Title
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ABSTRACT

More than 2000 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) have been described that confer a range of molecular cell biological and functional phenotypes. Most of these mutations lead to compromised anion conductance at the apical plasma membrane of secretory epithelia and cause cystic fibrosis (CF) with variable disease severity. Based on the molecular phenotypic complexity of CFTR mutants and their susceptibility to pharmacotherapy, it has been recognized that mutations may impose combinatorial defects in CFTR channel biology. This notion led to the conclusion that the combination of pharmacotherapies addressing single defects (e.g., transcription, translation, folding, and/or gating) may show improved clinical benefit over available low-efficacy monotherapies. Indeed, recent phase 3 clinical trials combining ivacaftor (a gating potentiator) and lumacaftor (a folding corrector) have proven efficacious in CF patients harboring the most common mutation (deletion of residue F508, ΔF508, or Phe508del). This drug combination was recently approved by the U.S. Food and Drug Administration for patients homozygous for ΔF508. Emerging studies of the structural, cell biological, and functional defects caused by rare mutations provide a new framework that reveals a mixture of deficiencies in different CFTR alleles. Establishment of a set of combinatorial categories of the previously defined basic defects in CF alleles will aid the design of even more efficacious therapeutic interventions for CF patients.
INTRODUCTION

Cystic fibrosis (CF), caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), is characterized by a multiorgan pathology affecting the upper and lower airway, gastrointestinal and reproductive tracts, and endocrine system (Riordan et al., 1989; Collins, 1992; Rowe et al., 2005; Cutting, 2015). CF is one of the most common lethal autosomal-recessive diseases, with a prevalence of one in 3500 in the United States and one in 2500 in the European Union (Farrell, 2008; Pettit and Fellner, 2014). Lack of functional CFTR expression at the apical membrane of secretory epithelia results in defective Cl− and bicarbonate secretion, coupled to enhanced Na+ absorption and mucus secretion, which in airway epithelia leads to dehydration and acidification of the airway surface liquid (Tarran et al., 2001; Chen et al., 2010; Derichs et al., 2011; Pezzulo et al., 2012). As a consequence, impaired mucociliary clearance provokes recurrent infection and uncontrolled inflammation culminating in lung damage, which is the primary cause of morbidity and mortality in CF (Ratjen and Doring, 2003; Boucher, 2007; Stoltz et al., 2015). CFTR is member of the ATP-binding cassette (ABC) subfamily C (ABCC7) (Kerr, 2002). It consists of two homologous halves, each containing a hexa-helical membrane-spanning domain (MSD1 and MSD2) and a nucleotide-binding domain (NBD1 and NBD2) that are connected by an unstructured regulatory domain (Riordan, 1993; Riordan et al., 1989).

BIOLOGY OF CFTR MUTATION: TRADITIONAL CLASSIFICATION

CF is caused by ~2000 mutations in the CFTR gene with a wide range of disease severity (www.genet.sickkids.on.ca/home.html; www.cfr2.org; Sosnay et al., 2013), which is further influenced by modifier genes (Collaco and Cutting, 2008; Cutting, 2010) and by the environmental and socioeconomic status of patients (Schechter et al., 2001; Barr et al., 2011; Taylor-Robinson et al., 2014; Kopp et al., 2015). The first classification of CF mutations into four classes according to their primary biological defect was proposed by Welsh and Smith in a landmark paper (Welsh and Smith, 1993). Currently, six major classes are distinguished (Rowe et al., 2005; Zielenksi and Tsui, 1995) (Figure 1).

Class I encompasses frameshift, splicing, or nonsense mutations that introduce premature termination codons (PTC), resulting in severely reduced or absent CFTR expression.

Class II mutations lead to misfolding, premature degradation by the endoplasmic reticulum (ER) quality-control system, and impaired protein biogenesis, severely reducing the number of CFTR molecules that reach the cell surface.

Class III mutations impaire the regulation of the CFTR channel, resulting in abnormal gating characterized by a reduced open probability.

Class IV mutations alter the channel conductance by impeding the ion conduction pore, leading to a reduced unitary conductance (Sheppard et al., 1993; Hammerle et al., 2001).

Class V mutations do not change the conformation of the protein but alter its abundance by introducing promoter or splicing abnormalities (Highsmith et al., 1994, 1997; Zielenksi and Tsui, 1995).

Class VI mutations destabilize the channel in post-ER compartments and/or at the plasma membrane (PM), by reducing its conformational stability (Haardt et al., 1999) and/or generating additional internalization signals (Silvis et al., 2003). This results in accelerated PM turnover and reduced apical PM expression (Haardt et al., 1999; Silvis et al., 2003).

For many of the identified mutations, the disease liability is unknown, but efforts are under way to assess their functional consequence and clinical severity (www.cfr2.org; Sosnay et al., 2013).

MUTATION CLASS–SPECIFIC PHARMACOTHERAPY

Defining the cellular and molecular pathology of CFTR mutations proved to be invaluable for development of small-molecule compounds targeting the underlying defect(s) in CF. The fact that some CFTR variants carrying class III or IV mutations can be expressed at the apical membrane of secretory epithelia at a density similar to that of the wild-type protein, although they are functionally impaired (e.g., G551D), led to the development of gating potentiators that increase the open probability and thereby the PM chloride conductance (Yang et al., 2003). VX-770 (ivacaftor) is the first potentiator drug to be U.S. Food and Drug Administration approved for CF treatment; it directly targets the gating defect of the class III mutation G551D-CFTR (Van Goor et al., 2009). This compound was developed by Vertex Pharmaceuticals in conjunction with Cystic Fibrosis Foundation Therapeutics, Inc. (CFFT), and shows remarkable clinical benefit in patients carrying the mutation in either one or two alleles (Van Goor et al., 2009; Accurso et al., 2010; Ramsey et al., 2011). The approval of VX-770 was extended to eight additional class III mutations (G178R, S549N, S549R, G551S, G1244E, S1251N, S1255P, and G1349D) (Yu et al., 2012; Vertex, 2014a) and recently to the class IV mutation R117H (Vertex, 2014b).

The prototypical class II mutation, ΔF508-CFTR (Phe508del), elicits a complex folding defect that compromises both NBD1 stability and the channel’s cooperative domain assembly (Du and Lukacs, 2009; Du et al., 2005; Mendoza et al., 2012; Rabeh et al., 2012). For many years, large-scale efforts have been under way to isolate correctors that act as pharmacological chaperones by directly binding to and promoting the biogenesis of class II CFTR mutations. The most promising corrector compound at present, VX-809 (lumacaftor), partially reverts the ΔF508-CFTR functional expression.
defect by stabilizing the NBD1-MSD1/2 interface (Farinha et al., 2013; Loo et al., 2013; Okiyoneda et al., 2013; Ren et al., 2013), leading to a marked correction from 3 to 15% of wild-type channel activity in vitro (Van Goor et al., 2011). A clinical trial, however, failed to observe significant clinical benefit in homozygous ΔF508-CFTR patients (Clancy et al., 2012). Acute addition of VX-770 to VX-809-corrected ΔF508-CFTR doubled the PM activity in vitro (Van Goor et al., 2011), and the combination therapy showed modest but significant clinical improvement (Boyle et al., 2014; Wainwright et al., 2015). Based on these results, the combination treatment has been approved for CF patients 12 years and older with two copies of the ΔF508 mutation (Vertex, 2015). Other class II mutations that can be corrected by VX-809 in vitro include E556K, P67L, E92K, R1207G, L206W, V232D, F508G, and A561E (Caldwell et al., 2011; Okiyoneda et al., 2013; Ren et al., 2013; Veit et al., 2014; Awata et al., 2015).

Ribosomal read-through allows synthesis of full-length CFTR carrying class I mutations. To this end, ataluren (PTC124) was developed as a drug that promotes near-cognate aminoacyl-tRNA incorporation at PTCs (Lentini et al., 2014; Welch et al., 2007). Ataluren partially restores G542X-CFTR (class I) expression in a mouse model and modestly corrects CFTR function in nasal epithelia in patients (Du et al., 2008; Sermet-Gaudelus et al., 2010; Wilschanski et al., 2011). In a recent phase 3 clinical trial, however, ataluren treatment failed to produce significant clinical benefit, perhaps due to an adverse drug–drug interaction with tobramycin, which is a commonly administered, inhaled antibiotic used to treat lung infections in CF patients (Kerem et al., 2014).

**Limitations of CF Mutation Classification**

The efficacy of available monotherapies for some mutant alleles, which have been designated as class I, class II, or class III/IV mutations, is currently limited. This could be partly explained by the pleiotropic molecular defects caused by single mutations. Thus comprehensive mapping of the multiple molecular defects caused by a single or combination of mutant alleles could offer considerable advantage for improving therapeutic interventions and for future development of drug combinations. In the following list, we present a subset of mutations that display combinational molecular defects.

- ΔF508: The most prevalent class II mutation impairs CFTR conformational maturation and leads to its targeting for premature ER-associated degradation (Cheng et al., 1990; Cyr, 2005; Kim and Skach, 2012; Lukacs et al., 1994). However, ΔF508-CFTR molecules that either constitutively or following rescue procedures escape the ER quality control and accumulate at the PM of airway epithelia exhibit a channel-gating defect, which is a hallmark of class III mutations (Dalemans et al., 1991), as well as accelerated turnover in post ER compartments and at the PM, a class VI mutation characteristic (Lukacs et al., 1993). Unless the folding and conformational dynamics of the rescued ΔF508-CFTR are fully restored to that of the wild-type protein by pharmacological treatment, this mutation remains partially defective and requires correction of its gating and/or peripheral stability defect. Rescue of the gating defect can be achieved with potentiators (e.g., VX-770) (Van Goor et al., 2009). Peripheral stabilization of the ΔF508-CFTR could be attained by 1) the peptide inhibitor iCAL36 (Cushing et al., 2010), 2) preventing post-Golgi ubiquitination (Fu et al., 2015; Okiyoneda et al., 2010), 3) restoring autophagosome formation (Luciani et al., 2012), or 4) modulating cellular protein homeostasis (Hutt et al., 2010). Thus the most common mutant has multiple defects that extend beyond the features of a class II mutation.

- W1282X: This PTC represents a class I mutation, though recent studies suggest a more complex phenotype. First, the level of the W1282X transcript is reduced by nonsense-mediated RNA decay (Hamosh et al., 1992; Linde et al., 2007). Second, the PTC deletes part of the NBD2, which likely compromises NBD1-NBD2 dimerization and W1282X-CFTR folding and activity. Moreover, if the primary defect is corrected either with spontaneous or drug-induced read-through, some of the fully translated channel will contain nonconservative amino acid substitutions. These missense mutations may cause structural defects (class II characteristic), as suggested by the phenotype of CF patients with a missense mutation at the W1282 residue (Faucz et al., 2007; Ivaschenko et al., 1993; Visca et al., 2008), as well as a gating defect (class III characteristic), which can be inferred based on W1282X-CFTR channel activation after exposure to VX-770 (Xue et al., 2014).

- P67L: P67L is a mild class II mutation that results in attenuated CFTR biogenesis, as indicated by the reduced ratio between post-ER complex-glycosylated (band C) and ER-resident core-glycosylated protein (band B) (Ren et al., 2013; Sosnay et al., 2013; Van Goor et al., 2014). Treatment with the corrector VX-809 increases the abundance of the complex-glycosylated form and PM density to nearly the level of WT-CFTR (Ren et al., 2013; Veit et al., 2014). However, the mutant channel is also sensitive in vitro to potentiator treatment (a class III characteristic), both in the presence and absence of corrector (Van Goor et al., 2014; Veit et al., 2014). Accordingly, treatment with VX-770 ameliorated the CF lung disease in a heterozygous P67L/ΔF508 patient (Yousef et al., 2015).

- R117H: This mutation in conjunction with the 5T variant in the polymorphic tract in intron 8 was originally categorized as a class IV mutation, but it also exhibits a gating defect (class III trait) that, at least in part, can be rectified by VX-770 treatment (Sheppard et al., 1993; Van Goor et al., 2014). The R117H mutation also results in reduced complex-glycosylated CFTR expression, which is a class II characteristic (Fanen et al., 1997; Sheppard et al., 1993). This potentially explains the limited success of VX-770 treatment in patients carrying this mutation (Char et al., 2014; Moss et al., 2015).

**An Expanded Classification of Mutant CFTR Biology**

We propose a modification of the current classification scheme, which would entail permutations of the traditional class I–VI CF mutations. This expanded classification of the major mechanistic categories (Welsh and Smith, 1993; Zielenksi, 2000; Rowe et al., 2005) accommodates the unusually complex, combinatorial molecular/ cellular phenotypes of CF alleles. It consists of 31 possible classes of mutations, including the original classes I, II, III/IV, V, and VI, as well as their 26 combinations, as depicted in the Venn diagram shown in Figure 2. For the sake of simplicity, class III and IV mutations, representing functional (gating and conductance, respectively) defects, are combined. For example, according to the expanded classification, G551D will be designated as a class III mutation as before (Welsh and Smith, 1993), while ΔF508 will be classified as class II–III–VI, W1282X as class I–II–III–VI, P67L as class II–III, and R117H as class II–III/IV, reflecting the composite defects in mutant CFTR biology (Figure 2 and Table 1).
A recent study by Vertex Pharmaceuticals successfully demonstrated that 24 of 54 tested missense mutations display both a processing (class II) and gating (class III) defect in the Fischer rat thyroid expression system (Van Goor et al., 2014). Characterization of several rare CF mutations is ongoing in laboratories of the CFTR2 Consortium, the CFTR Folding Consortium, CFFT, Vertex Pharmaceuticals, and many others (Caldwell et al., 2011; Yu et al., 2012; Sosnay et al., 2013; Harness-Brumley et al., 2014; Hong et al., 2014; Van Goor et al., 2014; Wang et al., 2014; Awatade et al., 2015). This work will likely provide further examples of combinatorial mechanistic defects exhibited by CF mutants.

### THERAPEUTIC SUSCEPTIBILITY OF CF MUTATIONS WITH COMPLEX BIOLOGICAL DEFECTS

In-depth analysis of the biology of CF mutants distinguishes them according to their complex molecular pathology and suggests drug combinations for treatment of different patient populations. This process, called

### TABLE 1: Examples for CF mutations with complex or classical cellular phenotypes.

<table>
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<tr>
<th>Refined classification</th>
<th>Mutation</th>
<th>I</th>
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<th>III/IV</th>
<th>V</th>
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**TABLE 1:** Examples for CF mutations with complex or classical cellular phenotypes.

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**TABLE 1:** Examples for CF mutations with complex or classical cellular phenotypes.

Continues
“theratyping” (Cutting, 2015), will pave the way to personalized medicine in CF. However, reliable prediction of the responsiveness of a mutant phenotype to pharmacotherapy could be challenging and is dependent on the cellular model system (Pedemonte et al., 2010).

Emerging evidence also suggests that the efficacy of approved and preclinical drugs may vary with different mutations within the same class. For example, while nearly complete processing correction of P67L- and R170G-CFTR (class II) was achieved with VX-809 treatment (Okiyoneda et al., 2013; Ren et al., 2013; Veit et al., 2014), VX-809 only partially reversed the folding defect of some other class II mutants; for example, N1303K and ΔF508 (Okiyoneda et al., 2013; Awatade et al., 2015). This differential susceptibility to correction is attributed to the nature of the primary folding/structural defect. According to one hypothesis, robust folding correction of ΔF508-CFTR requires corrector combinations to avert its NBD1-MSD2/1 interface and NBD1 stability defects (Mendoza et al., 2012; Rabeh et al., 2012; He et al., 2013; Okiyoneda et al., 2013). The N1303K mutation in NBD2 was not rescued by VX-809, and only modest processing was observed by targeting both the NBD1/MSDs and NBD2 interfaces with C4 and C18 (a VX-809 analogue) (Okiyoneda et al., 2013; Rapino et al., 2015).

Some of the class III mutations also respond differently to the gating potentiator VX-770. Although R347H- and T338I-CFTR cause severe functional defects with no or modest loss of protein expression, only R347H-CFTR is potentiated by VX-770 to near wild-type-like conductance (Van Goor et al., 2014). Likewise, the P5 potentiator activates ΔF508-CFTR, but it has no effect on G551D-CFTR chloride permeation (Yang et al., 2003). Thus identification of mutation-specific novel potentiators or their combinations may further optimize channel rescue for specific class III/IV mutations. Additive enhancement of G551D-CFTR activity by the combination of the potentiators genistein and curcumin supports the feasibility of combining potentiators (Yu et al., 2011). Likewise, we envision that mutation-specific read-through drugs will ultimately need to be combined with other correctors and potentiators, based on the pleiotropic defects associated with this class of mutations (as illustrated for W1282X above).

**CONCLUDING REMARKS**

The ultimate goal of theratyping is to achieve optimal correction of a specific mutant defect by selecting the most efficacious CFTR modulator(s), including corrector(s), potentiator(s), and/or read-through drugs, or a combination of these drugs. Based on accumulating observations, however, mechanistic subdivisions of some of the major classes of mutations (classes I, II, and III) may be necessary to further improve the success of drug-selection strategies. This will facilitate the theratyping of CF alleles and their combinations and expedite the identification and approval process for combination therapies. Theratyping has already proven successful in identifying class III mutations that are responsive to VX-770 (Yu et al., 2012), leading to the approval of this drug for eight rare mutations besides G551D (Vertex, 2014a). In fact, the results of large-scale theratyping could be overlaid as a third dimension on the Venn diagram presented in Figure 2.

Thus, during the 22 years following the initial classification of CF mutations (Welsh and Smith, 1993), our understanding of the molecular complexity of CF alleles has evolved remarkably, establishing the need for an advanced mutation classification scheme in conjunction with personalized CF therapy.

**ACKNOWLEDGMENTS**

We thank the members of the CFTR Folding Consortium, the CFTR Theratype Group, C. M. Penland, and K. Tuggle (Cystic Fibrosis Foundation, Bethesda, MD) for their valuable support. The work described here was supported by the following institutions and grants: National Institutes of Health (NIH) NO1-HL28187 and IAA-A-HL-14-007.001 to H.B.P.; Cystic Fibrosis Foundation (CFF), NIH DK51870, TRDRP23RT-0012, and HL095524 to W.E.B.; NIH R01 DK, CFF CUTT13A1, and CUTTXX0 to G.R.C.; CFFT SHEPPA14XX0 and Cystic Fibrosis Trust to D.N.S.; NIH R01-DK068196, P30-DK072506, 27CHO 27Bompadre et al., 2007.

### TABLE 1: Examples for CF mutations with complex or classical cellular phenotypes. Continued

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Superscript numbers refer to references in far-right column.
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- Does not exhibit a gating or conductance defect in this cell model.
- J.S.H. and E.J.S., unpublished observations.
- Does not exhibit a biogenesis defect in this cell model.
- Z.C. and D.N.S., unpublished observations.
- Does not exhibit a peripheral stability defect in this cell model.

### ACKNOWLEDGMENTS

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and CFFT FRIZE05X0 to R.A.F.; NIH RO1 GM56981 and CFFT CVR13XX0 to D.M.C.; the CFF Research Development Program, CFFT SORCH05XX0, and SORCH14XX0 to E.J.S.; CFFT BROD-SK13XX0 and NIH GM75061 to J.L.B.; CF Canada, CFFT Lukac-s13XX0, NIH DK075302, and Canadian Institutes of Health Research to G.L.L. R.G.A. was supported by CF Canada Studentship; G.L.L. is a recipient of a Canadian Research Chair.

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