Ivacaftor potentiation of multiple CFTR channels with gating mutations

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Abstract

Background: The investigational CFTR potentiator ivacaftor (VX-770) increased CFTR channel activity and improved lung function in subjects with CF who have the G551D CFTR gating mutation. The aim of this in vitro study was to determine whether ivacaftor potentiates mutant CFTR with gating defects caused by other CFTR gating mutations.

Methods: The effects of ivacaftor on CFTR channel open probability and chloride transport were tested in electrophysiological studies using Fischer rat thyroid (FRT) cells expressing different CFTR gating mutations.


Conclusion: These in vitro data suggest that ivacaftor has a similar effect on all CFTR forms with gating defects and support investigation of the potential clinical benefit of ivacaftor in CF patients who have CFTR gating mutations beyond G551D.

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1. Introduction

The underlying cause of cystic fibrosis (CF) is the loss of epithelial chloride transport due to mutations in the CF transmembrane conductance regulator gene (CFTR) that encodes the CFTR protein (CFTR) [1]. CFTR is an epithelial chloride channel that is composed of two membrane spanning domains that form the chloride channel pore, two nucleotide-binding domains (NBD) that bind and hydrolyze ATP, and a regulatory domain with several protein kinase A (PKA) phosphorylation sites [1]. The opening and closing of the CFTR channel pore, or channel gating, is a tightly regulated process, requiring phosphorylation of the regulatory domain by PKA and subsequent ATP binding and hydrolysis by the NBDs [2]. Normally, CFTR is present at the epithelial cell surface where it allows chloride transport across the epithelial cell membrane to maintain salt, fluid, and pH balance in multiple organs [3]. In CF, CFTR mutations cause a loss of chloride transport through CFTR that results in the accumulation of thick, sticky mucus in the bronchi of the lungs, loss of exocrine pancreatic function, impaired intestinal absorption, reproductive dysfunction and elevated sweat chloride concentrations [3–4].

There are over 1800 CFTR mutations, resulting in a wide spectrum in the severity of the loss in chloride transport and disease phenotype [4]. Evaluation of the molecular defect caused by some of the more common CFTR mutations has shown that the loss of chloride transport can be due to a decrease in the quantity and/or function of CFTR channels at the cell surface [4–5]. For example, the most common CFTR mutation, F508del-CFTR which accounts for two-thirds of all CFTR alleles in patients with CF [4], impairs CFTR processing in the endoplasmic reticulum (ER), greatly reducing the quantity of F508del-CFTR protein at the cell surface [5]. In contrast, approximately 4–5% of patients with CF carry a missense CFTR mutation that results in CFTR protein which is present at the cell surface but does not open and close properly (defective channel gating), resulting in minimal chloride transport
as measured in vitro or in vivo [6–8]. Such CFTR mutations are called CFTR gating mutations (also known as Class III mutations) [5]. The most common CFTR gating mutation in patients with CF is G551D [6–7]. Other known CFTR gating mutations include G178R, G551S, G970R, G1244E, S1255P, and G1349D [9–11].

One potential strategy to treat CF is to increase chloride transport by restoring or enhancing CFTR function with small molecule drugs known as CFTR modulators. A CFTR potentiator is a type of CFTR modulator that acts by increasing CFTR channel gating to enhance chloride transport. For a CFTR potentiator to act, CFTR at the cell surface must first be activated by PKA-dependent phosphorylation [12]. Experimentally, the effect of a CFTR potentiator on channel gating can be quantified using patch-clamp electrophysiology to directly measure the fraction of time the channel is open, which is called the channel open probability. Ivacaftor (VX-770), an investigational CFTR potentiator [12], increased the channel open probability of G551D-CFTR to enhance chloride transport in vitro [12]. In patients with CF who have the G551D mutation, ivacaftor increased clinical measures of chloride transport through CFTR, and improved clinical measures of lung function [13]. Ivacaftor has also been shown to potentiate normal CFTR and F508del-CFTR, suggesting that the compound is not specific for G551D-CFTR. We therefore hypothesized that ivacaftor might potentiate multiple mutant CFTR forms with CFTR gating mutations.

In this set of in vitro studies, the ability of ivacaftor to potentiate mutant CFTR was examined in a panel of Fischer rat thyroid (FRT) cells engineered to express previously reported CFTR gating mutations or CFTR mutations believed to be located in the ATP binding sites required for normal channel gating [2]. The results presented here indicated that ivacaftor potentiated CFTR channel gating and enhanced chloride transport in all examples of CFTR gating mutations tested. These in vitro data support the investigation of the clinical benefit of CFTR potentiators such as ivacaftor, in patients with CF who have CFTR gating mutations beyond G551D.

2. Results

2.1. Generation and characterization of cell lines expressing CFTR gating mutations

To systematically compare the effects of ivacaftor on mutant CFTR with defective channel gating, a panel of stable cell lines was generated using FRT cells. Each cell line was engineered to express a mutant CFTR form with a specific CFTR gating mutation or CFTR mutation believed to be located in the ATP binding sites required for normal channel gating [2]. These included G551D-, G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1251N-, S1255P-, and G1349D-CFTR [4,7,9–11]. No significant difference (P > 0.05; ANOVA followed Tukey’s multiple comparisons test; n = 3–6) in the level of CFTR mRNA was observed between normal CFTR and the individual mutant CFTR forms expressed in FRT cells (Fig. 1A), suggesting similar CFTR protein levels.

In order to further characterize these cell lines, the delivery of mutant CFTR protein to the cell surface was indirectly assessed in immunoblot studies that measure an increase in the molecular mass of CFTR (from a 135–140 kDa band to a 170–180 kDa band) due to glycosylation (Fig. 1B–D), an indicator of CFTR exit from the endoplasmic reticulum and passage through the Golgi. After CFTR is processed by the Golgi, the glycosylated CFTR (mature CFTR) is delivered to the cell surface [5]. The ratio of mature to total CFTR (mature + immature CFTR) was used to estimate the fraction of CFTR synthesized that was processed normally and delivered to the cell surface (Fig. 1C). In addition, the steady-state level of mature mutant CFTR at the cell surface was expressed as a percentage of that measured in four separate FRT cell lines expressing normal CFTR (% normal CFTR; Fig. 1D). F508del-CFTR was used as a reference to calibrate severe defects in CFTR processing and delivery to the cell surface [5]. This analysis showed that, as expected for known CFTR gating mutations (G551D, G178R, G551S, G970R, G1244E, S1255P, and G1349D) [5,9–11], the amount of CFTR delivered to the cell surface was generally similar between CFTR with gating defects and normal CFTR. In contrast, only a small amount of F508del-CFTR was delivered to the cell surface (Fig. 1B–D). Interestingly, there was significantly more G551S-CFTR at the cell surface than normal CFTR, although the total amount of G551S-CFTR synthesized and the ratio of mature to total CFTR were similar to normal CFTR.

2.2. Ivacaftor increased the channel gating of mutant CFTR with defective channel gating

The effect of ivacaftor on CFTR channel gating was monitored by quantifying the channel open probability by patch-clamp electrophysiology using membrane patches excised from FRT cells expressing the known CFTR gating mutations, G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, or G1349D-CFTR. To activate CFTR prior to ivacaftor addition, PKA (75 nM) and ATP (1 mM) were added to the cytoplasmic surface (bath solution) of the membrane patch. Under these conditions, the baseline CFTR channel open probability of G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR was ≤ 5% of normal CFTR (Fig. 2, B; Table 1). For most mutant CFTR forms, the single channel current amplitude, a measure of channel conductance, was similar to normal CFTR (between 77% and 122% of normal CFTR), although a small but statistically significant difference in single channel current amplitude was observed for S1255P-CFTR (Table 1). Acute (5 min) addition of 10 μM ivacaftor increased the channel open probability of all CFTR gating mutations tested, reaching levels equivalent to 30% to 118% of normal CFTR (Fig. 2B; Table 1). No change in the single channel current amplitude was observed following ivacaftor addition (Table 1). These data indicated that ivacaftor increased the channel open probability of all mutant CFTR forms with defective channel gating tested to a similar or greater extent than G551D-CFTR.
2.3. Ivacaftor enhanced chloride transport through mutant CFTR with defective channel gating

The impact of the increase in CFTR channel gating by ivacaftor on total chloride transport was assessed in Ussing chamber studies using FRT cells expressing the known CFTR gating mutations, G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR. To activate CFTR prior to ivacaftor addition, 10 μM forskolin was added to the bath to increase the intracellular levels of cAMP and activate PKA. The level of chloride transport for each mutant CFTR form was expressed as a percentage of that measured in four separate FRT cell lines expressing normal CFTR (% normal-CFTR; 204.5±29.8 μA/cm²). Under these conditions, the baseline level of chloride transport in FRT cells expressing G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR was <10% of normal CFTR (Fig. 3; Table 2), which was consistent with the low CFTR channel open probability of these mutant CFTR forms (Table 1). Acute (5-min) ivacaftor addition caused a >10-fold increase over baseline chloride transport (Fig. 3; Table 2). Compared to all CFTR gating mutations tested, the ivacaftor response in FRT-cells expressing F508del-CFTR was minimal (Fig. 3). The EC50 of ivacaftor for all mutant CFTR forms tested was similar to G551D-CFTR (range: 161 to 594 nM) (Fig. 3C; Table 2).

2.4. Identification of additional CFTR gating mutations and effects of ivacaftor

Several of the known CFTR gating mutations cause defects in one of the two ATP binding pockets formed by the two NBDs known to regulate CFTR channel gating [2]. The CF-associated CFTR mutations, S549N, S549R, and S1251N, are located in the ABC signature sequence (Fig. 4; LSGGQ; S549N,R in NBD1) and Walker A motif (Fig. 4; S1251N in NBD2), that form the ATP binding sites [14]. Immunoblot and Ussing chamber studies indicated that, although the maturation of S549N- and S1251N-CFTR was similar to normal, the baseline level of chloride transport was <10% of normal CFTR (Figs. 1 and 3; Table 2), which did not account for the <1% of normal CFTR function (Fig. 3; Table 2). These results indicated that the function of S549N-, S549R-, and S1251N-CFTR at the cell surface was minimal. Patch-clamp studies confirmed that the channel open probability of S549N-, S549R-, and S1251N-CFTR was <5% of normal CFTR, whereas the single channel current amplitude was minimal. Patch-clamp studies confirmed that the channel open probability of S549N-, S549R-, and S1251N-CFTR was <5% of normal CFTR.
In FRT cells expressing S549N-, S549R-, and S1251N-CFTR, acute addition of 10 μM ivacaftor increased the channel open probability and total chloride transport by >10-fold over baseline levels (Figs. 2 and 3; Tables 1 and 2). The EC50 for ivacaftor in FRT cells expressing S549N-, S549R-, and S1251N-CFTR were similar to G551D-CFTR (Fig. 3C; Table 2). These results indicated that S549N-, S549R-, and S1251N-CFTR were potentiated by ivacaftor in vitro.

3. Discussion

CFTR gating mutations result in CFTR which is present at the cell surface but does not open or close normally, resulting in a low CFTR channel open probability and the loss of epithelial chloride transport as determined in electrophysiological studies. In this study we focused on CFTR gating mutations resulting in minimal channel function and generally associated with severe CF. The most common CF-causing CFTR gating mutation is G551D [6] but several other CFTR gating mutations have been previously reported [9–11]. In a panel of FRT cells expressing G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR, we confirmed that all these mutant CFTR forms shared similar in vitro functional characteristics that were consistent with a defect in channel gating. In addition, we showed that the 3 additional mutations, S549N, S549R, and S1251N also have characteristics consistent with gating defects. These characteristics included the delivery of CFTR to the cell surface, a low (<5%) CFTR channel open probability, and minimal (<10% normal CFTR) baseline levels of chloride transport.

Ivacaftor addition caused a >10-fold increase in CFTR-mediated chloride transport in FRT cells expressing G551D-, G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR. For all CFTR gating mutations tested, the level of chloride transport achieved in the presence of ivacaftor was >10% of normal CFTR, which exceeds the level of chloride transport associated with lower sweat chloride levels and a mild CF phenotype [15]. Moreover, in patients with CF who have the G551D CFTR gating mutation, administration of ivacaftor was associated with improvements in CFTR function, as determined by a reduction in sweat chloride concentrations, and improvements in lung function, as determined by an increase in the FEV1 [13]. Taken together, these in vitro results provide a rationale for testing the potential benefit of ivacaftor in individuals with CF who have a CFTR gating mutation other than G551D, including the G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1255P-, and S1251N- CFTR. For all CFTR gating mutations tested, the level of chloride transport achieved in the presence of ivacaftor was >10% of normal CFTR, which exceeds the level of chloride transport associated with lower sweat chloride levels and a mild CF phenotype [15]. Moreover, in patients with CF who have the G551D CFTR gating mutation, administration of ivacaftor was associated with improvements in CFTR function, as determined by a reduction in sweat chloride concentrations, and improvements in lung function, as determined by an increase in the FEV1 [13]. Taken together, these in vitro results provide a rationale for testing the potential benefit of ivacaftor in individuals with CF who have a CFTR gating mutation other than G551D, including the G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR. For all CFTR gating mutations tested, the level of chloride transport achieved in the presence of ivacaftor was >10% of normal CFTR, which exceeds the level of chloride transport associated with lower sweat chloride levels and a mild CF phenotype [15]. Moreover, in patients with CF who have the G551D CFTR gating mutation, administration of ivacaftor was associated with improvements in CFTR function, as determined by a reduction in sweat chloride concentrations, and improvements in lung function, as determined by an increase in the FEV1 [13]. Taken together, these in vitro results provide a rationale for testing the potential benefit of ivacaftor in individuals with CF who have a CFTR gating mutation other than G551D, including the G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR. For all CFTR gating mutations tested, the level of chloride transport achieved in the presence of ivacaftor was >10% of normal CFTR, which exceeds the level of chloride transport associated with lower sweat chloride levels and a mild CF phenotype [15]. Moreover, in patients with CF who have the G551D CFTR gating mutation, administration of ivacaftor was associated with improvements in CFTR function, as determined by a reduction in sweat chloride concentrations, and improvements in lung function, as determined by an increase in the FEV1 [13].
normal CFTR and did not appear to be sufficient to account for the <1% normal CFTR chloride transport in FRT cells expressing this CFTR mutation. Patch-clamp studies showed that the channel open probability of S549R-CFTR was less than 1% of normal CFTR. These results indicated that although the predominant defect caused by the S549R CFTR mutation was defective CFTR channel gating, a mild processing defect was also observed. The apparent mild processing defect in S549R-CFTR may account for the lower ivacaftor response in FRT cells expressing S549R-CFTR compared to the other mutant CFTR forms with gating defects and normal CFTR maturation.

The low baseline levels of chloride transport in FRT cells expressing mutant CFTR forms encoded by the CFTR gating mutations studied here were consistent with the clinical characteristics of patients with CF who carry one of these CFTR gating mutations. Patients with CF who carry the G551D CFTR gating mutation have minimal amounts of CFTR function, as determined by high (~100 mmol/L) sweat chloride concentrations, and a severe CF phenotype [8,17]. Although there are limited case studies on patients with CF who have CFTR gating mutations beyond G551D, they also appear to have high sweat chloride concentrations and severe CF [18–20]. This was consistent with the low CFTR channel open probability and minimal (<10% normal CFTR) baseline levels of chloride transport measured in FRT cells expressing these CFTR gating mutations. A milder CF clinical phenotype has been associated with the G551S CFTR gating mutation, as demonstrated by a lower sweat chloride concentration (75–94 mmol/L) and lower incidence of pancreatic insufficiency compared to patients with CF who carry the G551D CFTR gating mutation [21]. In the present study, although the expression and channel open probability of G551S-CFTR were similar to G551D-CFTR, the baseline chloride transport was higher (~9% normal CFTR). This may be due to the increased level of mature G551S-CFTR delivered to the cell surface compared to normal CFTR, as determined by immunoblot studies in FRT cells.

The potency (EC50) of ivacaftor for the CFTR gating mutations tested was similar to G551D in vitro, suggesting that a similar dose of ivacaftor as that used in clinical trials of patients with CF who carry the G551D CFTR gating mutation may be appropriate for most other CFTR gating mutations. Previous in vitro studies using recombinant cells and primary human airway cultures have shown that higher concentrations of ivacaftor were required to potentiate G551D-CFTR compared to normal- and F508del-CFTR [12]. The potency of other CFTR potentiators has also been shown to be lower for G551D-CFTR compared to normal- or F508del-CFTR [12,22–25]. This suggests that some CFTR mutations may alter interaction of CFTR potentiators with CFTR to affect channel gating. Further studies will be needed to identify the ivacaftor binding site on CFTR to test this hypothesis.

CFTR potentiators from structural classes distinct from ivacaftor have also been shown to potentiate mutant CFTR with defective channel gating. These include derivatives of 1,4-dihydropyridine and phenylglycine, such as ivacaftor and phloxine B strongly potentiated G551D-CFTR [23,25]. In contrast, sulfamoyl-4-oxoquinoline-3-carboxamides were weakly effective on G551D-, G970R-, and S549R-CFTR [25]. This suggests that some CFTR mutations may alter interaction of CFTR potentiators with CFTR to affect channel gating. Further studies will be needed to identify the ivacaftor binding site on CFTR to test this hypothesis.

The gating of the CFTR channel pore is a tightly regulated process, requiring phosphorylation of the regulatory domain by PKA and subsequent ATP binding and hydrolysis by the NBDs [2]. Like G551D, the G551S, G1244E, S1255P, and G1349D CFTR gating mutations, as well as the S549N, S549R, and S1251N CFTR gating mutations identified in the
A

- Baseline
- With 10 μM Ivacaftor

Normal

Forskolin

G178R
G551D
G178R

S549N
S549R
G551S
G970R

G1244E
S1251N
S1255P
G1349D

90 μA/cm²
1 min

B

Baseline
With 10 μM Ivacaftor

Chloride Transport (µA/cm²)

CFTR Mutation

C

Chloride Transport (µA/cm²)

Ivacaftor [Log M]

G551D
G178R
S549N
S549R
G551S

Chloride Transport (% Normal CFTR)

G970R
G1244E
S1251N
S1255P
G1349D
The present study, cause protein alterations in the ATP binding pockets formed by the two NBDs required for normal CFTR channel gating (Fig. 4) [2]. The G178R and G970R CFTR gating mutations alter the intracellular cytoplasmic loops that are believed to link the ATP-driven conformational changes in the NBDs to the opening of the CFTR channel pore formed by the membrane spanning domains [27]. Given the importance of these regions in driving CFTR channel opening and closing, it was not surprising that CFTR mutations associated with these regions caused severe defects in CFTR channel gating. In vitro, ivacaftor potentiated mutant CFTR forms associated with alterations in the ATP binding pockets and the cytoplasmic loops linking the CFTR channel pore to the movement of the NBDs, suggesting that ivacaftor may bypass or augment ATP-dependent channel gating of mutant CFTR to potentiate the function of PKA-activated CFTR. Additional studies are needed to determine the ATP dependence and molecular mechanism of CFTR potentiation by ivacaftor.

In previous in vitro studies ivacaftor potentiated chloride transport by normal and F508del-CFTR [12]. Along with the data presented here on multiple mutant CFTR forms with defective channel gating, ivacaftor appeared to be a broad-acting CFTR potentiator. This suggests that ivacaftor may act on other mutant CFTR forms that are delivered to the cell surface in sufficient amounts. For example, R117H-CFTR is delivered to the cell surface in normal amounts, but exhibits a ~20% reduction in CFTR channel conductance and a ~75% reduction in the channel open probability, resulting in residual CFTR function both in vitro and clinically [28]. Other CFTR gene mutations associated with residual CFTR function include A445E, R347H, D1152H, and certain splice mutations (3849 +10kbC→T) [4,29–30]. Further in vitro studies and clinical measurements are needed to evaluate the ivacaftor response and potential clinical benefit for CFTR mutations beyond CFTR gating mutations.

In conclusion, ivacaftor increased the channel gating activity of all mutant CFTR forms associated with CFTR gating mutations tested in this study, resulting in enhanced chloride transport. This suggested that ivacaftor is not a mutation-specific CFTR potentiator, as it overcomes the underlying molecular defect caused by G551D and a number of other CFTR gating mutations. As a group these CFTR gating mutations shared the following characteristics; 1) defective CFTR channel gating as the predominant molecular defect, 2) residual or normal amounts of CFTR present at the cell surface, 3) minimal baseline chloride transport in vitro (<10% normal), and 4) a pronounced (>10-fold) increase over baseline chloride transport in response to ivacaftor. The in vitro data presented here suggest that ivacaftor has a similar effect on all CFTR forms with gating defects and support the investigation of ivacaftor in patients with CF who have CFTR gating mutations beyond G551D, including G178R, S549N, S549R, G551S, G970R, G1244E, S1251N, S1255P, and G1349D.

4. Methods

4.1. Cell line generation

A panel of FRT cell lines expressing different CFTR mutations was generated using a host cell line with a single integration site as described in the supplementary material.

4.2. RNA analysis

Total RNA was isolated and real-time PCR assays were performed as described in the Supplementary material.

4.3. Electrophysiology

Ussing chamber techniques using FRT cells were used to record the I\textsubscript{T} due to CFTR-mediated chloride transport. The single-channel activity of CFTR was measured using excised inside-out membrane patch recordings (see Supplementary material).

4.4. CFTR immunoblot analysis

Immunoblot techniques using the monoclonal CFTR antibody 769 (J. Riordan, University of North Carolina) were used to measure CFTR maturation in FRT, HEK-293, or HBE cells expressing wild-type- or F508del-CFTR (see Supplementary material).

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Fig. 3. Effect of ivacaftor on chloride transport in FRT cells expressing CFTR gating mutations associated with CF. A) Representative recordings of the forskolin-stimulated chloride transport in the absence (baseline; dotted line) and presence (solid line) of 10 µM ivacaftor in FRT cell expressing normal CFTR or the CFTR gating mutation indicated. F508del-CFTR was included as a reference. Forskolin (10 µM; arrows) was added to the bath to activate CFTR by increasing the intracellular concentrations of cAMP. B) The mean (±SEM; n=4–6) forskolin-mediated chloride transport (left y-axis) with 10 µM forskolin in the absence (baseline; open bars) or in the presence (filled bars) of 10 µM ivacaftor in FRT cells expressing normal CFTR and the mutant CFTR proteins indicated. To compare to normal CFTR function, the forskolin-stimulated chloride transport in FRT cells expressing mutant CFTR was normalized to the forskolin-stimulated chloride transport in 4 separate FRT cell lines expressing normal CFTR (204.5±29.8 µA/cm\textsuperscript{2}) and expressed as % normal CFTR (right y-axis). Asterisks indicate significant difference compared to baseline (p<0.05; paired t-test; n=4–6). C) Concentration response relationship for ivacaftor in FRT cells expressing the CFTR gating mutations indicated (mean±SEM; n=4–6).
Table 2
Baseline chloride transport and pharmacological effect of ivacaftor in cultured FRT cells expressing CFTR gating mutations.

<table>
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<tr>
<th>Mutation</th>
<th>Chloride transport (μA/cm²)</th>
<th>Chloride transport (% normal)</th>
<th>Fold change a</th>
<th>EC50 b</th>
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<td>Baseline</td>
<td>With ivacaftor</td>
<td>Baseline</td>
<td>With ivacaftor</td>
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<td>79.3±4.1 c</td>
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a The fold change in chloride transport was determined by dividing the level of chloride transport (% normal) in the presence of ivacaftor by the baseline chloride transport.
b No significant differences (P > 0.05; unpaired t-test, n = 4–6) compared to G551D-CFTR was observed for any of the mutant CFTR forms tested.
c Significant difference compared to baseline (p < 0.05; paired t-test; n = 4–6).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jcf.2011.12.005.

References


