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## **A chemical corrector modifies the channel function of F508del-CFTR**

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**Running Title: Modification of F508del-CFTR by a chemical corrector**

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*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-325: Vertex compound 325  
VRT-186: Vertex compound 186  
CFTR<sub>inh</sub>-172: CFTR inhibitor-172

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**Abstract:** The deletion of Phe-508 (F508del) constitutes the most prevalent Cystic Fibrosis causing mutation. This mutation leads to CFTR misfolding and retention in the endoplasmic reticulum, as well as altered channel activity in mammalian cells. This folding defect can however be partially overcome by growing cells expressing this mutant protein at low (27°C) temperature. Chemical “correctors” have been identified that are also effective in rescuing the biosynthetic defect in F508del-CFTR, thereby permitting its functional expression at the cell surface. The mechanism of action of chemical correctors remains unclear but it has been suggested that certain correctors (including VRT-325) may act to promote trafficking by interacting directly with the mutant protein. To test this hypothesis, we assessed the effect of VRT-325 addition on the channel activity of F508del-CFTR after its surface expression had been “rescued” by low temperature. Interestingly, acute pre-treatment with VRT-325 (but not an inactive analog (VRT-186)), caused a modest, but significant inhibition of cAMP-mediated halide flux. Further, VRT-325 decreased the apparent ATP affinity of purified and reconstituted F508del-CFTR in our ATPase activity assay, an effect which may account for the decrease in channel activity by temperature rescued F508del-CFTR. These findings suggest that biosynthetic rescue mediated by VRT-325 may be conferred (at least in part) by direct modification of the structure of the mutant protein, leading to a decrease in its ATP dependent conformational dynamics. Therefore, the challenge for therapy discovery will be the design of small molecules which bind to promote biosynthetic maturation of the major mutant without compromising its activity in-vivo.

## Introduction:

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) functions as a phosphorylation and nucleotide regulated chloride channel on the apical surface of epithelial tissues (Gadsby et al., 2006). Defects in CFTR lead to defective fluid transport by the epithelia lining the airways, gastrointestinal tract, pancreatic duct and the reproductive tract (Amaral, 2005; Cheung et al., 2008). The most common CF mutation, present on at least one allele in 90% of CF patients is the deletion of phenylalanine 508 (F508del-CFTR) (Kerem et al., 1989; Riordan et al., 1989). This mutation causes protein misfolding and retention in the Endoplasmic Reticulum (ER) where it is subsequently polyubiquitinated and targeted for degradation by the proteasome (Cheng et al., 1990; Gelman et al., 2002; Younger et al., 2006).

Previous studies by Denning *et al.* demonstrated that the trafficking defect of F508del-CFTR can be partially “corrected” in cell culture by incubation at low temperatures (27°C) (Denning et al., 1992). Importantly, temperature-rescued F508del-CFTR exhibits functional expression at the cell surface, albeit with altered gating relative to the normal protein (Aleksandrov and Riordan, 1998; Cui et al., 2006; Denning et al., 1992; Pissarra et al., 2008; Wang et al., 2000). Once at the cell surface, the open probability of the F508del-CFTR channel can be enhanced to near “normal” values following the addition of “potentiator” compounds (Hwang et al., 1997; Pedemonte et al., 2005; Van Goor et al., 2006).

Large scale screening efforts for small molecule compounds which could rescue the biosynthetic defect of F508del-CFTR (correctors) or enhance its regulated function once rescued to the

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surface (potentiators) have been very fruitful. Such screens led to the identification of several small molecule compounds which showed efficacy in modifying the trafficking and/or function of F508del-CFTR in heterologous expression systems as well as in human tissues in some cases (Pedemonte et al., 2005; Van Goor et al., 2006)

Studies of the molecular mechanism of action of “potentiator” compounds are ongoing. The acute effect of potentiators on mutant CFTR channel activity in detailed patch clamp studies suggests that they may interact directly with the mutant protein (Ai et al., 2004; Cai and Sheppard, 2002). Consistent with this idea, we showed recently that the acute addition of VRT-532 to isolated membranes expressing the F508del-CFTR protein modified its conformation and susceptibility to trypsin mediated proteolysis (Wellhauser et al., 2009). Finally, we showed that VRT-532 reduced the intrinsic ATP turnover by partially purified and reconstituted F508del-CFTR, an effect which confirms a direct interaction and provides insight into the mechanism underlying the potentiation of channel open time (Wellhauser et al., 2009).

Similarly, the molecular mechanism of action for “corrector” compounds remains unclear. Such compounds could conceivably modify aberrant interactions of F508del-CFTR with chaperone or co-chaperone proteins and/or components of the degradative pathway (Wang et al., 2006; Younger et al., 2006). On the other hand, “corrector” compounds could interact directly with the mutant protein to partially repair its inherent structural defects thereby preventing the aberrant interactions described above. In support of the latter hypothesis, studies by Clarke *et al.*, using “engineered” versions of CFTR or CFTR “half- molecules” have shown that crosslinking of non-native cysteine residues located at the interface between the first and second membrane

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spanning domains is altered by addition of the well studied corrector compound: VRT-325 (Loo et al., 2008; Loo et al., 2009; Van Goor et al., 2006; Wang et al., 2007a).

These findings prompted us to further investigate the consequences of VRT-325 binding to F508del-CFTR, hypothesizing that a direct interaction may lead to changes in protein activity. In the current work, we examined the consequences of adding VRT-325 acutely to the channel activity of the temperature rescued F508del-CFTR (bearing no suppressor mutations) in intact cells. Furthermore, we determined the effect of VRT-325 binding to the intrinsic ATPase activity of the partially purified mutant protein.

## **Methods and Materials:**

### **Materials and Cell Lines:**

Baby hamster kidney (BHK) cells stably expressing F508del-CFTR (BHK-F508del-CFTR) were obtained from Dr. G.L. Lukacs and maintained as previously described (Du et al., 2005).

Human embryonic kidney (HEK) GRIPTITE 293MSR cells stably expressing F508del-CFTR (HEK-F508del-CFTR) were obtained from Dr. D. Rotin and maintained as previously described (Kim Chiaw et al., 2009). All chemicals were purchased from Sigma-Aldrich (Oakville, Canada) unless otherwise noted. Genistein and VRT-325 (4-cyclohexyloxy-2-(1-[4-(4-methoxybenzenesulfonyl)-piperazin-1-yl]-ethyl)-quinazoline) were provided by the Cystic Fibrosis Foundation Therapeutics (CFFT), VRT-186 (4-hydroxy-2-(1-[4-(4-methoxybenzenesulfonyl)-

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piperazin-1-yl]-ethyl)-quinazoline) by Vertex Pharmaceuticals (Cambridge, MA, USA). All compounds were dissolved in DMSO.

### **Immunoblotting:**

HEK-F508del-CFTR cells were treated with VRT-325 or VRT-186 (10 or 25  $\mu$ M), or vehicle DMSO at 37°C for 48 hours. Following treatment, cells were washed with PBS 1X and lysed with a modified RIPA lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, Complete Mini EDTA-free protease inhibitor tablet (Roche Applied Sciences, Laval, Canada)). Protein was separated using a 6% SDS-polyacrylamide gel. CFTR was detected using a 1/1000 dilution of MAB3480 antibody (monoclonal M3A7) (Millipore, Billerica, Maine) and enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Baie d'Urfe, Canada).

### **Continuous Recording iodide efflux assay:**

Continuous recording iodide efflux assays were performed using BHK cells stably over-expressing F508del-CFTR as previously described (Wellhauser et al., 2009). Briefly, BHK-F508del-CFTR was cultured at low temperatures (27°C) for 24 hours to increase surface expression of the mutant protein. Cells were loaded with NaI, external iodide was removed and cells were resuspended in an iodide free buffer as previously described (Wellhauser et al., 2009). Surface channels were stimulated by addition of cAMP agonist forskolin (10  $\mu$ M). To assess if VRT-325 has the ability to further potentiate channel function, VRT-325 (10 or 25  $\mu$ M) was added after addition of forskolin. In order to investigate the effect of acute pre-incubation of VRT-325 on CFTR channel function, the following modifications were employed. During the

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equilibration period, suspensions of temperature-rescued F508del-CFTR expressing cells were incubated with test compounds or the vehicle DMSO for 10 minutes prior to measuring iodide efflux. F508del-CFTR expressing cells were then stimulated with a cyclic-AMP stimulation cocktail containing forskolin (10  $\mu$ M) and isobutyl-methyl-xanthine (IBMX) (200  $\mu$ M). This cAMP cocktail was used in order to elicit a maximal efflux response. Channel function (% Activation) was quantified as the % iodide efflux released after addition of cAMP agonist divided by the total % iodide efflux released upon addition of Triton X-100.

### **Purification and reconstitution of F508del-CFTR:**

A frozen Sf9 insect cell pellet from one half liter of expression culture was thawed and resuspended in 50 mL PBS containing 2% Triton X-100, DNase1 (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 100,000g 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 F508del-CFTR were similar to wild type-CFTR-His as described elsewhere (Ramjeesingh et al., 2008; Wellhauser et al., 2009). Phosphorylated samples were pelleted using an Airfuge ® ultracentrifuge, washed twice with 50 mM Tris-HCl, 50 mM NaCl, 2.5 mM MgCl<sub>2</sub>, and 1 mM DTT at pH 7.5 and then dialyzed overnight against 4L of washing buffer.



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### **ATPase assay of purified F508del-CFTR protein:**

F508del-CFTR was purified from Sf9 insect cells transfected using the baculovirus system as previously described (Wellhauser et al., 2009). ATPase activity was measured as the production of [ $\gamma^{32}$ ] Pi from [ $\gamma^{32}$ P]ATP (Gross et al., 2006; Ramjeeasingh et al., 2008; Wellhauser et al., 2009). In order to assess the effect of VRT-325 on ATPase activity, the ATP stock solution was prepared with or without VRT-325 (or an identical volume of the vehicle DMSO alone). The buffer for this solution was: 50 mM Tris-HCl, 50 mM NaCl, 2.5 mM MgCl<sub>2</sub> 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 37°C. The reaction was quenched and Pi separated from ATP by thin layer chromatography.

**Statistics:** Data are shown as mean  $\pm$  SEM of “n” observations. GraphPad Prism 4 software was used and statistical paired or non-paired “t” tests and One Way Analysis of Variance (ANOVA) were performed as appropriate. “p” values of less than 0.05 were considered significant and indicated using an asterisk (\*), “p” values less than 0.01 and 0.001 were indicated using (\*\*) and (\*\*\*) respectively.

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## Results:

### **VRT-325 inhibits cAMP dependent activation of F508del-CFTR at the cell surface.**

On the basis of biochemical studies suggesting that the corrector compound: VRT-325 (4-cyclohexyloxy-2-(1-[4-(4-methoxy-benzenesulfonyl)-piperazin-1-yl]-ethyl)-quinazoline) induces a change in the conformation of F508del-CFTR protein (Loo et al., 2009; Wang et al., 2007b), we were prompted to determine whether binding of VRT-325 could also cause an acute change in protein function. Clearly, a compound capable of correcting the trafficking defect of F508del-CFTR as well as potentiating its channel activity at the cell surface would be predicted to be highly efficacious as a potential CF therapy. As in our previous studies (Wellhauser et al., 2009), we monitored channel activity of the major mutant following its biosynthetic rescue by low temperature (27°C) incubation (see the Methods section). Channel activity was monitored by iodide efflux from a suspension of baby hamster kidney (BHK) cells stably expressing F508del-CFTR and loaded with iodide. We previously demonstrated that this efflux assay reports the activity of small molecules modifiers of F508del-CFTR, such as the potentiator compounds VRT-532 and genistein (Wellhauser et al., 2009).

Figure 1A shows a representative trace of iodide efflux from cells expressing F508del-CFTR rescued by low temperature incubation (27°C). The activation of cAMP dependent protein kinase A (PKA) by addition of forskolin (Fsk) did not significantly stimulate iodide efflux (Figure 1) relative to wild type CFTR stimulated under similar conditions (data not shown). This likely reflects the altered channel gating of the mutant protein (Dalemans et al., 1991). As

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expected, the subsequent addition of the CFTR potentiator VRT-532 (but not DMSO, the solvent), significantly enhanced efflux (Figure 1A, B and D). This is consistent with its known ability to increase the channel open probability (Van Goor et al., 2006). Interestingly, acute addition of 10  $\mu$ M or 25  $\mu$ M of corrector VRT-325 after forskolin stimulation did not significantly increase the iodide efflux rate (Figure 1C and D). These results suggest that acute addition of VRT-325 (10 or 25  $\mu$ M) does not act like VRT-532 to potentiate the activity of cell surface expressed F508del-CFTR.

We were then prompted to determine if the binding of VRT-325, could exert an inhibitory effect on F508del-CFTR channel gating. In order to evaluate this possibility, we modified the design of our iodide efflux assay so that it could detect inhibition of cAMP-dependent activation. In proof of concept studies (Figure 2A), we showed that pre-incubation of cells expressing F508del-CFTR for 10 minutes with the well known inhibitor; CFTR<sub>inh</sub>-172 (Caci et al., 2008; Ma et al., 2002; Taddei et al., 2004), prevented subsequent cAMP-dependent activation mediated by a cAMP “cocktail” of forskolin plus isobutyl methyl xanthine (IBMX), a cocktail known to maximally activate cell surface expressed F508del-CFTR channels. Given these “proof of concept studies”, we reasoned that this experimental design would be effective in determining whether VRT-325 exerts an inhibitory effect on cAMP dependent activation of F508del-CFTR.

Figure 2B-D, illustrates representative traces of temperature rescued F508del-CFTR pre-treated with varying concentrations of VRT-325 for 10 minutes as previously described for the studies of CFTR<sub>inh</sub>-172. Pre-treatment of F508del-CFTR with concentrations of VRT-325 up to and including 10  $\mu$ M did not significantly alter the agonist (10  $\mu$ M forskolin/ 200  $\mu$ M IBMX)

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response relative to vehicle (DMSO) pre-treated samples (Figure 2B,C and E). Interestingly, a significant decrease in the rate of agonist mediated efflux was observed when F508del-CFTR was pre-treated with a higher concentration of VRT-325 (25  $\mu$ M) (Figure 2C, gray line). While significant, the inhibitory response to VRT-325 was relatively modest compared with the inhibitory response caused by acute pre-treatment with CFTR<sub>inh</sub>-172 (Caci et al., 2008; Ma et al., 2002; Taddei et al., 2004) as seen in Figure 2E. This was the highest concentration of VRT-325 that could be studied, due to the limited solubility of VRT-325 in aqueous buffers.

To determine if the inhibitory effect of VRT-325 on F508del-CFTR channel activation correlates with its' activity as a corrector of the biosynthetic defect of F508del-CFTR, we were prompted to test the effect of a structurally related compound which lacks corrector activity (VRT-186 (4-hydroxy-2-(1-[4-(4-methoxy-benzenesulfonyl)-piperazin-1-yl]-ethyl)-quinazoline). The chemical structure of the active corrector (VRT-325) and the structurally related compound lacking corrector ability (VRT-186) are shown in Figure 3A. The only difference between the two structures is that the inactive compound lacks the hydrophobic cyclohexyl ether on the quinazoline ring. While there is no quantitative structure-activity relationship data published for these compounds, it does seem that a hydrophobic moiety at this region of the molecule is necessary for activity (Makings et al., 2005).

As shown in figures 3B and 3C, we confirmed that VRT-325, but not VRT-186, acts as a partial corrector of the biosynthetic trafficking defect in F508del-CFTR. Correction of this primary defect is evident as the appearance of complex glycosylation (ie. an increase in the ratio of the Band C to the Band B form of the protein). Biosynthetic rescue typically requires treatment of

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cells expressing F508del-CFTR with small molecules for 48 hours. Interestingly, using the iodide efflux assay, we determined that pre-treatment of temperature rescued F508del-CFTR with 25  $\mu$ M VRT-186 (for 10 min) also failed to inhibit agonist mediated efflux relative to vehicle (DMSO) treated samples (Figure 3D). These data are summarized in the bar graph of Figure 3E.

### **The small molecule- VRT-325 directly modifies the ATPase activity of reconstituted F508del-CFTR.**

The results of the flux-based assay shown in figure 2 suggest that the acute treatment of F508del-CFTR with VRT-325 at a concentration of 25  $\mu$ M, after surface expression of the mutant protein was rescued, prevents activation of its chloride channel activity. As channel gating between the closed and open conductance states requires the binding and hydrolysis of ATP (Ramjeesingh et al., 2008; Vergani et al., 2005), we were prompted to determine if VRT-325 mediated its inhibitory effect via a direct effect on the ATPase activity of the mutant protein.

As the intrinsic ATPase activity of CFTR is low, its measurement requires extraction from other cellular ATPases (Ramjeesingh et al., 2008). We developed a method for the partial purification and functional reconstitution of F508del-CFTR (possessing a carboxy terminal histidine tag) from Sf9 insect cells transiently expressing this protein (Wellhauser et al., 2009). The ATPase activity was measured as the production of  $[\gamma^{32}]$  Pi from  $[\gamma^{32}\text{P}]\text{ATP}$  by reconstituted F508del-CFTR as previously described (Ramjeesingh et al., 2008; Wellhauser et al., 2009). Figure 4A shows that partially purified and reconstituted F508del-CFTR conferred ATPase activity and that

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treatment of F508del-CFTR with VRT-325 at a concentration of 25  $\mu$ M resulted in a decrease in ATPase activity. The ATP dependence of F508del-CFTR ATPase activity ( $\pm$ VRT-325) was fitted using the Michaelis Menten function and the analysis revealed that treatment with VRT-325 caused a decrease in the apparent affinity ( $K_m$ ) of the mutant protein for MgATP, from  $\sim$ 0.2 mM to  $\sim$ 3.0 mM MgATP with a minor effect on  $V_{max}$ . (Figure 4B and D). The bar graph in figure 4C shows a summary of our data. We observed that the ATP dependence of F508del-CFTR in the presence of 25  $\mu$ M VRT-325 was significantly decreased by approximately 75% relative to DMSO treated samples (Figure 4C). Interestingly, the ATP dependence of F508del-CFTR was also significantly diminished in the presence of 10  $\mu$ M VRT-325 but to a lesser extent than 25  $\mu$ M VRT-325 (Figure 4C). These findings are consistent with the hypothesis that VRT-325 (25  $\mu$ M) prevents channel activation by reducing affinity of the mutant protein for MgATP binding.

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## Discussion:

The activity of VRT-325 as a corrector of the trafficking defect of F508del-CFTR has been validated in multiple laboratories yet its mechanism of action remains unknown and its specificity for the major CF causing mutation has been challenged (Van Goor et al., 2006; Wang et al., 2007b). The lack of mechanistic insight for this and other effective corrector compounds hinders future development in defining the molecular and cellular defects that lead to F508del-CFTR mistrafficking. Our study supports the original claim by Clarke *et al.* (Loo et al., 2009; Wang et al., 2007a) that F508del-CFTR is one of the molecular targets of VRT-325 and hence, this small molecule can serve as a tool for future studies of the molecular defects caused by this mutation. Importantly, we found that at relatively high concentrations, this direct interaction reduced apparent ATP affinity for partially purified F508del-CFTR as well as its' ATPase activity and exerted a modest but significant inhibition of its channel activity.

### **The ATPase activity assay provides insight into the mechanism of action of VRT-325.**

VRT-325 has been previously shown to enhance the biosynthetic maturation of F508del-CFTR with an EC<sub>50</sub> of approximately 6-7  $\mu$ M over extended treatment times of 24-48 hours. Further, it has been shown that prolonged treatment is associated with enhanced functional expression at the cell surface in mammalian heterologous expression systems and in bronchial epithelial monolayers obtained from CF patients homozygous for this mutation (Van Goor et al., 2006). In the current studies, we assessed whether there may also be an effect on the channel activity of F508del-CFTR (after its mistrafficking defect had been corrected) as would be predicted if VRT-325 directly interacts with the mutant protein. We found that there was no potentiation of

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cAMP dependent activity (although this would have been beneficial for optimizing the functional expression of F508del-CFTR) following treatment with VRT-325 (25  $\mu$ M). On the other hand, there was a significant inhibitory effect on cAMP-dependent channel activation induced by VRT-325 (25  $\mu$ M). There was also a trend toward this inhibitory effect at 10  $\mu$ M VRT-325, but it did not achieve statistical significance. Importantly, this inhibitory effect was not observed following addition of the structurally related VRT-186 (25  $\mu$ M) lacking correcting ability, pointing to a possible relationship between the activity of VRT-325 as a corrector and as an inhibitor.

We employed our purification and reconstitution system to evaluate the mechanism of action of VRT-325 on F508del-CFTR (Wellhauser et al., 2009). After addition of VRT-325 (25  $\mu$ M) or VRT-325 (10  $\mu$ M) to a lesser degree, we found that the specific ATPase activity of partially purified and reconstituted F508del-CFTR (in the presence of 1 mM MgATP) was significantly reduced. A similar inhibitory effect was mediated by CFTR<sub>inh</sub>-172 binding to F508del-CFTR in our previous studies (Wellhauser et al., 2009). Comparison of the ATP dependence of the ATPase activity of F508del-CFTR protein in the absence or presence of VRT-325 (25  $\mu$ M), suggests that binding of this small molecule reduces the specific ATPase activity of the mutant protein by altering its apparent affinity for ATP. As it has been suggested that nucleotide binding initiates opening of the CFTR channel gate (Vergani et al., 2005), a decrease in apparent ATP affinity may account for the inhibition of the cyclic AMP dependent iodide efflux rate (relative to control) caused by pre-treatment with VRT-325. As expected, this effect is different from that measured in our previous studies of the potentiator VRT-532. In contrast to the effects of VRT-325, VRT-532 decreased ATPase activity by reducing the  $V_{\max}$  - or the rate of ATP



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turnover, a metabolic event predicted to promote closure of the CFTR channel gate (Vergani et al., 2005). Together, these findings suggest that VRT-532 promotes channel activity by binding to the mutant protein to prevent ATP turnover (or ATPase activity), whereas VRT-325 inhibits channel activity by reducing apparent ATP affinity at the catalytic site formed at the interface between NBD1 and NBD2. This interpretation needs to be tested in studies of single channel kinetics as it is also possible that VRT-325 binding may not impair the rate of channel opening but rather, may reduce the stability of the open state or alternatively- modify both gating transitions. Further studies are also required to determine if difference between the potentiator: VRT-532 and the corrector compound: VRT-325 in terms of their effects on ATPase activity and channel activity is dependent on concentration. Interestingly, the well known potentiator: genistein, has been shown to act as inhibitor of channel activity at relatively high concentrations (Lansdell et al., 2000) and this may also be true for VRT-532.

Molecular modelling studies by Moran *et al.* previously highlighted the potential role for the nucleotide binding domains of CFTR in mediating small molecule “modifier” activity (Moran et al., 2005; Zegarra-Moran et al., 2007). However, we cannot conclude from the current studies that VRT-325 competes with ATP binding to the catalytic site as we are measuring apparent nucleotide affinity rather than direct binding. Further, the binding site for VRT-325 may be located in a region distant from the NBDs but functionally coupled to their activity through allosteric interactions. Recent studies using a split molecule approach, wherein the first half of the molecule (residues 1-633) bearing the F508del mutation was co-expressed with the second half (634-1480) revealed a related effect of the small molecules VRT-325 and Corrector 4a on promoting the interaction between the two halves of the mutant (Loo et al., 2009). Hence, VRT-

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325 may bind to one or multiple sites comprising the interfaces between the first and second half of the full length protein.

The effect of VRT-325 on ATP binding and hydrolysis by reconstituted F508del-CFTR supports a model wherein this small molecule acts as an allosteric inhibitor. As discussed in a recent review (Li and Sheppard, 2009), allosteric inhibitors are proposed to interact at a site distinct from the pore, possibly binding to the canonical catalytic site formed at the interface of NBD1 and NBD2. Further, the authors of this review, postulated that allosteric inhibitors of channel opening may act by preventing ATP binding to this site and modifying heterodimerization of the NBDs, events associated with channel opening. The biochemical studies presented here provide direct evidence in support of such a mechanism of inhibition for VRT-325. Interestingly, we found that at high concentrations, VRT-325 exerted a similar effect on apparent ATP affinity and ATPase activity as CFTR<sub>inh</sub>-172, an inhibitor studied biochemically in our previous work (Wellhauser et al., 2009) and previously suggested to have an allosteric mechanism of action (Ma et al., 2002). Recent studies by Robert *et al.* regarding a distinct small molecule corrector, the anthranilic acid derivative: glafenine, also showed that high concentrations (e.g. 100  $\mu$ M) resulted in a decrease in channel function as measured by iodide efflux assays (Robert et al., 2010). Future studies are required to determine if glafenine or other corrector molecules act directly as allosteric inhibitors.

**Relationship between efficacy of VRT-325 as a corrector of F508del-CFTR maturation and its modest inhibition of channel activity.** Our observations are reminiscent of the inhibitory effect induced by chemical crosslinking between the two halves of CFTR (Serohijos et al.,

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2008). Specifically, single channel studies by Serohijos *et al.* revealed that crosslinking of non-native cysteine residues engineered at domain-domain interfaces in the “cys-less” version of CFTR led to inhibition of channel opening. Hence, correctors which act by stabilizing certain inter-domain interactions within F508del-CFTR may also have a negative impact on ATP dependent channel gating transitions which are conferred by dynamic conformational changes. If this concept is confirmed in future experimentation, the challenge for therapy discovery will be the design of small molecules which modify the multi-domain assembly required for correction of the primary misassembly defect in F508del-CFTR yet exert minimal inhibitory effects on channel activity.

There is an extensive literature documenting the efficacy with which small molecules correct the misfolding of disease-causing mutations in other membrane proteins, such as the gonadotropin releasing hormone (GnRH) and the vasopressin 2 receptor (V2R) (Bichet, 2008; Conn et al., 2007; Ulloa-Aguirre et al., 2006). Many of these small molecules were designed to mimic the natural peptide ligands for these receptors and their efficacy as correctors point to the role of ligand binding in promoting a protein conformation conducive to ER exit. Importantly, there is also an obligate requirement for dissociation of such ligand mimetics from the receptor once it is correctly trafficked to the cell surface to ensure receptor function (Conn et al., 2007; Ulloa-Aguirre et al., 2006). Hence, as in the case of misfolded GnRH and V2 receptors, optimization of the functional expression of F508del-CFTR protein may require fine tuning of the bioavailability and affinity of small molecule “correctors” not only to ensure their interaction in the biosynthetic compartments but also their release from the properly trafficked protein at the cell surface.

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To summarize, the results of these studies show that VRT-325 directly binds F508del-CFTR and further, support the hypothesis that this interaction produces an allosteric effect which induces a more wild type conformation in the mutant protein, possibly enabling ER exit. Clearly, direct support for this hypothesis requires that the binding site (or sites) for VRT-325 in F508del-CFTR be identified in future work. Furthermore, this putative binding must be validated in mutagenesis studies. We expect that the definition of binding site in F508del-CFTR will not only provide insight into the mechanisms underlying the “correction” effect by VRT-325 of the major CF mutant, but also the correction caused by this small molecule in other misfolded mutant membrane proteins (Van Goor et al., 2006; Wang et al., 2007b).

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## Footnotes:

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## Figure Legends:

**Figure 1: VRT-325 does not potentiate channel function of F508del-CFTR after cyclic AMP-dependent activation.** As expected, there is little detectable activation of the rate of halide (iodide) efflux through F508del-CFTR channels by addition of an agonist of cAMP (Fsk) even though the trafficking defect was partially corrected by low temperature incubation (27°C) for 24 hours. However, as previously reported, the “potentiator” compound: VRT-532 (B) but not the vehicle alone: DMSO (A) was effective in enhancing the rate of halide flux following cAMP activation. The initial decrease in efflux observed upon addition of agonist or vehicle is an artifact of our assay and reflects a disturbance to the local environment of the iodide sensitive electrode resulting from addition of a 2  $\mu$ l volume. Addition of the detergent Triton X-100 confirmed equal halide loading in each case. C) VRT-325 does not act like VRT-532 to potentiate F508del-CFTR. The addition of VRT-325 at 10 or 25  $\mu$ M (see figure 1D) failed to enhance the rate of activation of F508del-CFTR after cAMP activation. D) Bars showing mean  $\pm$ SEM of iodide efflux rates after forskolin activation and addition of either Vertex compound. Acute addition of 10  $\mu$ M VRT-532 significantly increased the iodide efflux rate ( $11 \pm 2$  nM/s, n=6) relative to vehicle (DMSO) treated samples ( $0.6 \pm 0.6$  nM/s, n=3), whereas acute addition of 10  $\mu$ M or 25  $\mu$ M VRT-325 failed to significantly increase the iodide efflux rate ( $1.2 \pm 0.3$  nM/s, n=3 and  $1.4 \pm 0.4$  nM/s, n=5 respectively). \*\*\*  $p < 0.001$ , Analysis of Variances ANOVA statistical test employed.

**Figure 2: VRT-325 inhibits cAMP dependent activation of F508del-CFTR channels.** As in figure 1, cyclic AMP dependent iodide efflux was measured to assess channel activity mediated by F508del-CFTR, after biosynthetic rescue by low temperature incubation (27°C) for 24 hours. Cells in suspension were pre-treated for 10 minutes with A) 5  $\mu$ M CFTR<sub>inh</sub>-172, B) 2.5  $\mu$ M VRT-325, C) 10  $\mu$ M VRT-325 or D) 25  $\mu$ M VRT-325. Channels expressed at the cell surface were maximally stimulated with a cAMP agonist cocktail containing 10  $\mu$ M forskolin and 200  $\mu$ M IBMX. Panels A-D show representative traces of the activity in the presence of CFTR<sub>inh</sub>-172/VRT-325 or vehicle (DMSO) alone. The trace obtained in the presence of DMSO was a from a single representative experiment. E) Graph displays mean  $\pm$  SEM % activation (calculated as the % iodide released after addition of agonist divided by total iodide released after addition of

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Triton X-100). Addition of cyclic AMP agonist (forskolin and IBMX) to vehicle (DMSO) treated samples yielded a  $61 \pm 3\%$  ( $n=7$ ) activation, whereas cells pre-treated with  $5 \mu\text{M}$  CFTR<sub>inh</sub>-172 displayed a significant reduction in the agonist mediated iodide efflux response ( $22 \pm 4\%$ ,  $n=3$ ). Pre-treatment with VRT-325 led to a concentration dependent reduction in the agonist mediated iodide efflux response, with a significant reduction in function when pre-treated with  $25 \mu\text{M}$  VRT-325 ( $47 \pm 2\%$ ,  $n=6$ ). \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , ANOVA statistical test employed.

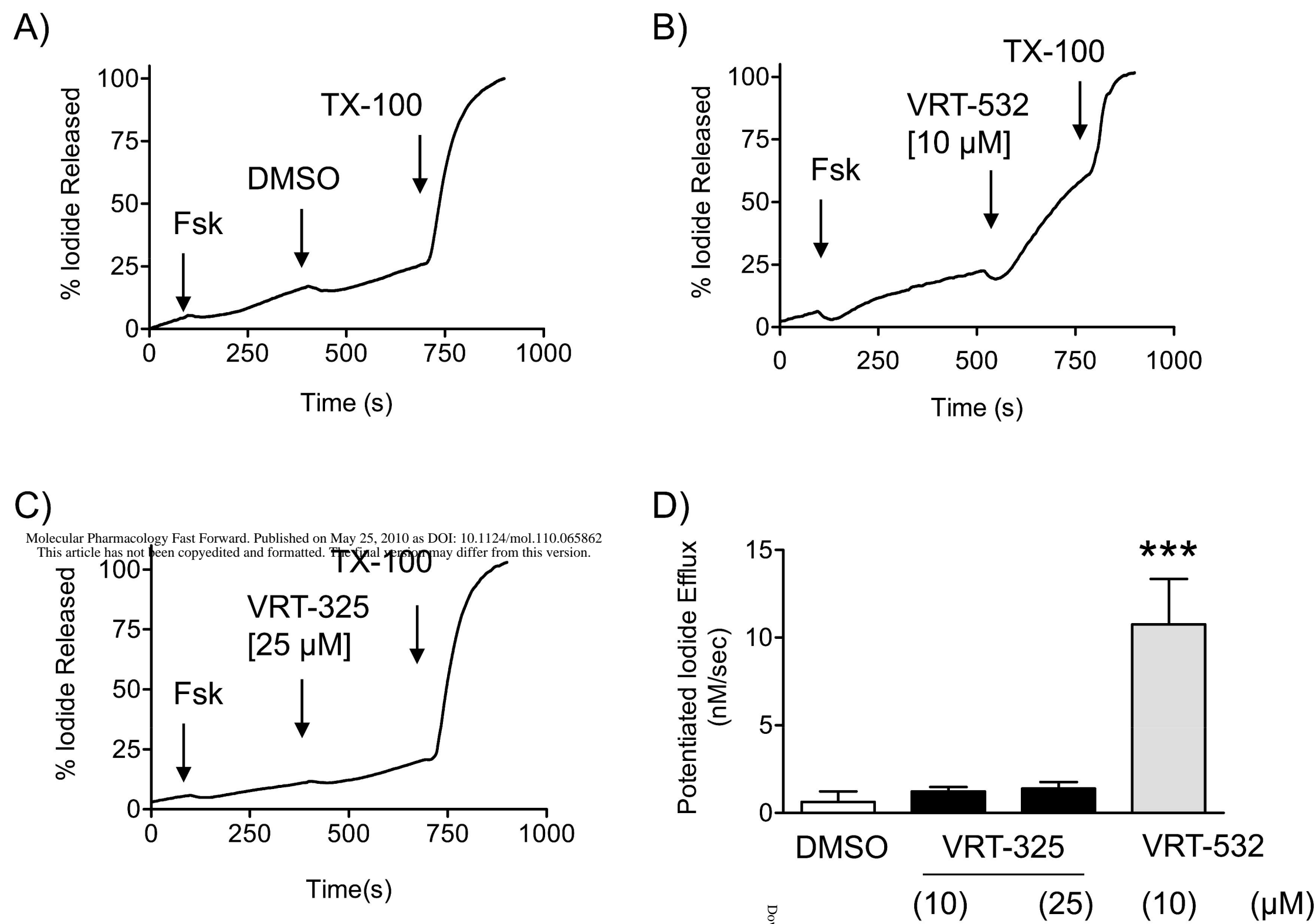
**Figure 3: Inhibition of F508del-CFTR channel activation by VRT-325 related to efficacy as a corrector.** A) Chemical structure of the active corrector VRT-325 (4-cyclohexyloxy-2-(1-[4-(4-methoxy-benzenesulfonyl)-piperazin-1-yl]-ethyl)-quinazoline) (left) and the structurally related compound VRT-186 (4-hydroxy-2-(1-[4-(4-methoxy-benzenesulfonyl)-piperazin-1-yl]-ethyl)-quinazoline) lacking corrector ability (right). B) Representative immunoblots illustrating maturation of F508del-CFTR in the presence of VRT-325 but not with VRT-186. Core-glycosylated form is denoted by the letter B; complex glycosylated form denoted by the letter C. C) Bar graph shows mean of several experiments wherein treatment of F508del-CFTR with  $10$  or  $25 \mu\text{M}$  VRT-325 significantly increased the Band C/B ratio  $0.40 \pm 0.02$  ( $n=7$ ) and  $0.54 \pm 0.04$  ( $n=3$ ) respectively relative to vehicle (DMSO) treated samples ( $0.10 \pm 0.02$  ( $n=7$ )). Treatment of F508del-CFTR with  $25 \mu\text{M}$  of VRT-186 failed to significantly increase the Band C/B ratio relative to vehicle (DMSO) treated samples ( $n=3$ ). D) Representative continuous recording iodide efflux trace of temperature rescued F508del-CFTR treated with  $25 \mu\text{M}$  VRT-186. E) Graph displays mean  $\pm$  SEM % activation (calculated as the % iodide released after addition of agonist divided by total iodide released after addition of Triton X-100) of DMSO (white bar), VRT-325 ( $25 \mu\text{M}$ ) (black bar), or VRT-186 ( $25 \mu\text{M}$ ) (gray bar) treated samples. Acute addition of  $5 \mu\text{M}$  CFTR<sub>inh</sub>-172 ( $n=3$ ) or  $25 \mu\text{M}$  VRT-325 ( $n=6$ ) significantly inhibited % activation of CFTR channel function whereas acute addition of  $25 \mu\text{M}$  VRT-186 ( $n=4$ ) failed to inhibit CFTR channel function. \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , ANOVA statistical analysis employed.

**Figure 4: VRT 325 inhibits the ATPase activity of F508del-CFTR by decreasing its apparent affinity for ATP.** A) Thin Layer Chromatography plate: lane 1 – purified and reconstituted F508del-CFTR in the absence of VRT-325; lane 2 – purified and reconstituted

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F508del-CFTR in the presence of 25  $\mu$ M VRT-325. In the presence of VRT-325, a reduction in ATPase activity by F508del-CFTR is observed. B) Graph illustrating the effect of VRT-325 (25  $\mu$ M) on ATP dose dependence of the ATPase activity for F508del-CFTR. Data obtained in the presence ( $\blacktriangle$ ) and absence ( $\blacksquare$ ) of VRT-325 were fitted using the Michaelis–Menten equation. (( $\blacksquare$ ) n=4 different protein preparations,  $r^2=0.92$  & ( $\blacktriangle$ ) n=2,  $r^2=0.99$ ). C) Quantification showing the % ATPase activity relative to vehicle (DMSO) control. Purified and reconstituted F508del-CFTR in the presence of 0.5 mM ATP after addition of 10  $\mu$ M VRT-325 (gray bar) displayed  $89 \pm 3\%$ , (n=3) activity, whereas 25 $\mu$ M VRT-325 (black bar) displayed  $25.8 \pm 0.3\%$ , (n=4) activity relative to DMSO treated controls. D) Table summarizing the effect of 25  $\mu$ M VRT-325 on F508del-CFTR ATPase activity. Addition of 25  $\mu$ M VRT-325 alters the apparent affinity for Mg-ATP ( $K_m = 2.87 \pm 0.33$  mM) relative to vehicle (DMSO) control ( $K_m = 0.22 \pm 0.06$  mM) without affecting the  $V_{max}$ .

Figure 1



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Figure 2

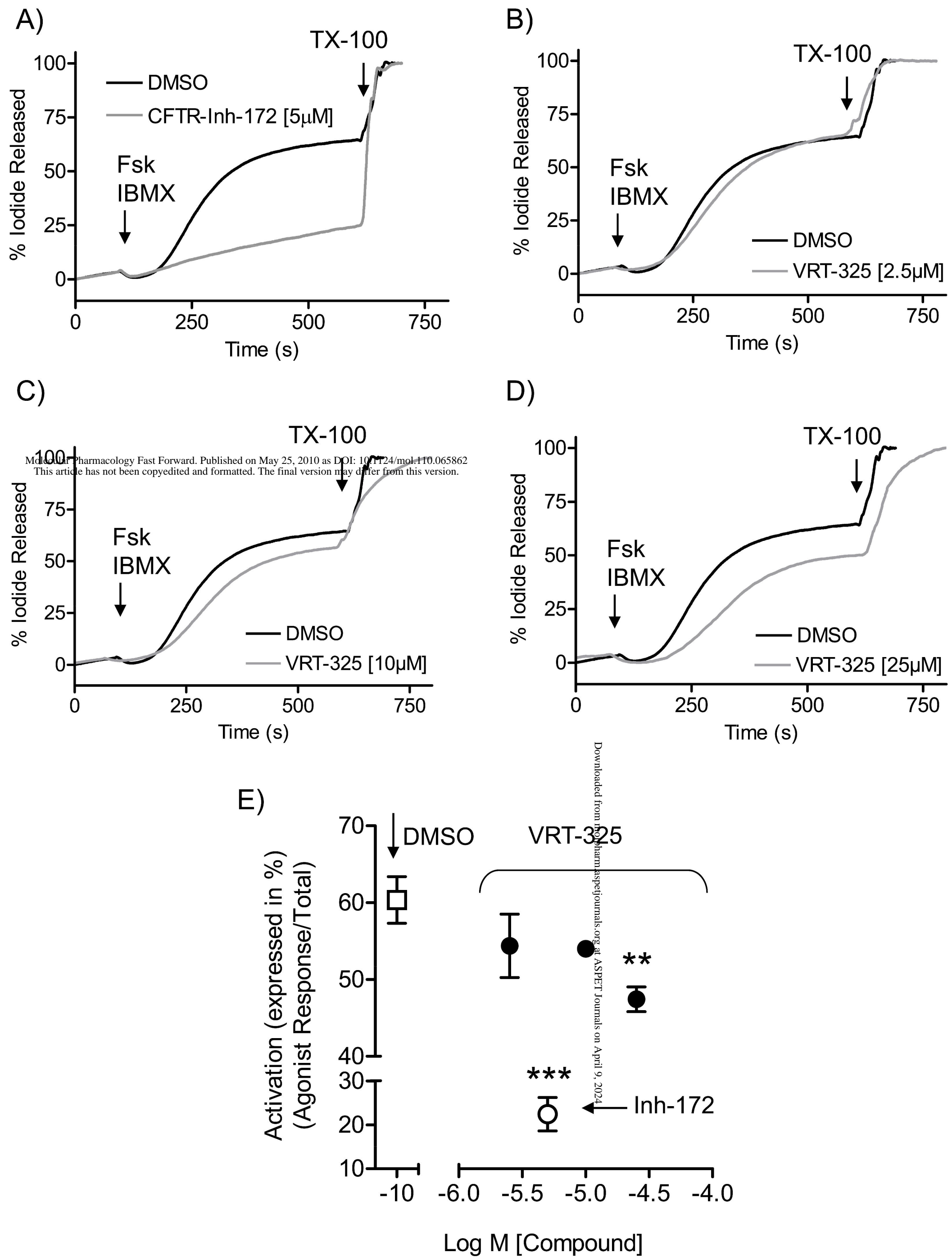


Figure 3

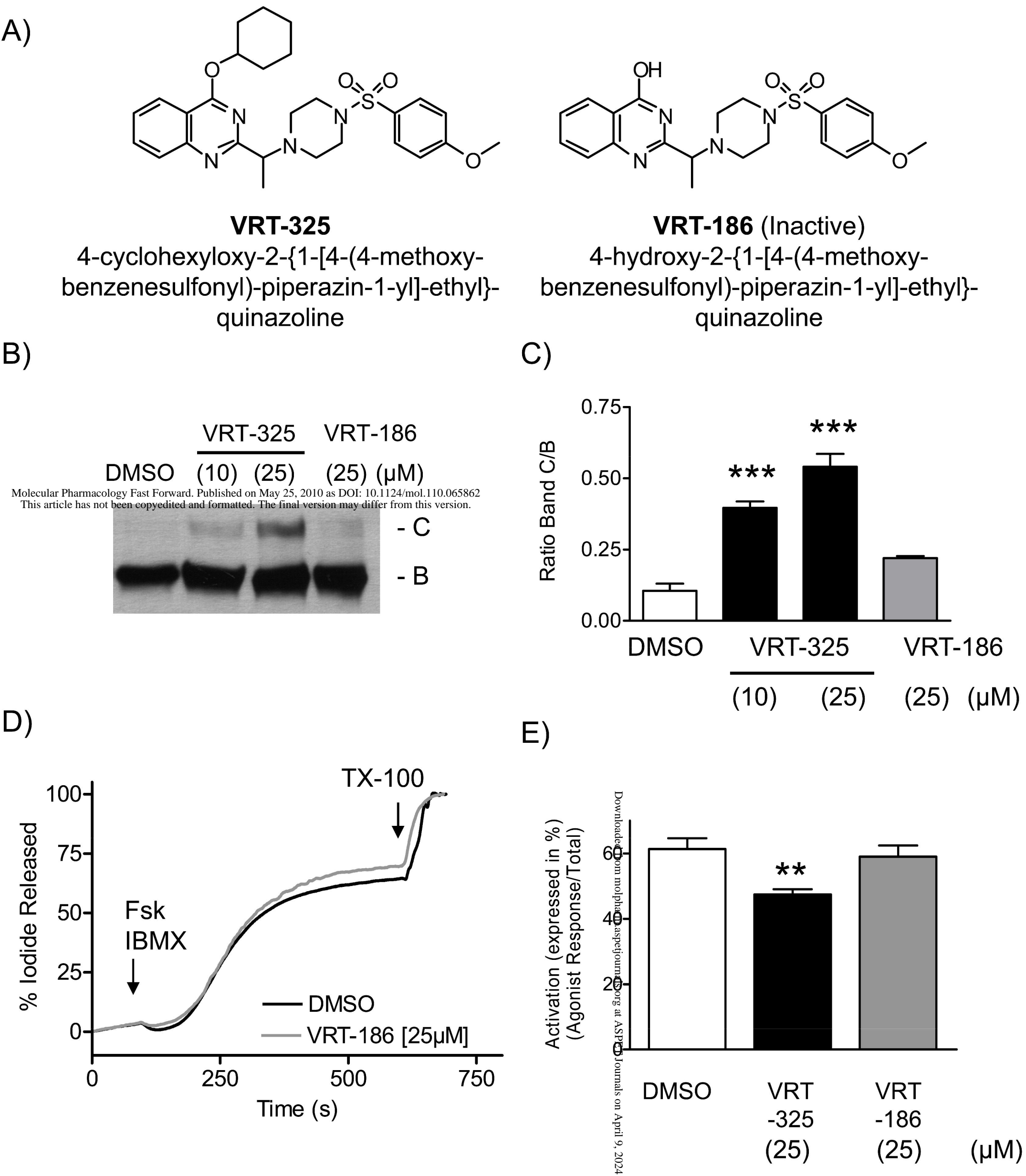


Figure 4

