Expanding Approved Patient Populations for Rare Disease Treatment Using *In Vitro* Data

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In vitro cell-based data can be used to support the extension of pharmaceutical approval to patient subsets with unique genetic variants. A set of conditions should be satisfied to support the extension of approval. The disease mechanism should be well described, and the impact of variants on protein function should be reasonably understood. The incidence data should show that clinical trials for the variants in question are not practical. The overall safety and efficacy of the drug should be clear in adequate and well-controlled clinical trials. The clinical trial should include patients found to be responders and nonresponders so that both positive and negative predictive power of the *in vitro* assay may be measured. The mechanism of action of the drug should be clearly defined and should be consistent with the disease mechanism. The assay system should be qualified, including the following points: (i) each variant construct should be confirmed by bidirectional sequencing; (ii) the *in vitro* assay should directly measure the variant protein function in comparison with the reference protein; (iii) the assay should be formally validated to the extent possible, clearly demonstrating precision, reproducibility, and sensitivity used to support the efficacy claim; and (iv) the primary data should be available for inspection and analytical validation. The overall goal is a robust and validated cell-based system that can be shown to predict the outcome of targeted therapy.

Developing treatments for rare diseases is one of the most scientifically complex health challenges of our time. Among the ~ 7,000 rare diseases, 95% have no US Food and Drug Administration (FDA)–approved treatment. FDA approval of a pharmaceutical product requires demonstration of its safety and efficacy in adequate and well-controlled clinical trials. For efficacy, approaches that enrich a trial's ability to demonstrate an effect have been encouraged. Enrichment can be accomplished in several ways, including selecting patients who are more likely to respond to the drug.[1] Over the past decade, numerous therapies, often together with companion *in vitro* diagnostic tests, have been approved for subsets of patients characterized by specific molecular features. Such predictive enrichment strategies have been applied in the development of treatments for cancer and for some rare genetic disorders. However, clinical trials for rare diseases are generally challenging to conduct, even without prospective biomarker-based enrichment strategies. Further, for targeted therapies that are expected to work only in patients with certain molecular features, it is important to understand how clinical trial data apply to patients not included in trials. In 2018, the FDA released guidance for using experimental evidence to identify eligible patient populations for clinical trials as well as considerations for narrowing or expanding the indication.[2] Here we describe key considerations for generating and using cell-based *in vitro* data to support

drug efficacy in the absence of clinical trial data as applied to the approvals of ivacaftor and migalastat for the treatment of cystic fibrosis (CF) and Fabry disease (FD), respectively. In both cases, an *in vitro* cell-based approach was used to assess the functional and biochemical response of mutated or dysfunctional protein(s) in the presence of drug to make inferences about the potential for response *in vivo*.

DISEASE-RELATED CONSIDERATIONS

There should be a clear understanding of disease mechanism and how genetic variants affect gene/protein structure and function. For both CF and FD, the mutated or dysfunctional protein is established and well characterized, and more than 1,000 distinct genetic variants (https://cftr2.org; http://www.hgmd.org) have been identified between the two conditions. CF is an autosomal recessive disease that results from variants in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which can abolish or diminish the number of and/or activity of the CFTR chloride channel at the surface of epithelial cells.[3] Approximately 360 pathogenic *CFTR* variants are known to cause CF (CFTR2: https://cftr2.org). The reduced chloride channel activity can affect many organ systems, but mainly leads to viscous pulmonary airway mucous resulting in recurrent infections, inflammation, and damage to lung tissue that ultimately results in respiratory

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failure. FD is a rare X-linked inherited lysosomal storage disorder that is caused by variants in *GLA,* which encodes the enzyme α-galactosidase-A (α-Gal A). The α-Gal A deficiency results in accumulation of intralysosomal globotriaosylceramide (GL-3) deposits in various cell types and organs including the heart, kidneys, and central and peripheral nervous systems.[3] The majority of *GLA* variants (55–59%) are missense variants. To date, 865 variants are categorized as disease-causing (pathogenic), per HGMD; http://www.hgmd.org.

It should be established that adequately powered clinical trials are not feasible for the patient subpopulations harboring the specific sequence variants. Both CF and FD are rare single-gene disorders for which many disease-causing variants are low frequency (< 5%), making it difficult to recruit enough patients to conduct adequately powered clinical trials to support a drug's efficacy. CF affects ~ 30,000 people in the United States.[3,4] Ivacaftor increases the activity of variant CFTR chloride channels that exhibit abnormal channel gating, which are estimated to be present in only 6% of CF patients; G551D is the most common variant, with an allele frequency of 2% per the CFTR2.org database. Other similar *CFTR* variants are rare; only a handful were studied in more than 10 clinical trial subjects.[4,5] FD currently affects an estimated 3,800 males in the United States,[6] while the prevalence in females remains unknown. FD demonstrates considerable genetic diversity; data indicates that only six variants are found in ≥ 2% of the FD population (N215S: 4.8%; R227X: 3.2%; A143P: 2.6%; R342Q: 2.2%; R112C: 2.1%; R227Q: 2.0%).[6] In the migalastat trial,[7] 63 patients with 40 distinct *GLA* variants were included, of which R342Q (12.7%) and I253T (6.3%) were the most common.

DRUG-RELATED CONSIDERATIONS

The safety and efficacy of the drug should be established based on clinical trial data from adequate and well-controlled trials. Ivacaftor was initially approved in 2012. In 2017, when expansion of use was sought, ivacaftor had previously been studied in three clinical trials with a total of 175 CF patients with 1 of 10 distinct *CFTR* variants.[5] Migalastat received accelerated approval in 2018 based on clinical trial data from a phase III clinical trial that included 67 patients with Fabry disease with 1 of 40 different *GLA* variants.[7]

The mechanism of drug action should be known and should be consistent with the disease mechanism. Ivacaftor potentiates chloride transport across the CFTR ion channel by increasing gating probability in both wild-type (WT) and variant CFTR. The overall level of ivacaftor-mediated CFTR chloride transport depends on the number of CFTR channels at the cell surface and how responsive a variant CFTR channel is to ivacaftor. In single-channel studies, ivacaftor increased the channel open probability of WT CFTR and ten *CFTR* gating variants.[5,8] Migalastat acts as pharmacological chaperone that selectively binds wild-type (WT) and variant α-Gal A enzyme to increase protein stability, lysosomal trafficking, and cellular activity. Migalastat targets misfolded and, thus, unstable mutant a-GAL A enzymes which are predominantly caused by *GLA* missense variants.[3,7]

Clinical trial data should be available for responders and nonresponders so the predictive power of the in vitro *assay can be evaluated.* To determine whether *in vitro* assay data can serve as a suitable bridge to inform similarities or differences in response across genetic variants, it is critical to have both clinical and experimental data from representative responsive and nonresponsive variants. For ivacaftor, data for the mean absolute change from baseline in percent predicted forced expiratory volume was available for 3 or more patients with 1 of 12 different *CFTR* variants across 222 CF patients. Of the 67 FD patients treated in the migalastat phase III trial, 50 patients had amenable *GLA* variants (responsive to migalastat as determined by the GLP-HEK *in vitro* assay) while the remaining 17 had nonamenable (nonresponsive) *GLA* variants.[7] No patients with nonamenable *GLA* variants experienced a reduction in kidney interstitial capillary GL-3 inclusions, the trial's primary end point for demonstration of efficacy. In both cases, clinical data were used to establish that the *in vitro* models used were suitable to make inferences about clinical responses in patients with variants predicted to respond. Additional clinical trials for each drug are ongoing as part of postmarketing commitments and may provide additional data to refine the evaluation of the *in vitro* assays.

ASSAY VALIDATION

The selection of the *in vitro* system is multifactorial. In general, the simplest system that accurately and reproducibly measures variant-specific drug effect is desirable. Complex systems including induced pluripotent stem cells, organoids, or microphysiological systems could be acceptable provided they meet the criteria below. Formal assay validation is especially important for these more complex systems.

Prior to transfection, the construct variant as well as the integrity of the surrounding context in the regulatory sequence should be confirmed by bidirectional sequencing. Because *in vitro* studies may be the only information that directly establishes drug effects for many variants, it is critical to confirm that the variant is actually present in the cells being tested. This would also include patient-derived cells such as induced pluripotent stem cells. For both ivacaftor and migalastat, the WT regulatory region or complementary DNA (cDNA) of the gene of interest was inserted into an expression construct. Variants were generated using site-directed mutagenesis of the WT cDNA construct and confirmed by bidirectional sequencing of the new constructs. For stable cell systems, the integration of the regulatory sequence randomly across the genome can cause differential effects on expression and activity unique to that clone. Wild-type and independent variant isogenic stable cell lines can be generated from a parental host cell line engineered through site-specific integration of the cDNA into the same genomic locus. For ivacaftor, a commercial integration system was used to generate stably expressing Fischer rat thyroid (FRT) cell lines and allow selection of cell lines with similar expression levels. When using a transient cell-based system, transfection efficiency and expression should be optimized for the cell type. For migalastat, a nonstandard real-time quantitative polymerase chain reaction (qPCR) method was used to confirm successful transfection (and productivity). The *GLA* expression construct(s) did not contain a unique reporter gene that would have allowed transfection efficiency to be independently determined in the cell medium. This approach was acceptable because cotransfection and measurement of a separate reporter gene (e.g., green fluorescent protein (GFP)) would

probably have interfered with the fluorescent quantitation of α-Gal A activity *in vitro* and/or required an independent reporter plate to be used in parallel with every test plate, which is not reliable.

The in vitro *assay should directly measure protein function, and a comparison between variant and WT activity should be determined*. Two types of *in vitro* data were generated for ivacaftor: (i) Ussing chamber data measuring chloride channel activity in FRT cells stably expressing WT or variant CFTR channels or human bronchial epithelial (HBE) cells; and (ii) Western blots showing the levels of immature and mature CFTR in variant and WT FRT stable cell lines following drug treatment.[5] Concordance of changes in CFTR activities between FRT and HBE cells was also measured. In the case of migalastat, the GLP-HEK293 assay measured the ability of protein lysates extracted for HEK293 cells transiently expressing WT and variant α-Gal A and grown in the presence or absence of migalastat to cleave a fluorogenic substrate.[9] Data evaluation included assessment of variant *GLA* construct quantity and quality, total protein extracted from WT and variant α-Gal A transfected cells, and enzyme activity in variant and WT α-Gal A protein lysates.[7] For *in vitro* CFTR ion channel activity and α-Gal A enzyme activity, thresholds were established to categorize a variant as responsive. Stable *CFTR* variant cells showing an increase of ≥ 10% of WT chloride current after ivacaftor treatment were classified as responsive. Variant α-Gal A enzymes exhibiting increased cellular levels and enzymatic activity of ≥ 3% WT, and a relative increase in enzyme activity ≥ 1.2-fold over baseline following incubation with migalastat were classified as responsive. The assay parameters will be specific to the drug, assay, and disease and should be generally consistent with *in vivo* values. As noted above, the *in vitro* assay categorization should be correlated with clinical trial response data from patients with the same variant to assess the assay's predictive performance.

The in vitro *cell-based assay should be formally validated to the extent possible*. Assay validation establishes that a method does what it is intended to do in terms of the seven key criteria: accuracy, precision, reproducibility, recovery, selectivity, specificity, and stability.[10] The sponsor and FDA review division responsible for product review should discuss the design of validation studies for the assay in question. Western blots and electrophysiology methods were used to assess the levels and effect of ivacaftor on variant CFTR stably expressed in FRT cells because CFTR is a membrane protein and is active in its mature form. Critical measures included replicate Westerns (n = 5 to 9) showing the ratio of mature to immature protein in stable cell lysates, and the ratio of mature CFTR in variant to wild-type (WT) cell lysates. Ussing chamber assays were used to measure short-circuit forskolin-activated current (I_{SC}) or the transmembrane conductance–mediated chloride secretion across WT and variant CFTR stable cell lines. Following a period of control or baseline recording for the individual cell line, ivacaftor was bath-applied to determine whether I_{SC} could be increased. An increase in I_{SC} was always mirrored by a reduction in transmembrane resistance, consistent with increased ion channel/transporter activity. For this assay, a full blocker should be added at the end of each recording to determine the degree of residual, non-CFTR background current. For migalastat, a GLP-validated *in vitro* assay (excluding DNA sequencing) was developed. This

assay was a multistep system that employed molecular, cellular, and enzymatic methods. These steps included construction of the variant-*GLA* expression construct(s); culturing HEK-293 cells in the presence or absence of migalastat following transient transfection with the recombinant (rh) *GLA* constructs to express variant α-Gal A; and extraction and quantitation of total protein from transfected cells and assessment of transfection efficiency. The activity of rhα-Gal A enzyme in cell lysates was measured across five independent experiments with four replicates and normalized to total protein content and dilution factor. Residual enzyme activity was then compared with WT. Assay performance metrics for protein, enzyme activity, and qPCR steps included assessment of precision, accuracy, linearity, and reproducibility.

Primary data should be available for analytical validation. For ivacaftor, Western blot scan data were obtained to assess quantitative changes in mature and immature CFTR. Primary electrophysiology recordings from Ussing chamber experiments of FRT and HBE cells and laboratory records of experimental parameters were reanalyzed by the FDA to confirm efficacy. For migalastat, primary data for both responsive and nonresponsive variants *in vitro* were analyzed, including restriction endonuclease maps of expression constructs, sequence chromatograms and text files, and data for protein levels, enzyme activity, and qPCR. FDA recapitulated the sponsor's analysis for estimation of final α-Gal-A enzyme activity. For both drugs, reanalysis of primary data confirmed the applicant's results and on-site inspections supported the data integrity.

SUMMARY

The Developing Targeted Therapies in Low-Frequency Molecular Subsets of a Disease Guidance[2] provides a scientific framework that enables the evaluation of proposals for approval of a targeted drug in subsets of a disease population with very rare genetic variants who may not be well represented in clinical trials. This framework is based on several considerations, including knowledge of the drug mechanism, the disease pathogenesis, and the performance characteristics of the *in vitro* assays used to predict clinical responses (and nonresponses) to treatment. This approach takes into account the statistical and biological uncertainties of classifying a variant as responsive or nonresponsive based solely on *in vitro* data supplemented by clinical data obtained from only a few patients treated in clinical trials.

The 2017 approval of ivacaftor for patients with 23 responsive *CFTR* gating variants (based on *in vitro* data) allowed ~1,500 new patients access to the drug based on *in vitro* data that predicted the clinical responsiveness of patients not included in clinical trials.[3] A similar estimation about the extent of patient access to migalastat based on the recent approval is not yet available, but a similar increase in access to the drug is expected over the coming years.

The effects of human genome variations on human health, including drug response, is complex. Functional assays that are clinically relevant may not always be available. Isolating differences in drug response in these cases has benefited from the availability of experimental models. However, assumptions or uncertainties still exist. Determining acceptability criteria of an *in vitro* assay is multifactorial. The FDA is continuing to gain experience with the development of genetically targeted therapies, including the many

oncology drugs that have been developed that target specific oncogenic variants. In addition, novel data sources may provide evidence that bolsters *in vitro* findings, such as those generated from real-world data sources.[11] The FDA will continue to evaluate circumstances in which approaches relying on *in vitro* data are or are not feasible. Drug developers are encouraged to interact with regulatory authorities early in the course of development to discuss using an *in vitro* approach to extend approval. In summary, laboratory data from validated and robust *in vitro* cell-based assays that can predict response to a targeted therapy with confidence, when supplemented with relevant clinical trial data, can provide the scientific basis for establishing a drug's effectiveness in patients with very rare variants not included in trials due to feasibility constraints.

CONFLICT OF INTEREST
All authors declared no competing interests for this work.

DISCLAIMER
The contents of this paper reflect the views of the authors and should not be construed to represent the US Food and Drug Administration's views or policies.

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