SUPPORTING INFORMATION

Affinity-Guided Design of Caveolin-1 Ligands for Deoligomerization

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SUPPORTING INFORMATION CONTENTS:

EXPERIMENTAL MATERIALS AND METHODS

Materials

Fmoc-protected amino acids for peptide synthesis were obtained from multiple suppliers, including NovaBiochem, ChemImpex, Anaspec, and Aroz Tech. All other reagents and materials were obtained through Sigma Aldrich or Fisher Scientific unless otherwise noted.

Protein expression and purification

Caveolin (1-104) (CAV(1-104)) and the full-length, soluble caveolin variant (CAV (FLV)) proteins were expressed and purified as described previously.[1]

Synthesis and screening of SPOT arrays

Peptides were synthesized as *C*-terminal adducts to cellulose as described in Hilpert et. al.[2] All sequences were synthesized and screened in duplicate. Purified CAV(1-104) was fluorescently labeled by adding a 15-molar excess of NHS-rhodamine to protein in phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4). The mixture was allowed to react 2 h, protected from light. Excess unreacted NHS-rhodamine was either removed by overnight dialysis using 3.5 kDa molecular weight cut-off Slide-a-Lyzer mini dialysis cups in PBS at 4 °C, or was removed using desalting column according to standard procedures. The completed peptide array was blocked with 0.2% bovine serum albumin fraction V (BSA) in PBS. The sheet was then washed five times with PBS containing 0.05% Tween20 detergent (PBST), then incubated with 0.2% BSA in PBS containing the dialyzed fluorescently-labeled CAV(1-104) for at least 2 h. After incubation, the sheet was washed four times with PBST and once with PBS containing no detergent. Sheets were dried flat overnight prior to fluorescence imaging.

Fluorescent imaging was performed using a GE Typhoon imager with the following fluorescence settings: Green (533 nm) excitation frequency, 580 nm BP 30 emission frequency window, normal sensitivity, 200 microns/px at 300 V laser strength. The resulting image files were analyzed by ImageQuantTL (.gel format) or ImageJ (.tiff format) to obtain quantitative data.

The methodology for selectivity screening was identical except rhodamine tagging was performed separately for CAV(1-104), bovine serum albumin, casein, and hen egg white lysozyme. These reactions were performed using the same NHS-rhodamine stock solution and treated identically. Replicate SPOT sheets carrying synthesized ligand **5**, ligand **6**, and **1** and blanks containing only the dual β-Ala linker were each treated with one of the four labeled proteins and quantified as described above. The fluorescence signal for the linker-only blanks for each protein screen was subtracted from the corresponding ligand **5**, ligand **6**, and **1** signals before plotting and analysis.

The suitability of this method for semi-quantitative comparison of binding affinity between peptides was confirmed by synthesizing a selection of peptide sequences and cutting out the individual peptide SPOTs. Each SPOT was subjected to a standard bicinchoninic acid (BCA) assay in 96-well plate format to determine its relative peptide concentration. The relative concentrations were measured through recording the absorbance of the BCA assay solutions at 562 nm. An approximately 20% variation in the concentrations of the SPOT-synthesized peptides was observed (Table S1).

Synthesis and purification of Peptides

Ligand **1** was synthesized by SPPS on a 0.1 mmol scale in 5-mL disposable syringe-type polypropylene reaction vessels (obtained from Torviq) with 70 micron polypropylene frit and Luer lock tip, using procedures adapted from Kirin et. al.[3] These vessels were outfitted with BD 16 gauge 1 inch needles for drawing up and expelling reagents. The following significant modifications were used: Swelling of resin and all coupling steps were performed in *N*-Methyl-2-pyrrolidone (NMP). The deprotection step was performed twice per cycle. Coupling steps were performed using HBTU and without DIPEA. Reaction progress was monitored continuously using bromophenol blue (0.01% final concentration) as described in Krchnak et al., with transition of reaction mixture color from deep blue to yellow indicating completion of coupling.[4] Coupling reactions were allowed to continue 10 min additional after visual monitoring of color change indicated reaction completion. After lyophilization, crude peptide product was purified by preparative HPLC using standard methods. Product presence was confirmed by MALDI-TOF and product purity confirmed by analytical HPLC.

Fluorescence anisotropy binding affinity assay

A 15 µl volume containing a range of CAV(1-104) concentrations was combined with a stock solution of Mantyl-**1** in PBS with 0.25% sarkosyl detergent (w/v) to yield 11 samples containing 24 nM Mantyl-**1** (12 nM dimerized Mantyl-**1**) and CAV(1-104) in a range of 0 to 585.98 nM. Fluorescence data was collected for each of these samples on a Cary Eclipse Spectrofluorometer at an excitation wavelength of 340 nm and emission range of 400 to 500 nm with Savitzky-Golay smoothing at filter size 15. Intensity (*I*) measurements were taken with four polarization filter conditions: vertical excitation with vertical emission (I_{VV}), vertical excitation with horizontal emission (I_{VH}), horizontal excitation with vertical emission (I_{HV}), and horizontal excitation with horizontal emission (I_{HH}). Anisotropy (r) was calculated by the following standard anisotropy equations.[5]

$$r = \frac{I_{VV} - G*I_{VH}}{I_{VV} + 2*G*I_{VH}} \quad (2)$$

and

$$G = I_{HV}/I_{HH} \quad (3)$$

The emission range in conjunction with the indicated smoothing yielded 87 distinct subsets of anisotropy data from 407-493 nm. The greatest magnitude of anisotropy was observed at wavelength 417 nm. This data set was converted to fractional ligand saturation by first calculating the percent change in binding by dividing each value in the subset by the minimum value for the same subset and subtracting 1, then converting these values to fractional saturation by dividing each percent change value by the maximum percent change value for each subset. This data set was fit to equation 1 using a weighted method of least squares that more heavily weights those data points with smaller variance. R^2 for the fit equation was 0.94. The K_D was calculated to be 23 nM, within a potential range of 44 to 3 nM with 95% confidence. A Hill plot was constructed using the same data set, with log(Y/(1-Y)) on the vertical axis and log(X) on the horizontal axis, where Y is the fractional saturation and X is the concentration in nM of CAV(1-104). The slope of the curve at log(Y/(1-Y)) = 0 provides the Hill coefficient, n_H. This coefficient is an indicator of binding cooperativity for binding at multiple sites, with values < 1 indicating negative cooperativity and values > 1 indicating positive coopertivity up to a limit of $n_H = z$ where z is the number of binding sites. The n_H for this binding interaction is 1.97 with two binding sites, indicating near complete cooperativity. In this case, binding of two molecules of ligand **1** happens simultaneously as a single binding event. Thus, we assume that dimerized ligand **1** functions essentially as a single ligand.

Deoligomerization shown by Dynamic Light Scattering

A Malvern Zetasizer ZS Nano DLS instrument was used in backscatter mode to determine particle size of oligomers composed of CAV(FLV). Readings were obtained for oligomers in PBS with or without ligand **1** under both reducing (5 mM TCEP) and non-reducing conditions. The General Purpose (non-negative least squares analysis) distribution analysis algorithm provided with the Zetasizer software was used to automatically calculate particle sizes and distribution. Average diameters for each condition were plotted along with the average plus or minus half the calculated polydispersity width to represent the distribution of observed diameters.

A second experiment with a different batch of the soluble caveolin variant was performed in triplicate using a range of ligand **1** to CAV(FLV) ratios to demonstrate that the deoligomerization effect of ligand **1** is dose-dependent.

Table S1. Variation in peptide concentration between SPOTs.

Sequence	Average Absorbance	Standard Error
CSWMRLK	0.189	0.0010
LRKSWMC	0.150	0.0026
KCWRSLM	0.163	0.0007
CMWKLRS	0.178	0.0017

Maximum % difference between SPOTs	20.46%

Table S2. Sequences of the shuffled peptides in the library corresponding to **Figure 4** in the main text.

R	W	G	S	M	L	K	C	F	Ligand 6
		R	W	S	M	L	K	C	Ligand 6(ΔGF)
		C	S	W	M	R	L	K	Shuffle 1
		C	L	S	K	R	M	W	Shuffle 2
		W	S	L	K	R	M	L	Shuffle 3
		C	M	S	W	R	L	K	Shuffle 4
		L	R	K	S	W	M	C	Shuffle 5
		R	K	S	C	L	M	W	Shuffle 6
		R	K	L	W	C	S	M	Shuffle 7
		K	C	W	R	S	L	M	Shuffle 8
		K	L	S	W	C	R	L	Shuffle 9
		M	R	S	C	W	L	K	Shuffle 10
		W	C	L	M	R	S	K	Shuffle 11
		R	C	M	W	K	L	S	Shuffle 12
		R	W	K	S	M	L	C	Shuffle 13
		S	C	K	M	L	W	R	Shuffle 14
		C	M	W	K	L	R	S	Shuffle 15
		S	L	C	R	W	K	M	Shuffle 16
		R	W	S	M	L	K	C	Shuffle 17
		C	M	W	K	L	R	S	Shuffle 18
									Blank

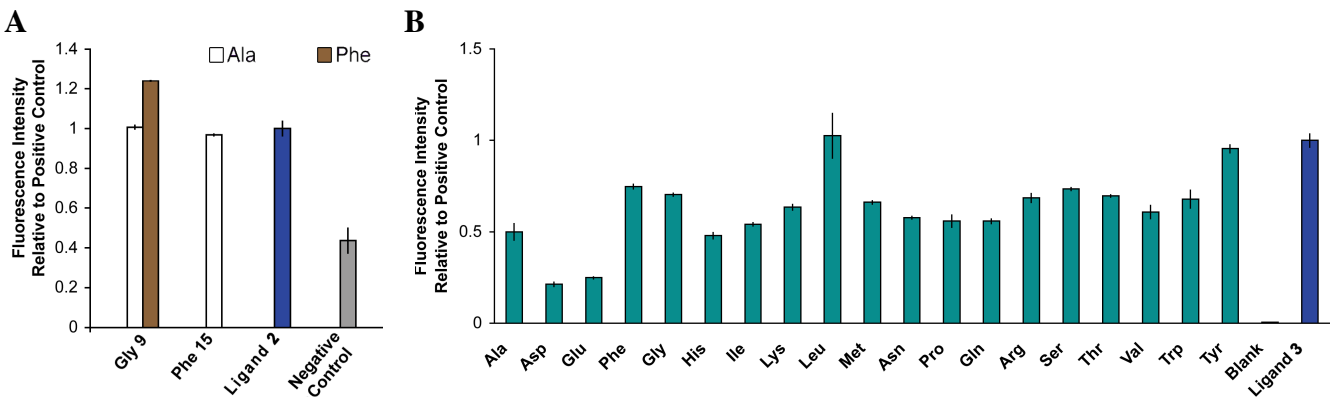
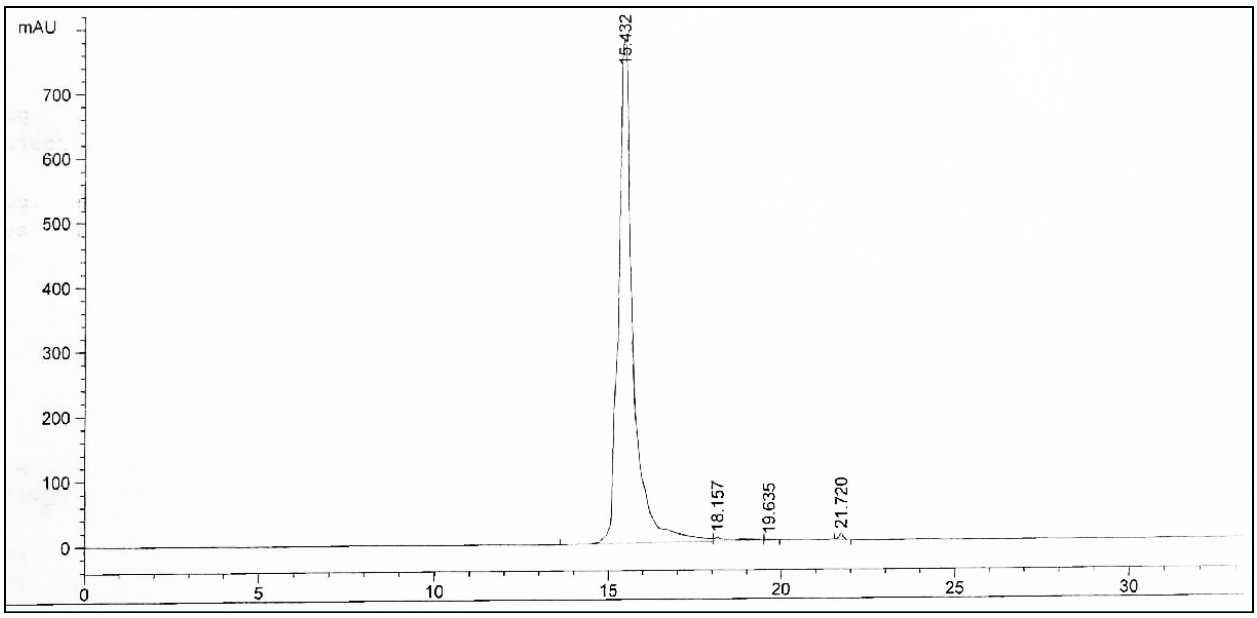


Figure S1. Rationale for removal of Gly9 and Phe15, and investigation of Cys14 supporting dimerization hypothesis. **a)** From the library of ligand **5** variants with similar and dissimilar substitutions of amino acids, Ala substitution at either the Gly9 and Phe15 residues was shown to have no significant effect on ligand binding. Gly cannot contribute to ligand binding through sidechain contacts, but often allows greater flexibility for a ligand. Replacement of Gly9 with the slightly less flexible Ala showed no reduction in ligand binding, and replacement of Gly9 with the bulky and significantly less flexible Phe also showed no reduction, only a minor, perhaps nonspecific, increase in apparent binding affinity. This data confirms that the flexibility of Gly is superfluous and the residue can be removed. Furthermore, substitution of Phe15 with Ala failed to alter the ligand's apparent ligand binding ability, making Phe15 a candidate for elimination, which was especially desirable given the higher likelihood of nonspecific binding when aromatic sidechains are surface-exposed. These results suggested that neither residue is essential for ligand binding. The negative control is identical to the negative control used in **Figure 3** in the main text. **b)** In the library of all amino acid substitutions for every site in ligand **6**, all substituents reduce ligand binding when replacing Cys14. This observation strongly suggested that ligand binding to CAV requires dimerization by a disulfide bond mediated by the Cys sidechain. Notably, both Leu and Tyr substitutions of Cys14 were well tolerated, suggesting these positions could accommodate hydrophobic residues capable of forming non-covalent interactions. This further suggests that ligand binding is dependent on dimerization.

A



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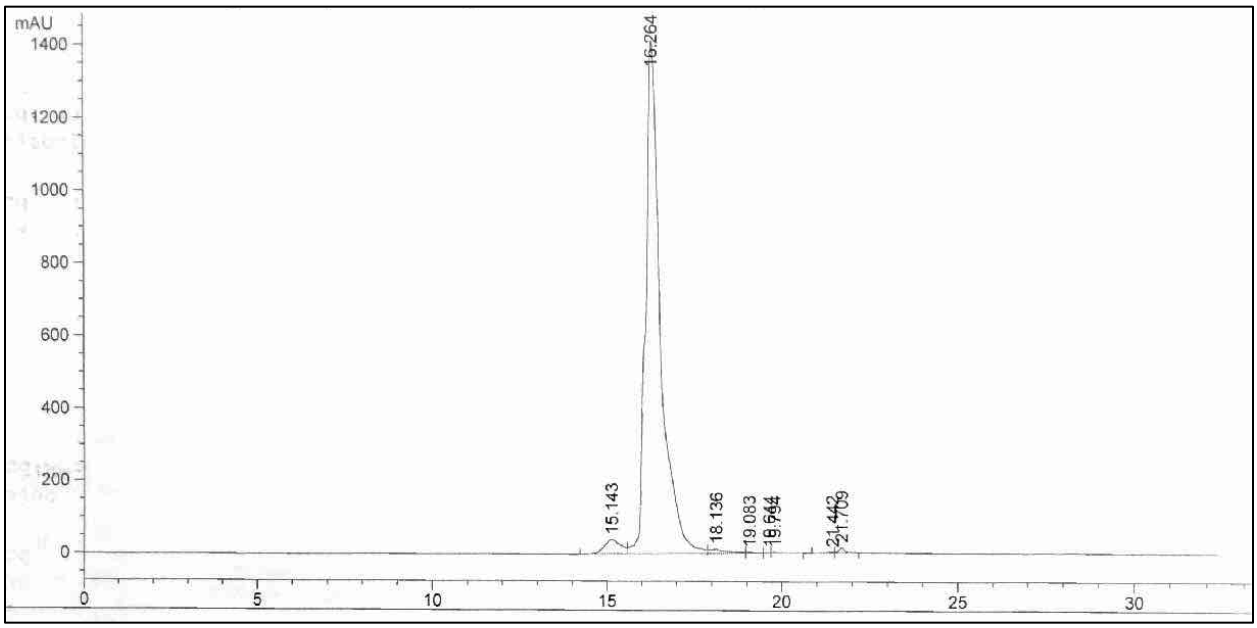

Figure S2. HPLC traces demonstrating **1** and Mantyl-**1** purity. **a)** Purity of ligand **1** demonstrated by analytical reversed-phase HPLC. The sample was injected onto a C18 column and eluted via water-methanol gradient. The major peak at 15.432 corresponds to Mantyl-**1**. Calcd *m/z* 922.17, found *m/z* 922.44 by MALDI-TOF**. b)** The purity of Mantyl-**1** demonstrated by analytical reversed-phase HPLC. The sample was injected onto a C18 column and eluted via water-methanol gradient. Major peak at 16.264 corresponds to Mantyl-**1**. Calcd *m/z* 1055.32, found *m/z* 1055.47 by MALDI-TOF. The minor peak at 15.143 represents minor quantities of ligand **1** that did not undergo mantylation.

SUPPLEMENTAL REFERENCES

(1) Majumdar, S.; Hajduczki, A.; Vithayathil, R.; Olsen, T. J.; Spitler, R. M.; Mendez, A. S.; Thompson, T. D.; Weiss, G. A. In Vitro Evolution of Ligands to the Membrane Protein Caveolin. J. Am. Chem. Soc. 2011, 133, 9855–9862.

(2) Hilpert, K.; Winkler, D. F.; Hancock, R. E. Peptide Arrays on Cellulose Support: SPOT Synthesis, a Time and Cost Efficient Method for Synthesis of Large Numbers of Peptides in a Parallel and Addressable Fashion. Nat. Protoc. 2007, 2, 1333–1349.

(3) Kirin, S. I.; Noor, F.; Metzler-Nolte, N.; Mier, W. Manual Solid–Phase Peptide Synthesis of Metallocene–Peptide Bioconjugates. J. Chem. Educ. 2007, 84, 108–111.

(4) Krchnák, V.; Vágner, J.; Lebl, M. Noninvasive Continuous Monitoring of Solid-Phase Peptide Synthesis by Acid-Base Indicator. Int. J. Pept. Protein Res 1988, 32, 415–416.

(5) Lakowicz, J.R. Principles of Fluorescence Spectroscopy, 3rd ed.; Springer Science+Business, New York, 2010.