LISBOA
UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA LISBOA

TRABALHO FINAL
MESTRADO INTEGRADO EM MEDICINA

Genética / Laboratório

Additive Potentiation of R334W-CFTR Function by Novel Small Molecules

Mafalda Rita Avó Bacalhau

Orientado por:

Doutor Miquéias Lopes Pacheco

Outubro' 2023

“O trabalho final é da exclusiva responsabilidade do seu autor, não cabendo qualquer responsabilidade à FMUL pelos conteúdos nele apresentados.”

Financial Support

Work supported by the UIDB/04046/2020 and UIDP/04046/2020 center grants (to BioISI) and by 2018 Research Scholar for CF from Gilead Sciences, USA and Rep2CFTR (LOPES21I0) from Cystic Fibrosis Foundation, USA (both to ML-P).

ACKNOWLEDGEMENTS

A realização desta tese não teria sido possível sem a colaboração, apoio e incentivo de várias pessoas a quem gostaria de expressar o meu agradecimento.

Em primeiro lugar, quero agradecer ao Doutor Miquéias Lopes Pacheco por me ter dado a oportunidade de estar integrada nos seus projetos de investigação, permitindo que eu continuasse a trabalhar na área das ciências da saúde, mesmo sabendo que eu estava a fazer um curso de grande exigência. Gostava também de agradecer por toda a compreensão e apoio que me permitiram chegar até esta fase, e todo o conhecimento que pude adquirir com alguém que é um exemplo de superação e um investigador de referência. Não menos importante, agradeço-te pela nossa amizade, e por teres feito todos os esforços que estavam ao teu alcance para que eu permanecesse nesta equipa até ao final desta etapa. Nunca terei palavras suficientes para agradecer!

Não posso deixar de agradecer à Professora Doutora Margarida Amaral e a toda a equipa do laboratório de Genómica Funcional e Proteostase (FunGP) do Instituto de Biossistemas e Ciências Integrativas (BioISI), da Faculdade de Ciências da Universidade de Lisboa, por todas as partilhas de conhecimento que contribuíram para enriquecer este trabalho. Também é importante agradecer à Sofia Correia pelo seu empenho como *Lab manager* que nos facilita a gestão do trabalho em equipa. À Iris Silva gostaria de agradecer pela colaboração na realização deste trabalho e, além disso, também pelas palavras de apoio que me encorajaram e que me proporcionaram a oportunidade de trabalhar com esta equipa. À Filipa Ferreira agradeço a amizade, companheirismo, apoio, compreensão e toda a disponibilidade para tornar o meu dia-a-dia mais fácil. Sem esta equipa, não tenho dúvidas de que o meu percurso durante estes anos de trabalhadora-estudante teria sido muito mais difícil de superar.

À Doutora Camilla Buarque agradeço a colaboração, a síntese dos compostos usados neste estudo e a partilha de ideias que permitiram realizar este trabalho.

Agradeço a todos os amigos que a FMUL me proporcionou, em especial ao João Aguiar e à Soraia Ribeiro que partilharam comigo esta aventura desde o início, e também

à Joana Melo, Ana Rocha, Joana Ferreira, Joana Torrejais e tantos outros, sem os quais os meus dias teriam sido muito menos animados.

Às minhas amigas Joana Balça Silva e Maria Inês Fonseca, também elas nesta aventura, por partilharem as suas experiências, angústias e sucessos, por me ouvirem e nunca me desampararem. À Carolina Ribeiro, Rita Reis e Vanessa Mendes, que mesmo longe, fizeram questão de estar sempre perto.

À Fernanda gostaria de agradecer o apoio, a preocupação e a compreensão em todos os momentos.

Agradeço aos meus pais por continuarem a apoiar as minhas ambições e a celebrar as minhas superações.

Ao Marcos, que está sempre ao meu lado para comemorar as minhas conquistas, mas também para me amparar nos dias menos bons, agradeço a compreensão, a paciência infinita e o amor.

RESUMO

A variante c.1000C>T (p.Arg334Trp) no gene *CFTR* é uma mutação rara que causa fibrose quística, para o qual não há uma terapia dirigida aprovada atualmente. Esta mutação provoca uma redução significativa da condutância do canal regulador da condutância transmembranar (CFTR), mas permite que o canal tenha uma função residual. Potenciadores são moléculas pequenas que interagem com a proteína CFTR na membrana plasmática para estimular o transporte de cloreto dependente da CFTR.

Neste trabalho, foi desenvolvida uma linha celular epitelial brônquica (CFBE) para testar uma coleção de compostos derivados de triazol de forma a identificar novos potenciadores para a p.R334W-CFTR.

Os compostos com atividade foram validados através de ensaios de eletrofisiologia e os seus efeitos aditivos em combinação com o VX-770, genisteína ou VX-445 foram testados numa linha celular, e posteriormente confirmados em organóides intestinais.

Quatro compostos (LSO-24, LSO-25, LSO-38 e LSO-77) demonstraram atividade na avaliação funcional inicial, e a sua capacidade de estimular o transporte de cloreto pelo canal CFTR foi confirmada através de ensaios de eletrofisiologia. A análise *in silico* ADME demonstrou que estes compostos seguem a regra de Lipinski, sugerindo que são biodisponíveis oralmente. No entanto, a relação da dose-resposta revelou uma eficácia abaixo do ideal e uma potência fraca. O VX-770 e a genisteína também exibiram um pequeno estímulo da função da p.R334W-CFTR e, por outro lado, o VX-445 não demonstrou qualquer atividade potenciadora. Na linha celular que expressa a p.R334W-CFTR, a função da proteína foi ainda potenciada pela combinação de LSO-24, LSO-25, LSO-38 ou LSO-77 com VX-770, mas não com genisteína. A eficácia do potenciador VX-770 com os compostos LSO foi confirmada em organóides intestinais (genótipo R334W/R334W).

Estas moléculas potenciam a função da p.R334W-CFTR por um mecanismo diferente do usado pelo VX-770, gerando um ponto de partida para o desenvolvimento de análogos com maior atividade potenciadora da CFTR.

Palavras-chave: Moduladores da CFTR; fibrose quística; organóides intestinais; mutação rara; triazol.

ABSTRACT

The c.1000C>T (p.Arg334Trp) *CFTR* variant is a rare cystic fibrosis (CF)-causing mutation for which no causal therapy is currently approved. This mutation leads to a significant reduction of CF transmembrane conductance regulator (CFTR) channel conductance that still allows for residual function. Potentiators are small molecules that interact with CFTR protein at the plasma membrane to enhance CFTR-dependent chloride secretion. Here, we generate a new CF bronchial epithelial (CFBE) cell line to screen a collection of triazole compounds and identify novel potentiators for R334W-CFTR.

The active compounds were validated by electrophysiological assays and their additive effects in combination with VX-770, genistein or VX-445 were exploited in cell line and further confirmed in intestinal organoids.

Four compounds (LSO-24, LSO-25, LSO-38 and LSO-77) were active in the functional primary screen and their ability to enhance R334W-CFTR-dependent chloride secretion was confirmed using electrophysiological measurements. *In silico* ADME analyses demonstrated that these compounds follow the Lipinski's rule of five and are thus suggested to be orally bioavailable. Dose-response relationships revealed nevertheless suboptimal efficacy and weak potency exerted by these compounds. VX-770 and genistein also displayed a small potentiation of p.R334W-CFTR function, while VX-445 demonstrated no potentiator activity for this mutation. In R334W-expressing cell line, CFTR function was further enhanced by the combination of LSO-24, LSO-25, LSO-38 or LSO-77 with VX-770, but not with genistein. The efficacy of potentiator VX-770 combined with active LSO compounds was further confirmed in intestinal organoids (R334W/R334W genotype).

Taken together, these molecules demonstrated to potentiate p.R334W-CFTR function by a different mechanism than that of VX-770. They may provide a feasible starting point for the design of analogues with improved CFTR potentiator activity.

Keywords: CFTR modulators; cystic fibrosis; intestinal organoids; rare mutation; triazole.

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

CF	Cystic Fibrosis
CFBE	CF bronchial epithelial
CFTR	CF transmembrane conductance regulator
CFTRinh-172	CFTR channel inhibitor
Clnx	Calnexin
DMSO	Dimethyl suloxide
FBS	Fetal bovine serum
FIS	Fsk-Induced Swelling
Fsk	Forskolin
Gen	Genistein
HS-YFP	Halide Sensitive-Yellow Fluorescent Protein
LogP	Calculated logarithm of the octanol-water partition coefficient
LSO	Laboratório de Sintese Orgânica
MEM	Minimum essential medium
MW	Molecular weight
nHBA	Number of H-bond acceptors
nHBD	Number of H-bond donors
nRB	Number of rotatable bonds
PAINS	Pan-assay interfence compounds
PBS	Phosphate-buffered saline
PKA	Protein Kinase A
PwCF	Patient with cystic fibrosis
TPSA	Topological polar surface area

INDEX

1.INTRODUCTION

Cystic fibrosis (CF) is a life-shortening autosomal recessive inherited disease that leads to a multiorgan pathology (Cutting, 2015; Riordan et al., 1989). It is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, an ATP-gated chloride channel activated by protein kinase A (PKA)-dependent phosphorylation (Cutting, 2015). CFTR is expressed at the apical membrane of secretory epithelia and plays a fundamental role in fluid and electrolyte movements, thus controlling the composition and amount of epithelial secretions (Saint-Criq & Gray, 2017). In the airways, loss of CFTR function causes depletion of periciliary liquid and mucus accumulation that along with chronic inflammation and recurrent infections progressively impair lung function (Saint-Criq & Gray, 2017).

Over the last decade, CF therapies have been transformed by the approval of orally bioavailable drugs targeting the root cause of disease – so-called CFTR modulators (Lopes-Pacheco, 2020). The potentiator VX-770 (also termed ivacaftor) was the first CFTR modulator drug approved for clinical use initially for people with CF (PwCF) carrying the c.1652G>A (p.Gly551Asp) *CFTR* mutation (Ramsey et al., 2011). Subsequent studies led to label extensions of VX-770 monotherapy for several other gating and residual function mutations (Lopes-Pacheco et al., 2021; Van Goor et al., 2014; Yu et al., 2012). VX-770 acts by increasing the function of mutant CFTR channels present at the plasma membrane (Van Goor et al., 2009). Once CFTR is phosphorylated by PKA, VX-770 enhances the duration and frequency of channel opening in an ATP-independent channel gating, thus allowing for CFTR-dependent chloride secretion (Eckford et al., 2012). Clinically, VX-770 monotherapy demonstrated to promote sustained and long-term clinical benefit, including slower decline of lung function and improved nutritional status (Bessonova et al., 2018; Volkova et al., 2020). When combined with CFTR correctors, which rescue CFTR folding and trafficking to the plasma membrane, VX-770 also induced therapeutic benefit for PwCF carrying the prevalent p.Phe508del mutation (Heijerman et al., 2019; Taylor-Cousar et al., 2017; Wainwright et al., 2015).

Despite significant therapeutic progress, many PwCF carrying rare mutations remain with no modulator therapy approved (Lopes-Pacheco, 2020). The R334W-CFTR mutation (c.1000C>T, p.Arg334Trp) is found in ~0.3% of CF alleles worldwide

(https://cftr2.org, accessed on 11 October 2023), but has a relatively higher prevalence in Portuguese and Brazilian CF populations (2.8% and 2.3% of CF alleles, respectively) (Cística, 2019; World Health Organization, 2004). This mutation has minimal impact on CFTR folding and trafficking, but significantly affects chloride distribution at the mouth of the channel pore, thus resulting in either very low channel conductance or reduced channel open probability that still enables a residual CFTR-dependent chloride secretion (Sheppard et al., 1993; Smith et al., 2001). In cell models expressing R334W-CFTR, the results have been conflicting with respect to the effects of VX-770 (Phuan et al., 2019; Van Goor et al., 2014; Veit et al., 2020). While no significant response to VX-770 was detected in experiments using Fischer rat thyroid cells expressing this mutant (Phuan et al., 2019; Van Goor et al., 2014), a small but significant increase in CFTR function was observed in R334W-expressing CF bronchial epithelial (CFBE) cells (Veit et al., 2020). Furthermore, VX-770 does not fully restore CFTR gating defects in G551D- and F508del-expressing cells (Jih & Hwang, 2013; Van Goor et al., 2009), which indicates that there is still scope for further enhancement. Recent reports have found that combination of potentiators with complementary mechanisms (i.e., co-potentiators) can further enhance CFTR-dependent chloride secretion (Phuan et al., 2018, 2019; Veit et al., 2020, 2021), and several small molecules with a chemical structure distinct from that of VX-770 have been demonstrated to rescue the function of mutant CFTR channels at different potency and degrees of efficacy (Gees et al., 2018; Liu et al., 2022; Moran et al., 2005; Phuan et al., 2019).

Triazoles are well-known synthetic heterocycle compounds widely used in both industry and academic research (Bonandi et al., 2017; Wang et al., 2016). This scaffold can be obtained by various synthetic routes, although the copper-catalyzed azide-alkyne click chemistry reaction is the most commonly applied due to its simplicity, robustness, and versatility (Bonandi et al., 2017; Wang et al., 2016). The 1,4-disubstituted 1,2,3-triazole pharmacophoric group has been used in the design of drugs with antimicrobial (Kant et al., 2016), anti-inflammatory (Kim et al., 2015), anti-viral (Ferreira et al., 2014), and anti-tumor properties (da Silva et al., 2019; Naaz et al., 2018; Stefely et al., 2010), proving to be a successful strategy in medicinal chemistry and capable of mimicking the characteristics of different functional groups.

2.OBJECTIVES

The present study aimed to identify novel potentiators for the rescue of p.Arg334Trp-CFTR function.

In this regard, we address the following specific objectives:

1. Screen a collection of triazole compounds and derivatives using a novel CFBE cell line co-expressing p.R334W-CFTR and Halide-Sensitive Yellow fluorescent protein (HS-YFP).
2. Validate the functional results of active compounds by electrophysiological measurements.
3. Identify the potential utility as co-potentiators with VX-770, genistein and VX-445 using a cell line and intestinal organoids.

3.1 Chemicals and compounds

The triazole compounds and derivatives were synthesized in the *Laboratório de Síntese Orgânica* (LSO) and physico-chemically characterized as previously described (da Silva et al., 2019; 2020). The remaining compounds were purchased from commercial sources at the analytical grade: VX-770 (#HY-13017), Gen (#HY-14596), forskolin (Fsk, #HY-15371), and CFTR channel inhibitor (CFTRinh-172, #16671), from MedChemExpress (Monmouth Junction, NJ, USA), and VX-445 (#S8851) from Selleckchem (Houston, TX, USA). All compounds were diluted in dimethyl sulfoxide (DMSO).

3.2 Generation of a new cell line

A new CF bronchial epithelial (CFBE) cell line stably co-expressing the halide sensitive-yellow fluorescence protein (HS-YFP F46L/H148Q/I152L) and R334W-CFTR was generated. Briefly, the HS-YFP cloned into pcDNA3.1 expression vector was re-cloned into the lentiviral expression vector pLVX-Puro and then transfected into the HEK-293T cells to produce the lentiviral particles (Bacalhau et al., 2023). After 48 h, these particles were harvested and transduced into CFBE cells expressing R334W-CFTR (Lopes-Pacheco et al., 2022). The efficiency of transduction was assessed by fluorescence microscopy (Zeiss Axiovert 200, Jena, Germany) and then cells were sorted in a FACSAria III cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) to select a homogeneous population with the highest expression of the HS-YFP.

3.3 Cell culture

CFBE cell lines expressing CFTR variants (WT and R334W) with and without co-expression of the HS-YFP were cultured in minimum essential medium (MEM, #10-010-CV, Corning, VA, USA) supplemented with fetal bovine serum (FBS, #LTI 10270-106, Gibco, Carlsbad, CA, USA) and 2 $\mu g.mL^{-1}$ puromycin (#P8833, Sigma-Aldrich, St. Louis, MO, USA) (Bebok et al., 2005; Lopes-Pacheco et al., 2022). All cells were maintained at

37°C and 5% CO_2 in a humidified incubator, expect for the low temperature experiments, in which cells were cultured at 27°C for 24h.

3.4 HS-YFP assay on a plate reader

A microplate reader (Tecan Infinite 200 Pro) equipped with high-quality excitation (485 ± 20 ηm) and emission (535 ± 25 ηm) was used for compound screening (Lopes-Pacheco et al., 2022). Briefly, CFBE cells co-expressing the HS-YFP and R334W-CFTR were plated in 96-well black-walled, clear-bottom microplates (#655090, Greiner Bio-One, Kremsmünster, Austria) at a density of 50,000 cell/well. On the next day, cells were washed twice with phosphate-buffered saline (PBS) and incubated for 30 min with 60 µL PBS with Fsk (5 µM) and test compounds. All plates in the potentiator screen contained wells with Fsk (5 µM) plus positive (1 µM VX-770 or 50 µM Gen) or negative (DMSO) controls. For the co-potentiator analysis, CFBE cells co-expressing CFTR variants were incubated with Fsk (5 µM) plus VX-770 (1 µM), Gen (50 µM) or VX-445 (3 µM) and active LSO compounds for 30 min. The assay consists of 14-s fluorescence reading: 2-s of the initial fluorescence intensity and 12-s after injection of 100 µL of an iodide-containing solution (PBS with NaCl substituted by NaI, final iodide concentration: 100 mM). The initial iodide influx was computed from fluorescence intensity by single exponential regression (Pedemonte et al., 2011). All conditions were performed in triplicate in each microplate.

3.5 *In Silico* absorption, distribution, metabolism and excretion (ADME) analysis

ADME analysis was carried out using the free online software SwissADME (http://www.swissadme.ch, accessed on 10 October 2022) as previously described (Daina et al., 2017). The following physicochemical variables were assessed according to the Lipinski's rule of five (Lipinski, 2004): molecular weight (MW), number of H-bond donors (nHBD), number of H-bond acceptors (nHBA), number of rotatable bonds (nRB), calculated logarithm of the octanol-water partition coefficient (LogP), in addition to the topological polar surface area (TPSA). Hit compounds were also subjected to filters for the identification of pan-assay interfence compounds (PAINS), i.e., "promiscuous"

substructures that demonstrate potential response in bioassays independent of the target (Baell & Holloway, 2010).

3.6 Western-Blot analysis

For CFTR protein detection, CFBE cells were lysed using a lysis buffer (31.25 mM Tris-HCl pH 6.8, 1.5% SDS [w/v], 5% glycerol and 0.5 mM DTT) supplemented with complete protease inhibitor cocktail (#11697498001, Roche, Basel, Switzerland) and Laemmli sample buffer (#1610747, Bio-Rad, Hercules, CA, USA). Whole-cell lysates were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane (#88518, Millipore, Burlington, MA, USA). CFTR was detected using the monoclonal anti-human CFTR antibody 596 (1:3,000, CF Foundation Therapeutics) and the blotting-grade horseradish peroxidase secondary antibody (1:5,000, Bio-Rad, Hercules, CA, USA). Calnexin (Clnx) was detected using the monoclonal anti-calnexin antibody (1:3,000, #610523, BD Bioscences, Franklin Lakes, NJ, USA) as a loading control. Images were acquired using ChemiDoc XRS+ imaging system and analysed with the Image lab version 6.0 (Bio-Rad, Hercules, CA, USA).

3.7 Micro-ussing chamber measurements

Short-circuit current was measured on polarized CFBE cells expressing R334W-CFTR cultured on porous membrane filters (#734-1646, Corning, New York, NJ, USA), as described (Awatade et al., 2015). Briefly, the transepithelial electrical resistance of cells was measured with the Chopstick Electrode (STX2, WPI, Sarasota, FL, USA), and when it was ≥1,000 $\Omega.cm^2$, recordings were carried out in modified micro-Ussing chambers. The solution bathing the basolateral membrane contained (in mM): 145 NaCl, 1.6 K_2HPO_4, 5.0 D-glucose, 1.0 $MgCl_2$ and 1.3 Ca-gluconate. The solution bating the apical membrane the apical membrane contained (in mM): 32 NaCl, 0.4 KH_2PO_4, 1.6 K_2HPO_4, 5.0 D-glucose, 1.0 $MgCl_2$, 5.7 Ca-gluconate and 112 Na-glucose. After the equilibration period, baseline values were recorded and compounds were added sequentially: Fsk (0.128 µM), test compounds and CFTRinh-172 (30 µM). Transepithelial resistance (Rte) and the voltage

(Vte) were recorded, and equivalent cAMP-stimulated CFTR short-circuit currents (ΔI_{eq-sc}) were calculated by Ohm's law from Rte and Vte as follow: $\Delta I_{eq-sc} = Vte/Rte$.

3.8 Forskolin-induced swelling assay of intestinal organoids

Intestinal organoids were seeded in a flat-bottom 96-well microplates in 4 µL of matrigel (#356231, Corning, New York, NJ, USA) and in 50 µL of culture medium (Dekkers et al., 2013). After 24 h, the organoids were incubated for 20 min with 3 µM of calcein green (Invitrogen, Waltham, MA, USA). Before imaging, organoids were incubated with Fsk at different concentrations (0.02, 0.128, 0.8 and 5 µM) with and without test compounds. Images were acquired every 10 min for 60 min in a confocal live cell microscopy (Leica SP8, Leica Microsystems, Wetzlar, German) at 37°C with 5% CO_2. FIS was quantified using a CellProfiler-based algorithm and expressed as the absolute area under the curve (AUC) calculated from the normalized surface area increase (baseline = 100%, t = 60 min).

3.9 Statistical analysis

All conditions were carried out in at least three independent experiments. GraphPad Prism software version 8.3 (GraphPad Inc., San Diego, CA, USA) was used for all statistical analysis. Statistical comparisons between the conditions tested were assessed using One-way ANOVA followed by Dunnett's or Tukey's post-hoc tests, and values of $P < 0.05$ were considered significant.

4.1 Characterization of a new cell line co-expressing the HS-YFP and R334W-CFTR

The R334 residue is located on transmembrane segment 6 at the outer mouth of the CFTR channel pore **(Figure 1)**. Compared to WT-CFTR, the R334W mutation similarly exhibited the presence of both core-glycosylated immature (~140 kDa, band B) and fully-glycosylated mature (~180 kDa, band C) forms of CFTR. Furthermore, R334W-CFTR processing was increased by the incubation of cells at low temperature (1.35-fold increase) **(Figure 2A, B)**.

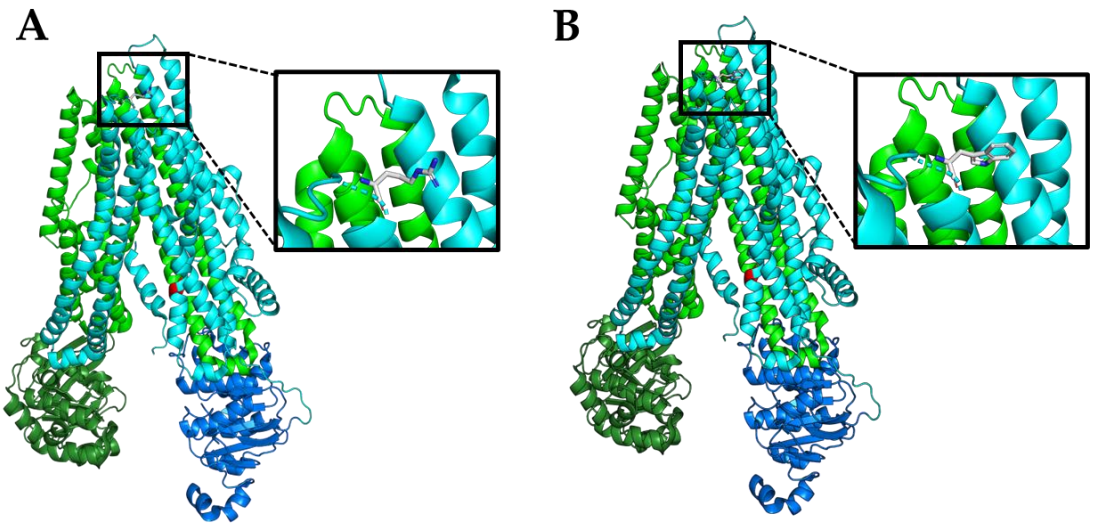

Figure 1. Ribbon diagram of the dephosphorylated, ATP-free human CFTR structure (PDB: 5UAK) demonstrating the location of the R334 residue in **(A)** WT-CFTR and **(B)** mutant R334W-CFTR.

Bright-field, fluorescence and merged images were acquired to confirm the transduction of the YFP sensor in CFBE cells stably expressing R334W-CFTR **(Figure 2C)**. These cells were then used for the functional assay to measure the HS-YFP quenching following addition of iodide-containing solution to the apical surface of cells **(Figure 2D)**. A decay in cell fluorescence was observed upon Fsk stimulation, consistent with a residual function in R334W-CFTR. Cell fluorescence quenching was further increased by Fsk stimulation together with VX-770 or Gen (1.37- and 1.38-fold increase, respectively)

(Figure 2E, F). Cells incubated at 27°C for 24 h also demonstrated an increase in cell fluorescence quenching upon Fsk stimulation compared to those cultured at 37°C (1.76-fold increase).

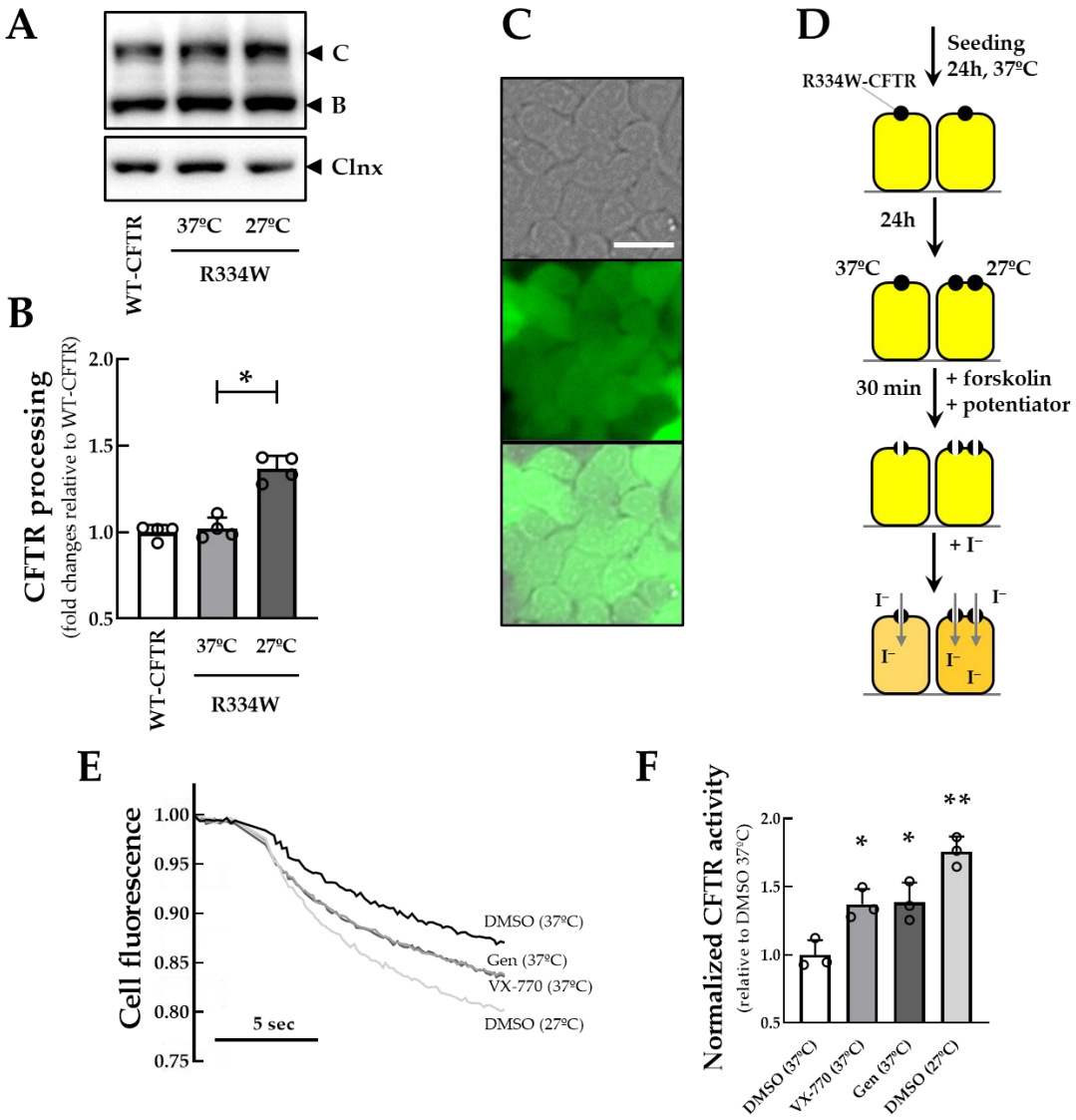

Figure 2. **Characterization of a new cell line co-expressing the HS-YFP and R334W-CFTR**. **(A)** Representative WB images of CFBE cells expressing WT- and R334W-CFTR incubated for 24 h at 37 °C or 27 °C. **(B)** CFTR processing [band C/bands B+C] was quantified and normalized to calnexin (Clnx) levels (loading control). Data are represented as means + SD: * $p < 0.05$ vs. R334W 37 °C. **(C)** R334W-CFTR expressing CFBE cells demonstrating cytosolic fluorescence of the HS-YFP (F46L/H148Q/I152L). Scale bar: 50 µm. **(D)** Schematic flow of the HS-YFP assay. CFBE cells co-expressing R334W-CFTR and the HS-YFP were incubated for 24 h at 37 °C or 27 °C and then acutely stimulated (30 min) with Fsk (5 µM) plus DMSO (negative control), VX-770 (1 µM) or Gen (50 µM). **(E)** Representative cell fluorescence recording on a plate reader. **(F)** CFTR function was assessed based on the cell fluorescence quenching rate and normalized to the negative control. Data are represented as means + SD: * $p < 0.05$ and ** $p < 0.01$ vs. DMSO (37 °C).

4.2 Identification of novel potentiators for the rescue of R334W-CFTR function

To assess the potential utility of the investigational compounds as novel potentiators for R334W-CFTR, cells were acutely incubated (30 min) with a test compound together with Fsk prior to assay **(Figure 3A)**. Among 46 compounds, four demonstrated a small increase, albeit significant, in the HS-YFP quenching rate compared to DMSO (1.21- to 1.42-fold increase) **(Figure 3B)**. Furthermore, the effect of LSO-24, LSO-25, LSO-38 and LSO-77 was closely similar to that of VX-770 or Gen. **Figure 3C** depicts the chemical structure of the active compounds in the screening.

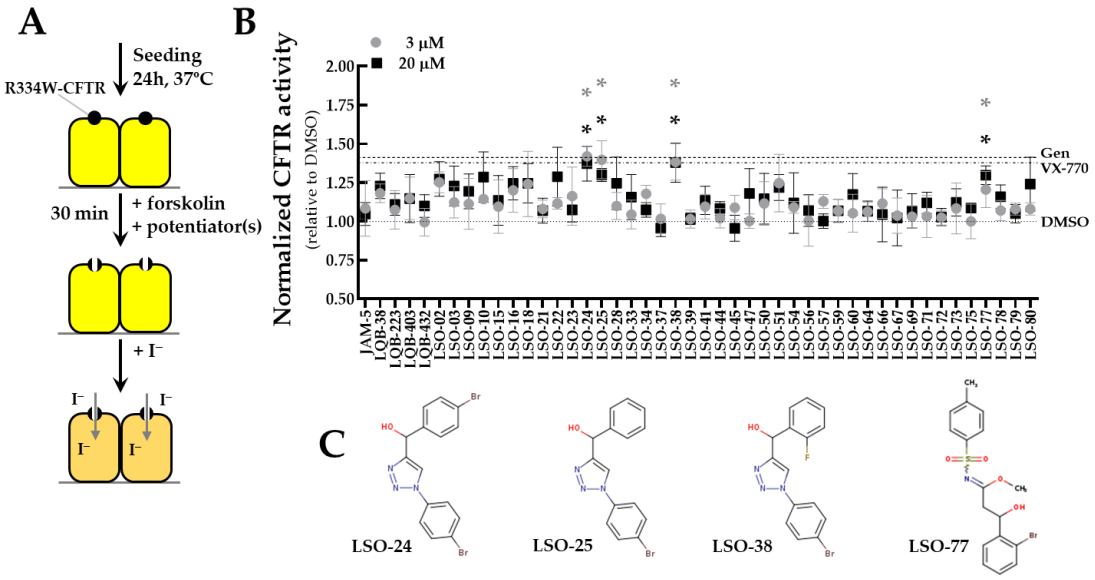

Figure 3. Search of novel R334W-CFTR potentiators by a high-throughput screening assay and dose-response of active compounds. (A) Schematic flow of the HS-YFP assay. CFBE cells co- expressing R334W-CFTR and the HS-YFP were acutely stimulated (30 min) with Fsk (5 µM) plus test compound. DMSO, VX-770 (1 µM) and Gen (50 µM) were used as negative and positive controls, respectively. **(B)** CFTR function was assessed based on the cell fluorescence quenching rate and normalized to the negative control. Data are represented as means + SD of 4 independent experiments: * $p < 0.05$ vs. DMSO. **(C)** Chemical structure of active compounds: LSO-24, LSO-25, LSO-38 and LSO-77.

Efficacy and potency of these compounds were further assessed by the incubation of cells with various concentrations in the range of 0.03 to 30 µM and the HS-YFP quenching rate was measured **(Figure 4A-D)**. **Table 1** depicts EC_{50} and E_{max} for these compounds.

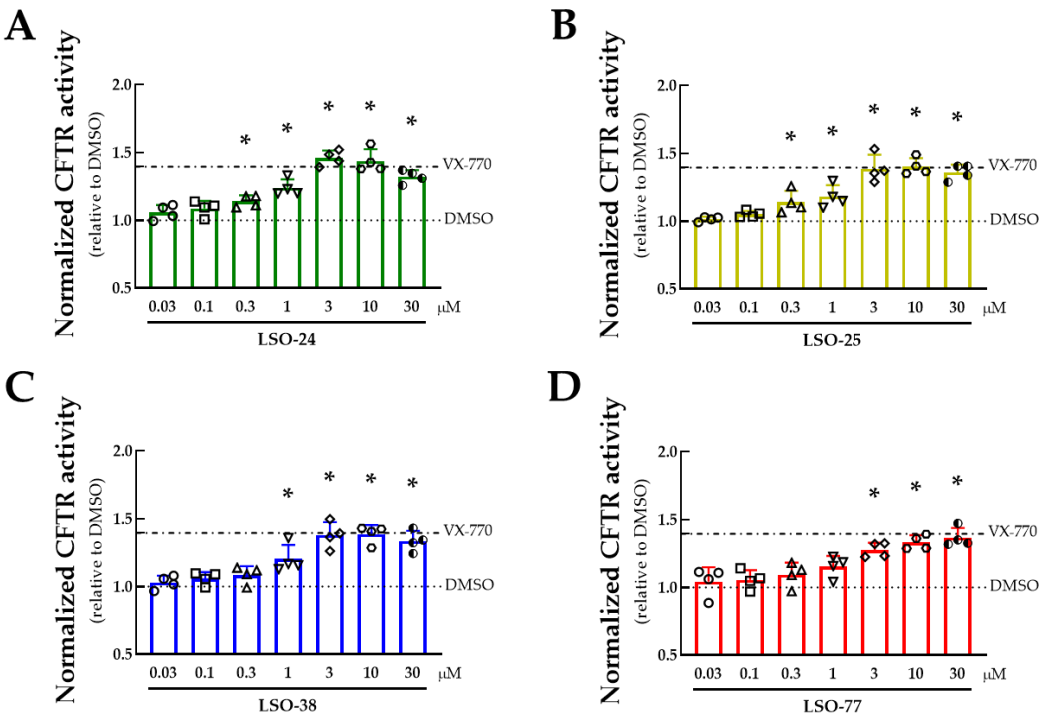

Figure 4. Dose-response relationships of the compounds (A) LSO-24, **(B)** LSO-25, **(C)** LSO-38 and **(D)** LSO-77 were determined by the HS-YFP assay on a plate reader in CFBE cells expressing R334W-CFTR. * $P < 0.05$ *vs.* DMSO.

Table 1. EC_{50} and E_{max} of Active LSO Compounds.

Compound	**EC_{50}** (μM)	**E_{max}** (μM)
LSO-24	1.24	2.6
LSO-25	1.41	3.3
LSO-38	1.81	2.7
LSO-77	2.96	5.9

The theoretical ADME properties of the active LSO compounds were determined by *in silico* analysis according to the Lipinski's rule of five, which state that a small molecule should have MW ≤ 500 Da, nHBD ≤ 5, nHBA ≤ 5, nRB ≤ 10 and LogP ≤ 5 to be orally active. TPSA should also be ≤ 140 $Å^2$, since it influences absorption and membrane permeability. **Table 2** depicts the values for compounds LSO-24, LSO-25, LSO-38 and LSO-77, in addition to those for the reference compound VX-770. All four LSO compounds obey the Lipinski's rule of five and have a TPSA < 140 $Å^2$, suggesting they

can be well absorbed in the gastrointestinal tract. Moreover, during the *in silico* analysis, these compounds were not recognized as PAINS.

Table 2. *In Silico* Absorption, Distribution, Metabolism and Excretion (ADME) Analysis

Comp	MW	nHBD	nHBA	nRB	LogP	TPSA (Å)	GI absorption	PAINS alert
LSO-24	409.08	1	3	3	3.54	50.94	High	0
LSO-25	330.18	1	3	3	2.77	50.94	High	0
LSO-38	348.17	1	4	3	3.11	50.94	High	0
LSO-77	412.30	1	5	6	3.60	84.34	High	0
VX-770	392.49	3	3	5	4.47	82.19	High	0

Comp: compound; MW: molecular weight; nHBD: number of H-bond donors; nHBA: number of H-bond acceptors; nRB: number of rotatable bonds; LogP: values correspond to Consensus $LogP_{w/o}$; TPSA: topological polar surface area; GI: gastrointestinal; PAINS: pan-assay interference structures.

To validate the results obtained by the HS-YFP assay on a plate reader, short-circuit current was measured in polarized monolayers of CFBE cells expressing R334W-CFTR to quantitatively assess CFTR-dependent chloride current **(Figure 5)**. Activation of cAMP-dependent CFTR-mediated chloride secretion by Fsk promoted a small increase in equivalent short-circuit current (Isc_{eq}), which was further increased by the subsequent addition of the potentiator LSO-24, LSO-25, LSO-38, LSO-77 or VX-770 (4.1, 4.3, 4.1, 2.6 and 4.7 $\mu A/cm^2$, respectively). These responses were thus inhibited by addition of CFTRinh-172, indicating they are CFTR specific.

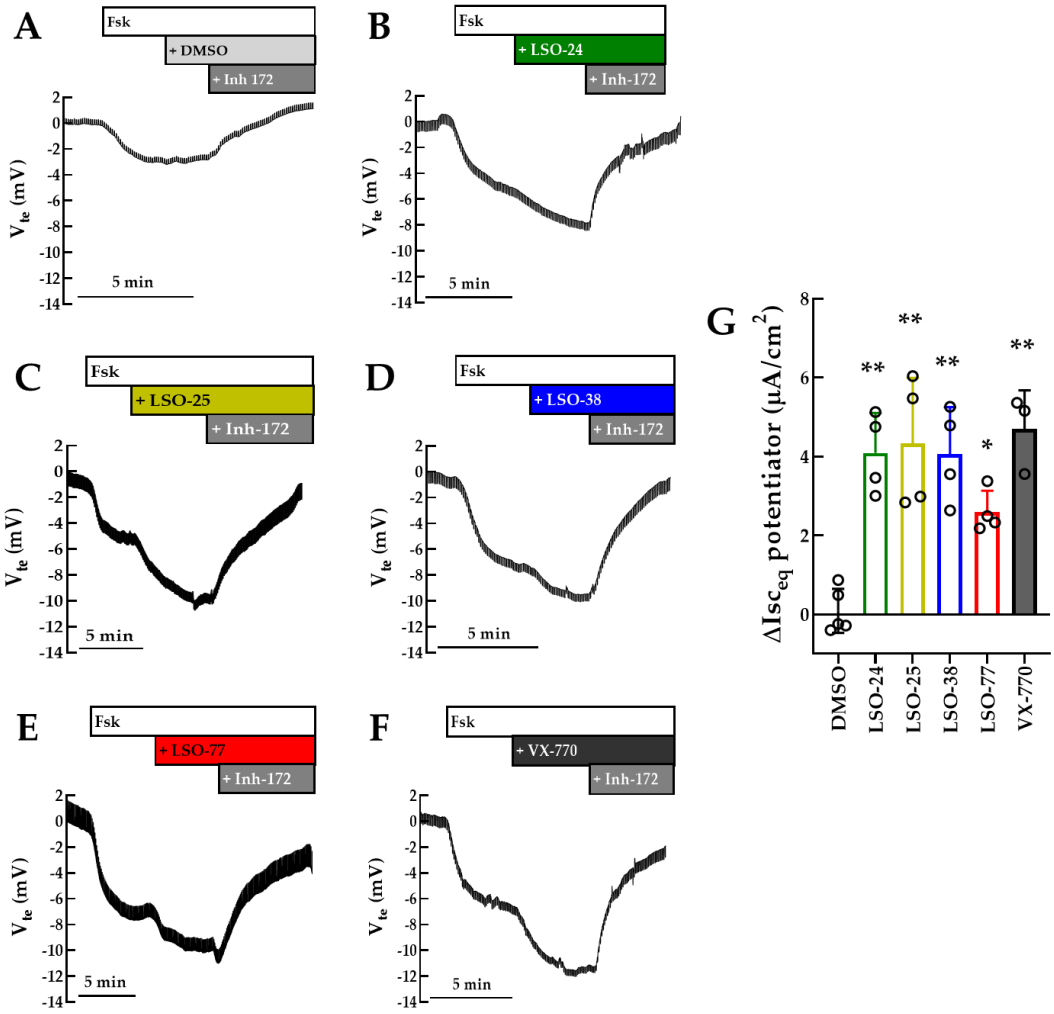

Figure 5. Assessment of R334W-CFTR function in micro-Ussing chamber measurements. **(A–F)** Representative Ussing chamber (open-circuit) recording representing transepithelial voltage measurements (Vte) of polarized monolayers of CFBE cells expressing R334W-CFTR. Cells were acutely stimulated with Fsk (0.128 μM), test compounds (DMSO, 3 μM LSO-24, 3 μM LSO-25, 3 μM LSO-38, 10 μM LSO-77, and 3 μM VX-770) and CFTRInh-172 (30 μM). **(G)** Data are represented as mean (+ SD) increase in I_{eq} promoted by test potentiator: * $p < 0.05$ and ** $p < 0.01$ vs. DMSO.

4.3 Assessment of co-potentiator activity for R334W-CFTR in cell line and intestinal organoids

In order to investigate the co-potentiator activity of the active LSO compounds, cells were acutely incubated (30 min) with LSO-24, LSO-25, LSO-38 and LSO-77 together with VX-770, Gen or VX-445 and the HS-YFP assay on a plate reader was carried out

(similarly to the scheme presented in **Figure 3A)**. Upon Fsk stimulation, a greater fluorescence quenching was observed in cells acutely treated with VX-770 in combination with LSO-24, LSO-25, LSO-38 or LSO-77 compared to DMSO (1.91- to 2.15-fold increase) or any compound individually **(Figure 6)**. However, combination of LSO compounds with Gen demonstrated an equivalent cell fluorescence quenching to that of Gen alone. VX-445 was unable to increase cell fluorescence quenching compared to DMSO, and its combination with LSO-24, LSO-25, LSO-38, or LSO-77 displayed similar effects of that for each compound individually **(Figure 6)**.

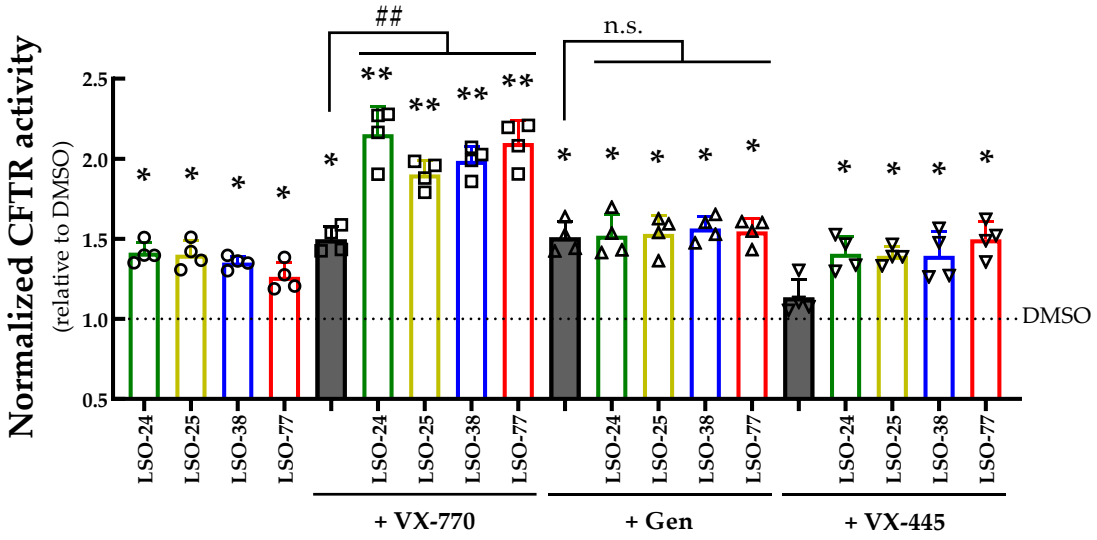

Figure 6. Assessment of additivity of potentiators activity on R334W-CFTR function in a cell line. CFBE cells co-expressing R334W-CFTR and the HS-YFP were acutely stimulated (30 min) with Fsk (5 µM) plus test compounds (individually or in combination): LSO-24 (5 µM); LSO-25 (5 µM); LSO-38 (5 µM); LSO-77 (10 µM); VX-770 (1 µM); Gen (50 µM); and VX-445 (3 µM). CFTR function was assessed based on the cell-fluorescence quenching rate and normalized to the negative control (DMSO). Data are represented as means + SD: * $p < 0.05$ and ** $p < 0.01$ vs. DMSO. Other symbols indicate the statistical significance of groups of data vs. VX-770 alone: ## $p < 0.01$; n.s.—not significant.

The ability of LSO-24, LSO-25, LSO-38 and LSO-77 with and without VX-770 in potentiating R334W-CFTR function was further assessed using the FIS assay of intestinal organoids from an individual with CF (R334W/R334W genotype) **(Figure 7A, B)**. The incremental Fsk concentration in organoids acutely incubated with DMSO led to increasing FIS values, compatible with a residual function in R334W-CFTR. Upon

stimulation with 0.128 µM Fsk plus LSO-24, LSO-25, LSO-38, LSO-77, or VX-770, organoid FIS values demonstrated a small but significant increase compared to that of DMSO (1.32- to 1.81-fold increase) **(Figure 7A, B)**.

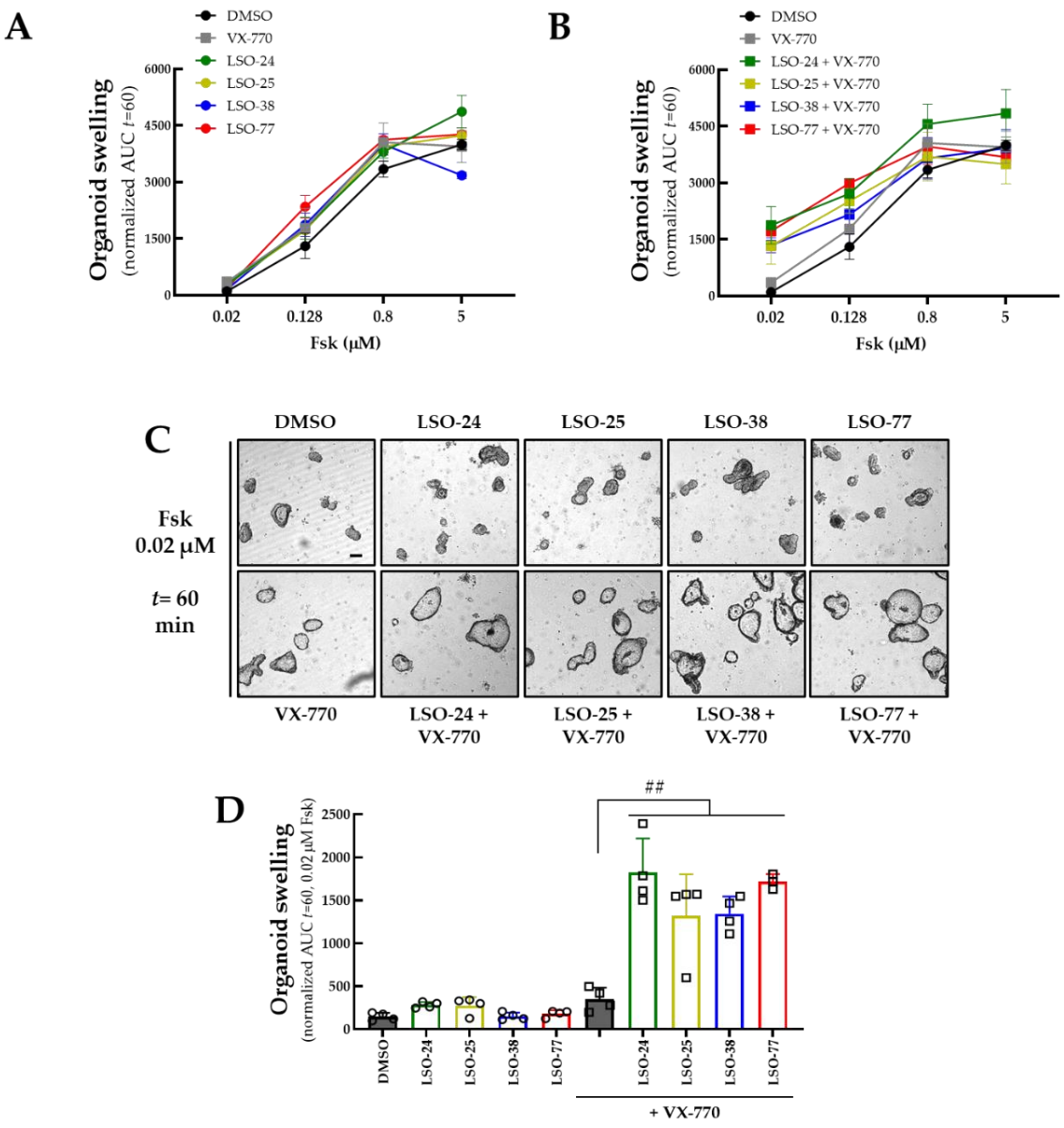

Figure 7. Assessment of additivity of potentiators activity on R334W-CFTR function in intestinal organoids. (A, B) Quantification of FIS in intestinal organoids from an individual with CF (R334W/R334W genotype) for all test compounds **(A)** individually and **(B)** in combination with VX-770 at Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, *t* = 60 min). **(C)** Representative images intestinal organoids (Scale bar: 50 µm) and **(D)** quantification at 0.02 µM Fsk for test compounds individually or in combination. Data are represented as means ± SD: $^{\#\#}$ $p < 0.01$ vs. VX-770 alone.

On the other hand, organoid FIS values were similar to those of DMSO upon stimulation with 0.02 µM Fsk with any single potentiator **(Figure 7C, D)**. However, significantly higher FIS values were observed for organoids incubated with LSO-24, LSO-25, LSO-38, or LSO-77 in combination with VX-770, compared to DMSO (3.78- to 5.36-fold increase vs. VX-770 alone), upon stimulation with 0.02 µM Fsk **(Figure 7C, D)**.

5.DISCUSSION

This study aimed to screen a collection of compounds to identify novel potentiators for R334W-CFTR, a residual function mutation that is reported to have no to suboptimal susceptibility to VX-770 monotherapy (Phuan et al., 2019; Van Goor et al., 2014; Veit et al., 2020). Because the four active compounds identified in the functional primary screening only demonstrated a small potentiation of R334W-CFTR function, their additivity with VX-770, genistein, or VX-445 was assessed in a new cell line and then further validated in intestinal organoids. Moreover, LSO-24, LSO-25, LSO-38, and LSO-77 were found to obey Lipinski's rule of five for drug-like molecules (Lipinski, 2004) and are thus suggested to have good bioavailability.

Similar to WT-CFTR, the R334W mutation enables CFTR to be fully glycosylated and traffic to the plasma membrane. However, in contrast to WT-CFTR that is a functional protein, R334W-CFTR presents a significantly reduced channel conductance (Sheppard et al., 1993; Smith et al., 2001). When R334W-expressing cells were shifted from 37^{o}C to 27^{o}C, there was an increase in CFTR processing, indicating that this mutant is temperature sensitive, similar to previously reported for WT- and F508del-CFTR (Denning et al., 1992; Lopes-Pacheco et al., 2015; Varga et al., 2008). Furthermore, the increased cell fluorescence quenching rate suggests that low-temperature incubation resulted in higher R334W-CFTR-dependent anion transport, most likely by increasing the number of CFTR channels at the plasma membrane rather than enhancing channel gating.

Numerous screening campaigns have been carried out to identify CFTR potentiators in libraries of drug-like small molecules, which resulted in the discovery of several compounds with distinct chemical structures that are able to potentiate CFTR function (Gees et al., 2018; Moran et al., 2005; Phuan et al., 2018; Van Der Plas et al., 2021; Van Goor et al., 2009). Nevertheless, few potentiators achieved clinical investigation and up to recently, only the potentiator VX-770 was clinically approved for PwCF carrying specific mutations (De Boeck et al., 2014; Lopes-Pacheco et al., 2021; Ramsey et al., 2011; Silva et al., 2022; Van Goor et al., 2014; Yu et al., 2012). ABBV-974 (formerly GLPG-1837) has the same binding site to VX-770 in the transmembrane domains of CFTR (Liu

et al., 2019; Yeh et al., 2019) and acts by facilitating the channel gating in a phosphorylation-dependent and ATP-independent fashion (Eckford et al., 2012; Yeh et al., 2017). GLPG-2451 has improved potency (Van Der Plas et al., 2021) but is expected to share the same mechanism to VX-770 and ABBV-974 (Gees et al., 2018). A previous study has attempted to substitute the amide bioisostere in the VX-770 structure to a 1,2,3-triazole scaffold and demonstrated reduced efficacy and potency in potentiating WT-, F508del- and G551D-CFTR function by this compound series (Doiron et al., 2019). The active triazole compounds identified in our functional screening (LSO-24, LSO-25, and LSO-38) also demonstrated a suboptimal efficacy and weak potency, but they appear to potentiate R334W-CFTR function by a different mechanism than that of VX-770.

Genistein and its analog apigenin also potentiate CFTR channel gating by a distinct mechanism to that of VX-770 (Moran et al., 2005; Phuan et al., 2018). They are presumable to bind at the nucleotide-binding domain 1 and 2 interface and promote dimerization, thus accelerating channel opening and slowing down its closure (Moran et al., 2005). ASP-11 is another compound that is suggested to potentiate CFTR function by this mechanism (Phuan et al., 2018). Furthermore, VX-445 (also termed elexacaftor) was initially identified as a CFTR corrector and approved for clinical use in the triple combo therapy VX-445/VX-661/VX-770 (Heijerman et al., 2019), but is now recognized as a compound with dual corrector and potentiator activity (Laselva et al., 2021; Veit et al., 2021). Although the mechanism by which VX-445 promotes CFTR potentiation is not elucidated, it is suggested to be different from that exerted by VX-770 and apigenin (Ensinck et al., 2022; Veit et al., 2021).

Despite the therapeutic success of VX-770 monotherapy (Ramsey et al., 2011), it achieves only a partial restoration of the CFTR gating activity for several mutations, including G551D and F508del (Jih & Hwang, 2013; Van Goor et al., 2009; Veit et al., 2021), and PwCF still experience a progressive decline of their lung function and pulmonary exacerbations albeit at reduced frequency (Flume et al., 2018; Volkova et al., 2020). As different potentiators may promote distinct conformational alterations in the CFTR protein to increase channel gating/conductance (Liu et al., 2019; Yeh et al., 2019), co-potentiators have emerged as a strategy to enhance CFTR-dependent chloride secretion for mutations that do not respond well to a single potentiator (Phuan et al.,

2018, 2019; Veit et al., 2020, 2021). Combinatorial profiling has shed some light on the mechanism of co-potentiation by clustering compounds into three mechanistic classes (Phuan et al., 2019; Veit et al., 2021). Nevertheless, CFTR mutations are not equally responsive to combined potentiators and some mutations were demonstrated to be sensitive (e.g., G551D, G1244E, and S1251N) while others were unresponsive (e.g., R347P, V520F, and L1077P) (Phuan et al., 2019; Veit et al., 2020, 2021). N1303K-CFTR function was also demonstrated to synergistically respond to a triple potentiator combo (VX-770, apigenin, and VX-445) (Ensinck et al., 2022). Our investigational compounds (LSO-24, LSO-25, LSO-38, and LSO-77) demonstrated an additive potentiation of R334W-CFTR function in combination with VX-770, but not with genistein. These results suggest that they may share a common mechanism with genistein. ASP-11 is also suggested to share a common mechanism with genistein, but was not additive to potentiate R334W-CFTR function in combination with VX-770 (Phuan et al., 2019). This probably occurred because this mutation is associated with residual CFTR function and the usage of a high concentration of Fsk (20 µM) already saturated CFTR activity (Phuan et al., 2019), thus hindering further potentiation of CFTR channel activity. Indeed, such fact is also observed by our investigational compounds in the FIS assay of intestinal organoids (R334W/R334W genotype). At the lowest concentration of Fsk (0.02 µM), there is a clear difference between the negative control and single potentiators with the co-potentiator treatments. However, upon the incremental concentration of Fsk, such differences could no longer be easily detected due to high residual function. Similar behavior was also reported for D614G, another mutation with residual CFTR function (Silva et al., 2021), reinforcing thus the need for assessing the concentration of Fsk (or other CFTR activators) when investigating a residual function mutation or when testing a 'highly effective' modulator combination.

Although the R334W mutation causes neither folding nor trafficking impairment, some studies have demonstrated that R334W-CFTR-dependent chloride secretion can be enhanced by increasing the number of R334W-CFTR channels at the plasma membrane with correctors, such as VX-809 and VX-661 (Bacalhau et al., 2023; Lopes-Pacheco et al., 2022; Lopes-Pacheco et al., 2020; Van Willigen et al., 2019). In a recent report, intestinal organoids from an individual with CF (1677delTA/R334W) were incubated with VX-770, VX-809/VX-770, VX-661/VX-770 and VX-445/VX-661/VX-770

(Mitropoulou et al., 2022). Upon Fsk stimulation, an increase in FIS values was observed with the different modulator combinations but to a comparable extent to that of VX-770 alone (Mitropoulou et al., 2022). Another report assessed the efficacy of VX-445/VX-661/VX-770 in F508del/R334W intestinal organoid-derived epithelial monolayers and demonstrated rescue of CFTR-mediated anion transport (Ciciriello et al., 2022). However, it has been suggested that the triple combo therapy only modestly improved R334W-CFTR function and most of the gain in function was attributed to F508del-CFTR rescue (Ciciriello et al., 2022). While the corrector activity of VX-445 on R334W-CFTR needs to be further elucidated, our data indicate that this compound does not potentiate R334W-CFTR function when used alone or in combination with LSO molecules.

6.CONCLUSION

In summary, the present study identified four small molecules that are able to potentiate R334W-CFTR function, a mutation for which no modulator therapy is currently approved. Despite the suboptimal efficacy of LSO-24, LSO-25, LSO-38 and LSO-77, they have a different scaffold from that of previous potentiators and may thus represent a valuable starting point to design analogue molecules with improved CFTR potentiator activity. These compounds also demonstrated an additive potentiation of CFTR function in combination with VX-770 in both R334W-heterologously expressing cells and intestinal organoids from a R334W homozygous CF subject. The additive potentiation effects support the idea that they act by a mechanism distinct from that of VX-770, and their potential utility to enhance CFTR function for other mutations should be exploited in upcoming studies.

REFERENCES

Awatade, N. T., Uliyakina, I., Farinha, C. M., Clarke, L. A., Mendes, K., Solé, A., Pastor, J., Ramos, M. M., & Amaral, M. D. (2015). Measurements of Functional Responses in Human Primary Lung Cells as a Basis for Personalized Therapy for Cystic Fibrosis. *EBioMedicine*, *2*(2), 147–153. https://doi.org/10.1016/j.ebiom.2014.12.005

Bacalhau, M., Ferreira, F. C., Kmit, A., Souza, F. R., da Silva, V. D., Pimentel, A. S., Amaral, M. D., Buarque, C. D., & Lopes-Pacheco, M. (2023). Identification of novel F508del-CFTR traffic correctors among triazole derivatives. *European Journal of Pharmacology*, *938*, 175396. https://doi.org/10.1016/j.ejphar.2022.175396

Baell, J. B., & Holloway, G. A. (2010). New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *Journal of Medicinal Chemistry*, *53*(7), 2719–2740. https://doi.org/10.1021/jm901137j

Bebok, Z., Collawn, J. F., Wakefield, J., Parker, W., Li, Y., Varga, K., Sorscher, E. J., & Clancy, J. P. (2005). Failure of cAMP agonists to activate rescued ΔF508 CFTR in CFBE41o- airway epithelial monolayers. *Journal of Physiology*, *569*(Pt 2), 601–615. https://doi.org/10.1113/jphysiol.2005.096669

Bessonova, L., Volkova, N., Higgins, M., Bengtsson, L., Tian, S., Simard, C., Konstan, M. W., Sawicki, G. S., Sewall, A., Nyangoma, S., Elbert, A., Marshall, B. C., & Bilton, D. (2018). Data from the US and UK cystic fibrosis registries support disease modification by CFTR modulation with ivacaftor. *Thorax*, *73*(8), 731–740. https://doi.org/10.1136/thoraxjnl-2017-210394

Bonandi, E., Christodoulou, M. S., Fumagalli, G., Perdicchia, D., Rastelli, G., & Passarella, D. (2017). The 1,2,3-triazole ring as a bioisostere in medicinal chemistry. *Drug Discovery Today*, *22*(10), 1572–1581. https://doi.org/10.1016/j.drudis.2017.05.014

Ciciriello, F., Bijvelds, M. J. C., Alghisi, F., Meijsen, K. F., Cristiani, L., Sorio, C., Melotti, P., Fiocchi, A. G., Lucidi, V., & De Jonge, H. R. (2022). Theratyping of the Rare CFTR Variants E193K and R334W in Rectal Organoid-Derived Epithelial Monolayers. *Journal of Personalized Medicine*, *12*(4), 632. https://doi.org/10.3390/jpm12040632

Cística, G. B. de E. de F. (2019). *Registro Brasileiro de Fibrose Cística - Relatório Anual de 2019*. http://portalgbefc.org.br/ckfinder/userfiles/files/REBRAFC_2019.pdf

Cutting, G. R. (2015). Cystic fibrosis genetics: from molecular understanding to clinical application. *Nature Reviews Genetics*, *16*(1), 45–56. https://doi.org/10.1038/nrg3849

da Silva, Veronica D., de Faria, B. M., Colombo, E., Ascari, L., Freitas, G. P. A., Flores, L. S., Cordeiro, Y., Romão, L., & Buarque, C. D. (2019). Design, synthesis, structural characterization and in vitro evaluation of new 1,4-disubstituted-1,2,3-triazole derivatives against glioblastoma cells. *Bioorganic Chemistry*, *83*, 87–97. https://doi.org/10.1016/j.bioorg.2018.10.003

da Silva, Verônica Diniz;, Silva, R. R. ., Gonçalves Neto, J., López-Corcuera, B., Guimarães,

M. Z. ., Noël, F., & Buarque, C. D. (2020). New α-Hydroxy-1,2,3-triazoles and 9H-Fluorenes-1,2,3-triazolesSynthesis and Evaluation as Glycine Transporter 1 Inhibitors. *Journal of Brazilian Chemistry Society*, *31*(6), 1258–1269. https://doi.org/10.21577/0103-5053.20200011

Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, *7*, 42717. https://doi.org/10.1038/srep42717

De Boeck, K., Munck, A., Walker, S., Faro, A., Hiatt, P., Gilmartin, G., & Higgins, M. (2014). Efficacy and safety of ivacaftor in patients with cystic fibrosis and a non-G551D gating mutation. *Journal of Cystic Fibrosis*, *13*(6), 674–680. https://doi.org/10.1016/j.jcf.2014.09.005

Dekkers, J. F., Wiegerinck, C. L., De Jonge, H. R., Bronsveld, I., Janssens, H. M., De Winter-De Groot, K. M., Brandsma, A. M., De Jong, N. W. M., Bijvelds, M. J. C., Scholte, B. J., Nieuwenhuis, E. E. S., Van Den Brink, S., Clevers, H., Van Der Ent, C. K., Middendorp, S., & Beekman, J. M. (2013). A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nature Medicine*, *19*(7), 939–945. https://doi.org/10.1038/nm.3201

Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., & Welsh, M. J. (1992). Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature*, *358*(6389), 761–764. https://doi.org/10.1038/358761a0

Doiron, J. E., Le, C. A., Ody, B. K., Brace, J. B., Post, S. J., Thacker, N. L., Hill, H. M., Breton, G. W., Mulder, M. J., Chang, S., Bridges, T. M., Tang, L., Wang, W., Rowe, S. M., Aller, S. G., & Turlington, M. (2019). Evaluation of 1,2,3-Triazoles as Amide Bioisosteres In Cystic Fibrosis Transmembrane Conductance Regulator Modulators VX-770 and VX-809. *Chemistry*, *25*(14), 3662–3674. https://doi.org/10.1002/chem.201805919

Eckford, P. D. W., Li, C., Ramjeesingh, M., & Bear, C. E. (2012). Cystic fibrosis transmembrane conductance regulator (CFTR) potentiator VX-770 (ivacaftor) opens the defective channel gate of mutant CFTR in a phosphorylation-dependent but ATP-independent manner. *Journal of Biological Chemistry*, *287*(44), 36639–36649. https://doi.org/10.1074/jbc.M112.393637

Ensinck, M. M., De Keersmaecker, L., Ramalho, A. S., Cuyx, S., Van Biervliet, S., Dupont, L., Christ, F., Debyser, Z., Vermeulen, F., & Carlon, M. S. (2022). Novel CFTR modulator combinations maximise rescue of G85E and N1303K in rectal organoids. *ERJ Open Research*, *8*(2), 00716–02021. https://doi.org/10.1183/23120541.00716-2021

Ferreira, M. D. L. G., Pinheiro, L. C. S., Santos-Filho, O. A., Peçanha, M. D. S., Sacramento, C. Q., Machado, V., Ferreira, V. F., Souza, T. M. L., & Boechat, N. (2014). Design, synthesis, and antiviral activity of new 1H-1,2,3-triazole nucleoside ribavirin analogs. *Medicinal Chemistry Research*, *23*(3), 1501–1511. https://doi.org/10.1007/s00044-013-0762-6

Flume, P. A., Wainwright, C. E., Elizabeth Tullis, D., Rodriguez, S., Niknian, M., Higgins,

M., Davies, J. C., & Wagener, J. S. (2018). Recovery of lung function following a pulmonary exacerbation in patients with cystic fibrosis and the G551D-CFTR mutation treated with ivacaftor. *Journal of Cystic Fibrosis*, *17*(1), 83–88. https://doi.org/10.1016/j.jcf.2017.06.002

Gees, M., Musch, S., Van Der Plas, S., Wesse, A. S., Vandevelde, A., Verdonck, K., Mammoliti, O., Hwang, T. C., Sonck, K., Stouten, P., Swensen, A. M., Jans, M., Van Der Schueren, J., Nelles, L., Andrews, M., & Conrath, K. (2018). Identification and characterization of novel CFTR potentiators. *Frontiers in Pharmacology*, *9*, 1221. https://doi.org/10.3389/fphar.2018.01221

Heijerman, H. G. M., McKone, E. F., Downey, D. G., Van Braeckel, E., Rowe, S. M., Tullis, E., Mall, M. A., Welter, J. J., Ramsey, B. W., McKee, C. M., Marigowda, G., Moskowitz, S. M., Waltz, D., Sosnay, P. R., Simard, C., Ahluwalia, N., Xuan, F., Zhang, Y., Taylor-Cousar, J. L., & McCoy, K. S. (2019). Efficacy and safety of the elexacaftor plus tezacaftor plus ivacaftor combination regimen in people with cystic fibrosis homozygous for the F508del mutation: a double-blind, randomised, phase 3 trial. *Lancet*, *394*(10212), 1940–1948. https://doi.org/10.1016/S0140-6736(19)32597-8

Jih, K. Y., & Hwang, T. C. (2013). Vx-770 potentiates CFTR function by promoting decoupling between the gating cycle and ATP hydrolysis cycle. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(11), 4404–4409. https://doi.org/10.1073/pnas.1215982110

Kant, R., Kumar, D., Agarwal, D., Gupta, R. D., Tilak, R., Awasthi, S. K., & Agarwal, A. (2016). Synthesis of newer 1,2,3-triazole linked chalcone and flavone hybrid compounds and evaluation of their antimicrobial and cytotoxic activities. *European Journal of Medicinal Chemistry*, *113*, 34–49. https://doi.org/10.1016/j.ejmech.2016.02.041

Kim, T. W., Yong, Y., Shin, S. Y., Jung, H., Park, K. H., Lee, Y. H., Lim, Y., & Jung, K. Y. (2015). Synthesis and biological evaluation of phenyl-1H-1,2,3-triazole derivatives as anti-inflammatory agents. *Bioorganic Chemistry*, *59*, 1–11. https://doi.org/10.1016/j.bioorg.2015.01.003

Laselva, O., Bartlett, C., Gunawardena, T. N. A., Ouyang, H., Eckford, P. D. W., Moraes, T. J., Bear, C. E., & Gonska, T. (2021). Rescue of multiple class II CFTR mutations by elexacaftor+tezacaftor+ ivacaftor mediated in part by the dual activities of elexacaftor as both corrector and potentiator. *European Respiratory Journal*, *57*(6), 2002774. https://doi.org/10.1183/13993003.02774-2020

Lipinski, C. A. (2004). Lead- and drug-like compounds: The rule-of-five revolution. *Drug Discovery Today: Technologies*, *1*(4), 337–341. https://doi.org/10.1016/j.ddtec.2004.11.007

Liu, F., Zhang, Z., Levit, A., Levring, J., Touhara, K. K., Shoichet, B. K., & Chen, J. (2019). Structural identification of a hotspot on CFTR for potentiation. *Science*, *364*(6446), 1184–1188. https://doi.org/10.1126/science.aaw7611

Liu, J., Berg, A. P., Wang, Y., Jantarajit, W., Sutcliffe, K. J., Stevens, E. B., Cao, L., Pregel, M. J., & Sheppard, D. N. (2022). A small molecule CFTR potentiator restores ATP-

dependent channel gating to the cystic fibrosis mutant G551D-CFTR. *British Journal of Pharmacology*, *179*(7), 1319–1337. https://doi.org/10.1111/bph.15709

Lopes-Pacheco, M, Bacalhau, M., Ramalho, S. S., Silva, I. A. L. ., Ferreira, F. C. ., Carlile, G. W. ., Thomas, D. Y. ., Farinha, C. M. ., Hanrahan, J. W. ., & Amaral, M. D. (2022). Rescue of Mutant CFTR Trafficking Defect by the Investigational Compound MCG1516A. *Cells*, *11*(1), 136. https://doi.org/10.3390/cells11010136

Lopes-Pacheco, M. (2020). CFTR Modulators: The Changing Face of Cystic Fibrosis in the Era of Precision Medicine. *Frontiers in Pharmacology*, *10*, 1662. https://doi.org/10.3389/fphar.2019.01662

Lopes-Pacheco, M, Boinot, C., Sabirzhanova, I., Morales, M. M., Guggino, W. B., & Cebotaru, L. (2015). Combination of Correctors Rescue ΔF508-CFTR by Reducing Its Association with Hsp40 and Hsp27. *Journal of Biological Chemistry*, *290*(42), 25636–25645. https://doi.org/10.1074/jbc.m115.671925

Lopes-Pacheco, M, Pedemonte, N., & Veit, G. (2021). Discovery of CFTR modulators for the treatment of cystic fibrosis. *Expert Opinion on Drug Discovery*, *16*(8), 897–913. https://doi.org/10.1080/17460441.2021.1912732

Lopes-Pacheco, M, Silva, I. A. L., Turner, M. J., Carlile, G. W., Sondo, E., Thomas, D. Y., Pedemonte, N., Hanrahan, J. W., & Amaral, M. D. (2020). Characterization of the mechanism of action of RDR01752, a novel corrector of F508del-CFTR. *Biochemical Pharmacology*, *180*, 114133. https://doi.org/10.1016/j.bcp.2020.114133

Mitropoulou, G., Brandenberg, N., Hoehnel, S., Ceroni, C., Balmpouzis, Z., Blanchon, S., Dorta, G., Sauty, A., & Koutsokera, A. (2022). Rectal organoid-guided CFTR modulator therapy restores lung function in a CF patient with the rare 1677delTA/R334W genotype. *European Respiratory Journal*. https://doi.org/10.1183/13993003.01341-2022

Moran, O., Galietta, L. J. V, & Zegarra-Moran, O. (2005). Binding site of activators of the cystic fibrosis transmembrane conductance regulator in the nucleotide binding domains. *Cellular and Molecular Life Sciences*, *62*(4), 446–460. https://doi.org/10.1007/s00018-004-4422-3

Naaz, F., Preeti Pallavi, M. C., Shafi, S., Mulakayala, N., Shahar Yar, M., & Sampath Kumar, H. M. (2018). 1,2,3-triazole tethered Indole-3-glyoxamide derivatives as multiple inhibitors of 5-LOX, COX-2 & tubulin: Their anti-proliferative & anti-inflammatory activity. *Bioorganic Chemistry*, *81*, 1–20. https://doi.org/10.1016/j.bioorg.2018.07.029

Pedemonte, N., Zegarra-moran, O., & Galietta, L. J. V. (2011). High-throughput screening of libraries of compounds to identify CFTR modulators. *Methods in Molecular Biology*, *741*, 13–21. https://doi.org/10.1007/978-1-61779-117-8

Phuan, P. W., Son, J. H., Tan, J. A., Li, C., Musante, I., Zlock, L., Nielson, D. W., Finkbeiner, W. E., Kurth, M. J., Galietta, L. J., Haggie, P. M., & Verkman, A. S. (2018). Combination potentiator ('co-potentiator') therapy for CF caused by CFTR mutants, including N1303K, that are poorly responsive to single potentiators. *Journal of Cystic Fibrosis*, *17*(5), 595–606. https://doi.org/10.1016/j.jcf.2018.05.010

Phuan, P. W., Tan, J. A., Rivera, A. A., Zlock, L., Nielson, D. W., Finkbeiner, W. E., Haggie, P. M., & Verkman, A. S. (2019). Nanomolar-potency 'co-potentiator' therapy for cystic fibrosis caused by a defined subset of minimal function CFTR mutants. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-54158-2

Ramsey, B. W., Davies, J. C., McElvaney, N. G., Tullis, E., Bell, S. C., Drevinek, P., Griese, M., McKone, E. F., Wainwright, C. E., Konstan, M. W., Moss, R., Ratjen, F., Sermet-Gaudelus, I., Rowe, S. M., Dong, Q., Rodriguez, S., Yen, K., Ordoñez, C., & Elborn, J. S. (2011). A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *New England Journal of Medicine*, *365*(18), 1663–1672. https://doi.org/0.1056/NEJMoa1105185

Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N. O. A., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S. I., Plavsic, N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., & Tsui, L. C. (1989). Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science*, *245*(4922), 1066–1073. https://doi.org/10.1126/science.2475911

Saint-Criq, V., & Gray, M. A. (2017). Role of CFTR in epithelial physiology. *Cellular and Molecular Life Sciences*, *74*(1), 93–115. https://doi.org/10.1007/s00018-016-2391-y

Sheppard, D. N., Rich, D. P., Ostedgaard, L. S., Gregory, R. J., Smith, A. E., & Welsh, M. J. (1993). Mutations in CFTR associated with mild-disease-form Cl- channels with altered pore properties. *Nature*, *362*(6416), 160–164. https://doi.org/10.1038/362160a0

Silva, I. A. L., Laselva, O., & Lopes-Pacheco, M. (2022). Advances in Preclinical In Vitro Models for the Translation of Precision Medicine for Cystic Fibrosis. *Journal of Personalized Medicine*, *12*(8), 1321. https://doi.org/10.3390/jpm12081321

Silva, I. A. L., Railean, V., Duarte, A., & Amaral, M. D. (2021). Personalized Medicine Based on Nasal Epithelial Cells: Comparative Studies with Rectal Biopsies and Intestinal Organoids. *Journal of Personalized Medicine*, *11*(5), 421. https://doi.org/10.3390/jpm11050421

Smith, S. S., Liu, X., Zhang, Z. R., Sun, F., Kriewall, T. E., Mccarty, N. A., & Dawson, D. C. (2001). CFTR: Covalent and noncovalent modification suggests a role for fixed charges in anion conduction. *Journal of General Physiology*, *118*(4), 407–431. https://doi.org/10.1085/jgp.118.4.407

Stefely, J. A., Palchaudhuri, R., Miller, P. A., Peterson, R. J., Moraski, G. C., Hergenrother, P. J., & Miller, M. J. (2010). N -((1-benzyl-1 H -1,2,3-triazol-4-yl)methyl)arylamide as a new scaffold that provides rapid access to antimicrotubule agents: Synthesis and evaluation of antiproliferative activity against select cancer cell lines. *Journal of Medicinal Chemistry*, *53*(8), 3389–3395. https://doi.org/10.1021/jm1000979

Taylor-Cousar, J. L., Munck, A., McKone, E. F., van der Ent, C. K., Moeller, A., Simard, C., Wang, L. T., Ingenito, E. P., McKee, C., Lu, Y., Lekstrom-Himes, J., & Elborn, J. S. (2017). Tezacaftor–Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del. *New England Journal of Medicine*, *377*(21), 2013–2023.

https://doi.org/10.1056/nejmoa1709846

Van Der Plas, S. E., Kelgtermans, H., Mammoliti, O., Menet, C., Tricarico, G., De Blieck, A., Joannesse, C., De Munck, T., Lambin, D., Cowart, M., Dropsit, S., Martina, S. L. X., Gees, M., Wesse, A. S., Conrath, K., & Andrews, M. (2021). Discovery of GLPG2451, a Novel Once Daily Potentiator for the Treatment of Cystic Fibrosis. *Journal of Medicinal Chemistry*, *64*(1), 343–353. https://doi.org/10.1021/acs.jmedchem.0c01796

Van Goor, F., Hadida, S., Grootenhuis, P. D. J., Burton, B., Cao, D., Neuberger, T., Turnbull, A., Singh, A., Joubran, J., Hazlewood, A., Zhou, J., McCartney, J., Arumugam, V., Decker, C., Yang, J., Young, C., Olson, E. R., Wine, J. J., Frizzell, R. A., … Negulescu, P. (2009). Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(44), 18825–18830. https://doi.org/10.1073/pnas.0904709106

Van Goor, F., Yu, H., Burton, B., & Hoffman, B. J. (2014). Effect of ivacaftor on CFTR forms with missense mutations associated with defects in protein processing or function. *Journal of Cystic Fibrosis*, *13*(1), 29–36. https://doi.org/10.1016/j.jcf.2013.06.008

Van Willigen, M., Vonk, A. M., Yeoh, H. Y., Kruisselbrink, E., Kleizen, B., Van Der Ent, C. K., Egmond, M. R., De Jonge, H. R., Braakman, I., Beekman, J. M., & Van Der Sluijs, P. (2019). Folding-function relationship of the most common cystic fibrosis-causing CFTR conductance mutants. *Life Science Alliance*, *2*(1), 2201800172. https://doi.org/10.26508/lsa.201800172

Varga, K., Goldstein, R. F., Jurkuvenaite, A., Chen, L., Matalon, S., Sorscher, E. J., Bebok, Z., & Collawn, J. F. (2008). Enhanced cell-surface stability of rescued DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) by pharmacological chaperones. *Biochemical Journal*, *410*(3), 555–564. https://doi.org/10.1042/BJ20071420

Veit, G., Da Fonte, D. F., Avramescu, R. G., Premchandar, A., Bagdany, M., Xu, H., Bensinger, D., Stubba, D., Schmidt, B., Matouk, E., & Lukacs, G. L. (2020). Mutation-specific dual potentiators maximize rescue of CFTR gating mutants. *Journal of Cystic Fibrosis*, *19*(2), 236–244. https://doi.org/10.1016/j.jcf.2019.10.011

Veit, G., Vaccarin, C., & Lukacs, G. L. (2021). Elexacaftor co-potentiates the activity of F508del and gating mutants of CFTR. *Journal of Cystic Fibrosis*, 3–6. https://doi.org/10.1016/j.jcf.2021.03.011

Volkova, N., Moy, K., Evans, J., Campbell, D., Tian, S., Simard, C., Higgins, M., Konstan, M. W., Sawicki, G. S., Elbert, A., Charman, S. C., Marshall, B. C., & Bilton, D. (2020). Disease progression in patients with cystic fibrosis treated with ivacaftor: Data from national US and UK registries. *Journal of Cystic Fibrosis*, *19*(1), 68–79. https://doi.org/10.1016/j.jcf.2019.05.015

Wainwright, C. E., Elborn, J. S., Ramsey, B. W., Marigowda, G., Huang, X., Cipolli, M., Colombo, C., Davies, J. C., De Boeck, K., Flume, P. A., Konstan, M. W., McColley, S. A., McCoy, K., McKone, E. F., Munck, A., Ratjen, F., Rowe, S. M., Waltz, D., & Boyle,

M. P. (2015). Lumacaftor–Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *New England Journal of Medicine*, *373*(3), 220–231. https://doi.org/10.1056/nejmoa1409547

Wang, X., Huang, B., Liu, X., & Zhan, P. (2016). Discovery of bioactive molecules from CuAAC click-chemistry-based combinatorial libraries. *Drug Discovery Today*, *21*(1), 118–132. https://doi.org/10.1016/j.drudis.2015.08.004

World Health Organization. (2004). *The molecular genetic epidemiology of cystic fibrosis. Report of a joint meeting of WHO/ECFTN/ICF(M)A/ECFS*. https://apps.who.int/iris/handle/10665/68702

Yeh, H.-I., Qiu, L., Sohma, Y., Conrath, K., Zou, X., & Hwang, T.-C. (2019). Identifying the molecular target sites for CFTR potentiators GLPG1837 and VX-770. *Journal of General Physiology*, *151*(7), 912–928. https://doi.org/10.1085/jgp.201912360

Yeh, H.-I., Sohma, Y., Conrath, K., & Hwang, T.-C. (2017). A common mechanism for CFTR potentiators. *Journal of General Physiology*, *149*(12), 1105–1118. https://doi.org/10.1085/jgp.201711886

Yu, H., Burton, B., Huang, C. J., Worley, J., Cao, D., Johnson, J. P., Urrutia, A., Joubran, J., Seepersaud, S., Sussky, K., Hoffman, B. J., & Van Goor, F. (2012). Ivacaftor potentiation of multiple CFTR channels with gating mutations. *Journal of Cystic Fibrosis*, *11*(3), 237–245. https://doi.org/10.1016/j.jcf.2011.12.005

APPENDIX

List of articles published in peer-reviewed scientific journals by Mafalda Bacalhau and co-authors, in the last 2 years:

1. **Mafalda Bacalhau**, Mariana Camargo, Grace A V Magalhães-Ghiotto, Sybelle Drumond, Carlos H M Castelletti, Miquéias Lopes-Pacheco. “Elexacaftor-Tezacaftor-Ivacaftor: A Life-Changing Triple Combination of CFTR Modulator Drugs for Cystic Fibrosis”. *Pharmaceuticals* (2023); 16(3):410.
2. **Mafalda Bacalhau**, Filipa C. Ferreira, Iris A. L. Silva, Camilla D. Buarque, Margarida D. Amaral, Miquéias Lopes-Pacheco. “Additive Potentiation of R334W-CFTR Function by Novel Small Molecules”. *Journal of Personalized Medicine* (2023); 13(1):102.
3. **Mafalda Bacalhau**; Filipa C. Ferreira; Filipe R. Souza; Arthur Kmit; Verônica D. da Silva; André S. Pimentel; Margarida D. Amaral; Camilla D. Buarque; Miquéias Lopes-Pacheco. "Identification of Novel F508del-CFTR Traffic Correctors Among Triazole Derivative Compounds". *European Journal of Pharmacology* (2023); 938:175396.
4. Miquéias Lopes-Pacheco; **Mafalda Bacalhau**; Sofia S. Ramalho; Iris A. L. Silva; Filipa C. Ferreira; Graeme W. Carlile; David Y. Thomas; *et al.* "Rescue of Mutant CFTR Trafficking Defect by the Investigational Compound MCG1516A". *Cells* (2022); 11(1):136.
5. Lúcia Santos; Karen Mention; Kader Cavusoglu-Doran; David J. Sanz; **Mafalda Bacalhau**; Miquéias Lopes-Pacheco; Patrick T Harrison; Carlos M Farinha. "Comparison of Cas9 and Cas12a CRISPR editing methods to correct the W1282X-CFTR mutation". *Journal of Cystic Fibrosis* 21 1 (2022): 181-187.