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EDGE ARTICLE

Supramolecular hosts that recognize methyllysines and disrupt the interaction between a modified histone tail and its epigenetic reader protein†

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Post-translational modifications of proteins (including phosphorylation, acetylation and methylation, among others) frequently carry out their biological functions by serving as 'on' switches for protein–protein interactions. As highly localized and perfectly defined hot-spots for protein–protein binding, they are a diverse set of motifs that collectively offer great promise as targets for therapeutic intervention and fundamental studies of chemical biology. Recent years have seen the discovery of a very large number of such modification sites on the unstructured tails of proteins, including histones and the tumor suppressor p53. These unstructured protein elements do not present concave binding pockets, and as such cannot be targeted by the conventional small-molecule agents of chemical biology and medicinal chemistry. We report here a family of calixarene-based supramolecular hosts that bind selectively and with high affinity to histone trimethyllysine motifs that are relevant to gene regulation and oncogenesis. We show that these compounds constitute a novel class of protein–protein interaction disruptors and that they can operate selectively against their targeted trimethyllysine sites even in highly complex protein substrates bearing a background of many unmethylated lysines and arginines.

Post-translationally modified amino acids function as dynamic sites that often achieve biochemical signaling by turning on protein–protein interactions with partners that recognize and bind to the site of modification (Fig. 1a and b). Lysine methylation is a post-translational modification of proteins that plays a central role in controlling gene expression.[1] Its importance in the gene (mis)regulation that drives human cancers is increasingly the target of therapeutic interventions.[2–7] The combined actions of methyltransferases and demethylases in the cell control the location of methylation (which lysine on which protein is methylated) and the degree of methylation (mono-, di- or tri-methylation of lysine) with a high degree of specificity.[8] The downstream signaling that occurs when specific lysine residues are methylated always takes the same basic form: methylation of a lysine turns on a protein–protein interaction with a partner that specifically recognizes and binds to that particular post-translational mark. In the cell, lysine methylation sites are most frequently identified on unstructured protein tails, including the *N*-terminal tails of histones 3 and 4[1] and the unstructured *C*-terminal tail of p53.[9] This means that, although they are well-defined hot spots, they do not present *any* form of structured, concave binding pocket that might be targeted by conventional small-molecule binding partners. We envisioned that a supramolecular approach, using capacious hosts programmed to bind selectively to post-translationally methylated lysines, would provide selective histone-binding agents not accessible by the conventional approaches of medicinal chemistry and chemical biology.

Calixarenes are aromatic macrocycles that can be programmed to bind a variety of guests depending on synthetic decorations of the macrocycle.[10–13] Host–guest complexes between sulfonated calix[4]arenes and quaternary ammonium groups (like that of trimethyllysine) have been well studied.[12,14–19] We have found that *p*-sulfonatocalix[4]arene (PSC, **1**, Fig. 1c and d) binds the free amino acid trimethyllysine with good selectivity over unmethylated lysine and all other amino acids.[20] This host, and others like it, have a rich history of broad-spectrum binding of cationic proteins and peptides.[12,19,21] Our earlier results suggested the possibility of developing **1** into an agent with biochemically relevant affinities and specificities for post-translationally modified trimethyllysines. We started this work by preparing a series of peptides, containing either trimethylated or unmethylated lysines, representing histone methylation sites that are known to be important for gene regulation (Table 1). Such peptides are routinely used for testing histone-binding "reader proteins", as they adequately represent the unstructured nature of the different histone tail sites from which their sequences are

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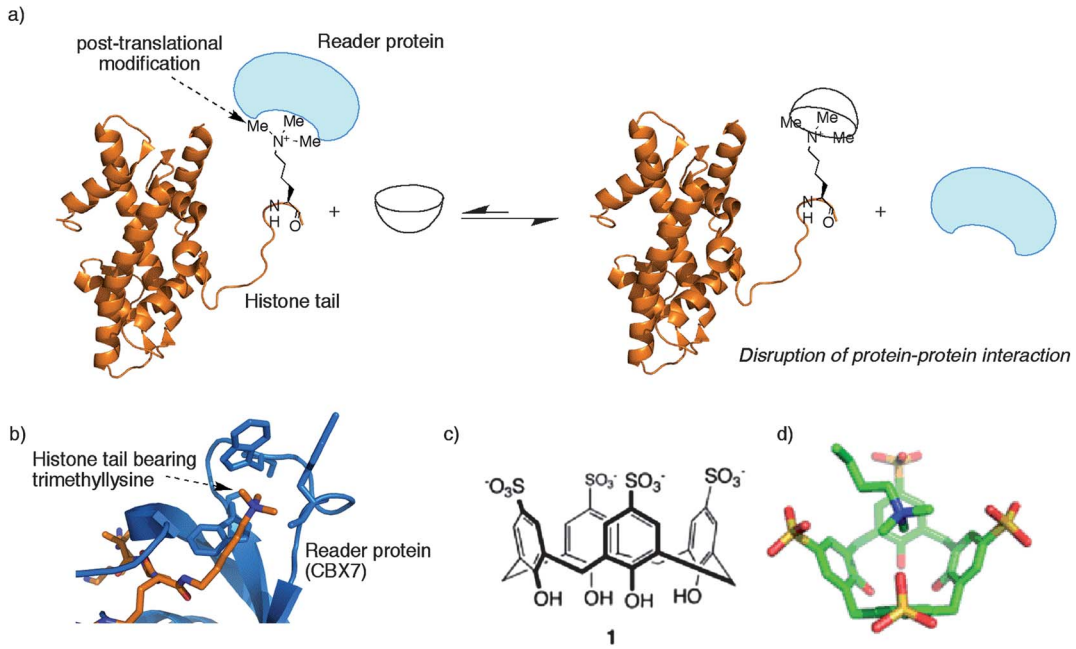

Fig. 1 (a) Schematic showing how post-translational modifications (*e.g.* lysine methylation) on unstructured protein tails serve as recruitment sites for protein–protein interactions (PPIs), and how competition from a supramolecular host can disrupt the PPI. (b) Close up of the complex between trimethyllysine on a histone tail (orange) and its aromatic-rich binding pocket on the surface of the reader protein CBX7 (pdb code 2L1B). (c) *p*-Sulfonatocalix[4]arene (PSC, **1**). (d) Model of the trimethyllysine side chain bound within the cavity of **1**.

derived.[22] Binding of each peptide by **1** was first studied by isothermal titration calorimetry (ITC), and the data were analyzed to provide K_d, ΔH and ΔS of binding. Compound **1** binds to each peptide with single-digit micromolar affinities for the trimethyllysine-containing peptides, and selectivities for the trimethylated state relative to the unmethylated state that range from 9- to 41-fold. Regardless of sequence, the binding of Kme3 peptides is primarily driven by favorable enthalpies of binding, suggesting that electrostatics and/or the non-classical, enthalpically driven hydrophobic effect (known to operate for small binding pockets)[23] are primary drivers of these recognition events.

We found, unsurprisingly, little inherent selectivity of **1** between the different methylated histone tail fragments. NMR data arising from titration of **1** into methylated peptides showed that only the lysine *N*–CH_3 signal was shifted upfield (by 2.0 ppm) upon complexation—consistent with expectations for protons entering the highly shielding environment of a calix[4]arene's binding pocket (Fig. 1 and 3a).[20] The selectivity for methylated peptides and the structural cues from NMR data both support the idea that the binding of trimethylated side chain within the calixarene's pocket (as opposed to the "side-on" binding[20,24] of the methylenes of unmodified lysine within the pocket of **1**) is an important part of the observed complexation events. Despite the simplicity of **1**, its dissociation constants for Kme3-containing peptides (5–9 μM) compare favorably with those of their naturally evolved "reader protein" binding partners (0.7–110 μM).[22,25–27]

We sought to improve the binding properties of **1** by rational synthetic modifications. The first such change was to introduce methoxyethyl ether lower rim substituents (**2**, Fig. 2), which we supposed would favor a cone conformation, facilitate further

Table 1 Thermodynamic data for the binding of methylated and unmethylated peptides by **1**

Peptide[a]	K_d[b]/μM	ΔH[b]/kJ mol^{-1}	$-T\Delta S$[b]/kJ mol^{-1}	Kme3/K Selectivity (-fold)
H3K4	46 ± 1	−34.9 ± 0.6	9.7	
H3K4me3	5.0 ± 0.2	−38.7 ± 0.2	7.9	9
H3K9	101[c] ± 8	−20.9 ± 0.6	−4.4	
H3K9me3	7.2 ± 0.1	−30.5 ± 0.1	0.6	14
H3K27	220[c] ± 7	−13.2 ± 0.2	−7.9	
H3K27me3	5.4 ± 0.1	−35.3 ± 0.2	4.7	41
H3K36	128[c] ± 10	−7.9 ± 0.5	−14.5	
H3K36me3	9.1 ± 0.2	−32.7 ± 0.2	6.9	14

[a] Peptide sequences are as follows: H3K4 = ^+H_3N–ART**K**QTAY–C(O)NH_2; H3K9 = Ac–TAR**K**STGY–C(O)NH_2; H3K27 = Ac–AAR**K**SAPY–C(O)NH_2; H3K36 = Ac–GGV**K**KPHY–C(O)NH_2. [b] Values determined by duplicate ITC titrations at 303 K in buffered H_2O (40 mM Na_2HPO_4/NaH_2PO_4, pH 7.4). See ESI† for details. [c] Stoichiometry fixed to 1.00 to obtain fits for weak host–guest interactions.

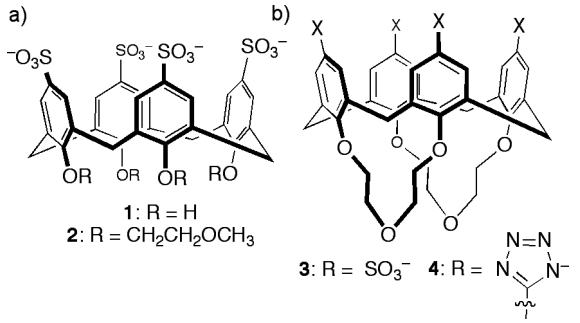

Fig. 2 (a) Hosts **1** and **2**. (b) Covalently 'strapped' hosts **3** and **4**.

synthetic modifications by masking the macrocycle's phenols, and maintain water solubility. Much to our surprise this subtle change completely ablated the ability to bind the H3K27me3 peptide (Table 2). We found binding was too weak to be observed by ITC or NMR, setting a K_d limit of >500 μM. One explanation for this failure is provided by prior work on different calixarene hosts, which showed that tetraether calixarenes analogous to **2** can adopt a collapsed pinched-cone conformation[28] in water which is prevented by the intramolecular network of OH···OH hydrogen bonding that exists in parent compound **1**.[17] Many calix–crown hybrids have been explored as hosts with modified properties relative to their calixarene parents.[21,29] Short crown ether straps, such as those present in **3**, have been suggested in other calix[4]arenes to have a rigidifying effect that prevents the formation of pinched-cone conformations.[18,30] We prepared host **3**, which presents four sulfonates in a disposition similar to **1** and **2**, but that has ethylene glycol lower rim 'straps'

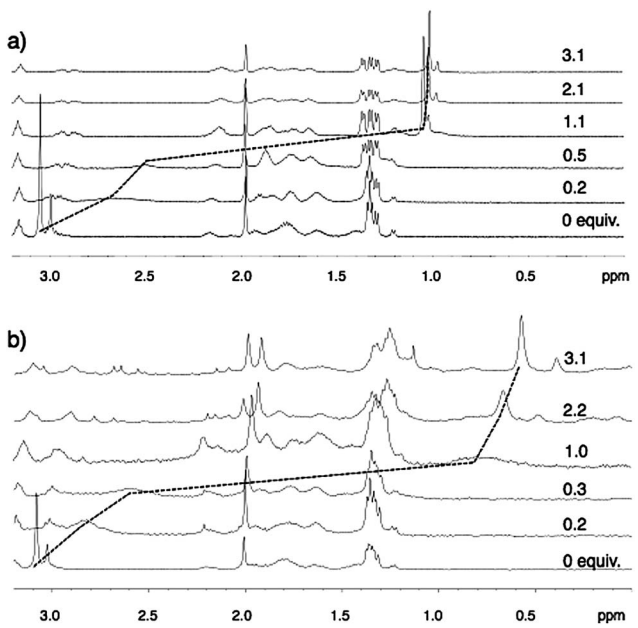

Fig. 3 ^{1}H NMR titrations of host (a) **1** and (b) **4** into guest H3K27me3 peptide. Equivalents of host relative to peptide are shown on right. Dashed line follows the shift of the trimethyllysine N–CH_3 resonance upon binding to hosts. Broadening of methyl signals is normally observed for these types of complexes.[20] The identity of the N–CH_3 resonance in (b) was confirmed by 2D-HSQC NMR (see ESI†).

Table 2 Thermodynamic data for the binding of methylated and unmethylated H3K27 peptides by all hosts

Host	Peptide	K_d[a]/μM	ΔH[a]/kJ mol^{-1}	$-T\Delta S$[a]/kJ mol^{-1}
1	H3K27	220[b] ± 7	−13.2 ± 0.2	−7.9
1	H3K27me3	5.4 ± 0.1	−35.3 ± 0.2	4.7
2	H3K27	>500[c]	n.d.[c]	n.d.[c]
2	H3K27me3	>500[c]	n.d.[c]	n.d.[c]
3	H3K27	>500[c]	n.d.[c]	n.d.[c]
3	H3K27me3	85 ± 6	−10.6 ± 0.4	−13.5
4	H3K27	>500[c]	n.d.[c]	n.d.[c]
4	H3K27me3	20[d] ± 1	−12.5 ± 0.1	−14.7

[a] Values determined by duplicate ITC titrations at 303 K in buffered H_2O (40 mM Na_2HPO_4–NaH_2PO_4, pH 7.4). See ESI† for details. [b] Stoichiometry fixed to 1.00 to obtain fits for weak host–guest interactions. [c] Host–guest interaction too weak to be observed by ITC. [d] Obtained a stoichiometry of 4.10 from curve fit.[33]

that are electronically identical to the glycol ethers of **2** but that should encode a more rigid structure less prone to hydrophobic collapse.[18,30] Much of the binding affinity lost in **2** was recovered in **3**, validating our supposition that the flexibility of **2** is critically important to its inactivity. We attribute the remainder of the difference to the different overall charge states between **1** (−5) and **3** (−4) at neutral pH.[31] We maintained the lower-rim straps in our final host, **4**, while substituting heterocyclic tetrazolates for sulfonates. Tetrazolates, as relatively hydrophobic anionic heterocycles, should be good complements to the cationic and hydrophobic cations like the side chain of Kme3, although we can not say with certainty that all four tetrazoles in **4** are deprotonated under these conditions.[32] Titration of host **4** with the H3K27me3 peptide shows that binding was stronger than **3** but weaker than our original host, **1**.[33] As with host **1** we subjected **4** to NMR studies and observed similarly that incremental addition of **4** to H3K27me3 peptide causes significant upfield shifts for only the N–CH_3 resonance (upfield shifted by 2.6 ppm, see Fig. 3b). This confirms that host **4** primarily engages the trimethyllysine side chain. Interestingly, rigidified hosts **3** and **4** participate in entropically driven binding of H3K27me3, unlike host **1**, which binds this and many other guests in an enthalpically driven manner.[17,34] Unfortunately, it is impossible to disentangle possible entropic contributions from host rigidity (which should favor **3** and **4** relative to the rapidly inverting host **1**) and (de) hydration effects (which are much more difficult to predict).

To explore the abilities of these hosts to disrupt the native protein–protein interactions that are encoded by Kme3 residues, we used a FRET biosensor consisting of the chromodomain CBX7 and its partner H3K27 peptide flanked by the FRET donor mTFP and FRET acceptor mCitrine at *N*- and *C*-termini, respectively (Fig. 4a).[35] This construct displays a 55% increase in FRET signal upon addition of *S*-adenosyl methionine (SAM) and vSET1, a methyltransferase that methylates H3K27 to make H3K27me3 (Fig. 4b). The biosensor, in methylated form, undergoes a transition to a more compact, higher-FRET state because of intramolecular binding between the H3K27me3 sequence and adjacent CBX7 domain.[35] The intramolecular FRET assay used here has been previously explored to test disruption of naturally occurring enzymatic and protein–protein interactions based on post-translationally modified amino acids.[36] The FRET biosensor was treated with increasing

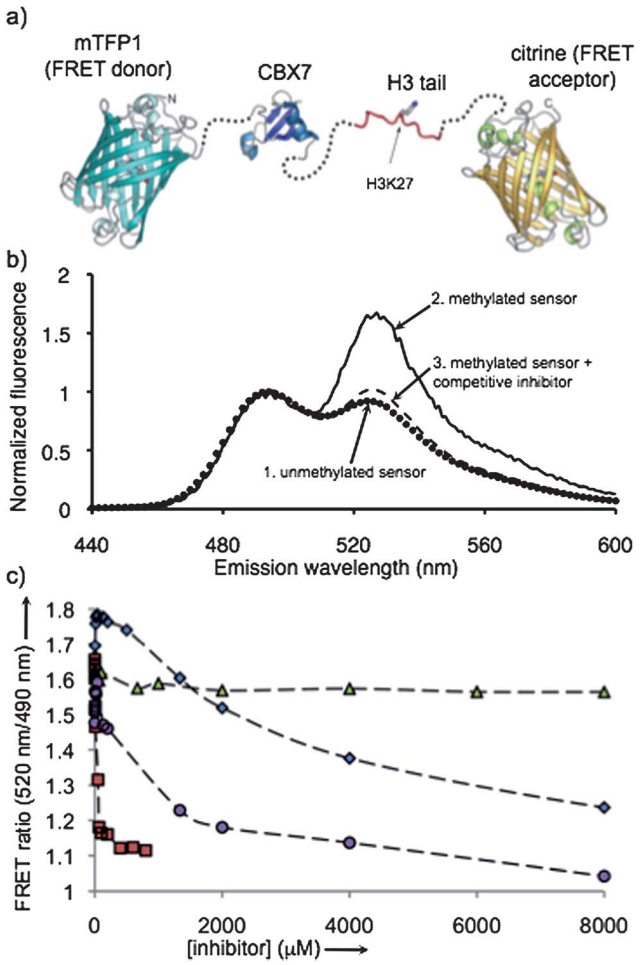

Fig. 4 Disruption of a methyllysine-dependent protein–protein interaction. (a) Graphical representation of the intramolecular FRET biosensor; (b) normalized fluorescence emission of; unmethylated sensor (low FRET), methylated sensor (high FRET), and methylated sensor + inhibitor (low FRET); (c) plot of FRET ratio *vs.* increasing inhibitor concentration (circles = **1**, IC_{50} : 800 μM; triangles = **2**, IC_{50} : >8000 μM; diamonds = **3**, IC_{50} : 1000 μM; squares = **4**, IC_{50} : 50 μM).

concentrations of calixarenes **1–4** while keeping the protein concentration constant (Fig. 4c). Each calixarene was also titrated into unmethylated FRET biosensor as a control for nonspecific photochemical/photophysical effects that might arise. Except for inactive compound **2**, addition of saturating concentrations of calixarenes produced a dose-dependent return to the initial FRET ratio, indicating disruption of the protein–protein interaction between H3K27me3 and CBX7. IC_{50} values generally track with the order of affinities for H3K27me3 determined by ITC. Compound **2** is again inactive, while the parent PSC (**1**) has an IC_{50} of 800 μM. Compound **4** has a higher potency (IC_{50} = 50 μM) than **1** in this assay, likely because the highly polar sulfonates of **1** cause some degree of off-target binding to the peripheral cationic residues of the protein, which the more selective tetrazolates of **4** avoid.[19,22] These IC_{50} values are generally higher than the K_d values determined by ITC for H3K27me3 binding. This difference is inherent to this type of assay, and is explained by the known difficulty of competing with the *intra*molecular protein–protein interaction encoded by unimolecular FRET biosensors.[35,36] IC_{50} results were highly reproducible even when using FRET biosensor obtained from separate expression trials.

These calixarenes are crude reproductions of the natural aromatic cage motifs that have evolved to bind trimethyllysine, which consists of a pocket made up of 2–4 aromatic side chains (Fig. 5).[37–40] The structure–function relations for **1–4** also instruct us on this particular biomolecular recognition event. The complete inactivity of **2** and rescue of binding in **3** suggests that preorganization of these artificial aromatic pockets into open conformations is of paramount importance. Investigation of known protein structures in the Protein Data Bank shows that this lesson also applies to the naturally evolved reader proteins that bind di- or trimethyllysine. Eight pairs of structures exist for which the reader proteins have been crystallized both with and without their methylated binding partners (Fig. 5 and S13, ESI†). In all of these structures, the core aromatic cage residues remain rigidly held open and immobile even in the unbound state.

These compounds are among the very first reported to disrupt any of the entire class of trimethyllysine-mediated protein–protein interaction.[41–44] Their mode of action — binding modified histone tails — is unique and is unlikely to be reproduced by any typical small-molecule antagonist. Because the examples here do not have extended structures beyond the Kme3-binding motifs, they lack specificity for the sequence context of methylation sites. This *selectivity* is complementary to the behaviors of antibodies, which have extremely high *specificity* for single methylation states at single sites, but which can not be used as general trimethyllysine binding tools that are ignorant to sequence context. Agents that display broad selectivity of this type are finding increasing use in the creation of intelligent sensors for various applications in chemical biology and biotechnology.[45–49] While small molecules are limited to biological targets that contain well defined binding pockets, supramolecular hosts are uniquely able to address and bind to protein-based signaling motifs that are unstructured or that are of unknown structure. Indeed, they can even do so with good selectivity for individual, modified functional groups in the context of whole proteins. We look forward to exploiting this general principle for the development of new families of biochemical tools that bind post-translationally modified residues of many types.[49,50]

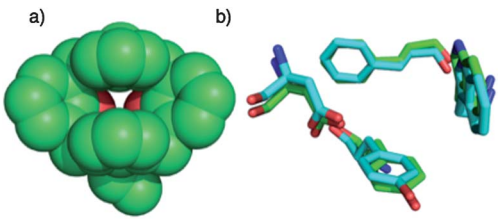

Fig. 5 Aspects of synthetic and natural binding pockets. (a) A calculated model of a pinched-cone (collapsed) conformer of calix[4]arene of the type that we assume is contributing to inactive calixarene **2** in aqueous solution. Hosts of types **1**, **3** and **4** are known to avoid this collapsed conformation.[18] (b) Aromatic cage residues from the crystal structures of the free and bound states of the MBT domain of L3MBTL1 show that the pocket is held rigidly open even in the absence of binding partner (teal = bound, 2RJD; green = unbound, 2PQW). See ESI† for more examples.

Acknowledgements

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