Supplementary Material for:

Cysteine modifiers suggest an allosteric inhibitory site on the CAL PDZ domain

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Figure S1: CAL-specific RNA interference increases cell-surface and WCL abundance of WT-CFTR

Polarized CFBE cells expressing WT-CFTR (WT-CFBE cells) were treated with CAL-specific (siCAL) or negative control (siNEG) siRNA. Cells were grown on filters at an air-liquid interface for three days, and then surface proteins were biotinylated. Cells were lysed and biotinylated protein were captured by streptavidin beads and probed to determine CFTR levels. Ezrin: loading control marker. The knockdown efficiency of siCAL was 86 ± 11% (n=3).

Following normalization to an ezrin loading control, surface biotinylated and whole-cell lysate (WCL) CFTR siCAL-treated cells showed statistically significant average increases of 2.33-fold ($p = 0.00007$) and 1.65-fold ($p = 0.00021$), respectively, compared to siNEG-treated controls. Statistics were calculated from three independent experiments each with two or four replicates, using a linear mixed-effects model in R (package nlme).
Figure S2: The overall screening strategy

(A) The flow chart of the screening strategy. In a FRET screen (red box and circle) of the St. Jude bioactive collection (5600 wells), a Cerulean domain fused to CALP acts as a donor to the TMR label attached to reporter peptide iCAL36. Inhibitors reduce the FRET signal measured as the ratio of fluorescence intensities $F_{575 \text{ nm}}/F_{475 \text{ nm}}$. In the Alpha-Screen (blue box and circle), a biotinylated peptide attached to streptavidin donor beads interacts with polyhistidine-tagged CerCALP attached to NiNTA acceptor beads, permitting proximity-based exchange of singlet oxygen. Inhibitors disrupt the coupling, decrease acceptor-bead emission. Only 12 compounds were identified by both screens, one of which was eliminated as a likely fluorescence artifact. In the NMR secondary screen, 12 FRET+/AS+ compounds, 119 FRET+/AS- and 30 AS+/FRET- compounds were tested using single-point NMR $^1\text{H}-^1\text{N}$ HSQC spectra. Among the compounds that disrupted binding, five from the FRET+/AS+ set, two from the FRET+/AS- set, and one from the AS+/FRET- set acted as protein aggregators. Tests for saturable binding and ability to inhibit endogenous CAL in a pull-down assay eliminated an additional compound. Three candidates demonstrated saturable binding and site-specific footprints. Biocompatibility assays validated MD as the primary lead. (B) The chemical identities and schematics of the four HSQC validated site-specific interactors.
Figure S3. Ussing-chamber measurements show that MD does not stimulate CFTR chloride currents in CFBE-ΔF cells

MD was applied to polarized CFBE-ΔF cells at a final concentration of 12.5 µM (A; n=6) or 50 µM (B, n=8). The change in short-circuit current (I_{sc}) was measured in response to CFTR_{inh172} (ΔI_{sc}). No significant changes were seen in either case, although a trend to lower current was observed at the higher dose.
Figure S4. Hetero-trimer formation

(A) Due to the non-competitive mode of inhibition, high concentrations of peptide ligand (yellow triangles) and MD inhibitor (red dots) can drive formation of a hetero-trimer with the CAL PDZ domain (blue notched circle). The hetero-trimer corresponds to the structure determined by co-crystallization in (B). (B) PyMOL views of the hetero-trimer CALP:HPV18E6:MD (PDB ID 5IC3). CALP (blue); peptide HPV18E6 (yellow); MD (red).
Figure S5. Alignments of the models of CALP complexes

(A) CALP:MD:peptide model 5IC3 (red, chain A) aligned with CALP:peptide model 4JOR (blue, chain A) (RMSD = 0.213 Å; 504 atoms). Black arrow points to the loop movement seen between the two models.

(B) CALP^{C319A}:peptide model 5K4F (black, chain A) aligned with CALP:peptide model 4JOR (blue, chain A) (RMSD = 0.213 Å; 472 atoms). Black arrow points to the loop movement seen between the two models.

(C) CALP^{C319A}:peptide model 5K4F (black, chain A) aligned with the CALP:MD:peptide model 5IC3 (red, chain A) (RMSD = 0.119 Å; 445 atoms). Black arrow points to the very close overlay of the loops in the two models.

PyMOL was used to perform all the alignments.
**Figure S6. Mass spectrometry confirms the covalent attachment of MD**

(A) Intact electrospray ionization Orbitrap analysis at 240,000 resolution (FWHM @ 200 m/z) shows the intact CAL-PDZ domain by itself (upper) and modified by MD (lower). The left panels show charge states from \((\text{M+H}^+)_{7^+}\) to \((\text{M+H}^+)_{10^+}\), ranging from 875 to 1,500 Thomson. The right panels show zoomed views of the \((\text{M+H}^+)_{7^+}\) isotopic envelopes that illustrate the shift in mass of CAL-PDZ by covalent addition of MD. Asterisks indicate non-specific MD background ions not related to CAL-PDZ \((z < 4)\).

(B) Annotated MS² spectra are shown for native (upper) or adducted (lower) CAL-PDZ peptides derived from proteinase-K digests and containing Cys\(^{319}\) with or without the MD covalent modification. In addition to differences in parent ion mass < 2.5 parts-per-million (ppm) from theoretical, the mass of the modification is identified in the \(b_{11}^{2^+}\) and \(y_{12}^{2^+}\) fragment ions from CAL-PDZ + MD. Additionally, the presence of the modification was inferred in fragment ions \(b_{14}^{2^+}\), \(y_6^{2^+}\), \(y_9^{2^+}\), \(y_{10}^{2^+}\), \(y_{11}^{2^+}\), and \(y_{12}^{2^+}\) wherein ions consistent with the additional loss of \(\text{H}_2\text{S}\) are observed.

MALDI-TOF confirmed that (C) under crystallization conditions, over 85% of CALP was modified by MD\((n=3)\); (D) under the NMR condition (with 125 \(\mu\text{M}\) MD), about 13% of CALP was modified by MD; (E) under FRET screening conditions, CALP was not modified by MD\((n=3)\).
Figure S7. CALP-MD adduct formation with different MD concentrations under NMR conditions

Percentage of CALP-MD adduct formation after co-incubation was measured by MALDI-TOF. Peak mass was determined for signals corresponding to either CALP or CALP-MD (n=3). 50 µM CALP was incubated with different concentrations of MD. Significant CALP-MD adduct formation was observed for MD concentrations at or above 250 µM.
Figure S8. NMR HSQC titration of MD with CALP<sup>C319A</sup>

HSQC spectra are shown for 50 µM <sup>15</sup>N- CALP<sup>C319A</sup> with 500 µM MD (orange) overlaid with vehicle control (1% DMSO, black). No significant chemical shift perturbations were observed upon addition of 500 µM MD, suggesting that no observable binding occurred between MD and CALP<sup>C319A</sup> under the condition tested. A two-fold HSQC titration was performed using concentrations of MD between 15 µM and 1 mM, and similar results were observed (data not shown).
**Figure S9. FP measurements of the inhibition effects of MD, ED and EM**

(A) FP competition assay to measure the $K_i$ of MD, ED and EM at pH6.8 (25 mM sodium phosphate [pH 6.8], 150 mM sodium chloride, 0.1 mM TCEP, 0.02% sodium azide) that disfavor covalent modification of CAL PDZ (see Figure S6D). DMSO was the negative control; peptide inhibitor iCAL36 was the positive control. The figure represents one of three independent
measurements. (B) FP binding assay of DMSO-, MD-, ED- or EM- treated CALP. The figure represents three independent measurements. (C) FP binding assay DMSO-, MD-, ED- or EM- treated CALP$^{C319A}$. The figure represents three independent measurements. (D-F) MALDI-TOF curves show the modification status of CALP following incubation with MD (D), ED (E), or EM (F) used in the FP binding assays shown in (B). (G-I) MALDI-TOF curves show the modification status of CALP$^{C319A}$ following incubation with MD (G), ED (H), or EM (I) used in the FP binding assays shown in (C).