Novel Correctors and Potentiators Augment Efficacy of Translational Readthrough in CFTR Nonsense Mutations

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Introduction

•Premature termination codons (PTCs) in the *CFTR* gene result in nonfunctional CFTR protein and are the proximate cause of ~11% of CF causing alleles. Use of aminoglycosides has been shown to induce readthrough (RT) of PTCs, restoring CFTR expression and function.

•To achieve therapeutic levels, we have previously shown that a combination approach of using the CFTR potentiator ivacaftor (VX-770) with the corrector lumacaftor (VX-809) is beneficial for enhancing RT of premature stop mutations.

•Using novel correctors and potentiators from Galapagos, we examined if these modulators had synergistic effect with RT agents that could enhance CFTR function to achieve therapeutic levels.

Methods

Cell Culture
- Fisher Rat Thyroid (FRT) cells: FRT's were stably transduced with CFTR G542X, W1282X and R1162X cDNA using the Flp-In system (Invitrogen).
- Primary cells: Human bronchial epithelial cells derived from lung explants carrying a nonsense alleles G542X or W1282X in trans with F508del CFTR (G542X/ΔF508,W1282X/ΔF508) were seeded on to permeable supports, grown in differentiating media for at least 6-8 weeks until terminally differentiated.

Conductance (transepithelial chloride conductance, TECC) Assay
- For conductance (Gt) measurements cells were grown to confluence and treated with correctors, corrector 1 (C1 or early corrector, 0.5 μM) and corrector 2 (C2 or late corrector (complementary to C1), 3 μM) or RT agent G418 (250 μg/ml) for 48 hrs.
- CFTR activity was measured as a change from baseline conductance following the addition of forskolin (10 μM), GLPG1837 (GP-5; 10 μM), VX-770 (10 μM) and then $CFTR_{Inh}$-172 (10 μM).

Horse Radish Peroxidase (HRP) Assay
- FRT cells expressing the fusion protein of G542X CFTR and HRP were treated with the correctors or G418 for two days at 37C. The cells were washed three times with D-PBS supplemented with $CaCl_2$(0.1mM) and $MgCl_2$(1mM). HRP is detected via a luminescent in three replicates.

Ussing chamber
- Short circuit current (Isc) in primary cells was measured, where baseline measurement was taken before the apical addition of 100 uM Amiloride. Forskolin (FSK) was administered to stimulate CFTR activity before Ivacaftor (VX-770) followed by the addition of CFTR specific inhibitor, CFTR$_{inh}$-172.

Dose response of GP-5 & VX-770 in FRT cells with CFTR PTC mutations following readthrough

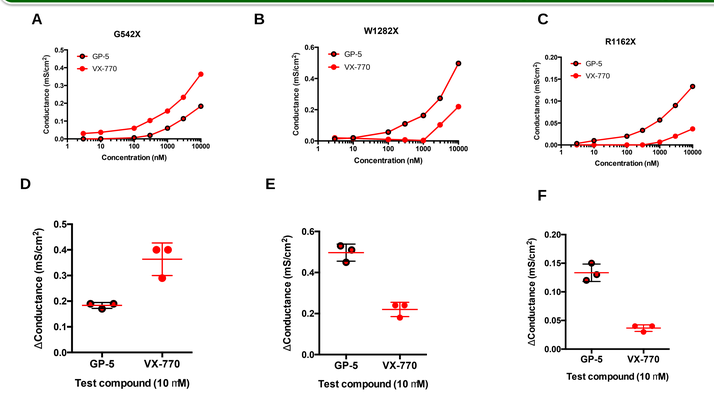

Fig.1. Effect of potentiators on FRT cells with stopcodon mutants pretreated with G418 : FRT cell monolayers with PTC constructs (G542X, W1282X, R1162X) were pretreated with G418 for 48 hrs followed by conductance (Gt) measurements with acute addition of FSK, potentiators, and $CFTR_{Inh}$-172 **(A)** Dose response (ranging from 3 nM to 10,000 nM) of GP-5, VX-770 in FRT G542X **(B)** FRT W1282X and **(C)** FRT R1162X cells. Scatter plots representing maximum efficacy of potentiators at 10 μM in **(D)** FRT G542X **(E)** FRT W1282X and **(F)** R1162X cells. Potentiators had no effect on FRT parental cells without CFTR (data not shown) indicating specificity. **** P<0.0001

Correctors + Potentiator + RT agent: Enhanced readthrough in FRT cells with CFTR PTC mutations

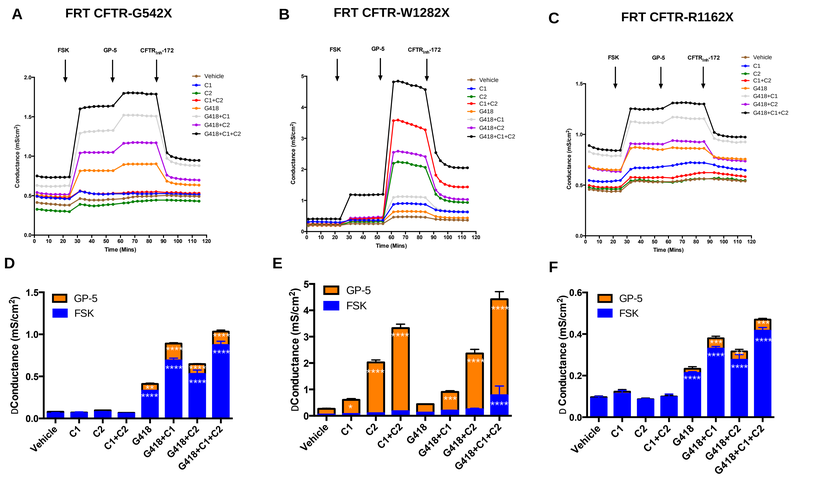

Fig. 2. Effect of correctors and potentiator treatment on FRT cells with PTC mutations. Representative conductance (Gt) tracings of FRT cell monolayers (n=6) expressing mutant **(A)** CFTR-G542X **(B)** CFTR-W1282X **(C)** CFTR-R1162X pretreated for 48 hrs with correctors (C1, C2 or C1+C2) or G418 or Correctors+G418 combination followed by Gt measurements using FSK and GP-5. FSK+GP-5 levels of different treatment combinations in **(D)** G542X **(E)** W1282X and **(F)** R1162X cells. The combination of G418 with C1+C2 induced significantly higher readthtrough CFTR activity compared G418 alone or C1+C2 alone in all the three PTC mutant cell lines.
* P<0.05, ** P<0.001, **** P<0.0001

Correctors + Potentiator+ RT agent: Enhanced readthrough in Primary cells

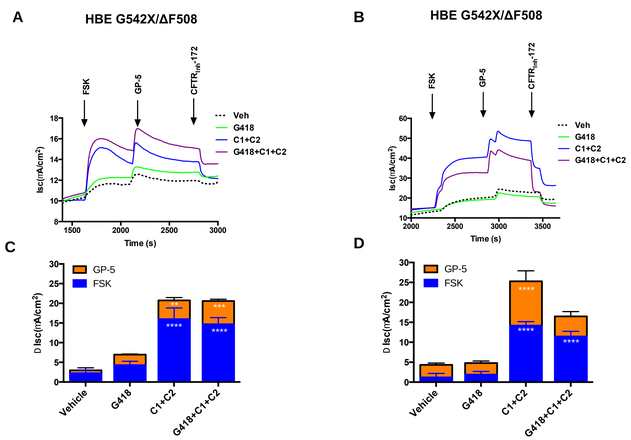

Fig. 3. Effect of correctors and potentiator in primary human bronchial epithelial cells. HBE cells were pretreated with either G418 (250 μg/mL) alone and correctors, C1 (0.5 μM) + C2 (3 μM) alone or in combination with G418 for 48 hrs followed by Isc measurements showing raw tracings in **(A)** HBE G542X/ΔF508 cells and **(B)** HBE G542X/ΔF508 cells. **(C)** FSK+ GP-5 stimulated Isc in HBE G542X/ΔF508 was significantly higher in C1+C2 alone or combination of G418+C1+C2 **(D)** FSK+ GP-5 stimulated Isc in HBE W1282X/ΔF508 cells was significantly higher in C1+C2 combination compared to G514+C1+C2.
* P<0.05, ** P<0.001, **** P<0.0001

Enhanced CFTR cell surface and protein expression, when RT agent combined with correctors and potentiator

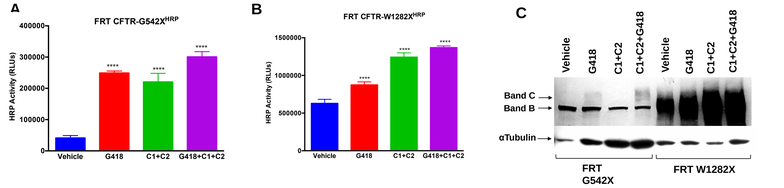

Fig. 4. CFTR cell surface expression using HRP assay and total protein expression in FRT cells. FRT cell monolayers pretreated for 48 hrs with DMSO vehicle, G418 or correctors (C1+C2) alone or combination of G418 and C1+C2 followed by cell surface expression assay in **(A)** FRT CFTR-G542X^{HRP} cells **(B)** FRT CFTR-W1282X^{HRP} cells. Both the cell lines demonstrate enhanced CFTR cell surface expression, when an RT agent is combined with correctors **(C)** Western blot of CFTR protein expression in FRT CFTR-G542X and FRT CFTR-W1282X cells. G418+C1+C2 combination showed increased protein expression of both glycosylated form (Band C) and native form (band B) of CFTR.
* P<0.05, ** P<0.001, **** P<0.0001

Potential readthrough agent in combination with correctors and potentiator

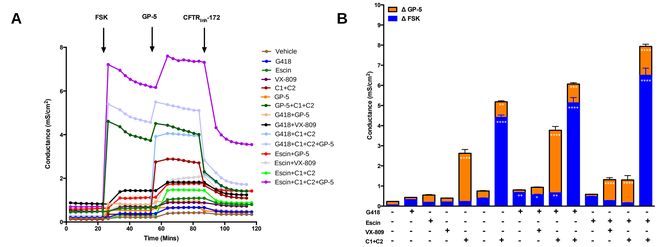

Fig. 5. Effect of RT agent (G418 or Escin) in combination with correctors (VX-809 or C1+C2) and potentiator in FRT W1282X cells: FRT W1282X cells were pretreated with RT agents (Escin or G418), correctors (VX-809 or C1+C2) or potentiator (GP-5) for 48 hrs followed by Gt measurements by acute addition of FSK and GP-5. **(A)** Raw Gt tracings showing the effect of different treatment combinations **(B)** FSK+ GP-5 induced CFTR activity. In this combination approach, C1+C2 showed significant synergy with G418 or Escin as compared to VX-809. Chronic treatment of GP-5 with C1+C2 and Escin had showed enhanced CFTR activity compared to other combination treatments
* P<0.05, ** P<0.001, **** P<0.0001

Conclusions

- ❖ **CFTR potentiator activity is significantly enhanced by RT agent and correctors as compared to RT alone or correctors alone**
- ❖ **The combination of novel correctors and a potentiator show significant synergy with RT agents.**
- ❖ **The combination of correctors (C1+C2) exhibited a significant benefit for the W1282X mutation as corrector therapy was efficacious on its own (without RT), especially when combined with GLPG1837 (GP-5).**
- ❖ **Combination therapy (CFTR modulators + RT agents) may be a useful approach to augment repair of CFTR nonsense mutations and deserves exploration in human subjects with nonsense mutations.**

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