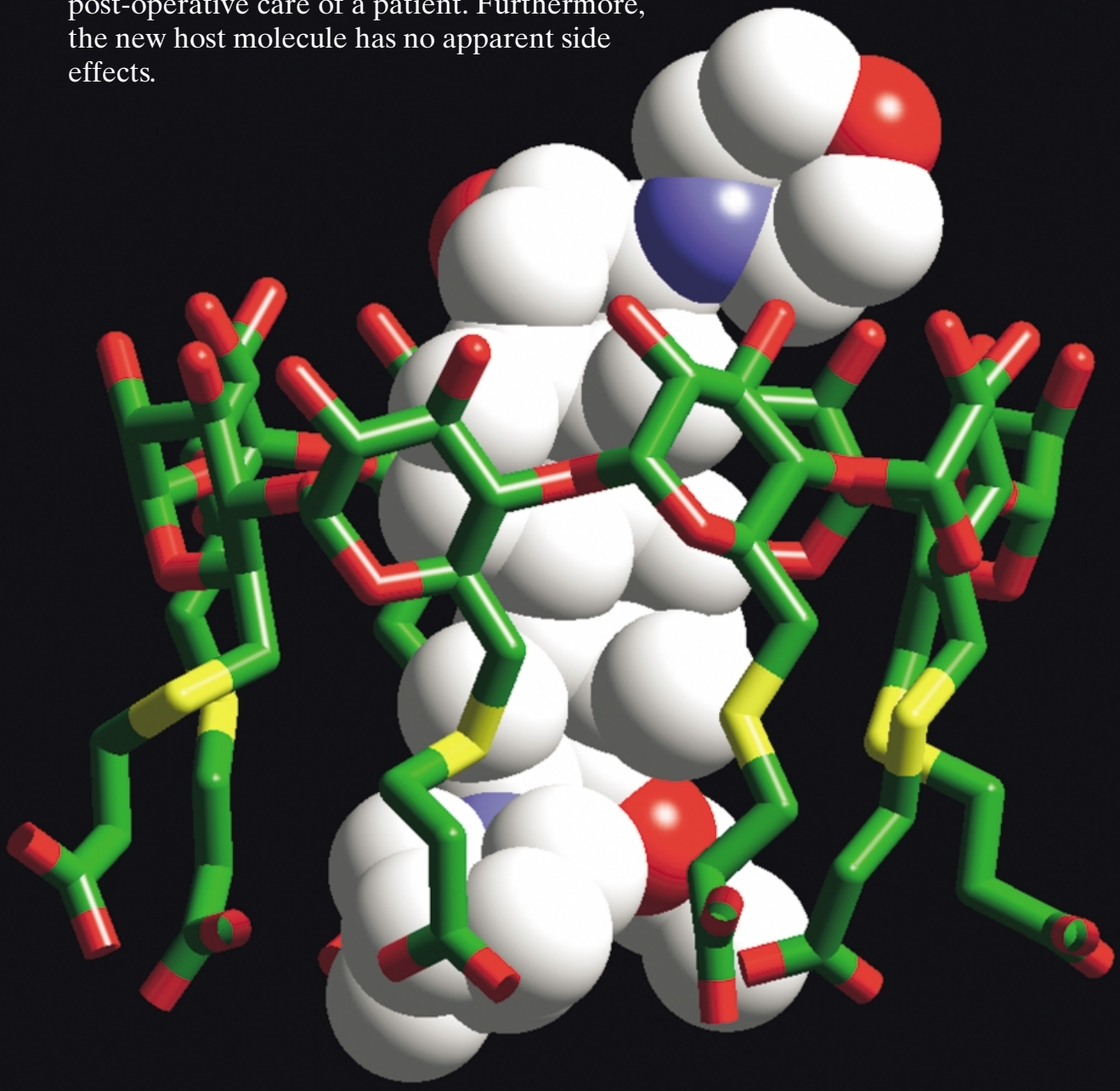
The steroid rocuronium, the most widely used neuromuscular blocking agent in anaesthesia, is completely encapsulated into the cavity of Org 25969, an extended γ-cyclodextrin host. The high affinity ($K_a = 10^7\ \text{M}^{-1}$) with which Org 25969 complexes rocuronium results in it reversing the biological activities of the steroid, thus it can be used to speed up the post-operative care of a patient. Furthermore, the new host molecule has no apparent side effects.



For more information see the following pages.

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A Novel Concept of Reversing Neuromuscular Block: Chemical Encapsulation of Rocuronium Bromide by a Cyclodextrin-Based Synthetic Host**

Anton Bom, Mark Bradley, Ken Cameron, John K. Clark, Jan van Egmond, Helen Feilden, Elizabeth J. MacLean, Alan W. Muir, Ronald Palin, David C. Rees, and Ming-Qiang Zhang*

Neuromuscular blockers (NMBs, also known as skeletal muscle relaxants) are widely used during anaesthesia to facilitate endotracheal intubation and to allow surgical access to body cavities, in particular the abdomen and thorax, without hindrance from voluntary or reflex muscle movement.[1, 2] Most of these drugs achieve their effect by blocking the physiological effects of acetylcholine (ACh) at nicotinic acetylcholine receptors (nAChR) on striated muscle cells. A reversal agent is frequently administered to patients to facilitate rapid neuromuscular recovery after surgery and to prevent residual blockade.[3, 4] At present, all clinically used reversal agents, such as neostigmine, pyridostigmine, and edrophonium, are inhibitors of acetylcholinesterase (AChE). They reverse the effect of NMBs by inhibiting the breakdown of ACh, thus increasing the concentration of ACh at the neuromuscular junction. However, this increase in ACh levels induced by AChE inhibition does not remain limited to the neuromuscular junction, but also leads to nonselective activation of muscarinic acetylcholine receptors (mAChR), thus causing many side-effects, for example, bradycardia, hypotension, increased salivation, nausea, vomiting, abdominal cramps, diarrhoea, and bronchoconstriction. Therefore, in clinical practice these drugs are usually used in combination with a mAChR antagonist such as atropine or glycopyrrolate, which themselves also have a number of side-effects, for example, dry mouth, blurred vision, tachycardia.

We hypothesized that chemical encapsulation (or chelation, complexation, sequestration) of NMBs by an exogenous host molecule would promote dissociation of NMBs from their site of action, and result in the reversal of neuromuscular blockade.[5] This mechanism of action does not involve direct interaction with cholinergic systems, and therefore, it should circumvent the undesired side-effects attendant with AChE inhibitors and furthermore eliminate the need for concomitant use of a mAChR antagonist such as atropine. We decided to investigate our hypothesis further with cyclodextrins (CDs), a group of cyclic oligosaccharides well-known for their capability to encapsulate lipophilic guest molecules such as steroids.[6] Compared with most small synthetic host molecules (including cyclophanes), CDs not only have a well-defined lipophilic cavity for host–guest complex formation, but are also generally more water soluble and biologically better tolerated.[7] In fact, several CDs have been successfully used as pharmaceutical excipients to increase water-solubility, stability, or bioavailability of lipophilic drugs.[8, 9]

The in vitro potency of the natural α-, β-, and γ-CDs to reverse rocuronium-induced neuromuscular block in isolated mouse hemi-diaphragm[10] correlates with their cavity sizes (Table 1). Only γ-CD, with the largest cavity size (diameter: 7.5-8.3 Å), showed reasonable reversal activity in vitro against rocuronium (concentration that is effective for 50% recovery of muscle contraction (EC_{50}): 34.6 μM, maximum reversal of 94.1% at 144 μM); the α- and β-CDs with the smaller lipophilic cavities (diameter: <6.5 Å) form less stable complexes with the bulky aminosteroid (molecule width ca. 7.5 Å). By using the NMR titration technique[11] we observed complexation-induced changes in the chemical shifts of protons from both the guest rocuronium molecule (for example, the axial methyl group (19-CH_3) and H-9α) and the hosts β- and γ-CDs (for example, the internal H-3 atom). The determined association constant of γ-CD with rocuronium ($K_a = 17000 - 20400\,M^{-1}$) by NMR titration is four- to fivefold higher than that of β-CD with rocuronium ($K_a = 3500 - 3900\,M^{-1}$), which is consistent with their rank order in reversal potency. More detailed analysis of complexation-induced changes in the chemical shifts revealed that the protons on the steroid with the largest chemical shift changes were all located on rings A, B, and C, which indicates there is partial encapsulation of the guest.

Two strategies were adopted to increase the binding affinity of the CD host to rocuronium. The first was to extend the cavity depth of γ-CD in order to achieve full encapsulation of all four steroidal rings within the lipophilic cavity. The most important contributions to complexation thermodynamics of CDs are believed to originate from: 1) penetration of the hydrophobic part of the guest molecule into the CD cavity and 2) dehydration of the organic guest,[12] that is, the principle factors involved in the binding of CDs with organic guests are van der Waals and hydrophobic interactions. The van der Waals interactions are highly dependent on the size and shape complementarity between the guest and the CD

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[**] The discovery of Org 25969 is the result of teamwork with contributions from a number of scientists both inside Organon and outside the company. We would like in particular to acknowledge the following scientists for their invaluable contributions: E. Hutchinson, D. Stevenson, R. Roy, and J. Pick for scaling-up the synthesis; F. Hope, S. Miller, and R. Mason for various in vitro and in vivo pharmacological testing; R. Watson, B. Montgomery, P. Desmond, and A. Osprey for all their analytical effort; and R. McGuire and J. Mestres for graphical presentation of the X-ray crystal structure of the Org 25969–rocuronium complex (Figure 3). We would also like to thank Prof. A. Cooper of Glasgow University who has critically reproduced our calorimetry data of Org 25969–rocuronium complexation (Figure 2).

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Table 1. In vitro reversal activity of the studied cyclodextrins against rocuronium-induced neuromuscular block in mouse hemidiaphragm.[10]

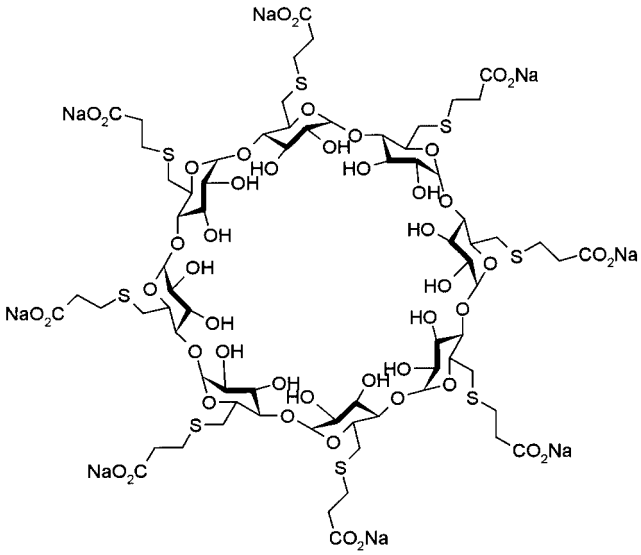
Compound	No. of glucose units in the ring (at conc. [μM])	Cavity dimension of the cyclodextrin		EC_{50} [μM][a] mean ± standard error	Max reversal [%][b] mean ± standard error
		diameter [Å]	height [Å]		
α-cyclodextrin	6	4.7 – 5.3[c]	7.9 ± 0.1[c]	> 360.0	9.7 ± 3.0 (360.0)
β-cyclodextrin	7	6.0 – 6.5[c]	7.9 ± 0.1[c]	> 360.0	29.0 ± 15.4 (360.0)
γ-cyclodextrin	8	7.5 – 8.3[c]	7.9 ± 0.1[c]	34.6 ± 10.4	94.1 ± 2.0 (144.0)
Org 25969	8	[d]	ca. 11[d]	1.2± 0.8	95.1 ± 2.3 (3.6)
neostigmine	–	–	–	0.9± 0.1	74.4 ± 9.5[e]

[a] Concentration that produces 50 % recovery of muscle twitch compared with prereversal twitch height. Data are presented as mean ± standard error of four independent determinations. [b] Maximum twitch recovery achieved with highest concentration tested. Data are percentage of the prereversal twitch height ($n = 4$). [c] Literature data[7] for free and uncomplexed CDs. [d] The cavity depth is estimated from the X-ray crystal structure of the Org 25969 – rocuronium complex. Since the modified CD ring is somewhat puckered and some of the propionic acid side chains have been modeled as being disordered over two sites, the precise diameter of the extended cavity cannot be given. [e] Cumulative administration of neostigmine bromide caused variable results. In some preparations neostigmine caused complete reversal, whereas in other preparations only limited reversal occurred.

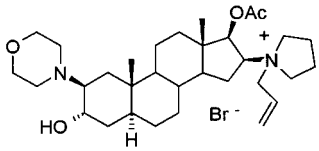
cavity (the size – fit concept). This means that to fully encapsulate rocuronium it is necessary to increase the cavity depth of γ-CD from 7.9 Å to about 11 Å, the approximate distance between the C-3 atom on ring A of the steroid and the C-16 atom on ring D. One way to achieve this is through perfacial substitution of all eight 6-hydroxyl groups of γ-CD with lipophilic groups. Such a per-6-modified γ-CD derivative would not only have a cavity with an increased depth but also an increased total area for hydrophobic interaction inside the cavity and, hence an improved hydrophobic interaction with rocuronium.

The second strategy was to introduce anionic functional groups, for example, carboxyls, at the rim of the cavity. These negatively charged groups would provide electrostatic interaction with the positively charged nitrogen atom of rocuronium, and also very importantly maintain the high water solubility of the resulting host molecule.

The biggest challenge in chemical modification of CDs is the presence of a large number of similarly reactive hydroxyl groups. Direct chemical modifications on natural CDs often result in heterogenous products, namely, mixtures of isomers.[8] To circumvent this, we chose a thioether as the linking unit for the construction of an extended cavity since this would allow us to use a nucleophilic substitution reaction involving a good nucleophile such as 3-sulfanylpropionic acid on the readily prepared per-6-iodo- or bromo-γ-CD.[13] This modification strategy provided us with a number of chemically pure per-6-deoxy-per-6-sulfanyl-γ-CD derivatives.[14, 15] Org 25969 (**1**, Scheme 1) is one of these derivatives, in which the γ-CD cavity has been extended from the primary side by three carbon atoms through a thioether linkage.



1, Org 25969



2, rocuronium bromide

Scheme 1. Structures of the synthetic cyclodextrin Org 25969 (**1**) and the steroidal neuromuscular blocker rocuronium bromide (**2**).

As shown in Table 1, Org 25969 has excellent in vitro reversal activity against rocuronium-induced neuromuscular block in isolated mouse hemi-diaphragm[10] with an EC_{50} value (1.2 ± 0.8 μM) similar to that of neostigmine. In this assay, Org 25969 produced almost complete reversal (95 %) of the muscle relaxant effects of rocuronium. Furthermore, the concentration of Org 25969 required to achieve this effect was the same as the concentration of rocuronium (3.6 μM). Org 25969 showed no observable effect on contractions of the isolated muscle when it was incubated with mouse hemi-diaphragm without pretreatment with rocuronium. Org 25969 did not cause any significant changes in the height of the electrically induced twitch response or the resting baseline tension at concentrations up to 100 μM, thus indicating its lack of intrinsic activity.

Consistent with its chelation mechanism of action, Org 25969 has been found to form a very tight binding complex with rocuronium. The association constant (K_a) of Org 25969 to rocuronium as determined by isothermal titration calorimetry (ITC) is about $10^7\,M^{-1}$ (Figure 1), with a negative ΔH and positive ΔS value, that is, the complexation is both enthalpy and entropy favored. Indeed, this level of binding affinity is rarely seen in small molecule host – guest interactions and has reached the range that is normally reserved for binding to biological macromolecules, for

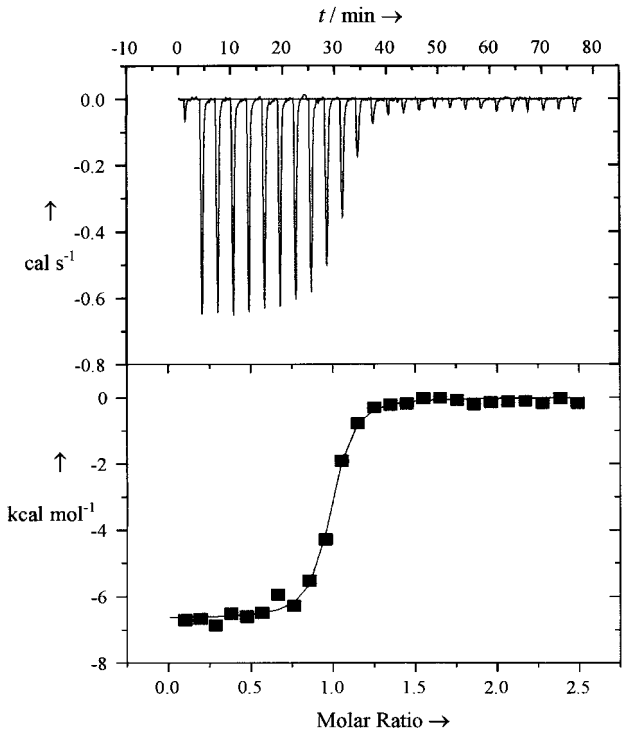


Figure 1. Isothermal calorimetry titration of Org25969 with rocuronium bromide at 25 °C. Instrument: Microcal VP-ITC (cell volume = 1.4 mL). Samples were made up by weight in 50 mM sodium phosphate buffer (pH 7.0) and degassed briefly at room temperature before loading. Standard injection sequence comprised an initial 1-μL injection followed by 25 × 10-μL titration injections at 3 minute intervals. A solution of Org25969 solution (21.6 μM) was in the cell, which was titrated with rocuronium bromide solution (278.5 μM) in the syringe. One binding site model was used to fit the data, which gives an association constant K_a of $1.05(\pm 0.16) \times 10^7\,\text{M}^{-1}$ (ΔH: -6658 ± 60 cal mol^{-1}, ΔS: 9.8 cal mol^{-1}, N = 0.9).

example, ligands to proteins. In fact, this is one of the most stable complexes of a CD with an organic guest ever reported in the literature![12]

The structure of the Org25969–rocuronium complex has been revealed by X-ray crystallography (Figure 2). In the crystalline form, rocuronium is encapsulated inside the CD cavity, with ring A, which contains the tertiary amine, pointing towards the opening of secondary hydroxyl groups and the polar 3-OH and 2-morpholine groups protruding outside the cavity and exposed to solvent. The CD ring is somewhat puckered, with the carboxyl-containing alkyl side chains forming the walls of an extended part of the cavity. The depth of the cavity is up to 11.5 Å, as estimated from the distances between the lowest hydroxyl oxygen atom on the secondary face and the uppermost carboxyl oxygen atom in the side chains. All four steroidal rings of rocuronium are in close contact with the lipophilic area of the cavity and the quaternary ammonium group on ring D is loosely surrounded by the carboxyl groups of Org25969. No direct interaction, however, is observed between the quaternary nitrogen atom and the carboxyl groups because the quaternary nitrogen atom is sterically shielded by the alkyl substituents and most of the formal charge will have been distributed through the surrounding alkyl substituents. A presentation of the complex with full van der Waals radii showed that the two structures are highly complementary to each other and form many close contacts (Figure 2C).

We have tested Org25969 in several animal models including guinea pigs, cats, and monkeys to investigate its potential clinical use for the reversal of rocuronium-induced neuromuscular block. Figure 3 shows the in vivo efficacy of Org25969 in anaesthetized Rhesus monkeys.[16] At 1.0 mg kg^{-1} (0.5 μmol kg^{-1}, intravenous (IV)) Org25969 reversed the rocuronium-induced block (block depth 90%) in a rapid and efficacious fashion to produce 90% recovery of muscle contraction within three minutes. By contrast, the standard treatment with neostigmine (40 μg kg^{-1}) and atropine (15 μg kg^{-1}) only produced the same level of recovery in more than six minutes. The action of Org25969 is therefore more than twice as fast as the standard treatment. Moreover,

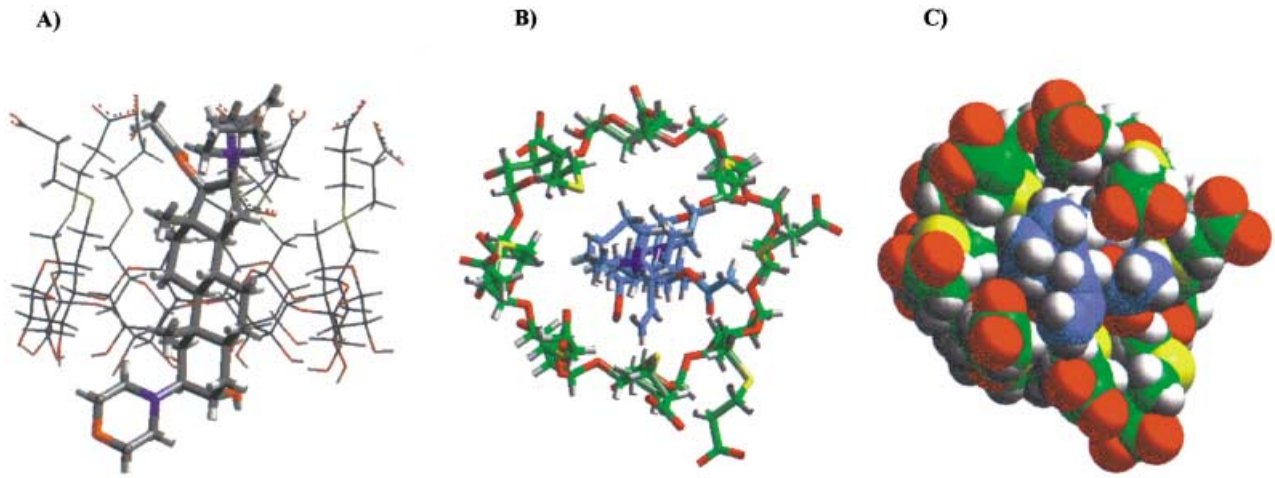


Figure 2. X-ray crystal structure of the Org25969–rocuronium complex. A) Side view showing polar substituents on ring A protruding outside the cavity from the opening of the secondary OH side and exposed to solvents. All four steroidal rings are encapsulated within the lipophilic cavity extended by per-6-alkyl substitution. B) Top-down view from the carboxyl side showing that the CD ring is somewhat puckered, with the carboxyl-containing alkyl side chains loosely surrounding the acetate and quaternary ammonium group on ring D. No direct interaction is observed between the quaternary nitrogen atom and the carboxyl groups. C) Same view as (B) but with a filled van der Waals surface showing that the two structures have many close contacts and are highly complementary to each other.

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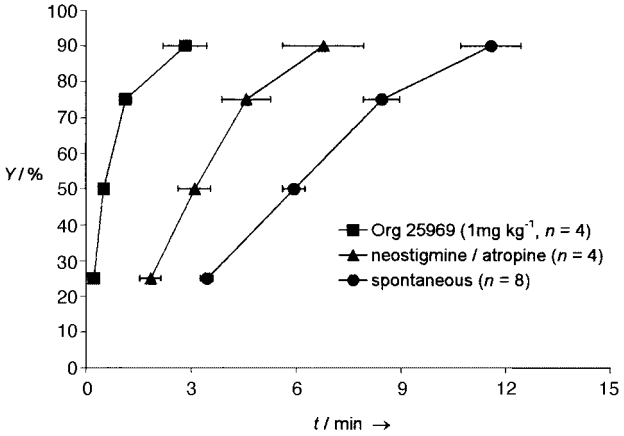

Figure 3. Reversal of rocuronium-induced neuromuscular block in anaesthetized monkeys by Org25969.[16] Ulnar nerve stimulation of anaesthetized Rhesus monkeys resulted in M. adductor pollicis contractions. A steady state 90% block of contractions was induced for 15–20 min by intravenous infusion of rocuronium. The infusion was then stopped to allow spontaneous recovery of muscle contractions. After complete recovery, a steady state 90% block was induced again. The infusion of rocuronium was stopped again and the reversal agent was given. The recovery of muscle contractions was measured again and the times of 25, 50, 75, and 90% recovery (Y) were determined. The results obtained with Org25969 (1.0 mg kg^{-1}) and neostigmine/atropine (40/15 μg kg^{-1}) are shown here as mean ± standard errors.

no significant changes in hemodynamic parameters, for example, blood pressure, heart rate, were observed during and after the treatment with Org25969 up to 10 mg kg^{-1}, IV (10 times the effective dose). All animals recovered completely without any complications. The compound is currently under development in human clinical trials.

In summary, we have designed a cyclodextrin-based synthetic receptor of rocuronium bromide with high affinity as shown by ITC and X-ray crystallography data. This synthetic cyclodextrin, Org25969, reverses the NMB effect of rocuronium bromide in vitro (mouse hemi-diaphragm) and in vivo (anaesthetized monkeys). We believe that the reversal of this biological activity is mediated by chemical encapsulation of the blocker, which is a novel therapeutic approach. This cyclodextrin derivative (Org25969) and its supramolecular mechanism of action appear to be superior to currently clinically used reversal agents in terms of speed and side effects. This approach appears to be a particularly good example of using a small synthetic host molecule as a reversal agent (or antidote, antagonist) to a biologically active drug.

Experimental Section

Org25969:[14] 3-sulfanylpropionic acid (1.22 mL, 14.0 mmol) was dissolved in dry DMF (45 mL) under N_2 at room temperature. Sodium hydride (1.23 g, 30.8 mmol, 60%) was added to this solution in three portions and the mixture was stirred for a further 30 min. A solution of 6-per-deoxy-6-per-iodo-γ-cyclodextrin[13] (3.12 g, 1.40 mmol) in dry DMF (45 mL) was then added dropwise to this mixture. After addition, the reaction mixture was heated at 70 °C for 12 h. After cooling, water (10 mL) was added to the mixture and the volume was reduced to 40 mL in vacuo. Addition of ethanol (250 mL) resulted in precipitation. The solid precipitate was collected by filtration and dialyzed for 36 h. The volume was then reduced to 20 mL in vacuo. Ethanol was then added, and the precipitate was collected by filtration and dried to afford Org25969 as a white solid (1.3 g, 43%). ^{1}H NMR (400.13 MHz, D_2O with sodium 3-trimethylsilyl propionate as reference): $\delta = 2.43-2.58$ (m, 16H), 2.88 (t, 16H), 3.01 (dd, 8H), 3.15 (d, 8H), 3.61–3.73 (m, 16H), 3.97 (t, 8H), 4.07 (m, 8H), 5.19 (d, 8H); MS (flow injection analysis, negative ion), run as the acid: m/z: 2000.5 $[M - 8\,\mathrm{Na} + 7\,\mathrm{H}]^-$.

X-Ray crystallography of the Org25969–rocuronium complex: An equimolar mixture of Org25969 (726 mg) and rocuronium (203 mg) was suspended in a DMF/H_2O mixture (2.5/1, 20 mL) and heated to about 90 °C. Water was added dropwise till complete solution was achieved and a further three drops were added. The solution was allowed to cool to room temperature slowly, to afford the complex as small prisms which were recrystallized once by the same procedure. The crystal ($0.30 \times 0.08 \times 0.02$ mm) was mounted on the end of a two-stage glass fibre with perfluoropolyether oil, and cooled by a Cryostream nitrogen-gas stream.[17] The wavelength was calibrated by measurement of the unit cell parameters of a standard crystal of known structure. Single-crystal data (65420 reflections) were collected on a Bruker AXS SMART CCD area-detector diffractometer on station 9.8 at the Daresbury Laboratory.[18] The unit cell determined at 150 K is $a = 30.9459(21)$, $b = 39.2446(27)$, $c = 13.3952(9)$ Å^2, $\alpha = 90$, $\beta = 90$, $\gamma = 90°$, space group $P2_12_12$, $\rho_{calcd} = 1.106$ g cm^{-3}, final R indices: $R1 = 0.1037$, $wR2 = 0.2720$. The structure was solved using the SnB program[19] which implements Shake and Bake methods (more commonly applied to protein crystallography) and refined by least-squares refinement on all unique measured F^2 values.[20] Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-172247. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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[10] The hemi-diaphragm with its phrenic nerve from male mice (20–30 g) was mounted on a tissue holder in a 20-mL tissue bath filled with a modified Krebs–Henseleit buffer (pH 7.4) at 37 °C, bubbled with 95% oxygen and 5% carbon dioxide. The buffer contains the following composition: NaCl: 118, KCl: 5, KH_2PO_4: 1, $MgSO_4$: 1, $NaHCO_3$: 30, $CaCl_2$: 2.5, and glucose: 20 mM. The phrenic nerve was stimulated continuously using a Grass S88E stimulator (rectangular pulses of 0.2 ms every 20 s at a supra-maximal voltage of 2.5 V) and isometric force was recorded using Grass FT03 transducers and a Grass 79D recorder. After a stimulation period of at least 30 min, rocuronium bromide was added to the bath (final concentration of rocuronium 3.60 μM) to produce approximately 90% twitch block. Twenty minutes later, increasing concentrations of reversal agents were added in a cumulative fashion, at intervals of 10 min. The concentrations of compounds producing 50% and maximum recovery of twitch height were determined.
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A Ring-in-Ring Complex**

Sheng-Hsien Chiu, Anthony R. Pease, J. Fraser Stoddart,* Andrew J. P. White, and David J. Williams*

Over the past two decades a vast array of interlocked and intertwined molecular compounds—specifically, catenanes, rotaxanes, and knots[1]—have been assembled using supramolecular assistance (templation[2]) as the key element in their synthesis[3] under either kinetic[4] or thermodynamic[5] control. Although these synthetic protocols have been implemented more recently for the construction of more intricate variants, such as oligocatenanes,[6] molecular necklaces,[7] and cyclic daisy chains[8] to name but a few, the topological challenge of the de novo synthesis of Borromean ring compounds[9] still presents a considerable hurdle to be overcome. Our way of addressing this challenge is to employ recognition motifs to create initially stable ring-in-ring superstructures[10] which could serve as templates for subsequent catenation. Here, we report 1) the design and noncovalent synthesis of a prototype, mutually orthogonal, partially preorganized, ring-in-ring complex, together with 2) the solid-state characterization of the 1:1 complex in the context of the X-ray crystal structures of its two separate ring components, and 3) the solution-state behavior of the 1:1 complex.

Previously, we have shown[11] that bisparaphenylene[34]-crown-10 (BPP34C10) can encircle two dibenzylammonium (DBA^+) ions simultaneously by locating the two NH_2^+ centers 6.9 Å apart in the polyether loops of BPP34C10 and relying upon N^+–H···O hydrogen bonds to form a stable 1:2 ($DBA^+ \subset BPP34C10 \supset DBA^+$) complex. Thus, it seemed not unreasonable to us that BPP34C10 might also be able to complex (Figure 1) with a suitably proportioned dicationic

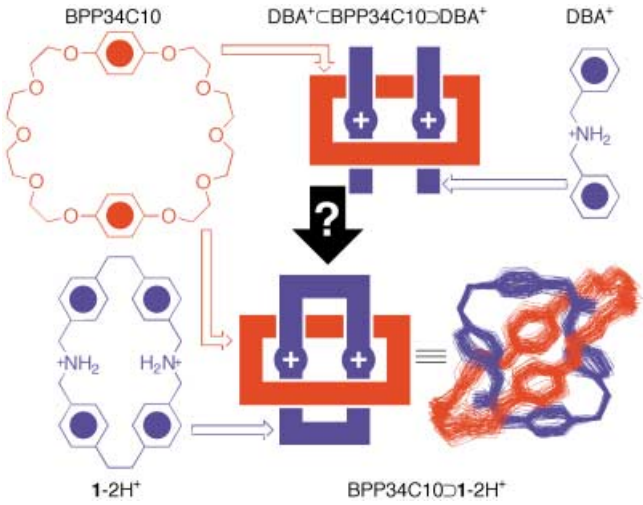

Figure 1. Employing precedent in the design and synthesis of a proposed 1:1 complex formed between BPP34C10 and **1**-2H^{2+}, together with an overlaid set of 70 co-conformations of BPP34C10 · **1**-2H^{2+} sampled from the last 70 ps of a 100-ps MD experiment.

macrocycle containing two NH_2^+ centers. After inspection of the solid-state superstructure[11] of the 1:2 complex formed between BPP34C10 and DBA^+, and construction of CPK space-filling molecular models, we concluded that the macrocycle **1**-2H^{2+} was a suitable candidate for further investigation. Our intuition was given a considerable boost by the results of the molecular dynamics (MD) calculations.[12] They predicted the existence of a stable co-conformation[13] in which the BPP34C10 ring encircles the dicationic macrocycle, such that the polyether loops surround the NH_2^+ centers, namely, the 1:1 (BPP34C10 ⊃ **1**-2H^{2+}) complex (illustrated in Figure 1) might constitute a discrete supermolecule.

The synthesis (Scheme 1) of the dicationic macrocycle **1**-2H · 2PF$_6$ was achieved by employing a bis-Wittig reaction in the key cyclization step. The bisphosphonium salt[14] **2**-H · 3PF$_6$ was protected (Boc_2O/Et_3N/MeOH) to give **3** · 2PF$_6$

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