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Supramolecular Hosts as *in Vivo* Sequestration Agents for Pharmaceuticals and Toxins

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Abstract

Pharmaceutical agents, drugs of abuse, and toxic substances have a large impact, positive and negative, on modern society. Efforts to mitigate the side effects of pharmaceuticals and counteract the life threatening effects of drugs of abuse and toxins can occur either by pharmacodynamic (PD) approaches based on bioreceptor•drug antagonism or by pharmacokinetic (PK) approaches that seek to reduce the concentration of free drug. In this tutorial review, we present the use of supramolecular hosts (cyclodextrins, calixarenes, (acyclic) cucurbiturils, and pillararenes) as *in vivo* sequestration agents for neuromuscular blockers, drugs of abuse (methamphetamine and fentanyl), anesthetics, neurotoxins, the pesticide paraquat, and heparin anti-coagulants by the PK approach. The review presents the basic physical and molecular recognition features of the supramolecular hosts and some of the principles used in their selection and structural optimization for *in vivo* sequestration applications. The influence of host•guest complexation on other relevant *in vivo* properties of drugs (e.g. distribution, circulation time, excretion, redox properties) are also mentioned. The article concludes with a discussion of future directions.

Introduction

Human biology rests on a complex network of reactions and interactions between endogenous (bio)molecular entities. Every day, either intentionally or unintentionally, human beings eat, drink, inhale, and otherwise ingest a variety of exogenous substances that may prove beneficial or detrimental to human health. For example, prescription drugs and medications are designed to interfere with specific biomolecular processes within this complex network to directly combat ongoing illness and reduce morbidity and mortality. Other prescription drugs are used pre- and post-operatively to optimize surgical conditions, to reduce pain, and to speed the recovery of the patient. Indeed, the increased longevity and higher quality of life achieved over the past century can be attributed in part to the great advances made by the pharmaceutical industry. However, there are many situations where the beneficial effects of a drug have ended and the residual deleterious effects remain. Chronic abuse of pharmaceutical agents can lead to poor quality of life, associated health risks of drug abuse, and an increased economic burden on the health care system. The rise of

Conflicts of interest

L.I. and S.M. are inventors on patents held by the University of Maryland on the use of supramolecular hosts as sequestration agents in biomedical applications.

the abuse of prescription opioids, synthetic opioids, and other drugs of abuse presents a prime example; 47,000 people died in the US in 2017 alone due to overdose with prescription opioids.¹ The abuse of illicit drugs such as cocaine, methamphetamine, and synthetic opioids lead to a series of health consequences such as acute discomfort, higher incidence rates for infectious disease, other chronic episodic disorders, and death.² Conversely, humans may be exposed to exogenous toxins from the environment. Such toxins include pesticides (e.g. paraquat), radiological hazards (e.g. radon), and chemical warfare agents (e.g. organophosphates nerve agents, synthetic opioids). Other toxins can originate endogenously. For example, the intracellular accumulation of abnormal amounts of toxic metabolites (product of abnormal secretion or metabolism) can lead to severe organ damage and the development of various pathological conditions and diseases.³ The immediate and long term impact of such exposures is dependent on the duration and route of exposure (e.g. inhalation, oral, topical) and whether the effects of the agent can be mitigated. One strategy to mitigate the ever-growing health crisis associated with drug toxicity relies on the development of molecules that sequester the drug or toxin thereby turning off its biological activity, reestablishing normal metabolite levels, and promoting clearance from the body. Herein, we focus on the use of supramolecular hosts (e.g. cyclodextrins (CD), cucurbiturils (CB[n]), pillararenes, calixarenes, Figure 1) in such applications.

Current treatments for drug overdose and toxicity rely on either pharmacodynamic (PD) or pharmacokinetic (PK) approaches (Figure 2). The PD approach focuses on manipulating the biological mechanism of action. In such studies, the chemist typically designs and synthesizes small molecule antagonists (generally presenting convex functionality) that bind non-covalently *inside* the primary binding site or an allosteric receptor binding site to competitively block and disrupt the effects of these drugs.⁴ To be effective, the antagonist must bind to the receptor selectively and preferentially with a higher K_a than the receptor•drug complex; although higher dosing can overcome weaker binding by mass action. Accordingly, PD approaches focus on receptor•antagonist non-covalent interactions and require intense effort, time, and financial expense to design, synthesize, evaluate, and optimize a new antagonist.

On the other hand, the PK approach seeks to reduce the concentration of available drug and thereby modulate its biological properties. In some examples, the PK approach involves the molecular recognition of the drug or toxin by a protein or supramolecular host coupled with its destruction (e.g. hydrolysis).⁵ In other examples, the PK approach relies on Le Chatelier's principle to sequester the drug of interest as a non-covalent complex which reduces the concentration of *free uncomplexed drug* below the efficacious dose and thereby reduces or eliminates the biological effect of the drug or toxin.⁶ Conventional PK agents mainly rely on general purpose decontaminants such as orally administered activated charcoal and extracorporeal procedures such as hemodialysis, whole bowel irrigation, or correction of electrolyte disturbances.⁷ In contrast to the PD approach, the PK approach does not require a precise knowledge of the biological mechanism of action or the 3D structure of the biomolecular receptor which enables initial *in vitro* binding affinity measurements as effectiveness measures. Recently, significant progress has been made towards injectable nanostructured biomaterials and nanoparticle (NP)-based systems, immunotherapies and enzymatic biodetoxifiers.^{8, 9} These systems fall outside the scope of this review.

As the ability of supramolecular chemists to design and synthesize aqueous host•guest systems whose affinities rival natural systems has increased over the past two decades, the great potential of the use of molecular containers as *in vivo* sequestration agents *via* the PK approach has come into focus. The use of synthetic supramolecular hosts in such applications offer some potential advantages over related biomolecular systems like proteins and antibodies. First, because supramolecular hosts are prepared by organic synthesis, their size, shape, and functionality can be modified to better recognize a specific molecular target or class of molecules. Host structure can also be iteratively modified to balance biocompatibility, solubility, rate of excretion, and potency to meet the requirements for effective *in vivo* sequestration. Improved experimental and computational methods to streamline the host design and optimization process would advance the field. Second, supramolecular hosts generally possess high thermal and chemical stability which relative to biomolecular detoxifiers (e.g. antibodies and enzymes) can result in longer shelf life. Third, given their abiotic nature, supramolecular hosts have a lower risk of evoking an immunogenic response. One potential drawback of supramolecular hosts as *in vivo* sequestrants is their lower selectivity relative to antibodies, although the ability of supramolecular hosts to recognize a class of structurally related drugs or toxins might prove useful in the creation of broad-spectrum sequestration agents. In this tutorial review we discuss the principles for the design of supramolecular hosts and evaluation of their use as *in vivo* sequestrants for pharmaceuticals and toxins.

1 General Considerations

In this section we present some of the guiding principles used in the design and refinement of supramolecular host systems for *in vivo* sequestration applications. Several criteria must be fulfilled regardless of the molecule targeted for sequestration. First, the supramolecular host system must display high *in vitro* and *in vivo* biocompatibility. Typical *in vitro* assays performed include cell viability and cell death assays, the Ames test to evaluate mutagenicity, and hERG ion channel inhibition assay to flag potential cardiotoxicity. Typical *in vivo* assays include maximum tolerated dose studies, monitoring of the effect of the host on blood gas, blood pH, and heart rate, and determination of the adsorption, distribution, metabolism, and excretion properties of the host. Hosts that do not pass these tests cannot be translated to the clinic. Second, the host should display comparable affinity (K_a , M^{-1}) toward the target molecule as its cognate biological receptor in biologically relevant aqueous medium. Ideally, the host should be highly selective for the target – that is – the host•target complex should be among the tightest complexes known for a given host. This high selectivity for the target and discrimination against other biomolecules prevents the filling of the host with other compounds (drugs) that might be present in the body. The influence of the ionic strength of the buffer and the presence of serum proteins (e.g. albumin, globulins, and fibrinogen) on the binding affinity should be ascertained. Third, the first two criteria argue against the *de novo* design of new host systems with unknown toxicity profile and baseline host•guest affinity and suggest the selection of a well known supramolecular host that can be synthetically functionalized to tailor its recognition, physical, and biological properties. Well known host systems that meet these criteria include CDs, calixarenes, CB[n], and most recently pillararenes.¹⁰ Each of these host systems uses the hydrophobic

effect (either classical or non-classical) as a major driving force toward target complexation based on the release of intracavity water molecules upon host guest complexation (Figure 3) which provides a good baseline affinity level.¹¹ Fourth, the supramolecular host should have high solubility in water and should not undergo significant self-association or other aggregation that might decrease its affinity toward its target. The solubility of supramolecular host systems can often be improved by the addition of ionic or other hydrophilic groups including ammonium, carboxylate, sulfonate, phosphonate, and poly ethylene glycol functionality. The presence of water solubilizing groups reduces overall host hydrophobicity and can thereby reduce binding to plasma proteins which would decrease the efficiency of the host as *in vivo* sequestrants. Fifth, modifications to the host structure designed to enhance target affinity must not negatively impact other properties like aqueous solubility and self-association. For example, it is straightforward to imagine that appending a hydrophobic aromatic substituent to a known host might increase target affinity by enhancing the hydrophobic driving force toward complexation but it might also decrease water solubility and promote aggregation. Lastly, if the host system is to be translated to the clinic then the synthetic route for its preparation must be amenable to large scale synthesis from cheap starting materials.

The selection of the targets for *in vivo* sequestration applications must be done carefully. First, of course, there must be an unmet health outcomes based need for a sequestering agent and ideally one that reduces overall healthcare costs. Second, the chemical properties of the target (e.g. conformation and protonation state under physiological conditions) must be known in order to guide design of the host system. For example, large hydrophobic targets can be bound strongly inside the hydrophobic hosts like cyclodextrins whereas ionic targets will benefit from complementary ionic groups that provide an electrostatic driving force for complexation. Polar functional groups on the target can be complemented by H-bonding groups on the host, however, such H-bonds are often weak in water and the introduction of intracavity functionality can be challenging in synthetic host systems. Ideally, a synthetic sequestration agent should complement as many of the unique structural aspects of the target as possible to provide highest levels of binding affinity and target selectivity. In the following sections, we will summarize recent progress in the development of synthetic sequestrants for a variety of drugs and toxins. This tutorial review aims to provide a roadmap for the design and testing of supramolecular hosts as sequestration agents for drugs and toxins and stimulate the development of next generation systems for real world applications.

2 Reversal of neuromuscular blockers

The concept of using supramolecular hosts as *in vivo* reversal agents was first popularized by the development of γ -cyclodextrin derivative Sugammadex which is marketed by Merck as Bridion™ for the reversal of the neuromuscular blocking agents (NMBA) rocuronium (roc) and vecuronium (vec) (Figure 4).^{12, 13} NMBAs are a class of molecules that are widely administered by anesthesiologists before surgery to prevent the patient from moving on the surgical table and to optimize surgical conditions. NMBAs bind to the nicotinoyl acetylcholine receptor (nAChR) at the neuromuscular junction and thereby influence neuromuscular transmission (Figure 4) normally mediated by the binding of acetylcholine (ACh) to nAChR and the hydrolysis of ACh to choline and acetate mediated by

acetylcholine esterase (AChE). NMBAAs can be divided into two categories based on their neurotransmission blocking mechanisms. Non-depolarizing NMBAAs (e.g. rocuronium (roc), pancuronium (pan), vecuronium (vec), cisatracurium (cis)) act as competitive antagonists that bind to the nAChR receptors but do not induce an action potential and are not hydrolyzed by AChE. Depolarizing NMBAAs (e.g. succinylcholine (SCh)) exhibit agonist behavior (e.g. generate an action potential) upon binding to the AChR. It should be noted that the steroidal NMBAAs roc, pan, and vec feature a central hydrophobic region with two flanking cationic ammonium ions which provides a roadmap for the design of supramolecular hosts for *in vivo* sequestration.

At the end of surgery, reversal of the residual effects of neuromuscular blockade is necessary in order for the return of adequate respiration and upper respiratory tract muscular function. To date, AChE inhibitors such as neostigmine and edrophonium are commonly used in the clinic for this purpose (Figure 4). AChE inhibitors speed up recovery by blocking hydrolysis of ACh which improves competition with NMBA to bind to the nAChR. However, the utility of AChE inhibitors is somewhat limited because they exhibit moderate reversal efficiency and are accompanied by a series of adverse side events due to non-specific binding at receptors beyond the NMJ.¹⁴ Accordingly, the development of alternative strategies to reverse neuromuscular block are warranted. Figure 4 also shows an alternative mechanism of reversal of neuromuscular block that is illustrated by the development of sugammadex. In this approach, a patient is given an intravenous (IV) injection of a supramolecular host that forms a tight host•NMBA complexes that outcompetes the nAChR. The formation of the host•NMBA complex creates a concentration gradient between the bloodstream and the neuromuscular junction which ideally results in the excretion of the host•NMBA complex. As the mechanism of action for host•NMBA encapsulation does not stimulate or bind with the targeted receptors, it has the potential to eliminate the undesired adverse effects that plague for AChE inhibitors. To date several classes of macrocyclic hosts (CDs, CB[n], pillararenes) have been used to reverse neuromuscular block as detailed in the following section.

2.1 Cyclodextrin-based NMBA Reversal Agents

Cyclodextrins, (CDs), are a family of macrocycles constructed from naturally occurring oligosaccharides consisting of D-glucose units connected by α -1,4-glucosidic bonds. The most commonly used CDs are α -, β -, and γ -CDs containing six, seven, and eight glucose units, respectively (Figure 1).¹⁵ In terms of their molecular shape, CDs resemble a truncated cone with the wider rim bearing secondary OH groups and the narrower rim bearing primary OH groups. The aperture sizes, cavity volumes, and physical properties of the CDs are dependent on the number of repeating glucose units (Figure 1). In CDs, the rims are hydrophilic due to the presence of the outward pointing polar hydroxyl groups, while the methine protons are directed inward toward the cavity, resulting in a hydrophobic cavity. CDs generally bind to hydrophobic guest molecules like derivatives of aromatics (e.g. benzene, naphthalene, anthracene), (poly)cyclic hydrocarbons (e.g. adamantane, steroids), and *n*-alkanes but the binding constants for CD•guest complexes exceed 10^5 M^{-1} only in very rare cases.¹⁶ The formation of unmodified CD•guest complexes are driven mainly by the hydrophobic effect. CDs are generally recognized as safe by the FDA, commercially

available, and inexpensive due to their enzymatic synthesis from renewable precursors. Accordingly, this remarkable host family and its derivatives have found extensive use as the active ingredient in household deodorizing products (e.g. Febreze™) and in the pharmaceutical field for the solubilization, controlled release, toxicity reduction, and bioavailability enhancement of drugs.¹⁷

Given the confluence of advantageous properties of CDs, the scientists at Organon decided to create a CD based NMBA reversal agent for roc and vec. Although both β -CD and γ -CD can bind steroids, the larger γ -cyclodextrin host was selected as the base scaffold. Given that the hydrophobic steroidal region of roc and vec are flanked by cationic N-atoms, the addition of electrostatically complementary carboxylate sidearms were appended to the primary hydroxyls to create the structure of Sugammadex (Figure 5). Synthetically, Sugammadex is prepared by the reaction of readily available per-6-bromo- γ -CD with the corresponding thiolate nucleophile by S_N2 reaction. The secondary hydroxyls remain unmodified in Sugammadex. Figure 5b shows the x-ray crystal structure of the Sugammadex•roc complex which illustrates that the steroidal nucleus is bound within the γ -CD ring whereas the anionic carboxylate substituents complement the cationic quaternary N-atom of roc.¹³ The morpholino N-atom of roc does not benefit from electrostatic complementation. The $SCH_2CH_2CO_2^-$ sidechains enhance water solubility, deepen the cavity (≈ 11.5 Å) to match the N...N distance of roc (≈ 11 Å), and maintain its cylindrical shape. Isothermal titration calorimetry (ITC) measurements establishes that Sugammadex forms a tight host•guest complex with roc in water ($K_a = 1.8 \times 10^7$ M⁻¹), whereas vec and pan form weaker complexes (5.7×10^6 M⁻¹ and 2.6×10^6 M⁻¹, respectively). A detailed structure-activity relationship study demonstrated that the depth of the hydrophobic cavity and negatively-charged substituents at the narrow rim within this series of cyclodextrins are crucial for the high binding affinities observed toward NMBAs.¹² The NMBA reversal efficacy of Sugammadex was assessed in *ex vivo* tests using mouse hemidiaphragm preparations and *in vivo* in guinea pigs, cats, and Rhesus monkeys. Rhesus monkeys treated with Sugammadex (1 mg/kg) recovered 90% of muscle contraction within 3 minutes which was twice as fast as neostigmine/atropine. Subsequent clinical development of Sugammadex by Organon, Schering-Plough, and Merck demonstrated that it effectively reversed the neuromuscular blocking effects of roc and vec in humans. Sugammadex (Bridion™) was first approved by the European Union in 2008 but its approval by the United States FDA was delayed until 2015 due to concerns over hypersensitivity and anaphylaxis. Global sales of Bridion™ in 2019 amounted to \$1.1 billion.¹⁸

2.2 Cucurbit[n]uril-Type Receptors

CB[n] molecular containers are macrocycles comprising n glycoluril monomers linked by $2n$ methylene groups (Figure 1) that are readily prepared in high yield by the condensation of glycoluril and formaldehyde under strongly acidic conditions.¹⁹ The molecular structure of CB[n] features a central hydrophobic cavity that is guarded by two symmetry equivalent ureidyl carbonyl portals of highly negative electrostatic potential. Accordingly, CB[n] show a preference to bind to guest molecules that feature a central hydrophobic domain that is flanked by cationic (typically ammonium) groups.¹⁹ Compared to cyclodextrins, CB[n] typically display several orders of magnitude higher binding affinity toward their targets due

to the combined driving force of the hydrophobic effect and ion-dipole interactions. Individually, the hydrophobic driving force toward complexation is larger for CB[n] than for CDs due to the presence of high energy water molecules in the cavity of CB[n] that are displaced upon CB[n]•guest binding.¹⁹ Ultratight binding affinity ($K_a = 10^{12} \text{ M}^{-1}$) has been achieved for complexes of CB[7] and CB[8] with cationic derivatives of adamantane, diamantane, and ferrocene. Figure 1 presents some of the chemical and physical parameters of CB[n] that are relevant when considering the use of CB[n] as potential *in vivo* sequestration agents. CB[6] and CB[8] have low solubility in pure water, but display enhanced solubility in the presence of metal ions (e.g. Na^+ , K^+) which bind to the C=O portals and reduce CB[n]•guest binding constants. A comparison of the cavity volumes of CDs versus CB[n] (Figure 1) show a correspondence between α -CD and CB[6], β -CD and CB[7], γ -CD and CB[8]. Numerous studies have investigated the toxicity of CB[n] by a combination of *in vitro*, *ex vivo*, and *in vivo* methods and have reached the conclusion that CB[n] are generally very well tolerated.²⁰ Accordingly, CB[n] but especially CB[7] has been used in a variety of applications in biological and medicinal chemistry.

2.2.1 Acyclic Cucurbituril molecular containers—The high binding affinity of macrocyclic CB[n] toward hydrophobic cations suggests their utility as *in vivo* reversal agents for NMBAs. However, the cavity size of CB[7] is insufficient to engulf the steroidal skeleton or roc and vec (*vide infra*) whereas the poor water solubility of CB[8] could complicate *in vivo* use. Acyclic CB[n]-type receptors are known which are both highly water soluble and possess a flexible cavity which can expand to bind larger guests. Acyclic CB[n] maintain the essential features of macrocyclic CB[n] (e.g. tight binding toward hydrophobic cations) and can be easily modified synthetically. Figure 6 shows the structures of acyclic CB[n]-type receptors **M1** and **M2** which feature a central glycoluril tetramer to impart a C-shape and hydrophobic cation binding properties, two terminal aromatic sidewalls to engage in cation- π , CH- π , and π - π interactions with guests, and four sodium sulfonate arms to enhance water solubility and promote secondary electrostatic interactions between host and guest.²¹ Compounds **M1** and **M2** are synthesized by a convergent 6-step synthetic route from inexpensive starting materials on a large scale (60 g batches). **M1** (346 mM) and **M2** (18 mM) possess very good water solubility and do not undergo significant self-association in water as determined by dilution experiments monitored by ^1H NMR spectroscopy (**M1**: $K_s = 47 \text{ M}^{-1}$; **M2**: $K_s = 624 \text{ M}^{-1}$).²¹ *In vitro* assays (MTS cell viability and Adenylate Kinase release cell death) for **M1** and **M2** show very low levels of cytotoxicity whereas *in vivo* maximum tolerated dose studies in mice show that **M1** (MTD > 1.23 g/kg) and **M2** (MTD > 203 mg/kg) are very well tolerated *in vivo*.²² Due to their excellent water solubility, **M1** and **M2** enhance the solubility of a panel of poorly water-soluble anticancer drugs (e.g., paclitaxel, melphalan, clopidogrel, amiodarone and camptothecin) by factors up to 2750-fold.²¹

Given the excellent solubility, low self-association, and high biocompatibility of **M1** and **M2** we next turned our attention to the investigation of their host-guest recognition properties toward neuromuscular blocking agents. We envisioned that the acyclic but conformationally restricted framework of **M1** and **M2** would allow them to flex their structures to accommodate the bulky steroidal skeleton of roc and vec.²⁴ The distance between cationic

N-atoms of roc and vec amounts to ≈ 11 Å which exceeds the distance between CB[n] carbonyl O-atoms (≈ 6 Å). Advantageously, the sulfonated arms deepen the cavity of **M1** and **M2** and engage in secondary sulfonate $\cdots$ ammonium ion-ion interactions. Binding studies of **M1** and **M2** toward roc, vec, cis, ACh and other neuromuscular blockers were initially conducted by ^1H NMR spectroscopy. Large upfield shifts with slow kinetics of exchange on the ^1H NMR timescale were observed for **M2**•roc which suggested a tight complex was formed. Binding affinity measurements for **M1** and **M2** were conducted by direct or competitive UV/Vis titrations. Whereas **M1** exhibits K_a values toward roc ($8.4 \times 10^6 \text{ M}^{-1}$) and vec ($5.4 \times 10^6 \text{ M}^{-1}$) that are similar to Sugammadex ($1.8 \times 10^7 \text{ M}^{-1}$; $5.7 \times 10^6 \text{ M}^{-1}$), **M2** exhibited superior binding affinity toward roc ($3.4 \times 10^9 \text{ M}^{-1}$) and vec ($1.6 \times 10^9 \text{ M}^{-1}$) in 20 mM phosphate buffered water at pH 7.4 while maintaining high levels of discrimination against ACh (19000-fold weaker). **M2** also bound to cisatracurium with $K_a = 4.8 \times 10^6 \text{ M}^{-1}$ whereas Sugammadex does not bind cis. Encouraged by these outstanding binding properties, we proceeded to *in vivo* efficacy studies in collaboration with the Eikermann group. For this purpose, rats were anesthetized with isoflurane followed by instrumenting with intravenous lines and subcutaneous electrodes to supramaximally stimulate the femoral nerve. The rats were then dosed with roc (3.5 mg/kg) to reduce the twitch height 90% followed by treatment with placebo or with **M2** (30 mg/kg). Figure 7a shows that **M2** accelerated the recovery of both train-of-four (TOF) ratio to 0.9 (accelerated from 16 min to 26 s) and spontaneous breathing (decreased to 32 s from 12.5 min), compared to mice treated with placebo.

Follow up dose-response studies compared the reversal efficiency of **M2** relative to Sugammadex or neostigmine for animals treated with vec, roc, or cis. It was found that the time of TOF-recovery from vecuronium-induced neuromuscular blockade was significantly faster with **M2** (Figure 7b) than with neostigmine or placebo. At low doses the potency of **M2** to reverse the effects of vecuronium was higher than sugammadex.²⁵ Using a ^1H NMR based assay we monitored the excretion of the metabolically intact **M2**•roc in the urine of the animals; we observed rapid excretion ($t_{1/2} \approx 1$ hour) which we attribute to the strength of the complex and the tetraanionic nature of **M2**.^{25, 26} These studies established that **M1** and **M2** are promising candidates for further development as broad-spectrum reversal agents for neuromuscular blockers.

2.2.2 Macrocyclic Unmodified CB[n]—Subsequent to our report on the use of **M1** and **M2** to reverse roc and vec, Macartney and coworkers studied the interaction of CB[7] with the steroidal NMBA's roc, vec, pan.²⁷ Electrospray mass spectrometry and ^1H NMR spectroscopy studies showed that CB[7] formed a mixture of 1:1 and 2:1 CB[7]•guest complexes of modest affinity (roc: $1.5 \times 10^4 \text{ M}^{-1}$; vec: $2.2 \times 10^5 \text{ M}^{-1}$; pan: $1.3 \times 10^5 \text{ M}^{-1}$) where the CB[7] binds to the ammonium ion termini of the NMBA rather than engulfing the bulky steroidal skeleton. It should be noted the CB[7] also binds with comparable affinity to ACh ($K_a = 2.2 \times 10^5 \text{ M}^{-1}$). This lack of NMBA vs ACh selectivity is problematic and precludes further development of CB[7] for *in vivo* reversal of neuromuscular block. Conversely, the depolarizing neuromuscular blocking agent succinyl choline (SCh) binds more strongly to CB[7] ($K_a = 1.6 \times 10^6 \text{ M}^{-1}$) which suggests it may function *in vivo*. Unfortunately, however, when animals received lethal doses of SCh the subsequent

administration of CB[7] was not able to fully prevent mouse mortality even at high molar ratios of CB[7]:SCh.²⁸ Normally, bis(quaternary) ammonium ions have high K_a values toward CB[7]. The presence of the two polar ester functional groups that are not complemented by H-bond donors in the CB[7]•SCh complex are likely responsible and illustrate the importance of complementing all relevant functional groups to achieve the high affinity and selectivity needed for effective reversal agents. In 2016, Nau and co-workers reported that CB[8] forms very tight complexes with vec ($K_a = 6.2 \times 10^9 \text{ M}^{-1}$) and pan ($K_a = 2.0 \times 10^8 \text{ M}^{-1}$) in water.²⁹ Follow up *in vivo* work has not been reported. Wang's group has been investigating CB[7] as a sequestration agent in a variety of *in vivo* applications (*vide infra*).

2.3 Pillar[n]arenes

Pillar[n]arenes (PAs) are a popular new class of macrocyclic hosts composed of n aromatic rings (generally dialkoxy benzenes) connected by n methylene ($-\text{CH}_2-$) bridges at the para positions (Figure 8a). This substitution pattern creates a symmetric and relatively well defined pillar-like conformation with two identical portals.³⁰ The supramolecular chemistry of PAs has been investigated in both organic solution and in water. The smaller P[5]A generally binds to narrow n-alkane derived guest molecules whereas the larger P[6]A and P[7]A bind to larger guests including aromatics, viologens, and alicyclic guests. Synthetic modifications of PAs are well developed which allow the introduction of chromophores, recognition handles, and solubilizing groups. The most popular water-soluble PAs are WP5 and WP6 which feature $\text{OCH}_2\text{CO}_2\text{Na}$ solubilizing groups that bind nicely to cationic guests in water (Figure 8a). Pillar[n]arenes are found to be both nontoxic and biocompatible.³¹ Wang and co-workers recognized the structural and functional similarity between CB[7] and WP6 and decided to investigate WP6 for *in vivo* sequestration of the depolarizing NMBA SCh.²⁸ ITC was used to determine the binding affinity of WP6 toward SCh ($2.8 \times 10^5 \text{ M}^{-1}$), ACh ($3.5 \times 10^4 \text{ M}^{-1}$), and choline ($5.99 \times 10^4 \text{ M}^{-1}$) in the competitive medium of phosphate buffered saline (PBS). High IV doses of WP6 (300 mg/kg) were well tolerated by mice and no changes in weight, hematological parameters, or histopathology were seen. To test the ability of WP6 to reverse the effect of SCh *in vivo*, it was first determined that SCh (0.75 mg/kg) is lethal to mice within 1 minute. Remarkably, when WP6 (20 mg/kg) was administered immediately after SCh, 100% of the mice survived (Figure 8b). SC4A or CB[7] did not function as well as WP6 as a sequestration agent for SCh in this application which illustrates the need to achieve high levels of affinity and selectivity for effective *in vivo* sequestration. WP6 was also shown to reverse the SCh-induced plasma membrane potential changes (depolarization) and efflux of intracellular potassium at the cellular level. This study represents the first example of pillar[n]arene hosts as *in vivo* sequestrants and suggests they will prove complementary to CB[n] for such applications.

More recently, Stoikov and co-workers reported the synthesis of water-soluble pillar[5]arene **St1**, which is defunctionalized with $\text{S}(\text{CH}_2)_2\text{CO}_2^-$ functional groups just like Sugammadex.³² Unfortunately, the cavity of **St1** is too small to accommodate the steroidal skeleton of roc and therefore only a weak complex **St1**•roc ($K_a = 4.5 \times 10^3 \text{ M}^{-1}$) could be realized which is too low to function *in vivo*. Most recently, Isaacs and co-workers have reported the synthesis of sulfated pillararenes **P5AS** – **P7AS** (Figure 8a) and studied their

molecular recognition properties toward (di)ammonium ions in aqueous solution.³³ The structural change from carboxylate to sulfate should increase overall negative charge at pH 7 and brings the negative charge closer to the portal of the macrocycle. Compared to WP5 and WP6, **P5AS** and **P6AS** exhibit $10^2 - 10^4$ -fold higher binding affinity toward cationic (bis)quaternary (di)ammonium ions. Remarkably, **P6AS** displayed picomolar binding affinity toward roc ($K_a = 6.3 \times 10^{11} \text{ M}^{-1}$) and vec ($K_a = 1.0 \times 10^{12} \text{ M}^{-1}$) which even exceed the K_a values achieved by **M2** by ≈ 100 -fold. **P6AS** also showed excellent discrimination against ACh (10^4 -fold), which is also present in the neuromuscular junction. **P7AS** forms a tight complex with cis ($K_a = 1.5 \times 10^7 \text{ M}^{-1}$). The ultratight binding and good selectivity of **P5AS** – **P7AS** towards NMBAs suggests that they should be considered as prime candidates for reversing NMBAs *in vivo*.

3 Sequestration agents for other drugs

3.1 Drugs of abuse

According to the US Food and Drug Administration, drugs of abuse (DOAs) are molecules that are used in a manner or amount inconsistent with their intended medical usage. The problematic consumption of methamphetamine (meth), cocaine (coc), heroin, marijuana, hallucinogens (ketamine (ket) and phencyclidine (PCP)), inhalants, or prescription pharmaceuticals (sedatives, tranquilizers, stimulants and pain relievers) are associated with increased morbidity and mortality resulting in severe financial, medical, and socioeconomic burdens (Figure 9a). The costs of drug abuse associated with crime and lost work productivity in the US was estimated at \$193 billion per year.³⁴ Accordingly, the development of pharmacotherapies to combat drug overdose and addiction is of high societal importance. Current clinical treatments for the overdose and addiction to *opioids* are based on a PD intervention approach which relies on the opioid agonists methadone and buprenorphine or the opioid antagonists naloxone and naltrexone.⁶ Overdose with high potency opioids like fentanyl and carfentanil often require multiple doses of naloxone to save patients' lives which highlights the need for new and improved PK-based *in vivo* sequestration agents. Furthermore, although these PD approaches have proven successful for opioids, there is currently no approved medication for the specific treatment of overdose with or addiction to methamphetamine or cocaine.

Given the absence of effective PD pharmacotherapies for methamphetamine and cocaine overdose, significant efforts have been directed toward the development of *in vivo* sequestrants and catalytic degraders of meth and cocaine by the PK approach. Catalytic degraders are somewhat outside the scope of this review, but one example is presented here for illustration (Figure 9b). Butyrylcholine esterase (BChE) is known to recognize and catalyze the hydrolysis of cocaine into inactive ecgonine methyl ester and benzoic acid. Rounds of site-directed mutagenesis can be used to improve the catalytic efficiency. In animal studies, treatment with BChE sped up cocaine hydrolysis and decreased brain cocaine levels; pretreatment with BChE was capable of reducing the behavioral effects, cardiovascular effects, and toxicity of cocaine.³⁵ The immunotherapeutic PK approach (Figure 9c) can be split into either active vaccinations to stimulate the immune system into producing endogenous anti-DOA antibodies or passive immunization accomplished by the

administration of exogenous monoclonal antibodies (mAbs) with high affinity toward specific DOAs. Janda and co-workers have shown that high affinity antibodies for cocaine, methamphetamine, and fentanyl are capable of sequestering each drug in the bloodstream to form mAbs•drug complexes. The antibody•drug complexes are incapable of crossing the blood–brain barrier and cannot arrive at the stimulatory target in the brain.^{8, 36} Janda performed *in vivo* studies of a meth monoclonal antibody and found 83% of mice survived a lethal dose of meth compared to 20% survival for the control group. The PK approach based on monoclonal antibodies is quite appealing because the production of high affinity and selective binders is straightforward relative to the optimization of supramolecular hosts. However, these immunotherapies have some limitations relative to supramolecular hosts including high production costs, large doses (weight) of antibody required, possible immunogenicity, modest thermal stability, and shorter shelf-life. The drawbacks of enzyme and antibody-based therapeutics listed above has stimulated workers in the supramolecular chemistry field to investigate synthetic hosts as alternative treatments for DOA overdose.

Given the generally high binding affinity of CB[n]-type receptors toward hydrophobic cations and the fact that many drugs of abuse exist as hydrophobic ammonium ions in water lead us to consider the use of CB[n]-type receptors as sequestration agents for DOAs by a PK approach. Initially, we screened the binding affinity of molecular containers **M1** and **M2**, CB[7], **SC4A**, and HP- β -CD toward seven representative drugs of abuse including stimulants (meth, coc), hallucinogens (ket, PCP), and prescription type psychotherapeutics used for pain relief (fentanyl, morphine, hydromorphone).³⁷ **SC4A** (Figure 1) is a water soluble and biocompatible member of the calix[n]arene family of molecular containers that features *n* aromatic rings connected in the meta positions by *n* CH₂-bridges. The conformation landscape of calix[n]arenes is complex with calix[4]arenes exhibiting cone, partial cone, 1,2-alternate, and 1,3-alternate forms. The cone conformation features a hydrophobic cavity that binds to complementary aliphatic and alicyclic guests. Tetraanionic host **SC4A** displays good affinity toward hydrophobic cations in water and often binds methonium (Me₃N⁺R) ions in its bowl shaped cavity. Compared to HP- β -CD and **SC4A**, the acyclic CB[n]-type hosts **M1** and **M2** display an overall higher binding affinity toward the seven drugs ($K_a > 10^4$ M⁻¹). For the narrower drugs Meth and Fentanyl, which are a better match to the cavity width of uncomplexed **M1** and **M2**, the K_a values fall in the 10⁶–10⁷ M⁻¹ range. Interestingly, CB[7] showed very tight binding toward meth ($K_a = 1.2 \times 10^8$ M⁻¹) and fentanyl ($K_a = 1.8 \times 10^7$ M⁻¹) and could discriminate against the remaining sterically encumbered DOAs ($K_a < 4400$ M⁻¹) shown in Figure 9a. Figure 10a shows a stereoview of the X-ray crystal structure of **M1**•Meth. As expected, the aromatic moiety of Meth is buried in the central hydrophobic cavity driven by the hydrophobic effect and π – π interactions whereas the ammonium ion forms ion-dipole interactions with the ureidyl carbonyl portal of **M1**, and secondary ion-ion interactions with the four sodium sulfonate solubilizing groups of **M1**.

Given the high affinity binding of **M1**, **M2**, and CB[7] toward methamphetamine, we set out to perform *in vivo* efficacy experiments (Figure 10b). For this purpose, open field tests were performed to monitor the hyperlocomotive activity of rats that had been dosed with methamphetamine. Acyclic CB[n]-type receptor **M2** and CB[7] were evaluated as potential

reversal agents. Two types of experiments were performed: *prevention* in which the animals are given host before Meth, and *treatment* in which animals are given host after Meth. Figure 10b shows the distance travelled by the animals in the open field for placebo, Meth only, and Meth + **M2** at two doses whereas Figure 10c shows the tracking plots for representative animals in each treatment group. Both prevention and treatment approaches were effective at ameliorating the hyperlocomotive activity of rats induced by methamphetamine when high doses of **M2** (130 mg/kg) were used. A lower dosage of **M2** (65 mg/kg) is also effective at decreasing locomotion to baseline levels when given before Meth because Meth is sequestered in the bloodstream before it can cross the blood brain barrier (BBB). Quite interestingly, treatment of methamphetamine dosed rats with CB[7] – which possesses a higher binding affinity toward methamphetamine than **M2** – did not significantly affect the hyperlocomotive activity of the rats compared to the placebo+meth group. Given that CB[7] binds selected guests with $K_a > 10^{12} \text{ M}^{-1}$, we surmise that CB[7] gets filled with other guests in preference to Meth. Some CB[n]•guest complexes – even weak ones – display slow kinetics of dissociation, which suggests kinetic factors may also be at play. This negative result highlights a key design aspect for supramolecular hosts as *in vivo* sequestration agents, namely, that the target guest should be amongst the tightest known binders of the host. In subsequent work, Eikermann and Miczek demonstrated that **M2** is capable of significantly decreasing methamphetamine induced reinstatement in male Long-Evans rats and that **M2** holds potential therefore as an agent to reduce drug addiction relapse.³⁸ Most recently, we showed that **M1** could reverse the respiratory depression and central nervous system effects of rats dosed with fentanyl.³⁹ In combination, the results described above suggest the great potential of acyclic CB[n]-type receptors as *in vivo* sequestration agents for drugs of abuse, especially methamphetamine for which no pharmacotherapies are currently available in the clinic.

In parallel with the use of **M1** and **M2** as *in vivo* reversal agents for NMBAs and DOAs, we have deduced structure-binding affinity correlations in attempts to optimize binding affinity.⁴⁰ We have learned some lessons that we believe are instructive. In one line of inquiry, based on the known importance of the hydrophobic effect and the release of high-energy waters on the binding affinity of CB[n]-type receptors, we sought to increase the cavity volume by using triptycene sidewalls.²³ Unfortunately, based on x-ray crystallography and analysis of ¹H NMR chemical shifts, **TetramerTrip** does not adopt a larger open cavity but rather undergoes a self-folding phenomena where one blade of one triptycene wall folds into its own cavity (Figure 6b). Intramolecular self-complexation must be avoided at all costs to maximize binding affinity. Recently, we disclosed the anthracene walled host **M3** (Figure 6a) which possesses ≈ 10 -fold higher binding affinity toward roc and vec than **M2**.⁴¹ Apparently, the length of the anthracene walls of **M3** sterically precludes a self-folded conformation. In a second line of inquiry we focused on the nature of the arms (linker-solubilizing group combinations). We found that acyclic CB[n] featuring OCH₂CH₂NH₃, OCH₂CH₂OH, and OCH₂CH₂NHAc and OCH₂CH₂NMeAc arms are poor hosts relative to **M1** because these arms result in self-complexation by the arm folding back to the ureidyl portals driven by H-bonds, ion-dipole interactions, and the hydrophobic effect.⁴⁰ In a third line of inquiry, we studied hosts with differing sulfonate arms O(CH₂)_nSO₃Na (*n* = 2, 3, 4) and found that the longer armed host **M2C4** (Figure 6a) is a less potent receptor due to out-

of-plane distortion which allows the (CH₂)₄ groups to partially fill their own cavity. Most recently, we have found that hosts **P5AS** and **M1C0** (Figures 8 and 6a) where the (CH₂)_n linkers have been removed (n = 0) display higher binding affinity – in particular toward diammonium ions – than the analogous hosts with n = 3 presumably due to fixation of the charged groups at the portals of the receptors.^{33, 42} These studies taught us that intramolecular self-folding and complexation must be avoided and electrostatic effects captured to maximize binding affinity.

3.2 Anesthetics

Intravenous general anesthetics including ketamine and etomidate are frequently used in the clinic (Figure 11a). Ketamine is used to sedate the patient and provide analgesia during mechanical ventilation procedures. Etomidate is a rapid acting anesthetic that is commonly used in emergency procedures for sedation and to induce anesthesia. Current strategies for faster emergence from anesthesia target opposing arousal systems or the creation of short acting chemical analogues rather than degrading or chemical sequestering the anesthetic and promoting its clearance from the body. Accordingly, the investigation of PK strategies to sequester anesthetics are attractive.

In 2015, Wang and co-workers were the first to report the ability of CB[n]-type receptors to influence the biological function of anesthetics. Wang chose to study the reversal of the general anesthetic tricaine mesylate (**TM**, Figure 11a, commonly used in fish) in combination with a zebrafish *in vivo* model.⁴³ First, the formation of the CB[7]•**TM** complex was confirmed by ¹H NMR which showed characteristic upfield shifting of the resonances of **TM** upon complexation. Next, UV/Vis spectroscopy was used to measure the strength of the CB[7]•**TM** complex ($K_a = 8.0 \times 10^4 \text{ M}^{-1}$) in water and confirm the 1:1 stoichiometry by Job plot. To test the ability of CB[7] to accelerate the recovery from **TM** anesthesia, zebrafish were first allowed to swim in E3 medium containing 1 mM **TM** for 3 minutes to induce anesthesia. Subsequently, the medium was removed and replaced with E3 medium containing 0.5 mM CB[7] and the locomotion behavior of the zebrafish were monitored for 50 minutes. Figure 11a shows plots of swimming distance of the zebrafish over three time periods for the four treatment groups ($\pm$ CB[7] and $\pm$ **TM**). The group anesthetized with **TM** and treated with CB[7] recovered their swimming distance more rapidly than the group receiving only **TM** which demonstrates the reversal ability of CB[7] in this model system. Additional assays monitored the time required for the zebrafish to regain equilibrium (e.g. float upright) and to regain full cardiac function (e.g. stroke volume, cardiac output, and fractional shortening) which further confirmed the reversal ability of CB[7]. The authors assert that the host•guest complexation of **TM** by CB[7] facilitates the dissociation of **TM** from the Na channels, which generates a concentration gradient that favors the diffusion of **TM** away from the Na channel into the plasma in a manner similar to Sugammadex.

The anesthetic agent ketamine is too large and bulky to be effectively complexed by macrocyclic CB[7] ($K_a(\text{CB[7]•ketamine}) = 640 \text{ M}^{-1}$). Accordingly, Isaacs and Eikermann investigated the complexation of ketamine and etomidate by the acyclic CB[n] **M2** which is able to flex its glycoluril oligomer backbone to accommodate larger guests.²² ¹H and

UV/Vis competition binding assays were used to confirm the 1:1 stoichiometry of the **M2**•ketamine and **M2**•etomidate complexes and determine their binding constants (ketamine: $K_a = 2.1 \times 10^5 \text{ M}^{-1}$; etomidate: $K_a = 3.7 \times 10^4 \text{ M}^{-1}$) in phosphate buffered water. Next, the ability of **M2** to reverse the *in vivo* effects of ketamine and etomidate were tested using Sprague-Dawley rats. Figure 11c shows a plot of the time required for the animals to recover from the loss of righting reflex (LORR) induced by ketamine, etomidate, or propofol upon treatment with placebo or **M2** ($80 \text{ mg kg}^{-1} \text{ min}^{-1}$). The recovery time for animals treated with either etomidate or ketamine was significantly shorter after reversal with **M2** versus placebo. The median (ED_{50}) dose required to reverse etomidate bolus (4 mg.kg) was 984 mg/kg **M2** and to reverse ketamine bolus (30 mg/kg) was 167 mg/kg **M2**. This illustrates that high doses of **M2** can be used to compensate for low binding constants by fundamental mass action considerations. **M2** is capable of reversing the effects of ketamine and etomidate which bind inside the host, but not propofol (neutral molecule) which does not. Complementary electrographic measures of unconsciousness (e.g. burst suppression ratio and EEG power) and functional mobility assays (Combs score) were performed which also indicate the reversal of anesthesia by **M2**. This example provides a proof-of-concept that acyclic CB[n]-type receptors can function as sequestration agents for intravenous anesthetics, which have no pharmacologic alternative for reversal.

3.3 Heparin anticoagulants

Heparin is a widely used anticoagulant for the treatment and prevention of thrombotic diseases and blood clotting in extracorporeal devices. However, extraneous bleeding is a major life-threatening complication associated with heparin therapy. Therefore, continuous monitoring and careful adjustments to dose regimens are needed to increase the antithrombotic efficacy of heparin and reduce the risk to the patient. In cases when bleeding occurs, heparin neutralization with suitable antidotes is necessary. Currently, the only FDA approved medication to counteract heparin anticoagulants is protamine sulfate which is an arginine rich basic protein derived from fish sperm. The association between protamine and heparin is driven by the electrostatic interactions between the anion regions of heparin and the cationic arginine moieties of protamine. However, it is well-known that protamine often causes severe side effects, has unpredictable dose responsiveness, and suffers from a narrow therapeutic window. The discovery of new heparin reversal agents that are simultaneously safe and highly efficient would be valuable clinically. Heparin reversal agents have been reviewed⁴⁴ so we focus here on examples involving synthetic hosts.

Heparin is a helical anionic oligosaccharide of different chain lengths that is based on 1,4-linked sulfated iduronic acid and sulfated glucosamine units that result in an extremely high density of negative charge. Accordingly, the design of supramolecular hosts as reversal agents are logically based on the creation of complementary highly cationic receptors. Cunsolo and coworkers used calix[8]arene as a base scaffold onto which eight cationic (di)cationic groups (L-lysine or 6-amino heptanoic acid were attached by amide bond forming reactions to yield **C8A-1** and **C8A-2** as their CF_3CO_2^- (**a**) or Cl^- (**b**) salts (Figure 12a).⁴⁵ Polycations **C8A-1** and **C8A-2** exist in their 8^+ and 16^+ forms in biological media. Just like their smaller analogues, calix[8]arene derivatives can exist in a variety of conformational forms. Cunsolo performed molecular dynamic simulations of **C8A-1a** in the

presence of heparin and obtained the structure shown (Figure 12b). Host **C8A-1a** adopts a pinched conformation with two sets of cone-like regions and 1,5-repeat units displayed outward. The heparin binds into the cationic cleft of **C8A-1a** to form a complex with a geometry reminiscent of a taco. The binding of **C8A-1** and **C8A-2** to unfractionated or low molecular weight heparin could be monitored by an indicator displacement assay using the complexes of **C8A-1a** and **C8A-2a** with eosin Y and by ^1H NMR assays monitoring the loss of signals in phosphate buffered saline. Hexadecacationic host **C8A-1a** performs comparably (w:w) to protamine, but better than octacationic host **C8A-2a** in these assays which highlights the importance of ammonium $^{++}$ sulfate electrostatic interactions in the recognition process. To further validate the potential in a more realistic biological system, the activated partial thromboplastin time (aPTT) assay was performed. The decrease in aPTT time from that of heparinized blood toward normal blood is steeper for **C8A-1b** than protamine sulfate or **C8A-2b**. In subsequent work, Cunsolo attached **C8A-1** to carboxylate poly(vinyl chloride) with the goal of using the material as filters or membranes in extracorporeal applications (e.g. open-heart surgery).

4 Sequestration agents for toxins

4.1 Endogenous substances/toxins

4.1.1 Cholesterol.—As described above, CDs display a high affinity for hydrophobic species including steroids like cholesterol. Niemann Pick type C (NPC) disease is caused by mutations in the NPC1 and NPC2 genes which cause abnormal accumulation of cholesterol and lipids in cells. Accordingly, hydroxypropyl- β -cyclodextrin (HP- β -CD) has been developed through clinical trials as a therapeutic for Niemann-Pick type C (NPC) disease which operates by supramolecular complexation of the excess cholesterol in lysosomes. The use of HP- β -CD in Niemann Pick type C disease has been reviewed recently⁴⁶ so we focus here on different examples.

4.1.2. Lipofuscin bisretinoids.—The excessive accumulation of lipofuscin bisretinoids (LBs) in the retinal pigment epithelium (RPE) is associated with retinal degeneration and blindness. To date, there has been no approved therapeutic to prevent or reverse lipofuscin-driven retinal degenerative changes. **A2E** is a prototypical LB molecule that contains two polyene arms and a 2-hydroxyethyl pyridinium headgroup (Figure 13a). On the basis of fluorescence assays, Rodriguez-Boulan and co-workers reported that methylated β -cyclodextrin could bind weakly to the hydrophobic arms of **A2E** ($K_a = 250 \text{ M}^{-1}$) and hinder its photo-oxidation and spontaneous oxidation.⁴⁷ *In vitro* experiments conducted using monolayers of RPE cells in transwell plates established that methylated β -CD could reduce **A2E** levels by 49% according to fluorescence microscopy. Figure 13b and c shows the results of *in vivo* experiments performed with 9-month old Abca4-Rdh8 DKO mice that received four intraocular injections of methylated β -CD (1.5 μL , 100 mM) in their right eye (left eye control). An HPLC assay was used to quantify the 25% decrease in **A2E** levels observed in the cyclodextrin group (Figure 13b). Figure 13c shows the immunofluorescence results that demonstrate that methylated β -CD reduced both the number and intensity of LB granules in flat mounted eyecups from the animals. The authors remark that the rapid renal clearance and low ability of methylated β -CD to reach the back

of the eye precludes immediate progression toward the clinic. Related considerations apply to other *in vivo* sequestrants and should be carefully considered.

4.1.3. Deoxycholic Acid (DCA).—Bile acids (BA) are steroidal compounds that perform the useful function of enhancing intestinal absorption of dietary lipids and fat-soluble vitamins upon secretion into the duodenum. Conversely, BAs can be toxic and their accumulation intracellularly can result in cholestatic liver problems and hepatocellular carcinoma. Liu's group previously showed that zwitterionic L-tyrosine derived β -CD host CD-tyrosine binds to DCA as shown in Figure 14b where the CO_2^- group protrudes from the tyrosine functionalized face of β -CD. To improve the potential for *in vivo* DCA sequestration, the unfavorable electrostatic interaction between carboxylates in CD-tyrosine•DCA was eliminated with the creation of tyramine derived host CD-tyramine (Figure 14c).⁴⁸ CD-tyramine is cationic at pH 7.2 whereas DCA is anionic so the CD-tyramine•DCA complex benefits from favorable electrostatic interactions in addition to the hydrophobic effect of steroidal inclusion in the CD cavity. Host CD-tyramine displays a significantly higher binding affinity toward DCA ($K_a = 1.56 \times 10^4 \text{ M}^{-1}$) in water compared to CD-tyrosine ($K_a = 6.27 \times 10^2 \text{ M}^{-1}$) according to ITC measurements. Cell viability studies (MTT assay) were conducted in two human colorectal cell cancer cell lines (HT-29 and HCT-116) which demonstrated that the cytotoxic effects of DCA alone could be significantly reduced when the cells were treated with CD-tyramine•DCA. CD-tyramine also reversed the observed decrease in cellular ATP levels induced by treatment with DCA (300 μM). Finally, the *in vivo* function of CD-tyramine (Figure 14e) was confirmed by the treatment of female BALB/c mice (tail vein injection) with CD-tyramine alone, DCA, or CD-tyramine•DCA (250 μM) and monitoring the levels of total bile acid (TBA) in the blood and urine of the animals. The total BA levels decreased in the blood and increased in the urine which suggests that CD-tyramine or further optimized derivatives hold promise for intrahepatic cholestasis and other BA related diseases.

4.2 Exogenous Substances/Toxins

4.2.1 N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and N-methyl-4-phenylpyridine (MPP⁺).—MPTP and its active metabolite MPP^+ are neurotoxins, which are causally linked with Parkinson's disease (PD) in various vertebrates. In 2015, Wang and co-workers reported that CB[7] forms 1:1 encapsulation complexes with MPTP and MPP^+ as evidenced by ^1H NMR spectroscopy.⁴⁹ The binding constants of the $\text{CB}[7]\cdot\text{MPTP}$ ($K_a = 4.8 \times 10^4 \text{ M}^{-1}$) and $\text{CB}[7]\cdot\text{MPP}^+$ ($K_a = 1.0 \times 10^5 \text{ M}^{-1}$) complexes were determined by UV/Vis titrations in PBS buffer. CB[7] was shown to ameliorate the recession of tyrosine hydrolase in zebrafish larval brains by immunostaining. Finally, Figure 15 shows the results of *in vivo* experiments of zebrafish treated with MPTP (50 μM) alone or in combination with CB[7] (100 μM). The swimming distance of the zebrafish is significantly reduced by treatment with MPTP but reverts toward baseline levels upon treatment with CB[7]. The authors speculate that the neuroprotection afforded by CB[7] may be due to prevention of MPTP or MPP^+ crossing the BBB and by effectively competing with the biological targets (MAOB and DAT) which exhibit similar binding constants toward MPTP and MPP^+ . This work shows that high binding constants are not necessary if the biological targets are also weaker binders and shows that CB[7] has an expanding scope as an *in vivo* reversal agent.

4.2 Viologens

Viologens are a class of dicationic derivatives of bipyridines. Paraquat (**PQ**) and diquat (**DQ**) are prototypical viologens that are widely used herbicides. However, accidental or deliberate ingestion of **PQ** leads to acute poisoning *via* paraquat-induced rapid multi-organ failure and death.⁵⁰ The biochemical mechanism of **PQ** toxicity involves the elevation of intracellular levels of reactive oxygen species (ROS) such as $O_2^{\bullet-}$ and $HO^{\bullet}$ by redox cycling (Figure 16). The generated ROS cause cellular toxicity by the oxidation of lipids, proteins, and nucleic acids. Apart from the acute toxicity of **PQ**, the high mortality rate for viologen poisoning is primarily due to the lack of efficacious and specific detoxification treatments.

In 2009, Liu and co-workers reported a landmark study on **PQ** detoxification based on host-guest chemistry.⁵¹ They used 1H NMR and x-ray crystallography to show that **C4AS** and **C5AS** bind **PQ** and **DQ** within their cavities driven by π - π , electrostatic interactions, and the hydrophobic effect. ITC titrations showed that **C5AS** forms tight complexes with **PQ** ($K_a = 2.51 \times 10^5 M^{-1}$) and **DQ** ($K_a = 3.23 \times 10^6 M^{-1}$) at pH 7.2 in PBS. *In vivo* efficacy studies were performed to test the ability of **C5AS** to reduce the 90% mortality rate of animals poisoned with **PQ** (Figure 17b). Remarkably, when the mice were treated with **C5AS**•**PQ** complex only 10% of the animals died and weights and tissue pathology (lung, liver) were comparable to animals receiving saline or **C5AS** alone. Administration of **C5AS** is even effective at reducing the mortality of mice up to 2 hours after **PQ**! Interestingly, treatment with **C5AS** 1 hour after **PQ** fully prevents the death of the animals which the authors trace to a pharmacokinetic effect where **PQ** starts appearing in the plasma of the animals at 60 min. with a maximum at ≈ 90 min. The authors suggest that the effectiveness of **C5AS** in this application is due not only to its sequestration ability but also because the **C5AS**•**PQ** complex is harder to reduce which decreases ROS production. Finally, the phenolic OH groups of **C5AS** can deactivate the ROS by H-atom abstraction. This study highlights the importance of factors beyond binding constant in their *in vivo* performance. The very good performance of **C5AS** in this application has prompted further investigations of other supramolecular hosts as described below.

Given the excellent biocompatibility of CB[7] and its known ability to bind **PQ** in water prompted Wang and co-workers to investigate its potential as an oral treatment for **PQ** poisoning. First, the binding strength of CB[7]•**PQ** across the relevant gastrointestinal pH range (1.2–7.2) was determined by ITC ($K_a > 10^5 M^{-1}$).⁵² Subsequently, the ability of CB[7] to protect A549 and LO2 cells *in vitro* was demonstrated by cell viability assays. The oral administration of **PQ** in the presence of CB[7] in mice showed significantly decreased **PQ** concentrations in the plasma and the tissues of major organs. Figure 18 shows the survival curves for mice treated with supra-lethal levels of **PQ** and either CB[7] or activated carbon (AC) at different time points. Relative to AC, treatment with CB[7] reduces the mortality of the mice and mice who survive did not show abnormality by hematoxylin and eosin stained sections of the intestines and major organs. These encouraging results suggest CB[7] holds promise as an antidote for **PQ** poisoning.

As early as 2012, Huang and co-workers reported that WP6 forms a remarkably tight complex with **PQ** in water ($K_a = 1.0 \times 10^8 M^{-1}$).⁵⁴ *In vitro* cell viability experiments (Raw

264.7 cells) showed that WP6 encapsulation of **PQ** reduced the detrimental effects of **PQ**. However, WP6 possesses an overall high negative charge at the rim of the host, which could lead to fast *in vivo* clearance and the potential for systemic **PQ** toxicity to persist even when given large dose. Accordingly, Sun and coworkers have developed a “supramolecular hunter” in which the WP6 hosts were anchored on red blood cells (RBC) non-covalently *via* a Janus dendrimer amphiphile (JDA) linker to create a long circulating system (RBC•JDA•WP6) to continuously remove **PQ** from the blood (Figure 19).⁵³ By design, the WP6•**PQ** complex is stronger than that of JDA•WP6, so a noncovalent guest-exchange reaction ensues in the presence of **PQ** in the bloodstream to deliver WP6•**PQ** and thereby reduce ROS toxicity of **PQ**. Moreover, it was found that RBC•JDA•WP6 could easily reach the polluted organs and lower the **PQ** level in the lung and kidney (Figure 19b,c). As a result, this nano-sequestration method shows favourable protection and treatment efficacy for the target organs of **PQ**. Among all the examined therapeutic strategies, the RBC•JDA•WP6/1 h treatment showed the best therapeutic efficacy, as reflected by the improved survival rate of the poisoned rats (Figure 19d). This strategy suggests that rationally designed supramolecular nano-systems can actively, precisely, and continuously sequester toxicants *in vivo*.

Conclusion and perspective

Although the contributions of pharmaceuticals toward human health is unquestionable, there are situations where the side effects of prescribed drugs or the detrimental effects of illicit drugs need to be mitigated. Such effects can be mitigated pharmacodynamically by antagonism of the bioreceptor•drug complex (e.g. naloxone for opioid overdose) or pharmacokinetically by reduction of free drug concentration (e.g. protamine for heparin anti-coagulants). Herein, we have focused on the use of supramolecular host scaffolds to create *in vivo* sequestrants by the PK approach. Work in this field can be traced to the pioneering work at Organon on Sugammadex for the reversal of neuromuscular blockers. Sugammadex is remarkable because it is easy to synthesize inexpensively, is highly soluble in water, possesses excellent biocompatibility, displays high affinity and selectivity for roc and vec over ACh and many other drugs, and promotes the clearance of roc and vec from the body. In this tutorial review, we presented information on the physical and molecular recognition properties of hosts (calixarenes, (acyclic) cucurbiturils, and pillararenes) that can be used as high affinity core scaffolds to create new *in vivo* sequestrants for a variety of compounds including neuromuscular blockers, drugs of abuse, anesthetics, paraquat, neurotoxins, and heparin anti-coagulants. Strategies to improve host•guest binding affinity including the importance of hydrophobic driving force, π – π interactions, and electrostatic interactions were presented along with lessons learned along the way. Host intermolecular self-association and intramolecular self-folding must be avoided because they reduce target binding affinity. The prospects for the field of supramolecular *in vivo* sequestrants is bright considering that several new agents (CB[7], Calabadions, WP6, calixarenes) have demonstrated *in vivo* function preclinically and are under consideration for advancement toward the clinic. Many challenges remain including the development of host systems as sequestrants for guests more complicated than hydrophobic (di)cations (e.g. with intracavity functionality to complement polar guest functional groups), the development of hosts that

resist the effects of physiological salt and serum proteins, the development of efficient synthetic methods to access low-symmetry host systems, the reliable integration of computational methods for host screening for binding and physical properties, and rapid analytical methods to assess host selectivity against the wide variety of pharmaceuticals used clinically that must not be sequestered. Methods that covalently or non-covalently conjugate supramolecular hosts to more complex systems (e.g. RBC based supramolecular hunters) hold the promise of extended blood circulation time, potential for biological targeting, and improved physical properties. The clearance of these hurdles will not only dramatically enhance our understanding of molecular recognition in water but will enable the mitigation of the lingering (life threatening) effects of commonly used (abused) drugs for the betterment of human health.

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Key Learning Points.

- Antagonism of the *in vivo* effect of drugs can be achieved by pharmacodynamic or pharmacokinetic approaches.
- Supramolecular hosts form sufficiently tight complexes with drugs and toxins to function as *in vivo* sequestrants.
- When optimizing host•drug binding affinity it is important to minimize intermolecular self-association and intramolecular self-folding processes and fully exploit electrostatic interactions.
- Supramolecular hosts (cyclodextrins, calixarenes, (acyclic) cucurbiturils, and pillararenes) generally display very good biocompatibility which allows them to be used in excess to outcompete the bioreceptor•drug complex.
- Beyond binding, hosts influence other relevant properties of drugs including adsorption, distribution, metabolism, and excretion as well as chemical stability and redox properties.

Figure 1.

a-d) Chemical structure and cartoon illustration, and e) physical information for CDs, CB[n], pillar[n]arenes, and sulfonatocalix[n]arenes. – solubilizing group dependent.

Figure 2.
Illustration of pharmacokinetic (PK) and pharmacodynamic (PD) approaches to drug reversal.

Figure 3.

Changes in host and guest solvation including expulsion of high-energy water molecules upon host•guest complexation. (Adapted with permission from ref.¹¹. Copyright 2014, Wiley-VCH)

Figure 4.

(Top) Structures of NMBAs. (Bottom) Illustration of the neurotransmission pathway at the neuromuscular junction (NMJ) activated by ACh, blocked by NMBA, and reversal by treatment with AChE inhibitors or by hosts. (Adapted with permission from ref.¹². Copyright 2002, American Chemical Society)

Figure 5.
Structure of Sugammadex and the crystal structure of the Sugammadex•roc complex.

Figure 6.

a) Structures of **M1** – **M3**. b) Representation of the acyclic CB[n]-type receptor•drug complex. c) Features of acyclic CB[n] complexes. (Reproduced with permission from ref.²³. Copyright 2018, Wiley-VCH)

Figure 7.

a) Plots of the time required (o = times for different animals): left) to achieve a TOF ratio of 0.9 after administration of placebo or **M2**, and right) to achieve spontaneous breathing after administration of placebo or **M2**. b) Plot of the recovery of TOF ratio at different doses of **M2** or sugammadex. Corresponding values for animals given placebo or neostigmine (0.06 mg / kg) are including for comparison. Reproduced from refs.²⁴ and²⁵. Copyright 2012 and 2015, Wiley-VCH and American Society of Anesthesiologists.

Figure 8. Structures of water soluble pillar[n]arenes. (Reproduced with permission from ref.²⁸. Copyright 2019, Ivyspring International).

Figure 9.

a) Structures of DOAs, b) hydrolysis of cocaine by BChE. c) Illustration of sequestration of DOAs by an antibody.

Figure 10.

a) Cross eyed stereoview of the crystal structure of **M1**•meth. b) Bar graph showing distance travelled as a percentage of the placebo + meth locomotor activity level. c) Tracking plots illustrate the distance traveled by one rat within 20 min. Conditions: a) baseline, no meth; b) meth (0.30 mg/kg)+placebo; c) meth (0.30 mg/kg) + **M2** (65 mg/kg). e) meth (0.30 mg/kg) + **M2** (130 mg/kg). (Reproduced with permission from ref.³⁷. Copyright 2017, Wiley-VCH)

Figure 11.

a) Plots of swimming distance versus treatment group for zebrafish anesthetized with TM. b) Effect of infusion of **M2** ($80 \text{ mg kg}^{-1} \text{ min}^{-1}$) on time to recovery from loss of righting reflex (LORR) after administration of a single intravenous bolus of etomidate, ketamine, or propofol. (Reproduced with permission from refs.⁴³ and²². Copyright 2015 and 2016, Royal Society of Chemistry and American Society of Anesthesiologists)

Figure 12.

a) Structures of polycationic calix[8]arene derivatives **C8A-1** and **C8A-2**. b) Computational model of **C8A-1a**•heparin. Heparin: stick model; **C8A-1a**: space-filling model. (Reproduced with permission from ref.⁴⁵. Copyright 2006, Royal Society of Chemistry)

Figure 13.

a) Structure of **A2E** with bulky β -ionone head groups. b) CD treatment decreased the total **A2E** content as determined by HPLC. c) The CD treatment reduced the number and fluorescent intensity of lipofuscin granules. (Reproduced with permission from ref.⁴⁷. Copyright 2014, National Academy of Sciences)

Figure 14. Structures of a) DCA, and b) CD-tyrosine and CD-tyramine. c) Schematic illustration of host-guest interaction between CD-tyramine or CD-tyrosine with DCA, and d) the *in vivo* clearance of DCA by CD-tyramine in mice. e) The TBA data of blood and urine after injection with free DCA, CD-tyramine, and CD-tyramine•DCA complex. Reproduced with permission from ref.⁴⁸. (Copyright 2017, American Chemical Society)

Figure 15.

CB[7] attenuated **MPTP**-induced locomotion deficiency in zebrafish larvae: a) Plot of total swimming distance over 45 min. b) Representative swimming traces of the zebrafish larvae from the four treatment groups. Velocity color code: Red, >6 mm/s; green, 3 – 6 mm/s; black, <3 mm/s. (Reproduced with permission from ref.⁴⁹. Copyright 2015, American Chemical Society)

Figure 16. Biochemical mechanism of PQ toxicity.⁵⁰ HWR: Haber-Weiss reaction. Adapted with permission from ref.⁵¹. Copyright 2009, American Chemical Society)

Figure 17.

a) Structure of diquat (**DQ**) and x-ray crystal structure of **SC4A•DQ**. b) Survival curves for mice treated with **SC5A**, **SC5A•PQ**, or **PQ**. c) micrographs of the lungs of mice from the three treatment groups. (Reproduced with permission from ref.⁵¹. Copyright 2009, American Chemical Society)

Figure 18.

a) Schematic representation of the administration methods. AC: activated charcoal. b) Kaplan-Meier survival curves of mice orally administered with CB[7] at different time after the mice had ingested PQ. c) Kaplan-Meier survival curves of mice orally administered with AC at different time after the mice had ingested PQ. (Reproduced with permission from ref. ⁵². Copyright 2019, Ivyspring International Publisher)

Figure 19.

a) Schematic representation of the WP6 anchored on RBC for **PQ** detoxification in the blood. (b) The **PQ** levels in (left) lung and (right) kidney under different therapeutic strategies and injection frequency. (c) Curves of the survival rate under different therapeutic strategies. (d) The moving paths for post-poisoned rats given different treatments in an open-field. (Reproduced with permission from ref.⁵³. Copyright 2020, American Chemical Society)