lengthening of suppression periods and this can be quantified as the burst-suppression ratio (BSR), the percentage of time the EEG is silent (Brown et al., 2010). For ethical reasons the full reversal of anesthesia in these acutely instrumented animals was not aimed for. An

average dose of 183.9±28.4 µg/kg/min was used to maintain the BSR at a stable rate of 63% [95% confidence interval (CI), 62-65%], 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, 24-51%] (reversal agent*dose, P=0.001, Fig. 9A; n=10; likelihood ratio test (LRT) P<0.001; table 1A), while the mean arterial blood pressure (MAP) returned from 83% [95% CI, 80-86%] to 101% [95% CI, 96-105%] of pre-etomidate baseline (reversal agent*dose P=0.033, Fig. 9A; 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. No significant changes in BSR or MAP were observed during saline infusion.

Unlike etomidate, anesthetic doses of ketamine do not produce a pattern of burst suppression in rats or humans (Eikermann et al., 2012; Rosen et al., 1976). Instead, ascending levels of ketamine gradually increase EEG power, probably due to inhibition of N-Methyl-D-aspartic acid (NMDA)-mediated glutamatergic inputs to gamma-aminobutyric acid(GABA)-ergic interneurons, leading to aberrant excitatory activity in the cortex, the hippocampus and the limbic system (Nelson et al., 2002; Olney et al., 1999; Seamans, 2008). During continuous ketamine infusion titrated to abolish responses to a noxious stimulus (tail clamping, average dose of 122 µg/kg/min), calabadion 2 induced a dose-dependent decrease in total EEG power to 63% [95% CI, 54-72%] of steady-state-ketamine EEG-power, indicating that calabadion 2 reversed the typical effects of ketamine on the EEG (reversal agent*dose P<0.001, Fig. 9B; n=10; LRT P<0.001, table 1B). During both calabadion 2 (n=10) and saline (n=3), all frequency bands behaved very similarly, without significant differences between individual bandwidths (Fig. 10). In parallel, calabadion 2 injection resulted in a dose dependent increase in MAP to almost 130% [95% CI, 117-142%] compared to pre-ketamine baseline (96 mmHg) at the highest dose (n=10), also indicating reversal of anesthesia (reversal agent*dose P<0.001, Fig. 9B; n=10; LRT P<0.001).

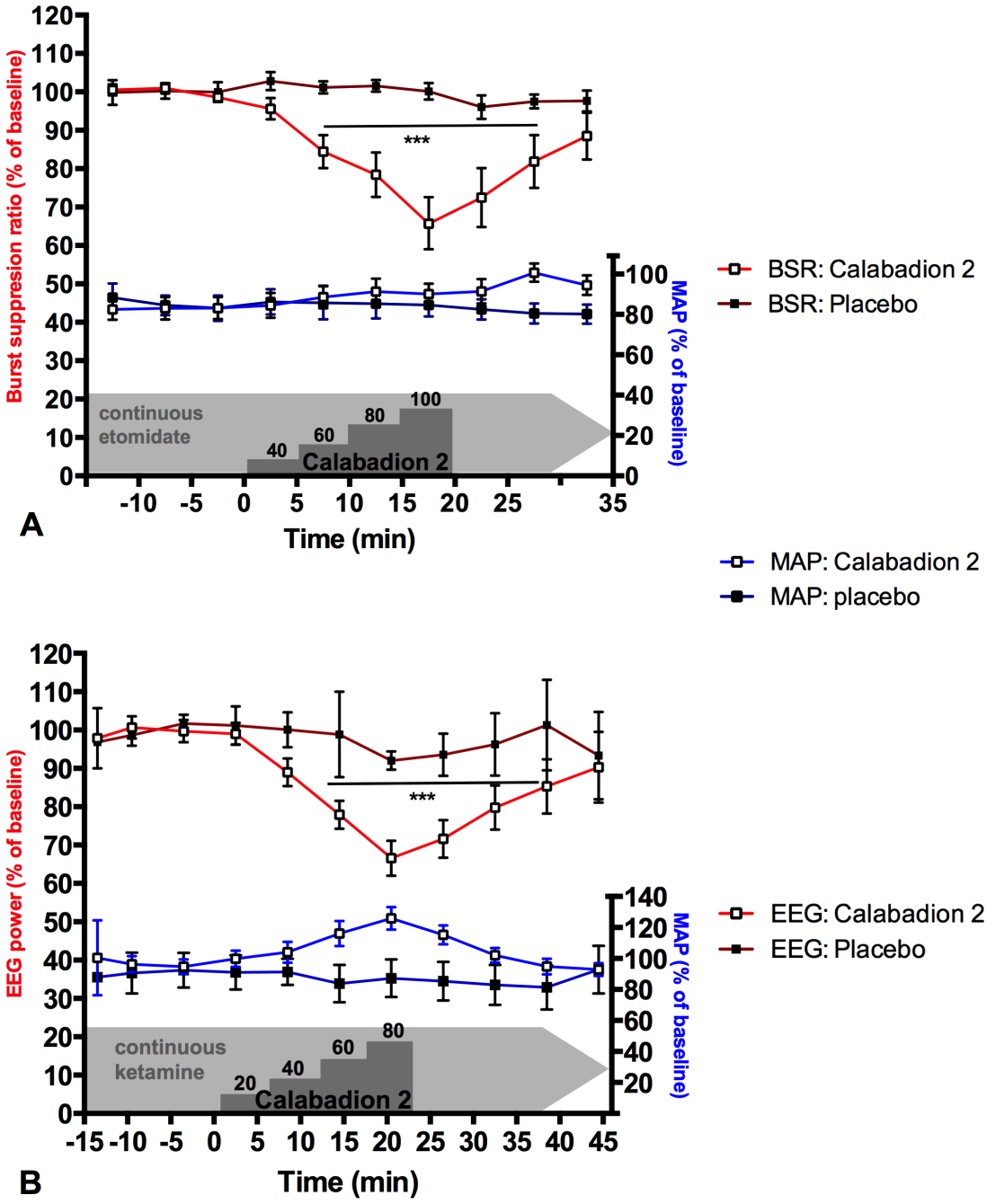


Figure 9. *Calabadion 2 decreases levels of unconsciousness during continuous administered anesthesia with etomidate and ketamine. Effect of an escalating calabadion 2 intravenous (i.v.) infusion on* ***(A)*** *burst suppression ratio (BSR) and mean arterial blood pressure (MAP) during continuous etomidate i.v. infusion (titrated to an average dose of 184 µg/kg/min; n=13) and on* ***(B)*** *electroencephalographic power (EEG-power) and mean arterial blood pressure (MAP) during continuous ketamine infusion (titrated to an average dose of 122 µg/kg/min; 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 % values from baseline (average value during continuous etomidate/ketamine infusion before test drug infusion). MAP is displayed as % of mean MAP before start of etomidate/ketamine administration. Error bars represent the 95% confidence intervals.*

Table 1. *Effect size of Fixed Effects – Calabadion 2 during continuous anesthesia*

A) *Effect of treatment and dose on BSR during continuous etomidate anesthesia*

Parameter	Effect size compared to baseline	P-Value	95% Confidence Interval	
			Lower Bound	Upper Bound
Calabadion	-34.44	0.001	-51.32	-17.56
Placebo	0			
Dose, 0 mg / V_{eq} Placebo	-0.20	0.956	-7.32	6.92
Dose, 40 mg/ V_{eq} Placebo	2.69	0.458	-4.43	9.81
Dose, 60 mg/ V_{eq} Placebo	1.04	0.774	-6.08	8.16
Dose, 80 mg/ V_{eq} Placebo	1.45	0.690	-5.67	8.57
Dose, 100 mg/ V_{eq} Placebo	0			
reversal agent * dose				
Calabadion 2, 0 mg	33.06	<0.001	24.93	41.19
Calabadion 2, 40 mg	27.25	<0.001	19.12	35.38
Calabadion 2, 60 mg	17.73	<0.001	9.60	25.85
Calabadion 2, 80 mg	11.30	0.007	3.17	19.42
Calabadion 2, 100 mg	0			

BSR=Burst suppression ratio, Veq= equivalent Volume

B) *Effect of treatment and dose on EEG power during continuous ketamine anesthesia*

Parameter	Effect size compared to baseline	P-Value	95% Confidence Interval	
			Lower Bound	Upper Bound
Calabadion	-25.47	<0.001	-35.26	-15.69
Placebo	0			
Dose, 0 mg / V_{eq} Placebo	9.64	0.007	2.72	16.56
Dose, 20 mg/ V_{eq} Placebo	9.17	0.010	2.25	16.09
Dose, 40 mg/ V_{eq} Placebo	8.05	0.023	1.13	14.97
Dose, 60 mg/ V_{eq} Placebo	6.83	0.053	-0.09	13.75
Dose, 80 mg/ V_{eq} Placebo	0			
reversal agent * dose				
Calabadion 2, 0 mg	23.51	<0.001	15.63	31.40
Calabadion 2, 40 mg	23.30	<0.001	15.42	31.19
Calabadion 2, 60 mg	14.37	<0.001	6.49	22.26
Calabadion 2, 80 mg	4.53	0.258	-3.35	12.42
Calabadion 2, 100 mg	0			

EEG=electroencephalogram, Veq= equivalent Volume

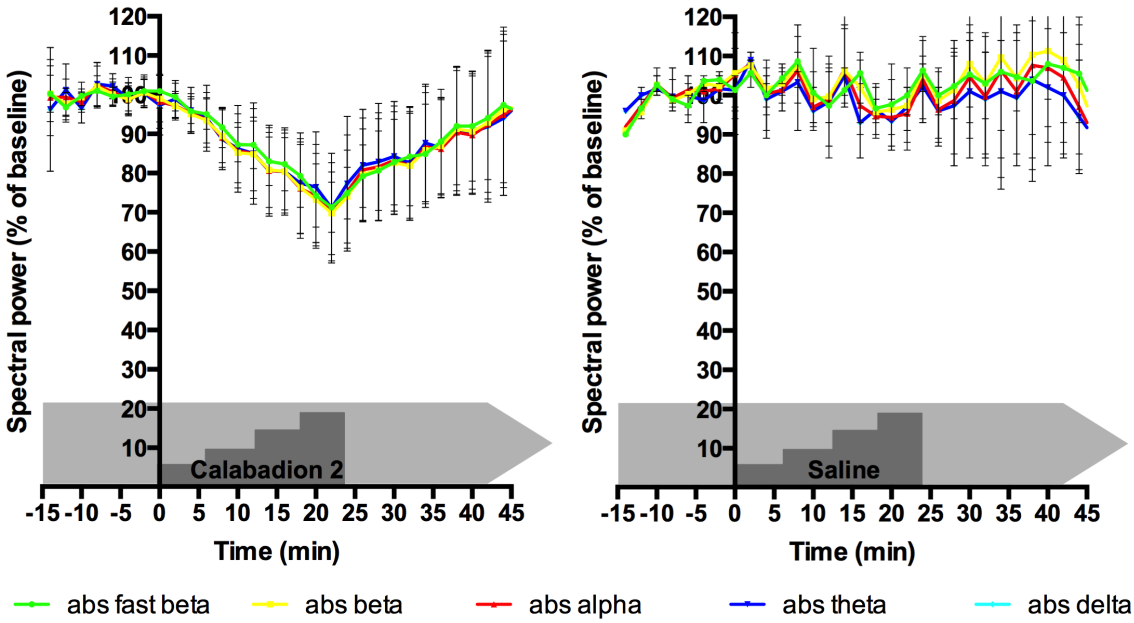


Figure 10. *Calabadion 2 effects on electroencephalographic frequency bands during continuous administered anesthesia with ketamine. During both calabadion 2 (n=10) and saline (n=3), all frequency bands (delta, 1–4 Hz; theta, 4–8 Hz; alpha, 8–12 Hz; beta, 12–25 Hz; and fast beta, 25–30 Hz) behaved very similarly, without significant differences between individual bandwidths. Error bars represent the 95% confidence intervals. (Abs=absolute)*

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 the saline infusion (Fig. 9A+B).

In contrast, a continuous phenylephrine infusion during steady state shallow ketamine anesthesia resulted in significant MAP increases without effects on EEG power (n=3, P=0.024). No effects of calabadion 2 were observed on EEG-power, BSR and MAP during and after the highest dose of the stepwise increasing calabadion 2 infusions 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 (Chemali et al., 2012; Solt et al., 2011). Anesthetic blood concentrations at recovery from LORR highly correlate with those at recovery of consciousness in humans, making it an useful

metric for recovery in animal models (Franks, 2008). Relative to saline, calabadion 2 significantly decreased the time to recovery from LORR by almost 50% in etomidate-anesthetized rats (15.2±1.4 min vs. 26.9±2.3 min, n=7, P<0.001, Fig. 11) and by about 30% in ketamine anesthetized rats (6.0±0.7 min vs. 8.4±1.6 min, n=7, P=0.023, Fig. 11). 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 976-991mg/kg] and 167 mg/kg [95%CI 161-173 mg/kg] for the reversal of a 4 mg/kg i.v. etomidate bolus and a 30 mg/kg i.v. bolus of ketamine, respectively.

Calabadion 2 did not affect the time to recovery from LORR after a single bolus of propofol compared to saline (13.0±1.3 min vs. 12.6±1.6 min, n=5, P=0.672, Fig. 11).

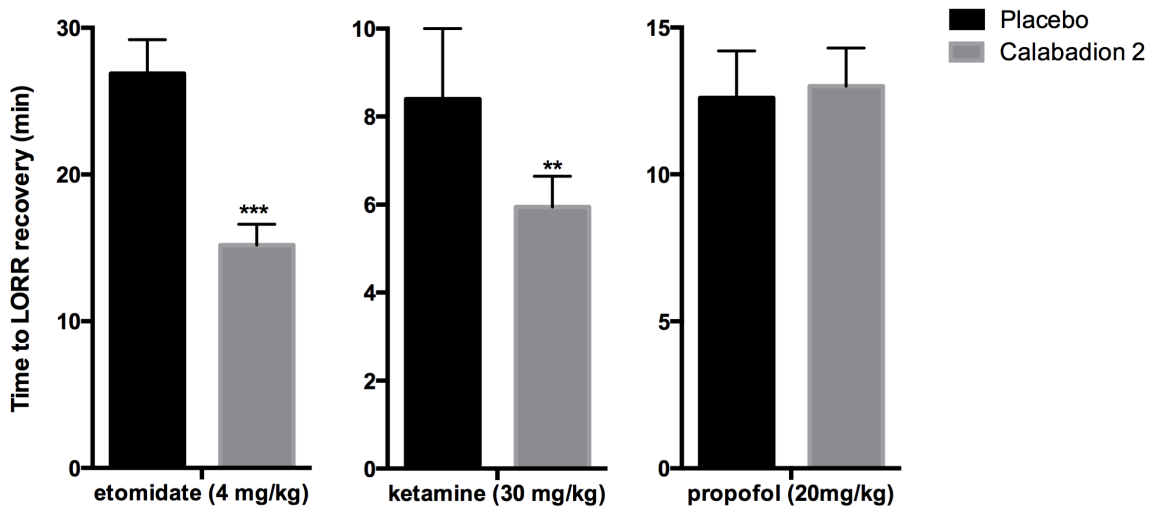


Figure 11. *Calabadion 2 accelerated recovery from single bolus anesthesia with etomidate and ketamine, and did not affect recovery from propofol anesthesia. Effect of calabadion 2 (80 mg/kg/min, intravenous (i.v.)) on time to recovery from loss of righting reflex (LORR) following administration of a single i.v. bolus of etomidate (4 mg/kg, n= 7), ketamine (30 mg/kg, n=7) or propofol (20 mg/kg, n=5). In the cases of etomidate and ketamine recovery time was significantly shorter following Calabadion 2 administration vs. 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 ± standard deviation.*

Effects of Calabadion 2 on Post-anesthesia Functional Mobility Impairment

Functional mobility was evaluated using the balance beam test to assess coordination and balance of rodents after anesthesia, which is believed to translate to the postoperative functional mobility of patients, including complex vestibulomotor activities,

such as walking, driving and operating machines (Diaz-Gil et al., 2014; X. He et al., 2014; Singleton et al., 2010). A significantly faster recovery of balance after anesthesia was observed, when injecting calabadion 2 compared to saline. Calabadion 2 significantly reduced the time slope of recovery by 4.9 min [95% CI, 1.1-8.6 min] (P=0.013; LRT P=0.002,) after 4 mg/kg etomidate i.v., by 3.9 min [95% CI, 1.5-6.3 min] (P=0.002; LRT P<0.001) after 30 mg/kg ketamine i.v. and by 15.7 min [95% CI, 9.4-22.0 min] (P<0.001; LRT P<0.001) after 50mg/kg ketamine i.m., as compared to saline (Fig. 12). The faster recovery of balance may suggest a faster recovery of muscle strength and/or motor coordination after Calabadion 2 injection for both anesthetics.

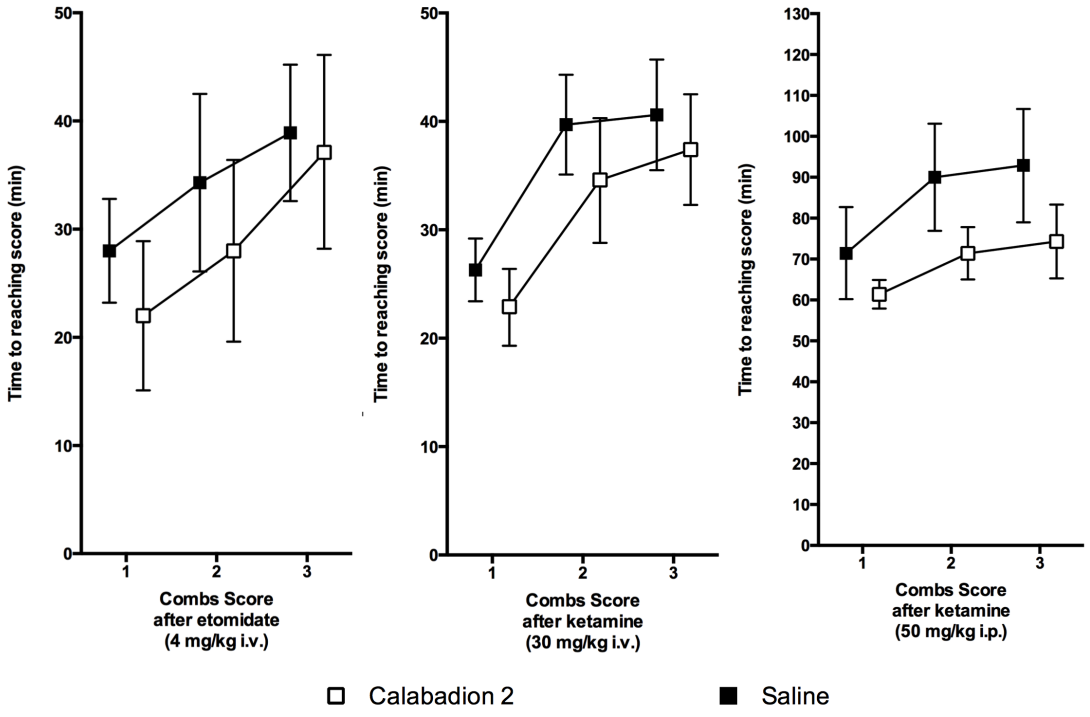


***Figure 12**. Calabadion 2 accelerated recovery from post-anesthetic functional mobility impairment. Effect of calabadion 2 on time to recovery of balance described by the Combs score following administration of a single bolus of etomidate (4 mg/kg intravenous (i.v.); n=7) or ketamine (30 mg/kg i.v.; n=7; 50 mg/kg intramuscular (i.m.); n=7) vs. 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 sec (1 point), 11 to 20 sec (2 points), or 21 to 30 sec or reaches support (3 points). Recovery time was significantly shorter following calabadion 2 vs. placebo. Following an intraperitoneal (i.p.) injection of calabadion 2 the effect in accelerating recovery time was observed to be significantly higher compared to i.v. administration (p <0.001). Error bars represent the 95% confidence intervals.*

Effects of Calabadion 2 on Etomidate-induced Adrenal Suppression

The baseline plasma corticosterone concentration was 9.8±8.0 ng/mL following dexamethasone treatment. The corticosterone levels following ACTH administration were observed to be significantly lower in rats receiving a bolus of etomidate 4 mg/kg followed by a saline infusion compared to the controls receiving a saline bolus instead of etomidate (144.2±60 ng/mL vs. 847.7±400.1 ng/mL, $p<0.001$, Figure 13). The plasma corticosterone levels after administration of calabadion 2, aiming to reverse these adrenal suppressive effects, did not significantly differ compared to the control group receiving a saline infusion after a bolus of etomidate 4 mg/kg (61.5±23.3 ng/mL vs. 144.2±60 ng/mL, $p = 0.074$; Figure 13).

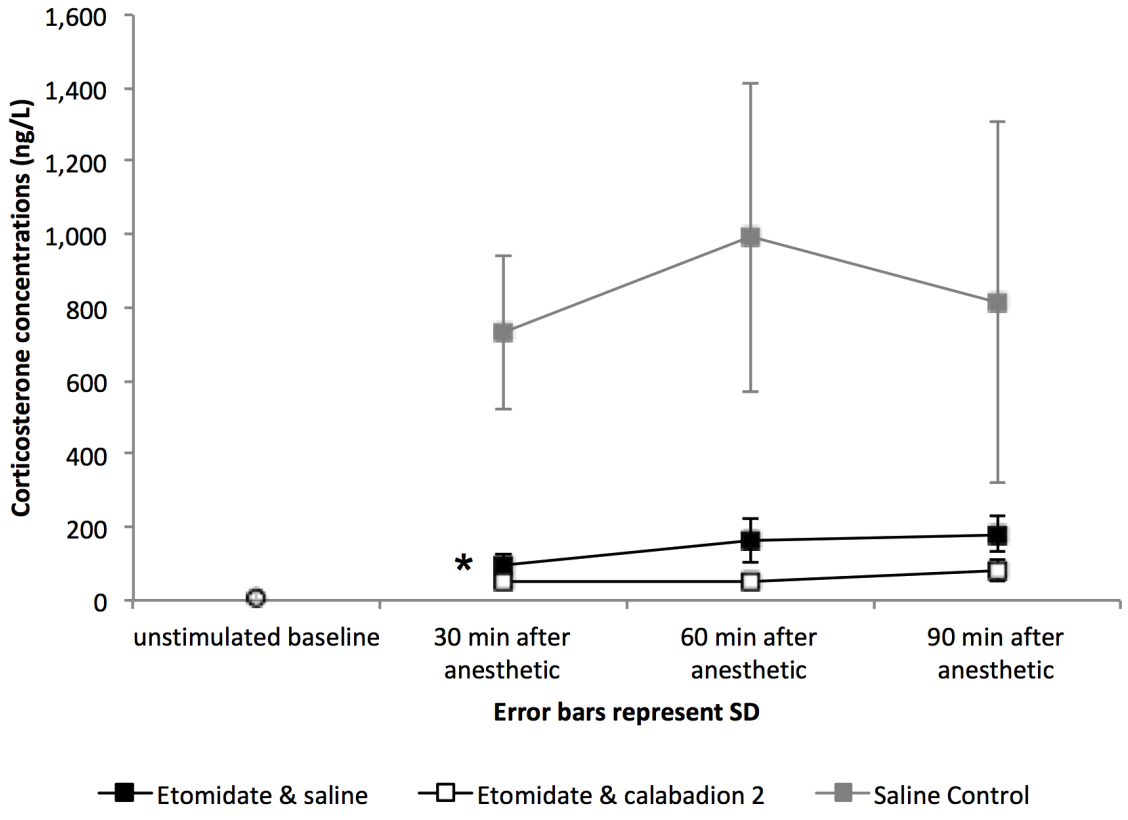


Figure 13. *Effects of calabadion 2 on etomidate-induced adrenal suppresion. After both 4 mg/kg of etomidate followed by a saline or a calabadion 2 infusion significantly lower responses to ACTH were observed during the full study period (* $p < 0.001$ for mean ACTH response after etomidate compared to placebo). No increase in ACTH response could be observed with calabadion 2 compared to saline after etomidate anesthesia. Data are ± standard deviation.*

Calabadion 2 is Not Toxic or Mutagenic

Even under stringent conditions with calabadion 2 up to 1 mM, no significant reduction in cell viability of THP-1 and HepG2-cells was observed. Only a slight dip on the HEK293 cells, and no cell lysis were observed (Fig. 14 A+B). These results were very comparable to the toxicity observed after incubation of the same cell lines with the antibiotic erythromycin and the cyclodextrin, HP-β-CD (Fig. 15).

The observed currents at the hERG channel with calabadion 2 treatment up to a concentration of 25μM (IC_{50}>25μM) did not significantly differ from baseline, 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 post-treatment current across the ion channel with increasing concentrations of the compound (IC_{50}=1.66μM, Fig. 14C).

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

	TA 1535		TA 1537		TA 98		TA 100	
Calabadion 2 concentration (mg)	-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	1101	282	1307

191-A = ICR 191 Acridine; 2-AA = 2-Aminoanthracene; D-myc = Daunomycin; SA = Sodium Azide; TA 98-1535=Strain number of Salmonella thyphimurium

Table 2. *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. 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.*

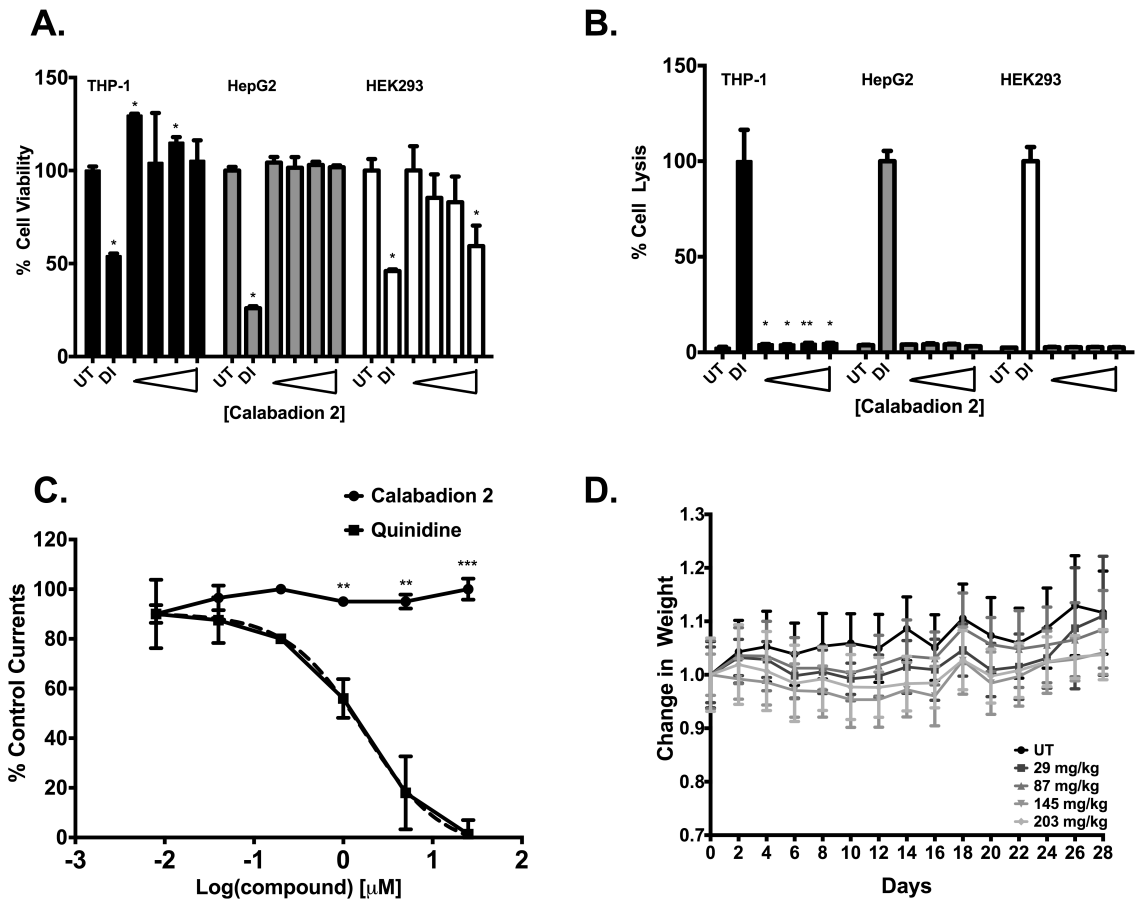


Figure 14. *Calabadion 2 does not induce acute cytotoxity, inhibit the human ether-à-go-go-related (hERG) channel or cause pathology in mice. The cell viability* ***(A)*** *or cell lysis* ***(B)*** *of human white blood cells (THP-1), liver cells (HepG2) and kidney cells (HEK293) after treatment with increasing doses (0.16 mM to 2.5 mM) of Calabadion 2 is shown and compared to untreated (UT) and deionised 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 positive control (IC_{50}=1.66μM; best-fit, nonlinear line of regression, Data are ± standard deviation).* ***(D)*** *Swiss Webster mice were injected intraperitoneally (i.p.) for 14 days either with increasing doses of calabadion 2 (mg/kg body weight) or left untreated (UT). The weight of each mouse was followed daily until 14 days post-treatment. The average and standard deviation of the change in weight of the mice per treatment group (n=7) is graphed. For* ***A-C*** *the values are an average of at least three replicates with corresponding standard deviation values (*p=0.01-0.05; **p=0.001-0.01; ***p<0.001).*

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. 14D).

Finally, a dose escalation study on ten male *Sprague-Dawley* rats suggested a median lethal dose of 2.7 g/kg (LD_{50}=2.7 g/kg [95%CI, 1.8-4.3]). Calabadion 2 did not induce apparent toxic effects in efficacy experiments. The histopathological 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 the escalating dose experiments, mild cellular necrosis of parts of the lungs with fluid in the alveolar spaces, and occasional distension of the pulmonary alveolar capillaries with red blood cells were observed, 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, 10-26:1) for the reversal of 30 mg/kg i.v. ketamine and 3:1 (95% CI, 2-5:1) for the reversal of 4 mg/kg i.v. etomidate. Calabadion 2 was well tolerated at effective doses. The detailed results of the histopathology studies are listed in table 3.

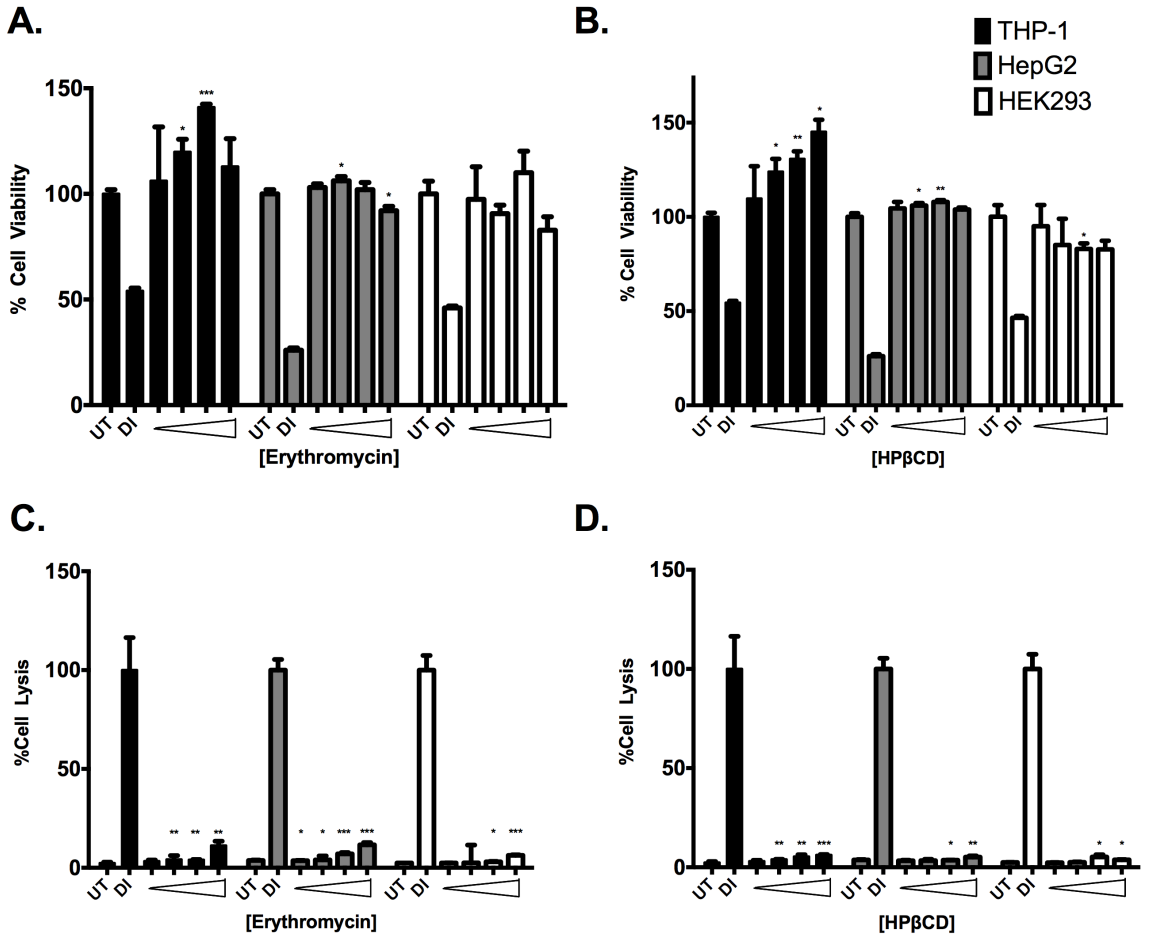


Figure 15. *Toxicity of Erythromycin and HPβCD in in vitro cell assays. Monocytes (THP-1), liver (HepG2) and kidney (HEK293) cell lines were incubated with indicated doses (0.16mM to 2.5mM) of erythromycin* ***(A+C)*** *and cyclodextrin (HPβCD)* ***(B+D)****. The untreated (UT) and cell death-inducing conditions (DI) are indicated as appropriate controls. The cell viability* ***(A+B)*** *and cell death* ***(C+D)*** *were analyzed and results were normalized to untreated groups or death induction controls, respectively. For* ***A-D*** *the values are an average of at least three replicates with corresponding standard deviation values (*p=0.01-0.05; **p=0.001-0.01; ***p<0.001).*

Organ	Calabadion 2 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 red blood cells 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, and one animal that received 1.6 g/kg	Mild to moderate vacuolation that is consistent with mild fat accumulation.
Kidney	Five of six animals that received the two highest doses. Not in the animals that received the 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

P1 and P2 = Segment 1 and 2 of the proximal tubule

Table 3. *Effects of lethal doses of calabadion 2 on rat organs. Pathological 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, and stained with hematoxylin and eosin (H&E), embedded in paraffin slides.*

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 ketamine anesthesia. These data indicate that calabadion 2 raises 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. The reversal effects were achieved dose-dependently by non-toxic concentrations of calabadion 2. This work provides the proof-of-concept that acyclic cucurbit[n]urils can function as true anesthesia reversal agents by reducing levels of etomidate and ketamine in rats through encapsulation followed by renal excretion (Diaz-Gil et al., 2016).

Comparison with the Literature

In clinical practice, emergence from general anesthesia is considered a passive process governed by anesthetic drug pharmacokinetics. Brown and Solt have described reanimation from general anesthesia as an active emergence with methylphenidate (Chemali et al., 2012; Solt et al., 2011). Methylphenidate inhibits reuptake transporters for dopamine and norepinephrine in the brain (Heal et al., 2009), and both neurotransmitters are known to promote arousal (Brown et al., 2011). A translational study in humans is currently being conducted by Solt and Brown (NCT02051452). Other follow-up experiments showed that the intravenous administration of a D1 dopamine receptor agonist (Taylor et al., 2013) as well as electrical stimulation of the ventral tegmental area (VTA) (Solt et al., 2014) also induce reanimation in anesthetized rodents, suggesting that dopamine release by VTA neurons causes a profound arousal response sufficient to reverse the behavioral effects of general anesthesia. Consequently, active emergence

with methylphenidate may be a viable approach to facilitate emergence from general anesthesia.

While reanimation from general anesthesia aims to overpower the anesthetics at the receptor level by the 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 eliminated renally with a short plasma half-life time in rodents (Haerter et al., 2015; Hoffmann et al., 2013).

In this work, emergence in rats was defined as recovery of etomidate and ketamine specific EEG measures to levels reflecting higher consciousness, reversal of blood pressure effects of the anesthetic agents, recovery of the righting reflex and recovery of coordination. Encapsulation of ketamine and etomidate by calabadion 2 was found to be consistent with higher levels of consciousness on EEG measures of brain function. Ketamine and etomidate both disrupt frontal-parietal communication, leading to unconsciousness (U. Lee et al., 2013). However, their molecular targets and neurophysiological 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 GABA types of anesthetics including isoflurane and propofol (Nelson et al., 2002). This effect can be actively attenuated by the microinjection of GABA receptor antagonists, such as gabazine in parts of the endogenous sleep pathway, e.g. in the tuberomamillary nucleus (Miller, 2010; Nelson et al., 2002). As opposed to anteriorization - the shift in occipital alpha activity to frontal alpha coherence also characteristic for GABA anesthetics - which develops rather abruptly as a function of anesthetic infusion (Ching et al., 2010; Vijayan et al., 2013), the BSR progressively and continuously increases with deeper levels of anesthesia, reflecting a decrease in cerebral metabolic rate coupled with the stabilizing

properties of ATP-gated potassium channels (Brown et al., 2010; Ching et al., 2012). Unlike etomidate, sedation with ketamine does not produce a pattern of burst suppression (Nelson et al., 2002). The mechanism of action to produce anesthesia differ substantially, shown by the unaffectedness of its sedative response by GABA receptor antagonists (Eikermann et al., 2012; Rosen et al., 1976). Instead, ascending levels of ketamine gradually increase EEG power likely due to inhibition of NMDA-mediated glutamatergic inputs to GABAergic interneurons, leading to aberrant excitatory activity in the cortex, hippocampus and limbic system (Seamans, 2008). Therefore, in this work, the electrographic depth of ketamine was quantified by measuring total EEG power (Eikermann et al., 2012). Calabadion 2 dose-dependently decreased both periods of suppression (BSR) during deep etomidate anesthesia and total EEG power in ketamine anesthetized rats, showing a reversal of these anesthetics' EEG effects.

LORR in rodents occurs over a very narrow range of anesthetic concentrations which correlate with anesthetic concentrations during loss of consciousness in humans (Franks, 2008). Thus, recovery from LORR is a useful composite index of emergence from general anesthesia. The accelerated recovery from LORR observed after injection of calabadion 2 can be interpreted as suggestive of faster emergence, which would translate to a recovery of full consciousness in humans.

Because lingering post-anesthetic effects may be caused by residual anesthetic molecules, drug encapsulation with calabadion 2 was hypothesized to mitigate post emergence motor impairment. Towards this end, the effects of calabadion 2 on functional mobility were evaluated with the balance beam test, which has previously been used as a predictor of pharmacologic impact on the recovery process (Goldstein et al., 1990). The balance beam test is indicative of subtle deficits in motor skills due to age, central nervous system lesions, and pharmacological manipulations with a higher sensitivity for coordination impairment than other motor tests, as for example the rotarod (X. He et al., 2014; Singleton et al., 2010). The balance beam test has also been used to differentiate the motor skills of wild-type and Huntington disease mouse models over time and to

assess improvements in Huntington disease mice with treatment (Luong et al., 2011). The finding that calabadion 2 mitigated the effects of both etomidate and ketamine in the balance beam test may translate to faster recovery of the patients' postoperative functional mobility including complex vestibulo-motor activities such as walking, driving, and operating machines. One group of experiments was conducted in order to analyze the encapsulation and reversal ability of calabadion 2 (i.p.) even when not administered by the same route as the anesthetic (ketamine i.m.). This could be of high clinical importance in emergency situations, when intravenous injection is not possible, e.g. after recreational ketamine overdose.

Calabadion 2 also dose-dependently reversed the etomidate-induced decrease in MAP, indicating a reversal of anesthesia-depth associated effects on the cardiovascular system. Also, an increase in MAP when reversing ketamine was observed. As opposed to the BSR-monitored experiments under deep etomidate anesthesia, a shallow ketamine anesthesia was titrated to achieve abolishment of the 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, a phenylephrine infusion was applied in 3 rats which were anesthetized with an equally titrated ketamine infusion. This did not affect any ketamine-induced EEG-patterns, indicating no reversal.

No changes in BSR, EEG or MAP were observed during or after the highest doses (80 mg/kg/min) of calabadion 2, when given during steady state isoflurane anesthesia, and no differences in recovery time during propofol anesthesia were encountered. This enforces the hypothesis that the observed effects are caused by the specific encapsulation and inactivation of etomidate and ketamine molecules.

The design of this study allows the conclusion that the 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 the 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, thus only relatively small doses could be applied without cardiovascularly compromising the animals. In contrast, at the recommended dose of etomidate used, the duration of action was longer, and more calabadion 2 could be titrated to accelerate recovery from LORR.

Clinical Implications

In clinical practice, the instances where one might desire to reverse etomidate and ketamine seem rare. Etomidate is usually given for anesthetic induction. When used as such, its duration of action is short. Thus, it is unlikely to delay recovery after surgery. Any delay is much more likely to come from the anesthetic maintenance agent (e.g. isoflurane) and/or other sedative drugs given during surgery (e.g. opioids). The same can be said for ketamine when used as an induction agent. Although ketamine is sometimes infused during surgery, it is at analgesic (not anesthetic) doses. Nevertheless, there is a clinical need to optimize the duration of action of ketamine and etomidate. Single boluses of both etomidate and ketamine are used during procedures of short duration, such as electroconvulsive therapy, or for emergency intubations (Jabre et al., Lancet 2009). Ketamine is also 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 non-anesthetists in situations where monitoring equipment is poor or absent (Bergman, 1999; Ketcham, 1990). Use of higher dosages are associated with longer hospitalization (Priestley et al., 2001), and typical complications described when used in pediatric patients include emesis, vomiting, rash, diplopia, salivation, as well as recovery agitation and transient airway complications, e.g. post sedative airway misalignment, or apnea (Green et al., 1998a; Green et al., 1998b; Priestley et al., 2001). Maybe even more importantly, ketamine is frequently abused (AddictionCenter, 2018). According to the

2013 National Survey on Drug Use and Health in the United States, an estimated 2.3 million people aged 12 or older used ketamine in their lifetimes, with 203,000 users in 2013 alone. Ketamine abusers are typically adolescents or young adults. According to the Department of Justice's National Drug Intelligence Center, individuals aged 12 to 25 accounted for 74 percent of emergency department visits due to ketamine abuse in the United States in the year 2000. Symptoms of ketamine intoxication include anxiety, altered mental status, palpitations, chest pain, and hallucinations (AddictionCenter, 2018). The ability to directly reverse their effects would not only result in reduced complication rates and time to discharge, but also decrease the costs of care in patients receiving etomidate and ketamine in such circumstances, and most importantly also provide an antidote in cases of abuse associated intoxication.

Even though the interest in reversing other anesthetics such as propofol and isoflurane is high, the indication of encapsulating agents will most likely not be extended into these areas. The reason for this is that the chemical structure of calabadion 2 features a glycoluril tetramer unit, which enables the compound to bind to hydrophobic and cationic species. Additionally, the aromatic sidewalls impart affinity due to p-p interactions toward targets that contain aromatic rings in their structures, and 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, which sets burdens on the clinical reversal of these compounds.

Considerations and Limitations

Maintenance of sedation with ketamine and etomidate has been achieved in humans at plasma concentrations of 2-3 μg/ml and 0.3-0.6 μg/ml, respectively (L. He et al., 2014),

and plasma concentrations in humans awakening from ketamine and etomidate were described at 1µg/ml and 0.28µg/ml (vs. 5µg/ml and 0.44µg/ml in rodents), respectively (Cohen et al., 1973; De Paepe et al., 1999; Fragen et al., 1983; Reich et al., 1989). Even though only one-fifth and half dosages of calabadion 2 are estimated to be needed in humans to achieve an equivalent reversal of ketamine and etomidate, respectively, a calabadion 2 excess concentration of 5.1-10.2 µmol/L and of 2.5-34.6 µmol/L is expected to achieve the 50-67 % anesthesia concentration reduction needed to reverse ketamine anesthesia and the 6-53 % reduction needed to reverse etomidate anesthesia in humans, respectively. Given the complex formation of calabadion 2 with different shaped molecules (ketamine, etomidate, rocuronium, vecuronium and cisatracurium) the relatively high concentrations of excess calabadion 2 raise the question of its interaction with other endogenous molecules. It is arguable, if the complex formation of calabadion 2 with such different drugs qualifies as narrow-spectrum reversal. However, the relationship between the binding affinity to the target drug that is to be reversed by encapsulation, and the binding affinity to other chemically similar compounds that are not to be inactivated (binding selectivity), has to be considered. For instance, sugammadex, which also acts by encapsulation, may bind to steroids used as anticontraceptive drugs, and also binds to coagulation factor X, which leads to increased thromboplastin time in patients receiving sugammadex. Administration of a bolus dose of sugammadex is considered to be equivalent to one missed daily dose of oral contraceptive steroids such that further contraceptive precautions are required if an oral contraceptive is taken on the same day that sugammadex is administered. In turn, calabadion 2 also binds to neuromuscular blocking agents. Therefore, it is prudent to titrate the dose of encapsulating agents to the minimally effective dose in order to avoid unspecific binding. At doses sufficient to reverse neuromuscular blockade, calabadion 2 has minimal effects on anesthetic depth or duration.

Given that plasma concentrations in rodents at recovery from the LORR are in the µM range, while adrenal suppression is already achieved in the nM range, calabadion 2 was

not expected to reverse etomidate-induced adrenal suppression at the doses used in this study to accelerate emergence from anesthesia. A more affine molecular container would have been needed to achieve a reversal of the etomidate-induced adrenal suppression.

Calabadion 2 reverses ketamine and etomidate with a narrow therapeutic index (LD_{50} to ED_{50} ratio) of 16:1 and 3:1, respectively, mainly explained by the design of calabadion 2 to reverse the neuromuscular blocking agents rocuronium, vecuronium and cisatracurium, which is achieved at about one-tenth of the doses used here (Haerter et al., 2015). The present 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 (Hoffmann et al., 2013), where subsequent medicinal chemistry optimization allowed the creation of a similar compound with higher affinity now used for drug development. Of note, the ED_{50} of calabadion 2 to reverse ketamine (166 mg/kg) is only about twice as high as the dose used to reverse cisatracurium, which might make the clinical use of calabadion 2 for the reversal of ketamine possible. The FDA defines drug products as narrow therapeutic index drugs, when small differences in dose or blood concentration may lead to serious therapeutic failures and/or adverse drug reactions that are life-threatening or result in persistent or significant disability (Yu et al., 2015). Typically this is the case when the therapeutic index of a drug is less than 2:1, providing a need for careful titration and patient monitoring to effectively use the drug (Tamargo et al., 2015). Two examples of drugs considered to have a narrow therapeutic index are the often clinically used digoxin and warfarin (Muller et al., 2012). Even though requiring narrow therapeutic drug monitoring, several randomized trials have demonstrated that digoxin confers benefits in patients with chronic heart failure related to improved exercise tolerance and quality of life (e.g. by reducing hospitalization rate), making it an important player in the treatment of highly symptomatic heart failure (DiBianco et al., 1989; Guyatt et al., 1988; V. C. Lee et al., 2004; Packer et al., 1993; Uretsky et al., 1993). Also, warfarin has traditionally been used for patients with atrial fibrillation and has been shown to reduce the risk of stroke and mortality, when optimally adjusted and regularly

monitored with help of the international normalized ratio (Hart, 2007; Hart et al., 2007). Considering that lower dosages will be required to reverse anesthesia in humans, and that there are plans to explore potential changes in the chemical structure to increase the affinity, the narrow therapeutic range in this study is not expected to be a limitation for the reversal of anesthesia by encapsulation of active anesthetic molecules.

Future Direction

Currently, calabadions are being developed 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 relationship studies in order to define the indications and contraindications, and to avoid side-effects from displacement.

Conclusions

Calabadion 2 accelerates emergence from etomidate and ketamine anesthesia and reverses evoked unconsciousness as well as the lingering effects of these anesthetics that impair motor coordination in rats by chemical encapsulation at non-toxic concentrations. The concept of reversing anesthesia at the end of the case may change the way we administer anesthetics and increase patient safety by not only helping avoid the residual effects of sub-anesthetic concentrations but also by providing an unprecedented ability to reverse accidental overdose in multiple settings, leading to reduced morbidity and associated healthcare costs.

Zusammenfassung

Gegenwärtig verwendete intravenöse Anästhetika wie Ketamin und Etomidat werden klinisch in einer Vielzahl von Situationen eingesetzt. Bis heute gibt es für diese Anästhetika keinen Mechanismus der pharmakologischen Wirkumkehr. Versuche einen schnelleren Austritt aus der Vollnarkose zu erreichen sind meist darauf ausgerichtet spezifischen physiologischen Sedierungseffekten entgegenzuwirken, indem sie gegensätzliche Systeme stimulieren. Eine innovative Möglichkeit, die Grenzen der Sedierungskontrolle mit diesen Anästhetika zu überwinden, indem eine tatsächliche Reduktion von Anästhetika erreicht wird, hat sich durch die Charakterisierung der Calabadions ergeben, einer besonders vielversprechenden neuen Untergruppe azyklischer Cucurbit[n]urilen (CB [n]), die eng und selektiv an eine Vielzahl von Kationen binden. In dieser Forschungsarbeit wurde die mögliche Verwendung von Calabadion 2 als Leitwirkstoff zur Inaktivierung von Ketamin und Etomidat untersucht.

Um die Wirkung von Calabadion 2 auf das Wiedererlangen des Bewusstseins nach Anästhesie zu untersuchen, wurde die Reaktion von Ratten auf Calabadion 2 nach kontinuierlicher und intravenöser Bolusgabe von Etomidat oder Ketamin und nach intramuskulärer Ketaminverabreichung getestet. Dabei wurden die Effekte auf elektroenzephalographische (EEG) Prädiktoren für die Anästhesietiefe (Burst Suppression Ratio und totale EEG-Power), auf funktionelle Mobilitätseinschränkungen in standardisierten Verhaltensanalysen, sowie auf physiologische Marker wie den Blutdruck getestet. Zusätzlich beinhaltet diese Arbeit Untersuchungen zur Toxikologie des Stoffes.

Dabei konnte gezeigt werden, dass Calabadion 2 dosisabhängig die Wirkung von Ketamin und Etomidat aufhebt, was durch die Reversierung der elektroenzephalographischen Prädiktoren der Narkosetiefe sowie der medikamenteninduzierten Hypotonie und der Verkürzung der Zeit bis zur Wiederherstellung des physiologischen Stellreflexes und der funktionellen Mobilität zu beobachten war. Diese Aufhebung erfolgte ohne toxische Effekte. Basierend auf der maximal tolerierbaren Dosis und der Beschleunigung der Wiederherstellung des physiologischen Stellreflexes ergab sich ein therapeutischer Index von Calabadion 2 von 16:1 (95% Konfidenzintervall [KI], 10-26: 1) für die Ketaminumkehr und 3:1 (95% KI, 2-5: 1) für die Umkehrung von Etomidat.

Diese Daten liefern damit den “Proof-of-concept”, dass azyklische Cucurbit [n]urile als echte „Reversal-Agents“ durch Reduktion der Etomidat- und Ketaminspiegel durch Enkapsulierung wirken können.

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Abkürzungsverzeichnis

191-A	ICR 191 Acridine
2-AA	2-Aminoanthracene
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
BIC	Bayesian information criterion
BSR	Burst suppression ratio
CI	Confidence interval
D-myc	Daunomycin
ED_{50}	Median dose of an effector to achieve an effect with a 50% probability
EEG	Electroencephalography
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
^{1}H NMR	Hydrogen-1 nuclear magnetic resonance
H&E	Hematoxylin and eosin
H_2O	Water
HEK293	Human kidney cell line
HepG2	Human liver cell line
hERG	Ether-à-go-go-Related Gene
HP-β-CD	Hydroxypropyl-β-cyclodextrin
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
IC_{50}	Half maximal inhibitory concentration
K_D	Dissociation constant/binding constant
LD_{50}	Median lethal dose
LORR	Loss of righting reflex
LRT	Likelihood ratio test
MAP	Mean arterial blood pressure
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NMDA	N-Methyl-D-aspartic acid
PACU	Postoperative anesthesia care unit
Q-Q	Quantile-quantile
SA	Sodium Azide
SD	Standard deviation
TA 98-1535	Strain number of Salmonella thyphimurium
THP-1	Human white blood cell line
UV/Vis	Ultraviolet-visible
VTA	Ventral tegmental area

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Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.