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Cysteine Reversal of the Novel Neuromuscular Blocking Drug CW002 in Dogs

Pharmacodynamics, Acute Cardiovascular Effects, and Preliminary Toxicology

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ABSTRACT

Background: CW002 is a neuromuscular blocking drug that is inactivated by endogenous L-cysteine. This study determined the exogenous L-cysteine dose–response relationship for CW002 reversal along with acute cardiovascular effects and organ toxicity in dogs.

Methods: Six dogs were each studied four times during isoflurane–nitrous oxide anesthesia and recording of muscle twitch, arterial pressure, and heart rate. CW002 (0.08 mg/kg or 9 × ED_{95}) was injected, and the time to spontaneous muscle recovery was determined. CW002 was then administered again followed 1 min later by 10, 20, 50, or 100 mg/kg L-cysteine (1 dose/experiment). After twitch recovery, CW002 was given a third time to determine whether residual L-cysteine influenced duration. Preliminary toxicology was performed in an additional group of dogs that received CW002 followed by vehicle (n = 8) or 200 mg/kg L-cysteine (n = 8). Animals were awakened and observed for 2 or 14 days before sacrificing and anatomic, biochemical, and histopathologic analyses.

Results: L-Cysteine at all doses accelerated recovery from CW002, with both 50 and 100 mg/kg decreasing median duration from more than 70 min to less than 5 min. After reversal, duration of a subsequent CW002 dose was also decreased in a dose-dependent manner. Over the studied dose range, L-cysteine had less than 10% effect on blood pressure and heart rate. Animals receiving a single 200-mg/kg dose of L-cysteine showed no clinical, anatomic, biochemical, or histologic evidence of organ toxicity.

Conclusion: The optimal L-cysteine dose for rapidly reversing the neuromuscular blockade produced by a large dose of CW002 in dogs is approximately 50 mg/kg, which has no concomitant hemodynamic effect. A dose of 200 mg/kg had no evident organ toxicity.

What We Already Know about This Topic

- The experimental neuromuscular blocking agent CW002 can be reversed by systemic administration of L-cysteine
- L-Cysteine can be neurotoxic at high doses in some species

What This Article Tells Us That Is New

- In dogs, the optimal dose of L-cysteine for reversal of a large dose of CW002 is 50 mg/kg
- At four times this dose, L-cysteine had no acute organotoxicity in dogs

RECENT studies of the neuromuscular blocking drug gantacurium have demonstrated an ultra-short duration of action largely due to rapid inactivation of the molecule by endogenous L-cysteine.[1–3] This chemical reaction occurs without an enzymatic catalyst at pH 7.4 and entails adduction of cysteine to the chlorofumarate component of the gantacurium molecule. The unique pharmacology of gantacurium has led to the development of related compounds with an intermediate duration of action that can be rapidly reversed by intravenous injection of L-cysteine, even in the absence of any spontaneous muscle recovery.[4]# One of these compounds is CW002, a nonhalogenated, symmetrical benzylisoquinolinium fumarate diester, which exhibits an intermediate duration and reversal by L-cysteine in rhesus monkeys and dogs.[5,6]

To date, the optimal intravenous L-cysteine dose for reversal of CW002 as well as any acute cardiovascular effects and systemic organ toxicity associated with the relevant dose range remain unclear. Toxicological evaluation in rats has indicated that the L-cysteine dose required to produce death in half of the study subjects (LD_{50}) is 1.89, 1.62, 1.55, and

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Savarese JJ, Sunaga H, Belmont MR, McGilvra J, Leung W. Immediate reversal of various olefinic isoquinolinium diester NMB's by cysteine in monkeys. Presented at the Annual Meeting of the American Society of Anesthesiologists, October 17–21, 2009, New Orleans, Louisiana; Abstract A855.

1.14 g/kg when administered orally, intraperitoneally, subcutaneously, and intravenously, respectively.[7] Although there are data indicating that L-cysteine can be neuroprotective,[8,9] when given to rats in doses close to the LD_{50}, L-cysteine has been deemed to be neurotoxic, both in neonatal animals with an incomplete blood–brain barrier[10] and in adults.[11] Other data have raised the prospect that sustained excess of L-cysteine within the central nervous system may be neuroexcitatory *via* a number of mechanisms and contribute to neurodegenerative processes.[12] Manifestations of acute neuroexcitation by L-cysteine injected directly into the brain or cerebrospinal fluid of rats include an increase in blood pressure modulated by both sympathetic nervous activation and vasopressin release.[13]

However, it remains unclear how studies in rodents using L-cysteine injected directly into the brain or administered in high systemic doses relate to the effects of smaller intravenous doses in large animals or human. L-Cysteine, a nonessential amino acid in adults, is conditionally essential in preterm infants because of enzymatic immaturity of the transsulfuration pathways that convert methionine to cysteine.[14] As such, L-cysteine has been commonly infused intravenously at doses up to 80 $mg \cdot kg^{-1} \cdot day^{-1}$ to supplement parenteral nutrition in preterm infants.[15] In both adults and children, the acetylated derivative (*N*-acetyl L-cysteine) has been administered to treat a wide variety of conditions (organ ischemia/reperfusion, hepatorenal injury, cocaine and amphetamine toxicity, and psychiatric illness) in which increased glutathione production and augmented antioxidant capacity are felt to be potentially beneficial.[16–21] Currently, both oral and intravenous *N*-acetyl cysteine preparations (Acetdote) are approved by the Food and Drug Administration for use in the treatment of acute acetaminophen toxicity.[22]

This study in dogs was designed with two goals. The first goal was to provide information about the optimal L-cysteine dose range for CW002 reversal and to broadly characterize any acute or sustained side effects. To accomplish this goal, the dose–response relationship for L-cysteine reversal of a fixed CW002 dose was determined from multiple L-cysteine injections at weekly intervals. These data were then interpreted in the context of both acute dose-related effects on blood pressure and heart rate (HR), and any physical or behavioral abnormalities evident in the interval between doses. The second goal was to more closely characterize the systemic toxicity of L-cysteine relative to the defined therapeutic dose range. This goal was accomplished by reversing a fixed CW002 dose with an amount of L-cysteine four times greater than the derived optimal dose and then observing the dogs for 2 or 14 days before necropsy and biochemical and histopathologic evaluation.

Materials and Methods

L-Cysteine Dose–Response and Acute Cardiovascular Side Effects

Six adult male beagles weighing between 9.1 and 12.5 kg were used for the study after approval of the protocol by the Institutional Animal Care and Use Committee of Weill Cornell Medical College (New York, New York). Each animal was studied four times at least 7 days apart, with a single L-cysteine dose evaluated during each procedure. For half the animals, the dose sequence was 10, 20, 50, and 100 mg/kg, and for the other half, it was 100, 50, 20, and 10 mg/kg.

Preparation. After an overnight fast, each dog was sedated with a subcutaneous injection of 0.1 mg/kg acepromazine, an intravenous catheter was inserted in a foreleg vein, and anesthesia was induced with 4–5 mg/kg intravenous propofol. The trachea was then intubated, and the lungs were ventilated with a mixture of 70% nitrous oxide, 28% oxygen, and 1.5–2.0% isoflurane. Stability of anesthesia was assessed by using a processed electroencephalogram (CSM-2; Danmeter a/s, Denmark). Ventilation was adjusted to maintain end-tidal carbon dioxide at 30–32 mmHg, and systemic arterial oxygen saturation was continuously monitored. Body temperature was measured with a rectal probe and maintained at 37°–38°C with a water-circulating heating blanket. Estimated fluid deficits and maintenance needs (5 $ml^{-1} \cdot kg^{-1} \cdot h^{-1}$) were provided with intravenous lactated Ringer's solution throughout the experiment. After placement of electrocardiographic leads, a femoral artery was percutaneously cannulated for the measurement of systemic arterial pressure. The electrocardiogram, HR, and mean arterial pressure (MAP) were continuously recorded in both analog and digital format throughout the experiment. A superficial segment of the tendon of the left tibialis anterior muscle was attached to a force transducer, preloaded with 50 g tension, and single twitch responses were elicited at 0.15 Hz with supramaximal square-wave pulses of 0.2 ms duration *via* stimulation of the peroneal nerve. Train-of-four stimulation, 2 Hz for 2 s, was interposed during the experiment whenever appropriate.

Reagents. Powdered CW002 was synthesized by Cedarburg Laboratories (Grafton, WI). L-Cysteine was purchased from Sigma-Aldrich (St. Louis, MO). Cysteine solution was prepared by dissolving the L-cysteine base in saline to a concentration of 100 mg/ml, which provided a solution with a pH of approximately 5.5.

Study Design and Data Acquisition. After surgical preparation, a 15- to 20-min stabilization period was allowed before initiating the protocol. During this time, end-tidal isoflurane concentration was allowed to equilibrate at approximately 1.3%, and consistency of twitch height was confirmed. CW002 was then administered as an intravenous bolus of 0.08 mg/kg (CW002 alone), a dose reflecting approximately nine times the ED_{95} for the beagle.[5] Fifteen minutes after complete recovery of twitch height and verification of normal train-of-four (defined as the fourth twitch being equal in amplitude to the first), the same dose of CW002 was repeated followed 1 min later by intravenous injection of 10, 20, 50, or 100 mg/kg L-cysteine (CW002 with cysteine). Fifteen minutes after complete recovery of twitch after the second dose, 0.08 mg/kg CW002 was given a third time (CW002 after cysteine) to determine the effect of residual

L-cysteine on CW002 pharmacodynamics. For each of the three CW002 injections, the duration of action (defined as the time from injection of CW002 to 95% recovery of twitch height) and recovery indices (defined as the time from 5 to 95% recovery of twitch height and the time from 5% recovery of twitch height to normal train-of-four) were determined. After return of neuromuscular function, the animals were awakened and the trachea was extubated, and activity was monitored for clinical signs of recurrent muscle weakness over the next 2 h. Between L-cysteine reversal experiments (separated by at least 7 days), dogs were observed daily for frank moribundity as well as changes in activity, behavior, and appetite by animal care personnel blinded.

To more fully assess the cardiac effects of high-dose L-cysteine, after completion of the full protocol in four animals, the chest was opened and additional instrumentation was placed for measurement of left ventricular (LV) volume and pressure (conductance/micromonometer catheter; Millar Medical Instruments, Dallas, TX) along with ascending aortic blood flow (electromagnetic flow probe; Carolina Medical, King, SC). From these data, stroke volume, systemic vascular resistance, and LV ejection fraction were recorded. In addition, LV inotropy was assessed as dP/dt normalized to LV end-diastolic volume, and lusitropy indexed from the time constant of isovolumic relaxation. For each experiment, 1 ml/kg of saline, the vehicle for L-cysteine, was first injected *via* peripheral vein to evaluate the effect of volume alone. Fifteen minutes later, the same amount of saline containing 100 mg/kg L-cysteine was injected. To shed light on the relative contributions of autonomic activation and vasopressin release to the effect of high-dose L-cysteine on blood pressure and HR, in four dogs, an additional dose of 100 mg/kg was given after ganglionic blockade with 20 mg/kg hexamethonium and 1 mg/kg atropine (n = 2), or pretreatment with 10 μg/kg of the vasopressin-1 receptor antagonist (β-mercapto-β, β-cyclopentamethylenepropionyl1, O-me-Tyr2, Arg8)-vasopressin (n = 2).

Assessment of L-Cysteine Systemic Toxicity

Preparation. Sixteen beagles weighing between 7.2 and 9.6 kg were used for this part of the study under a protocol approved by the animal care and use committee of Charles River Laboratories, Worchester, Massachusetts. As described earlier in the Cysteine Dose–Response and Acute Cardiovascular Side Effects section, animals were anesthetized with propofol for tracheal intubation and thereafter maintained with isoflurane. Body temperature was controlled with a circulating warm water blanket or forced air blanket. After sterile skin preparation, antibiotic administration (cefotaxime, 50 mg/kg, intravenously), and infiltration of 2% lidocaine, a femoral vessel was cannulated *via* small cut down for blood pressure measurement and blood sampling.

Study Design. Eight animals received 0.4 mg/kg CW002 followed 10 min later by 200 mg/kg L-cysteine; the CW002 dose was fivefold higher than that used for the dose ranging study, and the L-cysteine dose was 2- to 20-fold higher than the doses used in the dose–response analysis. The other eight dogs served as controls, receiving only CW002 and vehicle. For both groups, neuromuscular function was monitored, and after full recovery, the arterial catheter was removed and the wound was closed, the dogs were awakened, and the trachea was extubated. For postoperative analgesia, buprenorphine (0.01 mg/kg, subcutaneously) was administered every 8 h starting the afternoon/evening after the vascular access procedure and continued for a total of four injections.

Evaluation of Systemic Toxicity. All animals were observed twice daily for mortality/moribundity. Four animals from each group were sacrificed by pentobarbital overdose on day 2, and the remaining dogs from each group were sacrificed on day 14. Comparisons between groups were made with regard to hematology, coagulation, and serum chemistry. For this analysis, data collected at the time of initial treatment were compared with those obtained at the time of sacrificing. After sacrificing, gross necropsy was performed, which included examination of the carcass and musculoskeletal system; all external surfaces and orifices; the cranial cavity and external surface of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs. Tissues were embedded in paraffin, sectioned, and stained for histopathologic examination.

Data Analysis and Statistics

L-Cysteine Dose–Response for Reversal of CW002. The dose–response effects of L-cysteine on the duration of action and recovery indices were not normally distributed and therefore assessed by analysis of variance on ranks and the Newman–Keuls test when appropriate, with data presented as median with 25–75% confidence intervals. For all statistical analyses, a P value less than or equal to 0.05 was considered significant.

Acute Hemodynamic Effects of L-Cysteine. The magnitude and timing (relative to injection) of changes in blood pressure and HR produced by L-cysteine injection at each dose for each animal were assessed from continuous data sampled at 200 Hz and averaged over 5-s intervals. Pooled data were normally distributed and quantitatively assessed by analysis of variance for repeated measures and the Newman–Keuls test when appropriate and are presented as mean ± SD. Statistical analysis of cardiac performance variables was precluded by a low power because of the small sample size. Accordingly, dose-related qualitative variations in the cardiac response to L-cysteine were assessed by creating a composite representation of data from all animals. With this approach, drug injection was defined as time 0, and the mean and SD of data averaged over 5-s intervals for each dog were determined. These data were then plotted as a relative value reflecting a ratio of the steady-state data obtained just before injection of L-cysteine.

Toxicology.

Hematology, Coagulation, and Serum Chemistry. A single-factor analysis of variance was applied, with animal grouping as the factor, and Dunnett's test applied when appropriate.

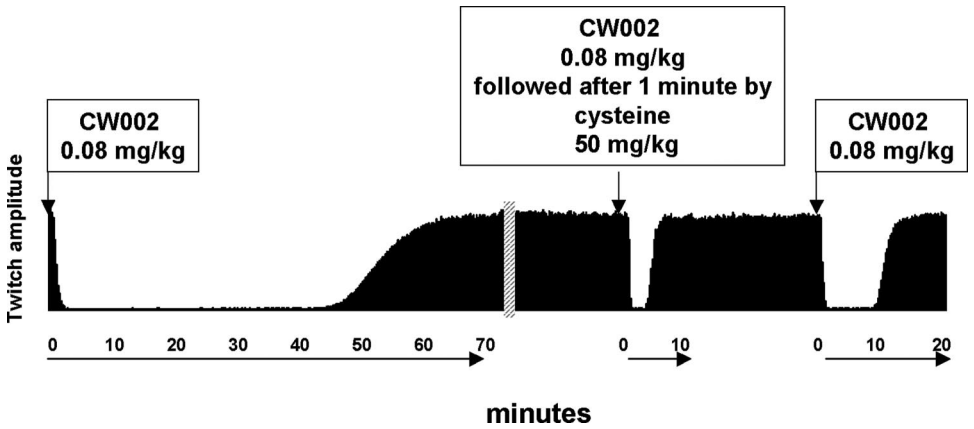

Fig. 1. Representative recordings of the change in tibialis anterior muscle twitch produced by 0.08 mg/kg CW002 given alone, in combination with L-cysteine, or after L-cysteine reversal of neuromuscular blockade.

For all analyses, a P value less than or equal to 0.05 was considered significant.

Results

L-Cysteine Dose–response for Reversal of CW002

Representative recordings of the change in muscle twitch produced by 0.08 mg/kg CW002 alone or in combination with L-cysteine are presented in figure 1. Pooled reversal data (table 1) show that, on average, 0.08 mg/kg CW002 alone produced neuromuscular blockade with a median duration of 75 min, a median time for 5–95% recovery of 23 min, and a median time for 5% recovery to normalized train-of-four of 40 min. L-Cysteine injected 1 min after CW002 markedly reduced the duration of muscle relaxation at all doses ($P < 0.001$), with both 50 and 100 mg/kg facilitating twitch recovery within 5 min. Similar dose-dependent effects of L-cysteine on recovery indices are also evident. As also shown in table 1, 15 min after complete recovery of twitch after reversal with L-cysteine, there was sufficient residual L-cysteine to reduce the duration of action for additional CW002 in a dose-dependent manner.

Acute Hemodynamic Effects of L-Cysteine

As shown in figure 2, increasing doses of L-cysteine tended to increase MAP and reduce HR. These changes were, on average, less than 10% even for 100 mg/kg. Table 2 depicts comparison of baseline values for MAP and HR between experiments (there were no differences), and the maximal change in each variable produced by L-cysteine. Of particular note is the fact that the changes in MAP were not clearly dose related: the pressor response to 20 mg/kg represented a significant increase, whereas 50 mg/kg did not change MAP. Furthermore, as depicted in figure 2, the pattern of change for both MAP and HR varied as a function of dose, with 100 mg/kg cysteine eliciting a multiphasic response with the peak increase in MAP occurring later than for other doses.

The temporal relationship between changes in cardiac performance variables in response to 100 mg/kg cysteine is shown in figure 3. Injection of saline vehicle alone had no effect on any variable (data not shown). Consistent with MAP and HR data, changes in LV pressure, stroke volume, contractility, and lusitropy after administration of 100 mg/kg cysteine were less than 10%. Nonetheless, the data do

Table 1. Dose–Response Data for L-Cysteine Reversal of CW002

		L-Cysteine Dose, mg/kg			
	CW002 Alone	10	20	50	100
Duration of action, min	75 (63–78)				
With cysteine		19 (17–21)*	11 (8–12)*†	4.6 (3.3–6.5)*†	3.8 (3.5–4.4)*
After cysteine		45 (43–46)*	33 (29–38)*†	11.6 (11–18.5)*†	5.9 (5.5–9.5)*†
5 to 95% recovery, min	23 (21–25)				
With cysteine		10 (7.0–11.0)*	5.3 (4.1–6.0)*†	2.0 (1.3–3.6)*†	1.5 (1.4–1.6)*
After cysteine		16 (12–18)*	11.3 (8.5–15)*	4.3 (4.0–7.5)*†	3.1 (3.0–3.7)*
5% to TOF recovery, min	40 (36–50)				
With cysteine		21 (15–22)*	12 (10.5–14)*†	6.4 (4.6–7.2)*†	4.7 (4.5–5.6)*
After cysteine		27 (25–30)*	20 (18–21)*†	8.2 (7.7–11.0)*†	7.8 (7.3–8.2)*

Values are expressed as median minutes with 25–75% confidence interval.

* Difference from CW002 alone. † Difference from preceding dose.

With cysteine = CW002 injection followed 1 min later by injection of 10, 20, 50, or 100 mg/kg intravenous L-cysteine; after cysteine = CW002 injection 15 min after complete recovery of twitch after reversal with L-cysteine; duration of action = time from drug injection to 95% recovery of twitch height; TOF = train-of-four; TOF recovery = point where amplitude of the fourth twitch in train-of-four stimulation equals amplitude of the first twitch.

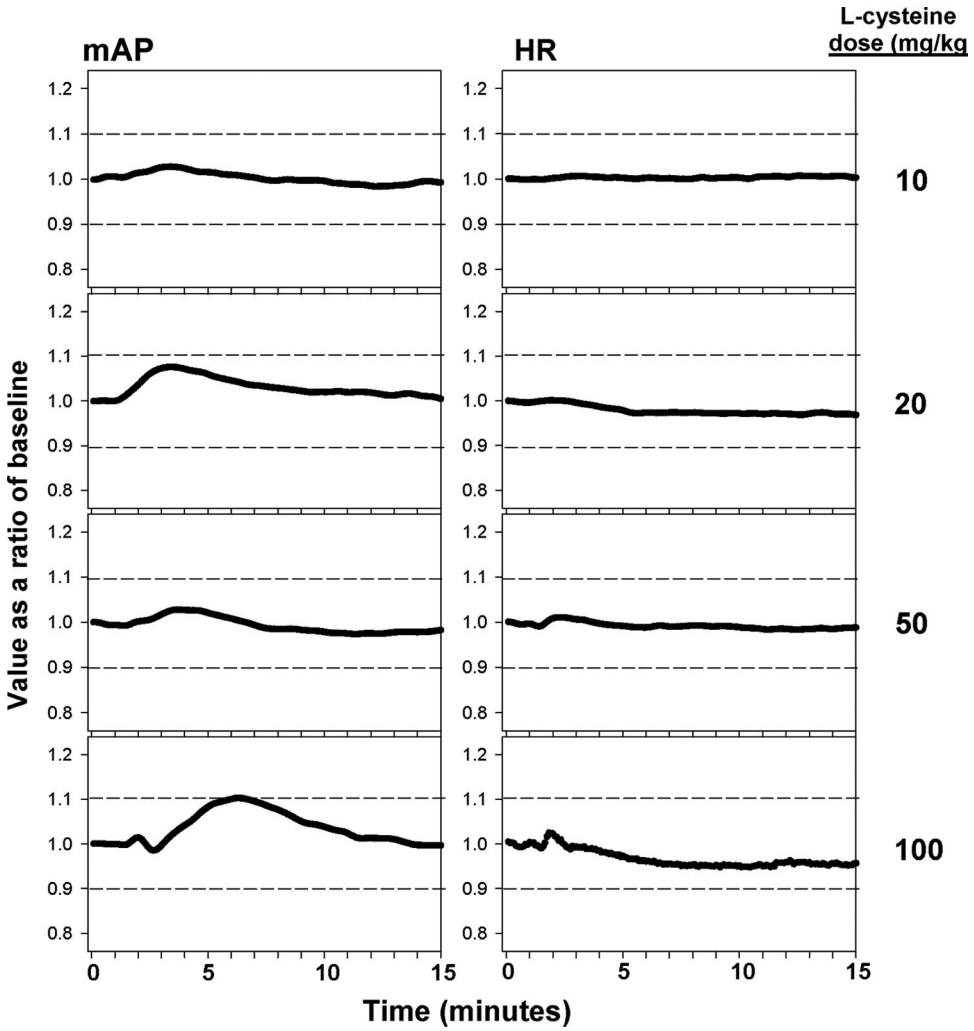

Fig. 2. The temporal relationship of dose-related changes in mean arterial pressure (MAP) and heart rate (HR) after injection of 0.08 mg/kg CW002 (time 0) followed 1 minute later by L-cysteine. *Lines* represent a composite of data averaged over 5-s intervals for each dog and are plotted as a ratio of baseline values recorded before drug injection; *dashed lines* depict 10% changes. In contrast to other doses, 100 mg/kg L-cysteine produced a biphasic response with a later peak in MAP.

suggest a multiphasic hemodynamic response to 100 mg/kg cysteine with an initial vasodilation followed by a more sustained vasoconstriction. Although the mechanism of this response remains unclear, as shown in figure 4, ganglionic blockade altered the initial fluctuation in MAP produced by 100 mg/kg cysteine but had no effect on the subsequent, more sustained pressor response. In contrast, antagonism of the vasopressin-1a receptor did not prevent the initial fluctuation in MAP evident under control conditions, but abolished the late pressor response.

Assessment of L-Cysteine Systemic Toxicity

In the animals that received repeated L-cysteine doses during a 4-week period for definition of the dose–CW002 reversal response, there were no fatalities or morbid events. All dogs maintained normal activity and behavior without change in appetite

Table 2. Changes in Blood Pressure and Heart Rate in Response to L-Cysteine

Cysteine Dose, mg/kg	Baseline MAP, mmHg	Peak MAP, mmHg	*P* Value	Baseline HR, beats/min	Peak HR, beats/min	*P* Value
10	84.1 ± 6.7	86.4 ± 6.9	0.26	159 ± 39	158 ± 38	0.6
20	83.7 ± 4.9	89.0 ± 4.8	0.03	154 ± 27	148 ± 29	0.08
50	89.4 ± 6.5	91.0 ± 6.9	0.07	158 ± 37	154 ± 34	0.5
100	87.5 ± 5.2	96.0 ± 4.4	0.005	159 ± 35	150 ± 34	0.03
P value	0.086	0.088		0.983	0.974	

Values are expressed as mean ± SD.
HR = heart rate; MAP = mean arterial pressure.

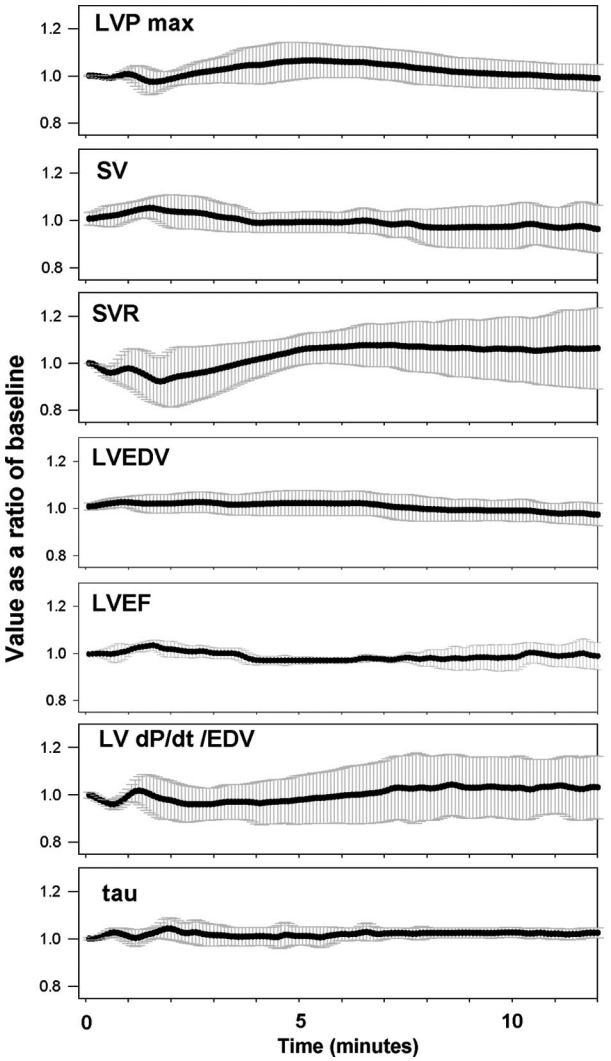

Fig. 3. The temporal relationship between changes in maximal left ventricular (LV) pressure (LVP max) after 100 mg/kg cysteine and simultaneous alterations in stroke volume (SV), systemic vascular resistance (SVR), left ventricular end-diastolic volume (LVEDV), left ventricular ejection fraction (LVEF), contractility index (LV dP/dt/EDV), and the time constant of isovolumic relaxation (tau). Injection of 100 mg/kg cysteine is defined as time 0. *Lines* represent a composite of data averaged over 5-s intervals for each dog with the mean and standard deviation plotted as a ratio of baseline values recorded before drug injection.

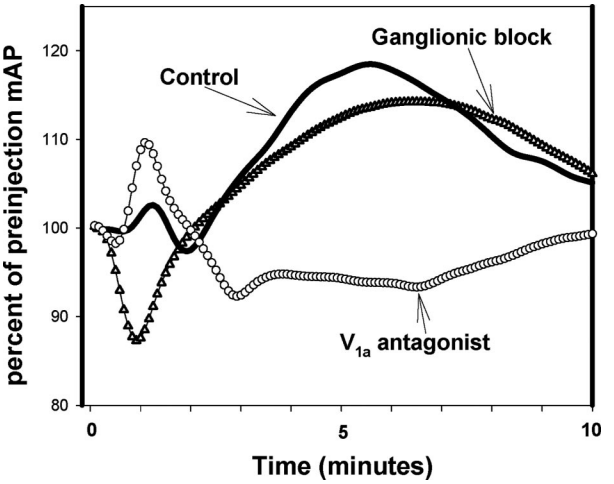

Fig. 4. Representative tracings showing the effect of ganglionic blockade (20 mg/kg hexamethonium and 1 mg/kg atropine), or selective V1a receptor antagonist (10 mg/kg [β-mercapto-β, β-cyclopentamethylenepropionyl1, O-me-Tyr2, Arg8]-vasopressin) before 100 mg/kg cysteine on changes in mean arterial pressure (MAP) relative to preinjection baseline. Injection of 100 mg/kg cysteine is defined as time 0 and the average data over every 5-s interval after injection for each animal combined to yield a discrete mean. *Solid line* = control, *circles* = V1a receptor antagonist, *triangles* = ganglionic blockade.

or weight loss. At the end of the study, two of the dogs were examined by veterinary staff and deemed suitable for adoption. Animals that received a single 200-mg/kg dose of L-cysteine and were sacrificed 2 or 14 days later also exhibited no mortality, moribundity, or weight loss during the observation period. Relative to control animals that did not receive L-cysteine, there were also no differences in hematology, coagulation, and serum chemistry values either at 2 or 14 days (data given in appendix 1). Similarly, neither gross nor histologic examination revealed differences among groups (data not shown, see appendix 2 for tissue list).

Discussion

CW002 is a fast-onset, intermediate duration, nondepolarizing neuromuscular blocking agent that is inactivated by nonenzymatic adduction of cysteine to the fumarate component of the molecule.[5] This study demonstrates that injection of exogenous L-cysteine facilitates the adduction process and can rapidly reverse the neuromuscular blockade produced by CW002, even in the absence of any spontaneous recovery. The optimal L-cysteine dose for reversal of 0.08 mg/kg CW002 ($9 \times ED_{95}$) in the dog is approximately 50 mg/kg, a concentration that elicits no significant cardiovascular effect.

The concept of reversing the effect of neuromuscular blocking drugs by molecular inactivation has recently been highlighted by the introduction of sugammadex, a cyclodextrin designed to bind the steroid compound rocuronium.[6] This idea of displacing a molecule from an active site by providing alternative, high-affinity binding sites has fostered a paradigm shift away from the conventional approach of reversing neuromuscular blockade by giving a cholinesterase inhibitor to increase the concentration of acetylcholine at the motor end plate once there is evidence of spontaneous muscle recovery. It is now possible to reverse the effects of a nondepolarizing muscle relaxant even in the absence of any spontaneous recovery, thus rendering intermediate duration drugs "ultrashort" if needed.

In contrast to the sugammadex–rocuronium interaction, data indicate that CW002 is modified by L-cysteine adduction in such a way that affinity of the molecule for skeletal muscle nicotinic receptors is reduced 60-fold.[5] Recent *in vitro* studies by our research group (unpublished) have provided a putative pathway for CW002 metabolism. Within this construct, CW002 undergoes a slow background hydrolysis (reaction half time of 495 min). Addition of L-cysteine

produces an adduction product with a reaction half time of 11.4 min, and subsequent hydrolysis of the adduct occurring with a reaction half time of 56 min. Importantly, these reactions proceed only in a forward direction, and as such, there is no potential for dissociation of cysteine and regeneration of CW002. These *in vitro* data support the concept that duration of action for CW002 *in vivo* can be shortened by dose-related increases in the amount of L-cysteine available for adduction with the molecule. Our data confirm that injection of exogenous L-cysteine rapidly facilitates inactivation of the CW002 molecule, with 50 mg/kg L-cysteine administered 1 min after CW002 markedly decreasing the median duration of neuromuscular blockade from 75 to 4.6 min. Our data also show that 15 min after complete reversal of CW002, there is sufficient residual L-cysteine to substantially reduce the duration of a subsequent CW002 dose.

Results of the current study indicate that there are no differences between 50 and 100 mg/kg L-cysteine in terms of how each dose shortens the duration of action, 5–95% recovery time, or time to normalization of the train-of-four response. From these data, we conclude that a dose in the 50-mg/kg range is optimal for reversal of CW002 under the experimental conditions. However, this conclusion needs to be interpreted in light of the fact that the only dose of CW002 given was 0.08 mg/kg, or approximately nine times the ED_{95} in beagles.[4] Use of this dose in the current study was based on previous observations in rhesus monkeys indicating that the potency of CW002 in primates is roughly one fifth that in dogs.[5] To provide a molar mass of CW002 that would approach a clinically relevant concentration in primates without producing an inordinately long duration of neuromuscular blockade in dogs, the 0.08 mg/kg dose was chosen. Importantly, even at this relatively high CW002 dose for dogs, 50 mg/kg L-cysteine injected 1 min after CW002 was remarkably effective in reversing neuromuscular blockade. Preliminary data indicate that reversal of $3 \times ED_{95}$ (0.15 mg/kg) CW002 by 50 mg/kg cysteine in rhesus monkeys is similar, if not more rapid, to that observed in dogs.[23]

Previous work in our laboratory has demonstrated that even at $100 \times ED_{95}$, CW002 produces only modest and transient hemodynamic changes.[4] Consistent with these data, 0.08 mg/kg CW002 alone caused no hemodynamic changes in the current study. When injected as an intravenous bolus 1 min after 0.08 mg/kg CW002, L-cysteine also had relatively little hemodynamic effect reflecting on average, changes in MAP and HR less than 10%, even at the highest dose of 100 mg/kg. However, blood pressure effects in particular were not clearly dose related because 20 and 100 mg/kg L-cysteine produced a significant pressor response, whereas 50 mg/kg did not. The reason for this effect remains unclear, but may reflect, at least in part, the emergence of counterbalancing effects on blood pressure, that is, simultaneous stimulation of both vasopressor and vasodilator responses. This process is most apparent after 100 mg/kg L-cysteine in which changes in both MAP and HR were multiphasic (direction of change shifting over time) with the peak increase in MAP occurring later after 100 mg/kg cysteine than for the other doses. More extensive evaluation of cardiac performance in a subset of dogs indicates that 100 mg/kg also produces multiphasic fluctuations in LV pressure, stroke volume, afterload, contractility, and relaxation. When considered in the context of simultaneous changes in MAP and HR, these data suggest an initial vasodilation and sympathetic nervous system activation followed by a more sustained vasoconstriction with a reflex parasympathetic response.

Although clearly demonstrable, the overall hemodynamic change produced by L-cysteine was relatively modest. Nonetheless, previous reports raise points of possible mechanistic and toxicological interest. In a study examining the cardiovascular effects of amino acid infusions in dogs, Hallberg and Soda[24] found that L-cysteine infusion increased hepatic and portal vein blood flow but did not change systemic arterial blood pressure. For this study, 100 ml of a solution containing 6.058 mg/ml cysteine was infused over 6.25 min for a total dose of approximately 40 mg/kg. In contrast, for the current study, L-cysteine in doses up to 100 mg/kg was given as an intravenous bolus. Potential implications of this include the possibility of entry into the central nervous system.[25] Previous reports indicate that injection of L-cysteine into the cerebrospinal fluid of conscious rats elicits a dose-related increase in arterial pressure because of autonomic nervous system activation combined with systemic vasopressin release.[13] Because the concentrations of L-cysteine in the brain and blood were not measured in our study, we cannot directly compare these results with our data obtained after intravenous dosing. Nonetheless, our preliminary observations indicating that the late pressor response to 100 mg/kg L-cysteine can be blocked by a vasopressin antagonist suggest that sufficient amounts of L-cysteine were achieved in the central nervous system to mimic effects noted for central injection of the compound. Given previous reports that high intraperitoneal doses of L-cysteine can produce acute neurotoxicity in both neonatal and adult rodents,[10,11] entry of L-cysteine into the brain may have potential consequences. However, it is important to note that the doses used in these studies—1,200 mg/kg in infants and 1,500 mg/kg in adults—are close to the reported LD_{50} of 1,620 mg/kg for intraperitoneal L-cysteine in the rat[7] and 24–30 times higher than the optimal dose for CW002 reversal found in the current study.

To more specifically characterize the toxicology of L-cysteine relative to its potency for reversal of CW002, a separate group of dogs received CW002 followed by 200 mg/kg L-cysteine, a dose fourfold higher than that deemed optimal in dose–reversal response studies. Animals were then sacrificed 2 or 14 days later. The study dogs exhibited no clinical, biochemical, or anatomic evidence of drug-related toxicity compared with control animals that did not receive L-cysteine. However, these results need to be interpreted in the context of the limitations inherent to preclinical research. In particular, although the data indicate that L-cysteine in the

doses administered is without adverse side effects to the dogs, they do not independently establish that L-cysteine is safe for clinical use. Currently, L-cysteine is added to the parenteral nutrition provided to preterm infants at a dose of approximately 80 mg · kg^{-1} · day^{-1},[14,15] and intravenous *N*-acetyl L-cysteine is approved by the Food and Drug Administration for the treatment of acute acetaminophen toxicity at a dose of 150 mg/kg over 60 min, followed by 50 mg/kg over 4 h, and then 100 mg/kg over 16 h.[22] Formulated in a preparation containing EDTA and sodium hydroxide as stabilizing agents, Acetadote® (Cumberland Pharmaceuticals Inc., Nashville, TN) has been associated most prominently with nausea and cutaneous anaphylactoid reactions related, at least in part, to histamine release.[22] The relative roles played by the acetyl and L-cysteine moieties along with the EDTA and sodium hydroxide excipients in reactions to intravenous administration remain uncertain.[22] However, in the current study of L-cysteine given as bolus injections, we did not observe the cutaneous flushing, hypotension, and reflex tachycardia commonly seen with histamine release in dogs, suggesting that L-cysteine alone may not be the cause of cutaneous anaphylactoid reactions. Nonetheless, clinical trials involving L-cysteine will be required to verify safety in human.

In conclusion, intravenous L-cysteine can rapidly reverse the neuromuscular blockade produced by a CW002 dose nine times greater than the ED_{95} in the beagle. The optimal dose for reversal seems to be approximately 50 mg/kg, with no added benefit to 100 mg/kg. After L-cysteine administration for reversal of CW002, the duration of a subsequent CW002 dose is also decreased in a dose-related manner. Over the dose range evaluated, L-cysteine has minimal hemodynamic effects inducing a small ($\leq$ 10%), but consistent, increase in MAP at 20 and 100 mg/kg and modest, but significant, decrease in HR at 100 mg/kg. Preliminary toxicology studies of reversing CW002-induced neuromuscular blockade with 200 mg/kg L-cysteine (fourfold higher than that deemed optimal) did not demonstrate clinical, biochemical, or anatomic evidence of drug-related toxicity.

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Appendix 1. Hematology, Coagulation, and Serum Chemistry Data from Control Dogs (CW002 + Vehicle) and Those That Received CW002 + 200 mg/kg L-Cysteine

	Control—2 Days	CW 002 + 200 mg/kg Cysteine—2 Days	Control—14 Days	CW 002 + 200 mg/kg Cysteine—14 Days
Hematology				
Erythrocyte count, $10^6/\mu l$	6.7 ± 0.4	6.4 ± 0.3	6.8 ± 0.5	6.4 ± 0.5
Hemoglobin concentration, g/dl	15.1 ± 1.0	14.6 ± 0.6	14.9 ± 0.9	14.2 ± 1.1
Hematocrit, %	44.8 ± 3.5	43.3 ± 2.0	45.3 ± 3.0	43.0 ± 3.0
Mean corpuscular volume, fL	66.7 ± 2.3	67.1 ± 2.4	67.1 ± 0.8	67.5 ± 1.6
Mean corpuscular hemoglobin concentration, g/dl	33.6 ± 0.7	33.8 ± 0.8	32.9 ± 0.4	33.0 ± 0.4
Mean corpuscular hemoglobin, pg	22.5 ± 0.5	22.6 ± 0.7	22.1 ± 0.3	22.3 ± 0.6
Reticulocyte count, 10^9/l	53.1 ± 25.0	37.9 ± 8.0	51.7 ± 9.4	57.0 ± 16.9
Platelet count, $10^3/\mu l$)	366.0 ± 83.0	320.0 ± 73.0	378.0 ± 31.0	329.0 ± 56.0
Leukocyte count, $10^3/\mu l$	13.6 ± 2.6	12.1 ± 1.7	7.6 ± 1.3	9.4 ± 0.9
Neutrophil count, $10^3/\mu l$	8.7 ± 2.2	7.6 ± 1.4	4.5 ± 0.8	5.7 ± 1.0
Lymphocyte count, $10^3/\mu l$	3.7 ± 0.9	3.4 ± 0.4	2.5 ± 0.6	2.9 ± 0.3
Monocyte count, $10^3/\mu l$	0.8 ± 0.2	0.76 ± 0.20	0.37 ± 0.14	0.52 ± 0.11
Eosinophil count, $10^3/\mu l$	0.3 ± 0.2	0.24 ± 0.10	0.19 ± 0.07	0.15 ± 0.04
Basophil count, $10^3/\mu l$	0.07 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
Coagulation				
Prothrombin time, s	7.3 ± 0.3	7.1 ± 0.4	7.7 ± 0.4	7.2 ± 0.2
Activated partial thromboplastin time, s	13.3 ± 0.5	13.0 ± 0.6	13.3 ± 0.7	12.3 ± 0.7
Fibrinogen, mg/dl	300.0 ± 36.0	275.0 ± 21.0	173.0 ± 27.0	186.0 ± 21.0
Serum chemistry				
Alanine aminotransferase, U/l	32.8 ± 11.7	28.1 ± 6.4	28.8 ± 8.5	27.0 ± 6.7
Aspartate aminotransferase, U/l	41.1 ± 22.5	31.3 ± 7.4	32.3 ± 2.8	33.5 ± 2.9
Alkaline phosphatase, U/l	122.3 ± 24.4	116.4 ± 44.4	106.3 ± 14.7	107.0 ± 43.7
Gamma-glutamyltransferase, U/l	3.3 ± 0.5	3.4 ± 0.5	3.0 ± 0.0	3.0 ± N/A
Total bilirubin, mg/dl	0.16 ± 0.05	0.18 ± 0.05	0.18 ± 0.10	0.18 ± 0.05
Urea nitrogen, mg/dl	17.0 ± 7.5	16.4 ± 7.0	16.0 ± 1.6	17.5 ± 4.5
Creatinine, mg/dl	0.49 ± 0.14	0.51 ± 0.06	0.45 ± 0.06	0.50 ± 0.08
Calcium, mg/dl	11.3 ± 0.6	11.2 ± 0.5	10.8 ± 0.2	11.0 ± 0.4
Phosphorus, mg/dl	6.7 ± 0.5	6.3 ± 0.9	5.7 ± 0.2	6.2 ± 0.3
Total protein, g/dl	5.9 ± 0.2	5.9 ± 0.4	5.7 ± 0.2	5.9 ± 0.3
Albumin, g/dl	3.1 ± 0.5	3.2 ± 0.3	3.2 ± 0.2	3.3 ± 0.2
Globulin, g/dl	2.8 ± 0.4	2.7 ± 0.2	2.5 ± 0.2	2.6 ± 0.1
Albumin/globulin ratio (ratio)	1.1 ± 0.3	1.20 ± 0.15	1.3 ± 0.2	1.25 ± 0.06
Glucose, mg/dl	96.9 ± 10.6	97.5 ± 9.8	89.8 ± 11.5	83.0 ± 9.7
Cholesterol, mg/dl	165.9 ± 25.3	165.8 ± 25.7	142.3 ± 26.1	184.0 ± 31.5
Triglycerides, mg/dl	43.0 ± 11.0	49.5 ± 25.0	37.0 ± 6.7	43.8 ± 19.3
Sodium, mEq/l	146.1 ± 2.2	146.8 ± 2.8	144.8 ± 1.0	145.0 ± 0.8
Potassium, mEq/l	4.7 ± 0.3	4.5 ± 0.2	4.5 ± 0.2	4.5 ± 0.2
Chloride, mEq/l	109.5 ± 0.9	110.5 ± 1.1	109.3 ± 0.5	107.8 ± 1.3

Appendix 2. Tissues Collected and Examined at Necropsy

Adrenal gland (paired)	Lymph node, mandibular
Animal identification	Lymph node, mesenteric
Aorta	Mammary gland
Bone, femur	Nerve, optic (paired)
Bone, sternum	Nerve, sciatic
Bone marrow, sternum	Ovary (paired)
Bone marrow smear	Pancreas
Brain (cerebrum, cerebellum, brainstem)	Parathyroid gland
	Pituitary gland
Cervix	Prostate gland
Epididymis (paired)	Salivary gland, mandibular (paired)
Esophagus	Skeletal muscle
Eye (paired)	Skin
Gallbladder	Spinal cord (cervical, thoracic, lumbar)
Heart	Spleen
Intestine, cecum	Stomach (cardiac, fundic, pyloric)
Intestine, colon	Testis (paired)
Intestine, duodenum	Thymus
Intestine, ileum (with Peyer's patch)	Thyroid gland (paired)
Intestine, jejunum	Tongue
Intestine, rectum	Trachea
Kidney (paired)	Urinary bladder
Lacrimal gland (paired)	Vagina
Liver	Uterus
Lung	Gross lesions/masses

Reviewed by a board-certified veterinary pathologist and did not reveal evidence of gross or histologic differences in any tissue between control animals and those that received 0.08 mg/kg CW002 followed by 200 mg/kg L-cysteine.