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Acyclic Cucurbit[*n*]uril-Type Molecular Containers Bind Neuromuscular Blocking Agents In Vitro and Reverse Neuromuscular Block In Vivo**

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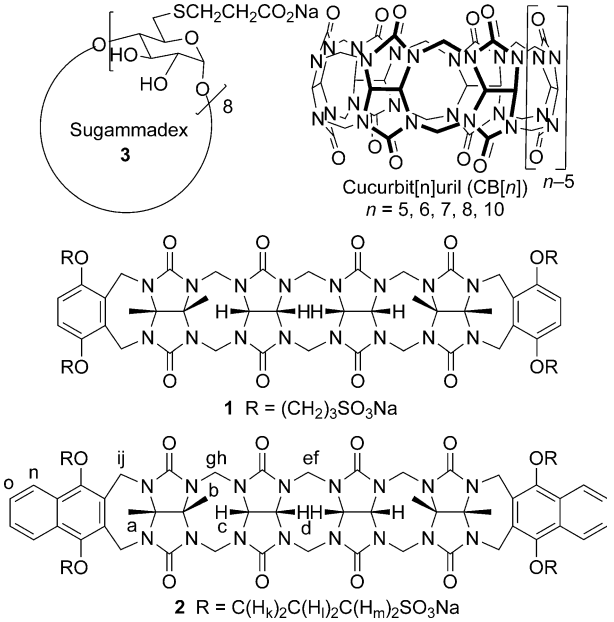
Annually, more than 400 million patients receive curare-type neuromuscular blocking agents (NMBAs) during anesthesia in operating rooms, intensive care units, and emergency medicine departments. NMBAs that are widely used in the clinical practice of anesthesia include rocuronium, pancuronium, vecuronium, atracurium, and cisatracurium.[1] To speed up the recovery of the patient's muscle function and to prevent residual neuromuscular block, it is often necessary to reverse the biological effect of NMBAs at the end of the surgery.[2] Conventional reversal agents, such as neostigmine and edrophonium, exert their activity by increasing the levels of acetylcholine at the neuromuscular junction by competitive inhibition of acetylcholine esterase.[3] Unfortunately, these conventional reversal agents may cause cardiovascular side effects owing to their nonselective potentiation of muscarinic acetylcholine receptors and may even induce a (depolarizing) neuromuscular block in clinical practice when given in the absence of a NMBA.[4] A major advance in clinical anesthesia was made by the introduction of a γ-cyclodextrin-derived molecular container known as Sugammadex (**3**, marketed as Bridion by Merck with sales of more than $100 million in 2010; Scheme 1), which binds rocuronium with high affinity ($K_a = 1.05 \times 10^7\ M^{-1}$) in water and reverses the effects of rocuronium and vecuronium in vivo.[5] Sugammadex reverses neuromuscular block by sequestering rocuronium and vecuronium in the bloodstream, thereby depleting their concentration at the neuromuscular junction.[6] The sugammadex•rocuronium complexes are subsequently excreted in the urine. Sugammadex has had a major impact on the clinical practice of anesthesia in Europe but is not yet approved for use in the United States because of potential allergic reactions and hemorrhagic side effects.[7] As a result, there is a real need to develop alternative classes of molecular containers that function as reversal agents for the full range of clinically important NMBAs.



Scheme 1. Chemical structures of sugammadex, CB[*n*], **1**, and **2**.

We, and others, have been studying the synthesis and supramolecular chemistry of a new family of molecular containers known as cucurbit[*n*]urils (CB[*n*]), which comprise *n* glycoluril rings linked by 2*n* CH_2 bridges.[8] The defining structural features of CB[*n*] molecular containers are a hydrophobic cavity guarded by two symmetry-equivalent electrostatically negative ureidyl C=O portals. Remarkably, CB[*n*] compounds display unusually high affinity (K_a routinely exceeds $10^9\ M^{-1}$) toward alkane (di)ammonium ions in water.[9] Accordingly, CB[*n*] compounds have been used in a variety of applications including stimuli-responsive molecular machines, sensing ensembles, biomimetic processes, supramolecular polymers, and (targeted) drug delivery.[10] Given the high K_a values typically observed for CB[*n*]•guest

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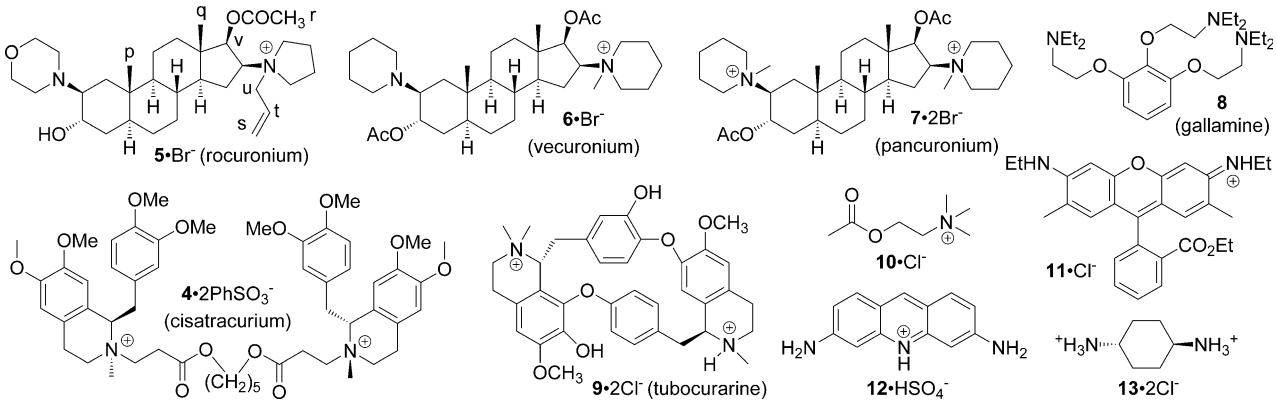
complexes, we realized that CB[*n*]-type receptors represent a potential alternative to γ-cyclodextrin derivatives for the reversal of neuromuscular block. Unfortunately, the water solubilities of CB[8] and CB[10], which are large enough to encapsulate the steroidal nucleus of NMBAs like rocuronium, are poor (< 100 μM), thereby severely limiting their potential to function as in vivo reversal agents for NMBAs. Conversely, water-soluble CB[7] is neither voluminous enough to encapsulate the steroidal ring system nor long enough (O···O distance ca. 6.1 Å) to electrostatically complement the N···N separation (ca. 11.0 Å) of steroidal NMBAs **5**–**7** (Scheme 2). Recently, we developed two acyclic CB[*n*]-type molecular containers (**1** and **2**), which have very good solubility characteristics, that solubilize insoluble pharmaceutical agents (e.g. paclitaxel) by up to 2750-fold in water and are well-tolerated (maximum tolerated dose > 1230 mg kg^{-1}) in mice.[11] Because **1** and **2** are acyclic, they are able to flex their methylene-bridged glycoluril oligomer backbone, expand their cavity, and thereby accommodate large guests.[12]

Accordingly, we thought that containers **1** and **2** would display excellent affinity both toward steroidal (e.g. **5**–**7**) and benzyl isoquinoline (e.g. **4** and **9**) NMBAs (Scheme 2) by π–π interactions and the hydrophobic effect.[13] Furthermore, we surmised that the distance between the anionic SO_3^- solubilizing groups on **1** and **2** (ca. 14 Å) would selectively complement the N···N separation within **5**–**7**. Herein we report the molecular recognition properties of containers **1** and **2** toward steroidal and benzyl isoquinoline NMBAs **4**–**9** and acetylcholine (**10**) in water and establish that container **2** acts as a potent reversal agent for rocuronium (**5**) in rats.

Initially, we investigated the interactions of **1** and **2** with NMBAs **4**–**9** and **10** by ^{1}H NMR spectroscopy. For complexes between container **1** and compounds **4**–**10** we observed upfield shifting of guest resonances indicative of cavity binding, but all of the complexes exhibit fast kinetics of exchange relative to the chemical shift timescale (Supporting Information). These results suggested that complexes between container **1** and compounds **4**–**10** are of moderate stability in water. In contrast, complexes between container **2** and NMBAs **4**–**6** exhibit slow exchange on the chemical shift timescale, whereas complexes with compounds **7**–**10** exhibit fast or intermediate kinetics of exchange. Figure 1 shows the ^{1}H NMR spectra recorded for **5**, **2**, **2**·**5** mixture, and a mixture of **2** and **5** with excess **5** present. These ^{1}H NMR spectra display a number of interesting features, which provide insight into the nature of the **2**·**5** complex. For example, the four symmetry-equivalent protons H_n and H_o become nonequivalent within the **2**·rocuronium complex, because the guest **5** is chiral and enantiomerically pure. Furthermore, these aromatic H atoms (H_n–$H_{n'''}$ and H_o–$H_{o'''}$) are shifted downfield within the **2**·**5** complex; in free container **2** the naphthalene rings undergo edge-to-face π–π interactions,[11] which result in upfield shifts that are reversed upon expansion of the cavity of **2** to form the **2**·**5** complex. The axial steroidal CH_3 groups ($C(H_p)_3$ and $C(H_q)_3$) of guest **5** undergo significant (0.5–1 ppm) upfield shifts, whereas the $O(C{=}O)C(H_r)_3$ group of **5** undergoes a slight downfield shift. The well-established shielding nature of the CB[*n*] cavity and the deshielding nature of the region outside the ureidyl C=O portals[8,9a] allows us to formulate a binding model where **2** engulfs the B–D rings of the steroid, which positions one ammonium ion near the portal of **2** and one near an anionic SO_3^- solubilizing group. An MMFF-minimized model of the **2**·**5** complex is shown in Figure 2. As expected on the basis of this model the protons of the steroidal ring system experience remarkable upfield shifts in the **2**·**5** complex because of the shielding nature of the glycoluril backbone and naphthalene rings of **2**. For example, in the spectrum of free guest **5** (Figure 1 a) these protons resonate from 1–2 ppm, whereas in that of the **2**·**5** complex (Figure 1 c) resonances appear in the 0.5 to −2.0 ppm region. Similar trends are observed for the **2**·**6** and **2**·**7** complexes (Supporting Information).

After having established the inclusion binding of guests **4**–**10** inside containers **1** and **2** by ^{1}H NMR spectroscopy, we decided to measure the binding affinity (K_a, M^{-1}) for these complexes. For this purpose, we performed a direct UV/Vis titration of Rhodamine 6G (**11**) with **1** (Figure 3 a). Fitting of the change in UV/Vis absorbance at 550 nm (Figure 3 b) to a standard 1:1 host·guest binding model allowed us to determine the binding affinity for complex **1**·**11** ($K_a = (4.8 \pm 0.1) \times 10^5$ M^{-1}, Table 1). Subsequently, we performed competitive binding assays[14] by treating solutions containing fixed



Scheme 2. Chemical structures of NMBAs **4**–**9** and other guests **10**–**13**. The counterions present in the salt form of the used guest molecules are indicated in the scheme.

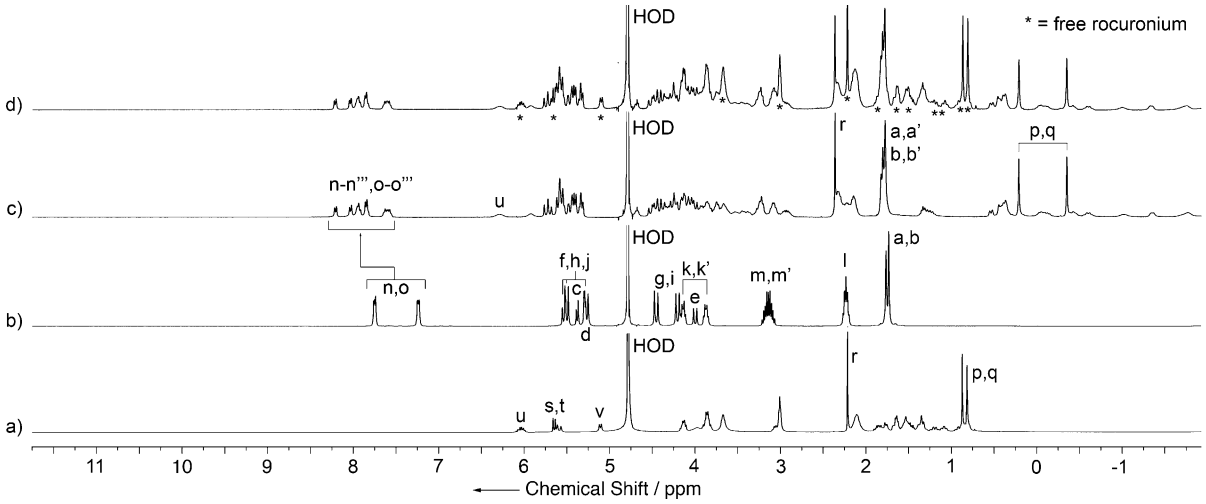


Figure 1. ^{1}H NMR spectra recorded (400 MHz, room temperature, 20 mM phosphate-buffered D_2O) for: a) **5** (5 mM), b) **2** (5 mM), c) **2·5** complex (2.5 mM), and d) a mixture of **2·5** (1.25 mM) and excess **5** (3.75 mM). *= resonances for unbound **5**.

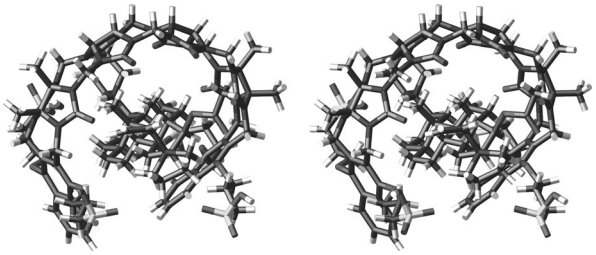


Figure 2. Cross-eyed stereoview of the MMFF minimized model of the **2·5** complex. MMFF = Merck Molecular Force Field. See the Supporting Information for a color version of this figure.

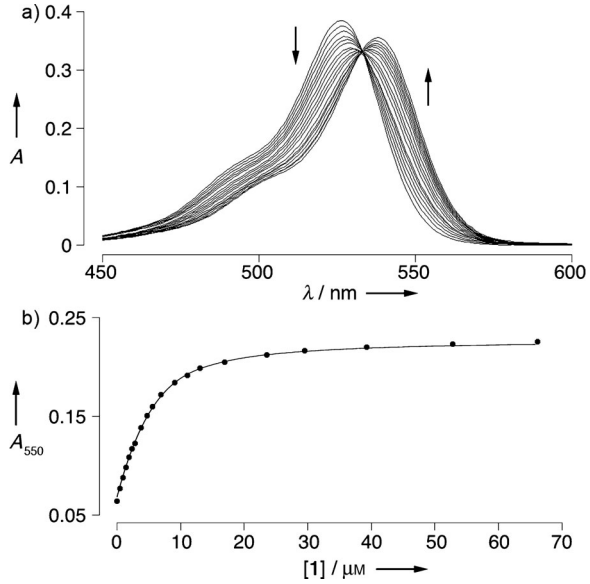


Figure 3. a) UV/Vis spectra recorded during the direct titration of a fixed concentration of **11** (4.94 μM) and container **1** (0–64 μM) in 20 mM sodium phosphate buffered D_2O (pH 7.4, 298 K); b) plot of absorbance at 550 nm (A_{550}) versus the concentration of **1**, and the fitting of the data to a 1:1 binding model allowed a determination of $K_a = (4.8 \pm 0.1) \times 10^5\ M^{-1}$.

Table 1: Binding constants (K_a) determined by direct and competitive UV/Vis assays for the interaction between containers **1** and **2** and guests **4**–**13**.[a]

Guest	Binding constants K_a [M^{-1}]		
	Host **1**	Host **2**	Sugammadex
4	$(9.7\pm0.8)\times10^5$	$(4.8\pm0.9)\times10^6$	
5	$(8.4\pm0.9)\times10^6$	$(3.4\pm0.6)\times10^9$	$(1.1\pm0.2)\times10^7$ [5a]
6	$(5.8\pm0.9)\times10^6$	$(1.6\pm0.2)\times10^9$	
7	$(4.5\pm0.1)\times10^5$	$(5.3\pm0.5)\times10^8$	
8	$(6.2\pm0.5)\times10^6$	$(3.2\pm0.4)\times10^8$	
9	$(4.7\pm0.2)\times10^5$	$(2.2\pm0.3)\times10^5$	
10	$(2.4\pm0.1)\times10^4$	$(1.8\pm0.2)\times10^5$	
11	$(4.8\pm0.1)\times10^5$	$(2.3\pm0.2)\times10^6$	
12	–	$(7.8\pm0.8)\times10^8$	
13	–	$(2.1\pm0.2)\times10^6$	

[a] Conditions: 20 mM sodium phosphate buffered H_2O, pH 7.4, room temperature.

concentrations of container **1** and Rhodamine 6G (**11**) with increasing concentrations of guests **4**–**10** and measuring the UV/Vis response (Supporting Information). By fitting the change in UV/Vis absorbance to a competitive binding model—with the known K_a value for the **1·11** complex as input—we could determine the K_a values for the **1·4**–**1·10** complexes (Table 1, Supporting Information). In a similar manner, we measured the K_a value for the **2·11** complex by direct UV/Vis titration ($K_a = (2.3 \pm 0.2) \times 10^6\ M^{-1}$) and then performed competitive UV/Vis titrations with solutions containing **2** and **11** to determine the K_a values for the **2·4**, **2·9**, and **2·10** complexes (Table 1, Supporting Information). Because the K_a values for the **2·5**–**2·8** complexes are significantly larger than the known K_a value for the **2·11** complex, we needed to measure a K_a value for an even tighter-binding dye that could be used in a competition assay with guests **5**–**8**. For this purpose, we measured the K_a value for the **2·13** complex ($K_a = (2.1 \pm 0.2) \times 10^6\ M^{-1}$, Table 1, Supporting Information) by competition with **11**. We then used the known value of K_a for the UV/Vis-silent complex **2·13** to measure the K_a value for the tightly bound complex

2·12 (Table 1, $K_a = (7.8 \pm 0.8) \times 10^8\,M^{-1}$, Supporting Information) by a competitive binding assay. Finally, competitive binding assays using fixed concentrations of host **2** and guest **12** and increasing concentrations of guests **5–8** were used to determine the K_a values for the **2·5–2·8** complexes (Table 1, Supporting Information).

The binding constants (K_a) measured for the interaction of host **1** with guests **4–10** range from 2.4×10^4 to $8.4 \times 10^6\,M^{-1}$. Quite interestingly, the binding constants measured for complexes between container **1** and the steroidal NMBAs **5–7** all exceed $10^5\,M^{-1}$ and that measured for the complex **1**·rocuronium (**1·5**, $K_a = 8.4 \times 10^6\,M^{-1}$) is comparable to that measured for the complex sugammadex·rocuronium (**3·5,** $K_a = 1.1 \times 10^7\,M^{-1}$).[5a] Container **1** also displays high affinity toward the benzyl isoquinoline type NMBA cisatracurium (**4**; $K_a = (9.7 \pm 0.8) \times 10^5$, Table 1). Importantly, container **1** binds 19–350-fold weaker to acetylcholine (**10**; $K_a = 2.4 \times 10^4\,M^{-1}$) than to NMBAs **4–9**, thereby ensuring that **1** will preferentially sequester the NMBA rather than acetylcholine (**10**). Container **2**—with its naphthalene walls, which result in a larger cavity because of the additional π surfaces—binds with even higher affinity toward guests **4–10**. For example, the affinities of container **2** toward steroidal NMBAs **5–7** fall in the range of $0.53–3.4 \times 10^9\,M^{-1}$, which is up to 300-fold larger than that measured toward sugammadex (**3**)! Container **2** also binds to benzyl isoquinoline type NMBA **4** with an affinity of $4.8 \times 10^6\,M^{-1}$, which approaches the $10^7\,M^{-1}$ level of affinity that forms the basis of the NMBA reversal agent sugammadex. Finally, container **2** also binds extremely tightly to gallamine (**8**; $K_a = 3.2 \times 10^8\,M^{-1}$; $K_d = 3$ nM). Importantly, these higher levels of affinity are also accompanied by good levels of selectivity against acetylcholine (**10**); container **2** binds NMBAs **4–8** 27–19000-fold more tightly than acetylcholine (**10**).

Given the high affinity of **1** and **2** toward NMBAs **4–9** (in some cases these affinities meet and even exceed those measured for the sugammadex·rocuronium (**3·5**) complex), we decided to test the ability of these acyclic CB[*n*]-type molecular containers to reverse neuromuscular block in vivo. For this purpose, we anesthetized eight rats with isoflurane, tracheotomized and instrumented them with intraveneous (iv) and arterial lines, as well as subcutaneous electrodes to supramaximally stimulate the femoral nerve. After continuous nerve stimulation for at least ten minutes, the TOF-Watch SX, an instrument to measure neuromuscular blockade, was recalibrated, and complete neuromuscular blockade (estimated twofold ED90, ED90 is the effective dose of rocuronium that is calculated to decrease the twitch height by 90 per cent) was induced with rocuronium ($3.5\,mg\,kg^{-1}$), as described previously.[3b] Rats were then ventilated and either placebo ($n = 5$) or compound **2** ($30\,mg\,kg^{-1}$) were given at maximum twitch depression (T1 = 0, T1 is the magnitude of the first muscular contraction in response to supramaximal (2 Hz) train-of-four stimulation of the femoral nerve. Values are given in percent of baseline T1 prior to injection of rocuronium.) to reverse neuromuscular block. Compound **2** accelerated recovery of both spontaneous breathing (placebo: (12.5 ± 1) min; **2**: (0.4 ± 0.1) min, $p < 0.0001$) and train-of-four (TOF) ratio to 0.9 (placebo: (21.3 ± 12) min; **2**: (0.4 ± 0.02) min, $p < 0.0001$; Figure 4). For comparison, we note that reversal with sugammadex (**3**, $15\,mg\,kg^{-1}$) to TOF = 0.9 was slower and required 2.5 min.[3b] Recurarization, which is a decrease in muscle function following initial reversal, did not occur during a two-hour observation period after reversal with compound **2**.

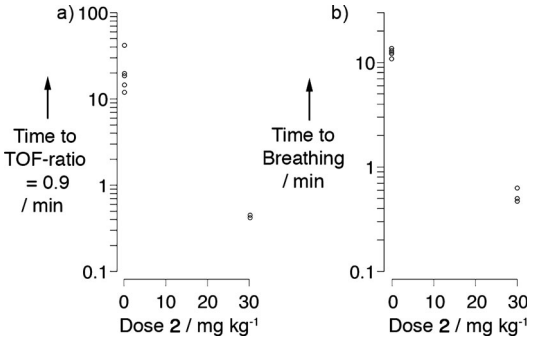


Figure 4. Results of the in vivo experiments with rats anesthetized with isoflurane and then treated with **5** ($3.5\,mg\,kg^{-1}$). Plots of the time required: a) to achieve a train-of-four ratio of 0.9 after administration of placebo or **2**, and b) to achieve spontaneous breathing after administration of placebo or **2**.

In summary, we have described the recognition properties of acyclic CB[*n*]-type molecular containers **1** and **2** toward NMBAs **4–9** and acetylcholine (**10**) in water. We find that containers **1** and **2** form 1:1 host·guest complexes with steroidal and benzyl isoquinoline type NMBAs **4–9** with values of K_a that range from $2.2 \times 10^5–3.4 \times 10^9\,M^{-1}$ as determined by direct and competitive UV/Vis titrations. Most striking is the outstanding affinity displayed by **2** toward rocuronium (**5**; $K_a = 3.4 \times 10^9\,M^{-1}$); this affinity is 19000-fold tighter than toward acetylcholine (**10**). We found that container **2** ($30\,mg\,kg^{-1}$) is able to reverse deep rocuronium-induced neuromuscular block in rats. Remarkably, the rats recover to a TOF = 0.9 level within 26 s versus 21 min for rats treated with placebo. Recovery of spontaneous breathing is also much faster (32 s versus 12.5 min) for rats treated with container **2** relative to placebo. The results herein establish acyclic CB[*n*] containers as a new class of in vivo reversal agents for rocuronium-induced neuromuscular block. The studies performed in water with steroidal NMBAs **6** and **7** and benzyl isoquinoline-type NMBA cisatracurium (**4**) strongly suggest that containers **1** and especially **2** should function as reversal agents in vivo for neuromuscular block induced by NMBAs **4**, **6**, and **7**.[15] As such, acyclic CB[*n*]-type molecular containers have the potential to function as broad-spectrum reversal agents for neuromuscular block in vivo. When that occurs, the impact on the 400 million patients treated annually with NMBAs will be significant.

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