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In Vitro and In Vivo Sequestration of Phencyclidine by Me_4Cucurbit[8]uril**

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Abstract: We report investigations of the use of cucurbit[8]uril (CB[8]) macrocycles as an antidote to counteract the in vivo biological effects of phencyclidine. We investigate the binding of CB[8] and its derivative Me_4CB[8] toward ten drugs of abuse (**3**–**9**, **12**–**14**) by a combination of ^{1}H NMR spectroscopy and isothermal titration calorimetry in phosphate buffered water. We find that the cavity of CB[8] and Me_4CB[8] are able to encapsulate the 1-amino-1-aryl-cyclohexane ring system of phencyclidine (PCP) and ketamine as well as the morphinan skeleton of morphine and hydromorphone with K_d values $\leq$50 nM. In vitro cytotoxicity (MTS metabolic and adenylate kinase cell death assays in HEK293 and HEPG2 cells) and in vivo maximum tolerated dose studies (Swiss Webster mice) which were performed for Me_4CB[8] indicated good tolerability. The tightest host·guest pair (Me_4CB[8]·PCP; $K_d = 2$ nM) was advanced to in vivo efficacy studies. The results of open field tests demonstrate that pretreatment of mice with Me_4CB[8] prevents subsequent hyperlocomotion induction by PCP and also that treatment of animals previously dosed with PCP with Me_4CB[8] significantly reduces the locomotion levels.

Introduction

Drug abuse is a major societal problem in the United States and deaths due to overdose are common.[1] Estimates of the costs associated with decreased work productivity and emergency room visits due to illicit drug use exceed $200 billion per year.[2] Illicit drugs are used by an estimated 10.2% of the US population aged 12 and older each month.[3] Commonly abused illicit drugs include methamphetamine, fentanyl, cocaine, heroin, hallucinogens (phencyclidine and ketamine), and marijuana along with abuse of prescription medicines. Accordingly, the development of therapeutics to treat drug overdose is a pressing societal need. Currently, naloxone—which acts by a pharmacodynamic effect at the opioid receptor—is available to treat overdose with opioids[4] but is ineffective at treating the effects of non-opioids like methamphetamine, cocaine, phencyclidine (PCP), and ketamine.[5] As a powerful alternative, researchers are exploring the use of pharmacokinetic approaches to decrease the freely circulating drug concentration by catalytic destruction or sequestering them in the bloodstream. Human butyrylcholine esterase, for example, which hydrolyzes cocaine to ecgonine methyl ester is being explored as a therapeutic for cocaine.[6] Similarly, antibody-based therapeutics that bind to and sequester methamphetamine, cocaine, and fentanyl in the bloodstream and thereby prevent their passage through the blood brain barrier have been investigated.[7] Our group has been interested in combating death due to drug overdose by implementing a pharmacokinetic approach based on the in vivo sequestration of drugs as their molecular container·drug complexes.[8]

Macrocycles have long occupied a central role in the field of supramolecular chemistry. Macrocycles enjoy this privileged status because the preorganization inherent to macrocycles leads to higher binding constants and often highly selective interactions with their target guests. Among the most popular macrocyclic host families are the cyclodextrins, calixarenes, cyclophanes, cucurbit[*n*]urils (CB[*n*]), and most recently pillararenes (Figure 1).[9] Molecular container compounds which also include systems self-assembled by H-bonds, metal–ligand interactions and the hydrophobic effect[10] bind to and sequester guests compounds within their cavities and thereby change their chemical and physical properties. Popular in vitro applications of these molecular containers include their use to prepare sensing ensembles,[11] supramolecular catalysts,[10d, 12] supramolecular materials,[13] chiral separations phases,[14]

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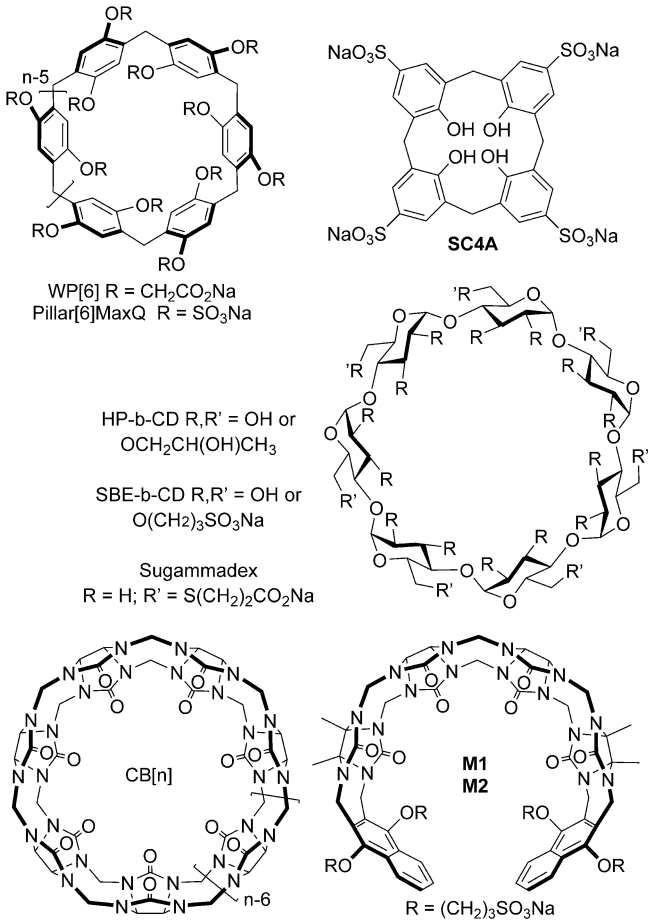

Figure 1. Structure of CB[*n*] and acyclic CB[*n*]-type receptors **M1** and **M2**.

household deodorizers,[15] and molecular machines.[16] For molecular containers with excellent biocompatibility and sufficient affinity, in vivo applications become feasible. For example, the sulfonated calix[4]arene derivative **SC4A** exhibits excellent biocompatibility[17] and has been investigated as an in vivo (mice) reversal agent for the toxic effects of paraquat (methyl viologen).[18] Squaraine rotaxanes have been used for in vivo imaging and theranostic applications.[19] Most significantly, the cyclodextrin derivatives HP-β-CD and SBE-β-CD (Figure 1) are widely used as solubilizing excipients for insoluble drugs for parental administration to humans[20] whereas Sugammadex is used as an in vivo reversal agent for the post-operative side effects of the neuromuscular blocking agents rocuronium and vecuronium.[21] Recently, water soluble pillararenes have been investigated as in vitro hosts and in vivo reversal agents for neuromuscular blockers and as solubilizing excipients for insoluble drugs.[22]

Figure 1 shows the structure of CB[*n*] which features *n* glycoluril rings connected by 2*n* methylene bridges which define a hydrophobic cavity rimmed by two symmetry equivalent ureidyl carbonyl portals.[23] Within the field of molecular containers, CB[*n*] ($n=5$, 6, 7, 8, 10; Figure 1) have distinguished themselves because of their remarkably tight binding toward hydrophobic (di)cations in water with K_a values that regularly exceed $10^6\ M^{-1}$, often exceed $10^9\ M^{-1}$, and even reach $10^{17}\ M^{-1}$ in special cases.[24] The remarkable binding affinity of CB[*n*] toward their guests has been traced to their highly electrostatically negative C=O portals which constitute cation binding regions juxtaposed with a hydrophobic cavity that contains high energy water molecules that provide an enthalpic driving force upon complexation.[24a, c, 25] Although unfunctionalized macrocyclic CB[*n*] exhibit excellent biocompatibility,[26] only CB[7] exhibits both good solubility (> 5 mM) and a cavity large enough to encapsulate biologically relevant guests.[25a] Accordingly, the group of Professor Ruibing Wang has demonstrated the use of CB[7] as an in vivo sequestration agent in several applications including to counteract the toxic effects of paraquat,[27] to alleviate blood coagulation induced by hexadimethrine bromide (mice),[28] to reverse paralysis induced by succinyl choline (mice),[22a] to reverse general anesthesia in zebrafish,[29] and to mask the bitter taste and toxicity of various species.[30] Over the past decade, we and others,[31] have been developing the synthesis and studying the molecular recognition properties of acyclic CB[*n*]-type receptors (e.g. **M1** and **M2**, Figure 1) as a means to enhance their water solubility while maintaining binding affinity and tailoring their binding selectivity toward specific classes of biologically active guests. Along this line of inquiry, we have demonstrated the ability of **M1** and **M2** as solubilizing excipients for insoluble drugs and as in vivo sequestration agents for neuromuscular blockers, the general anesthetics etomidate and ketamine, and drugs of abuse (methamphetamine and fentanyl).[8, 32] In a separate line of inquiry, the Isaacs group has developed a building block approach to functionalize macrocyclic CB[*n*] ($n=6$, 7, 8)[33] and showed that these CB[*n*] derivatives often possess enhanced water solubility. Accordingly, we saw an opportunity to expand the range of drugs that can be efficiently sequestered in vivo through the use of a water soluble derivative of macrocyclic CB[8] that displays enhanced binding affinity toward sets of drugs that are less efficiently sequestered by the smaller CB[*n*] homologues CB[6], CB[7], and acyclic CB[*n*] **M1** and **M2**. In this paper, we explore the in vitro binding affinity of CB[8] and its water soluble derivative Me_4CB[8] (Figure 2) toward a panel of drugs of abuse and demonstrate that Me_4CB[8] acts as an in vivo sequestration agent to reverse the hyperlocomotion observed in mice treated with PCP.

Results and Discussion

This results and discussion section is subdivided into sections as follows. First, we determine the binding properties of CB[8] and Me_4CB[8] toward a panel of commonly used and abused drugs (Figure 3) by a combination of ^{1}H NMR spectroscopy and isothermal titration calorimetry (ITC). Subsequently, we detail the results of in vitro cytotoxicity and in vivo maximum tolerated dose studies conducted for Me_4CB[8]. Finally, we demonstrate the ability of Me_4CB[8] to sequester PCP in vivo and thereby reduce the hyperlocomotion observed for mice that had been treated with PCP.

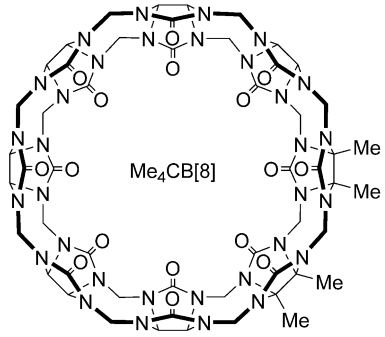

Figure 2. Structure of the water soluble CB[8] derivative ($Me_4CB[8]$) that was used in this study.

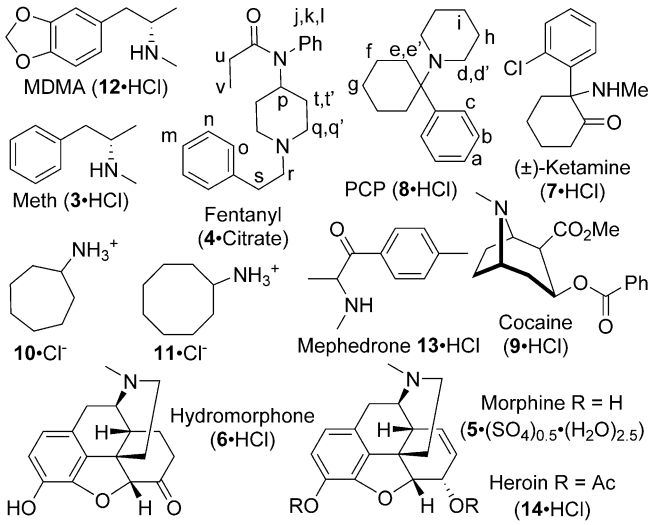

Figure 3. Chemical structures of competitive guests and drugs of abuse used in this study.

Host selection

In previous research,[8,32d] we found that the water soluble hosts **M1**, **M2**, CB[7], **SC4A**, and HP-β-CD exhibit poor binding affinity $K_a \approx 10^2$–$1\times10^6\ M^{-1}$ toward several members of the drug panel (morphine, hydromorphone, ketamine, PCP, cocaine). The non-opioids cocaine, PCP, and ketamine cannot be reversed by naloxone and no other specific therapeutics are used clinically to rescue patients in overdose cases. As mentioned above, CB[8] possesses such poor water solubility ($<10\ \mu M$)[25a] that it would not be possible to administer sufficient doses of CB[8] (e.g. limited by CB[8] concentration and maximum volume) to act as an in vivo sequestration agent toward drugs of abuse like PCP. Accordingly, we resynthesized CB[8] and the previously reported water-soluble host $Me_4CB[8]$ (3.1 mM) by the literature procedures.[23,33c]

Qualitative ^{1}H NMR host–guest recognition study

Initially, we performed qualitative host·guest binding studies between CB[8] and its water soluble derivative $Me_4CB[8]$ and the drug panel (**3**–**9**, **12**–**14**) by ^{1}H NMR spectroscopy (Supporting Information) to determine whether their cavities are large enough to bind drugs containing the morphinan ring system (**5**, **6**, **14**), the 1-aryl-1-amino cyclohexane moiety (**7** and **8**), and the aza-bicyclo[3.2.1]octane ring system of cocaine (**9**). For example, Figure 4a–c shows the ^{1}H NMR spectra recorded for uncomplexed PCP (**8**) as well as 1:1 and 1:2 mixtures of $Me_4CB[8]$ with **8**. At a 1:1 ratio of $Me_4CB[8]$:**8** we observe significant complexation induced upfield shifting of the resonances for the phenyl and piperidinium moieties of **8**. These observed upfield shifts constitute good evidence for the inclusion of the phenyl and piperidinium moieties inside the cavity of $Me_4CB[8]$ within the $Me_4CB[8]$·**8** complex. In the spectrum recorded at 1:2 ratio of $Me_4CB[8]$:**8** (Figure 4c), the presence of separate but broadened resonances for free **8** indicates that the guest exchange process is in the slow to intermediate regime on the chemical shift timescale. As expected based on symmetry, the spectrum for unsubstituted CB[8]·**8** displays two pairs of doublets for the diastereotopic CH_2 groups on the top and bottom rim of CB[8] that become different in the complex (Supporting Information). The inclusion of two 6-membered rings of **8** inside CB[8] and $Me_4CB[8]$ and the slow to intermediate guest exchange rate provided a first glimmer that high affinity binding of PCP was achievable. In contrast, CB[7] and acyclic CB[*n*]-type receptors **M1** and **M2** simply bind weakly to the aromatic

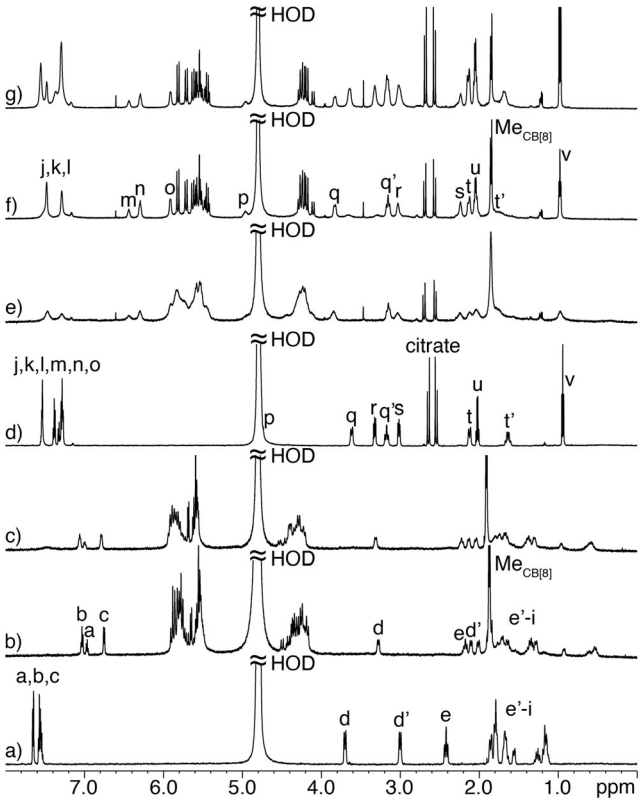

Figure 4. ^{1}H NMR spectra recorded (600 MHz, RT, D_2O) for a) PCP **8** (0.4 mM), b) an equimolar mixture of $Me_4CB[8]$ and **8** (0.2 mM), c) a mixture of **8** (0.4 mM) and $Me_4CB[8]$ (0.2 mM), d) fentanyl **4** (0.4 mM), e) a equimolar mixture of **4** (0.2 mM) and $Me_4CB[8]$ (0.2 mM), f) a mixture of **4** (0.4 mM) and $Me_4CB[8]$ (0.2 mM), g) a mixture of **4** (0.8 mM) and $Me_4CB[8]$ (0.2 mM).

residue of **8**. Figure 5 shows a cross-eyed stereoview of an MMFF minimized model of the $Me_4CB[8]$·**8** complex which illustrates the simultaneous penetration of the phenyl and the piperidinium moieties into the cavity of $Me_4CB[8]$ whereas the cyclohexyl ring remains at the ureidyl carbonyl portal of $Me_4CB[8]$.

Figure 4 d–g shows the ^{1}H NMR spectra recorded for **4** (fentanyl) alone and 1:1, 1:2, and 1:4 mixtures of $Me_4CB[8]$ and **4**. At a 1:1 ratio of $Me_4CB[8]$:**4** the resonances for most protons of **4** become broadened indicating complexation is occurring and that guest exchange is in the intermediate exchange regime on the chemical shift timescale. At a 1:2 ratio of $Me_4CB[8]$:**4** (Figure 4 f), a single set of sharp resonances is observed for guest **4** which is consistent with the formation of a discrete $Me_4CB[8]$·$\mathbf{4}_2$ complex. The three aryl resonances for the phenethylammonium ion moiety of **4** are shifted about 1 ppm upfield upon complexation whereas the resonances for the (C=O)NPh moiety do not shift. The observation of a single pair of diastereotopic CH_2 resonances for the CB[8] unit within the CB[8]·$\mathbf{4}_2$ complex (Supporting Information) dictates a head-to-tail orientation of the cavity bound phenethylammonium ion groups which presumably exhibit π–π stacking interactions. The ability of CB[8] to promote homo and hetero-ternary complexation of aromatic resonances is well precedented in the literature.[16b,34] Finally, at a 1:4 ratio of $Me_4CB[8]$:**4** we observe separate resonances for complexed and uncomplexed guest **4** which indicates slow kinetics of guest exchange on the ^{1}H NMR timescale which is typically observed for tight complexes. Related ^{1}H NMR experiments were performed for the remaining guests (**3**, **5**, **6**, **7**, **9** and **12**–**14**) with CB[8] and $Me_4CB[8]$ and are presented in the Supporting Information. Interestingly, complexes of CB[8] and $Me_4CB[8]$ with **5** and **6** display upfield shifting of most of the protons of the morphinan ring system which indicates that they fit in the cavity of CB[8] and $Me_4CB[8]$; they also displays slow kinetics of guest exchange which suggests strong binding. Similarly, the ^{1}H NMR spectrum of CB[8] and $Me_4CB[8]$ with ketamine **7** displays upfield shifting for both the aromatic and cyclohexyl resonances of **7** which indicates the 1-aryl-1-amino cyclohexyl moiety is fully bound inside CB[8] and $Me_4CB[8]$ (Supporting Information). Lastly, the ^{1}H NMR spectra of CB[8] and $Me_4CB[8]$ with cocaine **9** shows small upfield shifts for the benzoyl group and little shifting for the remaining protons which shows that the benzoyl group is preferentially bound by CB[8].

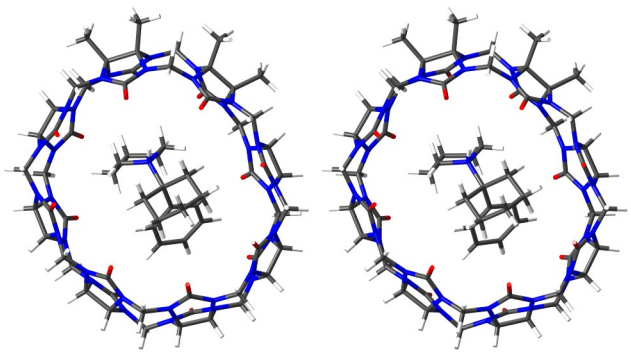

Figure 5. Cross-eyed stereoview of an MMFF minimized geometry of the $Me_4CB[8]$·**8** complex. Color code: C, grey; H, white; N, blue; O, red.

Measurement of the thermodynamic parameters of complex formation by ITC

With these promising qualitative results in hand and the likelihood of tight binding complexes, we turned our attention to measuring the thermodynamic parameters of binding by ITC. ITC provides invaluable data to supramolecular chemists such as (K_a, M^{-1}), ΔH, and stoichiometry of binding and has been previously used to study the complexation of guests **3**–**9** with CB[7] and acyclic CB[*n*]-type receptors **M1** and **M2**.[8,32d] This data set will provide a comparison to evaluate the potential of CB[8] and $Me_4CB[8]$ as a sequestering agents for drugs **3**–**9** and **12**–**14**. Direct ITC titrations of **3**, **4**, **9**, and **12**–**14** in the syringe into solutions of either CB[8] or $Me_4CB[8]$ in the sample cell was performed and the resulting thermodynamic parameters are presented in Table 1. The K_a values for these complexes range from $K_a = (2.98 \pm 0.47) \times 10^4\ M^{-1}$ for $Me_4CB[8]$·**3** to $K_a = (1.9 \pm 0.09) \times 10^7\ M^{-1}$ for CB[8]·**4**. The ITC results for guest **4** with CB[8] and $Me_4CB[8]$ confirm the overall 1:2 stoichiometry observed by NMR and show a negative cooperativity in the formation of the ternary complexes. The remaining drugs displayed binding constants that exceed the range that can be

Table 1. Thermodynamic parameters (K_a (M^{-1}), ΔH° (kcal mol^{-1})) determined for the complexes of CB[8] and $Me_4CB[8]$ with **3**–**14** by ITC. Conditions: 20 mM NaH_2PO_4 buffer (pH 7.4), 298 K.

Guest (G)	K_a (CB[8]·G) [M^{-1}] ΔH° [kcal mol^{-1}]	K_a ($Me_4CB[8]$·G) [M^{-1}] ΔH° [kcal mol^{-1}]	*n*
Meth **3**[a]	$(1.47 \pm 0.09) \times 10^5$ (-7.84 ± 0.1)	$(2.98 \pm 0.47) \times 10^4$ (-4.61 ± 0.04)	1
Fentanyl **4**[a]	$(1.9 \pm 0.09) \times 10^7$ (-10.8 ± 0.06) $(3.7 \pm 0.04) \times 10^5$ (-7.67 ± 0.04)	$(1.98 \pm 0.02) \times 10^6$ (-3.58 ± 0.01) $(9.52 \pm 0.03) \times 10^4$ (-1.94 ± 0.01)	1 2
Morphine **5**[b]	$(3.41 \pm 0.15) \times 10^8$ (-13.6 ± 0.04)	$(4.67 \pm 0.17) \times 10^7$ (-8.24 ± 0.02)	1
Hydromorphone **6**[b]	$(1.7 \pm 0.11) \times 10^8$ (-15.8 ± 0.1)	$(2.14 \pm 0.05) \times 10^7$ (-8.43 ± 0.02)	1
Ketamine **7**[c]	$(1.09 \pm 0.07) \times 10^9$ (-17.3 ± 0.16)	$(2.81 \pm 0.67) \times 10^7$ (-9.37 ± 0.02)	1
PCP **8**[c]	$(2.1 \pm 0.2) \times 10^{10}$ (-14.9 ± 0.04)	$(5.35 \pm 0.19) \times 10^8$ (-8.39 ± 0.01)	1
Cocaine **9**[a]	$(6.45 \pm 0.43) \times 10^5$ (-8.26 ± 0.15)	$(2.77 \pm 0.15) \times 10^5$ (-6.90 ± 0.07)	1
10[a]	$(5.3 \pm 0.23) \times 10^6$ (-7.62 ± 0.03)	$(1.04 \pm 0.02) \times 10^6$ (-5.35 ± 0.01)	1
11[a]	$(3.2 \pm 0.26) \times 10^7$ (-8.26 ± 0.04)	$(2.79 \pm 0.17) \times 10^6$ (-5.63 ± 0.03)	1
MDMA **12**[a]	n.d.	$(3.13 \pm 0.32) \times 10^4$ (-13.8 ± 0.77)	1
Mephedrone **13**[a]	n.d.	$(3.07 \pm 0.39) \times 10^5$ (-9.89 ± 0.29)	1
Heroin **14**[a]	n.d.	$(7.94 \pm 0.07) \times 10^4$ (-15.1 ± 0.10)	1

Measured by [a] direct ITC titration, [b] competition ITC titration with **10**, [c] competition ITC titration with **11**, n.d. = not determined.

measured accurately by direct titrations and therefore required competition ITC experiments.[35] In competition ITC experiments, a solution of host and an excess of a weaker binding guest of known K_a and ΔH in the sample cell are titrated with a solution of the tighter binding guest in the syringe and the data is subsequently fit to a competition binding model by the PEAQ ITC data analysis software to extract the K_a and ΔH values of the tighter binding complex. As weaker binding competitors, we selected cycloalkylammonium ions **10** and **11** and first measured their K_a and ΔH values toward CB[8] and Me_4CB[8] by direct ITC titrations (Table 1). Subsequently, **10** and **11** were used as competitors to measure the thermodynamic parameters for the complexes between hosts CB[8] and Me_4CB[8] with drugs **5**–**8** by competition ITC measurements (Table 1). For example, Figure 6a shows the thermogram recorded during the titration of a solution of Me_4CB[8] (0.104 mM) and **11** (0.2 mM) in the cell with **8** (1.0 mM) in the syringe. Figure 6b shows the fitting of the integrated heat values to a competitive binding model to give the $K_a = (5.35 \pm 0.19) \times 10^8\ M^{-1}$ and $\Delta H = -8.39 \pm 0.01$ kcal mol^{-1} values for the Me_4CB[8]·**8** complex. The complexes are uniformly driven by favorable enthalpic contributions to free energy as expected based on the release of high energy water molecules[24c,25b] from the cavity of CB[8] and Me_4CB[8] upon complexation. Interestingly, the measured values of K_a for CB[8] are consistently larger than those measured for Me_4CB[8] by factors of 2.3-fold for cocaine **9** to 39.3-fold for PCP (**8**). We attribute this effect to the ellipsoidal deformation previously observed for Me_4CB[8] by X-ray crystallography.[33c] It is worth noting that morphine **5** and hydromorphone **6** bind with high affinity ($K_a > 10^7\ M^{-1}$), whereas the bulky diacetylated heroin **14** displays relative weak binding ($K_a = 7.94 \times 10^4\ M^{-1}$). Me_4CB[8] displays comparable affinity toward meth **4** and its methylenedioxy analog MDMA **12** which probably reflects counterbalancing effects of the larger but more hydrophilic ring system of MDMA **12**. Conversely, mephedrone with its larger and more hydrophobic tolyl moiety binds 10-fold more tightly to Me_4CB[8] than meth **3** does. We were particularly intrigued by the very high binding constants displayed by PCP and ketamine toward CB[8] and Me_4CB[8] which suggests that they may function as in vivo sequestration agents for these drugs. We selected the Me_4CB[8] host and PCP (**8**) drug pair for advancement toward in vivo studies.

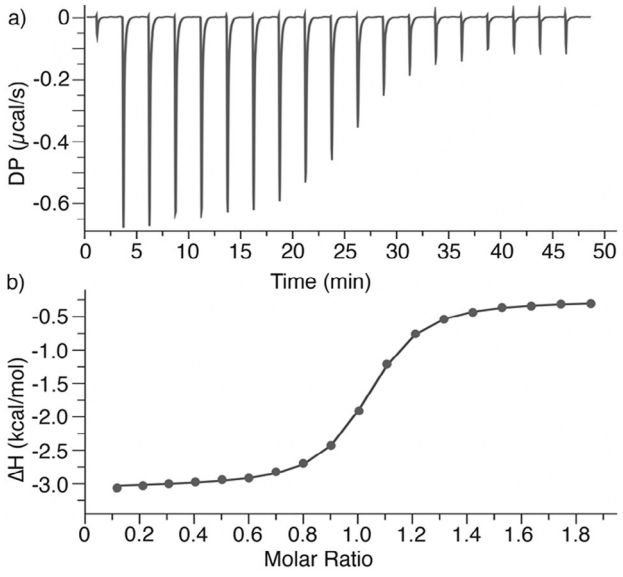

Figure 6. (a) Plot of DP vs. time from the titration of Me_4CB[8] (104 μM) and **11** (200 μM) in the cell with **8** (1.0 mM) in the syringe in 20 mM NaH_2PO_4 buffer (pH 7.4); (b) plot of ΔH as a function of molar ratio of Me_4CB[8] to **8**. The solid line represents the best non-linear fit of the data to the competition binding model ($K_a = (5.35 \pm 0.19) \times 10^8\ M^{-1}$ and $\Delta H = (-8.39 \pm 0.01)$ kcal mol^{-1}.

In vitro cytotoxicity and in vivo maximum tolerated dose studies

Previous studies of macrocyclic unfunctionalized CB[*n*] have shown that they possess high biocompatibility across a wide range of in vitro cytotoxicity and in vivo tolerability studies.[26a–c,e] Before proceeding to in vivo efficacy studies, we wanted to confirm the expected high biocompatibility of the Me_4CB[8] host. First, we performed in vitro cytotoxicity assays for Me_4CB[8] using the MTS metabolic and adenylate kinase (AK) release cell death assays that are well established in our lab (Figure 7). Human kidney (HEK293) and liver (HEPG2) cell

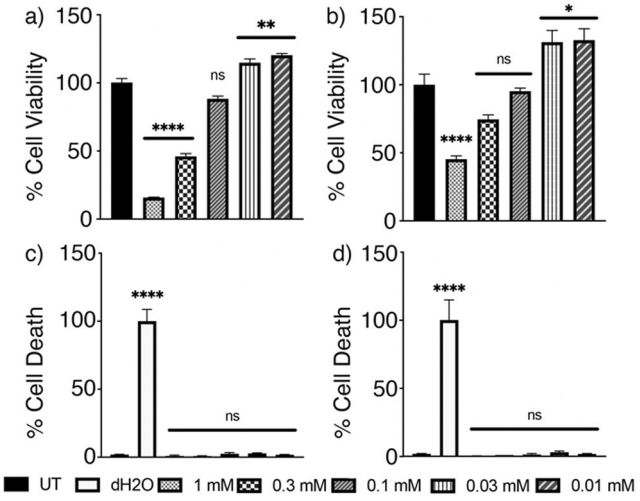

Figure 7. *In vitro* cytotoxicity experiments performed for Me_4CB[8]: a) HEPG2 cell viability assay after incubating the cells with Me_4CB[8] container for 24 h (UT = Untreated). This Figure is the average SEM values representative of two replicate experiments. Statistical analysis is one-way ANOVA with Dunnett's multiple comparisons test. $^{**}P = 0.001$–0.01; $^{****}P < 0.0001$. b) HEK293 cell viability assay performed after incubation with Me_4CB[8] container for 24 h (UT = Untreated). This figure is the average SEM values representative of two replicate experiments. Statistical analysis is one-way ANOVA with Dunnett's multiple comparisons test. $^{*}P = 0.01$–0.05; $^{****}P < 0.0001$. c) HEPG2 cell death after incubation with Me_4CB[8]. AK assay was performed using the supernatant from cells seeded for MTS assay (UT = untreated). This figure is the average and SEM values representative of two replicate experiments. Statistical analysis is one-way ANOVA with Dunnett's multiple comparisons test. $^{****}P < 0.001$. d) HEK293 cell death after incubation with Me_4CB[8]. AK assay was performed using the supernatant from cells seeded for MTS assay (UT = untreated). This figure is the average and SEM values representative of two replicate experiments. Statistical analysis is one-way ANOVA with Dunnett's multiple comparisons test. $^{****}P < 0.0001$.

lines were selected because they are commonly used in drug toxicity studies to determine liver cell and renal cell toxicity, respectively, and because the kidney and liver are where drugs accumulate for processing and clearance by the body. Distilled water was used as a positive control for the AK assay (set to 100% release) and untreated (UT) cells were used as a reference for the MTS assay (100% cell viability). HEK293 and HEPG2 cells treated with $Me_4CB[8]$ showed a dose-dependent response for cell viability. At the highest concentration tested (1 mM), the HEPG2 cells showed an ≈85% reduction in cell viability, and the HEK293 cells showed a ≈55% reduction (Figure 7a,b, respectively). This reduction of cell viability could be produced by cells that are dying but not yet lysed. The reduction in cell viability was absent at concentrations of $Me_4CB[8]$ less than 0.1 mM. Interestingly, the lower doses yielded cell viability values above 100% for both cell types. The observed slight increase in the percentage of viability at low doses of $Me_4CB[8]$ could be due to an increase in mitochondrial activity or to $Me_4CB[8]$ induced interference in the colorimetric assay. Neither the HEK298 or HEPG2 cells show any significant amount of lysis (Figure 7c,d) compared to the positive control.

With acceptable results from the cytotoxicity assays, we decided to demonstrate the in vivo compatibility of $Me_4CB[8]$ by a maximal tolerated dose (MTD) study. Swiss Webster mice were dosed via tail vein injection (6 mL kg^{-1}) of $Me_4CB[8]$ (3 mM (maximal solubility), 1.5 mM, and 0.7 mM) on days 0 and 2 (denoted by *) along with PBS as a control (Figure 8). The animals were weighed daily and monitored for a two-week period for signs of sickness or behavioral changes. Mice in all dosing groups showed no signs of sickness in terms of behavior or significant weight change over the course of the study (Figure 8). As such, we concluded that $Me_4CB[8]$ can be used at its maximum solubility (3 mM) for the greatest potential of PCP reversal without any significant risks of associated toxicity. All animal experiments were approved by the University of Maryland Animal Use and Care Committee (R-JAN-17-25 and R-AUG-18-42) and conformed to the guidelines set forth by the National Research Council committee for the Update of the Guide for the Use and Care of Laboratory Animals.

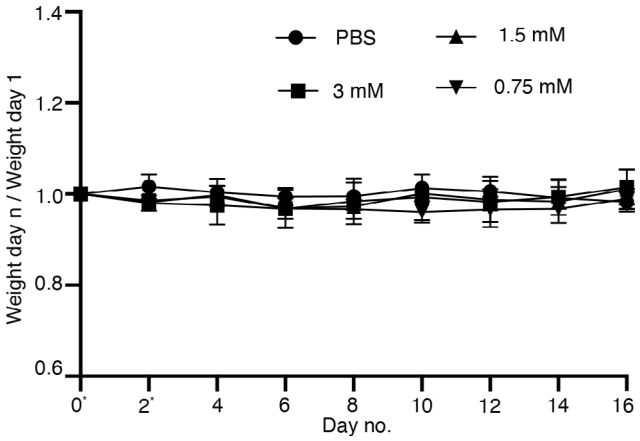

Figure 8. MTD study performed for $Me_4CB[8]$. Female Swiss Webster mice ($n=5$ per group) were dosed via tail vein injection (0.150 mL) on days 0 and 2 (denoted by *) with different concentrations of $Me_4CB[8]$ or phosphate buffered saline (PBS). The normalized average weight change per study group is indicated. Error bars represent SEM.

In vivo reversal of PCP-induced hyperlocomotion

Next, we set out to determine whether the high in vitro binding affinity of $Me_4CB[8]$ toward PCP could prevent or reverse the biological effects of PCP. As such, we used the known hyperlocomotive effects of PCP in mice as a way of monitoring its biological activity via open field tests.[36] A preliminary study was conducted on 8 male Swiss Webster (CFW) mice (weight, mean ± SD: 38.6 ± 2.3 g) in a randomized controlled crossover manner (Figure 9). Over four consecutive days, mice were treated with a 0.2 mL infusion of either sterile saline (0.9%), $Me_4CB[8]$ alone at (3 mM) in 1X PBS buffer, PCP (2 mg kg^{-1}) at (1.5 mM), or a premixed solution of $Me_4CB[8]$ plus PCP (2 mg kg^{-1}) at a ratio of ($Me_4CB[8]$:PCP) (2:1). Treatments were counterbalanced across the four treatment days. We predicted

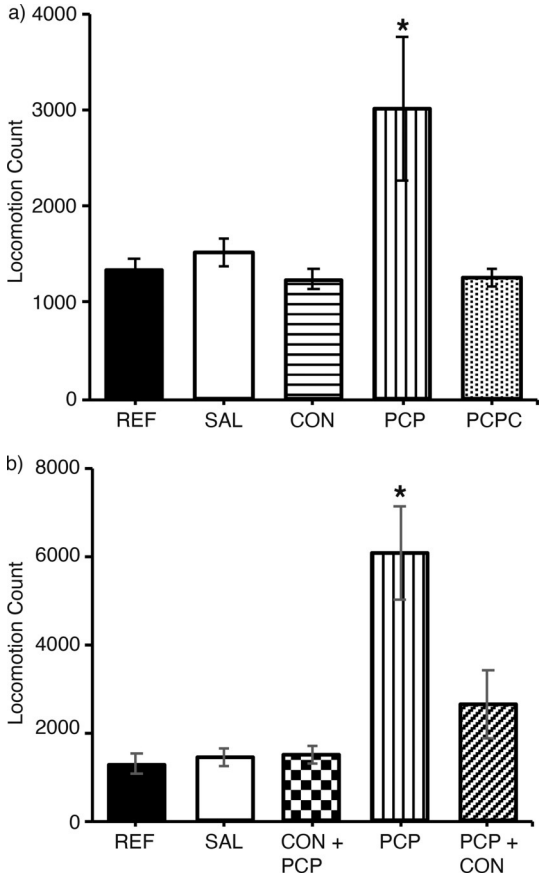

Figure 9. In vivo reversal of PCP-induced hyperlocomotion by $Me_4CB[8]$. a) Average locomotion counts for male Swiss Webster mice (N = 8) (black bars) treated with nothing (REF), saline (SAL), $Me_4CB[8]$ alone (CON), PCP (PCP, **8**) or a premixed solution of PCP and $Me_4CB[8]$ (PCPC). Error bars represent SEM. Star signifies significant increase in locomotion counts ($p<0.05$) for PCP compared to REF, SAL, CON and PCPC. b) Average locomotion counts for male Swiss Webster mice ($n=9$) (grey bars) treated with nothing (REF), saline (SAL), sequential administration of $Me_4CB[8]$ followed 30 s later by PCP (CON + PCP), sequential administration of PCP followed 30 s later by $Me_4CB[8]$ (PCP + CON), or PCP alone (PCP). Error bars represent SEM. Star signifies significant increase in locomotion counts ($p<0.05$) for PCP compared to REF, SAL, CPCP and PCPC.

that treatment with the premixed solution of Me_4CB[8] and PCP would not increase locomotor behavior relative to treatment with PCP. A one-way repeated measures ANOVA revealed a significant effect of treatment ($F(4,28)=4.331$, $p=0.0075$). Pairwise Bonferroni-corrected post-hoc comparisons of locomotion counts across treatments revealed that PCP treatment increased locomotion significantly more than treatments with saline ($p=0.049$), Me_4CB[8] alone ($p=0.011$), or treatment with the premixed solution of Me_4CB[8] and PCP ($p=0.012$), respectively. However, treatment with the premixed solution of Me_4CB[8] and PCP did not significantly increase locomotion when compared to treatment with saline ($p>0.05$) or treatment with Me_4CB[8] alone ($p>0.05$), suggesting that PCP remained bound to Me_4CB[8] in vivo and prevented PCP-induced increases in locomotor behavior. Moreover, we observed no effect of Me_4CB[8] alone on locomotion (saline vs. Me_4CB[8]; $p>0.05$).

Excited by these results, we turned our efforts toward determining if the molecular recognition event of Me_4CB[8] toward PCP could occur in the biological setting instead of the syringe. We attacked this question with a two-fold approach involving either *prevention* of PCP induced hyperlocomotion conducted by administering Me_4CB[8] *before* PCP, or *treatment* of PCP induced hyperlocomotion by administering Me_4CB[8] *after* PCP. The studies were performed using male Swiss Webster (CFW) mice ($N=9$; weight, mean ± SD: 37.9 ± 3.0 g) in a randomized controlled crossover manner. Over four consecutive days, mice were treated with either a 0.2 mL infusion of sterile saline (0.9%), PCP (1.95 mg kg^{-1} at 1.46 mM), a sequential infusion of Me_4CB[8] (2.93 mM) followed by PCP (1.95 mg kg^{-1}) at a 2:1 ratio of Me_4CB[8]:PCP, or a sequential infusion of PCP (1.95 mg kg^{-1}) followed by Me_4CB[8] (2.93 mM) at a Me_4CB[8]:PCP ratio of 2:1. For sequential infusions, the volume of both infusions totaled (0.2 mL), and were spaced 30 s apart (i.e., 30 s elapsed between the first and second infusions). Treatments were counterbalanced across the four treatment days. A one-way repeated measures ANOVA comparing locomotion counts across treatment conditions revealed a significant main effect of treatment ($F(4,32)=11.44$, $p<0.0001$). Pairwise Bonferroni-corrected post-hoc comparisons revealed that PCP significantly increased locomotion counts compared to treatment with saline ($p=0.0005$), sequential infusion of Me_4CB[8] followed by PCP ($p=0.0006$) as well as sequential infusion of PCP followed by Me_4CB[8] ($p=0.0034$). However, no differences in locomotion counts were observed when comparing the two sequential infusion treatments (CON + PCP; PCP + CON) to saline, respectively (p's >0.05) or when comparing the two sequential infusion treatments against one another ($p>0.05$). This suggests that independent of the order in which the infusion is administered, Me_4CB[8] is able to bind PCP in vivo and prevent PCP-induced hyperlocomotion.

Conclusions

In summary, we have measured the binding affinities of two CB[*n*] hosts—CB[8] and its water soluble derivative Me_4CB[8]—toward a panel of commonly used and abused drugs (**3**–**9**, **12**–**14**) by isothermal titration calorimetry. Water soluble host Me_4CB[8] displays remarkable binding affinity toward both ketamine (**7**, $K_d=36$ nM) and PCP (**8**, $K_d=2$ nM). ^{1}H NMR spectroscopy shows that the large Me_4CB[8] cavity is capable of simultaneously encapsulating the phenyl and cyclohexyl rings of **7** whereas the phenyl and piperidium rings of **8** are hosted in the Me_4CB[8] cavity. Similarly, the morphinan ring system of morphine (**5**, $K_d=21$ nM) and hydromorphone (**6**, $K_d=47$ nM) are encapsulated inside the CB[8] cavity quite efficiently. The water soluble Me_4CB[8] host displays low in vitro cytotoxicity below 100 μM toward HEK293 and HEPG2 cells according to standard MTS metabolic and AK release cell death assays and no deleterious effects in maximum tolerated dose studies in mice up to 3 mM. Finally, in vivo efficacy studies showed that PCP induced hyperlocomotion can be effectively controlled by either the prevention or treatment approaches. Given that CB[8] and Me_4CB[8] also bind strongly toward ketamine, morphine, and hydromorphone suggests that interventions based on these hosts or other water soluble CB[8] derivatives holds promise as a new general purpose treatment of overdose with a variety of drugs of abuse.

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Conflict of interest

L.I. and S.M. are named as inventors on a patent application related to this work.

Keywords: cucurbituril · hyperlocomotion · molecular recognition · phencyclidine · sequestration agents

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