

Hybrid Organic–Inorganic Inhibitors of a PDZ Interaction that Regulates the Endocytic Fate of CFTR**

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Protein–protein interactions (PPIs) play key roles in cellular processes and human disease, but the proteins involved often lack compact pockets accessible to traditional ligand-discovery methods. As first shown for enzyme inhibitors,^[1] multiple weak interactions can be combined to yield polyvalent^[2] ligands with enhanced potency and specificity, and this strategy has now been extended to “undruggable” PPI targets.^[3] Herein, we extend the ideas of fragment-based PPI inhibition to design hybrid structures utilizing cooperative organic–inorganic binding to a target protein (Figure 1A). In particular, we engineer a stable rhodium(II) metalloprotein that displaces representative peptide ligands from the PDZ domain of the cystic fibrosis transmembrane conductance regulator (CFTR)-associated ligand (CAL). PDZ domains are a family of peptide-binding PPI modules named for the first three members: PSD-95, Dlg, and ZO-1.

In an aqueous environment, metal–ligand interactions offer potentially dramatic increases in stability compared to noncovalent organic interactions, which are typically weak ($< 1 \text{ kcal mol}^{-1}$). However, taking advantage of this possibility is challenging. The recruitment of endogenous metal ions can stabilize a protein–inhibitor interface,^[4] but the low in vivo availability of transition-metal ions is a major limitation.^[5] Stable metal-based protein inhibitors have a significant history, most often based on exchange-inert, coordinatively

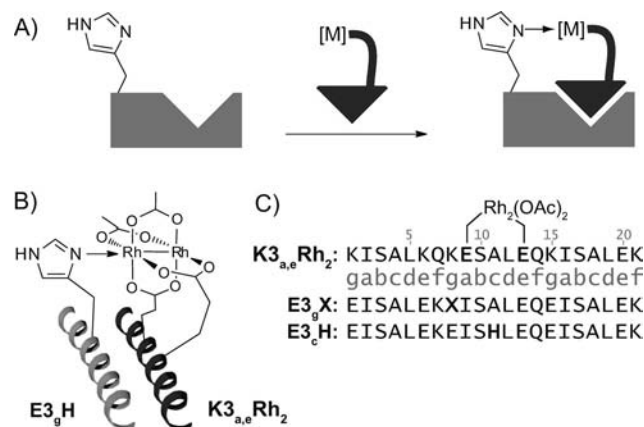


Figure 1. A) Conceptual depiction of a hybrid organic–inorganic inhibitor. B) Axial coordination in E3_gH–K3_{a,e}Rh₂ stabilizes the coiled coil. C) Sequences used in this study. Lower-case gray letters represent positions on a helical-wheel depiction (see Table 1).

saturated species that serve as structural scaffolds.^[6] With the exception of enzyme inhibitors that consist of a DNA oligonucleotide and a metal complex,^[7] few stable inhibitors have been able to exploit reversible coordination chemistry across the binding interface.^[8]

To do so, a discrete organic–inorganic complex must contain a stable linkage between the metal center and the organic part of the complex, while allowing ligand exchange at the metal center to bind targeted side chains. Fortunately, dimetal “pinwheel” structures, such as rhodium(II) tetracarboxylate, have well-differentiated ligand environments containing both kinetically inert, equatorial κ^2 -carboxylate ligands and kinetically labile axial ligand sites (e.g., Figure 1B),^[9] with a demonstrated capability to bind biologically relevant thiol and imidazole compounds in a reversible manner.^[10] The toxicity^[11] of rhodium(II) complexes and their interactions with nucleic acids^[12] and proteins^[13] have been reported.

We happened upon the idea that rhodium(II) centers could form stabilizing secondary contacts at the periphery of a protein binding interface while examining the coiled-coil assembly of rhodium(II) metalloproteins with histidine-containing peptides (Figure 1B).^[14] Based on established models,^[15] a rhodium(II) center linked to a coil at positions a and e of a heptad repeat (abcdefg, Table 1) would be proximal to position g of the complementary peptide, E3_gX. We found that coordination of appropriate side chains at position g strongly stabilizes the coiled coil. For example, thermal denaturation of a mixture of E3_gH and K3_{a,e}Rh₂ revealed a high melting temperature ($T_m = 66.1^\circ\text{C}$; Figure 2

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Table 1: Thermal denaturation of metallopeptide coiled coils.^[a]

Entry	Sequence	E3X	T_m [°C]
1		$E3_gF$	39.5
2		$E3_gE$	50.2
3		$E3_gH$	66.1
4 ^[b]		$E3_gH + 50$ mM imidazole	46.0
5		$E3_gM$	> 75
6 ^[c]		$E3_gM$	70.4
7		$E3_gC$	33.5
8		$E3_cH$	47.0

[a] Standard conditions: equimolar mixtures of E3X and $K3_{a,e}Rh_2$ in aqueous buffer (pH 5.9–6.2) were monitored by CD spectroscopy (222 nm) from –5 to 95 °C at 1 °C min^{–1}. All peptides were used at 100 μM unless otherwise noted. [b] CD signal was monitored at 225 nm. [c] Peptide concentration was 33 μM.

and Table 1, entry 3), in contrast to simple E3/K3 dimers^[15] and to control experiments with a mixture of $K3_{a,e}Rh_2$ and a noncoordinating phenylalanine analogue, $E3_gF$ (T_m = 39.5 °C, entry 1). This coiled-coil stabilization reflects a specific interaction of the rhodium center.

When the histidine residue was moved away from the interface, to position c, a drop in T_m to 47.0 °C was observed (Table 1, entry 8, and Figure 2). The addition of large

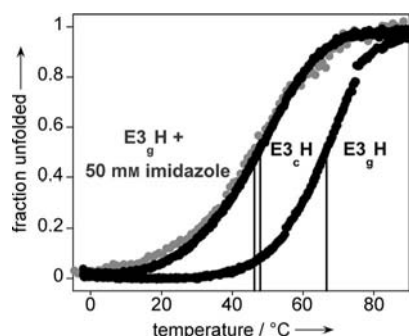


Figure 2. Selection of thermal denaturation profiles for stoichiometric mixtures of E3H and $K3_{a,e}Rh_2$. Vertical lines indicate melting temperatures (T_m). See Table 1 for sample conditions and T_m values.

concentrations of imidazole, either before or after coiled-coil assembly, also led to a significant drop in melting temperature (to 46 °C, Figure 2), thereby providing evidence for a reversible metal–ligand interaction (Table 1, entry 4). Finally, upon assembly with the $E3_gH$ coil, the metallopeptide $K3_{a,e}Rh_2$ exhibits a blue shift of the UV/Vis absorption peak from 587 to 567 nm, consistent with a rhodium(II) tetracarboxylate containing axial nitrogen or sulfur ligands.^[10a,16]

Other Lewis-basic side chains also facilitate stabilization. Coiled-coil assemblies with either glutamate ($E3_gE$) or methionine ($E3_gM$) peptides also exhibit elevated T_m values (50.2 °C and > 70 °C, respectively), consistent with carboxylate–rhodium or stronger thioether–rhodium interactions

(Table 1, entries 2, 5, and 6). So far as we are aware, T_m values of 65–70 °C represent the most stable intermolecular coiled coils reported to date for such a short peptide (21 amino acids);^[17] the stabilities are similar to those achieved with covalent crosslinking.^[18] In contrast, insertion of a cysteine residue at the same position lead to a coiled coil with decreased stability (Table 1, entry 7). Preliminary modeling indicates the cysteine side chain is too short to reach the rhodium center without disrupting the coiled coil.

To extend the concept of organic–inorganic cooperativity to the discovery of potent PPI inhibitors, we examined interactions between the CAL PDZ domain (CALP) and the cystic fibrosis transmembrane conductance regulator (CFTR). The C terminus of CFTR interacts with several proteins (e.g. CAL, NHERF1, NHERF2) through PDZ interactions that affect CFTR function in diverse ways.^[19] Despite its potential value as a target, inhibition of CALP is distinctly difficult owing to its broad specificity and comparatively low baseline affinity.^[20] We recently combined a screen of inverted peptide arrays with in vitro fluorescence polarization measurements to identify selective CALP inhibitors.^[20,21] However, the potency of these inhibitors remains modest, with $K_i \geq 1.3$ μM.

The CAL PDZ domain contains several histidine residues near the peptide-binding site, thereby making it an attractive target for a hybrid organic–inorganic approach to inhibitor design (Figure 3A).^[22] To test the potential contributions of rhodium-based interactions to CALP inhibitor affinity, we adapted known methods^[11a,23] to prepare metallopeptides based on sequences that are known to interact with CALP. PDZ binding requires free C-terminal carboxylates, and we found it convenient to metalate a peptide containing both C-terminal and side-chain carboxylates and then to isolate the side-chain-modified metallopeptide from the product mixture by HPLC (Scheme 1).

We measured inhibitor equilibrium dissociation (K_i) constants using fluorescence anisotropy to observe the displacement of a fluorescent reporter peptide (Figure 3B and Table 2). The VQDTRL peptide, derived from the native target CFTR, has weak CALP affinity. Direct incorporation of a rhodium(II) center at the aspartate side-chain carboxylate (VQD^{Rh}TRL) resulted in a decrease in the apparent inhibitory constant (K_i) relative to the parent peptide (Table 2, entries 1–2), but when the new value (6.3 μM) was compared to that of a simple rhodium complex, $[Rh_2(OAc)_4]$, the improvement was not statistically significant. The apparent affinity of VQD^{Rh}TRL was also comparable to that of a metallopeptide derived from a nonbinding, scrambled control sequence, QLD^{Rh}VTR (Table 2, entry 4). Together, these data suggest that the effects seen with rhodium(II) addition at the P^{–3} site (P⁰ = C-terminal residue) of VQDTRL are not specific.

We designed two metallopeptides having a site of rhodium attachment at the P^{–6} position, because structural analysis of the CALP domain^[22] indicated that the P^{–6} position should be proximal to the His301 residue in this target (Figure 3A). Both new metallopeptides exhibited statistically significant and reproducible affinity enhancement relative to controls (Table 2, entries 5–8). When a sequence (EWPTSII) carrying

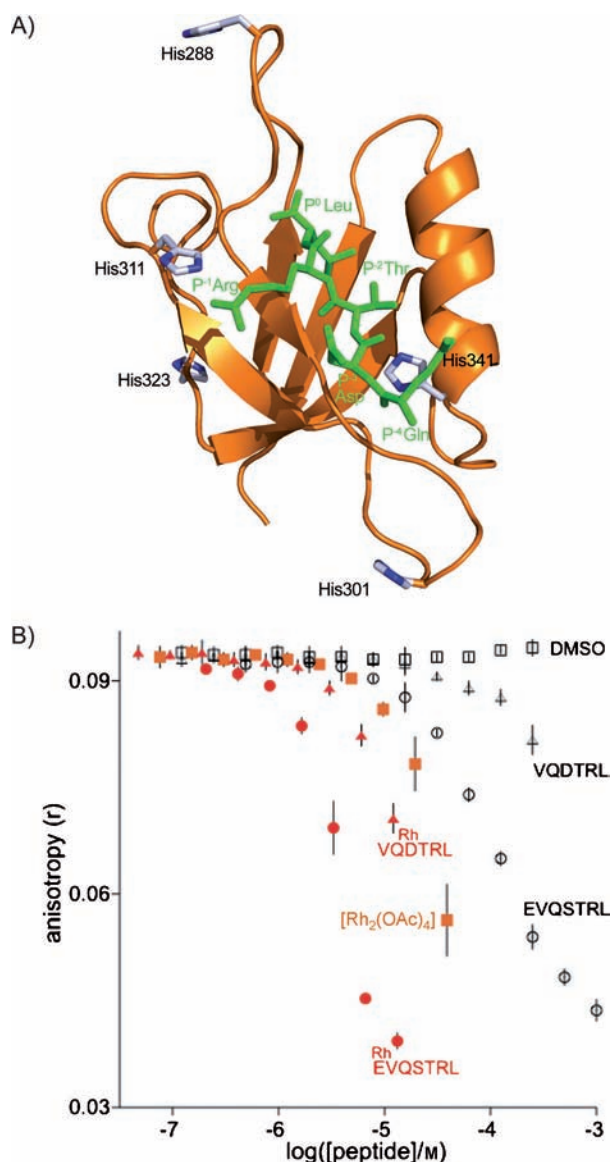
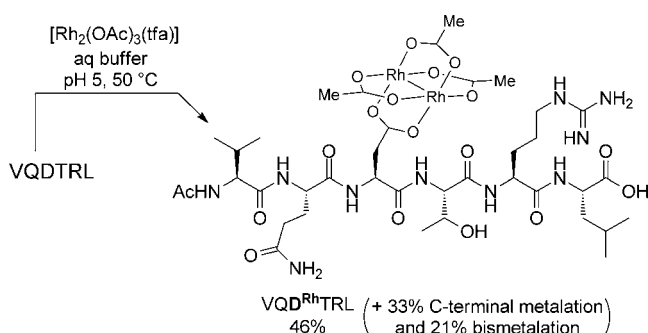


Figure 3. A) Structure of the CAL PDZ domain (orange ribbon) bound to a CFTR peptide (green stick figure).^[22] All CALP His side chains are shown explicitly (stick figures colored by element; gray = C, blue = N). B) Fluorescence anisotropy displacement isotherms for candidate CALP inhibitors. K_i values are reported in Table 2.



Scheme 1. Representative synthesis of a PDZ-binding metallopeptide.

a glutamate side chain at the P⁻⁶ position was used, metalation increased binding affinity from 65 to 1.7 μM , an

Table 2: Metallopeptide inhibitors of CALP.^[a]

Entry	Peptide	Sequence	K_i [μM]
1	1	VQDTRL	320 ± 43
2	1-Rh	VQD ^{Rh} TRL	$6.3 \pm 0.7^{[b]}$
3	2	QLDVTR	> 500
4	2-Rh	QLDV ^{Rh} TR	$10.0 \pm 0.9^{[b]}$
5	3	EWPTSII	65 ± 5
6	3-Rh	EW ^{Rh} PTSII	$1.7 \pm 0.2^{[c]}$
7	4	EVQSTRL	42 ± 5
8	4-Rh	EVQ ^{Rh} STRL	$0.56 \pm 0.08^{[c]}$

[a] Inhibitor equilibrium dissociation (K_i) constants were determined for cognate peptide/metallopeptide pairs. [b] All rhodium(II) complexes, including $[\text{Rh}_2(\text{OAc})_4]$ ($K_i = 13 \pm 5 \mu\text{M}$) exhibit nonspecific inhibition, establishing an upper bound for these measurements. [c] K_i value significantly different from that of $[\text{Rh}_2(\text{OAc})_4]$ ($p < 0.05$, $n = 3$).

approximately 40-fold enhancement. An even larger enhancement was seen with the sequence EVQSTRL, which contains the dominant C-terminal tetrapeptide identified by array screens.^[24] The metallopeptide E^{Rh}VQSTRL bound with the highest affinity reported to date for CALP (0.56 μM), a 75-fold change relative to the parent peptide (Table 2 and Figure 3B). HSQC footprinting spectra in the presence of either the peptide or metallopeptide (see the Supporting Information) were well-dispersed, thus confirming binding to the canonical PDZ site, with localized differences.

A CALP-H301A mutant was prepared to ascertain the role of residue His301 in metallopeptide affinity. This mutant binds the parent peptide EVQSTRL with a K_i value of 80 μM , only slightly (ca. twofold) weaker than the wild-type protein. However, the mutant binds the metallopeptide with an apparent K_i of 9.2 μM , a more than 16-fold loss of affinity relative to the wild-type, consistent with the predicted His301–rhodium ligation.

To provide an independent demonstration of rhodium-based affinity enhancement and to establish the efficacy of rhodium metallopeptides in a more complex environment, a pulldown inhibition assay was performed using epithelial cell lysate (Figure 4). Relative to the nonmetalated control, the metallopeptide E^{Rh}VQSTRL exhibits improved inhibition, demonstrating that the affinity gains are also observed in a more physiological environment.

The metallopeptide E^{Rh}VQSTRL ($K_i = 0.56 \mu\text{M}$) is the first reported inhibitor with submicromolar affinity for the CAL PDZ domain and is significantly shorter than decameric alternatives with single-micromolar affinities.^[21b] The ability to quickly generate a potent inhibitor for a recalcitrant protein target demonstrates that combining organic inhibitors with coordination chemistry is a viable strategy to inhibit protein–protein interactions. Furthermore, comparative binding studies with the CALP-H301A mutant and with P⁻³ metalated peptides indicate that rhodium mediates affinity enhancement through peripheral interactions that are specific to the given target binding site. This capability bodes well for the development of inhibitors not only for PDZ domains, but for other structural classes of protein regulators as well.

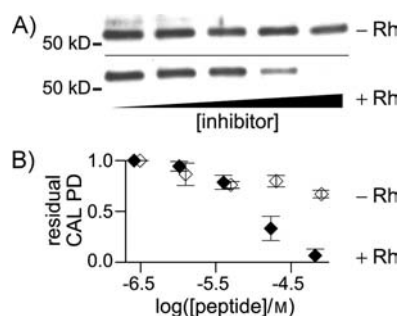


Figure 4. Metalation improves inhibitor potency. A) Western blot of native CAL captured from epithelial lysates,^[21b] in the presence of increasing concentrations of EVQSTRL peptide with (+Rh) or without (-Rh) rhodium. B) Quantification reveals dose-dependent inhibition of CAL pulldown (PD) by the metalated peptide ($n=3$).

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