Altering Metabolic Profiles of Drugs by Precision Deuteration 2: Discovery of a Deuterated Analog of Ivacaftor with Differentiated Pharmacokinetics for Clinical Development

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ABSTRACT

Ivacaftor is currently used for the treatment of cystic fibrosis as both monotherapy (Kalydeco; Vertex Pharmaceuticals, Boston, MA) and combination therapy with lumacaftor (Orkambi; Vertex Pharmaceuticals). Each therapy targets specific patient populations: Kalydeco treats patients carrying one of nine gating mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, whereas Orkambi treats patients homozygous for the F508del CFTR mutation. In this study, we explored the pharmacological and metabolic effects of precision deuteration chemistry on ivacaftor by synthesizing two novel deuterated ivacaftor analogs, CTP-656 (d18-ivacaftor) and d16-ivacaftor. Ivacaftor is administered twice daily and is extensively converted in humans to major metabolites M1 and M6; therefore, the corresponding deuterated metabolites were also prepared. Both CTP-656 and d16-ivacaftor showed in vitro pharmacologic potency similar to that in ivacaftor, and the deuterated M1 and M6 metabolites showed pharmacology equivalent to that in the corresponding metabolites of ivacaftor, which is consistent with the findings of previous studies of deuterated compounds. However, CTP-656 exhibited markedly enhanced stability when tested in vitro. The deuterium isotope effects for CTP-656 metabolism (\( V = 3.8, V/K = 2.2 \)) were notably large for a cytochrome P450–mediated oxidation. The pharmacokinetic (PK) profile of CTP-656 and d18-ivacaftor were assessed in six healthy volunteers in a single-dose crossover study, which provided the basis for advancing CTP-656 in development. The overall PK profile, including the 15.9-hour half-life for CTP-656, suggests that CTP-656 may be dosed once daily, thereby enhancing patient adherence. Together, these data continue to validate deuterium substitution as a viable approach for creating novel therapeutic agents with properties potentially differentiated from existing drugs.

Introduction

Deuterium labeling of compounds has a long history of in vitro and in vivo use, primarily as a method to probe enzymatic mechanisms and metabolic pathways or to allow nonradioactive isotopic tracing (Rittenberg and Schoenheimer, 1937; Bailie, 1981). Deuterated agents as pharmacologicals are an area of growing interest, as shown by the number of deuterated compounds entering clinical development (Halford, 2016). Additionally, the first deuterated compound, deuterobenazine (Austedo), has recently been approved. A key feature of this technology is that deuterium substitution generally has minimal impact on the structure, physical properties, or pharmacology compared with the all-hydrogen version of a drug. However, deuterium substitution can have a marked effect upon the pharmacokinetics (PK) profile of a compound due to the deuterium isotope effect (DIE), which can lower the rate of carbon–deuterium versus carbon–hydrogen bond cleavage (Kushner et al., 1999; Nelson and Trager, 2003; Harbeson and Tung, 2011). Although the theoretical limit for the DIE is 6-fold to 9-fold, effects this large are rarely observed for enzyme-catalyzed reactions (Northrop, 1975; Jenks, 1987). The magnitude of the DIE for a cytochrome P450–mediated oxidation is unpredictable (Fisher et al., 2006; Harbeson and Tung, 2014). Due to this unpredictability, deuterium-substituted analogs must be synthesized and tested in preclinical studies to assess the magnitude and direction of the DIE. Even then, preclinical observations may not quantitatively, or potentially even qualitatively, predict human clinical results.

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ABBREVIATIONS: AUC, area under the curve; AUC0–24hr, area under the curve from time 0 to 24 hours postdose; AUC0–inf, area under the curve from time 0 extrapolated to infinity; C24hr, concentration at 24 hours postdose; Cmax, maximum plasma concentration; CF, cystic fibrosis; CTP, cystic fibrosis transmembrane conductance regulator; DIE, deuterium isotope effect; DMSO, dimethylsulfoxide; GLP, good laboratory practice; HBE, human bronchial epithelial; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; Ieq, equivalent current; IS, internal standard; Kcon, concentration of substrate at one-half \( V_{max} \); LC, liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; PK, pharmacokinetics; SAD, single ascending dose; t1/2, half-life.
The first article in this series presented the data for CTP-347, a deuterated analog of paroxetine (Paxil; GlaxoSmithKline, Research Triangle Park, NC), which is a selective serotonin reuptake inhibitor for the treatment of a number of psychologic disorders (Paxil, 2012; Uttamsingh et al., 2015). Herein, we report the effects of selective deuterium substitution on another therapeutic agent, ivacaftor (Fig. 1). Ivacaftor (Kalydeco; Vertex Pharmaceuticals, Boston, MA) was approved in 2012 as the first drug to target specific mutational defects in the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein (Kalydeco, 2015). It is currently labeled for the treatment of CF patients 2 years and older who have at least one mutant allele from a group of nine gating mutations in the CFTR gene, with G551D being the most common (4.4% of CF patients in the United States) (Aditya, 2012; Kalydeco, 2015). In 2016, ivacaftor was approved as part of the fixed-dose combination drug Orkambi (Vertex Pharmaceuticals), which combines ivacaftor and lumacaftor for the treatment of CF patients 12 years and older homozygous for the F508del CFTR mutation (approximately 45% of CF patients in the United States) (Wainwright et al., 2015; Orkambi, 2016). Lumacaftor is a CFTR “corrector” that improves the formation of active CFTR at the epithelial surface of cells (Van Goor et al., 2011). The CFTR protein is an anion channel that regulates epithelial chloride and bicarbonate concentrations to maintain proper hydration and pH of secretory epithelia (Riordan, 2008). CFTR mutations reduce anion secretion, and the resulting dysregulation of anion concentrations promotes the accumulation of thick, viscous mucus. Ivacaftor is a CFTR “potentiator” and increases the channel open probability, thereby increasing anion conductance, improving mucus hydration and decreasing mucus viscosity (Van Goor et al., 2009). Viscous mucus is particularly problematic for lung function since it results in an inability to efficiently clear airways, which leads to chronic inflammation and infections that contribute to a decline in lung function over time (Ong and Ramsey, 2015).

In this study, deuterated analogs of ivacaftor (Fig. 1) were prepared and characterized with respect to pharmacology, in vitro metabolism, and in vivo PK. Since ivacaftor is extensively metabolized in humans to two major circulating metabolites, M1 and M6, these metabolites and the corresponding deuterated metabolites d8-M1 and d6-M6 were also prepared and studied (Fig. 2) (Zha et al., 2011). Two compounds—d9-ivacaftor (CTP-656) and d18-ivacaftor—showed increased metabolic stability versus ivacaftor in vitro. Deuterium modification did not impact pharmacology, as the two deuterated compounds demonstrated equivalent potency to ivacaftor in multiple in vitro studies. In vitro metabolic stabilization did translate to increases in in vivo exposure parameters when the compounds were orally dosed in rats and dogs, although species differences were observed. Subsequently, both compounds were dosed in healthy volunteers in a single-dose crossover study. The new chemical entity CTP-656 was identified as the agent for further clinical advancement because of its general trend of increased exposure versus d15-ivacaftor. The observed increase in exposure and half-life (t1/2) compared with that reported for ivacaftor supported the potential for CTP-656 to be dosed once daily. These data continue to validate deuterium substitution as a viable approach to creating novel therapeutic agents with properties potentially differentiated from existing drugs.

Materials and Methods

Reagents

Indiplon, CTP-656, d15-ivacaftor, ivacaftor, M1, M6, d9-M1, and d6-M6 were provided by Concert Pharmaceuticals, Inc. (Lexington, MA). Human liver microsomes (HLM; mixed gender, pool of 200, 20 mg/ml) were from Xenotech, LLC (Lenexa, KS). Cryopreserved human hepatocytes were purchased from In Vitro ADMET Laboratories (Malden, MA). Human cDNA expressed CYP3A4 Supersomes (Corning, Corning, NY) were obtained from BD Biosciences (Woburn, MA). Dimethylsulfoxide (DMSO), acetonitrile, MgCl2, and NADPH were from Sigma-Aldrich. Dimethylsulfoxide (DMSO), acetonitrile, MgCl2, and NADPH were from Sigma-Aldrich (St. Louis, MO) and PEG400 was from J.T. Baker (Center Valley, PA). CF-HBE (human bronchial epithelial) cells were obtained from the Cystic Fibrosis Foundation Therapeutics organization. Forskolin was obtained from LC Laboratories (Woburn, MA). Benzamil and bumetanide were obtained from Sigma-Aldrich. HBE studies were conducted in the laboratory of Dr. Robert Bridges at Rosalind Franklin University of Medicine and Science, Chicago Medical School (North Chicago, IL).

Synthetic Procedures for the Synthesis of CTP-656, d15-ivacaftor, d9-M1, d6-M6, M1, and M6

The synthetic methods for the preparation of CTP-656 and d15-ivacaftor have been previously described (Morgan, 2015). Analytical
characterization of CTP-656 can be found in the Supplemental Material. These include liquid chromatography (LC) purity assessment (Supplemental Fig. 1), mass spectrum (Supplemental Fig. 2), and the 1H-NMR spectrum (Supplemental Fig. 3). Analytical data for d18-ivacaftor in the Supplemental Material include LC purity assessment (Supplemental Fig. 4), mass spectra (Supplemental Fig. 5), and the 1H-NMR spectrum (Supplemental Fig. 6). Synthetic routes and methods for the preparation of CTP-656 metabolites d2-M1 and d6-M6 were also in the Supplemental Material (Supplemental Figs. 7 and 8, respectively). Analytical data for d4-M1 and d4-M6 in the Supplemental Material include LC purity assessments (Supplemental Fig. 9), mass spectra (Supplemental Fig. 10), and 1H-NMR spectra (Supplemental Fig. 11). The M1 metabolite of ivacaftor was prepared by a modification of a synthetic method previously described (Yang et al., 2010). The modified synthetic route (Supplemental Fig. 12) and the methods for the preparation of M1 can be found in the Supplemental Material. Ivacaftor metabolite M6 was prepared as previously described (Yang et al., 2010). Analytical data for M1 and M6 in the Supplemental Material include LC purity assessments (Supplemental Fig. 13), mass spectra (Supplemental Fig. 14), and 1H-NMR spectra (Supplemental Fig. 15).

Bioanalytical Methods

Preclinical Non–GLP Methods. All plasma samples, in vitro samples, quality control, and standards were treated with acetonitrile during protein precipitation. Acetonitrile containing internal standard (IS; indiplon) was then added, and samples were vortexed at 1450 rpm for 3 minutes and then centrifuged at 3500 rpm for 10 minutes. Ten microliters of the reprepared sample was injected onto a high-performance LC (HPLC)—tandem quadrupole mass spectrometer (LC-MS/MS), consisting of an Agilent (Santa Clara, CA) 1200 HPLC spectrometer with a Leap HTS PAL auto-sampler (Leap Technologies, Morrisville, NC) equipped with a C6 Gemini phenyl 2.1 × 50 mm column maintained at 30°C. Analytes were eluted from the column using a gradient, where mobile phase B was linearly increased from 0% to 100% over 1 minute and then returned to 0% over another minute. The composition of mobile phase B was 0.1% formic acid in acetonitrile, whereas the composition of mobile phase A was 0.1% formic acid in reverse osmosis–deionized water.

LC-MS/MS analysis was performed using atmospheric pressure chemical ionization, followed by multiple reaction monitoring (MRM) of each analyte, including the IS. Transitions monitored were 393.2/172.2 m/z for ivacaftor, 402.2/172.2 m/z for CTP-656, 411.2/172.2 m/z for d7-M1, and 409.2/172.2 m/z for M1, whereas the transition for indiplon (the IS) was 377.2/293.2 m/z. For all analytes, the method was linear, and in all cases the precision and accuracy met the criteria of the Food and Drug Administration and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use.

In Vitro Pharmacology Assessments

Chloride conductance was evaluated with an equivalent current (Iaq) assay system using fully differentiated primary cultures of CF-HBE cells grown at an air/liquid interface for 4–6 weeks. Transepithelial voltage and transepithelial conductance were measured under current-clamp conditions using a custom-designed 24-channel current clamp (TECC-24; ChanTest Corporation, Cleveland, OH) and a 24-well electrode manifold. For all experiments, cells were tested in symmetric chloride solution (HEPES-buffered Coon’s F-12 modified liquid media without serum or bicarbonate) at 36 ± 0.5°C, and all compounds and test articles were dissolved in DMSO. For chloride conductance measurements, epithelial sodium channels were blocked with 3 μM benzamid; potentiatior responses were measured after 10 μM forskolin addition. CPTP specificity was determined by blocking chloride secretion with 20 μM bumetanide. Potentiator responses were quantified by calculating the total peak area under the curve (AUC) of Iaq after addition of test article. For studies using a corrector, lumacaftor was added at a concentration of 3 μM 24 hours prior to chloride conductance measurements. All compounds were assayed in triplicate at a single concentration per well, and AUC values were fit to a Hill equation to yield EC50 values.

Metabolic Stability in CYP3A4 Supersomes

Stock solutions (10 mM) of CTP-656, d18-ivacaftor, and ivacaftor were prepared in DMSO. The stock solutions were diluted to 50 μM in acetonitrile. Human CYP3A4 Supersomes (1000 pmol/ml) were diluted to 62.5 pmol/ml in 0.1 M potassium phosphate buffer, pH 7.4, containing 3 mM MgCl2. Metabolic stability in the diluted Supersomes was assessed as previously described (Morgan, 2015).

Semiquantitative Metabolite Profile in Human Hepatocytes

Stock solutions (5 mM) of CTP-656, d18-ivacaftor, and ivacaftor were prepared in DMSO. Cryopreserved human hepatocytes were thawed, diluted in cold thawing media, and centrifuged. The supernatants were removed, and the cells were resuspended in Krebs-Henseleit buffer. The test articles were added to the hepatocytes in Krebs-Henseleit buffer to achieve a final concentration of 25 μM in 0.5 ml, containing 4 × 106 cells/ml hepatocytes. The reaction mixtures were incubated at 37°C in a cell culture incubator (5% CO2). Aliquots of the mixture were removed at 0 and 4 hours, and acetonitrile was added. Samples were transferred to microcentrifuge tubes and centrifuged at 10,000g for 8 minutes to pellet the precipitated proteins. Supernatants were analyzed for amounts of the parent remaining and metabolites formed by LC-MS/MS. UV-visible light at 254 nm was used for semiquantitative analysis, wherein metabolites were measured as percentage of parent peak area. Analysis was by UV-visible light since metabolite standards had not been synthesized at the time of this experiment.

Determination of DIE for CTP-656 in HLM

Stock solutions (50 mM) of test compounds were prepared in DMSO. The 20 mg/ml HLM were diluted to 0.625 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4, containing 3 mM MgCl2. Aliquots (375 μl) of the HLM and test compound mixtures were transferred to wells of a deep 96-well plate in triplicate and prewarmed for 7 minutes. Reactions were initiated by the addition of 125 μl of prewarmed NADPH solution. The final reaction volume was 0.5 ml and contained 0.5 mg/ml HLM; 1, 2, 5, 10, 20, 40, 60, 80, and 100 μM ivacaftor or CTP-656; 2 mM NADPH; and 3 mM MgCl2. The reaction mixtures were incubated at 37°C for 10 minutes, after which 50 μl aliquots were
removed and added to shallow 96-well plates. The reactions were stopped by the addition of 50 μl of ice-cold acetonitrile with IS. The plates were stored at 4°C for 20 minutes, after which 100 μl of water was added to the wells before centrifugation to pellet precipitated proteins. Supernatants were transferred to another 96-well plate and analyzed for parent and metabolite by LC-MS/MS using an AB Sciex API 4000 mass spectrometer.

The kinetic parameters $K_m$ (concentration of substrate at one-half $V_{max}$) and $V_{max}$ (maximum velocity) were calculated from the Michaelis-Menten equation. The enzyme velocity (enzyme velocity = concentration of hydroxyl methyl/time/protein concentration) from three separate experiments was analyzed by GraphPad Prism version 6.04 (GraphPad Software, San Diego, CA), and reported values for $K_m$ and $V_{max}$ were determined from the fit using the average value.

Preclinical PK Studies

All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the US National Institutes of Health, and were approved by the National Institutes of Health Animal Care and Use Committee or the local equivalent.

PK of CTP-656, $d_{18}$-Ivacaftor and Ivacaftor in Rats. Male Sprague-Dawley rats (234-276 g) were obtained from Harlan Laboratories (Indianapolis, IN) and housed at VivoPath, LLC (Worcester, MA), where the studies were conducted. The PK of CTP-656, $d_{18}$-ivacaftor, and ivacaftor in rats were assessed as previously described (Morgan, 2015). The plasma PK parameters for the dosed compounds were calculated by noncompartmental analysis using Phoenix 64 WinNonlin software (Certara, Princeton, NJ).

PK of CTP-656, $d_{18}$-Ivacaftor, and Ivacaftor in Dogs. Four male beagle dogs were housed at Agilux Laboratories (Worcester, MA), where the studies were conducted. The dogs were administered 3 mg/kg each test compound orally in a discrete-dose crossover study with a 1-week washout period between the dosing phases. All dogs were fasted overnight before the administration of each test article. Each compound was formulated as a solution in 100% PEG400 at a concentration of 1.5 mg/ml. Blood samples were collected from each dog at 15 and 30 minutes, and at 1, 2, 4, 6, 8, 16, 24, 48, 72, 84, and 96 hours postdose. Blood samples were centrifuged to obtain plasma samples that were analyzed for concentrations of the dosed compound at each time point using LC-MS/MS. The limit of quantitation of each compound was 1 ng/ml. The plasma PK parameters for the dosed compounds were calculated by noncompartmental analysis using Phoenix 64 WinNonlin software (Certara, Princeton, NJ).

Evaluation of PK Profiles of CTP-656 and $d_{18}$-Ivacaftor in Healthy Human Volunteers

The first-in-human study was a two-part open-label, randomized, sequential, crossover, single-dose study to evaluate the safety, tolerability, and PK of CTP-656 and $d_{18}$-ivacaftor amorphous dispersion aqueous suspension in healthy volunteers, with a PK comparison with ivacaftor. This article will report on the first part of this clinical study, which was a single-dose, randomized, two-period, two-treatment, crossover study conducted in healthy male and female volunteers to evaluate the safety, tolerability, and PK of CTP-656 and $d_{18}$-ivacaftor under fasted conditions. The study was performed at a single center (CMAX (a division of IDT Australia Limited), Adelaide, SA, Australia) under the Clinical Trial Notification scheme through the Therapeutic Goods Administration. The study was conducted in accordance with the Declaration of Helsinki (e.g., the clinical study protocol, amendments, and informed consent documents), and any other study-related documents were reviewed and approved by the Bellberry Human Research Ethics Committee. Informed consent was obtained prior to any study-related procedures.

Six healthy volunteers were enrolled in this part of the study. On Day 1, three subjects received a single oral dose of 25 mg of CTP-656 and three subjects received a single oral dose of 25 mg of $d_{18}$-ivacaftor as an aqueous suspension. On Day 8 after the first dose, subjects crossed over and the dosing was repeated. A total of 21 PK blood samples (4 ml each) were collected for the assessment of plasma concentrations of CTP-656 and $d_{18}$-ivacaftor. Time points for collections were immediately prior to dosing (0 hour), and 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, 24, 36, 48, 60, 72, 84, and 96 hours postdose. Plasma levels of CTP-656 and $d_{18}$-ivacaftor were measured by LC-MS/MS. The plasma PK parameters for the dosed compounds were calculated by noncompartmental analysis using Phoenix 64 WinNonlin software (Certara). Each subject was monitored for safety throughout the study, including obtaining vital signs, performing physical examinations, obtaining clinical laboratory measurements, and performing 12-lead electrocardiograms.

Results

Deuterium Substitution Does Not Alter the Pharmacology of Ivacaftor and Its Major Metabolites. The pharmacological properties of CTP-656 and its major metabolites, $d_{3}$-M1 and $d_{3}$-M6, were compared with ivacaftor, M1, and M6 using a chloride conductance assay with fully differentiated HBE cells expressing mutant CFTR. It has been reported for the G551D CFTR mutation that M1 has approximately one-sixth the potency and M6 less than 1/50 the potency of ivacaftor (Kalydeco, 2015). In G551D/F508del HBE cells, ivacaftor and CTP-656 had similar potencies for potentiating forskolin responses, with EC₅₀ values of 336 and 255 nM, respectively (Table 1). The potencies of the M1 and $d_{3}$-M1 metabolites were also similar (0.96 and 1.35 μM, respectively), and both metabolites were less potent than the parent: 3-fold for M1 and 5-fold for $d_{3}$-M1. The EC₅₀ values for M6 and $d_{3}$-M6 were 11 and 15 μM, respectively, which is in agreement with prior reports that the M6 metabolite does not contribute to the therapeutic efficacy of ivacaftor in CF patients (Kalydeco, 2015).

To compare the activities of CTP-656, ivacaftor, and their metabolites in the context of pharmacologically corrected F508del-CFTR, the potencies of all compounds for potentiating forskolin responses were tested in F508del/F508del HBE cells after a 24-hour incubation period with lumacaftor. Similar to results obtained with G551D/F508del HBE, the potencies of nondeuterated and deuterated parent and metabolite compounds were very similar (Table 2). $d_{18}$-ivacaftor was also tested in G551D-CFTR–expressing FRT (Fischer rat thyroid)

### TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC₅₀, nM (95% CI)</th>
<th>Compound</th>
<th>EC₅₀, nM (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivacaftor</td>
<td>336 (295–382)</td>
<td>CTP-656</td>
<td>255 (220–296)</td>
</tr>
<tr>
<td>M1</td>
<td>960 (756–1220)</td>
<td>$d_{3}$-M1</td>
<td>1346 (1040–1743)</td>
</tr>
<tr>
<td>M6</td>
<td>11,320 (8906–14,380)</td>
<td>$d_{3}$-M6</td>
<td>15,140 (10,390–22,050)</td>
</tr>
</tbody>
</table>

Current traces of representative data are shown in Supplemental Fig. 16. *Calculated using AUC potentiator $I_{eq} (\mu A/cm^2)$ after forskolin addition, $n = 3$.

### TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC₅₀, nM (95% CI)</th>
<th>Compound</th>
<th>EC₅₀, nM (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivacaftor</td>
<td>387 (275–545)</td>
<td>CTP-656</td>
<td>295 (151–575)</td>
</tr>
<tr>
<td>M1</td>
<td>390 (259–588)</td>
<td>$d_{3}$-M1</td>
<td>286 (164–500)</td>
</tr>
<tr>
<td>M6</td>
<td>2410 (927–6289)</td>
<td>$d_{3}$-M6</td>
<td>3048 (undetermined)</td>
</tr>
</tbody>
</table>

Current traces of representative data are shown in Supplemental Fig. 16. *Calculated using AUC potentiator $I_{eq} (\mu A/cm^2)$ after forskolin addition, $n = 3$.
cells and lumacaftor-corrected F508del/F508del HBE cells; no significant differences in potentiation activity or potency were observed among \(d_{18}\)-ivacaftor, CTP-656, and ivacaftor (data not shown).

Deuterated Ivacaftor Analogs Show Increased Metabolic Stability in In Vitro Assays. Metabolic stability was initially determined by incubating each compound in the presence of CYP3A4 Supersomes for 30 minutes and monitoring the decrease in compound versus time. Additionally, compounds were incubated with human hepatocytes, and the amounts of parent remaining and metabolites formed were measured. In CYP3A4 Supersomes, the in vitro \(t_{1/2}\) value for ivacaftor was 5.5 minutes, whereas the \(t_{1/2}\) values for CTP-656 and \(d_{18}\)-ivacaftor were both 8.1 minutes (average of two experiments for the three compounds tested), which is a 47% increase in \(t_{1/2}\) for CTP-656 and \(d_{18}\)-ivacaftor versus ivacaftor (Fig. 3A). The data from human hepatocytes showed an approximate 2-fold reduction of the M1 metabolites as a percentage of parent for both CTP-656 and \(d_{18}\)-ivacaftor with respect to ivacaftor (Table 3). Additionally, no new metabolites were identified in the human hepatocyte incubations of the two deuterium-modified compounds.

Deuterium-enhanced metabolic stability was also assessed by incubating increasing concentrations of ivacaftor and CTP-656 in the presence of HLM for 10 minutes and measuring the rate of formation for M1 and \(d_{8}\)-M1, respectively. The data were then fit to the hyperbolic form of the Michaelis-Menten equation to derive the kinetic parameters \(K_m\), \(V_{max}\) (enzyme velocity at saturating substrate: \([S] >> K_m\)), and \(V_{max}/K_m\) (enzyme velocity at low substrate concentrations: \([S] << K_m\)). The ratios of the kinetic parameters \(V_{max}\) and \(V_{max}/K_m\) for ivacaftor versus CTP-656 measure the deuterium kinetic isotope effects \(^{13}V\) (\(V_{max}/V_{maxD}\)) and \(^{13}V/K_i\) ([\(V_{max}/K_m\)]H/[\(V_{max}/K_m\)]D) for the CYP3A4-catalyzed formation of M1 in HLM (Northrop, 1975). Significant DIEs were measured for both kinetic parameters: \(^{13}V = 3.8\) and \(^{13}V/K_i = 2.2\) (Fig. 3B). The observed increase in \(t_{1/2}\) for CTP-656 and \(d_{18}\)-ivacaftor versus ivacaftor in CYP3A4 Supersomes, the decreased metabolism in human hepatocytes, and the isotope effects observed in HLMs supported progression of the compounds to in vivo PK studies in rats and dogs.

Deuterated Ivacaftor Analogs Show Differentiated Nonclinical PK Upon Oral Dosing. The PK profiles of the three compounds were initially assessed in male Sprague-Dawley rats. Each compound was dosed at 10 mg/kg to each of three rats, with a total of nine rats in the study. The plasma levels of compound were measured over 72 hours. The plasma concentration versus time curves for drug exposure of the compounds are shown in Fig. 4A (only the 0 to 24-hour data are shown for clarity). Although \(d_{18}\)-ivacaftor showed greater exposure than either CTP-656 or ivacaftor, the error bars for the time points are overlapping, indicating modest differentiation for \(d_{18}\)-ivacaftor versus ivacaftor. This modest differentiation is supported by the PK parameters for the three compounds (Table 4). Across the parameters \(C_{max}\), \(AUC_{0-24\ hour}\), \(C_{24\ hour}\) and \(t_{1/2}\), \(d_{18}\)-ivacaftor showed a trend to larger exposures in rats compared with both CTP-656 and ivacaftor.

The PK profiles of the three compounds were also studied in male beagle dogs. Each compound was dosed by oral gavage at 3 mg/kg to each of four dogs in a crossover fashion with a 1-week washout period between doses, and the plasma levels

![Fig. 3. In vitro metabolism. (A) \(t_{1/2}\) values of ivacaftor and \(d_{18}\)-ivacaftor analogs in human CYP3A4 Supersomes. (B) Deuterium kinetic isotope effect as measured by the rate of formation of M1 and \(d_{8}\)-M1 metabolites in HLM.](image)

| Table 3 | Semiquantitive analysis of M1 metabolites versus parent after incubation of ivacaftor, CTP-656, and \(d_{18}\)-ivacaftor in human hepatocytes |
|---|---|---|---|
| Compound | Parent Area (mAU) | M1 Area (mAU) | M1 Formed as % of Parent |
| Icvaftor | 3.32 | 0.749 | 23 |
| CTP-656 | 3.09 | 0.365 | 12 |
| \(d_{18}\)-ivacaftor | 3.28 | 0.298 | 9.1 |
of compound were measured over 96 hours. As shown by the plasma concentration versus time profiles for each compound (Fig. 4B), CTP-656 had much greater exposure than ivacaftor and appeared to exhibit greater exposure than \(d_{18}\)-ivacaftor (only the 0 to 24-hour data are shown for clarity). The PK parameters for CTP-656 versus ivacaftor also supported the differentiated profile versus ivacaftor in dogs (Table 4). The \(C_{\text{max}}\), AUC\(_{0-24\text{hr}}\), and \(C_{24\text{hr}}\) values show 62%, 69%, and 83% increases, respectively, versus ivacaftor.

Although the PK data for CTP-656 and \(d_{18}\)-ivacaftor in rats and dogs demonstrated deuterium-mediated slowing of ivacaftor metabolism, the data did not clearly identify which compound should be advanced to human studies. The Pharmacology Review section of the Summary Basis of Approval for ivacaftor noted that exposure to ivacaftor was significantly greater than M1 and M6 in nonclinical test species (mouse, rat, and dog) (Center for Drug Evaluation and Research, Pharmacology Review(s): Ivacaftor (Application Number: 203188Orig1s000); http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203188Orig1s000PharmR.pdf). However, in humans, the exposure to M1 and M6 is significantly greater than ivacaftor. In view of the extensive metabolism of ivacaftor that occurs in humans, but is not observed in either rats or dogs, both CTP-656 and \(d_{18}\)-ivacaftor were advanced to a first-in-human study to identify the preferred compound for continued clinical development.

CTP-656 Identified as the Deuterated Ivacaftor Analog for Clinical Advancement. This first-in-human study of CTP-656 and \(d_{18}\)-ivacaftor was designed as a two-part crossover study of a low (25 mg) single oral dose of the two compounds as an aqueous suspension of their respective solid, amorphous dispersions. The baseline demographics of the study participants are summarized in Table 5. In the first part, six healthy volunteers (4 male and 2 female) were administered study drug on Day 1: three received CTP-656 and three received \(d_{18}\)-ivacaftor. On Day 8, the subjects were crossed over and were administered the other compound. Blood samples were collected over a 96-hour period on Days 1 and 8, and the PK parameters were determined (Table 6). The plasma concentration versus time curves (0–24 hours shown for clarity) for each compound are also shown (Fig. 5).

The PK parameters reflect the general trend shown in the exposure curves in that \(C_{\text{max}}\) and AUC from time 0 extrapolated to infinity (AUC\(_{0-\infty}\)) values are greater for CTP-656 than for \(d_{18}\)-ivacaftor. The approximate 16-hour \(t_{1/2}\) of CTP-656 is also compatible with identifying a dose that would enable once-daily dosing. PK data for a 25 mg dose of ivacaftor have been previously reported as part of a single-ascending dose (SAD) study (Center for Drug Evaluation and Research, Clinical Pharmacology and Biopharmaceutics Review(s): Ivacaftor (Application Number: 203188Orig1s000); http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203188Orig1s000ClinPharmR.pdf). For comparative purposes, the \(t_{1/2}\) for ivacaftor was 11.1 hours, and the exposure (AUC\(_{0-\infty}\)) was 1627 h•ng/mL. These data for ivacaftor suggest that at the same dose CTP-656 may have greater exposure and a longer \(t_{1/2}\) versus ivacaftor in humans. In subsequent clinical studies, CTP-656 as a solid oral 150 mg dose was compared directly to a singledose of ivacaftor (150 mg tablet) and was shown to have an approximate 15-hour \(t_{1/2}\) (Uttamsingh et al., 2016).

### TABLE 4.

Mean PK parameters (%CV) for CTP-656, \(d_{18}\)-ivacaftor, and ivacaftor in male Sprague-Dawley rats (10 mg/kg) and male beagle dogs (3.0 mg/kg) after oral administration

<table>
<thead>
<tr>
<th>Compound</th>
<th>(C_{\text{max}}) (ng/mL)</th>
<th>AUC(_{0-24\text{hr}}) (h•ng/mL)</th>
<th>(C_{24\text{hr}}) (ng/mL)</th>
<th>(t_{1/2}) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTP-656</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ivacaftor</td>
<td>1913 (7%)</td>
<td>22,177 (24%)</td>
<td>346 (54%)</td>
<td>10.5 (17%)</td>
</tr>
<tr>
<td>CTP-656</td>
<td>1970 (15%)</td>
<td>24,260 (17%)</td>
<td>413 (19%)</td>
<td>13.2 (9%)</td>
</tr>
<tr>
<td>(d_{18})-Ivacaftor</td>
<td>2460 (28%)</td>
<td>31,556 (20%)</td>
<td>623 (24%)</td>
<td>14.8 (9%)</td>
</tr>
<tr>
<td><strong>Ivacaftor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ivacaftor</td>
<td>1913 (7%)</td>
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<tr>
<td>(d_{18})-Ivacaftor</td>
<td>2460 (28%)</td>
<td>31,556 (20%)</td>
<td>623 (24%)</td>
<td>14.8 (9%)</td>
</tr>
<tr>
<td><strong>PK parameters in male beagle dogs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ivacaftor</td>
<td>2255 (33%)</td>
<td>29,448 (29%)</td>
<td>774 (30%)</td>
<td>17.6 (6.9%)</td>
</tr>
<tr>
<td>CTP-656</td>
<td>3463 (9%)</td>
<td>49,782 (15%)</td>
<td>1418 (31%)</td>
<td>22.8 (62%)</td>
</tr>
<tr>
<td>(d_{18})-Ivacaftor</td>
<td>3030 (38%)</td>
<td>40,019 (33%)</td>
<td>1089 (43%)</td>
<td>18.0 (26%)</td>
</tr>
</tbody>
</table>
TABLE 5
Subject demographics

<table>
<thead>
<tr>
<th></th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n (%)</td>
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</tr>
<tr>
<td>Male</td>
<td>4 (67)</td>
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<tr>
<td>Female</td>
<td>2 (33)</td>
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<tr>
<td>Race, n (%)</td>
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</tr>
<tr>
<td>Caucasian</td>
<td>4 (67)</td>
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<tr>
<td>Asian</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Aboriginal/Torres Strait Islander</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Age, mean year (range)</td>
<td>23.0 (18–31)</td>
</tr>
<tr>
<td>Weight, mean kg (range)</td>
<td>74.4 (62.8–91.4)</td>
</tr>
<tr>
<td>BMI, mean kg/m² (range)</td>
<td>23.9 (18.8–27.0)</td>
</tr>
</tbody>
</table>

BMI, body mass index.

**Discussion**

This study reports the results of precision deuteration of ivacaftor (Kalydeco; Vertex Pharmaceuticals), which is the first approved CFTR modulator for the treatment of CF. Two deuterated analogs, d_9-ivacaftor (CTP-656) and d_{18}-ivacaftor were tested in vitro and in vivo to assess the pharmacology, PK, and metabolism of the compounds with respect to ivacaftor. The studies of the deuterated ivacaftor analogs were designed to assess whether a DIE could reduce metabolism in humans and provide an agent with a differentiated clinical profile. The data for the deuterated analogs of ivacaftor align with the previous article in this series (Uttam Singh et al., 2015) that assessed the effects of precision deuteration on paroxetine pharmacology and PK. Deuterated paroxetine (CTP-347) retained the intrinsic in vivo pharmacology profile of paroxetine with greatly reduced mechanism-based inactivation of CYP2D6 in vitro studies. This preclinical profile of CTP-347 translated to a reduced drug-drug interaction liability with dextromethorphan in a clinical study. However, further clinical development of CTP-347 as a nonhormonal therapy for hot flashes was stopped for commercial reasons.

To confirm that deuterium substitution did not affect the intrinsic pharmacology of ivacaftor, in vitro studies were performed in HBE cells derived from CF patients. The potency as a potentiator of CFTR with a gating mutation was measured in G551D/F508del HBE cells. For homozygous F508del HBE cells, the cells were first corrected with lumacaftor prior to measuring the potentiation of conductance. Ivacaftor is extensively metabolized in humans to two major metabolites, M1 and M6 (Zha et al., 2011). At steady state, the metabolites are the major circulating species in humans, with mean exposure ratios (metabolite/parent) of approximately 5-fold and 2-fold, respectively, for M1 and M6 (Center for Drug Evaluation and Research, Clinical Pharmacology and Biopharmaceutics Review(s); Ivacaftor (Application Number: 203188Orig1s000); http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203188Orig1s000ClinPharmR.pdf). These metabolites of ivacaftor and CTP-656 were, therefore, also evaluated in the HBE cells assays.

Overall, the in vitro pharmacology data confirmed that deuteration did not change the CFTR activity of ivacaftor and the major metabolites. The equivalent activity of CTP-656 and ivacaftor measured in G551D/F508del HBE cells supports the use of CTP-656 in the same patient population as ivacaftor. Likewise, the potentiation of CFTR function in lumacaftor-corrected F508del homozygous HBE cells supports the clinical use of CTP-656 in combination with lumacaftor and potentially other correctors currently in development.

Although precision deuteration of ivacaftor did not change the intrinsic pharmacology of the compound, decreases in metabolism for the deuterated analogs were shown both in vitro and in vivo. High protein binding, nonspecific binding, and low solubility prevented the metabolic stability assessment of ivacaftor in our standard HLM assay due to the slow apparent rate of metabolism (unpublished data). Since ivacaftor is metabolized primarily by CYP3A4 (Kalydeco, 2015), CYP3A4 Super-somes were used to assess metabolic stability. The two deuterated analogs exhibited t_{1/2} values approximately 50% greater than ivacaftor in this study, which signaled that precision deuteration could positively impact the PK of ivacaftor.

Once the respective M1 and d_{9}-M1 metabolites of ivacaftor and CTP-656 were synthesized, a more sensitive assay to measure metabolism in HLM was possible. By measuring the formation of metabolites instead of the loss of parent, a direct measurement of metabolite formation in HLM was possible and enabled calculation of the DIE using classic Michaelis-Menten kinetics. The measured V/K and V values for CYP3A4 catalyzed metabolism in HLM were large.

Although a V/K isotope effect of 2.2 was measured, the literature reports that an isotope effect on V/K should not be observed unless it has been unmasked, which can occur under certain conditions (Korzekwa et al., 1989; Nelson and Trager, 2003). Unmasking could arise due to oxidation at a different site on CTP-656 to produce an alternate metabolite (“metabolic switching”) (Horning et al., 1975). Preclinical and clinical data, however, do not suggest metabolic switching. Incubations of CTP-656 and d_{18}-ivacaftor in human hepatocytes showed only reductions in the formation of the M1 metabolites versus ivacaftor; no other metabolites were detected. Clinical data also showed the same plasma metabolites for CTP-656 and ivacaftor in humans (manuscript in preparation). Another possible mechanism for unmasking the isotope effect is direct reduction of the activated enzyme-substrate complex to produce free enzyme, substrate, and water (Atkins and Sligar, 1987). Studies were not conducted to assess this mechanism, but it appears to be a reasonable explanation for the observed V/K isotope effect for CTP-656 in the absence of metabolic switching.

A recent review (Guengerich, 2013) on the potential issues for deuterium in drug development notes than an isotope effect in vitro needs to translate to a PK effect in vivo to have a

**TABLE 6**

<table>
<thead>
<tr>
<th>Compound</th>
<th>C_{max} (ng/ml)</th>
<th>t_{1/2} (h)</th>
<th>AUC_{0-24h} (h*ng/mL)</th>
<th>C_{fa max} (ng/ml)</th>
<th>CL/F (l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP-656</td>
<td>270.5 (24%)</td>
<td>15.9 (18%)</td>
<td>3812 (26%)</td>
<td>52.6 (28%)</td>
<td>6.92 (25%)</td>
</tr>
<tr>
<td>d_{18}-Ivacaftor</td>
<td>232.7 (18%)</td>
<td>16.4 (16%)</td>
<td>3196 (15%)</td>
<td>42.3 (16%)</td>
<td>7.97 (15%)</td>
</tr>
</tbody>
</table>

CL/F: oral clearance.
practical application to drug development. Although slower metabolism was observed in vitro, the translation to in vivo is unpredictable since non–cytochrome P450 clearance mechanisms may predominate in vivo and clearance mechanisms may vary between species. PK studies in rats and dogs were particularly challenging since neither rats nor dogs metabolize ivacaftor as extensively as humans; therefore, these preclinical species may not be adequate models for human metabolism (Center for Drug Evaluation and Research; Pharmacology Review(s): Ivacaftor (Application Number: 203188Orig1s000); http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203188Orig1s000PharmR.pdf). The PK results in rats and dogs for CTP-656 were encouraging, with a longer $t_{1/2}$ and greater exposure versus ivacaftor in both species. $d_{18}$-Ivacaftor, however, showed increased exposure versus ivacaftor in rats, but not in dogs. These results emphasize the unpredictable effects of deuterium substitution. Although positive preclinical PK effects were very encouraging, these models were not sufficient to choose a preferred compound for clinical development. The more rigorous approach involved testing both compounds in humans and selecting a compound for advancement based upon human PK data.

The clinical crossover study of orally dosed CTP-656 and $d_{18}$-ivacaftor identified the preferred compound for further clinical development. CTP-656 exposure was only modestly increased versus $d_{18}$-ivacaftor; however, the increases were consistent across the PK parameters $C_{\text{max}}$, $t_{1/2}$, $AUC_{0-\text{inf}}$, $C_{24\text{hr}}$, and oral clearance (CL/F). These data, coupled with the reduced amount of deuterium in CTP-656, were sufficient to identify CTP-656 for continued clinical development.

Once-daily dosing may translate to improved adherence in real-world use. A small clinical study (Siracusa et al., 2015) assessed ivacaftor adherence in patients and found the adherence to be highly variable. The ivacaftor label (Kalydeco, 2015) specifies dosing every 12 hours; however, this study found that the median duration between doses was 16.9 hours, with a range of 12.1–43.7 hours. The overall adherence rate—calculated as (total doses taken)/total days monitored —was only 61%. It is also noteworthy that the 10.4% improvement in absolute percent predicted forced expiratory volume in 1 s observed in the clinical study ($\geq 12$ years old) is much greater than the 5.4% improvement observed over 12 months in a non–clinical trial patient population (Fink et al., 2015). Although this may be due to a number of factors, the suboptimal adherence could be an important contributing factor, which may be positively impacted by a once-daily potentiator.

CTP-656 has completed three phase I studies in healthy volunteers, including SAD, multiple–ascending dose, and food-effect studies. The tablet formulation was reported to have an average $t_{1/2}$ of 15 hours after a single oral dose and an average $t_{1/2}$ of 18 hours in the multiple–ascending dose studies (Uttamsingh et al., 2016). Of particular note is the reported CTP-656/$d_{18}$-M1 AUC ratio of 1.5:1 after a single dose and also at steady state. This is much greater than ivacaftor, which has a parent/M1 AUC ratio of 1:5 at steady state. With a longer $t_{1/2}$ and greater exposure to the more active parent, the PK profile for CTP-656 may translate into an improved clinical profile in CF patients with gating mutations. A phase II safety and efficacy study in CF patients is currently underway (NCT02971839).

Acknowledgments

We thank Doug Williams, Kinga Chojnacka, and Junfeng Huang (Adesis Inc., New Castle, DE) for metabolite synthesis; and David Norris (Ecosse Medical Communications, Falmouth, MA) for editorial assistance.

Authorship Contributions

Participated in research design: Harbeson, Liu, Aslanian, Brummel, Wu, Tung, Braman, and Uttamsingh.

Conducted experiments: Morgan, Aslanian, Nguyen, Bridson, Pilja, and Uttamsingh.

Contributed new reagents or analytic tools: Morgan, Liu, Bridson, and Brummel.

Performed data analysis: Aslanian, Nguyen, Brummel, and Uttamsingh.

Wrote or contributed to the writing of the manuscript: Harbeson, Liu, Aslanian, Brummel, Tung, Braman, and Uttamsingh.
Altering Metabolic Profiles of Drugs by Precision Deuteration 2: Discovery of a Deuterated Analog of Ivacaftor with Differentiated Pharmacokinetics for Clinical Development

Authors
Scott L. Harbeson, Adam J. Morgan, Julie F. Liu, Ara M. Aslanian, Sophia Nguyen, Gary W. Bridson, Christopher L. Brummel, Lijun Wu, Roger D. Tung, Lana Pilja, Virginia Braman, Vinita Uttamsingh

Journal of Pharmacology and Experimental Therapeutics

SUPPLEMENTAL DATA SECTION
Supplemental Figure 1. CTP-656 LC purity assessment

<table>
<thead>
<tr>
<th>Elution Time (min)</th>
<th>Peak Area</th>
<th>Percentage of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.823</td>
<td>5490.28857</td>
<td>100%</td>
</tr>
</tbody>
</table>

CTP-656 LC-purity: 100.0%
Supplemental Figure 2. CTP-656 MS identity assessment

CTP-656 MS m/z: 402.2744 [M+H]+
Supplemental Figure 3. CTP-656 $^1$H-NMR spectrum
Supplemental Figure 4. $d_{18}$-Ivacaftor LC purity assessment

<table>
<thead>
<tr>
<th>Elution Time (min)</th>
<th>Peak Area</th>
<th>Percentage of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.827</td>
<td>5049.94531</td>
<td>100%</td>
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</table>
Supplemental Figure 5. $d_{18}$-Ivacaftor MS identity assessment

$\tilde{d}_{18}$-Ivacaftor MS m/z: 411.3303 [M+H]+
Supplemental Figure 6. $d_{18}$-Ivacaftor $^1$H-NMR spectrum

Ethanol solvate
Supplemental Figure 7. Synthesis of CTP-656 Metabolite $d_8$-M1

XV

NaH, CD$_3$I
THF

XVI

DIBALD
Toluene

XVII

NaBD$_4$
MeOD

XVIII

H$_2$, Pd(OH)$_2$
EtOH

XIX

NBS
CH$_2$Cl$_2$

XX

ClCO$_2$Me
CH$_2$Cl$_2$

XXI

HNO$_3$
CH$_2$Cl$_2$

XXII

H$_2$, Pd/C
MeOH

XXIII

T$_3$P, pyridine
2-MeTHF

VII
\[
\text{XXIV} \xrightarrow{\text{NaOMe, MeOH}} \text{d}_8\text{-M1}
\]
Supplemental Materials: Synthesis of CTP-656 $d_8$-M1 Metabolite

2-(2-(Benzyloxy)-5-(tert-butyl)phenyl)-2-(methyl-$d_3$)propanenitrile-3,3,3-$d_3$ (XVI) – 60% dispersion of sodium hydride in mineral oil (4.3 g, 107.5 mmol, 6 equiv) was added to THF (75 mL, anhydrous) and stirred for 15 minutes. XV (5.0 g, 17.9 mmol, 1 equiv) was added and the mixture was stirred at room temperature for 1 hour. Iodomethane-$d_3$ (4.6 mL, 71.7 mmol, 4 equiv, Cambridge Isotopes) was added and the mixture was stirred at room temperature overnight. TLC analysis (30% ethyl acetate in heptanes, SM $R_f$ = 0.6, product $R_f$ = 0.65, stained with KMnO$_4$) indicated the reaction was complete. The mixture was cooled to 0 °C and quenched by the slow addition of 0.5 N HCl (200 mL). The biphasic mixture was transferred to a separatory funnel and extracted with ethyl acetate (2 x 100 mL). The combined organic layers were washed with brine (1 x 100 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude product was purified on an Analogix automated chromatography system, eluting with a gradient of 0 to 25% ethyl acetate in heptanes, over 45 minutes to give XVI (6.4 g, >100% yield) as a yellow oil.

2-(2-(Benzyloxy)-5-(tert-butyl)phenyl)-2-(methyl-$d_3$)propanal-1,3,3,3-$d_4$ (XVII) – A solution of XVI (6.4 g, 17.9 mmol, assumed 100 % yield in previous step, 1 equiv) in toluene (60 mL, anhydrous) was cooled to -78 °C. A 0.7 M solution of diisobutylaluminum deuteride (28.1 mL, 19.7 mmol, 1.1 equiv, Aldrich, 98 atom% D) was added drop-wise over 15 minutes. The mixture was stirred at -78 °C for 3 hours, then at room temperature overnight, at which time GC/MS analysis indicated ~40% conversion. The reaction was cooled to -78 °C and additional 0.7 M solution of diisobutylaluminum deuteride (28.1 mL, 19.7 mmol, 1.1 equiv) was added drop-wise. The mixture was warmed to room temperature and stirred for 6 hours at which time GC/MS analysis indicated the reaction was complete. The mixture was left stirring overnight then cooled to 0 °C and quenched by the addition of 1 N DCl in D$_2$O (100 mL). The mixture was
stirred for 30 minutes at room temperature, transferred to a separatory funnel and extracted with heptanes (100 mL). The layers were separated and the organic layer was washed with 1 N HCl (1 x 100 mL), water (1 x 100 mL), saturated aqueous sodium bicarbonate (1 x 100 mL) and brine (1 x 100 mL). The organic layer was dried over sodium sulfate and Celite (10 g), filtered, and concentrated under reduced pressure to give crude XVII (8.1 g) as a yellow oil which was used without purification.

2-(2-(Benzyloxy)-5-(tert-butyl)phenyl)-2-(methyl-d₅)propan-1,1,3,3,3-d₅-1-ol (XVIII) – A solution of XVII (8.1 g, 17.9 mmol, assumed 100% yield in previous step, 1 equiv) in methanol-OD (80 mL, CDN, 99.6 atom% D) was cooled to 0 °C and sodium borodeuteride (1.50 g, 35.8 mmol, 2 equiv, Cambridge Isotopes, 99 atom% D) was added in portions over 5 minutes. The mixture was stirred at room temperature for 90 minutes at which time TLC analysis (30% ethyl acetate in heptanes, SM Rf = 0.65, product Rf = 0.3) indicated the reaction was complete. The mixture was quenched by adding 1 N DCl in D₂O (100 mL, Cambridge Isotopes, 99 atom% D). The mixture was partially concentrated under reduced pressure to remove methanol. The remaining aqueous mixture was extracted with ethyl acetate (1 x 150 mL). The organic layer was washed with brine (1 x 100 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified on an Analogix automated chromatography system, eluting with 0 to 50 % ethyl acetate in heptanes, over 40 minutes to give XVIII (3.3 g, 58% yield over 2 steps) as a colorless oil which solidified upon standing.

4-(tert-Butyl)-2-(1-hydroxy-2-(methyl-d₃)propan-2-yl-1,1,3,3,3-d₅)phenol (XIX). A 500-mL Parr shaker bottle was charged with 20% Pd(OH)₂ on carbon (325 mg), XVIII (3.25 g, 10.2 mmol, 1 equiv), and methanol (50 mL). The mixture was shaken at 20 psi H₂ for 7 hours at which time GC/MS analysis indicated the reaction was complete. The mixture was filtered
through a pad of Celite (washing with methanol) and the filtrate was concentrated under reduced pressure to give \( \text{XIX} \) (1.77 g, 75% yield) as an off-white solid.

### 2-Bromo-4-(tert-butyl)-6-(1-hydroxy-2-(methyl-d\textsubscript{3})propan-2-yl-1,1,3,3,3-d\textsubscript{5})phenol (XX) –

\( N\)-bromosuccinimide (1.37 g, 7.70 mmol, 1 equiv) was added to a solution of \( \text{XIX} \) (1.77 g, 7.70 mmol, 1 equiv) in dichloromethane (30 mL) at 0 °C. The mixture was stirred for 2 hours while warming to room temperature, at which time GC/MS analysis indicated the reaction was complete. Additional dichloromethane (100 mL) was added and the mixture was washed with saturated aqueous sodium bicarbonate solution (1 x 100 mL). The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure to give \( \text{XX} \) (2.7 g, >100% yield) as a light yellow oil which solidified on standing.

### 2-Bromo-4-(tert-butyl)-6-(1-((methoxycarbonyl)oxy)-2-(methyl-d\textsubscript{3})propan-2-yl-1,1,3,3,3-d\textsubscript{5})phenyl methyl carbonate (XXI) –

A solution of \( \text{XX} \) (2.7 g, 7.7 mmol, 1 equiv, assumed 100% yield in previous step), triethylamine (3.2 mL, 23.1 mmol, 3 equiv) and DMAP (100 mg, 0.11 mmol, 0.1 equiv) in dichloromethane (75 mL) was cooled to 0 °C and methyl chloroformate (1.30 mL, 16.9 mmol, 2.2 equiv) was added drop-wise over 5 minutes. The mixture was stirred at 0 °C for 30 minutes, then at room temperature for 2 hours, at which time TLC analysis (20% ethyl acetate in heptanes, SM \( R_f = 0.3 \), product \( R_f = 0.35 \), stained with KMnO\textsubscript{4} \) indicated the reaction was complete. The reaction mixture was diluted with dichloromethane (100 mL), water (100 mL) and 1 N HCl (100 mL). The layers were separated and the aqueous layer was washed with dichloromethane (1 x 100 mL). The combined organic layers were dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified on an Analogix automated chromatography system, eluting with 0 to 40 % ethyl acetate in heptanes, over 45 minutes to give \( \text{XXI} \) (2.9 g, 89% yield over 2 steps) as a colorless oil.
2-Bromo-4-(tert-butyl)-6-((methoxycarbonyl)oxy)-2-(methyl-d3)propan-2-yl-1,1,3,3,3-d5)-3-nitrophenyl methyl carbonate (XXII) – A solution of XXI (2.8 g, 6.6 mmol, 1 equiv) in dichloromethane (50 mL) was cooled to 0 °C and sulfuric acid (2.97 g, 30.4 mmol, 4.6 equiv) added drop-wise over 1 minute. The mixture was stirred for 15 minutes at 0 °C before the drop-wise addition of nitric acid (1.19 g, 13.2 mmol, 2 equiv) over 5 minutes. The mixture was stirred at 0 °C for 3 hours, during which time a yellow/brown color developed. Proton NMR analysis of an aliquot indicated the reaction was incomplete; therefore, the mixture was stirred an additional 2 hours at 0 °C. The mixture was quenched by the addition of ice water (100 mL). Dichloromethane (100 mL) was added and the layers were separated. The organic layer was washed with saturated sodium bicarbonate (50 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure to give XXII (2.89 g, 93 % yield) as a yellow oil.

5-Amino-4-(tert-butyl)-2-((methoxycarbonyl)oxy)-2-(methyl-d3)propan-2-yl-1,1,3,3,3-d5)-phenyl methyl carbonate (XXIII) – A 500-mL Parr shaker bottle was charged with 10% Pd/C (720 mg, dry), ethanol (85 mL), XXII (2.89 g, 6.15 mmol, 1 equiv), and N,N-diisopropylethyl amine (1.2 mL, 6.76 mmol, 1.1 equiv). The mixture was shaken at 50 psi H2 overnight at which time TLC and LC/MS analysis confirmed reduction was complete. The mixture was filtered through a pad of Celite (washing with ethanol). The filtrate was partially concentrated under reduced pressure to a volume of approximately 15 mL. The remaining solution was cooled to 0 °C and water (50 mL) was added drop-wise. The resulting solid was filtered, washed with water and dried on the filter to give XXIII (1.60 g, 72% yield) as an off-white solid.

4-(tert-Butyl)-2-((methoxycarbonyl)oxy)-2-(methyl-d3)propan-2-yl-1,1,3,3,3-d5)-5-(4-oxo-1,4-dihydroquinoline-3-carboxamido)phenyl methyl carbonate (XXIV) – A 50% solution of propylphosphonic anhydride in ethyl acetate (5.73 g, 9.0 mmol, 2.5 equiv) and pyridine (570
mg, 7.2 mmol, 2 equiv) were added to a suspension of VII (680 mg, 3.6 mmol, 1 equiv) and XXIII (1.3 g, 3.6 mmol, 1 equiv) in 2-methyltetrahydrofuran (25 mL). The mixture was heated at 55 °C for 16 hours; after 2 hours most of the solids had dissolved. The mixture was cooled to room temperature and ethyl acetate (50 mL) was added. The mixture was washed with 10% aqueous sodium carbonate solution (2 x 50 mL) and brine (1 x 50 mL). The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure to give XXIV (1.9 g, 99% yield) as a tan foam which was used without purification.

\[ N-(2-(\text{tert-butyl})-5-hydroxy-4-(1-hydroxy-2-(\text{methyl-d}_3)\text{propan-2-yl}-1,1,3,3,3-d_5)\text{phenyl})-4-oxo-1,4-dihydroquinoline-3-carboxamide \ (d_8-M1) \] – A 25% solution of sodium methoxide in methanol (1.54 g, 7.2 mmol, 2 equiv) was added to a solution of XXIV (1.9 g, 3.6 mmol, 1 equiv) in 2-methyltetrahydrofuran (50 mL). The mixture was stirred at room temperature for 4 hours, during which time a dark brown color developed. LC/MS analysis indicated the reaction was complete. The mixture was cooled to 0 °C, 1 N HCl (50 mL) was added and the mixture was extracted with ethyl acetate (100 mL). The organic layer was washed with brine, dried over sodium sulfate, filtered and concentrated under reduced pressure to afford a tan foam. The foam was dissolved in ethyl acetate (50 mL) and stirred, resulting in the formation of white crystals. Heptanes (50 mL) were added, and the solids were filtered and dried in a vacuum oven at room temperature for 2 hours to give \( d_8-M1 \) (1.25 g, 83% yield) as white solid.
Supplemental Figure 8.  Synthesis of CTP-656 Metabolite $d_6$-M6

$D_3C\_CO_2H$ $\rightarrow$ n-BuOH, $H_2SO_4$ $\rightarrow$ LDA, TMSCl

$XXV$ $\rightarrow$ $XXVI$ $\rightarrow$ $XXVII$ $\rightarrow$ $XXVIII$, $Pd(t-Bu_3P)_2$, $ZnF_2$, $DMF$

$XXIX$ $\rightarrow$ XXX $\rightarrow$ XXXI

$VII$ $\rightarrow$ XXXII $\rightarrow$ $d_6$-M6

$XXXI$ $\rightarrow$ $XXXII$ $\rightarrow$ $d_6$-M6

$XXX$ $\rightarrow$ $XXXI$ $\rightarrow$ $d_6$-M6
Supplemental Materials: Synthesis of CTP-656 $d_6$-M6 Metabolite

**Butyl 2-(methyl-$d_3$)propanoate-2,3,3,3-d4 (XXVI)** – Methylpropionic-$d_7$ acid (XXV, 5 g, 52.6 mmol, 1.0 equiv, CDN, 98 atom% D) was dissolved in n-butanol (30 mL) and sulfuric acid (1 mL, 18.8 mmol, 0.35 equiv) was added. The mixture was stirred at 80 °C overnight then poured onto ice (300 g). The mixture was extracted with MTBE (3 x 50 mL). The combined organic layers were washed with saturated sodium bicarbonate solution (50 mL), water (50 mL), brine (50 mL), dried over sodium sulfate, filtered and concentrated to give crude XXVI (5.2 g, 65% yield) as a colorless liquid.

**((1-Butoxy-2-(methyl-$d_3$)prop-1-en-1-yl-3,3,3-d3)oxy)trimethylsilane (XXVII)** – Diisopropylamine (7.3 mL, 53 mmol, 1.2 equiv) was added to anhydrous THF (200 mL) and the solution was cooled to -40 °C. 2.5 M $n$-butyllithium in hexane (17.6 mL, 44 mmol, 1.1 equiv) was added over 15 minutes. The mixture was stirred at -30 to -20 °C for 30 minutes then cooled to -78 °C. A solution of XXVI (5.2 g, 40 mmol, 1.0 equiv) in anhydrous THF (10 mL) was added over 30 minutes. The mixture was stirred for 30 minutes at -78 °C then chlorotrimethylsilane (10 mL, 80 mmol, 2.0 equiv) was added over 15 minutes. The mixture was stirred at -78 °C for 2 hours, then at room temperature for 2 hours. The mixture was poured into a mixture of saturated sodium bicarbonate solution (300 mL) and ice (100 g) and extracted with hexanes (200 mL). The organic phase was washed with water (3 x 100 mL), dried over sodium sulfate, filtered and concentrated. The residue was distilled (60-70 °C at 15 mmHg) to give XXVII (6.2 g, 70% yield) as a colorless liquid.

**Butyl 2-(5-(tert-butyl)-2-hydroxy-4-nitrophenyl)-2-(methyl-$d_3$)propanoate-3,3,3-d3 (XXIX)** – To a solution of XXVIII (1.7 g, 6.2 mmol, 1.0 equiv) in anhydrous DMF (20 mL) was added zinc fluoride (640 mg, 6.2 equiv, 1.0 equiv). The mixture was sparged with nitrogen for 15
minutes, then fresh bis(tri-tert-butylphosphine)palladium(0) (160 mg, 0.31 mmol, 0.05 equiv) and XXVII (2.7 g, 12.4 mmol, 2.0 equiv) were added. The mixture was heated to 80 °C and stirred for 16 hours. The mixture was cooled to room temperature and MTBE (50 mL) and water (100 mL) were added. The mixture was stirred for 1 hour and extracted with MTBE (100 mL). The organic layer was washed with 1 N HCl (50 mL), saturated sodium bicarbonate solution (50 mL), and brine (50 mL), dried over sodium sulfate, filtered and concentrated. The residue was purified on an Analogix automated chromatography system, eluting with a gradient of 0 to 35% ethyl acetate in heptanes, to give XXIX (900 mg, 42% yield) as a white solid.

**Butyl 2-(5-(tert-butyl)-2-((methoxycarbonyl)oxy)-4-nitrophenyl)-2-(methyl-d3)propanoate-3,3,3-d3 (XXX)** – A solution of XXIX (900 mg, 2.6 mmol, 1.0 equiv) and triethylamine (0.76 mL, 5.4 mmol, 2.0 equiv) in dichloromethane (30 mL) was cooled to 0 °C and methyl chloroformate (0.51 mL, 6.5 mmol, 1.2 equiv) was added. The mixture was stirred at room temperature for 3 hours, quenched with 1 N HCl (50 mL) and stirred for 15 minutes. The organic layer was washed with 1 N HCl (50 mL), saturated sodium bicarbonate solution (100 mL), water (100 mL), and brine (100 mL), dried over sodium sulfate, filtered and concentrated. The residue was purified on an Analogix automated chromatography system, eluting with a gradient of 0 to 10% ethyl acetate in heptanes, to give XXX (950 mg, 90% yield) as a yellow solid.

**Butyl 2-(4-amino-5-(tert-butyl)-2-((methoxycarbonyl)oxy)phenyl)-2-(methyl-d3)propanoate-3,3,3-d3 (XXXI)** – A mixture of XXX (950 mg, 2.37 mmol, 1.0 equiv) and 10% palladium on carbon, 50 wt% wet (100 mg) in methanol (50 mL) was hydrogenated at 30 psi H₂ overnight. TLC analysis showed reduction was complete. The mixture was filtered through a pad of Celite and the filtrate was concentrated to give XXXI (810 mg, 91% yield) as a yellow oil.
Butyl 2-(5-(tert-butyl)-2-((methoxycarbonyl)oxy)-4-(4-oxo-1,4-dihydroquinoline-3-carboxamido)phenyl)-2-(methyl-d3)propanoate-3,3,3-d3 (XXXII) – To a solution of XXXI (810 mg, 2 mmol, 1.0 equiv) in anhydrous 2-methyltetrahydrofuran (10 mL) was added VII (455 mg, 2.4 mmol, 1.2 equiv), a 50% solution of propylphosphonic anhydride in DMF (1.9 g, 3 mmol, 1.5 equiv) and pyridine (0.35 mL, 4 mmol, 2.0 equiv). The mixture was stirred at 50 °C for 48 hours then cooled to room temperature. Water (40 mL) and ethyl acetate (40 mL) were added and the layers were separated. The organic layer was washed with saturated sodium bicarbonate solution (2 x 50 mL), water (50 mL), and brine (50 mL), dried over sodium sulfate, filtered and concentrated. The residue was purified on an Analogix automated chromatography system, eluting with a gradient of 0 to 35% ethyl acetate in dichloromethane, to give XXXII (800 mg, 74% yield) as a white solid.

2-(5-(tert-Butyl)-2-hydroxy-4-(4-oxo-1,4-dihydroquinoline-3-carboxamido)-phenyl)-2-(methyl-d3)propanoic-3,3,3-d3 acid (d6-M1) – 1 N sodium hydroxide solution (5.9 mL, 5.9 mmol, 4 equiv) was added to a solution of XXXII (800 mg, 1.47 mmol, 1.0 equiv) in methanol (30 mL) and the mixture was stirred for 6 hours at room temperature. The mixture was concentrated, diluted with water (30 mL), and extracted with dichloromethane (20 mL). The aqueous solution was acidified with 6 N HCl to pH 2 at 0 °C, and stirred for 30 minutes at 0 °C. The solid was filtered, washed with water (10 mL) and dried in vacuum oven at 60 °C to give d6-M1 (520 mg, 81% yield) as a white solid.
**Supplemental Figure 9.** \(d_8\)-M1 and \(d_6\)-M6 LC purity assessments

### \(d_8\)-M1 LC purity: 97.9%

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### \(d_6\)-M6 LC purity: 99.1%

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Supplemental Figure 10. $d_8$-M1 and $d_6$-M6 MS identity assessments

$d_8$-M1 MS m/z: 417.2 $[M+H]^+$

$d_6$-M6 MS m/z: 429.2 $[M+H]^+$
Supplemental Figure 11. $d_8$-M1 and $d_6$-M6 $^1$H-NMR spectra

$d_8$-M1 1H NMR DMSO-$d_6$ 300 mHz

$d_6$-M6 1H NMR DMSO-$d_6$ 300 mHz
Supplemental Figure 12. Synthesis of Ivacaftor Metabolite M1.

XXXIII

\[ \text{OH} + \text{NBS} \rightarrow \text{Br} \]

\[ \text{CH}_2\text{Cl}_2 \]

XXXIV

\[ \text{ClCO}_2\text{Me} \rightarrow \]

\[ \text{CH}_2\text{Cl}_2 \]

XXXV

\[ \text{OH} + \text{HNO}_3 \rightarrow \text{O}_2\text{N} \]

\[ \text{H}_2\text{SO}_4 \]

\[ \text{CH}_2\text{Cl}_2 \]

XXXVI

\[ \text{H}_2, \text{Pd/C} \rightarrow \]

\[ \text{MeOH} \]

XXXVII

\[ \text{NaOMe} \rightarrow \]

\[ \text{MeOH} \]

\[ \text{2-MeTHF} \]

XXXVIII

\[ \text{M1} \]
Supplemental Materials: Synthesis of Ivacaftor Metabolite M1

2-Bromo-4-(tert-butyl)-6-(1-hydroxy-2-methylpropan-2-yl)phenol (XXXIV) – N-bromosuccinimide (3.64 g, 20.5 mmol, 1 equiv) was added to a solution of XXXIII (4.54 g, 20.5 mmol, 1 equiv) in dichloromethane (190 mL) at 0 °C. The reaction mixture was stirred at 0-10 °C for 2 hours. GC/MS analysis indicated that the reaction was complete after 1 hour. The mixture was diluted with dichloromethane (200 mL) and washed with saturated aqueous sodium bicarbonate solution (150 mL). The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure to give XXXIV (6.20 g, quantitative yield) as a yellow oil that was used without purification.

2-Bromo-4-(tert-butyl)-6-(1-((methoxycarbonyl)oxy)-2-methylpropan-2-yl)phenyl methyl carbonate (XXXV) – A solution of XXXIV (6.16 g, 20.5 mmol, 1 equiv), triethylamine (8.00 mL, 61.5 mmol, 3 equiv) and DMAP (250 mg, 2.0 mmol, 0.1 equiv) in dichloromethane (150 mL) was cooled to 0 °C followed by the drop-wise addition of methyl chloroformate (3.48 mL, 45.0 mmol, 2.2 equiv) over 5 minutes. The reaction mixture was stirred at 0-10 °C for 2 hours at which time GC analysis indicated the reaction was complete. The reaction mixture was diluted with dichloromethane (100 mL), water (100 mL) was added and the mixture was stirred for 20 minutes. The layers were separated and the organic layer was washed with 1 N HCl (2 x 100 mL), water (100 mL), and brine (100 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified on an Analogix automated chromatography system, eluting with 0 to 40% ethyl acetate in heptanes, to give XXXV (6.12 g, 72% yield) as yellow oil.

2-Bromo-4-(tert-butyl)-6-(1-((methoxycarbonyl)oxy)-2-methylpropan-2-yl)-3-nitrophenyl methyl carbonate (XXXVI)– A solution of XXXV (6.12 g, 14.7 mmol, 1 equiv) in
dichloromethane (120 mL) was cooled to 0 °C and concentrated sulfuric acid (6.61 g, 67.5 mmol, 4.6 equiv) was added over 1 minute. The reaction mixture was stirred for 15 minutes at 0 °C followed by the drop-wise addition of nitric acid (1.85 g, 29.3 mmol, 2 equiv) over 5 minutes. The reaction mixture was stirred at 0 °C for 6 hours. Proton NMR analysis of reaction aliquots indicated that the reaction stopped at 60% conversion after approximately 3 hours. The reaction mixture was quenched by adding ice water (100 mL). Dichloromethane (200 mL) was added and the layers were separated. The organic layer was washed with saturated sodium bicarbonate (150 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The material was resubjected to the reaction conditions using the same amounts of acids as above. Proton NMR analysis after 3 hours showed that the reaction was complete. Ice water (100 mL) was added and the reaction was worked up as above to give XXXVI (6.17 g, 91% yield) as an orange oil that was used without purification.

**5-Amino-4-(tert-butyl)-2-(1-((methoxycarbonyl)oxy)-2-methylpropan-2-yl)phenyl methyl carbonate (XXXVII)** – A 500-mL Parr shaker bottle was charged with 10% Pd/C (1.54 g), ethanol (200 mL), XXXVI (6.16 g, 13.3 mmol, 1 equiv), and N,N-diisopropylethylamine (2.55 mL, 14.7 mmol, 1.1 equiv). The reaction was shaken at 50 psi H₂ overnight. The reaction mixture was filtered through a pad of Celite (washing with ethanol). The filtrate was partially concentrated under reduced pressure to a volume of approximately 30 mL. The remaining solution was cooled to 0 °C and water (60 mL) was added drop-wise. The mixture was extracted with dichloromethane and the organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude product was purified on an Analogix
automated chromatography system, eluting with a gradient of 0 to 15% ethyl acetate in heptanes, to give XXXVII (2.48 g, 53% yield) as an off-white solid.

4-(tert-Butyl)-2-(1-((methoxycarbonyl)oxy)-2-methylpropan-2-yl)-5-(4-oxo-1,4-dihydroquinoline-3-carboxamido)phenyl methyl carbonate (XXXVIII) – A 50% solution of propylphosphonic anhydride in ethyl acetate (6.5 mL, 11.0 mmol, 2.5 equiv) and pyridine (0.70 mL, 8.8 mmol, 2 equiv) were added to a suspension of powdered VII (0.82 g, 4.4 mmol, 1 equiv) and XXXVII (1.55 g, 4.4 mmol, 1 equiv) in 2-methyltetrahydrofuran (35 mL). The reaction mixture was heated at 55 °C for 16 hours. The reaction mixture was cooled to room temperature and ethyl acetate (300 mL) was added. The mixture was washed with 10% sodium carbonate solution (1 x 300 mL, 1 x 200 mL) and brine (200 mL). The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure to give XXXVIII (1.80 g, 78% yield) as a tan foam that was used without purification.

N-(2-(tert-Butyl)-5-hydroxy-4-(1-hydroxy-2-methylpropan-2-yl)phenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide (M1) – 25% sodium methoxide in methanol (1.56 mL, 6.82 mmol, 2 equiv) was added to a solution of XXXVIII (1.79 g, 3.41 mmol, 1 equiv) in 2-methyltetrahydrofuran (50 mL). The reaction mixture was stirred at room temperature for 2 hours. LC/MS analysis indicated the reaction was complete after 1 hour. The mixture was cooled to 0 °C and 1 N HCl (60 mL) was added. The layers were separated and the aqueous layer was extracted with ethyl acetate (3 x 35 mL). The combined organic layers were washed with brine (30 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure to give a tan foam. The foam was dissolved in ethyl acetate (30 mL), and heptanes (300 mL) was added to afford a solid. The solid was filtered, washed with heptane (15 mL) and dichloromethane (5 mL) and dried in a vacuum oven at room temperature overnight to give M1 (1.05 g, 76% yield) as a white solid.
Supplemental Figure 13. M1 and M6 LC purity assessments

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Supplemental Figure 14. M1 and M6 MS identity assessments

M1 MS m/z: 409.2 [M+H]⁺
M6 MS m/z: 423.2 [M+H]⁺
Supplemental Figure 15. M1 and M6 $^1$H-NMR spectra
Supplemental Figure 16. Current Traces Showing Potentiator Responses of Ivacaftor and CTP-656 in A) G551D/F508del HBE and B) VX-809-corrected F508del/F508del HBE

Mean ± SD, N = 3 replicates. Z = Benzamil (1 µM), F = Forskolin (10 µM); P = Potentiator (300 nM); B = Bumetanide (20 µM). Data for potentiator addition at 300 nM are shown, which is the approximate EC50 for VX-770 and CTP-656. Figures are representative of all dose-response comparisons.