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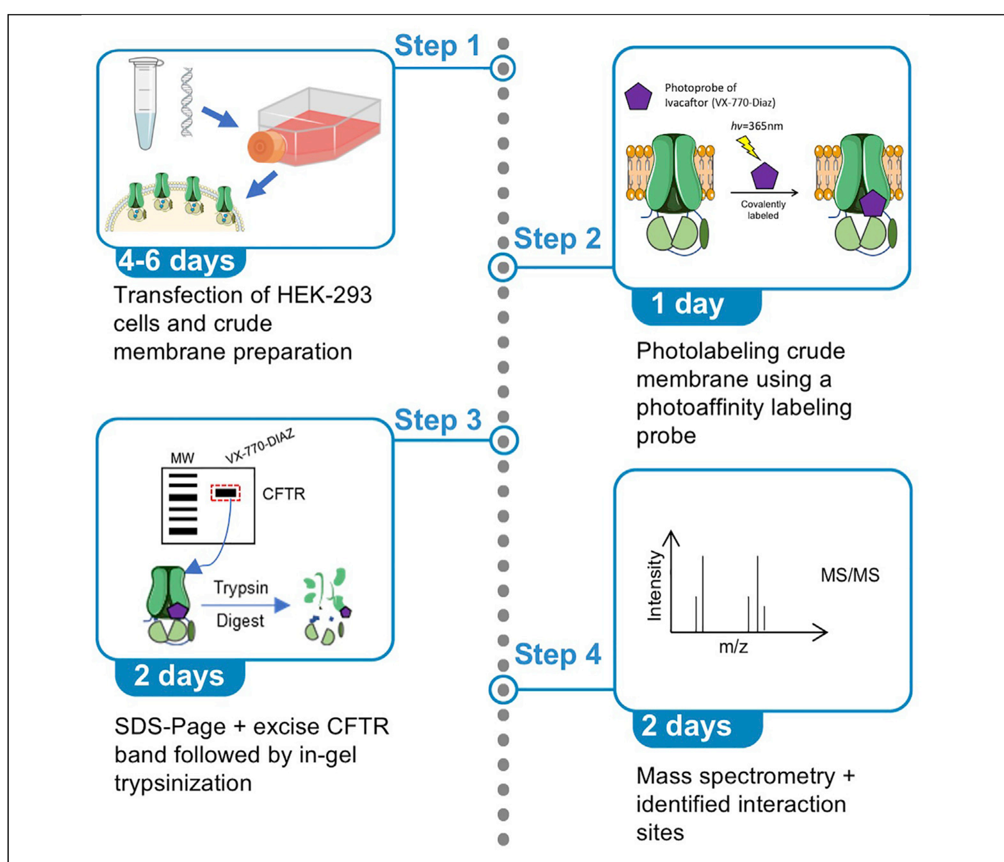
Protocol

A protocol for identifying the binding sites of small molecules on the cystic fibrosis transmembrane conductance regulator (CFTR) protein



We describe a protocol to identify the binding site(s) for a drug called ivacaftor that potentiates the CFTR chloride channel. We use photoaffinity probes — based on the structure of ivacaftor — to covalently modify the CFTR protein at the region that constitutes the drug binding site (s). We define the methods for photo-labeling CFTR, its membrane extraction, and enzymatic digestion using trypsin. We then describe the experimental methods to identify the modified peptides by using mass spectrometry.

Onofrio Laselva, Evgeniy V. Petrotchenko, C. Michael Hamilton, Zafar Qureshi, Christoph H. Borchers, Robert N. Young, Christine E. Bear

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Highlights

Protocol to define ivacaftor binding sites on CFTR using photo activatable probes

Detailed steps for analyzing drug-modified CFTR by mass spectrometry

Method for enriching full-length CFTR using biotinylated photoprobe

Functionally validated photo-probes enable insight into drug mechanisms

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A protocol for identifying the binding sites of small molecules on the cystic fibrosis transmembrane conductance regulator (CFTR) protein

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SUMMARY

We describe a protocol to identify the binding site(s) for a drug called ivacaftor that potentiates the CFTR chloride channel. We use photoaffinity probes—based on the structure of ivacaftor—to covalently modify the CFTR protein at the region that constitutes the drug binding site(s). We define the methods for photo-labeling CFTR, its membrane extraction, and enzymatic digestion using trypsin. We then describe the experimental methods to identify the modified peptides by using mass spectrometry.

For complete details on the use and execution of this protocol, please refer to Laselva et al. (2021).

BEFORE YOU BEGIN

Previously, studies using cryo-EM (Liu et al., 2019) indicated that Ivacaftor (VX-770) binds at the interface between the hinge-like discontinuity of CFTR protein in tm4, tm5 and tm8 and the lipid bilayer. However, these studies were conducted with CFTR protein solubilized with detergents. It has been demonstrated that associated lipids contribute to, and/ or stabilize, the regulated function of CFTR (Chin et al., 2019; Hildebrandt et al., 2017; Yang et al., 2014). Hence, we developed a protocol to define the VX-770 binding site(s) to CFTR in its natural environment of the phospholipid bilayer. Using photoactivatable probes we confirmed that VX-770 labeled CFTR at the site identified by cryo-EM (Liu et al., 2019) and also labeled a cytosolic region, intracellular loop 4 (IC4), previously revealed by Byrnes et al. (2018) using Hydrogen/Deuterium exchange.

We recommend that before you begin similar studies, the functional properties of the probes be confirmed. For example, as VX-770, or ivacaftor, is a potentiator of the CFTR channel (Van Goor et al., 2009) we first confirmed that this activity was retained in the photoactivatable probes (Hamilton et al., 2018).

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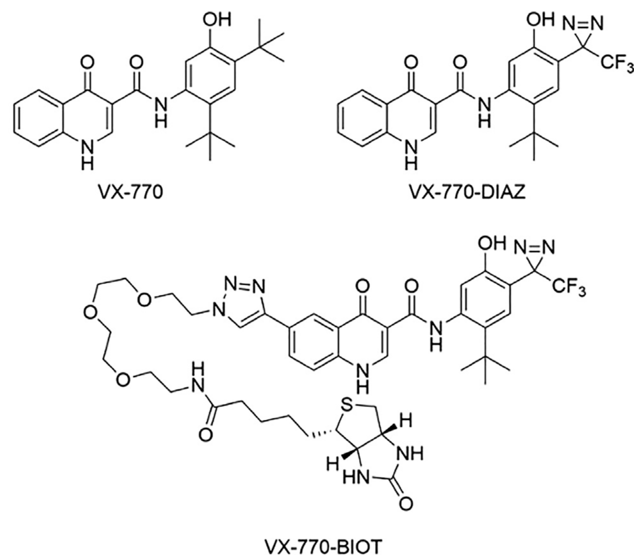

Figure 1. Chemical structures of VX-770, VX-770-DIAZ, and VX-770-BIOT

Preparation: handling of photolabeling probes

The detailed procedures for synthesizing the necessary probes, as shown in Figure 1, were previously described (Hamilton et al., 2018; Laselva et al., 2021). It is critical that the resulting probes be kept in stock solutions (0.2–2 mM in DMSO) at −80°C prior to future CFTR labeling studies. Longer term storage of stock solutions at −80°C requires assessment of quality. After 1 year of storage at −80°C, we determined that there was 10% degradation with conversion to the alcohol product resulting from reaction with water.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CFTR	University of North Caroline at Chapel Hill - CFTR antibodies https://cftrantibodies.web.unc.edu	ID: 596
Anti-Calnexin	Sigma-Aldrich	Cat#C4731, RRID:AB_476845
Chemicals, peptides, and recombinant proteins		
HEPES	Sigma-Aldrich	Cat# H3375
EDTA	Sigma-Aldrich	Cat# E9884
TRIS-HCl	Sigma-Aldrich	Cat# T5941
$MgCl_2$ (Magnesium chloride)	Sigma-Aldrich	Cat# M8266
complete™, Protease Inhibitor Cocktail (Roche)	Sigma-Aldrich	Cat# 4693159001
Ammonium bicarbonate (ABC)	Sigma-Aldrich	Cat# 09830
Acetonitrile (MeCN)	Sigma-Aldrich	Cat# 34851
Trypsin, sequence grade	Promega	Cat# V5117
C18 Zip-Tips	Sigma-Aldrich	Cat# ZTC18M096
VX-770-Biot	Must be prepared by synthesis (Laselva et al., 2021).	See adjacent reference
VX-770-Diaz	Must be prepared by synthesis (Hamilton et al., 2018). See preparation notes.	See adjacent reference
Critical commercial assays		
Pierce™ Monomeric Avidin Agarose	Thermo Fisher Scientific	Cat# 20228

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Micron-30 kDa Centrifugal filter Unit with Ultracel-30 membrane	Millipore	Cat# MRCF0R030
Nitrocellulose Membrane, Roll, 0.2 μm, 30 cm × 3.5 m	Bio-Rad	Cat# 1620112
10× Tris-Glycine SDS buffer	WISENT	Cat# 880-570-LL
10× Tris-Glycine buffer	WISENT	Cat# 880-560-LL
10× PBS (Phosphate Buffered Saline)	WISENT	Cat# 311-410-CL
Pierce™ Monomeric Avidin Agarose Kit	Thermo Fisher Scientific	Cat# 20227
ECL	GE Healthcare	Cat# 10308449
Experimental models: Cell lines		
Human Embryonic Kidney (HEK)-293 cells	Laboratory of Dr. Daniela Rotin	ID: CRL-1573
Software and algorithms		
ImageStudioLite	LI-COR Biosciences	https://www.licor.com/bio/image-studio-lite/download

MATERIALS AND EQUIPMENT

Cell Lysis Buffer

Reagent	Final concentration	Amount
HEPES	10 mM	1.19 g
EDTA	1 mM	0.226 g
ddH_2O	n/a	500 mL

Adjust pH to 7.2 at 25°C, add ddH_2O water up to 500 mL. The buffer can be stored at 4°C for 12 months.

Re-suspension Buffer

Reagent	Final concentration	Amount
Tris-HCl	40 mM	2.42 g
$MgCl_2$	5 mM	0.238 g
EGTA	0.1 mM	0.019
ddH_2O	n/a	500 mL

Adjust pH to 7.5 at 25°C, add ddH_2O water up to 500 mL. The buffer can be stored at 4°C for 12 months.

RIPA Buffer

Reagent	Final concentration	Amount
Tris-HCl	50 mM	3.94 g
NaCl	150 mM	4.38 g
EDTA	1 mM	0.14
SDS	0.2%	1 mL
Triton X-100	0.1%	0.5 mL

Adjust pH to 7.4 at 25°C, add ddH_2O water up to 500 mL. The buffer can be stored at 4°C for 12 months.

T-PBS Buffer

Reagent	Final concentration	Amount
10× PBS	1×	100 mL
ddH_2O	n/a	900 mL
Tween 20	n/a	1 mL

Running Buffer		
Reagent	Final concentration	Amount
10× Tris-Glycine SDS buffer	1×	100 mL
ddH_2O	n/a	800 mL

Transfer Buffer		
Reagent	Final concentration	Amount
10× Tris-Glycine buffer	1×	100 mL
Methanol	n/a	100 mL
ddH_2O	n/a	800 mL

STEP-BY-STEP METHOD DETAILS

Preparing the ivacaftor (VX-770) target: CFTR, in its native membrane environment

Timing: 4–6 days

1. Transfect Human embryonic kidney (HEK) 293 cells (HEK293).
   a. HEK293 cells were maintained in DMEM supplemented with non-essential amino acids, 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) pen-strep (10,000 U/mL) at 37°C.
   b. HEK293 cells were transfected in T75 flasks (~7.000.000 cells).
      i. HEK293 cells were seeded in one T75 flask (3.500.000 cells).
      ii. After ~24 h, at 60–80% of confluence, HEK293 cells were transfected with 15 μg cDNA expressing WT-CFTR using PolyFect following the manufacturer's protocol (https://www.abpbio.com/wp-content/uploads/2021/04/FP330.pdf). Briefly, aliquots 15 μg of cDNA was diluted in 150 μL of H_2O. 1,500 μL of DMEM (w/o FBS) + 150 μL of PolyFect was added to the diluted cDNA. After 8 min, 5,900 μL of DMEM (with FBS) was added.
      iii. 24 h after transfection, the DMEM media was changed with 15 mL of fresh DMEM media with 5 mM Sodium butyrate (NaB) at 37°C for 24 h to enhance protein expression.

2. Crude membrane purification.
   a. HEK293 cells were first trypsinized (0.0025% Trypsin for 5 min at 37°C and neutralized with HEK293 medium) and centrifuged for 5 min at 600 × *g*. Then the cell pellet was re-suspended in 20 mL of Cell Lysis Buffer (10 mM HEPES, pH 7.2 and 1 mM EDTA) plus protease inhibitors (1 tablet in 50 mL of cell lysis buffer). Cells were lysed using a cell disruptor (10,000 psi, 4°C for 5 min).

   Alternatives: other methods for cell disruption include sonication and the use of a French press.

   b. Unlysed cells were removed by centrifugation at 1,900 rpm for 10 min at 4°C.
   c. Crude membranes were isolated from the resulting supernatant after a 1 h spin at 45,000 rpm for 60 min at 4°C.
   d. The crude membrane pellet was re-suspended in 250 μL of re-suspension buffer (40 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 0.1 mM EGTA) using a 18 gauge needle (~20 times on ice). The solution was then passed through a 27-gauge needle (~20 times on ice).
   e. The total protein concentrations of the crude membranes were quantified using Nanodrop (expected concentration ~7–9 mg/mL).

   Alternatives: the total protein concentration could also be measured using the Bradford or BCA kit assay.

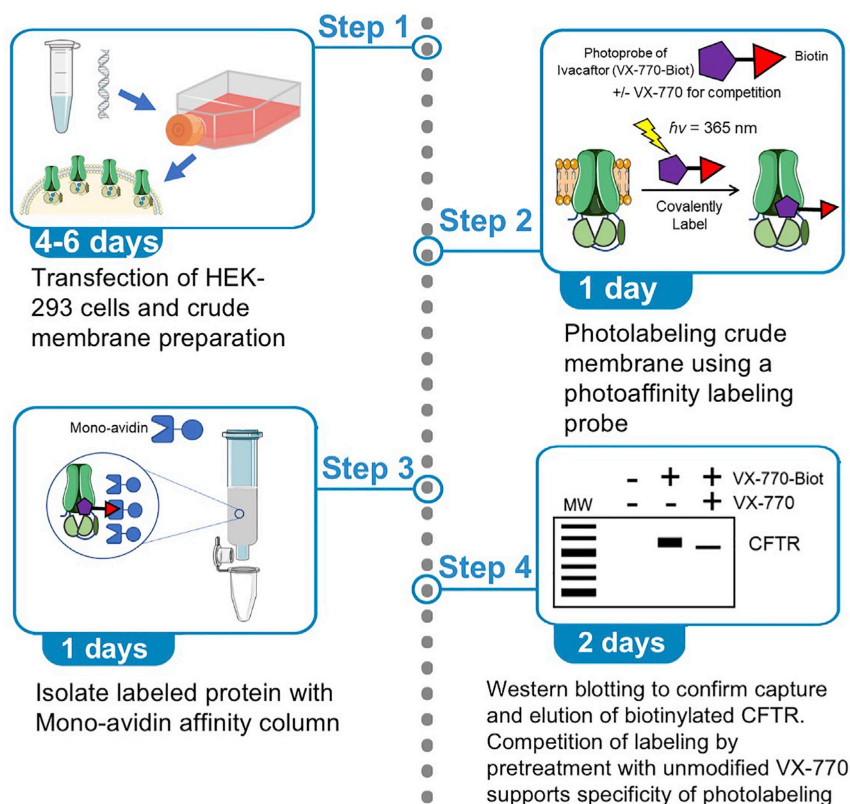

Figure 2. Major steps for confirming specific photolabeling of CFTR with VX-770-BIOT

f. Membranes were aliquoted (~20 μg in ~20 μL) and snap frozen in liquid nitrogen for storage at −80°C.

Specific photolabeling of CFTR with VX-770-biot

We employed the photoaffinity probe (VX-770-DIAZ, Figure 1) modified with a biotin group (VX-770-BIOT, Figure 1) to enable confirmation of efficient and specific labeling of CFTR in its native environment, the cellular membrane. The biotin tag can be employed to enrich labeled CFTR after solubilization by virtue of its affinity to a mono-avidin column. Specific labeling with the VX-770-BIOT probe can be confirmed by competition with the parental drug molecule (Figure 2).

3. Labeling of CFTR in cellular membranes using VX-770-BIOT.

Timing: ~10 h

a. Take 3 aliquots of crude membrane stocks (~20 μL) and thaw in ice. Then add up to 50 μL of the re-suspension buffer plus cocktail of protease inhibitors (100× stock , this mixture contains individual components including Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A, AEBSF which inhibit serine, cysteine and acid proteases, and aminopeptidases).
b. Add DMSO, 10 μM of VX-770 (control) or 0.1 μM of VX-770-Biot (photoaffinity labeling probe stock solution in DMSO) and keep in ice for 20 min, minimizing exposure to light as much as possible. The VX-770 as control, should be used at high concentration for competition studies.
c. Transfer the samples to 96 well plate (black sided, clear bottom, Corning) and keep on top of ice. If not accessible, the black sided plates are not required and clear sided plates can be employed.
d. Expose the samples to UV lamp (30–50 mW cm^{-2}) for 15 min on ice (Figure 3).

Figure 3. Exposure of sample to UV lamp in 96 well plate (black, clear bottom) on top of ice

4. Pull down the biotinylated protein and elution.
   a. After exposure of the samples to UV light, add 100 μL of RIPA buffer plus protease inhibitor to each well and transfer back the samples to the 1.5 mL microeppendorf tube. After UV irradiation, it is no longer necessary to protect the samples from ambient light.

   ⚠ CRITICAL: be sure to wash the well two more times with another 100 μL of RIPA buffer and transfer everything to the 1.5 mL microeppendorf tube (total final volume ~350 μL.

   b. Shake the samples using a tube shaker for 20–30 min in the cold room (4°C).

   Alternatives: any other shakers (i.e., rocker or rotator shaker) can be used.

   c. To wash the monomeric avidin agarose beads, transfer to the Eppendorf tube and add 300 μL of RIPA buffer (without Protease Inhibitors). Hand shake 5 times and centrifuge at 19,000 × *g* for 30 s. Then remove the supernatant and wash the beads adding 300 μL of RIPA plus PI twice.
   d. Transfer the samples to the Eppendorf tubes containing the washed monomeric avidin agarose beads. Shake the samples, using a tube shaker, at 21°C–25°C for 1 h.

   Alternatives: any other shakers (i.e., rocker or rotator shaker) can be used.

   e. Centrifuge the samples at 19,000 × *g* for 1 min, transfer the supernatant to the new 1.5 mL micro-Eppendorf tubes and keep in ice or cold room. These samples constitute "unbound" CFTR protein. Previous studies showed that the abundance of CFTR in "unbound" sample was consistently less than that in the "preloaded" sample, i.e., that sample tested before application to beads. CFTR protein is susceptible to being degraded and/or aggregated and this property likely accounts for its loss during these pull-down studies.
   f. Add 250 μL of RIPA buffer + PI and wash the agarose beads 3 times.
   g. Add 350 μL of Elution buffer (ThermoFisher kit, Cat# 20227) and linear shaking the samples at 21°C–25°C for 20 min. Any tube, rocker or rotator shaker can be employed.
   h. Centrifuge the samples at 19,000 × *g* for 1 min.
   i. Transfer the supernatant to concentrator tubes (30 kDa centrifugal filter unit with ultracel-30 membrane, Millipore) and centrifuge the samples at 19,000 × *g* for 7 min.

   ⚠ CRITICAL: The final amount of the samples should be ~30 μL. Therefore, centrifuge until you have that volume.

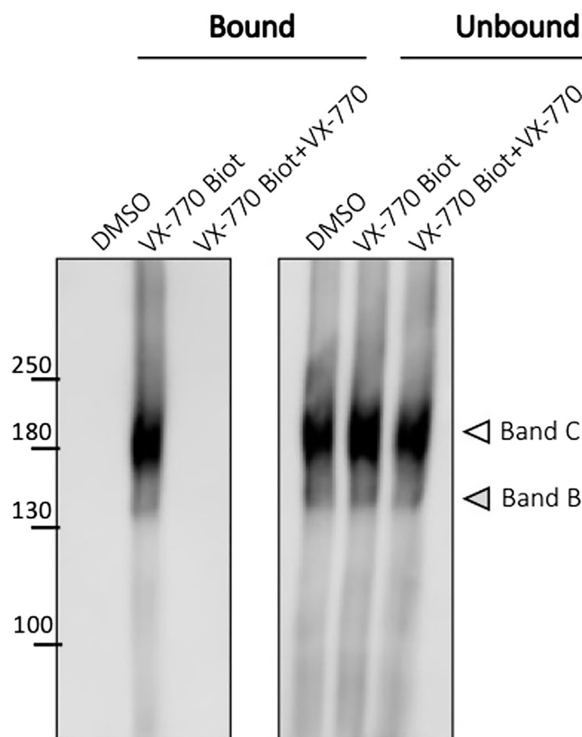

Figure 4. Immunoblots of labeled Wt-CFTR (bound) and unlabelled Wt-CFTR (unbound)
Left panel shows that only CFTR labeled with the photo-label (bearing a biotin) is bound to the mono-avidin matrix.

j. Flip down the centrifugal filter to a new Eppendorf tube and centrifuge at 13,000 rpm for 30 s.
k. Add 6 μL of the 5× Laemmli buffer to each of the samples and analyze by SDS-PAGE on a 6% gel for full-length CFTR in running buffer at 150 V for ~1.5 h. Good laboratory practice includes the addition of positive and negative controls for subsequent CFTR immunoblotting. A positive control includes lysate from HEK-293 cells transfected with CFTR cDNA and untransfected cells, the negative control.

5. Immunoblotting for CFTR in flow-through and eluate from mono-avidin affinity column.

Timing: 2 days

a. After electrophoresis, proteins are transferred to 0.2 μm nitrocellulose membrane at 100 V for 1 h in a Transfer buffer.
b. Incubate the nitrocellulose membrane (blot) with 5% milk in T-PBS for 1 h. Then cut the blot in two parts around 110 kDa. Incubate the top part (130–250 kDa) with human NBD2-CFTR-specific murine mAb 596 (1:5.000). For the bottom part (75–110 kDa) incubate with human Calnexin-specific rabbit (1:10,000) for overnight at 4°C.
c. Wash the blots 5 times for 5 min with T-PBS (Phosphate buffered saline with Tween 20).
d. Incubate the blots with anti-mouse and anti-rabbit secondary antibodies conjugated with HRP for 1 h at 21°C–25°C. Then wash the blots 5 times for 5 min with T-PBS.
e. Develop the blots with ECL (Amersham) using the Li-Cor Odyssey Fc (LI-COR Bioscience) in a linear range of exposure (0.5–5 min) (Figure 4).

Alternatives: the immunoblots can also be exposed using a ChemiDoc Imaging system.

Identification of binding site for VX-770 by mass spectrometry

Timing: 3 days

The peptides modified with the VX-770 photo-affinity labeling probe can be identified using mass spectrometric analysis. Following electrophoresis, the gel is stained by Coomassie Blue, the band corresponding to the 130–180 kD, the MW of CFTR is excised, and the proteins are in-gel digested with trypsin. The peptides obtained from CFTR labeled with VX-770-DIAZ are analyzed by nano-LC-MS for the detection and identification of the VX-770-modified peptides.

6. In-gel trypsin digestion is performed according to the standard protocol (Borchers et al., 2000).
   a. The excised band is cut into approximately 1 × 1 mm pieces.
   b. Gel pieces are destained with alternating washes of freshly prepared 50 mM ammonium bicarbonate (ABC) and 50 mM ABC 50% MeCN (Acetonitrile) until the color is removed.
   c. Following last wash with 50 mM ABC 50% MeCN, the solvent is removed and gel pieces are dried under vacuum in SpeedVac (Labconco) for 30 min–1 h (gel pieces should be easily detachable from the tube wall).
   d. Freshly prepared trypsin (sequence grade, Promega) solution in 50 mM ABC, at least equal to the gel band volume trypsin solution (for example 100 ul for excised 1 cm^2 gel piece of 1 mm thickness) is added to the dried gel pieces and is allowed to soak in (approximate enzyme:protein ratio of 1:10 to 1:5).
   e. Gel pieces are incubated 14–16 h at 37°C with orbital shaking at 450 rpm in a Thermomixer (Eppendorf).
   f. The peptides are extracted from the gel by three changes of gel band volume 0.1% formic acid (FA), and one gel volume of each 25%, 50% and 75% MeCN in 0.1% FA (total of 6 extractions).
   g. The peptide solution is dried under vacuum in SpeedVac, reconstituted in 0.1% FA and desalted using C18 ZipTip (Sigma).
   h. Elution from the ZipTip is placed into autosampler vial glass insert (Agilent), supplemented with an equal volume of the 0.1% FA and is briefly dried under vacuum in SpeedVac to approximately 20% of the original volume.
7. LC-MS analysis of the tryptic peptides:
   a. The glass insert with the obtained trypsin digest is placed into a vial and loaded into the autosampler.
   b. Nano-LC-MS analysis was performed with data dependent acquisition using an Easy-nLCTM 1200 System (Thermo Scientific) coupled to a Q Exactive Plus (Thermo Scientific) mass spectrometer as described (Borchers et al., 2000).
   c. Peptides were pre-concentrated on an AcclaimTMPepMapTM 100 C18 pre-column (3 mm particle size, 75 mm inner diameter × 2 cm length) and separated on an AcclaimTM PepMapTM 100 C18 main column (2 mm particle size, 75 mm inner diameter × 25 cm length) using a 0–50% B 50-min binary gradient (Buffer A: 0.1% FA, buffer B: 84% ACN in 0.1% FA), at a flow rate of 300 nL/min.
   d. Full MS scans were acquired in positive mode from m/z 350–1,500 at a resolution of 70,000 with an automatic gain control (AGC) target value of 1×10^6 and a maximum injection time of 120 ms.
   e. The 15 most intense precursor ions (charge states +2 - +4) were isolated with a window of m/z 1.2 and fragmented using a normalized higher-energy collisional dissociation energy of 28, and a dynamic exclusion of 40 s.
   f. The MS/MS spectra were acquired at a resolution of 17,500, using an AGC target value of 2×10^4 and a maximum injection time of 64 ms.
8. LC-MS data processing:
   a. Data are processed using the Proteome Discoverer 2.4 software suite (Thermo Scientific) using the Sequest search engine and Percolator validation nodes against the human proteome database using variable modification settings corresponding to the covalent modification with VX-770-DIAZ probe (elemental addition of H19C22F3N2O3, mass addition of 416.1348) at any residue.

b. Matched modified peptides are expected to be identified as having VX-770 modifications.
c. Additionally, data can be searched for the specific reporter fragment ions (246.11 and 417.14 m/z) in the MS/MS spectra, corresponding to the moiety of the photo-affinity probe attached to the peptides.
d. Detection of low intensity MS1 precursors signals can be additionally improved with BoxCar-type acquisition (Meier et al., 2018) over five 200 Da mass ranges.

EXPECTED OUTCOMES

Photoactivatable photoaffinity probes can identify drug binding sites on clinically relevant proteins like CFTR in their native membrane environment. The compound studied in this work, VX-770 or ivacaftor, is a potentiator that acts on the mature CFTR protein (Wt-CFTR or certain disease-causing mutants) to enhance their channel activity (Guerra et al., 2020). Using the above protocol, we showed that VX-770-DIAZ, specifically modified the CFTR membrane protein at two sites, enabling future biochemical and electrophysiological studies of the molecular consequences of these interactions on CFTR channel gating. Importantly, we expect that the major experimental steps defined here, can be employed to identify biologically relevant small molecule interactions with any membrane protein if the photolabel (PAL) probe has been optimized as we confirmed in our published studies of the VX-770-PALs (Hamilton et al., 2018; Laselva et al., 2021).

LIMITATIONS

Prolonged exposure to ambient conditions and light has the potential to degrade photoprobes used in this work. The probes, VX-770-Diaz and VX-770-Biot are both kept as the precursor ethoxymethylether for long term storage aliquoted in DMSO and kept at -80°C and shielded from light. They are converted to their activated forms by brief exposure to 4 M HCl in MeOH, then concentrated in vacuo to give the active probes. Following conversion to the active forms of the probes, both VX-770-Diaz and VX-770-Biot should be stored aliquoted in DMSO (0.1–1 mg/mL) in the dark at -80°C, to be used within 1–2 months. Upon thawing in the dark probes should be used immediately in the photolabeling experiments.

CFTR-derived, trypsin generated peptides that are modified by photoprobes may not be detected using mass spectrometry. Trypsin digestion of the full length CFTR resulted in approximately 60% coverage of the whole polypeptide chain. Despite this incomplete coverage, we did identify two peptides modified by the VX-770 DIAZ in the current study. However, it is possible that in future labeling reactions with different probes, we would not be able to identify the modified region of CFTR using this approach because that peptide was undetectable due to its physical properties.

TROUBLESHOOTING

Problem 1

Low CFTR protein expression in crude membrane preparation. For example, in comparison to the positive control in a cell lysate, the CFTR signal detected in membrane preparations following SDS-PAGE analysis and immunoblotting is less than half when expressed relative to the loading control, calnexin.

Potential solution

CFTR in membrane preparations may be degraded. It is critical to ensure that protease inhibitors are included during membrane preparation steps and then aliquots of the membrane preparation, are flash frozen in liquid nitrogen prior to longer term storage.

Problem 2

Poor labeling of CFTR by photoactivatable probe harboring biotin. In comparison to CFTR immunoreactivity detected in flow-through from mono-avidin column (Figures 2 and 4), the CFTR signal in eluted samples may be weak. This could reflect suboptimal conditions, i.e., suboptimal UV lamp intensity, for photoactivation and labeling of CFTR with probe.

Potential solution

Photoreactivity of VX-770 probe harboring biotin should be confirmed using BSA and exposure to UV light. This labeling will not be specific, i.e., not competed by the unmodified VX-770, but the conditions for optimal labeling can be determined using this convenient substrate.

Ensure optimal storage conditions. VX-770-Diaz and VX-770-Biot are both kept as the precursor ethoxymethylether for long term storage aliquoted in DMSO and kept at −80°C and shielded from light (i.e., Wrapped in foil). The VX-770-Diaz and VX-770-Biot probes are converted to their activated forms by brief exposure to 4 M HCl in MeOH, then concentration *in vacuo* to give the active probes. Following conversion to the active forms of the probes, both VX-770-Diaz and VX-770-Biot should be stored aliquoted in DMSO (0.1–1 mg/mL) in the dark at –80°C, to be used within 1–2 months. Upon thawing in the dark probes should be used immediately in the photolabeling experiments. Prolonged exposure to ambient conditions (i.e., room temperature and ambient light) will degrade the photoprobe.

Problem 3

Non-specific labeling of CFTR using biotinylated photoactivatable probe. The CFTR signal in the solution eluted from the mono-avidin column is similar regardless of the competition step, i.e., addition of excess unmodified VX-770 (Figure 4).

Potential solution

It is critical that the dose response for labeling of CFTR using the biotinylated VX-770 photoprobe be determined prior to competition studies. Once the EC50 is determined, prior incubation with a ten-fold excess of the unmodified VX-770 should be effective in specifically completing the photolabeling step.

Problem 4

Loss of solubilized CFTR. The full-length CFTR protein, like most large membrane proteins, is susceptible to aggregation after solubilization in detergents. CFTR aggregation, appearing as a smear close to the junction with the stacking gel in SDS-PAGE, will prevent specific labeling with the VX-770 photoprobe.

Potential solution

Aggregation of membrane proteins in detergent solutions is a concentration and time dependent phenomenon. The use of fresh membrane preparations that are not exposed to repeated freeze-thaw cycles will protect the protein from aggregation.

Problem 5

Coverage of the CFTR polypeptide by mass spectrometry following trypsin digestion is low (less than 50% of primary amino acid sequence).

Potential solution

In our studies, trypsin digestion of the full length CFTR resulted in approximately 60% coverage of the polypeptide chain. With this percent coverage, we did identify the two modified peptides described in Laselva et al. (2021). If the coverage is lower, it will be necessary to test additional proteases for better protein digestion and peptide coverage. In pilot studies, we found that CFTR digestion using a combination of proteases, namely trypsin and chymotrypsin, increased coverage of the CFTR polypeptide to approximately 80%, providing a potential solution for studies where protein sequence coverage is low.

Problem 6: No detection of modified CFTR peptides by mass spectrometry. Specific content of the modified with photoprobe peptides can be low compared to the non-modified tryptic peptides, which may lead to low intensity signals and therefore missed identification with database search programs.

Potential solution

Modification yield can be increased with performing irradiation of the flash-frozen samples. Additionally, extracted ion chromatograms can be searched for the combination of the reporter ions characteristic for the modified peptides. When detected, corresponding MS1 spectra can be manually inspected for the correct assignment of the monoisotopic peaks used in database search. If monoisotopic peaks were misassigned, data can be reacquired with BoxCar-like methods of narrow MS1 mass ranges around potential hits (Meier et al., 2018). This will produce better quality MS1 spectra and potentially correct assignment of the monoisotopic peaks of the modified peptides.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Christine E. Bear, bear@sickkids.ca.

Materials availability

Materials (plasmids) generated in this study will be made available upon reasonable request to the lead contact.

Limited availability of the photo-probes used in the study, can be provided by CMH and RNY.

Data and code availability

This protocol did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

O.L., C.M.H., R.N.Y., E.V.P., Z.Q., C.H.B., and C.E.B. conceived the project. O.L. and E.V.P. performed the experiments. O.L., C.M.H., R.N.Y., E.V.P., and C.E.B. wrote the manuscript. C.M.H., Z.Q., and R.N.Y. designed and synthesized the photoaffinity labeling probes used in the experiment.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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