

# Characterization of the Mechanism of Action of RDR01752, a Novel Corrector of F508del-CFTR

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## Abstract

**Background and Purpose:** Despite progress in developing pharmacotherapies to rescue F508del-CFTR, the most prevalent Cystic Fibrosis (CF)-causing mutation, individuals homozygous for this mutation still face several disease-related symptoms. Thus, more potent compound combinations are still needed. Here, we investigated the mechanism of action (MoA) of RDR01752, a novel F508del-CFTR trafficking corrector. **Experimental approach:** F508del-CFTR correction by RDR01752 was assessed by biochemical, immunofluorescence microscopy and functional assays in cell lines and in intestinal organoids. To determine the MoA of RDR01752, we assessed its additive effects to those of genetic revertants of F508del-CFTR, the FDA-approved corrector drugs VX-809 and VX-661, and low temperature. **Key Results:** Our data demonstrated that RDR01752 rescues F508del-CFTR processing and plasma membrane (PM) expression to similar levels of VX-809 in cell lines, although RDR01752 produced lower functional rescue. However, in functional assays using intestinal organoids (F508del/F508del), RDR01752, VX-809 and VX-661 had similar efficacy. RDR01752 demonstrated additivity to revertants 4RK and G550E, but not to R1070W, as previously shown for VX-809. RDR01752 was also additive to low temperature. Co-treatment of RDR01752 and VX-809 further increased F508del-CFTR function compared to each corrector alone, but not PM expression. **Conclusion and Implications:** The lack of additivity of RDR01752 with the genetic revertant R1070W suggests that this compound has the same effect as the insertion of tryptophan at 1070, i.e., filling the pocket at the NBD1:ICL4 interface in F508del-CFTR, similarly to VX-809. Combination of RDR01752 with correctors mimicking the rescue by revertants G550E or 4RK could thus maximize rescue of F508del-CFTR.

## Characterization of the Mechanism of Action of RDR01752, a Novel Corrector of F508del-CFTR

*Running title:* Rescue of F508del-CFTR by corrector RDR01752

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## Conflict of interest statement

There is no conflict of interest related to this work.

## ABSTRACT (248 words; max 250)

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**Experimental approach:** F508del-CFTR correction by RDR01752 was assessed by biochemical, immunofluorescence microscopy and functional assays in cell lines and in intestinal organoids. To determine the MoA of RDR01752, we assessed its additive effects to those of genetic revertants of F508del-CFTR, the FDA-approved corrector drugs VX-809 and VX-661, and low temperature.

**Key Results:** Our data demonstrated that RDR01752 rescues F508del-CFTR processing and plasma membrane (PM) expression to similar levels of VX-809 in cell lines, although RDR01752 produced lower functional rescue. However, in functional assays using intestinal organoids (F508del/F508del), RDR01752, VX-809 and VX-661 had similar efficacy. RDR01752 demonstrated additivity to revertants 4RK and G550E, but not to R1070W, as previously shown for VX-809. RDR01752 was also additive to low temperature. Co-treatment of RDR01752 and VX-809 further increased F508del-CFTR function compared to each corrector alone, but not PM expression.

**Conclusion and Implications:** The lack of additivity of RDR01752 with the genetic revertant R1070W suggests that this compound has the same effect as the insertion of tryptophan at 1070, i.e., filling the pocket at the NBD1:ICL4 interface in F508del-CFTR, similarly to VX-809. Combination of RDR01752 with correctors mimicking the rescue by revertants G550E or 4RK could thus maximize rescue of F508del-CFTR.

**Keywords:** cystic fibrosis, protein trafficking, revertants, low temperature, intestinal organoids, drug discovery.

## INTRODUCTION

Cystic fibrosis (CF) is the most common life-threatening autosomal recessive disease among Caucasians, affecting almost 50,000 individuals in Europe (ECFS Patient Registry, 2017). It is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, which functions as a chloride (Cl<sup>-</sup>) and bicarbonate (HCO<sub>3</sub><sup>-</sup>) channel at the apical plasma membrane (PM) of epithelial cells (Riordan,

2008). CF-causing mutations cause channel dysfunction, leading to abnormal ion transport and dehydration of epithelia in several tissues (De Boeck & Amaral, 2016; Lopes-Pacheco, 2016). Although CF is a multi-organ disease, the respiratory disorder represents the major cause of morbidity and mortality of individuals with CF due to airway obstruction by a thick mucus, chronic inflammation and persistent infections, which ultimately result in respiratory failure (De Boeck & Amaral, 2016; Lopes-Pacheco, 2016).

CFTR protein is composed of two transmembrane domains (TMD1/2), two nucleotide-binding domains (NBD1/2) and a regulatory domain (RD). The TMDs form the pore through which anions are conducted along their electrochemical gradient, while the NBDs regulate channel gating by binding and hydrolyzing ATP and after RD phosphorylation at multiple sites. Interdomain interactions are critical for this complex protein to achieve its native conformation state (Riordan, 2008).

Over 2,000 CFTR gene variants have been reported so far (<http://www.genet.sickkids.on.ca/>), with deletion of a phenylalanine at position 508 (F508del in NBD1) being the most prevalent and occurring in ~80% of individuals with CF in Europe, albeit with some geographic variability (ECFS Patient Registry, 2017). F508del causes CFTR protein misfolding that is recognized by the endoplasmic reticulum (ER) quality control (ERQC) machinery and prematurely degraded by the proteasome (Jensen *et al.*, 1995). Rescue of F508del-CFTR was first demonstrated by low temperature incubation of cells heterologously expressing this mutant (Denning *et al.*, 1992), thus proving that this mutant is both temperature sensitive and rescuable.

Over the past decade, significant efforts have been put into high-throughput screening (HTS) of small molecule libraries to identify compounds that rescue the F508del-CFTR protein to the PM. To date, there are three correctors approved for clinical use by the Food and Drug Administration (FDA), being two also approved by the European Medicine Agency (EMA) (all combined with potentiator VX-770/ivacaftor): VX-809/lumacaftor, VX-661/tezacaftor and VX-445/elexacaftor (only FDA-approved). In clinical trials, individuals who were F508del-homozygous and treated with either VX-809 or VX-661 plus VX-770 demonstrated a significant, albeit modest, improvement in lung function (Wainwright *et al.*, 2015; Taylor-Cousar *et al.* 2017). More recently, VX-445 was added to the co-treatment with VX-661/VX-770 and this triple combination demonstrated greater therapeutic benefit in phase 3 clinical trials (Heijerman *et al.*, 2019; Middleton *et al.*, 2019), thus leading to its FDA-approval in individuals with CF, aged [?]12 years and with the F508del mutation in at least one allele.

Despite such progress, individuals with CF still face several disease-related symptoms and complications, including a progressive deterioration of lung function, and thus novel correctors are still needed to achieve more potent combinations. Furthermore, there are other CFTR trafficking mutants that are not efficiently rescued by available correctors, including G85E and N1303K (Dekkers *et al.*, 2016; Lopes-Pacheco *et al.*, 2017). Along these lines, the novel RDR01752 compound was identified as a F508del-CFTR traffic corrector in a small-scale screen (Carlile *et al.*, 2007) and demonstrated to thermally stabilize purified murine F508del-NBD1 *in vitro* (Sampson *et al.*, 2011). However, its mechanism of action (MoA) remains to be elucidated.

Here, we investigated the MoA of RDR01752 in cell lines stably expressing either F508del-CFTR or other CFTR mutants, and in F508del/F508del intestinal organoids. The MoA of RDR01752 was explored by analyzing its additive effects to those of previously described CFTR genetic revertants. These are second-site mutations, i.e., in *cis* with F508del that partially rescue F508del-CFTR. One of these revertants results from removal of the arginine-framed motifs (AFT) acting as retention signals (4RK), thus allowing the mutant protein to escape the ERQC (Chang *et al.*, 1999; Roxo-Rosa *et al.*, 2006; Farinha *et al.*, 2013). Two others work by correcting folding at critical structural pockets present in the 3D-structure of F508del-CFTR that are absent in wild-type (WT)-CFTR. These include G550E that acts by stabilizing the NBD1:NBD2 dimer interface (Roxo-Rosa *et al.*, 2006) and R1070W that restores the NBD1:ICL4 interaction (Serohijos *et al.*, 2008; Thibodeau *et al.*, 2010; Farinha *et al.*, 2013). We also investigated the effects of RDR01752 on the DD/AA variant on the background of WT-CFTR, which lacks the double diacidic code necessary for Sec24-CFTR association and ER exit and thus is retained in the ER but is not misfolded (Wang *et al.*, 2004; Farinha *et al.*, 2013). Finally, we tested RDR01752 in combination with low temperature and the FDA-approved corrector drugs VX-809/lumacaftor and VX-661/tezacaftor (and compound C18) with and

without chronic exposure of the potentiator VX-770/ivacaftor.

## METHODS

*Cell culture.* The CF bronchial epithelial (CFBE) cell line stably expressing F508del-CFTR was cultured as before (Canato *et al.*, 2018). CFBE cells stably expressing mCherry-Flag-CFTR (WT, F508del, DD/AA variants or carrying G550E, R1070W, 4RK in *cis* with F508del) were cultured and CFTR expression was induced with doxycycline (Dox) 1  $\mu\text{g}/\text{mL}$  as described (Botelho *et al.*, 2015). Fischer rat thyroid (FRT) epithelial cells stably expressing CFTR variants (wt, G85E, R334W, T338I, R347P, F508del, V520F, S549F, G551D, M1101K, N1303K) were cultured as before (Turner *et al.*, 2020). All cell lines were maintained in a humidified incubator at 5%  $\text{CO}_2$  and 37°C, except during low temperature experiments, in which cells were incubated at 27°C for 24h.

*Chemicals.* All reagents were of the highest purity available. Corrector compounds were either commercially obtained: RDR01752 (STK001879, Vitas-M Lab., IL, USA), VX-809, VX-661 and VX-770 (S1565, S7058 and S1144, Selleckchem, TX, USA) or from CFFT: C18. Correctors were diluted in dimethyl sulfoxide (DMSO) and added to cells diluted in 1% FBS supplemented medium at indicated concentrations: 1 to 20  $\mu\text{M}$  RDR01752, 1 or 3.7  $\mu\text{M}$  VX-809, 5  $\mu\text{M}$  VX-661, 5  $\mu\text{M}$  C18, 3  $\mu\text{M}$  VX-770. Other reagents (all from Sigma, MO, USA, in DMSO solutions) were (final concentrations, unless otherwise stated): 2  $\mu\text{M}$  forskolin (Fsk), 50  $\mu\text{M}$  genistein (Gen), 100  $\mu\text{M}$  3-Isobutyl-1-methylxanthine (IBMX) and 30  $\mu\text{M}$  CFTR channel inhibitor CFTR<sub>Inh-172</sub>, (Inh<sub>172</sub>).

*Western blotting (WB).* Whole-cell lysates were subjected to SDS-PAGE 7% (w/v) gel analysis followed by CFTR detection using monoclonal anti-human CFTR antibody (596 [1:3000] from CFFT) as previously (Farinha *et al.*, 2013). Anti- $\alpha$ -tubulin antibody (1:10,000, Sigma) was used as a loading control. CFTR quantification was as described (Canato *et al.*, 2018) and its processing obtained by the ratio between the mature form and total CFTR (mature and immature forms).

*Immunostaining.* Twenty-four hours after seeding stably expressing mCherry-Flag-CFTR CFBE cells (carrying wt-, F508del-, DD/AA- or -G550E-F508del, R1070W-F508del, 4RK-F508del-CFTR) onto 384-well plates, compounds were concomitantly administered with Dox. After 48h, cells were immunostained and CFTR PM expression quantified as described (Botelho *et al.*, 2015).

*HS-YFP assay on the plate reader.* Measurements of CFTR activity were carried out on CFBE cells expressing both F508del-CFTR and HS-YFP (YFP-H148Q/I152L) as described (Sondo *et al.*, 2011). The assay consists of a continuous 14-s fluorescence read-out 2s before and 12s after injection of an iodide-containing solution (PBS with  $\text{I}^-$  at final concentration 100mM). Fluorescence quenching rate of  $\text{I}^-$  influx was determined for the final 11s of the data for each well and was fitted to an exponential function to extrapolate initial slope (Sondo *et al.*, 2011).

*FLIPR membrane potential (FMP) assay.* Measurements of FMP to assess CFTR function were performed in FRT cells expressing various CFTR mutants as described (Carlile *et al.*, 2018).

*Micro-Ussing chamber measurements.* Transepithelial electrical resistance (TEER) of CFBE cells growing on Snapwell inserts was measured as before (Awatade *et al.*, 2015). Briefly, monolayers with resistance [?]450  $\Omega\text{cm}^2$  were mounted in micro-Ussing chambers with perfusion for recordings under open-circuit conditions as described (Awatade *et al.*, 2015). Changes in transepithelial voltage ( $V_{\text{te}}$ ) were continuously recorded and equivalent Fsk/IBMX-stimulated short-circuit currents ( $I_{\text{eq-sc}}$ ) were calculated by Ohm's law from  $V_{\text{te}}$  and  $R_{\text{te}}$  ( $I_{\text{eq-sc}} = V_{\text{te}}/R_{\text{te}}$ ). For FRT cell lines, transepithelial voltage was measured at 37°C with continuous stirring by gassing with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  as described (Turner *et al.*, 2020).

*Organoid swelling assay.* The forskolin-induced swelling (FIS) assay was performed as described (Dekkers *et al.*, 2016). Twenty-four hours after seeding, organoids were stimulated with Fsk with or without a potentiator and live-cell imaging was performed using bright field microscopy (Leica DMI6000B) with a 5 $\times$  objective for 60 min at 37°C. For quantification of the area under the curve (AUC;  $t = 60$  min, baseline = 100%) a

CellProfiler-based algorithm was used (Hagemeyer *et al.*, in preparation). Experiments were performed in triplicate and repeated 3-4 times.

*Statistical analyses.* Statistical comparisons were made using one-way ANOVA followed by Dunnett's or Tukey's *post-hoc* tests for parametric data or using Kruskal-Wallis test followed by Dunn's *post-hoc* test for nonparametric data (GraphPad Prism v.6.01). *P* values <0.05 were considered significant.

## RESULTS

### *RDR01752 rescues F508del-CFTR processing, PM traffic and channel function*

Incubation of CFBE cells expressing F508del-CFTR with the RDR01752 compound rescued F508del-CFTR processing, resulting in the appearance of the fully-glycosylated form of CFTR (~180 kDa, band C) in a dose-dependent manner with the maximal correction achieved at 10  $\mu$ M (Fig. 1A,B). This effect was comparable to that obtained for VX-809 or VX-661, and in contrast to that obtained for DMSO (vehicle), which only led to the appearance of the core-glycosylated form of CFTR (~140 kDa, band B). This result was also confirmed by the immunofluorescence detection of the Flag-tag of mCherry-Flag-F508del-CFTR expressed in CFBE cells without cell permeabilization, only in cells treated with RDR01752 or VX-809, but not DMSO (Fig. 1C,D).

To assess the ability of RDR01752 to restore F508del-CFTR function, we first measured the rate of HS-YFP quenching induced by iodide influx into cells in CFBE cells stably co-expressing F508del-CFTR and the HS-YFP (Fig. 2A,B). Both RDR01752 (10  $\mu$ M) and VX-809 demonstrated rescue of F508del-CFTR function, although the efficacy of RDR01752 was lower than that of VX-809. In order to confirm these findings, we then investigated rescue of F508del-CFTR function by RDR01752 in polarized CFBE cells in the Ussing chamber (Fig. 2C-F). A significant increase in equivalent short-circuit current ( $\Delta I_{sc_{eq}}$ ) was observed upon Fsk/IBMX stimulation in cells incubated with either RDR01752 or VX-809 and stimulated with potentiator genistein (Gen) *versus* those incubated with DMSO alone. These data are consistent with the rate of HS-YFP quenching.

### *F508del/F508del intestinal organoids respond positively to RDR01752*

Next, we tested the effects of RDR01752 using the FIS assay in intestinal organoids with the F508del/F508del genotype. Organoids were incubated with RDR01752, VX-809 or VX-661 for 24h and then acutely stimulated (30 min) with Fsk with or without a potentiator (VX-770 or Gen) to further enhance CFTR function (Figs. 3 and S1). Significant swelling was observed in organoids incubated with RDR01752, VX-809 or VX-661 and acutely stimulated with either VX-770 or Gen, in contrast to absence of swelling in organoids without any potentiator or DMSO control. Interestingly, similar swelling values were observed for organoids incubated with any corrector plus potentiator combination.

### *RDR01752 increases the rescue of F508del-CFTR PM in cells expressing in cis the genetic revertants G550E and 4RK or low temperature, but not in R1070W nor DD/AA*

In order to characterize the mechanism of action (MoA) by which RDR01752 rescues F508del-CFTR, we investigated revertants of this mutant. To this end, CFBE cell lines stably expressing double tagged-F508del-CFTR *in cis* with the following genetic revertants: G550E, R1070W, or 4RK were incubated with this compound. In parallel CFBE cells expressing WT-CFTR or the traffic-null variant DD/AA (on a WT background) were also treated with RDR01752. Cells were incubated both at 37°C and low temperature (27°C), and PM expression of each CFTR variant was assessed by immunofluorescence (Fig. 4A,B, respectively) and normalized to cells incubated with DMSO at 37°C (upper heatmap, first row).

Each of the correctors tested RDR01752, VX-809 or VX-661 rescued F508del-CFTR to the PM with similar efficacy (Fig. 4A) which was further enhanced when incubated at 27°C (Fig. 4B). These compounds also increased WT-CFTR PM expression, although VX-809 and VX-661 showed higher efficacy than RDR01752. A small additive effect was found for PM levels of wt-CFTR with each of these compounds and low temperature.

Analysis of the effects on the revertants demonstrated that RDR01752, similarly to VX-809 and VX-661, is additive to G550E and 4RK in F508del-CFTR PM rescue (Fig.4A), and further additive to that of low temperature (Fig. 4B). In contrast, RDR01752, also similarly to VX-809 and VX-661, did not demonstrate additivity with R1070W (Fig. 4A), thus suggesting that, like VX-809 (Farinha *et al.*, 2013), it might share a common mechanism. Furthermore, an increase in PM expression of DD/AA-CFTR variant was not observed (Fig. 4A), except when cells were incubated at low temperature (Fig. 4B), as before (Farinha *et al.*, 2013), with no significant additive effect elicited by any of the correctors.

#### *Rescuing of F508del-CFTR traffic by RDR01752 is not additive to VX-809 or VX-661*

Next, we tested whether rescuing by RDR01752 is additive to that of other correctors, namely FDA-approved drugs VX-809 and VX-661 (Fig. 5) or C18 (Fig. S2). We also evaluated whether chronic exposure to VX-770 affects the rescue of F508del-CFTR by RDR01752 (Figs. 5 and S2). CFBE cells stably expressing mCherry-Flag-F508del-CFTR were incubated with each corrector for 48h and CFTR PM levels were quantified as above. None of the two-corrector combinations further enhance F508del-CFTR PM expression compared to each corrector alone (Fig. 5). Similarly to VX-809 or VX-661, rescue of F508del-CFTR by RDR01752 under chronic exposure to a relatively high concentration of VX-770 resulted in a decrease of F508del-CFTR PM expression (Fig. S2). The inhibitory effect of chronic exposure to VX-770 on F508del-CFTR rescue by VX-809 or VX-661 exposure observed here, as previously reported by others (Cholon *et al.*, 2014; Veit *et al.*, 2014), was even more pronounced for any combination of two correctors.

#### *Functional rescue of other CFTR mutants by RDR01752*

To evaluate the ability of RDR01752 to rescue other CFTR mutants, we used an FMP assay in FRT cells to measure the depolarization that occurs when CFTR PM channels are activated. The nine missense mutants studied are located across the different CFTR domains, namely in: TMD1 (G85E, R334W, T338I and R347P); NBD1 (V520F, S549F and G551D); TMD2 (M1101K); and NBD2 (N1303K). Cells expressing these CF-causing mutations were incubated with either RDR01752 or VX-809 for 24h (Fig. 6). An increase in the Fsk+Gen response was observed in F508del-expressing FRT cells treated with either RDR01752 or VX-809, indicating that CFTR function was rescued by RDR01752, consistent with both the  $\Delta I_{sc_{eq}}$  data and the rate of HS-YFP quenching observed in CFBE cells. Both RDR01752 and VX-809 also rescue CFTR function in R334W-, V520F- and M1101K-expressing cells. However, in this assay only RDR01752 (and not VX-809) demonstrated an effect on G85E- and T338I-expressing cells, while R347P-, S549F- and N1303K-expressing cells only responded to VX-809 treatment, albeit the latter at very low levels (Fig. 6). No significant differences were observed in CFTR function between G551D- and WT-expressing cells after treatment with either RDR01752 or VX-809 compared to control.

To confirm these findings, we investigated the effects of RDR01752 and VX-809, alone or combined, in polarized FRT cells expressing the mutants that showed rescue, namely F508del-, G85E-, N1303K- or R334W-CFTR by Ussing chamber measurements (Fig. 7). An increase of CFTR-dependent  $Cl^-$  secretion was observed in F508del-expressing cells incubated with either VX-809 or RDR01752, albeit much lower for the latter. Incubation with both compounds combined resulted in a significantly greater rescue of F508del-CFTR function in comparison to VX-809 alone. Furthermore, although the rescue of R334W-CFTR by RDR01752 was not higher than that by VX-809 (as in the FMP assay), co-administering the two compounds did increase CFTR-dependent  $Cl^-$  secretion. No significant effects were found in G85E- and N1303K-expressing cells in CFTR function after incubation with either RDR01752 or VX-809, alone or combined.

## **DISCUSSION AND CONCLUSIONS**

The aim of this study was to characterize the effects of corrector RDR01752 both in cell lines that stably express F508del-CFTR or other rare CFTR mutations and in intestinal organoids that are F508del/F508del. Furthermore, we investigated the MoA of RDR01752 by evaluating its additivity with available corrector drugs, genetic revertants of F508del-CFTR, and low temperature.

Most CF drug development programs for CFTR modulators have focused on the rescue of F508del-CFTR

as the most prevalent disease-causing mutant. Despite significant progress in restoring F508del-CFTR trafficking by corrector drugs, treatment of F508del-homozygous individuals with single correctors (VX-809 or VX-661) in combination with the potentiator VX-770 achieved only modest clinical improvements (Wainwright *et al.*, 2015; Taylor-Cousar *et al.* 2017). Significantly greater therapeutic response was recently achieved by adding a second corrector (VX-445) to the previous combination (VX-661/VX-770) (Heijerman *et al.*, 2019; Middleton *et al.*, 2019), indicating that combination of correctors acting by distinct mechanisms is needed for efficient correction of F508del-CFTR and for clinical benefit of individuals with CF carrying this mutation. Although the MoA of VX-445 is still unknown, its additivity to VX-661 on F508del-CFTR rescue suggests that these compounds act by different modes.

Here, we looked into the MoA of the recently described F508del-CFTR corrector RDR01752 (Carlile *et al.*, 2007). Our biochemical and immunofluorescence data demonstrated that RDR01752 rescues F508del-CFTR processing and PM expression in CFBE cells to levels similar to those of VX-809. However, in previous studies using baby hamster kidney (BHK) cells stably expressing F508del-CFTR, RDR01752 appeared to be less efficacious than VX-809 (Carlile *et al.*, 2007, Sampson *et al.*, 2011). Our functional data here, in both CFBE and FRT cell lines, also show that RDR01752 is less effective than VX-809 in restoring F508del-CFTR-mediated Cl<sup>-</sup> secretion, consistent with previous findings in human bronchial epithelial (HBE) cells (F508del/F508del) (Carlile *et al.*, 2018). Notwithstanding, as the validation of results in patient-derived specimens is an important step to provide a better prediction of the *in vivo* efficacy (Amaral and De Boeck, 2019; Lopes-Pacheco, 2020), we tested here this compound in intestinal organoids (F508del/F508del). Our data revealed that RDR01752 and VX-809/VX-661 can rescue F508del-CFTR function to similar levels in this assay. The higher efficacy observed in the organoids may derive from the fact that different cell systems were used and CFTR processing and function are influenced by the cell background and polarization state (Pedemonte *et al.*, 2010; Rowe *et al.*, 2010; Farinha *et al.*, 2015).

To investigate the MoA of RDR01752 we analyzed its additivity to revertants that rescue F508del-CFTR by different mechanisms. RDR01752 effects were additive to G550E and 4RK, but not to R1070W as determined by rescue of F508del-CFTR protein to the PM. G550E and R1070W were proposed to act at two distinct CFTR interdomain contact points that are disrupted by F508del (Farinha *et al.*, 2013): while G550E likely restores the NBD1:NBD2 dimerization interface (Roxo-Rosa *et al.*, 2006), R1070W restores the NBD1:ICL4 interaction (Serohijos *et al.*, 2008; Thibodeau *et al.*, 2010). Thus, RDR01752, like VX-809, might act similarly to R1070W, i.e., by restoring the anchoring of ICL4 to the NBD1 surface by filling a pocket generated by the absence of F508, as the lack of additivity of these two compounds indicates. Indeed, the replacement of an arginine with a tryptophan at position 1070 (R1070W) helps restore interactions among the aromatic residue that were impaired by the lack of F508del at the NBD1 surface (Serohijos *et al.*, 2008; Thibodeau *et al.*, 2010; Farinha *et al.*, 2013). RDR01752, like VX-809/VX-661, has a fused aromatic ring in its structure that can fit into this pocket left empty by F508del (Heet *et al.*, 2013; Farinha *et al.*, 2013). This terminal aromatic ring in RDR01752 was also found to be critical for the stabilization of isolated F508del-NBD1 with an additional stabilization effect in presence of ATP, thus suggesting that RDR01752 does not bind to the ATP binding site in NBD1 (Sampson *et al.*, 2011). This is also in agreement with the observed additivity of RDR01752 to G550E.

On the other hand, RDR01752 was also additive to 4RK. This variant (where four arginines in the AFT are simultaneously replaced to lysines) enables some F508del-CFTR protein to traffic to the PM by escaping the ERQC (Chang *et al.*, 1999; Roxo-Rosa *et al.*, 2006). Notably, although RDR01752, VX-809 and VX-661 rescued F508del-4RK-CFTR PM expression, their efficacy was distinct, with the additivity of RDR01752 to 4RK being higher than that of VX-809/VX-661. In turn, the latter correctors were more effective than RDR01752 in rescuing G550E-F508del-CFTR. Altogether, these data suggest that combinations of RDR01752 with compounds that mimic the correction induced by the G550E and 4RK revertants could maximize the rescue of F508del-CFTR.

Regarding the additivity to low temperature, RDR01752, VX-809 and VX-661 were similarly additive to 27°C incubation of F508del-expressing cells, consistent with previous reports demonstrating that VX-809

was unable to restore the thermostability of F508del-CFTR (He *et al.*, 2013). The three correctors, which were additive to G550E and 4RK, had an even greater effect when combined to low temperature. Although none of the correctors was additive to R1070W at 37°C, additivity was observed for all three in combination to low temperature for this revertant, which was however, less pronounced for RDR01752. Altogether, these data indicate that despite the double correction effect of revertants and compounds on F508del-CFTR, there is still scope for further enhancement as indicated by low temperature data.

As with VX-809 and VX-661, RDR01752 was unable to overcome a Sec24-COPII-ER export defect of the diacidic variant DD/AA (on WT-CFTR backbone). Low temperature nevertheless enables DD/AA-CFTR to exit the ER through the conventional ER-to-Golgi pathway, since the rescued DD/AA-CFTR was found to be fully-glycosylated (Farinha *et al.*, 2013). These data are compatible with the proposed mechanism for F508del-CFTR by low temperature (Farinha *et al.*, 2013).

Although RDR01752 did not further enhance F508del-CFTR PM expression rescue by VX-809 or VX-661 in the immunofluorescence assay, it further increased the rescue of F508del-CFTR function when combined with VX-809 *versus* each compound alone, which may be attributable to a weak potentiator activity of RDR01752 on F508del-CFTR channels (Sampson *et al.*, 2011). Notably, chronic VX-770 exposure reduced RDR01752-rescued CFTR PM expression in F508del-expressing cells, as described for VX-809 and VX-661 (Cholon *et al.*, 2014; Veit *et al.*, 2014). However, chronic exposure of F508del-expressing cells to a low free concentration of VX-770 prevents the negative effect on VX-809-rescued CFTR (Matthes *et al.*, 2016). Surprisingly, rescuing of CFTR PM expression by C18, a compound with a similar chemical structure to VX-809/VX-661 (Eckford *et al.*, 2014; Lopes-Pacheco *et al.*, 2016), was less affected by chronic VX-770 exposure. In fact, C18 and VX-809 possibly have a different MoA since in primary HBE cells, the former failed to rescue the trafficking mutant A561E-CFTR, in contrast to VX-809 (Awatade *et al.*, 2014).

Because there are several CFTR mutations with the same trafficking defect as F508del-CFTR, i.e., class II (De Boeck & Amaral, 2016, Lopes-Pacheco, 2020), but do not equally respond to the same CFTR corrector (Grove *et al.*, 2011; Rapino *et al.*, 2015, Dekkers *et al.*, 2016; Lopes-Pacheco *et al.*, 2017), we investigated whether RDR01752 could rescue some of these mutants. Functional assessment was initially performed by the FMP assay and then confirmed in Ussing chamber measurements of CFTR activity in polarized FRT cells stably expressing some of those CFTR mutations. As RDR01752 was demonstrated to be a weak potentiator of F508del-CFTR channels (Sampson *et al.*, 2011), we tested its effect on R334W-CFTR, which has minimal trafficking impairment but reduced channel conductance that still allows for residual function (Sheppard *et al.*, 1993). A slight but non-statistically significant increase in R334W-CFTR function was induced by RDR01752 whereas a greater effect was observed when combined with VX-809. We also tested RDR01752 on G85E and N1303K class II CFTR mutants which are temperature-insensitive despite having trafficking defects like F508del (Rapino *et al.*, 2015; Lopes-Pacheco *et al.*, 2017). N1303K was not rescued efficiently by RDR01752 or VX-809 when tested individually or in combination, consistent with previous findings in HBE cells (Awatade *et al.*, 2014) and intestinal organoids (Dekkers *et al.*, 2016). Regarding G85E, although it appeared to respond functionally to RDR01752 in the FMP assay, this was not confirmed in the Ussing chamber measurements. The lack of response of this mutant to several correctors has been previously reported (Grove *et al.*, 2011; Lopes-Pacheco *et al.* 2017). Altogether, these data demonstrate that both G85E and N1303K trafficking defects are difficult to rescue and alternative correctors remain an unmet need for these mutants.

In conclusion, these data show that RDR01752, like VX-809/VX-661, rescues F508del-CFTR, albeit at lower efficiency, and like those two approved corrector drugs it does not rescue the G85E and N1303K traffic mutants. Our studies with revertants, aimed at understanding the MoA of this novel corrector, help explain such similarity in pharmaco-therapeutic behavior. Indeed, the data suggest RDR01752 may share a binding site on F508del-CFTR with VX-809 and VX-661, i.e., at the NBD1:ICL4 interface. The fact that RDR01752, like VX-809/VX-661, is additive to correction by the revertants G550E and 4RK and also by low temperature indicate that there is still scope for correctors to further increase the rescue of F508del-CFTR.

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### Figure legends:

**Figure 1 – RDR01752 rescues F508del-CFTR processing and PM expression.** (A) CFBE cells stably expressing F508del-CFTR were incubated for 24 h with DMSO (negative control), VX-809 (3.7  $\mu$ M), VX-661 (5  $\mu$ M) or an increasing concentration of RDR01752. (B) CFTR processing (C/B+C) was quantified and normalized to tubulin levels (loading control). N = 4. *Vs.* DMSO: \* $P$  <0.05, \*\*\* $P$  <0.001. *Vs.* VX-809: # $P$  <0.05, ## $P$  <0.01. (C) CFBE stably expressing mCherry-Flag-F508del-CFTR were incubated for 48 h with DMSO (negative control), VX-809 (3.7  $\mu$ M) or an increasing concentration of RDR01752. (D) Immunostaining was performed and fluorescence images of extracellularly exposed Flag-tags were quantified to determine CFTR PM expression. Data are normalized to the negative control (DMSO). N = 4. *Vs.* VX-809: # $P$  <0.05, ## $P$  <0.01.

**Figure 2 – RDR01752 rescues F508del-CFTR function.** (A) Representative cell fluorescence recording acquired with a microplate reader. CFBE cells stably co-expressing F508del-CFTR and the HS-YFP were incubated for 24h with DMSO (vehicle), VX-809 (3.7  $\mu$ M) or increasing concentrations of RDR01752. Cells were then acutely (30 min) stimulated with Fsk (20  $\mu$ M) and Gen (50  $\mu$ M). (B) CFTR activity was quantified based on the rate of YFP quenching and normalized to the negative control (DMSO, dashed line). N = 4. *Vs.* VX-809: # $P$  <0.05. (C-F) Monolayers of CFBE cells stably expressing F508del-CFTR were incubated for 24 h with (C) DMSO (negative control), (D) VX-809 (3.7  $\mu$ M), or (E) RDR01752 (10  $\mu$ M). Original Ussing chamber (open-circuit) recordings depicting transepithelial voltage measurements ( $V_{te}$ ). There is an absence of response in cells treated with DMSO, while negative deflections are observed in cells treated with VX-809 or RDR01752 following the application of Fsk+IBMX and genistein, which are reverted by application of Inh<sub>172</sub>. (F) Data are expressed as I<sub>sc</sub> calculated from voltage deflections obtained for the responses to Fsk+IBMX+Gen. N = 3. *Vs.* DMSO: \* $P$  <0.05, \*\* $P$  <0.01. *Vs.* VX-809: # $P$  <0.05.

**Figure 3 – Intestinal organoids (F508del/F508del) respond positively to RDR01752.** (A) Bright-field images of organoids incubated for 24 h with DMSO (negative control), VX-809 (3.7  $\mu$ M), VX-661 (5  $\mu$ M) or RDR01752 (10  $\mu$ M) and acutely stimulated with forskolin (Fsk, 0.128  $\mu$ M) with VX-770 (3  $\mu$ M) or genistein (Gen, 50  $\mu$ M). (B) Data of FIS of organoids are expressed as the absolute area under the curve (AUC; baseline = 100%,  $t$  = 60 min, 0.128  $\mu$ M Fsk). Absence of bars indicates there was no swelling (NS). N = 3. *Vs.* DMSO: \* $P$  <0.05, \*\* $P$  <0.01, \*\*\* $P$  <0.001.

**Figure 4 – RDR01752 increases the rescue of F508del-CFTR PM expression in low temperature and in cells expressing in *cis* the genetic revertants G550E and 4RK, but not in R1070W.** CFBE stably expressing mCherry-Flag-CFTR (WT, F508del, DD/AA variants or carrying G550E, R1070W, 4RK in *cis* with F508del) were incubated for 24 h with DMSO (negative control), RDR01752 (10  $\mu$ M), VX-809 (3.7  $\mu$ M) or VX-661 (5  $\mu$ M) and then maintained for additional 24h at (A) 37°C or (B) in low temperature (27°C). Data are represented as heatmaps of the quantification from fluorescence images of extracellularly exposed Flag-tags to determine CFTR PM expression. The values for each cell line are normalized to DMSO at 37°C. N = 6.

**Figure 5 – RDR01752 is not additive to VX-809 or VX-661 in rescuing F508del-CFTR traffic.** (A) CFBE stably expressing mCherry-Flag-F508del-CFTR were incubated for 48 h with the following compounds individually or in combination: DMSO (negative control), RDR01752 (10  $\mu$ M), VX-809 (3.7  $\mu$ M), VX-661 (5  $\mu$ M) and VX-770 (3  $\mu$ M). (B) Immunostaining was performed and fluorescence images of extracellularly exposed Flag-tags were quantified to determine CFTR PM expression. Data are normalized to the negative control (DMSO). N = 4. Vs. single corrector (white bars): \* $P < 0.05$ , \*\*\* $P < 0.001$ . n.s.: no significant.

**Figure 6 – Assessment of RDR01752 effects on rare CFTR mutants by FLIPR membrane potential (FMP) assay.** FRT cells stably expressing CFTR variants (wt, G85E, R334W, T338I, R347P, F508del, V520F, S549F, G551D, M1101K or N1303K) were incubated for 24 h with DMSO (negative control), RDR01752 (10  $\mu$ M) or VX-809 (1  $\mu$ M). FMP assay was performed to monitor membrane depolarization induced by stimulation with forskolin (10  $\mu$ M) plus genistein (50  $\mu$ M) as a measurement of CFTR function. N = 3. Vs. DMSO (black bars): \* $P < 0.05$ .

**Figure 7 – Effect of RDR01752 and VX-809 individually or in combination on functional rescue of CFTR carrying F508del, G85E, N1303K or R334W.** (A) Monolayers of FRT cells stably expressing CFTR variants (F508del, G85E, N1303K or R334W) were incubated for 24 h with DMSO (negative control), VX-809 (1  $\mu$ M) and RDR01752 (10  $\mu$ M) alone or combined. Representative recordings of I<sub>sc</sub> measurements of Ussing chamber for each CFTR mutant. CFTR currents were stimulated using forskolin (FSK; 10  $\mu$ M) and genistein (GST; 50  $\mu$ M) and inhibited by CFTR<sub>inh-172</sub> (172; 10  $\mu$ M). ATP (100 $\mu$ M) was added at the end of each experiment as a positive control for viability. (B) Data are represented as mean increase in I<sub>sc</sub> induced by FSK+GST. N = 3-6. Vs. DMSO: \* $P < 0.05$ , \*\* $P < 0.01$ .

### Supplementary Figures:

#### Supplementary Figure 1 – Intestinal organoids (F508del/F508del) respond positively to RDR01752.

Organoids were incubated for 24 h with DMSO (negative control), VX-809 (3.7  $\mu$ M), VX-661 (5  $\mu$ M) or RDR01752 (10  $\mu$ M) and acutely stimulated with different concentrations of forskolin (Fsk, 0.128 to 20  $\mu$ M) with VX-770 (3  $\mu$ M) or genistein (Gen, 50  $\mu$ M). Graph summarizing data of FIS expressed as the absolute area under the curve (AUC; baseline = 100%,  $t = 60$  min). N = 3.

#### Supplementary Figure 2 – RDR01752 is not additive to C18 in rescuing F508del-CFTR traffic.

(A) CFBE stably expressing mCherry-Flag-F508del-CFTR were incubated for 48 h with the following compounds individually or in combination: DMSO (negative control), RDR01752 (10  $\mu$ M), C18 (5  $\mu$ M) and VX-770 (3  $\mu$ M). (B) Immunostaining was performed and fluorescence images of extracellularly exposed Flag-tags were quantified to determine CFTR PM expression compared to the DMSO. N = 4. Vs. single corrector (white bars): \* $P < 0.05$ , \*\*\* $P < 0.001$ . n.s.: not significant.











