Molecular Pharmacology Fast Forward. Published on April 1, 2009 as doi: 10.1124/mol.109.055608

MOL Manuscript# 55608

A small molecule modulator interacts directly with deltaF508-CFTR to modify its ATPase activity and conformational stability

Leigh Wellhauser, Patrick Kim Chiaw, Stan Pasyk, Canhui Li, Mohabir Ramjeesingh and Christine E. Bear

Programme in Molecular Structure and Function, Research Institute, Hospital for Sick Children and Departments of Biochemistry and Physiology, University of Toronto, CANADA

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

MOL Manuscript# 55608

Running Title: A small molecule modulator interacts with deltaF508-CFTR

Corresponding author:
Christine E. Bear, Ph.D.
Senior Scientist, Programme in Molecular Structure and Function
Research Institute
Hospital for Sick Children
Telephone: 416-813-5981
Fax: 416-813-5022
Email: bear@sickkids.ca

Statistics:
#text pages: 30
#figures: 6
#references: 41
#words in Abstract: 249
#words in Introduction: 748
words in Discussion: 1469

List of non-standard abbreviations:
CFTR: Cystic Fibrosis Transmembrane Conductance Regulator
ABC: ATP Binding Cassette
NBDs: nucleotide binding domains
MSDs: membrane spanning domains
BHK: Baby Hamster Kidney
VRT-532: Vertex compound 532
CFTRinh-172: CFTR inhibitor-172

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract: The deletion of Phe-508 (deltaF508) constitutes the most prevalent mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) causing Cystic Fibrosis (CF). This mutation leads to CFTR misfolding and retention in the endoplasmic reticulum, as well as impaired channel activity. Importantly, the biosynthetic defect can be partially overcome by small molecule "correctors" and once at the cell surface, small molecule "potentiators" enhance the channel activity of deltaF508-CFTR. Certain compounds, such as VRT-532, exhibit both corrector and potentiator functions. In the current studies, we confirmed that the inherent chloride channel activity of deltaF508-CFTR (after biosynthetic rescue) is potentiated in studies of intact cells and membrane vesicles. Importantly, we showed that the ATPase activity of the purified and reconstituted mutant protein is directly modulated by binding of VRT-532. ATP turnover by reconstituted deltaF508-CFTR is decreased by VRT-532 treatment, an effect which may account for the increase in channel open time induced by this compound. To determine if the modification of deltaF508-CFTR function caused by direct VRT-532 binding is associated with structural changes, we evaluated the effect of VRT-532 binding on the protease susceptibility of the major mutant. Interestingly, binding of VRT-532 to deltaF508-CFTR led to a minor, but significant decrease in the trypsin susceptibility of the full length mutant protein and a fragment encompassing the second half of the protein. These findings suggest that direct binding of this small molecule induces and/or stabilizes a structure which promotes the channel open state and may underlie its efficacy as a corrector of deltaF508-CFTR.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Cystic Fibrosis is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. CFTR is a member of the ATP Binding Cassette (ABC) Superfamily of membrane proteins (Riordan et al., 1989). Together, ABC family members mediate the transport of a diverse range of substrates, with some family members working as exporters and some as importers. These activities depend on regulated interactions between their cytosolic nucleotide binding domains (NBDs) and membrane spanning domains (MSDs). The NBDs of CFTR mediate ATP binding and ATPase activity like the other members of this family (Li et al., 1996; Ramjeesingh et al., 2008) . However, CFTR is unique in that its membrane spanning domains form a chloride selective pore to mediate chloride conduction (Bear et al., 1992; Linsdell, 2006). ATP binding at the interface between NBD1 and NBD2 in CFTR is thought to promote opening of the channel gate whereas ATPase activity promotes dissociation of the NBD heterodimer, leading to the closing of the channel (Aleksandrov et al., 2007; Lewis et al., 2004; Vergani et al., 2005a). Opening of the CFTR channel gate also requires phosphorylation of multiple serine residues within the unique "R domain" region by PKA, a modification which alters "R domain" interactions with other domains, notably NBD1 (Baker et al., 2007; Csanady et al., 2005; Seibert et al., 1999; Winter and Welsh, 1997). Overall, interactions between multiple domains are necessary to communicate signals between cytosolic domains and the chloride channel gate in the membrane.

The major mutation found in CFTR results in the deletion of phenyalanine at position 508. This mutation leads to a serious folding defect, causing deltaF508-CFTR to be retained in the ER and quickly degraded (Cheng et al., 1990). The first clues to the structural basis for this trafficking defect came from protease susceptibility studies by Zhang et al. (Zhang et al., 1998). The full

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

length deltaF508-CFTR protein exhibited an enhanced susceptibility to digestion by lower concentrations of trypsin relative to the normal CFTR protein, supporting the idea that the mutant protein is conformationally altered and tryptic digestion sites are aberrantly exposed (Du et al., 2005; Sharma et al., 2004). More recently, structural studies of protein fragments corresponding to NBD1 (Lewis et al., 2005) and molecular models of the full length CFTR protein (Mornon et al., 2008; Serohijos et al., 2008), suggest that F508 resides at the interface between NBD1 and the MSDs and that the deletion of this residue may perturb native intra-molecular interactions. Chemical crosslinking studies, using wild type and deltaF508-CFTR proteins, lacking all 18 endogenous cysteine residues and possessing non-native cysteine pairs strategically inserted into different domains, support the idea that deletion of F508 perturbs domain:domain interactions (Serohijos et al., 2008). To date, evaluation of the consequences of F508 deletion in the context of the native CFTR protein sequence represents a significant experimental hurdle, preventing a comprehensive understanding of the molecular basis for CF disease.

The biosynthetic defect in deltaF508-CFTR is temperature-sensitive. Denning and colleagues have shown that incubating cells expressing the recombinant mutant protein at 27ºC for 24 to 48 hours partially rescues its biosynthesis and cell surface expression (Denning et al., 1992). Several small molecules that are also effective in rescuing the mistrafficking defect exhibited by deltaF508-CFTR in cells have been defined through high-throughput screens (Pedemonte et al., 2005; Van Goor et al., 2006). Following biosynthetic rescue and delivery to the cell surface, deltaF508-CFTR exhibits phosphorylation dependent and ATP regulated chloride channel activity, albeit with altered gating in mammalian cells (Wang et al., 2000). Other small

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

molecules, such as genistein (Hwang et al., 1997), capsaicin (Ai et al., 2004) and more recently VRT-532 (Van Goor et al., 2006), were found to enhance or "potentiate" the open probability of deltaF508-CFTR following its biosynthetic rescue and phosphorylation at the cell surface. The latter compound was subsequently shown to also function as a "corrector" rescuing the surface expression of deltaF508-CFTR (Wang et al., 2006). Wang and colleagues suggested that VRT-532 may directly interact with the mutant protein to induce structural modifications which are both conducive to enhanced channel function and correct trafficking through the biosynthetic pathway (Wang et al., 2006). The purpose of the current study is to determine whether VRT-532 directly interacts with deltaF508-CFTR to alter its conformation and function. Toward this goal, we describe novel experimental approaches which enable us to study the structure-activity relationships for deltaF508-CFTR with its native primary sequence. These studies provide insight into the molecular lesion inherent in this mutant protein and the mechanisms underlying its repair.

Methods and Materials:

Materials:

Baby hamster kidney (BHK) cells stably expressing the major mutant deltaF508-CFTR possessing an exofacial triple HA tag on the 4th extracellular loop were obtained from Dr. G.L. Lukacs (Du et al., 2005). The mouse monoclonal antibodies used in Western blotting, M3A7 and anti-HA, were obtained from Millipore (Billerica, MA) and Covance (Princeton, NJ) respectively. TPCK-treated trypsin was purchased from Promega (Madison, WI) and soybean trypsin inhibitor was obtained through Sigma (St. Louis, MI).

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Iodide efflux assay- cell based: BHK cells stably expressing the major mutant deltaF508-CFTR were grown in DMEM/F12 (50/50) media supplemented with 5% fetal bovine serum and methotrexate [200 µg/mL] (Sigma) to a density of approximately 10^6 cells per mL. Cells were incubated at 27^{o}C for 24 hours prior to measurement in order to increase cell surface expression of deltaF508-CFTR. In control studies, untransfected BHK cells were transferred to 27^{o}C, 24 hours prior to measurement to be consistent with deltaF508-CFTR expressing cells. Cells were loaded with NaI loading buffer (3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM Glucose, 20 mM HEPES, 136 mM NaI) at 27^{o}C for 1 hour (Du et al., 2005). NaI loading buffer was aspirated and cells were subsequently washed twice with PBS followed by iodide free efflux buffer (3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM Glucose, 20 mM HEPES, 136 mM $NaNO_3$). Cells were scraped in 500 µL of iodide free efflux buffer and collected by centrifugation (350xg for 3 min at 25°C). Iodide free efflux buffer was removed and the cell pellet was resuspended in 250 µL of fresh iodide free efflux buffer. Iodide efflux was measured at room temperature using an iodide sensitive electrode (Lazar Research Laboratories, Los Angeles, California). DeltaF508-CFTR at the cell surface was stimulated with 10 µM forskolin followed by addition of VRT-532 (10 µM), genistein (50 µM) or vehicle (DMSO). We quantified the maximal rate of iodide efflux as the one minute interval associated with the largest positive slope during the 4-5 minute time period after addition of the potentiator.

Vesicle-based assay: Iodide efflux from membrane vesicles was measured using a previously described method (Pasyk et al., 2008). In brief, inside-out vesicles were formed from the crude membrane pellets of deltaF508-CFTR infected Sf9 cells in an iodide containing buffer (150 mM KI, 20 mM MOPS, pH 7.4). Iodide was removed from the extravesicular solution by subjecting

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

vesicle suspensions to Sephadex G-50 gel filtration columns saturated in an iodide-free external solution (150 mM Kglutamate, 20 mM Mops, pH 7.4). Valinomycin, a potassium selective ionophore, was added (20 μM) to shunt changes in potential difference generated by CFTR-mediated iodide conductance. DeltaF508-CFTR channels were activated with 1mM MgATP and 200 nM of the catalytic subunit of PKA, followed by the addition of 10μM VRT-532. All measurements of iodide efflux were made using an iodide-selective electrode as described above. Traces were recorded using the Digidata 1320A Data Acquisition System with Clampex 8 software (Axon Instruments Inc., Sunnyvale, California). The method for analyzing for maximal rates of efflux was similar to that described for the cell-based assay.

Purification and reconstitution of deltaF508-CFTR:

A frozen Sf9 cell pellet from one half liter of expression culture was thawed and resuspended in 50 mL PBS containing 2% Triton X-100, DNAse 1 (40 units /ml), 2 mM magnesium chloride, 1mM dithiothreitol, and protease inhibitors (Roche Diagnostics) while shaking at room temperature for 1 hour. The mixture was then centrifuged for 2h at 100,000***g*** at 4°C to yield a Triton insoluble pellet. This pellet was solubilized for 2-4 hrs in 8% pentadecafluorooctanoic acid (PFO), 25 mM phosphate pH 8.0. Procedures for purification, reconstitution, and phosphorylation of purified deltaF508-CFTR were similar to wildtype-CFTR-His as described elsewhere (Ramjeesingh et al., 1997; Ramjeesingh et al., 2008). Phosphorylated samples were pelleted using an airfuge, washed twice with 50 mM Tris-HCl, 50 mM NaCl, 2.5 mM $MgCl_2$, and 1 mM DTT at pH 7.5 and then dialyzed overnight against 4L of washing buffer.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

ATPase assay of purified deltaF508-CFTR protein:

ATPase activity was measured as the production of [γ^{32}] Pi from [γ^{32}P]ATP as described by Gross and colleagues (Gross et al., 2006). The ATP stock solution was prepared with or without the small molecule compound in 50 mM Tris-HCl, 50 mM NaCl, 2.5 mM $MgCl_2$ and 1 mM DTT at pH 7.5. The ATPase reaction was initiated by mixing the ATP stock solution with the reconstituted protein (0.2-0.5 mg/ml) and incubating the mixture for 2 hours at 37ºC. Reaction was quenched and Pi separated from ATP by thin layer chromatography performed using the same conditions as described by Gross et al. (Gross et al., 2006).

Limited proteolysis of deltaF508-CFTR in mammalian membranes:

Isolation of crude membranes: Crude membranes were isolated from BHK cells stably expressing deltaF508–CFTR containing a triple HA tag in the 4th extracellular loop as previously described (Aleksandrov et al., 2001) . Briefly, cells were homogenized in 10 mM HEPES, pH 7.2, 1 mM EDTA in the presence of protease inhibitors and unbroken cells were removed by centrifugation for 10 minutes at 500 *g*. Crude membranes were isolated from the resulting supernatant after a 1 hour spin at 100 000 *g*.

Acute treatment with VRT-532 and trypsin digestion of crude membranes:

DeltaF508-CFTR crude membranes (containing a total of 100 µg of protein) were re-suspended in re-suspension buffer (40 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 mM EGTA). A final concentration of 5 µM of VRT-532 in DMSO or an equivalent volume of DMSO was added to the membranes, sonicated, and incubated at 32°C for 20 minutes. Samples were cooled on ice

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

for 5 minutes and trypsin was added (at indicated concentrations of 5 to 50 µg/mL) (Du et al. 2005). Crude membranes were again sonicated, incubated at 4°C for 15 minutes and proteolysis terminated with trypsin soybean inhibitor. Membranes were solubilized in modified RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1 % (v/v) Triton-100) for 15 minutes and the soluble fraction was analyzed by SDS-PAGE on a 4-20% gradient gel (Cui et al., 2007) and Western blotted with M3A7 or anti-HA antibodies to identify peptide fragments containing NBD2 or the exofacial HA tag in MSD2 respectively.

Quantification of proteolysis experiments:

The mean pixel intensity of the full length or proteolytic fragments resulting from the tryptic digestion of deltaF508-CFTR was measured using ImageJ software (National Institutes of Health, USA), which allowed for verification that the signal intensity was within the linear range. Mean pixel intensity of full length deltaF508-CFTR after tryptic digestion was normalized to the intensity prior to treatment (0 µg/mL trypsin). The 60 kDa proteolytic fragment detected by both anti-HA and M3A7 antibodies was quantified as above and the signal intensity at two trypsin concentrations were compared. Specifically, the protease sensitivity of this fragment was determined as the ratio of the band intensity measured at 10 µg/mL trypsin versus the band intensity at 5 µg/mL trypsin. These ratios were determined for control (DMSO-treated) cells and VRT-532 treated cells, with the ratio in the presence of DMSO assigned a value of one and the ratio obtained in the presence of VRT-532 normalized relative to DMSO.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Statistics: Data are shown as mean ± SEM of "n" observations. Statistical analyses, paired or non-paired "t" tests as appropriate, were conducted using GraphPad Prism 4 software and "p" values of less than 0.05 were considered significant and indicated using an asterix (*).

Results:

The small molecule VRT-532 enhances channel activity of deltaF508-CFTR in intact cells and in membrane vesicles. An assay of cAMP-activated iodide efflux from iodide-loaded cells has been used routinely to monitor the channel function of CFTR within a population of mammalian cells (Schmidt et al., 2008; Seavilleklein et al., 2008). We modified this assay so that it can be used to monitor iodide efflux continuously, rather than at one minute intervals as in the original protocol. In figure 1A, the basic features of this modified iodide efflux assay have been shown using control, untransfected BHK cells. As expected, iodide loaded BHK cells in suspension did not mediate iodide efflux after the addition of forskolin (10 μM), an activator of cAMP production, as they lacked CFTR expression. Furthermore, after a transient downward deflection, a deviation commonly observed after addition of solutions to the bath, there was no response to the addition of VRT-532 (10 μM), consistent with previous reports that this molecule specifically modifies the activity of CFTR. A representative trace is shown in figure 1A and we determined that there was no positive change in slope for 4-5 minutes after VRT-532 addition over three studies (mean slope=-0.96 ± 0.58 nM iodide /sec.). We verified that iodide had indeed been trapped in these cells by the addition of Triton X-100. As expected, a large increase in iodide was detected in the bath following lysis, confirming that the cells had been loaded and

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

supporting the claim that untransfected cells lack an endogenous response to cAMP and to VRT-532. We then evaluated the utility of this assay to monitor the efficacy of deltaF508-CFTR potentiation using the well known potentiator, genistein, as a positive control (Hwang et al., 1997). In these studies, BHK cells stably expressing deltaF508-CFTR were incubated at 27°C for 24 hours to partially correct the deltaF508 trafficking defect and were treated with forskolin to activate the surface expressed protein (as described in the methods). The addition of genistein (50 μM), but not the vehicle (DMSO) caused a significant increase in the rate of iodide efflux (figure 1B). We quantified the maximal rate of iodide efflux as the one minute interval associated with the largest positive slope during the 4-5 minute time period after addition of the potentiator. The mean±SEM of three studies is shown in the bar graph in figure 1B. The difference between the DMSO and genistein treatment groups was statistically significant ($p=0.01$).

As shown in figures 1C, addition of VRT-532 (10 μM) also caused potentiation of temperature-rescued and forskolin-pretreated deltaF508-CFTR channel activity. The maximal efflux rate after VRT-532 addition was significantly greater than the vehicle (DMSO) control (figure 1D, $p=0.02$). This latter finding suggests that VRT-532 binds directly to the mutant protein at the cell surface to act upon its gating mechanism. Alternatively, VRT-532 could be acting to promote channel activity of deltaF508-CFTR by binding to cellular proteins which regulate CFTR function and/ or endocytosis and recycling.

We developed a vesicle based assay in order to study the effect of VRT-532 on deltaF508-CFTR function in a system devoid of most cellular regulatory proteins (Guggino and Stanton, 2006;

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Pasyk et al., 2008). DeltaF508–CFTR was expressed in Sf9 insect cells in order to ensure a relatively high membrane expression of this mutant. Inside-out membrane vesicles were loaded with an iodide (KI) containing buffer and placed into an iodide-free solution (K glutamate) to impose an outward gradient for iodide. Valinomycin (20 μM) was added to ensure that iodide flux was not limited by permeability of the counter ion. As shown in figure 2A, the addition of the catalytic subunit of PKA (200 nM) along with MgATP (1 mM) caused no change in the rate of vesicular iodide efflux, consistent with a low level of functional expression of this mutant. The addition of VRT-532 (10 μM) caused a significant increase in the maximal rate of efflux relative to the effect of vehicle (DMSO) alone ($p=0.03$, figure 2B). As in the studies shown in figure 1, this maximal efflux rate was determined as the greatest slope after addition of the potentiator compound during the 4-5 minute evaluation period. The VRT-532 mediated activation is only observed in membrane vesicles expressing deltaF508-CFTR and not in membranes lacking CFTR. There was no increase in the rate of iodide efflux after addition of VRT-532 to membrane vesicles lacking CFTR (-0.1±0.5 nM/sec, n=3). These findings are consistent with the hypothesis that this small molecule interacts directly with the mutant protein to modify its conformation.

The small molecule- VRT-532 directly modifies the ATPase activity of reconstituted deltaF508-CFTR.

The flux-based assays described above support the electrophysiological studies by Van Goor et al. which showed that VRT-532 potentiates the channel activity of deltaF508-CFTR (Van Goor et al., 2006). Single channel experiments in the above study suggested that potentiation occurred

due to an increase in the mean channel open burst duration. These authors proposed that this effect may be mediated via inhibition of the intrinsic ATPase activity of deltaF508-CFTR, an activity which is associated with channel closure. Therefore, we were prompted to test this hypothesis directly.

The intrinsic ATPase activity of wildtype CFTR exhibits a very low Vmax (3-5 nmole/µg/hr), comparable to the low rate of GTPases (Ramjeesingh et al., 2008). This low activity necessitates purification of the CFTR protein away from other cellular ATPases (Li et al., 1996; Ramjeesingh et al., 2008). Therefore, a similar strategy was employed to study the intrinsic ATPase activity by deltaF508-CFTR. As in the case of the wildtype protein, deltaF508-CFTR bearing a carboxy terminal polyhistidine tag was expressed in Sf9 cells to ensure high levels of expression. Unlike the purification procedure for wildtype CFTR protein, wherein CFTR was detergent extracted from microsomal membrane preparations, deltaF508-CFTR was extracted from the entire cell lysate as its expression level is very low. Solubilized deltaF508-CFTR-His was bound to Ni-NTA and specifically eluted using a pH gradient. As shown in Figure 3A, deltaF508-CFTR protein was significantly enriched and could be visualized as the dominant silver stained band migrating as expected for the 140-150 kD protein in SDS-PAGE. As expected, this broad band was immunoreactive with CFTR specific antibodies (including M3A7, Figure 3A). The mutant protein can be purified to approximately 80% of the total silver stained protein as quantitated using NIH-Imaging. To date, efforts to further purify deltaF508-CFTR resulted in very low levels of protein, too low for subsequent analysis of ATPase activity.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

The ATPase activity of this partially purified deltaF508-CFTR protein was measured following its reconstitution in phospholipid liposomes and phosphorylation by PKA as described in our previous work with wildtype CFTR (Ramjeesingh et al., 2008). The production of P^{32} labelled Pi from P^{32} labelled ATP by reconstituted deltaF508-CFTR (or phospholiposomes lacking reconstituted protein as a control) was quantitated by phosphoimaging following separation of the product from its substrate by thin layer chromatography. As shown in figure 3B, the reconstituted deltaF508-CFTR conferred ATPase activity (ΔF), apparent as the enhanced production of inorganic phosphate from ATP relative to phospholiposomes lacking protein (-).

In order to confirm that the ATPase activity associated with partially purified and reconstituted deltaF508-CFTR is conferred by the mutant protein directly and not by a contaminant possibly co-purifying with it, we assessed its ATPase activity after pre-treatment of a specific CFTR inhibitor (CFTRinh-172) (Ma et al., 2002; Taddei et al., 2004). As shown in figure 3C, we found that the ATPase activity of reconstituted deltaF508-CFTR was inhibited by CFTRinh-172 in a dose-dependent manner, with an IC_{50} of approximately 2.6 μM, close to that observed for its inhibition of Wild type-CFTR channel activity (figure 3C). The extent of this inhibition was statistically significant at 2.5 μM (n=3, p=0.001), supporting our claim that ATPase activity is directly mediated by reconstituted deltaF508-CFTR.

The ATP dependence of deltaF508-CFTR mediated ATPase activity can be described by Michaelis-Menten kinetics, with an apparent affinity or K_m for ATP of 0.2 mM and a V_{max} of 0.3 nmole/μg/hr (Figure 3D). Although the estimate of Km (ATP) for the mutant protein is somewhat lower, the value is still within range of the wildtype CFTR protein (0.7 mM ATP)

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

(Ramjeesingh et al., 2008). Unfortunately, a direct comparison of the Vmax determined for the ATPase activity of deltaF508 and wild type CFTR proteins was not possible as the two proteins were purified differently and the yield of functionally reconstituted protein may be different. However, the present findings suggest that deltaF508-CFTR is capable of binding and hydrolyzing ATP.

Potentiation of deltaF508-CFTR chloride channel activity by VRT-532 in intact cells and in isolated membrane vesicles prompted us to study the effect of this small molecule on the ATPase activity of reconstituted deltaF508-CFTR. We found that treatment with 10 μM VRT-532, the same concentration effective in modulating its channel function, caused a significant decrease in its ATPase activity (Figure 4A). Furthermore, VRT-532 treatment also reduced the ATP dependence of this activity as observed through a three-fold decrease in Vmax (from 0.30 to 0.12 nmole/μg/hr), but only had a minor effect on the Km for ATP (from 0.18 to 0.22 mM ATP) (Figure 4A and B). Inhibition of ATPase activity was statistically significant and these findings are consistent with a model wherein VRT-532 binds directly to the mutant protein at a position distinct from the ATP catalytic site.

VRT-532 binding induces a change in conformational stability of the carboxy terminal half of deltaF508-CFTR.

Susceptibility of the deltaF508-CFTR protein in mammalian cell membranes to limited trypsin digestion has been used to monitor conformational changes induced by low temperature rescue

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

(Denning et al., 1992; Sharma et al., 2004). The carboxy terminus of deltaF508-CFTR was found to be "hyper-sensitive" to proteolytic digestion and low temperature rescue led to partial protection of this region. Our findings that VRT-532 treatment inhibited the ATPase activity of reconstituted deltaF508-CFTR is consistent with its possible role in promoting a tight ATP bound NBD heterodimer (Vergani et al., 2005b). Therefore, we were prompted to test whether an acute treatment of deltaF508-CFTR with the compound would alter its trypsin susceptibility.

Microsomal membranes prepared from BHK cells stably-expressing deltaF508-CFTR containing an exofacial HA tag in MSD2 were treated acutely with VRT-532 (5 μM for 20 minutes) and subjected to limited proteolysis in the presence of varying concentrations of trypsin. The full length and trypsin digested fragments were analyzed by SDS-PAGE and probed using domain-specific antibodies. Initially, we employed the monoclonal antibody M3A7 (which recognizes residues 1370-1380 in NBD2) as this antibody has been effective at revealing changes in protease susceptibility of the carboxy terminus of deltaF508-CFTR in previous studies (Du et al., 2005). In the presence of 5 μg/mL trypsin two dominant bands are detected, one corresponding to the full length protein and the other to the major proteolytic product which migrates as a ~60 kDa protein (indicated by an arrow and bracket respectively in Figure 5A). An increase in the relative abundance of full length (FL) protein at 5 μg/mL trypsin relative to its abundance prior to trypsin addition (0 μg/mL) was measured upon VRT-532 pre-treatment. This protective effect of VRT-532 on the integrity of the full length protein was small but significant as determined in three different preparations of BHK membranes (Figures 5A and 5B). VRT-532 treatment also induced protease resistance in the major fragment (the ~60 kDa band). The abundance of this 60 kD fragment at 10 μg/mL trypsin relative to its abundance at 5

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

μg/mL trypsin was significantly greater following VRT-532 treatment than in DMSO treated controls (Figure 5C).

The size of the 60 kDa fragment is consistent with it encompassing both NBD2 and MSD2. In order to test if this fragment contained both regions, we probed the proteolytic fragments using an anti-HA antibody to target the triple HA epitope in the fourth extracellular loop of MSD2 (Figure 6). Indeed, a dominant 60 kDa fragment could also be detected in immunoblots of trypsin digests using this antibody (Figure 6A). As in the case of the M3A7 immunoblots, the abundance of this fragment at 10 μg/mL trypsin relative to its abundance at 5 μg/mL (detected using the anti-HA antibody) was significantly greater in membranes pretreated with VRT-532 (Figures 6A and 6B). Together, the reduced susceptibility of MSDS and NBD2 to proteolytic digestion suggests the carboxy terminal half of deltaF508-CFTR undergoes a structural change or alternatively, a more compact conformation is stabilized, upon binding of VRT-532.

Discussion:

Studies of the ATPase activity of purified and reconstituted deltaF508-CFTR provide insight into the molecular defect caused by this mutation. There have been several informative studies on the molecular lesion inherent in the major CFTR mutant, deltaF508-CFTR. Protease susceptibility of the deltaF508-CFTR protein (with its native sequence) was enhanced relative to the wildtype protein indicating that it exhibits a “hyper protease-sensitive” conformation (Zhang et al., 1998). These authors suggested that deletion of F508 in NBD1

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

could lead to the perturbation of native domain:domain interactions in CFTR, thus accounting for the increased susceptibility to proteases observed for this mutant. This hypothesis was tested in chemical cross-linking studies employing "cys-less" wild type and deltaF508-CFTR proteins wherein non-native cysteines were strategically introduced at putative domain interfaces (Loo et al., 2008; Serohijos et al., 2008). The results of chemical crosslinking studies in the modified "wild type" CFTR supported the molecular models for CFTR based on the crystal structure of the prokaryotic ABC protein, Sav1866 (Serohijos et al., 2008; Mornon et al., 2008). Sav1866 exhibits an interesting "cross-over" pattern of domain:domain interactions wherein the NBD in one half of the molecule interacts primarily with the membrane spanning domain of the other half (Dawson and Locher, 2006). Interestingly, the research groups of Clarke and Riordan showed that deletion of F508 impairs the chemical cross-linking which normally occurs between a non-native cysteine residue incorporated into NBD1, in the proximity of F508 (V510C), and a non-native cysteine introduced into the fourth intracellular loop (ICL4, A1067C) extending from MSD2 (Loo et al., 2008; Serohijos et al., 2008). Clarke and co-workers also found that deletion of F508 disrupted chemical crosslinking which normally occurred between non-native cysteines of MSD1 (A274C) and NBD2 (Loo et al., 2008) and crosslinking between non-native cysteines introduced into transmembrane helix 6 (MSD1) and 7 or 12 (MSD2) (Wang et al., 2007). Together, these findings support the hypotheses that the deltaF508 mutation in NBD1 disrupts multiple domain:domain interactions within CFTR. The current study provides novel insight into the molecular defect conferred by the deltaF508 mutation in the context of the native CFTR sequence.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Evaluation of partially purified and reconstituted deltaF508-CFTR revealed that it retained measurable levels of ATPase activity, with a saturable ATP dependence that could be fitted by the Michaelis Menten equation. The Vmax determined for the ATPase activity of the mutant protein could not be compared to that of the wildtype CFTR, as these proteins were obtained using different purification protocols. The apparent affinity (Km) for ATP binding to the catalytic site is independent of the number of functionally reconstituted protein molecules and thus this parameter can be directly compared between the two genotypes. The apparent Km (ATP) for deltaF508-CFTR is approximately 0.2 mM which is less than that of the Wt protein (~ 0.7 mM) (Ramjeesingh et al., 2008). This difference could reflect an increase in ATP affinity and/or a decrease in the rate of ATP hydrolysis upon deletion of F508. A more detailed understanding of the consequences of the deltaF508-CFTR mutation on ATP hydrolysis will require accurate quantitation of the number of functionally reconstituted molecules, a major goal for our future work. However, the present biochemical findings support the hypothesis that the chloride channel gating defect described for deltaF508-CFTR (Roxo-Rosa et al., 2006) may not be due to a defect in ATP binding *per se*, but rather to a defect in the unknown conformational changes that occur subsequent to ATP binding and lead to the opening of the channel.

Molecular mechanism of VRT-532 action revealed in studies of the functional and structural consequences of binding.

As shown in figure 1 and 2, VRT-532 is effective in potentiating the channel activity of deltaF508-CFTR after temperature rescue in intact BHK cells overexpressing the protein, as well as in membrane vesicles prepared from Sf9 cells (an insect cell expression system). The present

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

experiments performed on membrane vesicles confirm previous electrophysiological studies showing that this small molecule potentiates the channel function of deltaF508-CFTR (Van Goor et al., 2006). Van Goor et al. suggested that this potentiation effect was likely mediated by inhibition of the intrinsic ATPase activity of the mutant protein, an activity associated with channel closure. However, until the current study, it was not known whether deltaF508-CFTR retained ATPase activity and if this activity was modulated by potentiators. The effect of VRT-532 on the ATPase activity of partially purified deltaF508-CFTR provides the most compelling evidence for direct binding to the protein and elucidates the mechanism underlying its potentiating effect on channel activity. VRT-532 specifically inhibited the intrinsic ATPase activity of deltaF508-CFTR not by preventing ATP binding to the NBD domains, but by reducing ATP turnover. As mentioned previously, ATP binding to the catalytic site at the NBD1:NBD2 interface is thought to be permissive to opening of the channel gate, whereas ATP hydrolysis by CFTR permits the gate to return to its closed state (Aleksandrov et al., 2007; Gadsby et al., 2006). Therefore, the inhibitory effect of VRT-532 binding on ATPase activity would be predicted to prevent closing of the channel by stabilizing the opened/ATP bound state. The molecular basis for binding of VRT-532 to deltaF508-CFTR has yet to be determined, but our ATPase studies suggest it is likely not at the nucleotide binding sites formed from the NBD1: NBD2 heterodimer as there was little change in the apparent ATP affinity (Km). Rather, it is possible that the binding site lies at an interface region between the NBDs and the MSDs, a region which is well positioned to modulate both the catalytic domains as well as the open gate structure in the membrane.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Molecular mechanism of VRT-532 action shares features with mechanisms proposed for other small molecule potentiators of deltaF508-CFTR. Analyses of the single channel activity of wild type and deltaF508-CFTR have been instrumental in providing insight into the possible mechanism of action of such well known small molecule potentiators as genistein and capsaicin (Ai et al., 2004). As in the case of VRT-532, both of these potentiators act to enhance the open time of deltaF508-CFTR single channels following pre-phosphorylation by protein kinase A. Ai and colleagues suggested that potentiators may act to stabilize the NBD1:NBD2 heterodimer, possibly by preventing ATP hydrolysis. The measurements of reduced ATP hydrolysis by deltaF508-CFTR following incubation with VRT-532 provides direct evidence to support this hypothesis.

The site at which potentiators (including VRT-532) bind to deltaF508-CFTR remains to be determined. The current findings suggest that it is unlikely that VRT-532 binds at the catalytic site as it does not inhibit the apparent affinity for ATP in this reaction. In agreement with this conclusion, Cai and Sheppard proposed that phloxine B, another potentiator which increases the channel open time and increases the affinity for ATP in ATP -dependent channel gating, interacted with the protein at a site which is distinct from the catalytic site (Cai and Sheppard, 2002). Molecular modelling and mutagenesis studies by Zegarra-Moran and colleagues also support a model wherein genistein and other potentiators act to stabilize the NBD1:NBD2 dimer interface by binding to a site which is proximal to, but distinct from, the primary catalytic site (Moran et al., 2005; Zegarra-Moran et al., 2007). Together, the biochemical assays described in the present study, previous single channel analyses and molecular modeling studies support a

common model wherein small molecule binding at a site distinct from the catalytic site, promotes enhanced stability of the NBD1:NBD2 heterodimer.

Finally, our studies of the protease susceptibility of deltaF508-CFTR expressed in mammalian membranes also suggest that binding of VRT-532 modifies intra-molecular interactions within this multi-domain protein. We showed that VRT-532 binding caused a structural change or stabilization of a particular conformation of deltaF508-CFTR in membrane vesicles. This effect was detected as a decrease in trypsin susceptibility of both the full length protein and a large fragment encompassing both NBD2 and MSD2. These findings could indicate that VRT-532 binds to either NBD2, MSD2, or to a site where these domains interact. However, it is also possible that the compound binds at a distinct location leading to the promotion of a more compact deltaF508-CFTR protein and protection of the second half the protein from trypsin digestion. These results are consistent with those of Zhang et al. who initially reported that "temperature-rescue" promotes a structural change associated with partial rescue of the protease susceptibility of the carboxy terminus of deltaF508-CFTR (Zhang et al., 1998).

In summary, the present study has demonstrated a direct interaction of a small molecule modulator, VRT-532, with the full length deltaF508-CFTR protein bearing its native sequence, with binding leading to direct modification of its structure. Furthermore, the development of methods for the partial purification and characterization of full length deltaF505-CFTR permits future detailed investigations of the binding site for VRT-532. Such studies will enable an improved understanding of the structural basis for the mistrafficking of deltaF508-CFTR as well as future structure-based therapy design.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements: VRT-532 was kindly supplied by Prof. Robert Bridges (Rosalind Franklin University), as a component of the CFTR Modulator Library. Pellets of Sf9 cells expressing deltaF508-CFTR protein were provided as a service through the Baculovirus/Mab Core of the Proteomics Shared Resource at Baylor College of Medicine, USA.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

References:

Ai T, Bompadre SG, Wang X, Hu S, Li M and Hwang TC (2004) Capsaicin potentiates wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride-channel currents. *Mol Pharmacol* **65**:1415-1426.

Aleksandrov AA, Aleksandrov LA and Riordan JR (2007) CFTR (ABCC7) is a hydrolyzable-ligand-gated channel. *Pflugers Arch* **453**:693-702.

Aleksandrov L, Mengos A, Chang X, Aleksandrov A and Riordan JR (2001) Differential interactions of nucleotides at the two nucleotide binding domains of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* **276**:12918-12923.

Baker JM, Hudson RP, Kanelis V, Choy WY, Thibodeau PH, Thomas PJ and Forman-Kay JD (2007) CFTR regulatory region interacts with NBD1 predominantly via multiple transient helices. *Nat Struct Mol Biol* **14**:738-745.

Bear CE, Li CH, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M and Riordan JR (1992) Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell* **68**:809-818.

Cai Z and Sheppard DN (2002) Phloxine B interacts with the cystic fibrosis transmembrane conductance regulator at multiple sites to modulate channel activity. *J Biol Chem* **277**:19546-19553.

Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR and Smith AE (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* **63**:827-834.

Cui L, Aleksandrov L, Chang X, Hou Y, He L, Hegedus T, Gentzsch M, Aleksandrov A, Balch WE and Riordan JR (2007) Domain interdependence in the biosynthetic assembly of CFTR. *J Mol Biol* **365**:981-994.

Csanady L, Seto-Young D, Chan KW, Cenciarelli C, Angel BB, Qin J, McLachlin DT, Krutchinsky AN, Chait BT, Nairn AC and Gadsby DC (2005) Preferential phosphorylation of R-domain Serine 768 dampens activation of CFTR channels by PKA. *J Gen Physiol* **125**:171-186.

Dawson RJ and Locher KP (2006) Structure of a bacterial multidrug ABC transporter. *Nature* **443**:180-185.

Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE and Welsh MJ (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature* **358**:761-764.

Du K, Sharma M and Lukacs GL (2005) The DeltaF508 cystic fibrosis mutation impairs domain-domain interactions and arrests post-translational folding of CFTR. *Nat Struct Mol Biol* **12**:17-25.

Gadsby DC, Vergani P and Csanady L (2006) The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature* **440**:477-483.

Gross CH, Abdul-Manan N, Fulghum J, Lippke J, Liu X, Prabhakar P, Brennan D, Willis MS, Faerman C, Connelly P, Raybuck S and Moore J (2006) Nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator, an ABC transporter, catalyze adenylate kinase activity but not ATP hydrolysis. *J Biol Chem* **281**:4058-4068.

Guggino WB and Stanton BA (2006) New insights into cystic fibrosis: molecular switches that regulate CFTR. *Nat Rev Mol Cell Biol* **7**:426-36.

Hwang TC, Wang F, Yang IC and Reenstra WW (1997) Genistein potentiates wild-type and delta F508-CFTR channel activity. *Am J Physiol* **273**:C988-C998.

Lewis HA, Buchanan SG, Burley SK, Conners K, Dickey M, Dorwart M, Fowler R, Gao X, Guggino WB, Hendrickson WA, Hunt JF, Kearins MC, Lorimer D, Maloney PC, Post KW, Rajashankar KR, Rutter ME, Sauder JM, Shriver S, Thibodeau PH, Thomas PJ, Zhang M, Zhao X and Emtage S (2004) Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. *Embo J* **23**:282-293.

Lewis HA, Zhao X, Wang C, Sauder JM, Rooney I, Noland BW, Lorimer D, Kearins MC, Conners K, Condon B, Maloney PC, Guggino WB, Hunt JF and Emtage S (2005) Impact of the deltaF508 mutation in first nucleotide-binding domain of human cystic fibrosis transmembrane conductance regulator on domain folding and structure. *J Biol Chem* **280**:1346-1353.

Li C, Ramjeesingh M, Wang W, Garami E, Hewryk M, Lee D, Rommens JM, Galley K and Bear CE (1996) ATPase activity of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* **271**:28463-28468.

Linsdell P (2006) Mechanism of chloride permeation in the cystic fibrosis transmembrane conductance regulator chloride channel. *Exp Physiol* **91**:123-129.

Loo TW, Bartlett MC and Clarke DM (2008) Processing mutations disrupt interactions between the nucleotide binding and transmembrane domains of P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR). *J Biol Chem* **283**:28190-28197.

Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galietta LJ and Verkman AS (2002) Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest* **110**:1651-8.

Mense M, Vergani P, White DM, Altberg G, Nairn AC and Gadsby DC (2006) In vivo phosphorylation of CFTR promotes formation of a nucleotide-binding domain heterodimer. *Embo J* **25**:4728-4739.

Moran O, Galietta LJ and Zegarra-Moran O (2005) Binding site of activators of the cystic fibrosis transmembrane conductance regulator in the nucleotide binding domains. *Cell Mol Life Sci* **62**:446-460.

Mornon JP, Lehn P and Callebaut I (2008) Atomic model of human cystic fibrosis transmembrane conductance regulator: membrane-spanning domains and coupling interfaces. *Cell Mol Life Sci* **65**:2594-2612.

Pasyk S, Li C, Ramjeesingh M and Bear CE (2008) Direct interaction of a small molecule modulator with G551D-CFTR, a cystic fibrosis causing mutant associated with severe disease. *Biochem J* **418**:185-190.

Pedemonte N, Lukacs GL, Du K, Caci E, Zegarra-Moran O, Galietta LJ and Verkman AS (2005) Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. *J Clin Invest* **115**:2564-2571.

Ramjeesingh M, Li C, Garami E, Huan LJ, Hewryk M, Wang Y, Galley K and Bear CE (1997) A novel procedure for the efficient purification of the cystic fibrosis transmembrane conductance regulator (CFTR). *Biochem J* **327 (Pt 1)**:17-21.

Ramjeesingh M, Ugwu F, Stratford FL, Huan LJ, Li C and Bear CE (2008) The intact CFTR protein mediates ATPase rather than adenylate kinase activity. *Biochem J* **412**:315-321.

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL and et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**:1066-1073.

Roxo-Rosa M, Xu Z, Schmidt A, Neto M, Cai Z, Soares CM, Sheppard DN and Amaral MD (2006) Revertant mutants G550E and 4RK rescue cystic fibrosis mutants in the first

nucleotide-binding domain of CFTR by different mechanisms. *Proc Natl Acad Sci U S A* **103**:17891-7896.

Schmidt A, Hughes LK, Cai Z, Mendes F, Li H, Sheppard DN and Amaral MD (2008) Prolonged treatment of cells with genistein modulates the expression and function of the cystic fibrosis transmembrane conductance regulator. *Br J Pharmacol* **153**:1311-1123.

Seavilleklein G, Amer N, Evagelidis A, Chappe F, Irvine T, Hanrahan JW and Chappe V (2008) PKC phosphorylation modulates PKA-dependent binding of the R domain to other domains of CFTR. *Am J Physiol Cell Physiol* **295**:C1366-C1375.

Seibert FS, Chang XB, Aleksandrov AA, Clarke DM, Hanrahan JW and Riordan JR (1999) Influence of phosphorylation by protein kinase A on CFTR at the cell surface and endoplasmic reticulum. *Biochim Biophys Acta* **1461**:275-283.

Serohijos AW, Hegedus T, Aleksandrov AA, He L, Cui L, Dokholyan NV and Riordan JR (2008) Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function. *Proc Natl Acad Sci U S A* **105**:3256-3261.

Sharma M, Pampinella F, Nemes C, Benharouga M, So J, Du K, Bache KG, Papsin B, Zerangue N, Stenmark H and Lukacs GL (2004) Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J Cell Biol* **164**:923-933.

Taddei A, Folli C, Zegarra-Moran O, Fanen P, Verkman AS and Galietta LJ (2004) Altered channel gating mechanism for CFTR inhibition by a high-affinity thiazolidinone blocker. *FEBS Lett* **558**:52-56.

Van Goor F, Straley KS, Cao D, Gonzalez J, Hadida S, Hazlewood A, Joubran J, Knapp T, Makings LR, Miller M, Neuberger T, Olson E, Panchenko V, Rader J, Singh A, Stack JH, Tung R, Grootenhuis PD and Negulescu P (2006) Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am J Physiol Lung Cell Mol Physiol* **290**:L1117-L1130.

Vergani P, Basso C, Mense M, Nairn AC and Gadsby DC (2005a) Control of the CFTR channel's gates. *Biochem Soc Trans* **33**:1003-1007.

Vergani P, Lockless SW, Nairn AC and Gadsby DC (2005b) CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. *Nature* **433**:876-880.

Wang F, Zeltwanger S, Hu S and Hwang TC (2000) Deletion of phenylalanine 508 causes attenuated phosphorylation-dependent activation of CFTR chloride channels. *J Physiol* **524 Pt 3**:637-648.

Wang Y, Bartlett MC, Loo TW and Clarke DM (2006) Specific rescue of cystic fibrosis transmembrane conductance regulator processing mutants using pharmacological chaperones. *Mol Pharmacol* **70**:297-302.

Wang Y, Loo TW, Bartlett MC and Clarke DM (2007) Correctors promote maturation of cystic fibrosis transmembrane conductance regulator (CFTR)-processing mutants by binding to the protein. *J Biol Chem* **282**:33247-33251.

Winter MC and Welsh MJ (1997) Stimulation of CFTR activity by its phosphorylated R domain. *Nature* **389**:294-296.

Zegarra-Moran O, Monteverde M, Galietta LJ and Moran O (2007) Functional analysis of mutations in the putative binding site for cystic fibrosis transmembrane conductance regulator potentiators. Interaction between activation and inhibition. *J Biol Chem* **282**:9098-9104.

Zhang F, Kartner N and Lukacs GL (1998) Limited proteolysis as a probe for arrested conformational maturation of delta F508 CFTR. *Nat Struct Biol* **5**:180-183.

Footnotes:

These studies were funded by operating grants awarded to Christine Bear by the Canadian Cystic Fibrosis Foundation and Canadian Institute of Health Research (*BREATHE* PROGRAMME 1) and the Cystic Fibrosis Foundation Therapeutics Foundation: #BEAR06DDS0. Leigh Wellhauser was supported by an NSERC Postgraduate Doctoral Award and Patrick Kim Chiaw was supported by a Doctoral Award provided by the Canadian Institutes of Health Research Strategic Training Programme in the Structure of Membrane Proteins and Disease.

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures:

Figure 1: VRT-532 stimulates channel function of deltaF508-CFTR in cells. A) Representative trace showing that untransfected BHK cells, loaded with iodide, did not mediate iodide efflux in response to VRT-532 (10μM). Cells were lysed with TritonX-100 (1% (v/v) resulting in an increase in iodide release, confirming that the cells were effectively loaded. (B) Iodide efflux was stimulated by addition of genistein (50 μM) to a suspension of BHK cells stably expressing deltaF508-CFTR, that had been subjected to temperature rescue and pre-treated with forskolin (10 μM). Bar graph shows mean±SEM of maximal rates of iodide efflux (nM/sec) for genistein treated cell suspensions (n=3) and vehicle (DMSO) treated cell suspensions (n=3). These groups are significantly different (*, $p<0.05$). (C) Tracing shows iodide efflux stimulated by addition of VRT-532 (10 μM) from BHK cells expressing deltaF508-CFTR, after temperature rescue and pre-treated with forskolin (10 μM). (D) Bar graph shows maximal efflux (mean±SEM) after the addition of VRT-532 (n=6), was greater than maximal efflux after DMSO (1.3 ± 0.4 nM/sec, n=4) (p=0.02, *). Baseline efflux rates, attributed to leak and electrode drift, are subtracted from final efflux values.

Figure 2: VRT-532 stimulates channel function of deltaF508-CFTR in inside out membrane vesicles. A. Representative tracing of iodide efflux from inside out membrane vesicles expressing deltaF508-CFTR, stimulated first by addition 1mM MgATP and 200nM PKA, followed by 10μM VRT 532. **B.** The bar graph shows mean ± SEM of maximal efflux rates from Sf9 membrane vesicles expressing deltaF508-CFTR and treated with DMSO or VRT-532 (10 μM). The maximal efflux rates are significantly different between this pair (p=0.03, *).

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

Baseline efflux rates, attributed to leak and electrode drift, are subtracted from final efflux values.

Figure 3: Partially purified and reconstituted deltaF508-CFTR exhibits CFTRinh-172 sensitive ATPase activity. (**A)** Silver stained (SS) gel and immunoblot (IB) of partially purified deltaF508-CFTR which migrates as a diffuse 140 kD protein indicated by a bracket. This band corresponds to the core glycosylated form of the protein. (**B**) ATPase activity of partially purified, reconstituted and phosphorylated deltaF508-CFTR measured as the production of radioactive Pi from radioactive ATP. Representative TLC showed significant inorganic phosphate production with proteoliposomes (ΔF) over liposomes without protein (-). (**C**) The ATPase activity of deltaF508-CFTR is inhibited by the CFTR-specific inhibitor inh-172 (CFTRinh-172) in a dose dependent manner. Activity was measured in the presence of 1 mM MgATP. Curve was fitted with an exponential decay function ($r^2 = 0.88$) yielding an IC_{50} of 2.6 μM. Bar graph shows significant inhibition at 2.5μM of inh-172 relative to control (C) (n=3 protein preparations, p=0.001). (**D**) ATP dose-dependence of the ATPase activity; Michaelis-Menton analysis gave an apparent affinity, Km (ATP) of 0.176 mM ($r^2 = 0.95$, each point represents the mean of duplicate measurements using two different protein preparations).

Figure 4: VRT-532 directly modifies ATPase activity of deltaF508-CFTR. Activity of reconstituted deltaF508-CFTR was measured in the presence or absence of 10 μM of the drug. **(A)** ATP dose-dependence curves are fitted using Michaelis-Menten equation (+VRT-532, Km (MgATP) = 0.22 mM, Vmax= 0.12 nmoles/μg/hr, $r^2 = 0.9$ versus control (no drug), Km (MgATP)=0.18 mM, Vmax=0.30 nmoles/μg/hr, $r^2 = 0.95$, n=2 different protein preparations).

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

(B) Bar graph show the relative ATPase activity of reconstituted deltaF508 after addition of 10 µM VRT-532 (n=3 protein preparations, p=0.001).

Figure 5: VRT-532 binding induces conformational change in the carboxy terminal half of deltaF508-CFTR. (A) Western blots of trypsin digested crude membranes expressing deltaF508 CFTR using the M3A7 antibody against NBD2 after a pre-treatment with vehicle (DMSO) or 5 µM VRT-532. Arrow indicates position of full length mutant protein, Band B (core glycosylated form). **(B)** The bars in B show significant protection of full length (FL) mutant protein at 5 µg/mL trypsin (expressed as a ratio relative to undigested protein at 0 µg/mL trypsin) (n=3, p=0.02, *). The intensity of the bands corresponding to full length CFTR protein was quantified using ImageJ software. **(C)** A proteolytic fragment (~ 60kDa band indicated by a bracket), exhibited an increase in resistance to digestion (assessed as the ratio of band intensity at 10 relative to 5 µg/mL trypsin) following VRT-532 treatment vs. vehicle (DMSO) treatment (n=3, p=0.02,*). The ratio in the presence of DMSO assigned a value of one and the ratio obtained in the presence of VRT-532 normalized relative to DMSO. The intensity of the bands corresponding to this fragment was quantified using ImageJ software as above.

Figure 6: VRT-532 binding induces conformational change in the carboxy terminal half of deltaF508-CFTR. (A) A proteolytic band (~ 60kDa and bracketed), is reactive to an anti-HA antibody which recognizes an epitope engineered into extracellular loop 4. This band was more resistant to trypsin digestion (ratio of abundance measured using ImageJ software at 10 µg/mL relative to 5 µg/mL trypsin) following VRT-532 treatment vs. DMSO treatment. **(B)** This

Molecular Pharmacology Fast Forward. Published on April 1, 2009 as DOI: 10.1124/mol.109.055608
This article has not been copyedited and formatted. The final version may differ from this version.

protective effect was observed for VRT-532 in three trials (p=0.032, *). The arrow indicates the migration of the full length protein, band B.

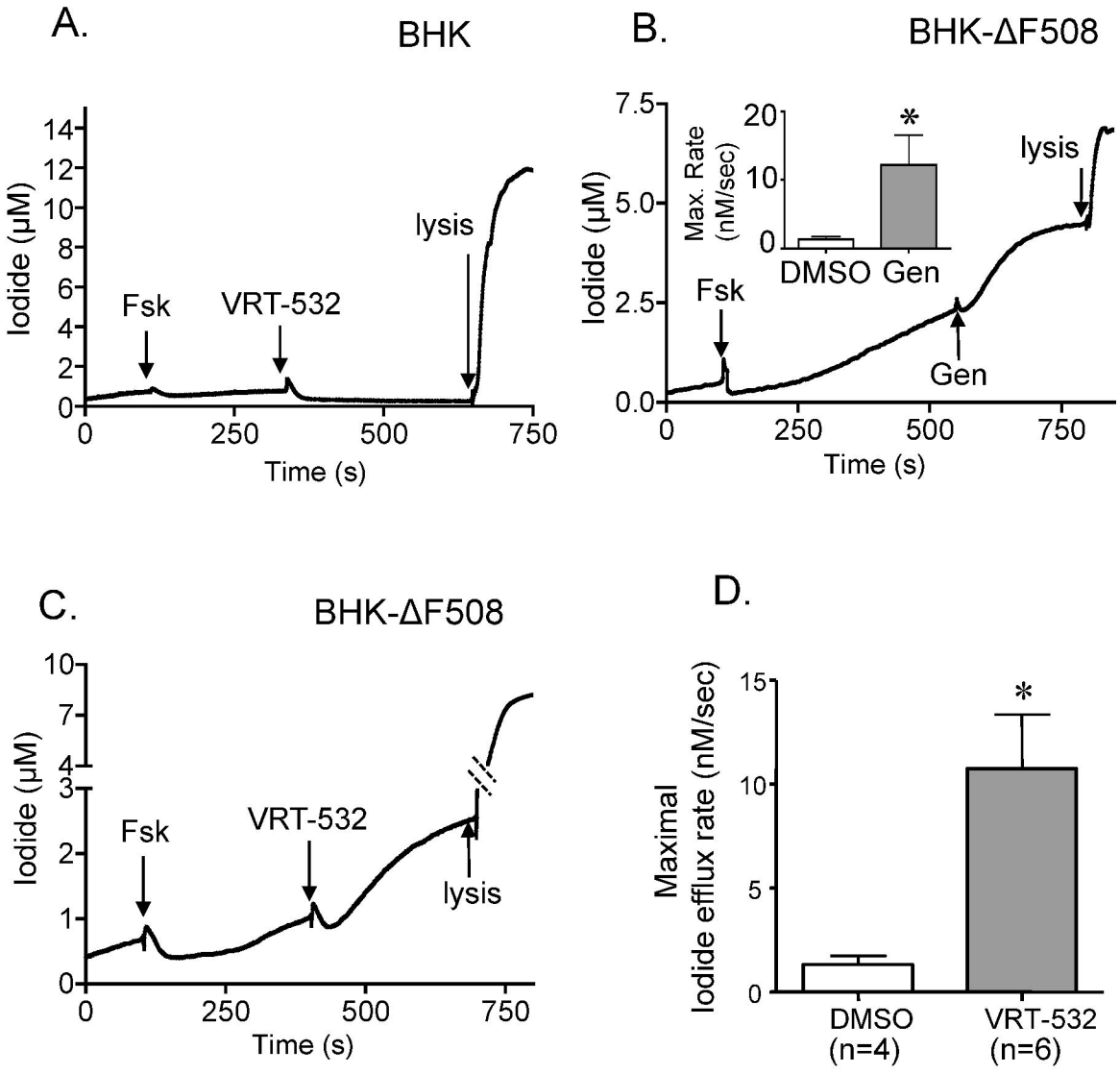

A.
BHK
Iodide (μM)
Fsk
VRT-532
lysis
Time (s)
B.
BHK-ΔF508
Max. Rate (nM/sec)
DMSO
Gen
*
Fsk
Gen
lysis
Time (s)
C.
BHK-ΔF508
Iodide (μM)
Fsk
VRT-532
lysis
Time (s)
D.
Maximal Iodide efflux rate (nM/sec)
*
DMSO (n=4)
VRT-532 (n=6)

Figure 1

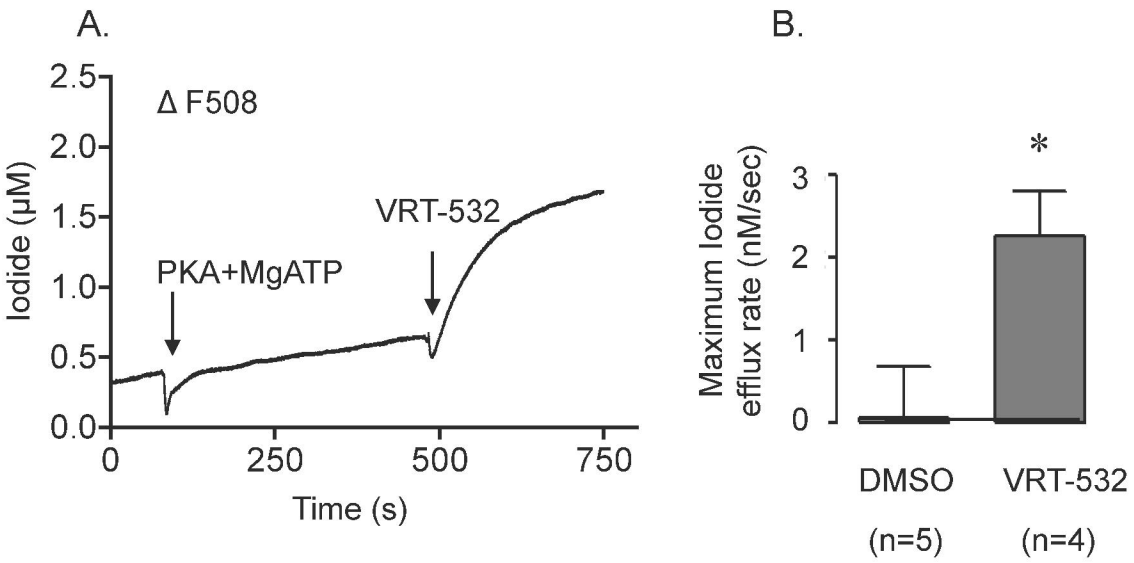
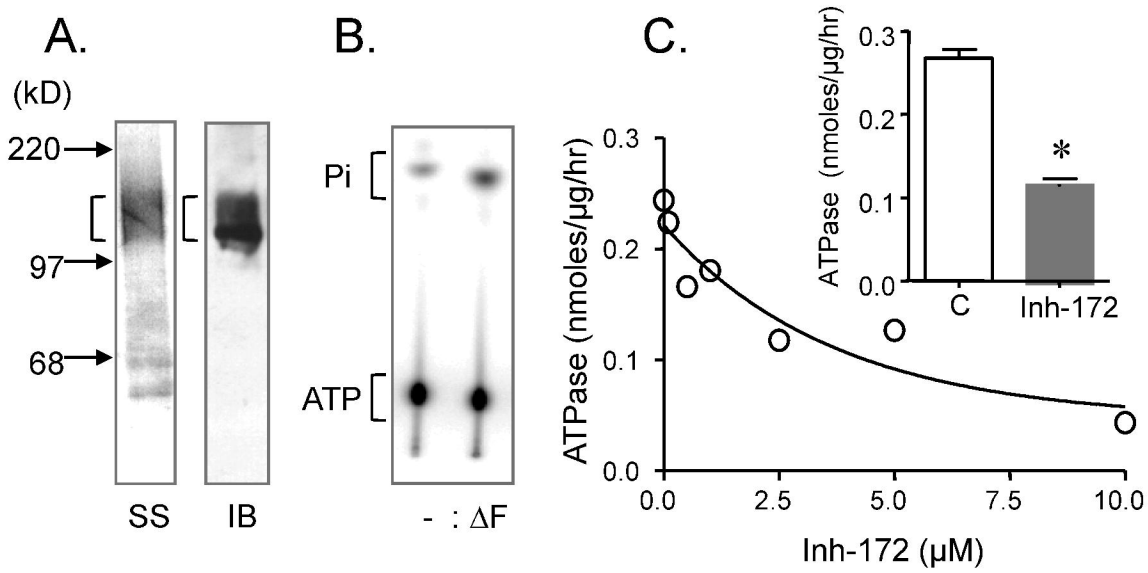

A.
Δ F508
Iodide (μM)
2.5
2.0
1.5
1.0
0.5
0.0
PKA+MgATP
VRT-532
0
250
500
750
Time (s)
B.
Maximum Iodide efflux rate (nM/sec)
3
2
1
0
*
DMSO
(n=5)
VRT-532
(n=4)

Figure 2


A.
(kD)
220
97
68
SS
IB
B.
Pi
ATP
- : ΔF
C.
ATPase (nmoles/µg/hr)
0.0
0.1
0.2
0.3
0.0
2.5
5.0
7.5
10.0
Inh-172 (µM)
ATPase (nmoles/µg/hr)
0.0
0.1
0.2
0.3
*
C
Inh-172

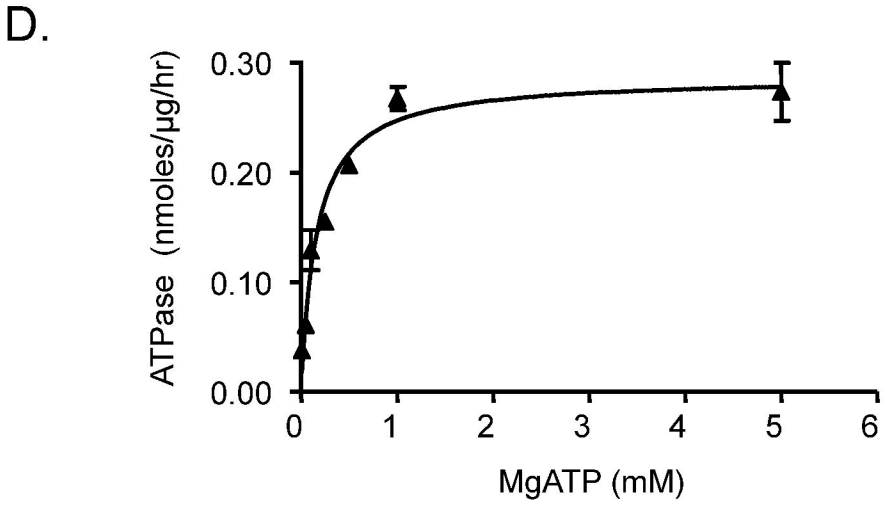

D.
ATPase (nmoles/µg/hr)
0.00
0.10
0.20
0.30
0
1
2
3
4
5
6
MgATP (mM)

Figure 3

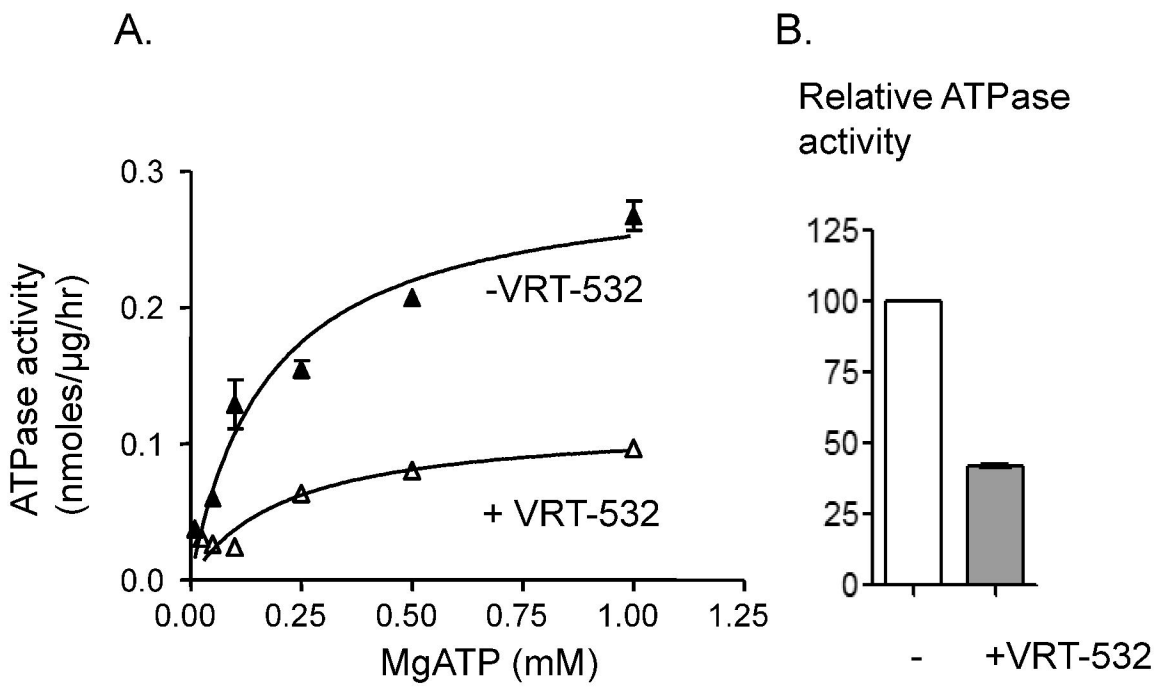

A.
B.
Relative ATPase activity
ATPase activity (nmoles/µg/hr)
0.3
0.2
0.1
0.0
0.00
0.25
0.50
0.75
1.00
1.25
MgATP (mM)
-VRT-532
+ VRT-532
125
100
75
50
25
0
-
+VRT-532

Figure 4

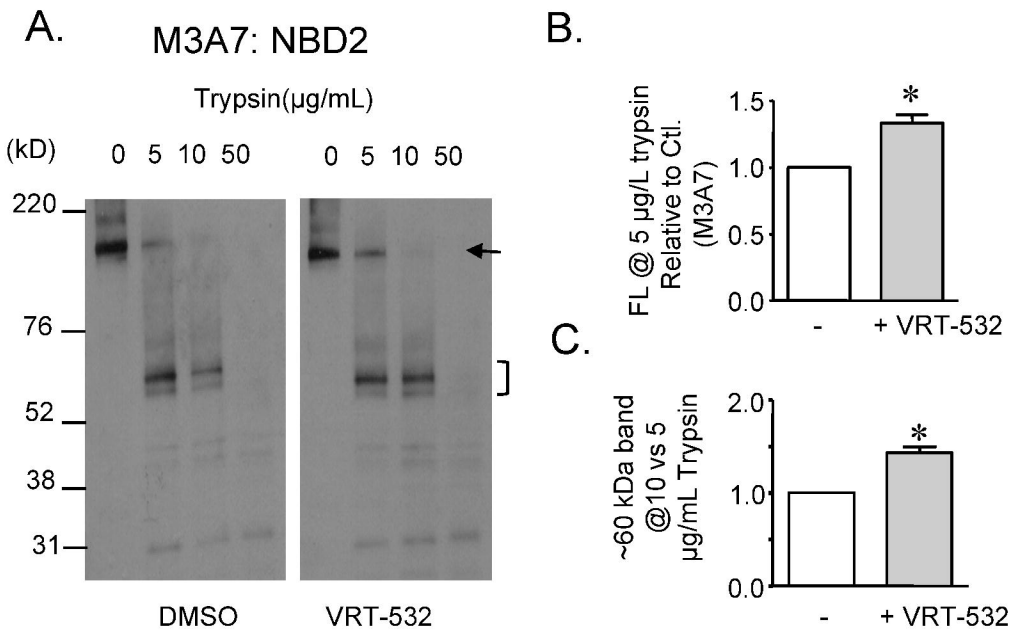

A.
M3A7: NBD2
Trypsin(µg/mL)
(kD)
0 5 10 50
0 5 10 50
220
76
52
38
31
DMSO
VRT-532
B.
FL @ 5 µg/L trypsin
Relative to Ctl.
(M3A7)
1.5
1.0
0.5
0.0
*
-
+ VRT-532
C.
~60 kDa band
@10 vs 5
µg/mL Trypsin
2.0
1.0
0.0
*
-
+ VRT-532

Figure 5

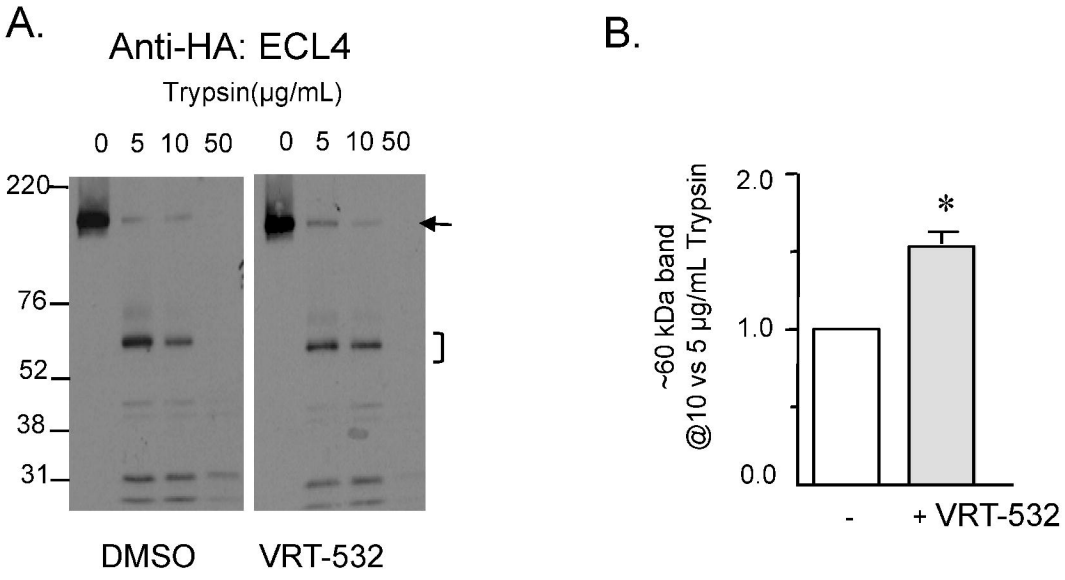

A.
Anti-HA: ECL4
Trypsin(µg/mL)
0 5 10 50 0 5 10 50
220
76
52
38
31
DMSO
VRT-532
B.
~60 kDa band
@10 vs 5 µg/mL Trypsin
2.0
1.0
0.0
*
-
+ VRT-532

Figure 6