Supplementary online materials for:

High-content Surface and Total Expression siRNA Kinase Library Screen with VX-809 Treatment Reveals Kinase Targets that Enhance F508del-CFTR Rescue.

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SUPPLEMENTAL MATERIAL

Synthesis and characterization of MGnBu:

Scheme 1: Protocol for the synthesis of MGnBu from a previously reported MG precursor. MG[H]ester is first transformed to MG[H]COOH using a base, followed by the activation of terminal carboxylic acid, using NHS, DCC, and coupling using nButylamine to obtain MGnBu.

Synthesis:

To a solution of MG[H]ester (460 mg, 1 mmol, 1 eq.) in acetonitrile (10 mL), 0.5 M sodium hydroxide (2 mL) was added and the mixture was stirred overnight. ESI-MS of the mixture confirmed the ester cleavage. Subsequently, the solvent was removed in vacuo and the dry, solid MG[H]COOH was dissolved in anhydrous dichloromethane (5 mL). To this solution, NHS (127 mg, 1.1 mmol, 1.1 eq.) and DCC (227 mg, 1.1 mmol, 1.1 eq.), were added and stirred at room temperature. After 6 hours, a mixture of nButylamine (118 uL, 1.2 mmol, 1.2 eq.) and triethyl amine (181 uL, 1.3 mmol, 1.3 eq.) was added and stirred overnight (14 h) at room temperature. The mixture was filtered, dried, taken up in acetonitrile and boiled. To the boiling mixture, chloranil (245 mg, 1 mmol, 1 eq.) was added and stirred for 15 mins. The mixture was then cooled, filtered, dried and taken up in a mixture of 30% acetonitrile in water (0.1 % TFA) for purification using reverse phase MPLC column. Yield: 72% (350 mg, 0.72 mmol)

\[^1H\text{ NMR (CD_3OD), 500 MHz:}\] 0.95 (t, 3H, J = 7.3 Hz), 1.37 (m, 2H), 1.49 (m, 2H), 2.16 (t, 2H, J = 6.9 Hz), 2.44 (t, 2H, J = 7.3 Hz), 3.21 (t, 2H, J = 7.0 Hz), 3.34 (s, 12H), 4.21 (t, 2H, J = 6.2 Hz), 7.06 (d, 4H, J = 9.3 Hz), 7.19 (d, 2H, J = 8.7 Hz), 7.39 (d, 2H, J = 8.7 Hz), 7.45 (d, 4H, J = 9.15 Hz)

\[^13C\text{ NMR (CD_3OD), 500 MHz:}\] 12.69, 19.69, 25.03, 31.15, 31.93, 38.77, 39.40, 67.60, 113.00, 114.62, 126.92, 131.85, 137.48, 140.47, 157.01, 164.31, 178.36

ESI-MS (positive mode, MeOH): expected: 486.31 obtained: 486.2
**MGNBu Photophysical properties:**

*The final dye solutions for spectroscopy and biological experiments were diluted from a 1000x stock solution in 1% acidic ethanol and prepared the same day of experiments.

**Quantum yield:** Quantum yield of the dyes were determined using MG2p-dL5** as a standard with a quantum yield of 20% in PBS 7.4. The fluorescence emission of dye-dL5** solutions containing the same O.D at 630 nm (λex) in PBS 7.4 were obtained. The fluorescence spectra of the solutions were measured and the ratio of quantum yield of standard and unknown sample is: \( F_x F_s = Q_x Q_s \) Where \( F \) – fluorescence intensity, \( Q \) – quantum yield; \( x \) – sample, \( s \) – standard

**Absorbance spectra:**

Supplementary Figure 1: Absorbance spectra of MGNBu and MG2p (3 µM) in phosphate buffered saline (PBS) pH 7.4. Each dye was normalized to the wavelength corresponding to the highest optical density. The spectral profile of MG2p and MGNBu are similar. The extinction co-efficient of MG at 607 nm is 74,250 M⁻¹ cm⁻¹ in PBS 7.4.¹
Supplementary Figure 2: Absorbance spectra of MGnBu and MG2p (3µM) complexed with dL5** (15 uM) in phosphate buffered saline (PBS) pH 7.4. Each dye-dL5** was normalized to the wavelength corresponding to the highest optical density. The spectral shift of MGnBu upon binding to dL5** is similar as MG2p-dL5** complex. The extinction co-efficient of MG – dL5** at 607 nm is 91,700 M⁻¹ cm⁻¹ in PBS 7.4.

Binding affinity (Kd measurement): Binding affinity of MGnBu was determined by titrating it against a known concentration of dL5**. Triplicate fluorescence response was determined using a 96-well plate on a TECAN Infinite M1000 96-well plate reader fluorimeter using ex/em of 636 nm/664 nm. Analysis of fluorescence response was determined using a non-linear regression using One site – Total, accounting for ligand depletion. The model, originally used for radioactivity measurements was modified to fit fluorescence data by fixing the volume at 0.2 mL and SpecAct was set to 1.00 on GraphPad Prism 7.0 software, and the maximum fluorescent intensity was set to 5.0, representing the maximum concentration of complex that can be formed (based on 5 nM protein concentration). The ligand depletion model assumes that changes in complex formation are associated with complementary changes in free ligand and free receptor, and are a typical model for ligand-receptor interactions when one has to work at protein concentrations that are near the Kd value. The original formula and fitting can be found at: http://www.graphpad.com/guides/prism/6/curve-fitting/index.htm?reg_one_site_total_depletion.htm
Supplementary Figure 3: Kd measurement of MGnBu and MG2p using purified dL5**. 5 nM dL5** was incubated with a serial dilution of 500 nM to 10 pM of the respective dye dissolved in phosphate buffered saline (PBS) pH 7.4. The dye fluorescence was subtracted from the dye-dL5** complex. MGnBu Kd (0.4 ± 0.35 pM); MG2p Kd (0.5 ± 0.35 pM)
**Excitation and emission spectra**

Supplementary Figure 4: Excitation spectra was recorded using 1 μM dye and 5 μM dL5** in phosphate buffered saline (PBS) pH 7.4. λem was set to 700 nm. MGnBu was compared to MG-2p and MG-B-Tau. Normalization for the respective dye-dL5** was done by setting the λ of dye-dL5** with highest fluorescence intensity to 1.0.

Supplementary Figure 5: Emission spectra was recorded using 1 μM dye and 5 μM dL5** in phosphate buffered saline (PBS) pH 7.4. λex was set to 600 nm to visualize direct excitation of MG-dL5** in MGnBu in comparison to MG-2p and MG-B-Tau.
**Analysis and Statistics**

**Pre-processing data:** All well measurements had the mean background fluorescence subtracted. The surface CFTR fluorescence signal and total fluorescence signal were normalized to the relative quantity of cells (Hoechst 33342 signal) per scanning point, 16 total, inside a well. All values were then transformed to log scale to achieve a normal distribution required for the subsequent statistical tests. For each well, the 16 intra-well values were subjected to outlier analysis using robust Z-score testing to identify and exclude significant anomalies within a well. These irregular measurements could arise from technical error or small debris. The final well sample measurement was calculated by the interquartile mean of the intra-well values.

\[
Z\text{-score} = \frac{Y_i - \bar{Y}_N(16 \text{ intra-well values})}{\text{MAD}(16 \text{ intra-well values})}
\]

(1)

- \(Y_i\): value of point within a well
- \(\bar{Y}_N\): median of 16 intra-well values
- \(\text{MAD}\): median absolute deviation of all 16 intra well measurements

**SiRNA screen quality assessment:** The quality control (QC) of the siRNA screen was assessed through strictly standardize mean difference (SSMD). This scoring method is favorable due to its ability to report consistent quality control results with positive controls that aren’t necessarily very strong. In our system, our positive control is siRNA that targets CFTR, and the total amount of CFTR is reduced due to the F508d mutation. Even with VX-809 treatment, knockdown effect can be moderate in terms of total protein. Our QC criterion was based off of a moderate control effect assessing the VX-809 treated plate of a transfection pair, and using the CFTR siRNA wells as a positive control for total protein knockdown and scrambled NC siRNA wells as a negative control. A plate passes QC if it has good or excellent quality.

\[
\text{SSMD QC} = \frac{\bar{x}_p - \bar{x}_N}{\sqrt{S_p^2 + S_N^2}}
\]

(2)

- \(\bar{x}_p\): Sample mean of positive control
- \(\bar{x}_N\): Sample mean of negative control
- \(S_p\): Standard deviation of positive control
- \(S_N\): Standard deviation of Negative control

**Hit selection:** Paired SSMD and mean fold change in CFTR surface expression were used to evaluate hits.

\[
\text{SSMD} = \frac{d_i}{S_i}
\]

(3)

**VX-809 treated plate:** The median of the negative siRNA VX-809 treated NC of a plate was subtracted from each sample of the same plate. The mean difference and standard deviation of an siRNA was calculated from the plate replicates. To select for strong siRNA effects with VX-809, we used a hit threshold of 2 for the kinase library screen, which used 3 replicates per siRNA. The siRNAs that were above the threshold were ranked based on their average fold change from the median VX-809 NC.

**Vehicle (DMSO) treated plate:** Without any chemical or temperature correction, there is near zero surface fluorescence in the NC wells alone. These vehicle treated plates are more sensitive to small increases in surface signal, which can potentially arise from media evaporation at the edges. In the kinase library screen, the negative controls are positioned in the first column, making it important to be able to identify and counter this potential
effect. The % difference between the median of the NC and the interquartile mean of the whole plate is determined. The interquartile mean is used only when there is a larger than 15% difference between the surface fluorescence of the negative controls and the interquartile mean of the plate. This large difference infers that the characteristic NC near zero signal was likely aggravated by possible edge effects, making it important to utilize the plate based normalization. Normalizing using most of the samples on the plate is powerful because there are only very few instances where siRNA treatments promotes F508del-CFTR surface expression with DMSO alone. In addition, the large amount of samples in a plate promotes a more accurate measurement of the baseline surface signal. The median of the negative control siRNA or interquartile mean of a DMSO treated plate was subtracted from each sample of the same plate. The mean difference and standard deviation of an siRNA was calculated from the plate replicates. To identify strong siRNA effects with DMSO, we used a hit threshold of 2 for the kinase library screen, which used 3 replicates per siRNA. The siRNAs that were above the threshold were ranked based on their average fold change from the median DMSO NC or plate interquartile mean. The primary purpose of the vehicle treated plate was to establish the effect of an siRNA without VX-809 to determine the interaction significance.

**Interaction testing:** Interaction was assessed using a linear model that included a siRNA-drug interaction effect.\(^6,7\)

Surface Signal = siRNA(yes/no) + VX-809(yes/no) + siRNA*VX-809

(yes,yes) siRNA+VX-809
(yes,no) Vehicle siRNA
(no,yes) VX-809 NC
(no,no) Vehicle NC

A two-way, balanced (3 replicates per condition) ANOVA was performed in Graphpad prism 6 to test for significant interaction using the log fold change from DMSO NC (NC well median or interquartile mean of plate). Equal variance was confirmed using Levene’s test in Minitab. However, due to the variable nature of siRNA knockdown efficiency between replicates and a bare minimum of replicates, there were instances where replicate variance was too broad to calculate significant interaction. To help mitigate variance between replicates in the VX-809 condition, where there is often some variability in the VX-809 treatment itself, the overall average of VX-809 NC log fold increase from the DMSO baseline surface expression of each transfection pair (VX-809 and DMSO treated plate of a single replicate) was used to rescale each replicate to the overall average.
Select siRNA kinase knock-down Immunofluorescence

**siRNA Transfection Method:**
Cherry picked CAMKK1 and RAF1 siRNA from library and performed knockdown according to Dharmacon’s Library transfection protocol, using same methods as those described in the manuscript.

**Immunofluorescence 96 well imaging format:**
Adapted from BD biosciences support protocols for bioimaging in 96 well plate.

Cell culture media was removed and 100 µL of 37°C 4% PFA was added per well and incubated at room temperature for 10 minutes. After PFA incubation, PFA was removed and wells were washed with 100 µL of PBS, followed by adding 100 µL of 0.1% Triton X-100/ 4% FBS solution to wells for 1 hour at room temperature. Permeabilization/blocking solution was removed and incubated with either 50 µL of Anti-RAF1 (abcam ab147435) (1:200 dilution) or Anti-CAMKK (abcam ab80066) (5 µg/ mL dilution) in 4% FBS for 1 hour at room temperature. Primary antibodies were washed off 3x with 100 µL PBS. 50 µL of secondary antibody, Goat Anti Rabbit IgG Alexa Fluor 488 (abcam ab15007) (1:500 dilution) was added to wells and incubated in dark at room temperature for 1 hour. After incubation, wells were washed 3x with 100 µL PBS and then 200 µL of PBS containing 2 µg/mL of Hoechst 33342 was added. Plate was imaged on Andor Revolution XD system (Andor technology) with Yokogawa CSU-X1 spinning disk confocal unit (Yokogawa Industries). Cells were imaged with a 40x, .95 air objective (Nikon) with (488ex:525/50em) and (405ex:435/25em).

Supplementary Figure 6. SiRNA knock-down of CAMKK1 and RAF. A) Immunofluorescence imaging. Matched LUT between NC and + siRNA for each antibody. B) Measured mean Alexa 488 fluorescence intensity in cell ROIs ± S.D. (7 ROIs/image). ****P≤ 0.0001; **P≤ 0.01. Scale bar: 10 µm
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