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2-*O*-Substituted Cyclodextrins as Reversal Agents for the Neuromuscular Blocker Rocuronium Bromide

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Abstract—A series of secondary face modified cyclodextrins (CDs) were synthesised with the aim of constructing host molecules capable of forming host–guest complexes with neuromuscular blockers, especially with rocuronium bromide. Perfacial 2-*O*-substitution of γ-CD with 4-carboxybenzyl resulted in a CD host molecule **1** that forms a 1:1 binary complex with rocuronium bromide (K_a 6.2×10^5 M^{-1}). The biological activities of this compound and other derivatives as reversal agents of rocuronium bromide were examined in vitro (mouse hemi-diaphragm) and in vivo (anaesthetized guinea pigs). The host molecule **1** was found to exert potent reversal activity (ED_{50} 0.21 μmol/kg, iv) against rocuronium-induced neuromuscular block, and thus proved the viability of using host molecules as antidotes of a biologically active compound.

Introduction

The reversal of neuromuscular block at the end of surgery is often necessary to speed up recovery of a patient's muscle function and to prevent residual neuromuscular block. Residual muscle weakness after surgery is still very common even with neuromuscular blocking agents (NMBAs) that have intermediate duration of action.[1] Therefore reversal of neuromuscular block contributes to a patient's safety.

All current clinically used reversal agents, such as neostigmine and edrophonium, exert their activity by inhibiting acetylcholine esterase (AChE), to increase the level of acetylcholine (ACh) at the neuromuscular junction.[2]

The use of AChE inhibitors as reversal agents leads to problems with selectivity, since neurotransmission at both nicotinic and muscarinic receptors involving ACh is potentiated by these agents. This lack of selectivity causes many side effects including bradycardia, hypotension, and so on. Therefore, in practice, these agents are only used after or together with the administration of atropine or glycopyrrolate to antagonise the muscarinic effects of ACh. Unfortunately, muscarinic antagonists themselves also cause a number of side-effects, for example, tachycardia, dry mouth, blurred vision, difficulties in emptying the bladder and so on, and, furthermore, may affect cardiac conduction.

We have therefore been active in developing compounds that act by means of an alternative mode of action.[3–5] We hypothesised that chemical encapsulation of a NMBA by a host molecule would promote its dissociation from the nicotinic receptor and therefore could be a viable method for NMB reversal. Since this mechanism of action does not involve direct interaction with the cholinergic system, it may circumvent the undesired side effects associated with AChE inhibitors. The lead compound chosen for initial studies was the cyclic oligosaccharide γ-cyclodextrin (γ-CD). In vitro studies of this compound showed that it acted as a weak reversal agent of rocuronium bromide (Fig. 1), the most widely used NMBA in anaesthesia. We believed that the reversal of rocuronium activity by γ-CD resulted from chemical encapsulation of the NMBA inside the cyclodextrin cavity. To optimise this lead and increase the binding affinity of a cyclodextrin derivative to the NMBA rocuronium, we carried out series of chemical modifications of cyclodextrins (CDs).[4,5] In this paper, we describe the

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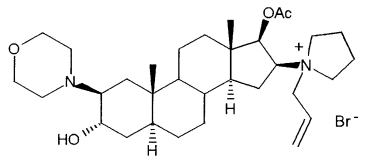


Figure 1. Chemical structure of rocuronium bromide, the target guest molecule of this study.

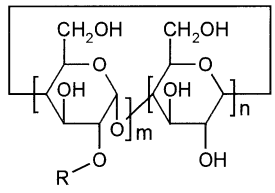


Figure 2. General formula of the modified CDs reported in this paper. For per-substituted γ-CDs $m=8$, $n=0$; for mono-substituted γ-CDs $m=1$, $n=7$. For per-substituted β-CDs $m=7$, $n=0$; for mono-substituted β-CDs $m=1$, $n=6$. R = alkyl or substituted benzyl groups.

modifications on the secondary face of CDs (Fig. 2) and their effects on the reversal activities of the resulting host molecules.

The design of these target compounds (Fig. 2, Table 1) was based around the size of the CD cavity with respect to the steroidal skeleton of rocuronium (necessitating β- or γ-CD) and the fact that rocuronium contains a positively charged quaternary nitrogen. We chose to mono and per substitute β- and γ-CDs with a series of aromatic and aliphatic side chains containing terminal acidic or polar functional groups. The lipophilic side chains were expected to increase hydrophobic interaction with rocuronium and the acidic functional groups were introduced to form electrostatic interaction with the positively charged nitrogen of the blocker. Furthermore, the salts of these acidic groups would contribute to the high water solubility of the resulting compounds. The length of side chains was varied in order to optimise the cavity depth and the position of electrostatic interaction, to achieve high binding affinity. For general background information on cyclodextrin–steroid complexation[6] and chemical modifications[7] of cyclodextrins, readers are referred to the cited literature reviews.

Synthesis

The target CDs (Fig. 2) were synthesised by either benzylation or alkylation of the 2-hydroxyls (Scheme 1) under basic conditions, or by radical addition of a thiol to 2-*O*-allyl CDs[8] (Scheme 2).

Typically, before 2-*O*-benzylation or alkylation, all 6-hydroxyls of the starting β- or γ-CD were protected with *tert*-butyldimethylsilyl (TBS).[9] Such a per-6-*O*-protected CD was then allowed to react with a benzyl or alkyl halide under basic conditions. The most effective protecting group for carboxyls of 3- or 4-carboxybenzyl halide under these conditions was found to be the 2-(trimethylsilyl)ethyl ester, which also offered the advantage of simultaneous deprotection with the per-6-*O*-TBS groups when treated with either tetrabutylammonium fluoride (TBAF) or boron trifluoride (Scheme 1). For the synthesis of compounds **9** and **10**, direct alkylation of the un-protected γ-CD also yielded the desired products although in general per-6-*O*-silylated CDs are preferred starting materials for ease of purification.

Attempts to per-substitute the 2-hydroxyls with aliphatic alkylating agents other than methyl or allyl iodide were found to give mixtures of products under various reaction conditions, which were difficult to isolate and purify. This might relate to the lower reactivity of these alkylating agents. There are few examples of 2-*O*-substituted CDs with aliphatic groups reported in the literature.[7,10]

To vary the chain length of the 2-*O*-aliphatic substituents, we chose to use the readily prepared 2-*O*-allyl-β- or -γ-CDs[11] as the starting materials. Radical addition[8] of thiols to the allyl double bond offered a series of 2-*O*-(3-mercaptopropyl) CD derivatives (Scheme 2). The terminal acid functions in the alkylating agents were protected either as simple alkyl esters or as the free acid. Aromatic thiols gave incomplete reactions due to dimerization and other side reactions of the thiols.

Purification of chemically modified CDs is always a challenge. For the current series of compounds, the intermediates before deprotection of 6-*O*-silyl groups were most easily purified by silica gel chromatography. The final per-2-*O* substituted derivatives were purified by removing low molecular weight (LMW) materials with dialysis, or G25 size exclusion Sephadex column (Aldrich). For mono-2-*O* substituted CDs, purification was performed by a DEAE (amine function) Sephadex column eluted with water to remove non-acid containing CD materials, followed by 0.1M NaOH to isolate the mono sodium salt of the acid.

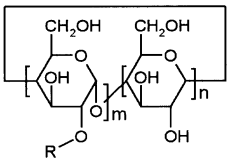
Results and Discussion

All compounds were screened for their reversal activity against rocuronium-induced neuromuscular block in isolated mouse hemi-diaphragm (Table 1). Those that showed reasonable activity in this in vitro assay were then tested in anaesthetized guinea pigs to evaluate their in vivo potency. The compounds in Table 1 are listed with the order of decreasing reversal potency. In this way it is clear to see which modifications improved the activity in comparison with the lead γ-CD (entry **8** in Table 1) and which modifications did not.

First of all, most of the compounds that are more potent than **8** are γ-CD derivatives (**1**–**3**, **5**–**7**) with negatively charged acidic groups at the end of the 2-*O* substituents. Compounds with neutral, basic or zwitterionic substituents (**9**, **11**, **12**, **15**–**21**) are mostly less active than γ-CD itself. The most notable exception to this is compound **4**.

Although β-CD (**13**) is less active than γ-CD (**8**), most probably due to its smaller cavity size, the glycerolpropyl thioether derivative **4** exhibited about 4-fold

Table 1. Reversal activity of 2-*O*-substituted CDs versus rocuronium bromide induced neuromuscular block



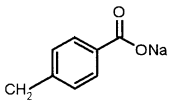
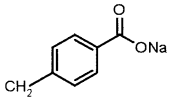
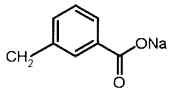
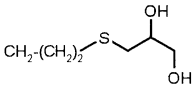
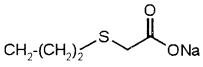
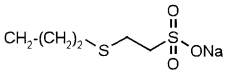
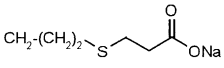
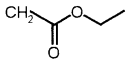
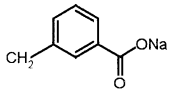
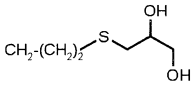
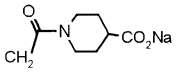
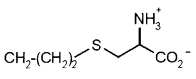
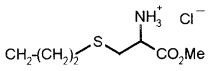
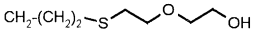
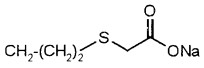
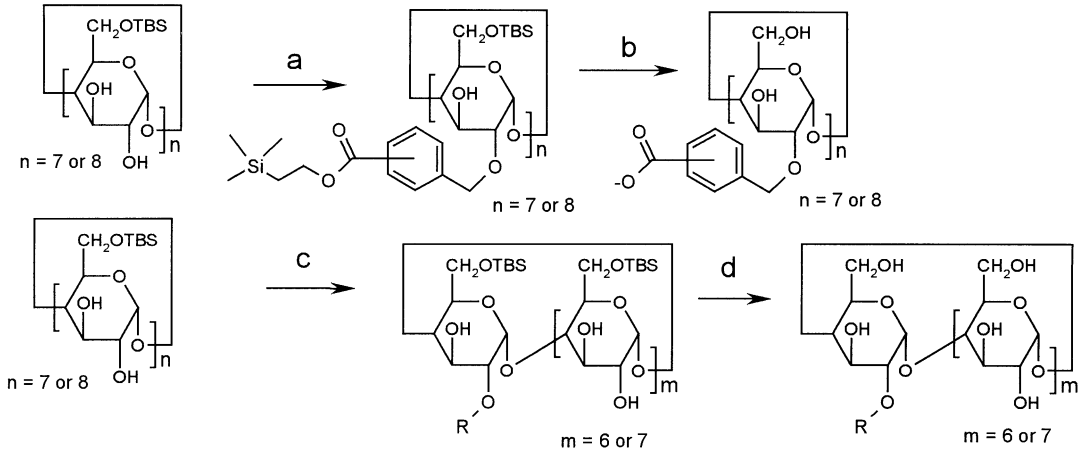
Compound	R	*m*	*n*	Purity [a]	In-vitro activity [b]		In-vivo activity [c]	
					EC_{50} (μM)	% max reversal (at μM)	ED_{50} (μM/kg)	% max reversal (at μmol/kg)
1		8	0	93	7.2±2.0	100.8±2.8 (5.4)	0.21±0.02	96.7±1.1 (0.8)
2		1	7	75	3.4±0.6	95.6±2.4 (9.0)	0.25±0.11	96.0±1.7 (2.6)
3		8	0	90	2.2±0.4	92.4±1.5 (7.2)	0.38±0.04	104.1±6.5 (4.8)
4		7	0	80	11.5±2.9	75.8±13.0 (18.0)	1.1	93.0 (6.4)
5		8	0	70	>18.0	24.3±19.8 (18.0)	1.1	90.8 (10.0)
6		8	0	70	>18.0	34.8±19.8 (14.4)	1.4	84.5 (4.5)
7		8	0	70	23.1±12.9	33.3±11.2 (12.6)	3.4	85.7 (16.0)
8	H	8	0	98[d]	34.6±10.4	94.1±2.0 (144.0)	4.0±0.0	104.7±8.6 (47.0)
9		1	7	85	56.3±14.5	98.6±5.2 (252.0)	3.9	93.5 (160.0)
10		1	7	90	67.0±21.7	48.4±7.2 (7.2)	5.5±0.7	95.8±3.0 (26.0)
11		1	7	70	21.8±7.3	51.0±16.5 (18.0)	8.8	55.5 (13.0)
12		8	0	90	32.0±8.6	24.0±9.3 (18.0)	8.9	55.9 (8.0)
13	H	7	0	98[d]	>360.0	29.0±15.4 (360.0)	20.0±7.0	92.9±10.3 (113.0)
14		8	0	85	>360.0	0.0±0.0 (360.0)	39.2	41.5 (32.0)
15		8	0	80	21.2±3.9	30.5±8.4 (18.0)	>1.6	4.3 (1.6)
16		8	0	88	>18.0	5.6±3.6 (5.4)	n.t. [e]	n.t.[e]

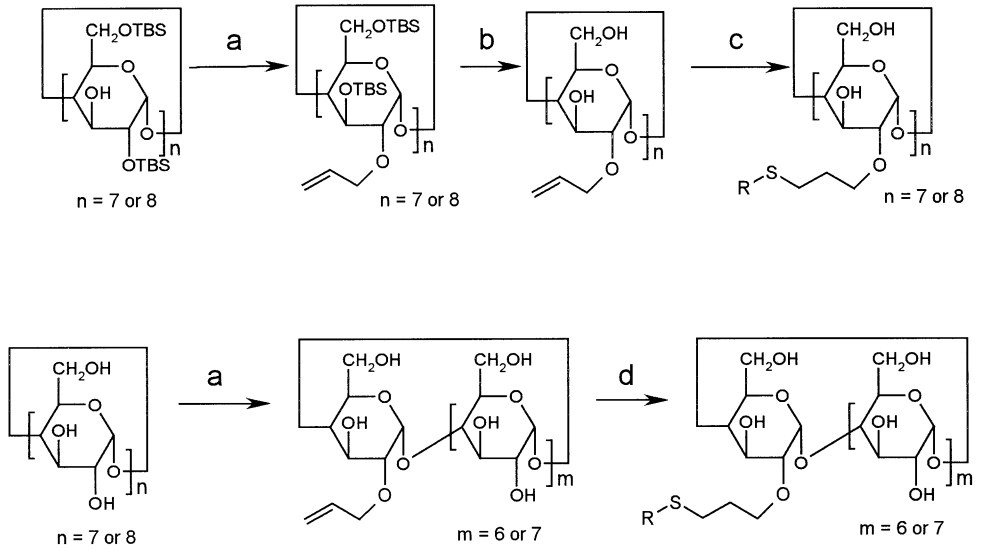
Table 1 (*continued*)

Compound	R	*m*	*n*	Purity [a]	In-vitro activity [b]		In-vivo activity [c]	
					EC_{50} (μM)	% max reversal (at μM)	ED_{50} (μM/kg)	% max reversal (at μmol/kg)
17		8	0	70	25.4±3.5	28.2±5.3 (18.0)	5.6	72.3 (12.0)
18		8	0	90	>360.0	0.0±0.0 (180.0)	n.t.[e]	n.t.[e]
19		8	0	90	>360.0	0.0±0.0 (360.0)	n.t.[e]	n.t.[e]
20		7	0	90	>360.0	0.0±0.0 (360.0)	n.t.[e]	n.t.[e]
21[f]	CH_3	7	0	90 70	>360.0 >18.0	0.0±0.0 (344.0)	n.t.[e]	n.t.[e]
22		1	6			0.0±0.0 (18.0)	n.t.[e]	n.t.[e]
Neostigmine bromide[g]	—	—	—	—	0.9±0.1	74.4±9.5	0.04±0.00	65.3±5.3 (0.08)

[a]Data are represented as the minimum purity determined by two diverse chromatography systems: HPLC-ELSD with a Phenomenex Aqua C18 (25×0.46 cm) column eluted with CH_3CN/H_2O 25/75+0.1% HCOOH) and a Time of Flight (TOF) LC–MS with a Jupiter C18 (150×4.6 mm) eluted with CH_3CN/H_2O gradient+0.1% HCOOH), and a 400 MHz ^1H NMR spectroscopy.
[b]Data are presented as means±SEM of at least four independent experiments. The concentration of rocuronium bromide in the organ bath was 3.6 μM, which produced ~90% reduction of the twitch height. EC_{50}=concentration that produces 50% recovery of muscle twitch compared with pre-reversal twitch height.% max reversal=Maximum twitch recovery achieved with highest concentration tested in bracket.
[c]Data are presented as means±SEM of at least two independent experiments or a single experiment when the potency was of low interests. An average of 90% (80–97%) neuromuscular block was achieved by continuous iv infusion (~10 nmol/kg/min) of rocuronium bromide and applying cumulative doses of CD. ED_{50}=dose (iv) that produces 50% recovery of muscle twitch compared with pre-block twitch height.% max reversal=-Maximum twitch recovery achieved with highest dose tested in bracket.
[d]Data provided by the commercial supplier Wacker-Chemie GmbH and were not checked by the authors.
[e]Not tested.
[f]Known compound (see reference 12).
[g]Cumulative administration of neostigmine bromide caused variable results. In some preparations neostigmine caused complete reversal, whereas in other preparations only limited reversal occurred.



Scheme 1. Synthesis of 2-*O* alkylated CDs; (a) *p*- or *m*-$(CH_3)_3SiCH_2CH_2OC(O)PhCH_2Br$ (or I), BaO, $Ba(OH)_2$, rt 2 days; (b) TBAF, THF, 3 h Δ; (c) NaH, RCH_2X rt, o/n R=alkyl or carboxybenzyl, (d) TBAF, THF, Δ.



Scheme 2. Synthesis of 2-*O* alkylated CDs; (a) Allyl iodide, NaH. o/n; (b)TBAF, 1 h; (c) RSH, AIBN Δ2–24h (d) RSH, AIBN, 1 h, Δ. Toluene, MeCN water or MeOH. 100% conversion.

increased in vitro and in vivo potency compared with the lead γ-CD. It is quite remarkable that its γ-CD analogue **12** showed much weaker activity. It is possible that this particular modification resulted in an extended cavity of β-CD that has better structural complementarity to rocuronium than the similarly extended γ-CD cavity.

The comparison between the two regioisomers of per-2-*O*-carboxybenzyl-γ-CDs **1** and **3** suggests that *para*-carboxyl is better positioned to interact with the positively charged nitrogen of rocuronium and hence better activity. This is further supported by the higher potency of the mono-2-*O*-*para*-carboxybenzylated γ-CD **2** than its *meta*-substituted analogue **10**.

The rigid benzene ring appears to be a better linker between the acidic group and the CD backbone, probably by contributing to the pre-organisation of the binding cavity. This may explain the weaker activity of 2-*O*-aliphalic alkylated γ-CDs (**5**–**7**), because the flexible alkyl linkers may be self-included within the cavity and thus restricting the entry of rocuronium to the cavity. Within this small group (**5**–**7**), the longer and the more flexible the linker is, the less potent is the reversal activity.

The formation of a host–guest complex between **1** (the most potent compound of the current series) and rocuronium bromide was confirmed by isothermal titration calorimetry (Fig. 3). The complexation was endothermic, suggesting that conformational changes of the host were necessary to accommodate the guest. Restriction of cavity entrance by π–π stacking (face-to-face and/or edge-to-face) between the benzene side chains may be the reason for such requirement of conformational change. The stoichiometry of the complex is 1:1 and the association constant (K_a) is about 62,000 M^{-1}. In a separate experiment using the NMR titration technique,[13] the lead γ-CD (**8**) was found to have an association constant of about 17,000 M^{-1} for the complexation with rocuronium. The rocuronium-

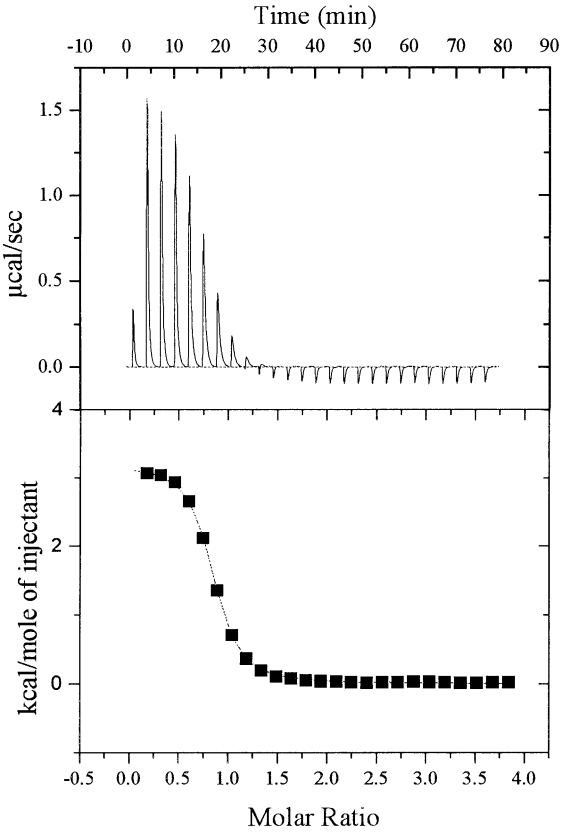


Figure 3. Isothermal titration calorimetry of compound **1** and rocuronium bromide. A buffer solution (pH 7.0) of rocuronium bromide (1.5 mM) was titrated into the same buffer solution of compound **1** (0.075 mM). The results show the endothermic complexation between **1** and rocuronium, $\Delta H = 3198 \pm 12.06$ cal/mol, $\Delta S = 37.20$ cal/mol, $N = 0.80$, and $K_a = 6.21 \pm 0.20 \times 10^5$ M^{-1}.

binding affinity difference between **1** and **8** is consistent with the proposed supramolecular mechanism of action of these compounds, that is, the CD (**1**) with higher binding affinity to rocuronium is more potent in reversing the activity of rocuronium than **8**.

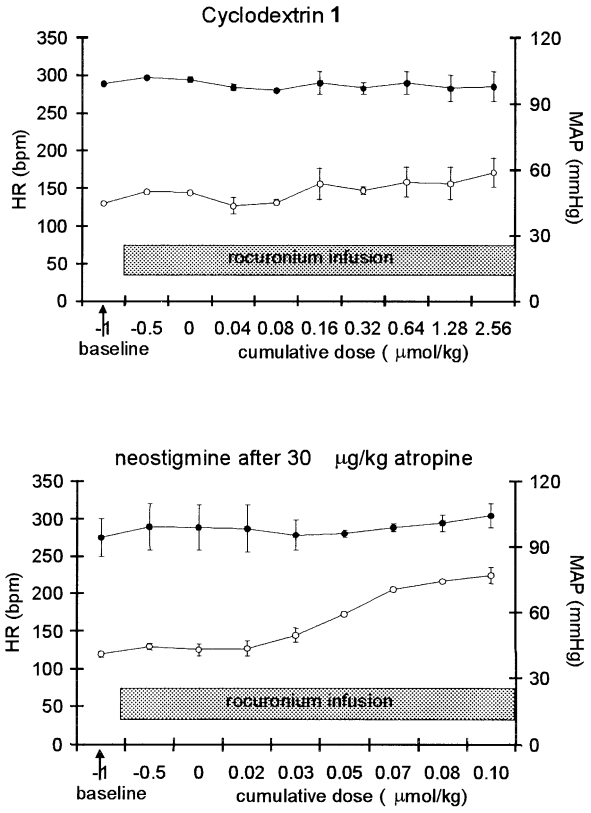


Figure 4. Cardiovascular effects of compound **1** in anaesthetised guinea pigs, in comparison with the combination of neostigmine plus atropine. During continuous iv infusion of rocuronium bromide, increasing doses of the reversal agents (**1** or neostigmine) were given by bolus iv injection. Data presented are means of two independent experiments. The open circles represent mean artery blood pressure (MAP) and the closed circles represent hear rate (HR).

Conclusion

In conclusion, we have shown that modification of γ-CD cavity by substitution of the 2-hydroxyls, especially with carboxybenzyls, resulted in compounds with improved reversal activity against rocuronium-induced neuromuscular block. We believe this increase in reversal activity is related to the improvement in host–guest complexation between the modified CD and rocuronium. The key factors contributing to this improvement in host-guest complexation are thought to be electrostatic interaction via the negatively charged terminal acidic groups and the better pre-organized binding cavity, for example, size, rigidity of the linker, the positioning of the acidic group, and so on.

The fact that some of these 2-O substituted CDs, for example **1**, showed potent in vitro and in vivo reversal activity against rocuronium bromide indicates that chemical encapsulation of NMBAs is a viable approach to design novel reversal agents with new mechanism of action.

In anaesthetised guinea pigs, compound **1** (coded Org 25819) was found not to cause any significant changes in heart rate (HR) and mean arterial blood pressure (MAP), during the entire process of cumulative dosing up to 2.56 μmol/kg, that is, >3-fold higher than the effective reversal dose of 0.8 μmol/kg (Fig. 4, upper graph). Pretreatment with the muscarinic receptor antagonist atropine (30 μg/kg) prevented the decrease in heart rate, normally observed after neostigmine administration (Fig. 4, lower graph). However, atropine does not block nicotinic receptors. The neostigmine-induced increase in ACh levels in the autonomic ganglia, followed by an increased activity of the postganglionic sympathetic nervous system, resulted in a dramatic increase in arterial blood pressure (Fig. 4, lower graph), from a baseline value of 43 mmHg to 74 mmHg at the effective reversal dose of 0.08 μmol/kg (or 25 μg/kg).

The data in Figure 4 further demonstrate the superiority of chemical encapsulation of a NMBA over AChE inhibition as a method of NMB reversal. Because this supramolecular mechanism of action does not involve direct interaction with the cholinergic systems, the use of a NMBA-encapsulating host molecule does not cause significant cardiovascular side effects.

Experimental

Chemistry

The starting materials β- and γ-CDs were purchased from Wacker-Chemie GmbH (Munich, Germany) and they were of the pharmaceutical grade with >98% purity. All other chemical reagents were obtained from commercial suppliers and used without further purification. All NMR spectra were recorded at 200 or 400 MHz on Bruker AM200 or 400 spectrometers, and chemical shifts are reported in ppm relative to TMS or sodium 3-trimethylsilylpropionate. Purity of all compounds was determined by at least two diverse chromatography systems: HPLC-ELSD with a Phenomenex Aqua C18 (25×0.46 cm) column eluted with CH_3CN/H_2O 25/75+0.1% HCOOH), and a Time Of Flight (TOF) LC-MS using a PerSeptive Biosystems Mariner TOF instrument with a Jupiter C18 (150×4.6 mm) eluted with CH_3CN/H_2O gradient+0.1% HCOOH)

2-*O*-Per-(4-carboxybenzyl) γ-CD sodium salt (1). To a solution of 4-(chloromethyl)benzoyl chloride (2.5 g, 13.22 mmol) in dichloromethane (100 mL) was added 2-(trimethylsilyl)ethanol (1.72 mL, 12.0 mmol). Triethylamine (9.21 mL, 66 mmol) was added and the reaction stirred overnight. The dichloromethane was evaporated and the crude material taken up in ether and washed with water. The ether layer was dried (sodium sulfate) and evaporated to give a slightly yellow mobile oil. The oil (2.7 g) was dissolved in acetone (30 mL) containing sodium iodide (1.5 g) and stirred for 2 h. The solvent was removed ether added and the reaction filtered. The filtrate was then evaporated to give 2-trimethylsilylethyl 4-iodomethylbenzoate as a brown temperature unstable oil, 3.58 g, 75%: ^1H NMR ($CDCl_3$) δ 7.88 (2H, d), 7.34 (2H, d), 4.38 (2H, s), 4.32 (2H, m), 1.04 (2H, m), 0.01 (9H, s). Mass spectrum (M-I) 235

To a solution of octakis-per-6-*O*-(*tert*-butyldimethylsilyl)-γ-CD (0.5 g, 0.225 mmol) in dimethylformamide (20 mL) was added barium hydroxide octahydrate (0.5 g, 1.58 mmol) followed by barium oxide (0.9 g, 5.87 mmol). 2-(Trimethylsilyl)ethyl 4-iodomethylbenzoate (2 equiv per hydroxyl) was added and the reaction stirred for 24 h. The reaction mixture was filtered to remove barium oxide and poured into water to remove barium hydroxide. The crude CD precipitate was dissolved in dichloromethane and dried (sodium sulfate). The solution was filtered and the solvent evaporated to a small volume. Methanol was added to triturate the compound which was then filtered and dried in vacuo.

Tetrabutyl ammonium fluoride (17.65 mL, 1.0 M solution in tetrahydrofuran, 17.6 mmol) was added to a solution of per-2-*O*-[4-{2-(trimethylsilyl)ethylcarboxy} benzyl]-6-*O*-(*tert*-butyldimethylsilyl)-γ-cyclodextrin (2.4 g, 0.59 mmol) in tetrahydrofuran (30 mL). The reaction mixture was refluxed for 2 h and allowed to cool to room temperature. The solvent was removed under reduced pressure and water (150 mL) added. The pH was adjusted to 4 by addition of 2 M hydrochloric acid and the solid formed was filtered off. Washing with acetone gave 1.2 g of per-2-*O*-(3-carboxybenzyl)-γ-cyclodextrin as a pale solid. To this was added methanol (100 mL) and sodium hydroxide (0.16 g, 4.1 mmol) and the suspension was stirred until dissolution had occurred. The solvent was removed under reduced pressure and water was added (30 mL). This solution was dialyzed and the water was removed under reduced pressure to give a gum. Stirring with acetone gave a solid which was filtered to give the title compound as a tan solid (1.1 g, 73%). ^{1}H NMR (D_2O) δ 7.9 (2H), 7.5 (2H), 5.1 (1H), 4.9 (2H), 4.7–3.95 (1H), 3.7 (3H), 3.55 (2H) ppm. Electrospray mass spectrum M^+ 2545.2.

2-*O*-Per-(3-carboxybenzyl) γ-CD sodium salt (3). This compound was prepared in the similar way as described above for compound 1, but with the alkylating agent 2-(trimethylsilyl)ethyl 3-iodomethylbenzoate. ^{1}H NMR (D_2O); δ 32;3.47 (t, 8H), 3.54–3.57 (m, 8H), 3.67–3.74 (m, 24H), 3.86 (t, 8H), 4.70–4.75 (m, 8H), 4.84 (d, 8H), 5.07 (d, 8H), 7.50 (t, 8H), 7.56 (d, 8H), 7.88–7.90 (m, 16H) ppm. Electrospray MS $(M+Na)^+$ 2393.1.

2-*O*-Mono-(4-carboxybenzyl) γ-CD sodium salt (2). To a solution of octakis-per-6-*O*-(*tert*-butyldimethylsilyl)-γ-cyclodextrin (3 g, 1.35 mmol) in tetrahydrofuran (90 mL) was added sodium hydride, (108 mg, 60% in oil, 2.7 mmol) the reaction was stirred for 15 min. (3-(Trimethylsilyl)ethyl)-4-(iodomethyl)benzoate (483 mg, 1.29 mmol) was added and the reaction stirred overnight. Methanol was added and the solvents removed. Water was then added and the compound precipitated. The compound was then dried in vacuo. The white solid (1 g, 0.42 mmol) was dissolved in dichlormethane (45 mL) and boron trifluoride diethyl etherate (1.33 mL, 10.1 mmol) was added. The reaction was stirred for 2 h and then dilute sodium carbonate was added to quench the reaction. The solvents were removed and acetone added to precipitate the compound. The compound was purified by chromatography on Sephadex DEAE A-25 resin eluting with pure water. The compound was dialysed overnight and the water evaporated to give the title compound. Analysis showed a 1:1 mixture of 2 and 3 substituted products, (100 mg, 3.3%): ^{1}H NMR (D_2O): δ 7.75 (m), 7.35 (m), 4.9 (m), 4.85 (m), 4.0–3.3 (m) ppm; ^{13}C NMR δ 130.0, 128.7, 102.1, 100.0, 80.6, 74.7, 73.7, 73.3, 72.7, 72.6, 72.0, 60.5 ppm. Electrospray mass spectrum M + Na 1475.3; IR ν (cm^{-1}) 1550 ($CO2^-$).

2-*O*-Mono-carboxymethyl-γ-CD ethyl ester (9). To a solution of γ-cyclodextrin (1 g, 0.77 mmol) in dimethylformamide (20 mL) was added sodium hydride (33.88 mg, 60% in oil, 0.845 mmol). The reaction was stirred for 15 min, ethyl-iodoacetate (100 μL, 0.84 mmol) was added and the reaction heated at 55 °C for 3 h. The reaction was cooled and the product precipitated by addition of acetone. The crude precipitate was filtered and dried in vacuo to give the title compound, (1 g, 94%): ^{1}H NMR (D_2O) δ 5.35 (H1′), 3.6 (H2′), 4.05 (H3′), 4.45 (H7′), 4.2 (H9′), 1.25 (H10′) ppm; ^{13}C NMR (D_2O): δ 102.0, 99.9, 80.7, 73.2, 72.7, 72.6, 72.2, 72.0, 69.2, 62.6, 60.6, 13.9 ppm. Electrospray mass spectrum M + Na 1399.7.

2-*O*-Mono-(3-carboxybenzyl)-γ-CD sodium salt (10). Sodium hydride (0.31 g, 60% dispersion in mineral oil, 7.75 mmol) was added to a solution of γ-cyclodextrin (10 g, 7.71 mmol) in dimethylformamide (100 mL) and the reaction mixture was stirred for 1 h. To this was added (dropwise) a solution of 2-(trimethylsilyl)ethyl-3-(iodomethyl)benzoate (2.79 g, 7.70 mmol) in dimethyl formamide (50 mL) and stirring was continued for 4 h. The solvent was removed under reduced pressure and the solid obtained was stirred in acetone. Filtration gave 10g of white solid which was dissolved in dimethyl sulphoxide (80 mL). To this was added tetrabutyl ammonium fluoride (13.06 mL, 1.0 M solution in tetrahydrofuran, 13.1 mmol) and the solution was stirred for 1 h. The solvent was removed under reduced pressure. Chromatography on Sephadex DEAE A-25 resin eluting with water followed by 0.01 M sodium hydroxide solution gave 1 g of white solid. This was dialyzed for 4 h and the water removed under reduced pressure. The solid obtained was stirred in acetone and filtered to give the title compound as a white solid (190 mg, 2%). ^{1}H NMR (D_2O); δ 3.50 (t, 1H), 3.59–3.67 (m, 15H), 3.83–3.94 (m, 23H), 4.09 (t, 1H), 4.85 (d, 1H), 4.93 (d, 1H), 5.09–5.14 (m, 8H), 7.53 (t, 1H), 7.67 (d, 1H), 7.92 (d, 1H), 7.98 (s, 1H) ppm. Electrospray MS $(M+H)^+$ 1453.4, $(M+Na)^+$ 1475.5.

2-*O*-Per-(4-carboxypiperidin-1-yl)carbonylmethyl-γ-CD sodium salt (14). Sodium hydride (2.72 g, 60% dispersion in mineral oil, 67.9 mmol) was added portionwise to a solution of per-6-*O*-(*tert*-butyldimethylsilyl)-γ-cyclodextrin (15 g, 6.79 mmol) in tetrahydrofuran (500 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and a solution of 4-piperidinecarboxylic acid, 1-(bromoacetyl)-, ethyl ester (18.88 g, 67.9 mmol) in tetrahydrofuran (30 mL) was added. The reaction mixture was stirred for 48 h and the solvent removed under reduced pressure. Chromatography

on silica gel with gradient elution (ethyl acetate to ethyl acetate:methanol; 10:1) gave 4.1 g of a foam which was dissolved in tetrahydrofuran (60 mL). To this was added tetrabutyl ammonium fluoride (17.33 mL, 1.0 M solution in tetrahydrofuran, 17.3 mmol) and the reaction mixture was refluxed for 3 h and allowed to cool to room temperature. The solvent was removed under reduced pressure and water (150 mL) added. The pH was adjusted to 4 by addition of 2 M hydrochloric acid which gave a gum. The water was decanted off and stirring with acetone (100 mL) gave a solid which was filtered off. To this was added methanol (60 mL) and a solution of sodium hydroxide (0.43 g, 10.8 mmol) in water (40 mL) and the reaction mixture was refluxed for 3 h. The solvent was removed under reduced pressure and water was added (30 mL). This solution was dialyzed and the water was removed under reduced pressure to give a gum. Stirring with acetone gave a solid which was filtered to give the title compound as a tan solid (550 mg, 2.9%).

General procedure for the radical addition of a alkylthiol to 2-*O*-allyl CDs. The 2-*O*-allyl-CD starting material and an appropriate alkylthiol (1.5–3 equiv per allyl group) were dissolved in a solvent selected from acetonitrile, water, methanol or toluene or a miscible mixture of the above. The reaction mixture was thoroughly degassed with either nitrogen or argon and then heated at 110 °C for 2–24 h in the presence of catalytic amounts of AIBN (10–20 mg per 250 mg of CD).

After the desired reaction time the crude reaction mixture was stripped of solvent and a NMR performed to check for remaining allyl functionality. If found this was usually due to reaction mixture precipitation and the reaction was resubmitted to thiol and AIBN in a solvent system in which complete solubility could be maintained, typically water/methanol.

The crude reaction mixture was stripped of solvent and purified of starting alkylthiol by dialysis in water using 1000 MWCO dialysis tubing. A G25 size-exclusion Sephadex column was used to remove trace amounts of alkylthiol to the point where no thiol odor was detectable. In cases where mono modifications were attempted, compound purification was performed using DEAE (amine function) Sephadex chromatography eluted with water to remove non-acid containing cyclodextrin followed by 0.1 M NaOH to isolate the mono sodium salt of the acid, dialysis and solvent removal effected purification.

The following compounds were prepared according to the above general procedure.

2-*O*-Per-[3-(1,2-dihydroxypropylthio)propyl]-β-CD (4). ^{1}H NMR (D_2O) δ 5.1 (1H), 3.95 (1H), 3.7–3.9 (1H), 3.45 (1H), 3.45 (4H), 3.4 (1H), 3.5–3.6 (1H) 2.5–2.7 (2H), 1.85 (1H) ppm. MS *m*/*z* M−H 2156.

2-*O*-Per-[3-(carboxymethylthio)propyl]-γ-CD sodium salt (5). ^{1}H NMR (D_2O) δ 32;5.3 (1H), 32;3.45 (1H), 32;4.0 (1H), 32;3.6 (1H), 32;3.85 (1H), 32;3.8–3.85 (2H), 2.7 (1H), 1.95 (1H) ppm. IR υ; 3417, 2932, 1587, 1391, 1164, 1037.

2-*O*-Per-[3-(2-sulfoethylthio)propyl]-γ-CD sodium salt (6). ^{1}H NMR (D_2O) δ 32;5.3 (1H), 4.0 (1H), 3.7–3.85 (3H), 3.6 (1H), 3.4 (1H), 3.1 (1H), 2.8–2.85 (1H), 2.65 (1H), 1.9 (1H) ppm.

2-*O*-Per-[3-(2-carboxyethylthio)propyl]-γ-CD sodium salt (7). ^{1}H NMR (D_2O) δ 32;5.4 (1H), 4.0 (1H), 3.75–3.95 (1H), 3.65 (3H), 3.45 (1H), 2.77 (1H), 2.68 (1H), 2.48 (1H). 1.9 (1H) ppm. MS *m*/*z* M-2H 1233. IR υ; 3400, 2966, 1567, 1454, 1409.

2-*O*-Per-[3-(1,2-dihydroxypropylthio)propyl]-γ-CD (12). ^{1}H NMR (D_2O) δ 32;5.35 (1H), 4.0 (1H), 3.9–3.8 (4H),3.65 (1H), 3.45 (1H), 3.6 (1H), 2.8–2.6 (2H), 1.9 (1H) ppm. MS *m*/*z* (M−H) 2481.2.

2-*O*-Per-[3-(2-carboxy-2-aminoethylthio)propyl]-γ-CD sodium salt (15). ^{1}H NMR (D_2O) δ 32;5.3 (1H), 3.95 (1H), 3.65–3.9 (3H), 3.6 (1H), 3.6 (1H), 3.35 (1H), 2.8–2.95 (1H), 2.6 (1H), 1.85 (1H) ppm.

2-*O*-Per-[3-(2-carboxy-2-aminoethylthio)propyl]-γ-CD methyl ester hydrochloride (16). ^{1}H NMR (D_2O) δ 32;5.33 (1H), 3.94 (1H), 3.63 (1H), 3.42 (1H), 3.28 (3H), 3.15 (1H), 2.66 (1H), 1.88 (1H) ppm. IR υ; 32;3390, 1747, 1633, 1519, 1442, 1039.

2-*O*-Per-{3-[2-(2-hydroxyethoxy)ethylthio]propyl}-γ-CD (17). ^{1}H NMR (D_2O) δ 32;5.15 (1H), 3.85–4.0 (2H), 3.6–3.9 (5H), 3.6–3.9 (1H), 3.4–3.5 (2H), 2.8 (1H), 2.7 (1H), 1.9 (1H) ppm. MS *m*/*z* M-CO_2H 2315.

2-*O*-Mono-[3-(carboxymethylthio)propyl]-β-CD sodium salt (22). ^{1}H NMR (D_2O) δ 4.97 (1H), 3.86 (1H), 3.55 (1H), 3.08 (1H), 3.77 (1H), 3.76 (1H), 3.58 (1H), 2.54 (1H), 1.8 (1H),) ppm. MS *m*/*z* M−H 1266.

Isothermal titration calorimetry

Isothermal titration calorimetry was carried out using a VP-ITC, cell volume 1.345 mL, and the results were analyzed using Origin software, both from Microcal Inc. Samples were made up by weight in 50 mM, pH 7.0 sodium phosphate buffer and titrations were carried out at 26 °C. The standard injection parameters comprised of an initial injection of 3 μL followed by 25×10 μL injections at 3 min intervals. For all measurements, the cyclodextrin solution was in the cell and rocuronium bromide in the syringe. The titrations were carried out with ~0.075 mM cyclodextrin solution in the cell and 1.5 mM rocuronium bromide in the syringe.

Pharmacology

Reversal of rocuronium-induced neuromuscular block in isolated mouse hemi-diaphragm in vitro. 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 following composition in mM: NaCl 118, KCl 5, KH_2PO_4 1, $MgSO_4$ 1, $NaHCO_3$ 30, $CaCl_2$ 2.5, and glucose 20. The phrenic nerve was stimulated continuously using a Grass S88E stimulator (rectangular pulses of 0.2 ms every 20 s at a supramaximal 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 ~90% twitch block. Twenty minutes later, increasing concentrations of reversal agents were added in a cumulative fashion, with intervals of 10 min. Concentrations of compounds producing 50% and maximum recovery of twitch height were determined.

Reversal of rocuronium-induced neuromuscular block in anaesthetised guinea pigs in vivo. Male Dunkin-Hartley guinea pigs (bodyweight: 600–900 g) were anaesthetized by ip administration of 10 mg/kg pentobarbitone and 1000 mg/kg urethane. After tracheotomy, the animals were artificially ventilated using a Harvard small animal ventilator. A catheter was placed into the carotid artery for continuous monitoring of arterial blood pressure and the taking of blood samples for blood gas analysis. Heart rate was derived from the blood pressure signal. The sciatic nerve was stimulated (rectangular pulses of 0.5 ms duration at 10 s (0.1 Hz) intervals at a supramaximal voltage, using a Grass S88 Stimulator) and the force of *M. gastrocnemius* contractions was measured using a Grass FT03 force-displacement transducer. Contractions, blood pressure and heart rate were recorded on a multichannel Grass 7D recorder. Catheters were placed in both jugular veins. One catheter was used for the continuous infusion of a neuromuscular blocking agent. The infusion rate of the neuromuscular blocking agent was increased until a steady-state block of 85–90% was obtained. The other catheter was used for administration of increasing doses of the reversal agent. During continuous infusion of the neuromuscular blocking agent, single doses of increasing concentration of reversal agent were given. At the end of the experiment, the measured force of muscle contractions was plotted against the concentration of reversal agent, and using regression analysis techniques, the 50% reversal concentration was calculated.

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