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N1303K: Leaving no stone unturned in the search for transformational therapeutics

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The cystic fibrosis (CF) community is witnessing great strides in the development of mutation-specific CF therapeutics. In 2017, the cystic fibrosis transmembrane conductance regulator (CFTR) potentiator ivacaftor (KalydecoTM; VX-770; Vertex Pharmaceuticals) [1,2] became available to individuals with 38 CF-causing mutations (<https://www.cff.org/News/News-Archive/2017/FDA-Approves-Ivacaftor-for-Five-Splice-Mutations/>), while early results of clinical trials of triple combination therapy using two CFTR correctors and ivacaftor for the most common CF mutation, F508del-CFTR, were highly encouraging (<https://investors.vrtx.com/news-releases/news-release-details/vertex-announces-positive-phase-1-phase-2-data-three-different>). It is anticipated that mutation-specific therapies will soon become available to most individuals living with CF (https://www.nacfconference.org/Content/Archives/2017/Plenary_Session_I_Thursday_November_2_2017/). But, what about those individuals with CF mutations currently refractory to small molecule CFTR modulators?

One such mutation is N1303K, a mutation that disrupts CFTR delivery to, and stability at, the plasma membrane and function as a regulated Cl⁻ channel, which is associated with a severe disease phenotype [3]. For several reasons, N1303K is highly deserving of attention from the CF community. First, N1303K is among the more common rare CF mutation, being particularly prevalent in Mediterranean populations [4]. Second, N1303K affects a residue in nucleotide-binding domain 2 (NBD2) located in a similar region to F508del in NBD1 [5]. Third, N1303K-CFTR appears refractory to rescue by small molecule CFTR correctors (e.g. [6,7]). Encouragingly, the current issue of the *Journal* publishes three N1303K studies, which advance greatly our understanding of how the mutation disrupts CFTR expression and function and inform therapeutic strategies to fully restore its function [8–10].

DeStefano and colleagues [8] comprehensively characterised the behaviour of N1303K-CFTR as a regulated Cl⁻ channel. Previous work had demonstrated that N1303K slows channel gating with the result that the frequency of channel openings is decreased greatly, but their duration noticeably prolonged [11]. They also suggest that N1303K moderately reduced open probability (P_o), one measure of CFTR activity [11]. However, calculating the P_o of mutant CFTR is frequently challenging because of uncertainty about the number of active channels present in cell-free membrane patches. To overcome this technical difficulty, DeStefano et al. [8] used the efficacious CFTR potentiator GLPG1837, which enhances channel gating by a similar mechanism to ivacaftor [12], to accurately determine the number of active channels in membrane patches. Using this approach, they found that N1303K-CFTR has a severe gating defect ($P_o \leq 0.03$), worse than that of F508del-CFTR, but akin to that of the gating mutation G551D-CFTR [13].

To understand better how N1303K disrupts CFTR channel gating, DeStefano et al. [8] used high-affinity ATP analogues that strongly stimulate channel gating and site-directed mutations that either impede channel opening or delay channel closure [13]. In contrast to their effects on F508del-CFTR, the site-directed mutations and one ATP analogue were without effect on N1303K-CFTR channel gating, while the other ATP analogue, a “super” stimulator of CFTR channel gating [14], surprisingly inhibited N1303K-CFTR channel activity. If these data were not enough, upon removal of ATP, N1303K-CFTR did not disappear in a flash like wild-type CFTR, but instead retained significant channel activity, reminiscent of the ATP-independent channel gating of G551D-CFTR (for review, see [13]). To explain these data, DeStefano et al. [8] employed the atomic-resolution structure of CFTR solved by Chen and colleagues using cryo-electron microscopy [5]. The authors

convincingly explain that N1303K causes uncoupling of the NBDs from the membrane-spanning domains (MSDs), which form the channel pore, by preventing the correct positioning of ATP in ATP-binding site 2 and hence, formation of the NBD1:NBD2 dimer. They further explain that F508del also disrupts NBD1:NBD2 dimer formation, albeit by a different mechanism [15]. Thus, DeStafano et al. [8] demonstrate that N1303K is the CF mutation in NBD2 functionally equivalent to F508del in NBD1, albeit its gating defect is more severe than that of F508del-CFTR. By distinct molecular mechanisms the two mutations disrupt communication between the NBDs and the MSDs, with the result that CFTR becomes a rusty gate.

Using CF bronchial epithelial cells (CFBE41o⁻) engineered to express low levels of N1303K-CFTR, Liu and colleagues [9] investigated how the mutation prevents CFTR delivery to the plasma membrane and the action of CFTR correctors, which rescue misfolded F508del-CFTR. In previous work [16], the authors found that short-term culture at low temperature fails to rescue the plasma membrane expression of N1303K-CFTR in contrast to the effects of this well-known laboratory trick on F508del-CFTR. Based on this result and other observations, Rapino et al. [16] speculated that misfolded F508del- and N1303K-CFTR are processed differently by cells.

F508del-CFTR is associated with defective autophagy, leading to the intracellular accumulation of misfolded CFTR protein and hence, lung inflammation [17]. Using pharmacological tools, Liu et al. [9] found that the N1303K mutation arrests autophagy. The authors discovered immature N1303K-CFTR protein associated with autophagosomes, but not degraded by autophagosome fusion with lysosomes and enzymatic digestion. Upon treatment with the CFTR correctors corr-4a (CF Foundation Therapeutics CFTR Compound

Program reference no. C4; [18]) and C18 (a lumacaftor analogue; [19]), which target distinct molecular defects in misfolded CFTR, the association of N1303K-CFTR with autophagosomes was reduced, leading to the production of more mature CFTR protein. However, this mature protein was degraded by lysosomes with the result that little functional N1303K-CFTR protein was detected at the plasma membrane [9]. Thus, the authors' data caution that different CF mutations have distinct effects on the biosynthesis of CFTR and its intracellular transport with implications for therapy development. They also emphasize the difficulty of developing small molecule CFTR modulators that fully restore function to N1303K-CFTR.

To squeeze as much activity as possible out of the few N1303K-CFTR Cl⁻ channels present at the plasma membrane, Phuan and colleagues [10] advanced the idea of combination potentiator ('co-potentiator') therapy using small molecules that act additively or synergistically with ivacaftor. Based on their previous success hunting for small molecule CFTR modulators of W1282X-CFTR [20], Phuan et al. [10] performed a synergy screen of 16,000 small molecules by acutely treating butyrate-rescued N1303K-CFTR with ivacaftor and test compounds. Although the authors identified five active small molecules, all were less effective co-potentiators of N1303K-CFTR than the arylsulfonamide-pyrrolopyridine ASP-11 (formerly called W1282X_{pot}-A15; [20]), which robustly synergised with ivacaftor to restore near wild-type levels of activity to W1282X-CFTR as measured with the Ussing chamber technique. Importantly, ivacaftor and ASP-11 co-potentiated N1303K-CFTR in human nasal epithelial (hNE) cells homozygous for the N1303K mutation, restoring levels of CFTR function equivalent to ~18% of those achieved in non-CF hNE cells, an impressive amount given the great difficulty of coaxing the mutant protein to the plasma membrane [10]. Because of the severity of the N1303K processing defect and its insensitivity to CFTR

correctors, the authors caution the Reader not to overinterpret the potential clinical benefit of their data. Nevertheless, their data are very encouraging for two further reasons. First, ASP-11 activated wild-type CFTR in the absence of the cAMP agonist forskolin, raising the possibility that it has a unique mechanism of action. Second, through their medicinal chemistry efforts the authors identified a more potent arylsulfonamide-pyrrolopyridine, ASP-08, with favourable drug-like properties to oil N1303K-CFTR's rusty gate.

In conclusion, the three studies of N1303K published in the current issue of the *Journal* remind us that careful studies of the molecular mechanisms of CFTR dysfunction are crucial to the development of transformational therapies for all individuals living with CF. We are confident that they will stimulate new research, which will ultimately directly benefit individuals living with CF and N1303K-CFTR. Let's take action today!

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