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

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

Concert Pharmaceuticals, Inc., Lexington, Massachusetts

L.P. and L.W. are former employees of Concert Pharmaceuticals, Inc.

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## **Running Title**

CTP-656: Deuterated Ivacaftor Analog with Differentiated PK

## **Corresponding Author**

Dr. Scott L. Harbeson

Concert Pharmaceuticals, Inc

99 Hayden Ave., Suite 500

Lexington, MA 02421

Phone: 781-674-5208, fax: 781-674-5308, e-mail: sharbeson@concertpharma.com

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## **List of Non-Standard Abbreviations**

APCI, atmospheric pressure chemical ionization; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator protein; CTP-656, N-(2-(tert-Butyl)-4-(tert-butyl-d<sup>9</sup>)-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide; CYP, cytochrome P450; DIE, deuterium isotope effect; FEV<sub>1</sub>, forced expiratory volume in 1 s; HBE, human bronchial epithelial, HLM, human liver microsomes; IS, internal standard; I<sub>sc</sub>, short circuit current; LC, liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MBI,

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mechanism-based inhibition; MRM, multiple reaction monitoring; MS, mass spectrometry; PK, pharmacokinetics; ppFEV<sub>1</sub>, percent predicted FEV<sub>1</sub>; RODI, reverse osmosis deionized

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### **Abstract**

Ivacaftor is currently used for the treatment of cystic fibrosis as both monotherapy (Kalydeco®) and combination therapy with lumacaftor (Orkambi®). Each therapy targets specific patient populations: Kalydeco treats patients carrying one of nine gating mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR), while 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 ( $d_9$ -ivacaftor) and  $d_{18}$ -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  $d_{18}$ -ivacaftor showed similar *in vitro* pharmacologic potency to ivacaftor, and the deuterated M1 and M6 metabolites showed equivalent pharmacology to the corresponding metabolites of ivacaftor, consistent with previous studies of deuterated compounds. However, CTP-656 exhibited markedly enhanced stability when tested *in vitro*. The deuterium isotope effects for CTP-656 metabolism ( $^D V = 3.8$ ,  $^D V/K = 2.2$ ) were notably large for a CYP-mediated oxidation. The pharmacokinetic (PK) profile of CTP-656 and  $d_{18}$ -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  $t_{1/2}$  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 potentially differentiated properties to existing drugs.

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## **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 non-radioactive isotopic tracing (Rittenberg and Schoenheimer, 1937; Baillie, 1981). Deuterated agents as pharmaceuticals are an area of growing interest as shown by the number of deuterated compounds entering clinical development (Halford, 2016). Additionally, the first deuterated compound, deutrabenazine (Austedo®), has recently been approved (Austedo, 2017). A key feature of this technology is that deuterium substitution generally has minimal impact on the structure, physical properties or pharmacology compared to the all-hydrogen version of a drug. However, deuterium substitution can have a marked effect upon the pharmacokinetic 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 6–9 fold, effects this large are rarely observed for enzyme-catalyzed reactions (Northrop, 1975; Jenks, 1987). The magnitude of the DIE for a cytochrome P450 (CYP)-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.

The first paper in this series presented the data for CTP-347, a deuterated analog of paroxetine (Paxil®), which is a selective serotonin reuptake inhibitor for the treatment of a number of psychological disorders (Paxil, 2012; Uttamsingh et al, 2015). Herein, we report the effects of selective deuterium substitution on another therapeutic agent, ivacaftor (Figure 1). Ivacaftor (Kalydeco) was approved in 2012 as the first drug to target specific mutational defects in the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Kalydeco, 2015). It is

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currently labeled for the treatment of cystic fibrosis (CF) patients 2 years and older who have at least one mutant allele from a group of 9 gating mutations in the CFTR gene, with G551D being the most common (4.4% of US CF patients) (Aditya, 2012; Kalydeco, 2015). In 2016, ivacaftor was approved as part of the fixed-dose combination drug Orkambi, which combines ivacaftor and lumacaftor for the treatment of CF patients 12 years and older homozygous for the F508del CFTR mutation (approximately 45% of the US CF patients) (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 (Figure 1) were prepared and characterized with respect to pharmacology, *in vitro* metabolism and *in vivo* pharmacokinetics (PK). Since ivacaftor is extensively metabolized in humans to two major circulating metabolites, M1 and M6, these metabolites and the corresponding deuterated metabolites  $d_8$ -M1 and  $d_6$ -M6 were also prepared and studied (Figure 2) (Zha et al., 2011). Two compounds --  $d_9$ -ivacaftor (CTP-656) and  $d_{18}$ -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

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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 due to its general trend of increased exposure versus  $d_{18}$ -ivacaftor. The observed increase in exposure and half-life compared to 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 potentially differentiated properties to existing drugs.

## Materials and Methods

### Reagents

Indiplon, CTP-656,  $d_{18}$ -ivacaftor, ivacaftor, M1, M6,  $d_8$ -M1 and  $d_6$ -M6 were provided by Concert Pharmaceuticals, Inc. (Lexington, MA). Human liver microsomes (mixed gender, pool of 200, 20 mg/mL) were from Xenotech, LLC (Lenexa, KS). Cryopreserved human hepatocytes were purchased from In Vitro ADMET Laboratories (IVAL, LLC), (Malden, MA). Human cDNA expressed CYP3A4 Supersomes<sup>TM</sup> were obtained from BD Biosciences (Woburn, MA). Dimethylsulfoxide (DMSO), acetonitrile,  $MgCl_2$  and NADPH were from Sigma-Aldrich (St. Louis, MO) and PEG400 was from J.T. Baker (Center Valley, PA). CF-HBE cells were obtained from 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, 3333 Green Bay Road, North Chicago, IL 60064.

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### **Synthetic Procedures for the Synthesis of CTP-656, $d_{18}$ -Ivacaftor, $d_8$ -M1, $d_6$ -M6, M1 and M6**

The synthetic methods for the preparation of CTP-656 and  $d_{18}$ -ivacaftor have been previously described (Morgan, 2015). Analytical characterization of CTP-656 can be found in the Supplemental Materials. These include liquid chromatography (LC) purity assessment (Supplemental Figure 1); mass spectrum (Supplemental Figure 2) and the  $^1\text{H-NMR}$  spectrum (Supplemental Figure 3). Analytical data for  $d_{18}$ -ivacaftor in the Supplemental Materials include LC purity assessment (Supplemental Figure 4); mass spectrum (Supplemental Figure 5) and the  $^1\text{H-NMR}$  spectrum (Supplemental Fig. 6). Synthetic routes and methods for the preparation of CTP-656 metabolites  $d_8$ -M1 and  $d_6$ -M6 are also in the Supplemental Materials (Supplemental Figures 7 and 8, respectively). Analytical data for  $d_8$ -M1 and  $d_6$ -M6 in the Supplemental Material include LC purity assessments (Supplemental Figure 9), mass spectra (Supplemental Figure 10), and  $^1\text{H-NMR}$  spectra (Supplemental Figure 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 Figure 12) and methods for the preparation of M1 can be found in the Supplemental Materials. 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 Figure 13), mass spectra (Supplemental Figure 14), and  $^1\text{H-NMR}$  spectra (Supplemental Figure 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 (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 prepared sample



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was injected onto an HPLC-tandem quadrupole mass spectrometer (LC-MS/MS) consisting of an Agilent 1200 HPLC with a Leap auto-sampler (Leap HTS-PAL) equipped with a C6 Gemini phenyl 2.1x50 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 while the composition of mobile phase A was 0.1% formic acid in reverse osmosis deionized (RODI) water.

LC-MS/MS analysis was performed using atmospheric pressure chemical ionization (APCI), followed by multiple reaction monitoring (MRM) of each analyte including the internal standard. 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  $d_{18}$ -ivacaftor, 417.2/172.2 m/z for  $d_8$ -M1 and 409.2/172.2 m/z for M1, while the transition for indiplon (the IS) was 377.2/293.2 m/z. For all analytes, a dwell time of 100 ms was used.

*Clinical GLP Method.* The determination of CTP-656 and  $d_{18}$ -ivacaftor in plasma was performed using a validated method under GLP by CPR Pharma Services (28 Dalgleish Street, Thebarton SA 5031, Australia) conducted in compliance with CPR Pharma Standard Operation Procedures. Plasma analysis was performed following protein precipitation with 99% acetonitrile and 1% formic acid using ivacaftor as the internal standard. The analytes were separated by HPLC using a C18 column (Phenomenex; Luna C18, 50 x2 mm, 3 $\mu$ m), where mobile phase A consisted of 10% acetonitrile and 90% water with 0.1 % formic acid and mobile phase B consisted of 50% acetonitrile, 50% methanol with 0.1% formic acid. Elution of the analytes from the column was performed at a flowrate of 0.3 mL/min using a gradient where the percent of mobile phase B was increased linearly from 55% to 100% over 2.5 minutes and held at 100% for 1 minute, after which the mobile phase composition was set to 10% B for 0.4 minutes and

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then returned to initial conditions (55% B) for re-equilibration. Total run time for the method was 6 minutes.

The analysis of the eluates was performed using an API4000 (PE Sciex) mass spectrometer in positive multiple reaction monitoring (MRM) mode. The MRM transitions monitored were 402.2/172.2 m/z for CTP-656 and 411.2/172.2 m/z for  $d_{18}$ -ivacaftor. Ivacaftor was the internal standard for both CTP-656 and  $d_{18}$ -ivacaftor. For all analytes, the method was linear and in all cases the precision and accuracy met FDA & ICH criteria.

### ***In Vitro* Pharmacology Assessments**

Chloride conductance was evaluated with an equivalent current ( $I_{eq}$ ) assay system using fully differentiated primary cultures of CF human bronchial epithelial (HBE) cells grown at an air liquid interface for four to six weeks. Transepithelial voltage ( $V_T$ ) and conductance ( $G_T$ ) were measured under current clamp conditions using a custom-designed 24-channel current clamp (TECC-24) and a 24-well electrode manifold. For all experiments, cells were tested in symmetric chloride solution (HEPES buffered Coon's F12 media without serum or bicarbonate) at  $36 \pm 0.5$  °C, and all compounds and test articles were dissolved in DMSO. For chloride conductance measurements, epithelial sodium channels were blocked with 3  $\mu$ M benzamil; potentiator responses were measured following 10  $\mu$ M forskolin addition. CFTR specificity was determined by blocking chloride secretion with 20  $\mu$ M bumetanide. Potentiator responses were quantified by calculating the total peak area (AUC) of  $I_{eq}$  following addition of test article. For studies using corrector, lumacaftor was added at 3  $\mu$ M 24 hr 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  $EC_{50}$  values.

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### Metabolic Stability in CYP3A4 Supersomes

Stock solutions (10 mM) of CTP-656, *d*<sub>18</sub>-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 MgCl<sub>2</sub>. Metabolic stability in the diluted Supersomes was assessed as previously described (Morgan, 2015).

**Semi-Quantitative Metabolite Profile in Human Hepatocytes** Stock solutions (5 mM) of CTP-656, *d*<sub>18</sub>-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 resuspended in Krebs-Henseleit Buffer (KHB). The test articles were added to the hepatocytes in KHB buffer to achieve a final concentration of 25 μM in 0.5 mL, containing 4 x 10<sup>6</sup> cells/mL hepatocytes. The reaction mixtures were incubated at 37°C in a cell culture incubator (5% CO<sub>2</sub>). Aliquots of the mixture were removed at 0 and 4 hours and acetonitrile was added. Samples were transferred micro-centrifuge tubes and centrifuged at 10,000 g for 8 minutes to pellet precipitated proteins. Supernatants were analyzed for amounts of parent remaining and metabolites formed by LC-MS/MS. UV/Vis @ 254nm was used for semi-quantitative analysis, wherein metabolites were measured as percentage of parent peak area. Analysis was by UV/Vis since metabolite standards had not been synthesized at the time of this experiment.

### Determination of Deuterium Isotope Effect for CTP-656 in Human Liver Microsomes

Stock solutions (50 mM) of test compounds were prepared in DMSO. The 20 mg/mL human liver microsomes (HLM) were diluted to 0.625 mg/mL in 0.1 M potassium phosphate buffer, pH 7.4, containing 3 mM MgCl<sub>2</sub>. Aliquots (375 μL) of the HLM and test compound mixtures were transferred to wells of a deep 96-well plate in triplicate and pre-warmed for 7 minutes. Reactions were initiated by addition of 125 μL of pre-warmed NADPH solution. The final reaction volume

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was 0.5 mL and contained 0.5 mg/mL HLM, and 1, 2, 5, 10, 20, 40, 60, 80 and 100  $\mu$ M of ivacaftor or CTP-656, 2 mM NADPH and 3 mM  $MgCl_2$ . The reaction mixtures were incubated at 37°C for 10 minutes after which 50  $\mu$ L aliquots were removed and added to shallow 96-well plates. The reactions were stopped by addition of 50  $\mu$ L of ice-cold acetonitrile with internal standard. The plates were stored at 4°C for 20 minutes, after which 100  $\mu$ L 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 Applied Bio-systems API 4000 mass spectrometer.

The kinetic parameters,  $K_m$  and  $V_{max}$ , were calculated from the Michaelis-Menten equation. The enzyme velocity (enzyme velocity=concentration of hydroxyl methyl/time/protein concentration) from three separate experiments was input into GraphPad Prism 6.04 and reported values for  $K_m$  and  $V_{max}$  were determined from the fit using the average value.

### Preclinical Pharmacokinetic 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 U.S. National Institutes of Health, and were approved by the Institution's Animal Care and use Committee or local equivalent.

*Pharmacokinetics of CTP-656,  $d_{18}$ -Ivacaftor and Ivacaftor in Rats.* Male, Sprague-Dawley rats (234-276 g) were obtained from Harlan Labs (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 was assessed as previously described (Morgan, 2015). The plasma PK parameters for the dosed compounds were calculated by non-compartmental analysis using Phoenix 64 WinNonlin software.

*Pharmacokinetics of CTP-656,  $d_{18}$ -Ivacaftor and Ivacaftor in Dogs.* Four male beagle dogs were housed at Agilux Labs (Worcester, MA) where the studies were conducted. The dogs were

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administered 3 mg/kg of each test compound orally, in a discrete-dose crossover study with one week wash out period between the dosing phases. All dogs were fasted overnight before 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 1, 2, 4, 6, 8, 16, 24, 48, 72, 84 and 96 hours post-dose. 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 non-compartmental analysis using Phoenix 64 WinNonlin software.

### **Evaluation of Pharmacokinetic 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 pharmacokinetics of CTP-656 and  $d_{18}$ -ivacaftor amorphous dispersion aqueous suspension in healthy volunteers, with a pharmacokinetic comparison to ivacaftor. This manuscript will report on the first part of this clinical study, which was a single-dose, randomized, 2-period, 2-treatment, crossover study conducted in healthy male and female volunteers to evaluate the safety, tolerability and pharmacokinetics 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, Australia) under the Clinical Trial Notification (CTN) scheme through Therapeutic Goods Administration. The study was conducted in accordance with the Declaration of Helsinki, e.g., the clinical study protocol, amendments, informed consent document(s), and any other study-related documents were reviewed and approved by the Bellberry Human Research Ethics Committee, and informed consent was obtained prior to any study-related procedures.

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Six healthy volunteers were enrolled in this part of the study. On Day 1, 3 subjects received a single oral dose of 25 mg CTP-656 and 3 subjects received a single oral dose of 25 mg  $d_{18}$ -ivacaftor as an aqueous suspension. On Day 8 following the first dose, subjects crossed over and the dosing was repeated. A total of 21 PK blood samples (4 mL each) were collected for assessment of plasma concentrations of CTP-656 and  $d_{18}$ -ivacaftor. Time points for collections were immediately prior to dosing (0) 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 post-dose. 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 non-compartmental analysis using Phoenix 64 WinNonlin software. Each subject was monitored for safety throughout the study, including vital signs, physical examinations, clinical laboratory measurements and 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,  $d_8$ -M1 and  $d_6$ -M6, were compared to 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 1/6<sup>th</sup> the potency and M6 less than 1/50<sup>th</sup> the potency of ivacaftor (Kalydeco, 2015). In G551D/F508del HBE cells, ivacaftor and CTP-656 had similar potencies for potentiating forskolin responses, with EC<sub>50</sub> values of 336 nM and 255 nM, respectively (Table 1). The potencies of the M1 and  $d_8$ -M1 metabolites were also similar (0.96  $\mu$ M and 1.35  $\mu$ M, respectively), and both metabolites were less potent than the parent: 3-fold for M1 and 5-fold for  $d_8$ -M1. The EC<sub>50</sub> values for M6 and  $d_6$ -M6 were 11  $\mu$ M and 15  $\mu$ M, respectively, in agreement with prior reports that the M6 metabolite does not contribute to the therapeutic efficacy of ivacaftor in CF patients (Kalydeco, 2015).

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To compare the activities of CTP-656, ivacaftor, and 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 following a 24-hour incubation with lumacaftor. Similar to results obtained with G551D/F508del HBE, the potencies of non-deuterated and deuterated parent and metabolite compounds were very similar (Table 2).  $d_{18}$ -Ivacaftor was also tested in G551D-CFTR-expressing FRT cells and lumacaftor-corrected F508del/F508del HBE cells; no significant differences in potentiation activity or potency were observed between  $d_{18}$ -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 min (average of two experiments for the 3 compounds tested), which is a 47% increase in  $t_{1/2}$  for CTP-656 and  $d_{18}$ -ivacaftor versus ivacaftor (Figure 3A). The data from human hepatocytes showed an approximate 2-fold reduction of the M1 metabolites as percent 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  $K_m$  (concentration of substrate at  $\frac{1}{2} V_{max}$ ),  $V_{max}$  (enzyme velocity at saturating substrate:  $[S] \gg K_m$ ) and  $V_{max}/K_m$

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(enzyme velocity at low substrate concentrations:  $[S] \ll K_m$ ). The ratios of the kinetic parameters  $V_{max}$  and  $V_{max}/K_m$  for ivacaftor versus CTP-656 measure the deuterium kinetic isotope effects  $^{D}V$  and  $^{D}V/K$  for the CYP3A4-catalyzed formation of M1 in HLM (Northrop, 1975). Significant deuterium isotope effects were measured for both kinetic parameters:  $^{D}V = 3.8$  and  $^{D}V/K = 2.2$  (Figure 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 HLM supported progression of the compounds to *in vivo* pharmacokinetic studies in rats and dogs.

### Deuterated Ivacaftor Analogs Show Differentiated Non-clinical Pharmacokinetics Upon Oral Dosing

The PK profiles of the 3 compounds were initially assessed in male Sprague-Dawley rats. Each compound was dosed at 10 mg/kg to each of 3 rats, with a total of 9 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 Figure 4A (only the 0–24 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 3 compounds (Table 4). Across the parameters  $C_{max}$ ,  $AUC_{0-24\text{ hr}}$ ,  $C_{24\text{hr}}$  and  $t_{1/2}$ ,  $d_{18}$ -ivacaftor showed a trend to larger PK parameters in rats compared to both CTP-656 and ivacaftor.

The PK profiles of the 3 compounds were also studied in male beagle dogs. Each compound was dosed by oral gavage at 3 mg/kg to each of 4 dogs in a cross-over fashion with a 1 week washout between doses, and the plasma levels of compound were measured over 96 hours. As shown by the plasma concentration versus time profiles for each compound (Figure 4B), CTP-656 had much greater exposure than ivacaftor and appeared to exhibit greater exposure than



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*d*<sub>18</sub>-ivacaftor (only the 0–24 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*<sub>max</sub>, *AUC*<sub>0–24 hr</sub> and *C*<sub>24hr</sub> show 62%, 69% and 83% increases vs. ivacaftor, respectively.

Although the PK data for CTP-656 and *d*<sub>18</sub>-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 non-clinical test species (mouse, rat and dog) (Center for Drug Evaluation and Research, 2012a). 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*<sub>18</sub>-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*<sub>18</sub>-ivacaftor was designed as a 2-part cross-over 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 (4M and 2F) were administered study drug on Day 1: three received CTP-656 and three received *d*<sub>18</sub>-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 (Figure 5).

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The PK parameters reflect the general trend shown in the exposure curves in that  $C_{\max}$  and  $AUC_{0-\infty}$  are greater for CTP-656 than for  $d_{18}$ -ivacaftor. The approximate 16-hour half-life of CTP-656 is also compatible with identifying a dose that would enable once-daily dosing. PK data for a 25mg dose of ivacaftor have been previously reported as part of a single-ascending dose study (Center for Drug Evaluation and Research, 2012b). For comparative purposes, the half-life for ivacaftor was 11.1 hr and the exposure ( $AUC_{0-\infty}$ ) was 1627 ng/mL-hr. These data for ivacaftor suggest that at the same dose, CTP-656 may have greater exposure and a longer half-life versus ivacaftor in humans. In subsequent clinical studies, CTP-656 as a solid oral 150 mg dose was compared directly to a single dose of ivacaftor (150 mg tablet) and was shown to have an approximate 15-hour half-life (Uttamsingh et al., 2016).

### Discussion

This study reports the results of precision deuteration of ivacaftor (Kalydeco), which is the first approved CFTR modulator for the treatment of cystic fibrosis. Two deuterated analogs,  $d_9$ -ivacaftor (CTP-656) and  $d_{18}$ -ivacaftor were tested *in vitro* and *in vivo* to assess the pharmacology, pharmacokinetics and metabolism of the compounds with respect to ivacaftor. The studies of the deuterated ivacaftor analogs were designed to assess whether a deuterium isotope effect 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 paper in this series that assessed the effects of precision deuteration on paroxetine pharmacology and pharmacokinetics (Uttamsingh et al., 2015). Deuterated paroxetine (CTP-347) retained the intrinsic *in vitro* pharmacology profile of paroxetine with greatly reduced mechanism-based inactivation of CYP2D6 in *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 non-hormonal therapy for hot flashes was stopped for commercial reasons.

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To confirm that deuterium substitution did not affect the intrinsic pharmacology of ivacaftor, *in vitro* studies were performed in HBE derived from CF patients. The potency as a potentiator of CFTR with a gating mutation was measured in G551D/F508del HBE. For homozygous F508del HBE, the cells were first corrected with lumacaftor prior to measuring the potentiation of conductance. Ivacaftor is extensively metabolized in humans to two major circulating 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 for M1 and M6, respectively (Center for Drug Evaluation and Research, 2012b). These metabolites of ivacaftor and CTP-656 were, therefore, also evaluated in the HBE 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 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 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, non-specific 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 Supersomes 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.

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Once the respective M1 and  $d_8$ -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 loss of parent, a direct measurement of metabolite formation in HLM was possible and enabled calculation of the DIE using classical Michaelis-Menten kinetics. The measured  $^D V/K$  and  $^D V$  values for CYP3A4 catalyzed metabolism in HLM were large.

Although a  $^D V/K$  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 vs. 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  $^D V/K$  isotope effect for CTP-656 in the absence of metabolic switching.

A recent review on the potential issues for deuterium in drug development notes that an isotope effect *in vitro* needs to translate to a PK effect *in vivo* to have a practical application to drug development (Guengerich, 2013). Although slower metabolism was observed *in vitro*, the translation to *in vivo* is unpredictable since non-CYP clearance mechanisms may predominate *in vivo* and clearance mechanisms may vary between species. PK studies in rats and dogs were particularly challenging since neither rat nor dog metabolize ivacaftor as extensively as humans; therefore, these preclinical species may not be adequate models for human

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metabolism (Center for Drug Evaluation and Research, 2012a). The PK results in rats and dogs for CTP-656 were encouraging with a longer half-life and greater exposure vs. ivacaftor in both species.  $d_{18}$ -Ivacaftor, however, showed increased exposure versus ivacaftor in rats, but not 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  $C_{max}$ ,  $t_{1/2}$ ,  $AUC_{0-inf}$ ,  $C_{24hr}$  and CL/F. These data, coupled with the reduced amount of deuterium in CTP-656, were sufficient to identify CTP-656 for continued clinical development.

The potential of CTP-656 to be dosed once-daily was predicted based on available information for ivacaftor including single-ascending dose (SAD) PK in healthy volunteers and PK/PD relationships in patients (Center for Drug Evaluation and Research, 2012b). In the SAD study of ivacaftor, which was conducted in the fasted condition, exposure increases were dose proportional from 25mg to 375mg. Additionally, the 150mg twice-daily dose of ivacaftor was selected based on simulations that showed a plasma  $C_{min}$  at steady-state of ~250 ng/mL achieved the predicted  $EC_{90}$  for  $FEV_1$  and the  $EC_{84}$  for sweat chloride. The 25mg dose of CTP-656 in the fasted state provided a  $C_{24hr}$  ( $C_{min}$ ) of 53 ng/mL in plasma. Assuming dose proportional increases in exposure for CTP-656, a once-daily dose of approximately 118mg in the fasted condition would achieve the target  $C_{24hr}$  of 250 ng/mL even in the absence of accumulation on multiple-dosing. Ivacaftor shows an increase in exposure of 2-3 fold when dosed with food; therefore, CTP-656 has the potential to exceed the 250 ng/mL  $C_{min}$  at steady-

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state if a similar food effect is observed. Based on this analysis of the 25mg dose, CTP-656 was projected to achieve the desired once-daily profile in future studies, which was confirmed in subsequent Phase 1 studies.

Once-daily dosing may translate to improved adherence in real-world use. A small clinical study assessed ivacaftor adherence in patients and found the adherence to be highly variable (Siracusa et al., 2015). The ivacaftor label (Kalydeco, 2015) specifies dosing every 12 hours; however, this study found the median duration between doses was 16.9 hours, with a range of 12.1 to 43.7 hours. The overall adherence rate—calculated as (total doses taken)/(total days monitored x 2 doses/day)—was only 61%. It is also noteworthy that the 10.4% improvement in absolute ppFEV<sub>1</sub> observed in the clinical study (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 1 studies in healthy volunteers, including single-ascending dose (SAD), multiple-ascending dose (MAD) and food effect studies. The tablet formulation was reported to have an average half-life of 15 hours after a single oral dose and an average half-life of 18 hours in the MAD studies (Uttamsingh et al., 2016). Of particular note is the reported CTP-656 to *d*<sub>6</sub>-M1 AUC ratio of 1.5 to 1 after a single dose and also at steady state. This is much greater than ivacaftor, which has a parent to M1 AUC ratio of 1 to 5 at steady state. With a longer half-life 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 2 safety and efficacy study in CF patients is currently underway (NCT02971839).

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### **Authorship Contributions**

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

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

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

Performed data analysis: Aslanian, Brummel, Nguyen, Uttamsingh

Wrote or contributed to the writing of the manuscript: Aslanian, Braman, Brummel, Harbeson, Liu, Tung, Uttamsingh



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### **Footnotes**

- \* Research supported by Concert Pharmaceuticals, Inc., Lexington, Massachusetts.

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## Figure Legends

**Figure 1.** Structures of CTP-656 ( $d_8$ -ivacaftor),  $d_{18}$ -ivacaftor and ivacaftor. D = deuterium atom.

**Figure 2.** CTP-656 and ivacaftor are metabolized in humans by CYP3A to two major metabolites.

**Figure 3.** *In Vitro* metabolism. (A)  $t_{1/2}$  values of ivacaftor and  $d$ -ivacaftor analogs in human CYP3A4 supersomes. (B) Deuterium kinetic isotope effect as measured by rate of formation of M1 and  $d_8$ -M1 metabolites in human liver microsomes.

**Figure 4.** *In Vivo* metabolism. (A) Exposure profiles in male Sprague-Dawley rats (n=3/group) following oral dosing of CTP-656,  $d_{18}$ -ivacaftor and ivacaftor. (B) Exposure profiles in male Beagle dogs (n=4) following oral dosing of CTP-656,  $d_{18}$ -ivacaftor and ivacaftor in a crossover study.

**Figure 5.** Exposure profiles in healthy volunteers (n=6) following oral dosing of CTP-656 and  $d_{18}$ -ivacaftor.

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## **Tables**

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**Table 1.** Potency of CFTR Potentiation in G551D/F508del HBE Cells by Ivacaftor and Deuterated Ivacaftor Analogs.

| <b>Compound</b>  | <b>EC<sub>50</sub>, nM (95% CI)<sup>a</sup></b> | <b>Compound</b>         | <b>EC<sub>50</sub>, nM (95% CI)<sup>a</sup></b> |
|------------------|---|-------------------------|---|
| <b>Ivacaftor</b> | 336 (295-382)                                   | <b>CTP-656</b>          | 255 (220-296)                                   |
| <b>M1</b>        | 960 (756-1,220)                                 | <b>d<sub>8</sub>-M1</b> | 1,346 (1,040-1,743)                             |
| <b>M6</b>        | 11,320 (8,906-14,380)                           | <b>d<sub>6</sub>-M6</b> | 15,140 (10,390-22,050)                          |

Current traces of representative data are shown in Supplemental Figure 16

a. Calculated using AUC potentiator  $I_{eq}$  ( $\mu\text{A}/\text{cm}^2$ ) following forskolin addition,  $n=3$ .



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**Table 2.** Potency of CFTR Potentiation in Lumacaftor-Corrected F508del/F508del HBE Cells by Ivacaftor and Deuterated Ivacaftor Analogs

| <b>Compound</b>  | <b>EC<sub>50</sub>, nM (95% CI)<sup>a</sup></b> | <b>Compound</b>         | <b>EC<sub>50</sub>, nM (95% CI)<sup>a</sup></b> |
|------------------|---|-------------------------|---|
| <b>Ivacaftor</b> | 387 (275-545)                                   | <b>CTP-656</b>          | 295 (151-575)                                   |
| <b>M1</b>        | 390 (259-588)                                   | <b>d<sub>8</sub>-M1</b> | 286 (164-500)                                   |
| <b>M6</b>        | 2,410 (927-6,269)                               | <b>d<sub>6</sub>-M6</b> | 3,048 (undetermined)                            |

Current traces of representative data are shown in Supplemental Figure 16

a. Calculated using AUC potentiator I<sub>eq</sub> (μA/cm<sup>2</sup>) following forskolin addition, n=3.

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**Table 3.** Semi-quantitative Analysis of M1 Metabolites vs. Parent Following Incubation of Ivacaftor, CTP-656 and  $d_{18}$ -Ivacaftor in Human Hepatocytes.

| Compound            | Parent Area (mAU) | M1 Area (mAU) | M1 Formed as % of Parent |
|---------------------|-------------------|---------------|--------------------------|
| Ivacaftor           | 3.32              | 0.749         | 23                       |
| CTP-656             | 3.09              | 0.365         | 12                       |
| $d_{18}$ -ivacaftor | 3.28              | 0.298         | 9.1                      |

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**Table 4.** Mean Pharmacokinetic Parameters (%CV) for CTP-656, *d*<sub>18</sub>-Ivacaftor and Ivacaftor in Male Sprague-Dawley Rats (10 mg/kg) and Male Beagle Dogs (3.0 mg/kg) Following Oral Administration.

| <b>PK Parameters in Male Sprague-Dawley Rats</b> |                                    |   |                                      |                                 |
|--|------------------------------------|---|--------------------------------------|---------------------------------|
| <b>Compound</b>                                  | <b>C<sub>max</sub><br/>(ng/mL)</b> | <b>AUC<sub>0-24 hr</sub><br/>(hr*ng/mL)</b> | <b>C<sub>24 hr</sub><br/>(ng/mL)</b> | <b>t<sub>1/2</sub><br/>(hr)</b> |
| Ivacaftor  | 1913 (7%)                          | 22,177 (24%)                                | 346 (54%)                            | 10.5 (17%)                      |
| CTP-656  | 1970 (15%)                         | 24,260 (17%)                                | 413 (19%)                            | 13.2 (9%)                       |
| <i>d</i> <sub>18</sub> -Ivacaftor                | 2460 (28%)                         | 31,556 (20%)                                | 623 (24%)                            | 14.8 (9%)                       |

| <b>PK Parameters in Male Beagle Dogs</b> |                                    |   |                                      |                                 |
|--|------------------------------------|---|--------------------------------------|---------------------------------|
| <b>Compound</b>                          | <b>C<sub>max</sub><br/>(ng/mL)</b> | <b>AUC<sub>0-24 hr</sub><br/>(hr*ng/mL)</b> | <b>C<sub>24 hr</sub><br/>(ng/mL)</b> | <b>t<sub>1/2</sub><br/>(hr)</b> |
| Ivacaftor                                | 2255 (33%)                         | 29,448 (29%)                                | 774 (30%)                            | 17.6 (6.9%)                     |
| CTP-656                                  | 3463 (9%)                          | 49,782 (15%)                                | 1418 (31%)                           | 22.8 (62%)                      |
| <i>d</i> <sub>18</sub> -Ivacaftor        | 3030 (38%)                         | 40,019 (33%)                                | 1089 (43%)                           | 18.0 (26%)                      |

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**Table 5.** Subject Demographics

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|                                     |                  |
|-------------------------------------|------------------|
| N                                   | 6                |
| Sex                                 |                  |
| Male, n (%)                         | 4 (67%)          |
| Female, n (%)                       | 2 (33%)          |
| Race, n (%)                         |                  |
| Caucasian                           | 4 (67%)          |
| Asian                               | 1 (17%)          |
| Aboriginal/Torres Strait Islander   | 1 (17%)          |
| Age, mean year (range)              | 23.0 (18–31)     |
| Weight, mean kg (range)             | 74.4 (62.8–91.4) |
| BMI, mean kg/m <sup>2</sup> (range) | 23.9 (18.8–27.0) |

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BMI, body mass index.

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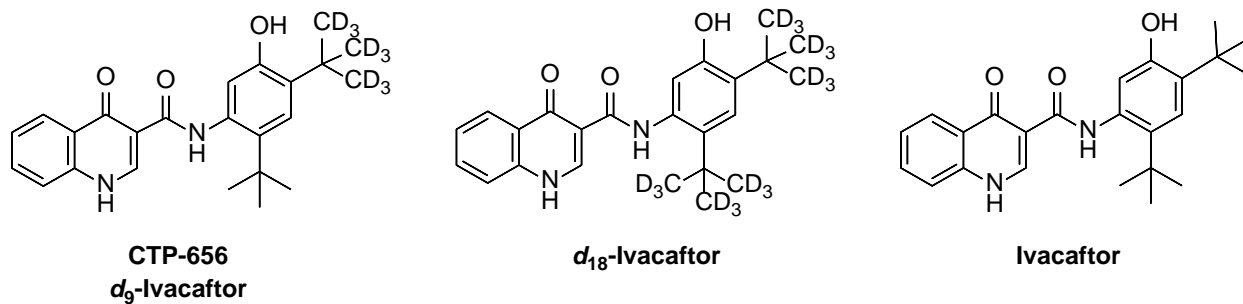
**Table 6.** Mean Pharmacokinetic Parameters (CV%) for CTP-656 and *d*<sub>18</sub>-Ivacaftor in Healthy Males and Females Following Oral Administration of a Single 25mg Dose.

| <b>Compound</b>                   | <b>C<sub>max</sub><br/>(ng/mL)</b> | <b>t<sub>1/2</sub><br/>(hr)</b> | <b>AUC<sub>0-inf</sub><br/>(ng/mL-hr)</b> | <b>C<sub>24hr</sub><br/>(ng/mL)</b> | <b>CL/F<br/>(L/hr)</b> |
|-----------------------------------|------------------------------------|---------------------------------|---|-------------------------------------|------------------------|
| CTP-656                           | 270.5 (24%)                        | 15.9 (18%)                      | 3812 (26%)                                | 52.6 (28%)                          | 6.92 (25%)             |
| <i>d</i> <sub>18</sub> -Ivacaftor | 232.7 (18%)                        | 16.4 (16%)                      | 3196 (15%)                                | 42.3 (16%)                          | 7.97 (15%)             |

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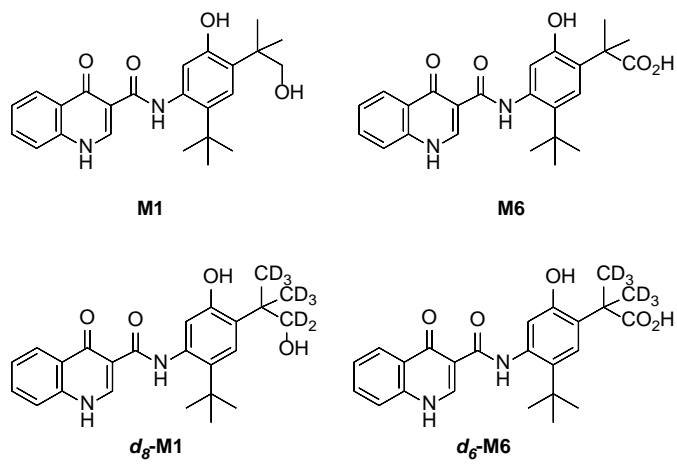
## Figures

Figure 1.



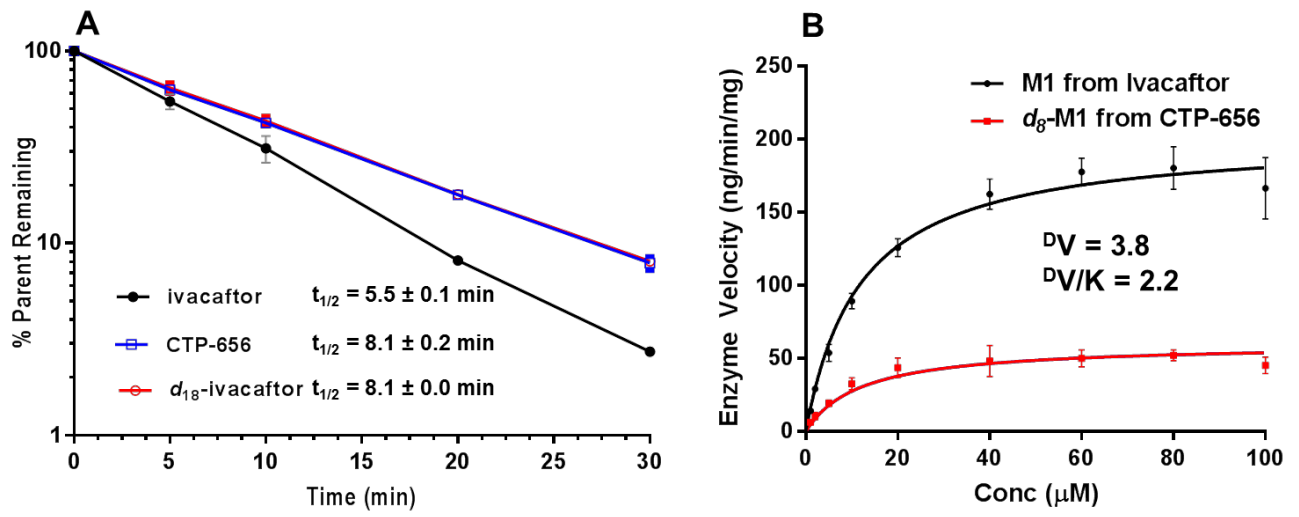
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Figure 2.



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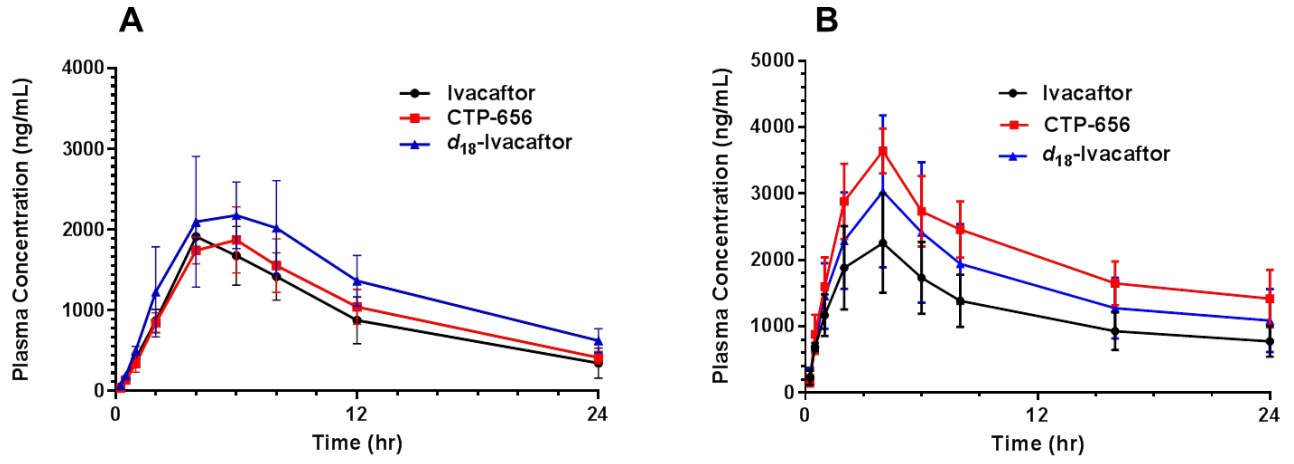
Figure 3.





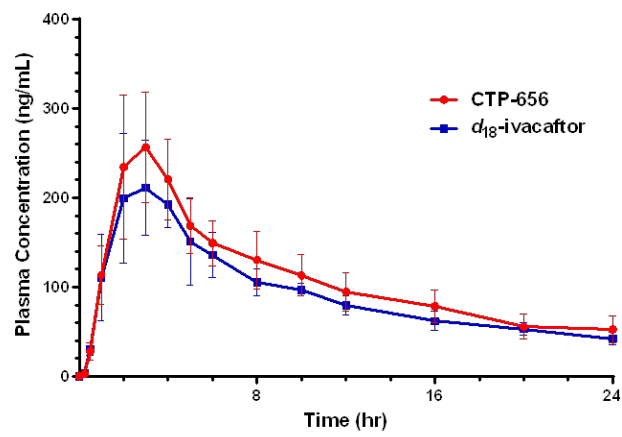
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Figure 4.



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Figure 5.



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

**Authors**

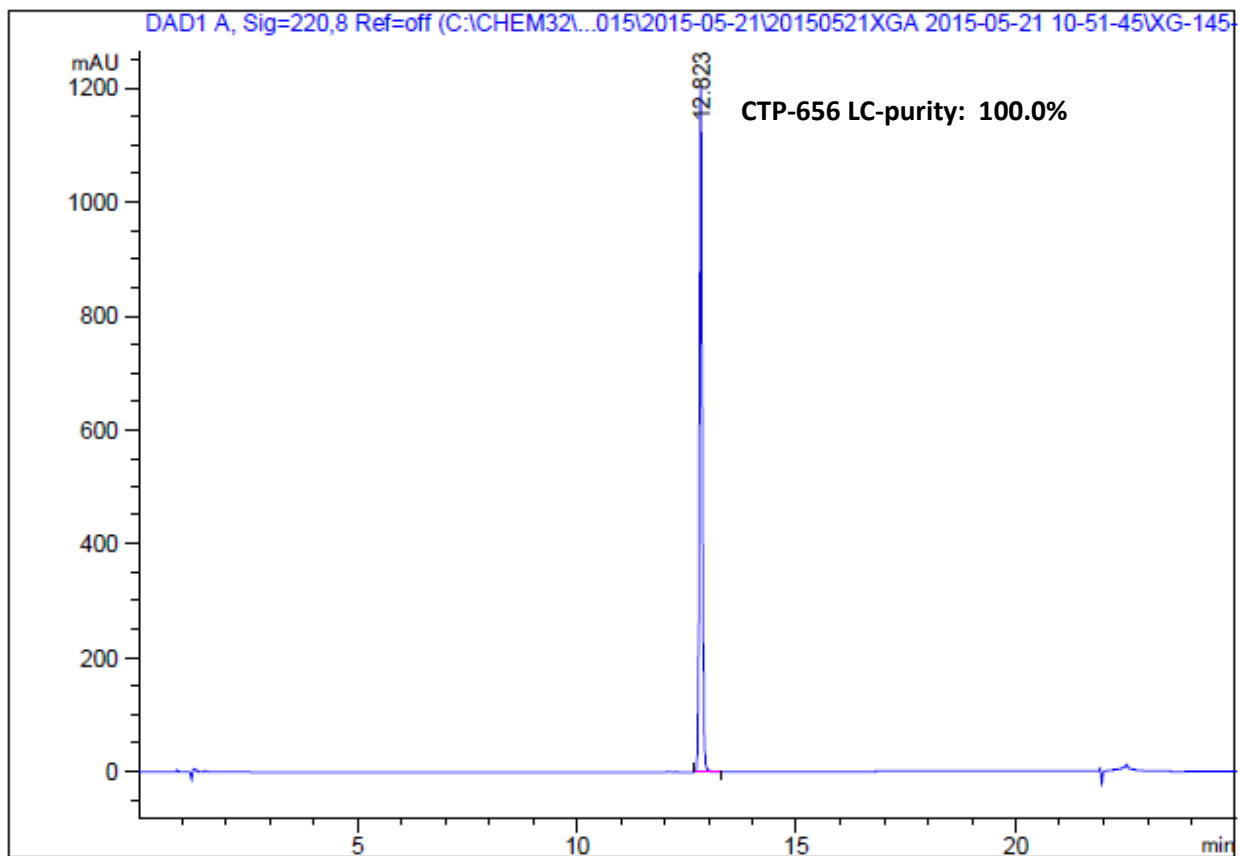
Scott L. Harbeson, Adam J. Morgan, Julie F. Liu, Ara M. Aslanian, Sophia Nguyen, Gary W.

Bridson, Christopher L. Brummel, Lijun Wu, Roger D. Tung, Lana Pilja, Virginia Braman, Vinita Uttamsingh

**Journal of Pharmacology and Experimental Therapeutics**

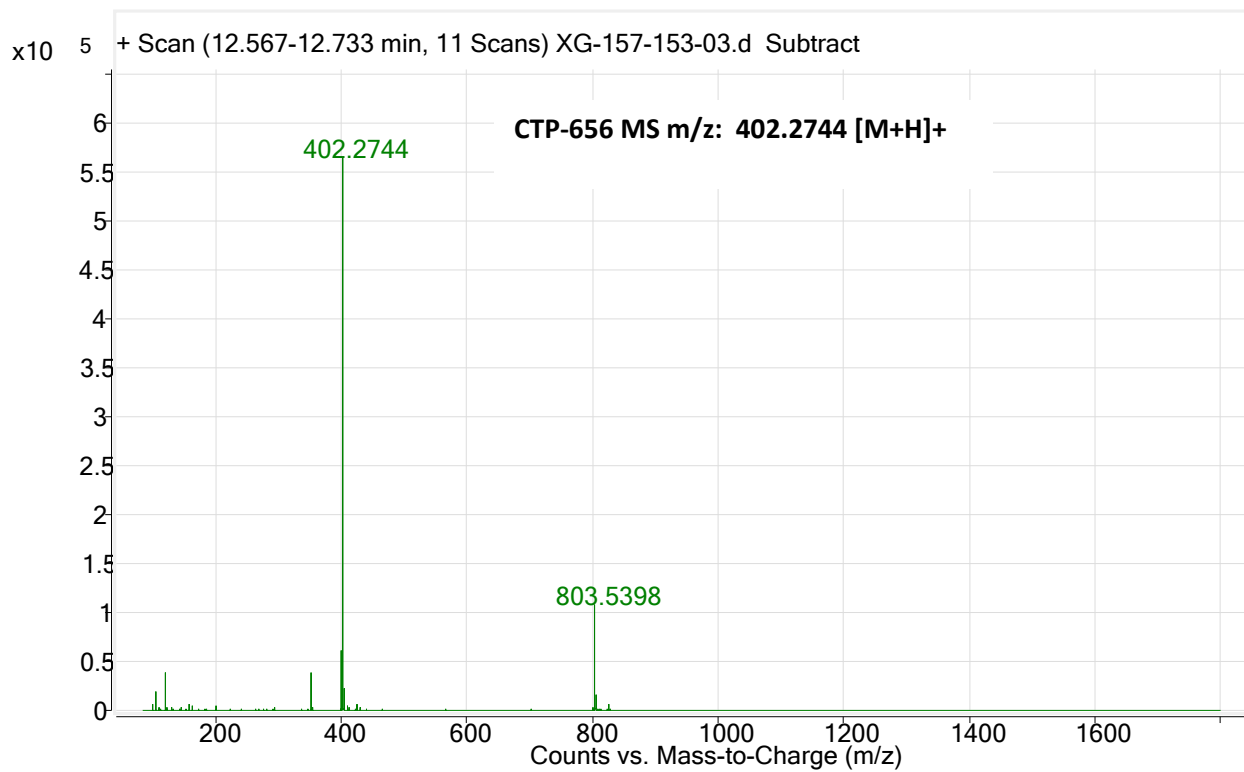
**SUPPLEMENTAL DATA SECTION**

**Supplemental Figure 1.** CTP-656 LC purity assessment



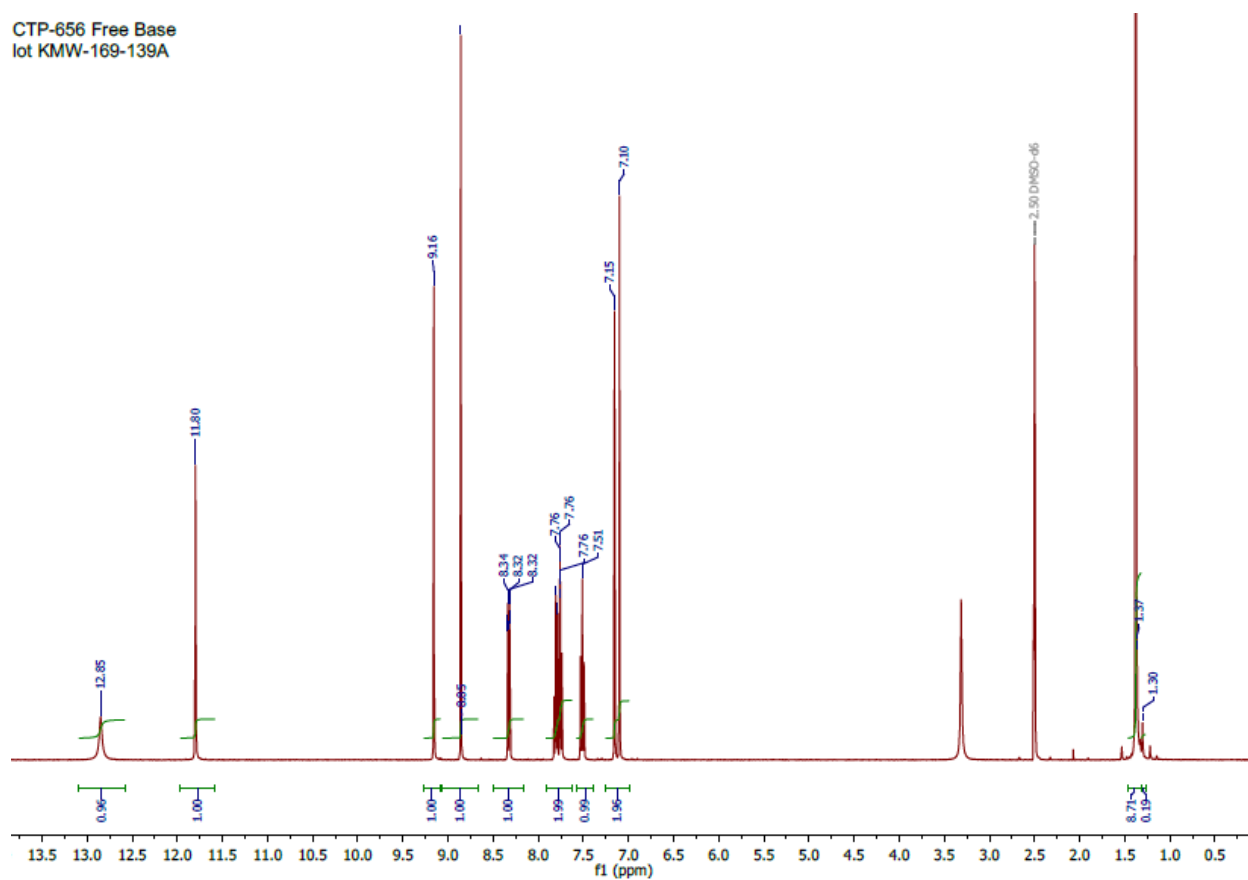
| Elution Time (min) | Peak Area  | Percentage of Total |
|--------------------|------------|---------------------|
| 12.823             | 5490.28857 | 100%                |

**Supplemental Figure 2. CTP-656 MS identity assessment**

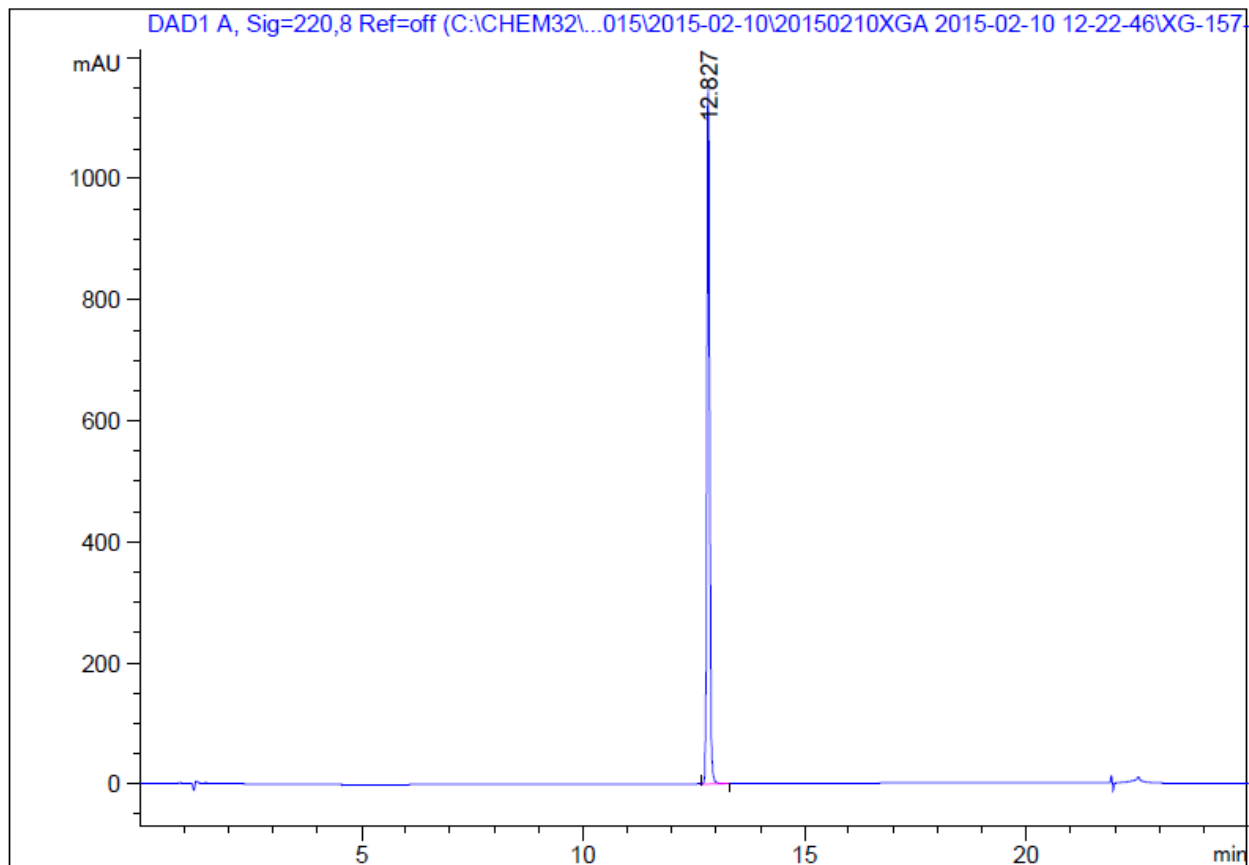


### Supplemental Figure 3. CTP-656 <sup>1</sup>H-NMR spectrum

CTP-656 Free Base  
lot KMW-169-139A

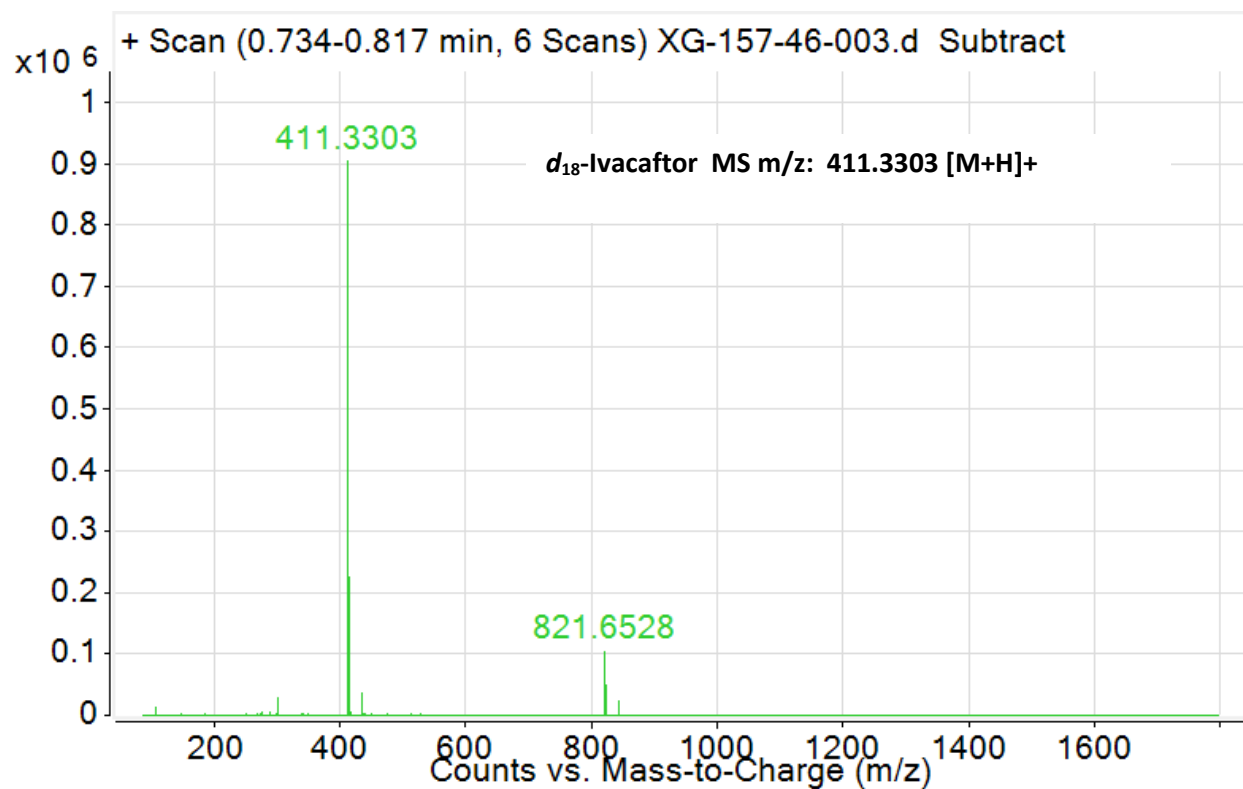


Supplemental Figure 4. *d*<sub>18</sub>-Ivacaftor LC purity assessment



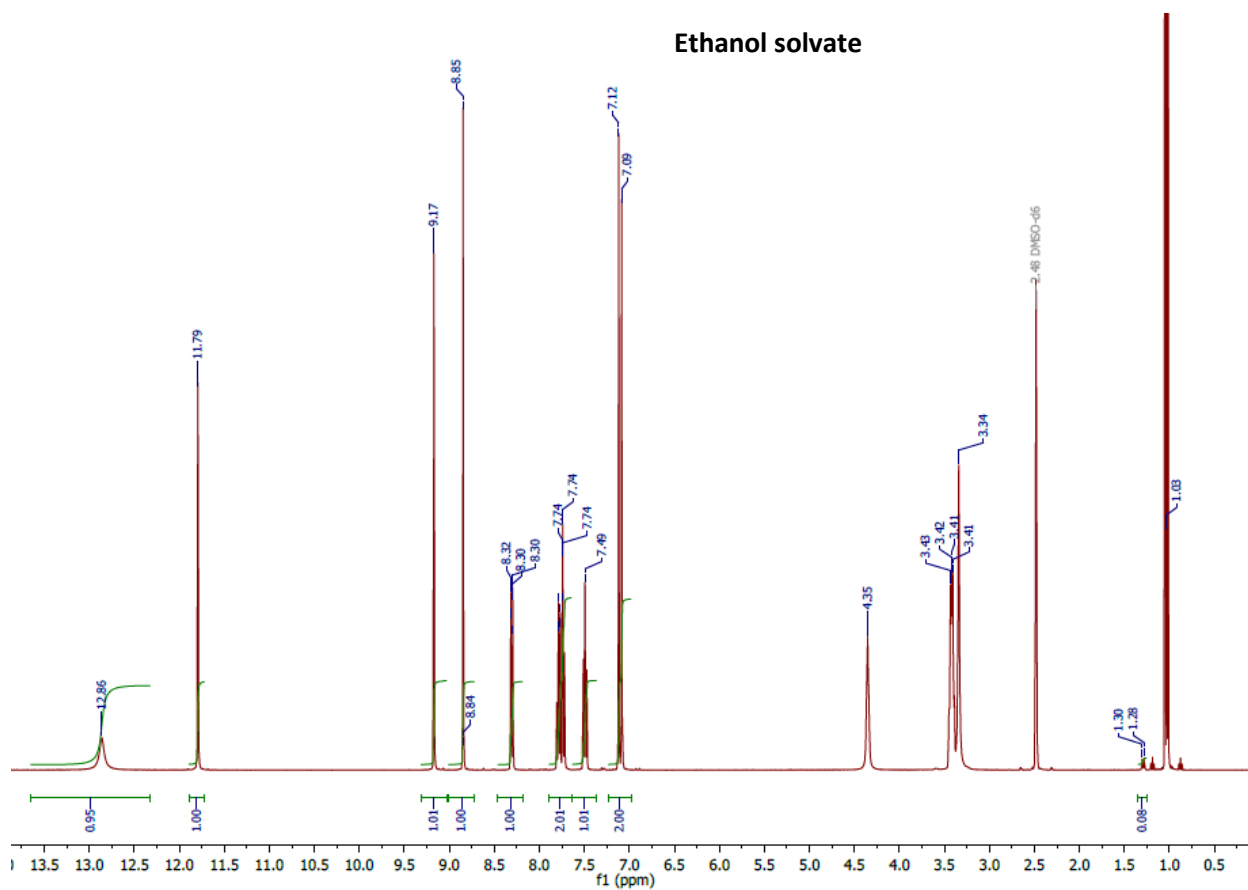
| Elution Time (min) | Peak Area  | Percentage of Total |
|--------------------|------------|---------------------|
| 12.827             | 5049.94531 | 100%                |

**Supplemental Figure 5.** *d*<sub>18</sub>-Ivacaftor MS identity assessment

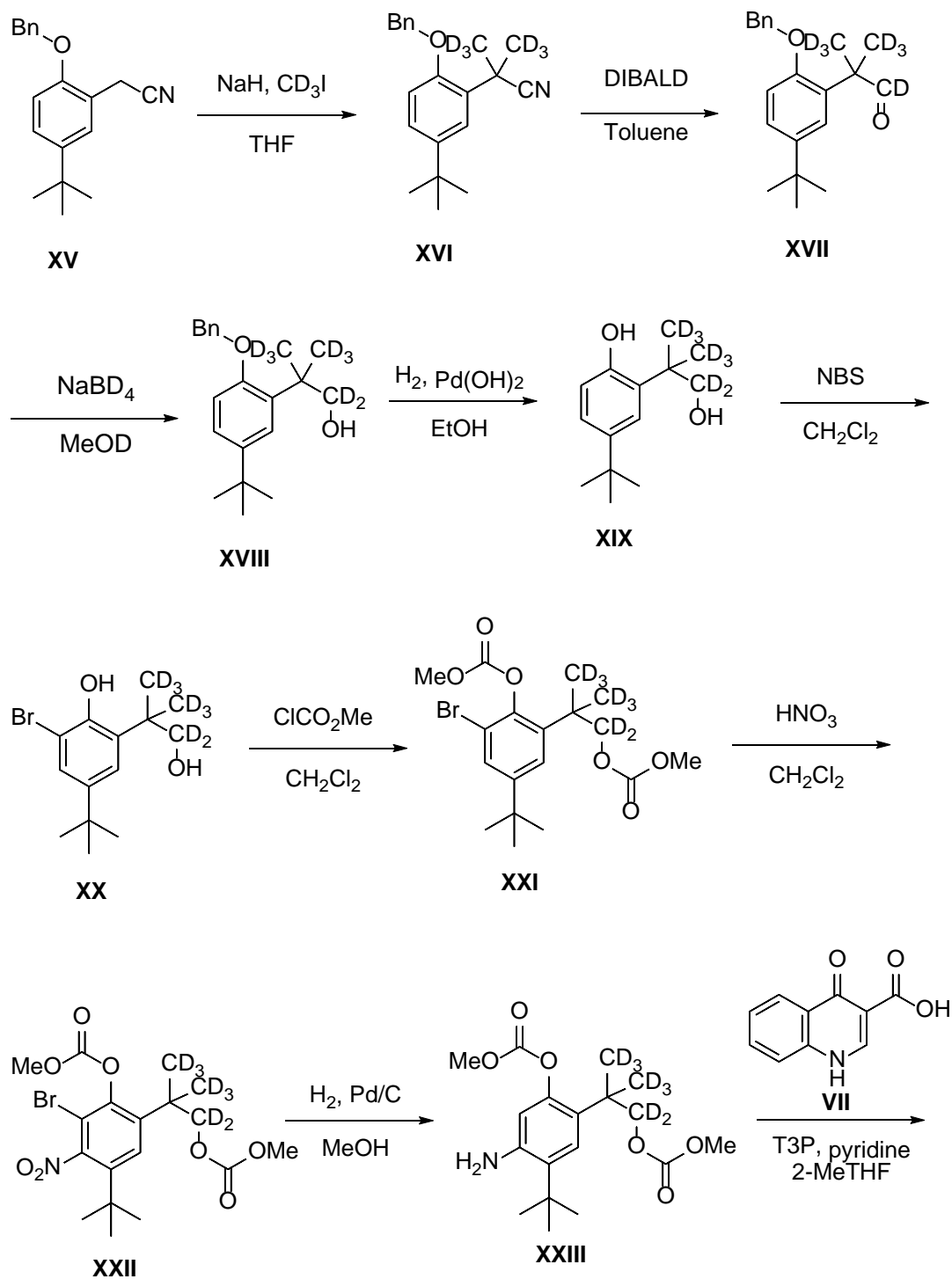


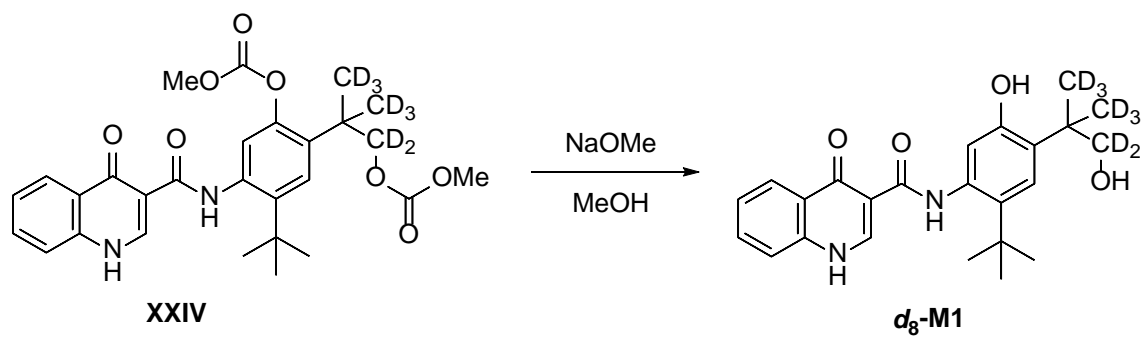


Supplemental Figure 6.  $d_{18}$ -Ivacaftor  $^1\text{H}$ -NMR spectrum



**Supplemental Figure 7.** Synthesis of CTP-656 Metabolite  $d_8$ -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  $D_2O$  (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- $d_3$ )propan-1,1,3,3,3- $d_5$ -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  $R_f$  = 0.65, product  $R_f$  = 0.3) indicated the reaction was complete. The mixture was quenched by adding 1 N DCl in  $D_2O$  (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- $d_3$ )propan-2-yl-1,1,3,3,3- $d_5$ )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  $H_2$  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 **XIX** (1.77 g, 75% yield) as an off-white solid.

**2-Bromo-4-(tert-butyl)-6-(1-hydroxy-2-(methyl-d<sub>3</sub>)propan-2-yl-1,1,3,3,3-d<sub>5</sub>)phenol (XX)** – *N*-bromosuccinimide (1.37 g, 7.70 mmol, 1 equiv) was added to a solution of **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 **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<sub>3</sub>)propan-2-yl-1,1,3,3,3-d<sub>5</sub>)phenyl methyl carbonate (XXI)** – A solution of **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<sub>f</sub> = 0.3, product R<sub>f</sub> = 0.35, stained with KMnO<sub>4</sub>) 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 **XXI** (2.9 g, 89% yield over 2 steps) as a colorless oil.

**2-Bromo-4-(*tert*-butyl)-6-(1-((methoxycarbonyl)oxy)-2-(methyl-d<sub>3</sub>)propan-2-yl-1,1,3,3,3-d<sub>5</sub>)-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-(1-((methoxycarbonyl)oxy)-2-(methyl-d<sub>3</sub>)propan-2-yl-1,1,3,3,3-d<sub>5</sub>)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 H<sub>2</sub> 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.

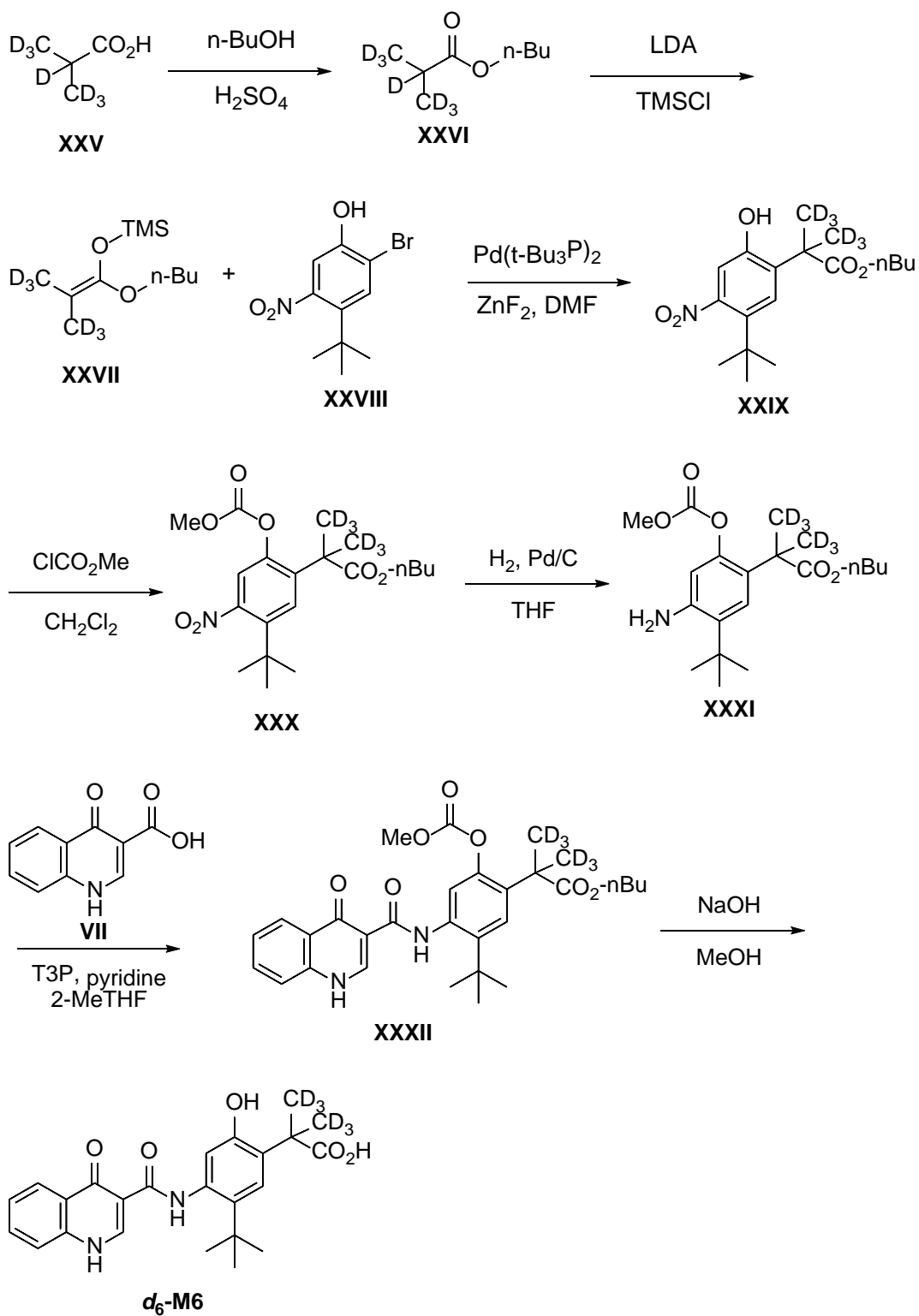
**4-(*tert*-Butyl)-2-(1-((methoxycarbonyl)oxy)-2-(methyl-d<sub>3</sub>)propan-2-yl-1,1,3,3,3-d<sub>5</sub>)-5-(4-oxo-1,4-dihydroquinoline-3-carboxamido)phenyl methyl carbonate (XXIV)** – A 50% solution of propylphosphonic anhydride in ethyl acetate (5.73 g, 9.0 mmol, 2.5 equiv) and pyridine (570

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-(*tert*-butyl)-5-hydroxy-4-(1-hydroxy-2-(methyl- $d_3$ )propan-2-yl-1,1,3,3,3- $d_5$ )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



**Supplemental Materials:** Synthesis of CTP-656  $d_6$ -M6 Metabolite

**Butyl 2-(methyl- $d_3$ )propanoate-2,3,3,3- $d_4$  (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- $d_3$ )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- $d_3$  (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.

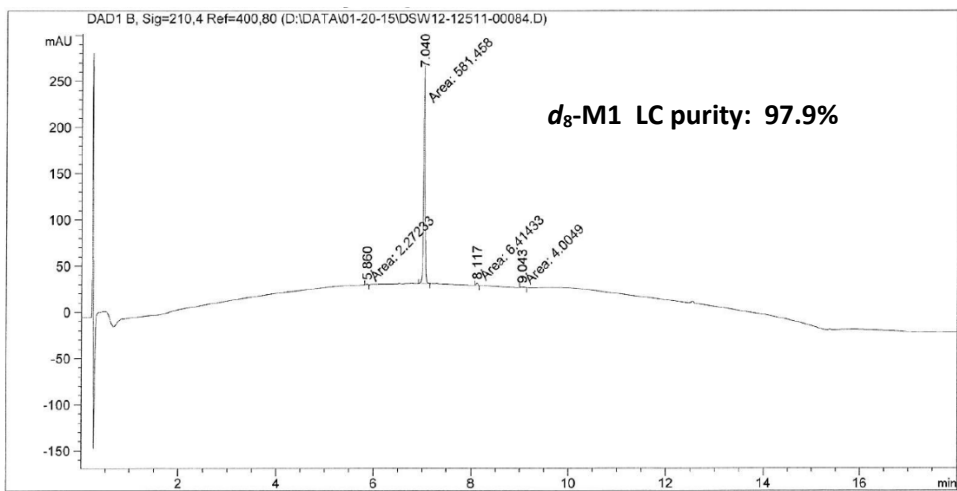
**Butyl 2-(5-(tert-butyl)-2-((methoxycarbonyl)oxy)-4-nitrophenyl)-2-(methyl-d3)propanoate-3,3,3-d3 (XXX)** – A solution of **XXIX** (900 mg, 2.6 mmol, 1.0 equiv) and triethylamine (0.76 mL, 5.4 mmol, 2.0 equiv) in dichloromethane (30 mL) was cooled to 0 °C and methyl chloroformate (0.51 mL, 6.5 mmol, 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 H<sub>2</sub> 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.

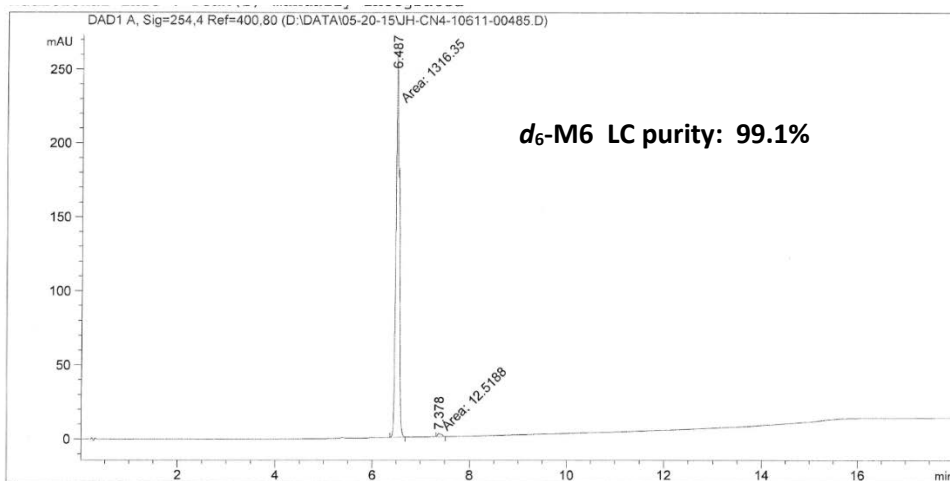
**Butyl 2-(5-(tert-butyl)-2-((methoxycarbonyl)oxy)-4-(4-oxo-1,4-dihydroquinoline-3-carboxamido)phenyl)-2-(methyl-d3)propanoate-3,3,3-d3 (XXXII)** – To a solution of **XXXI** (810 mg, 2 mmol, 1.0 equiv) in anhydrous 2-methyltetrahydrofuran (10 mL) was added **VII** (455 mg, 2.4 mmol, 1.2 equiv), a 50% 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 (d<sub>6</sub>-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 **d<sub>6</sub>-M1** (520 mg, 81% yield) as a white solid.

**Supplemental Figure 9.  $d_8$ -M1 and  $d_6$ -M6 LC purity assessments**

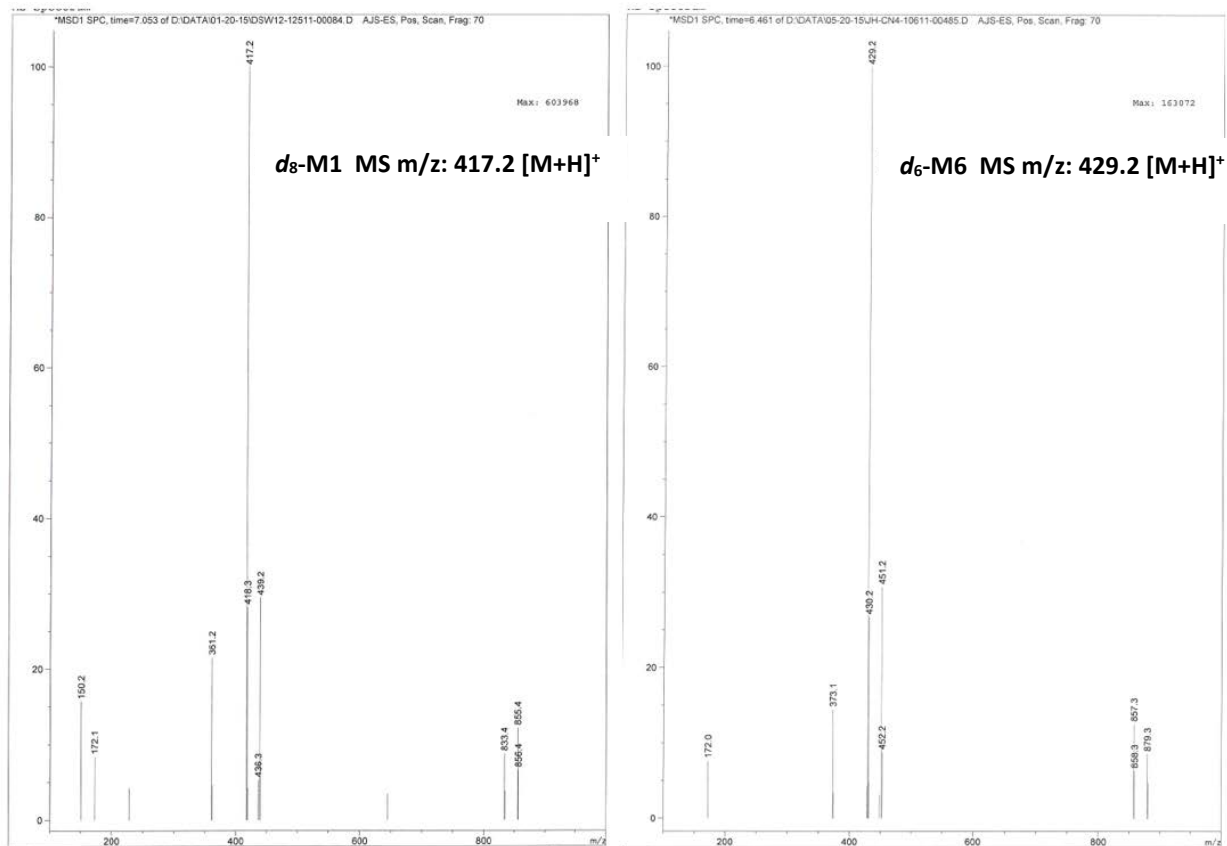


| Elution Time (min) | Peak Area | Percentage of Total   |
|--------------------|-----------|-----------------------|
| 5.860              | 2.27233   | 0.3825%               |
| 7.040              | 581.45795 | 97.8639% ( $d_8$ -M1) |
| 8.117              | 6.41433   | 1.0796%               |
| 9.043              | 4.00490   | 0.6741%               |

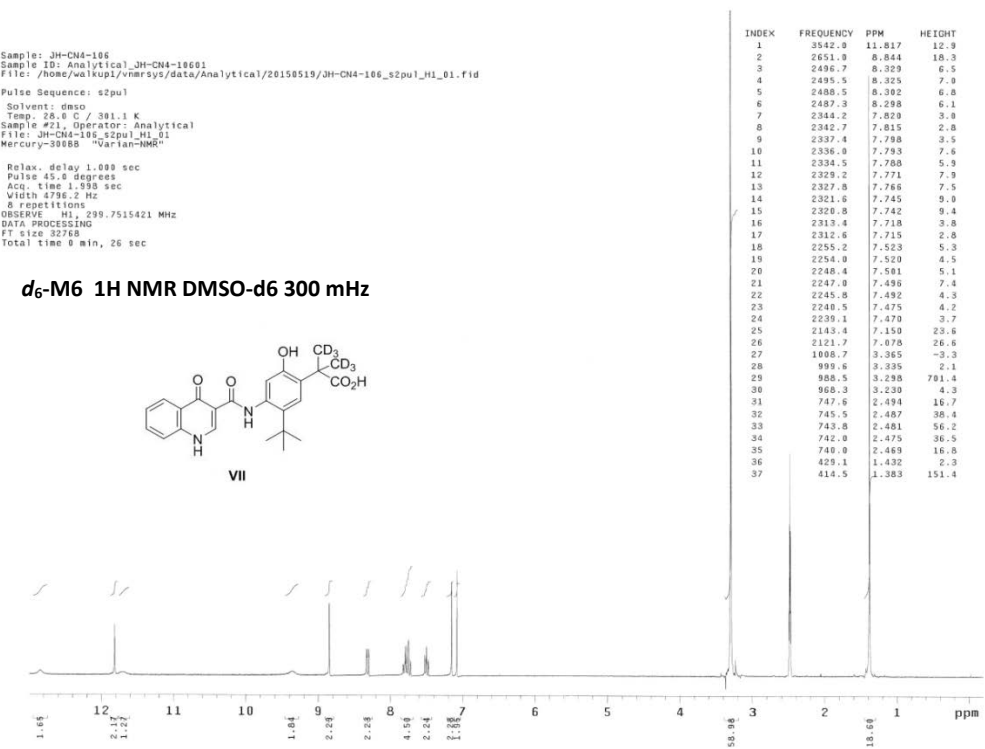
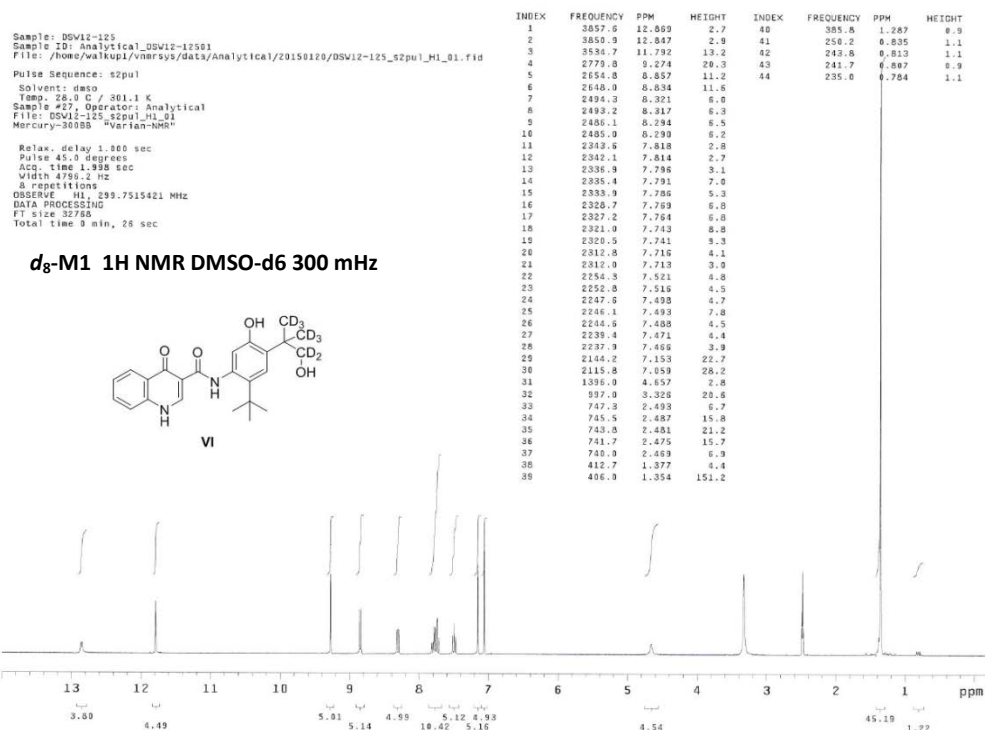


| Elution Time (min) | Peak Area  | Percentage of Total   |
|--------------------|------------|-----------------------|
| 6.487              | 1316.34900 | 99.0579% ( $d_6$ -M6) |
| 7.378              | 12.51883   | 0.9421%               |

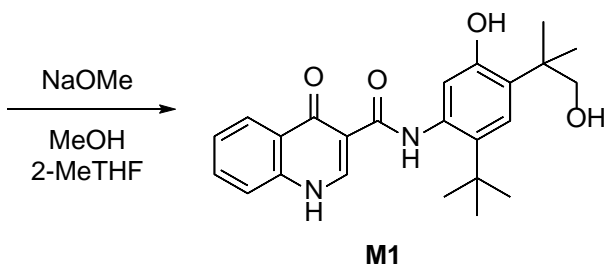
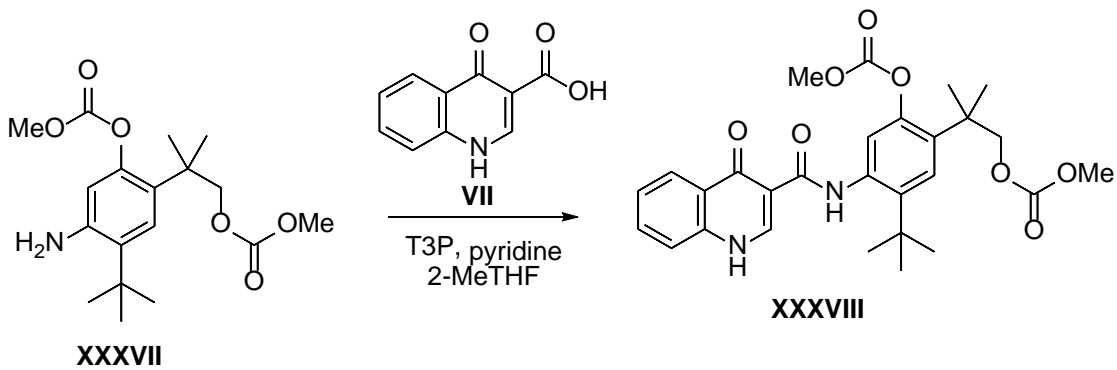
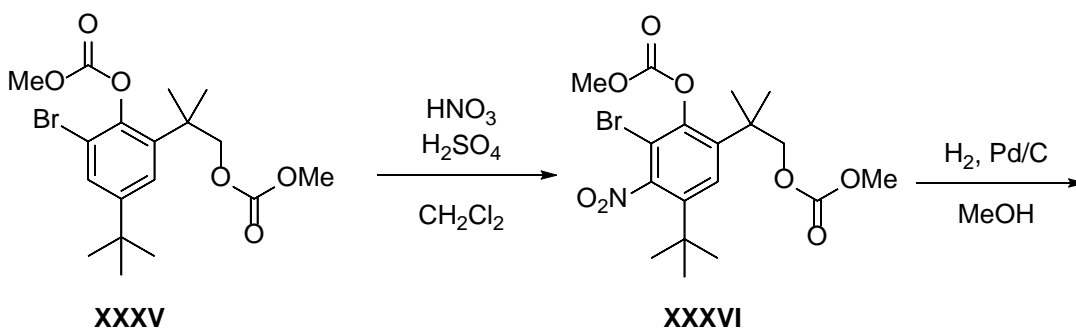
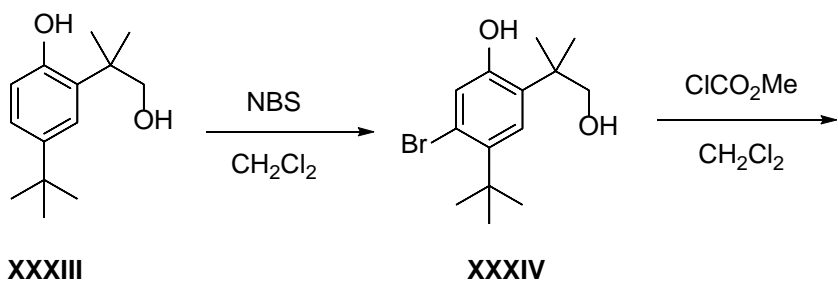
### Supplemental Figure 10. $d_8$ -M1 and $d_6$ -M6 MS identity assessments



## Supplemental Figure 11. $d_8$ -M1 and $d_6$ -M6 $^1\text{H-NMR}$ spectra



**Supplemental Figure 12.** Synthesis of Ivacaftor Metabolite 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.

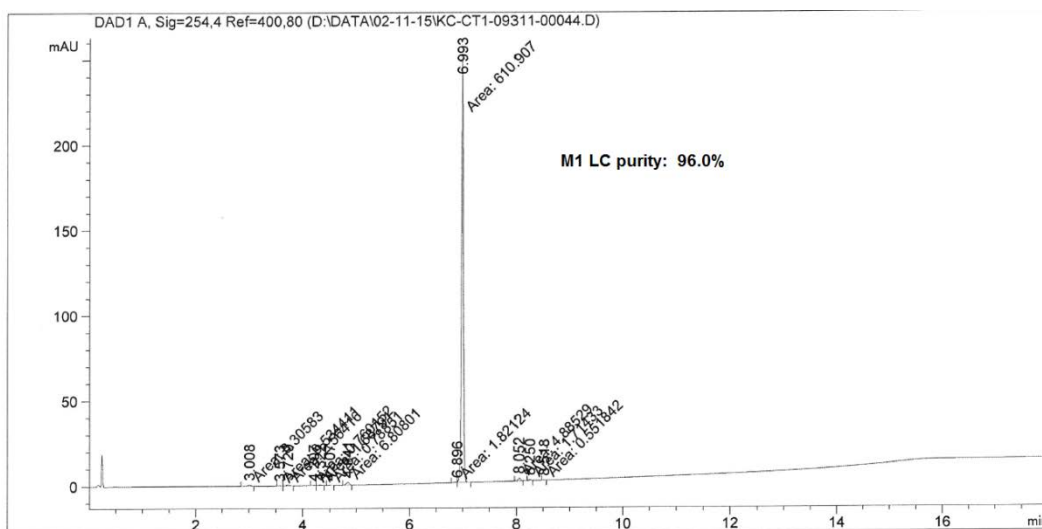
**5-Amino-4-(tert-butyl)-2-(1-((methoxycarbonyl)oxy)-2-methylpropan-2-yl)phenyl methyl carbonate (XXXVII)** – A 500-mL Parr shaker bottle was charged with 10% Pd/C (1.54 g), ethanol (200 mL), **XXXVI** (6.16 g, 13.3 mmol, 1 equiv), and N,N-diisopropylethylamine (2.55 mL, 14.7 mmol, 1.1 equiv). The reaction was shaken at 50 psi H<sub>2</sub> 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.

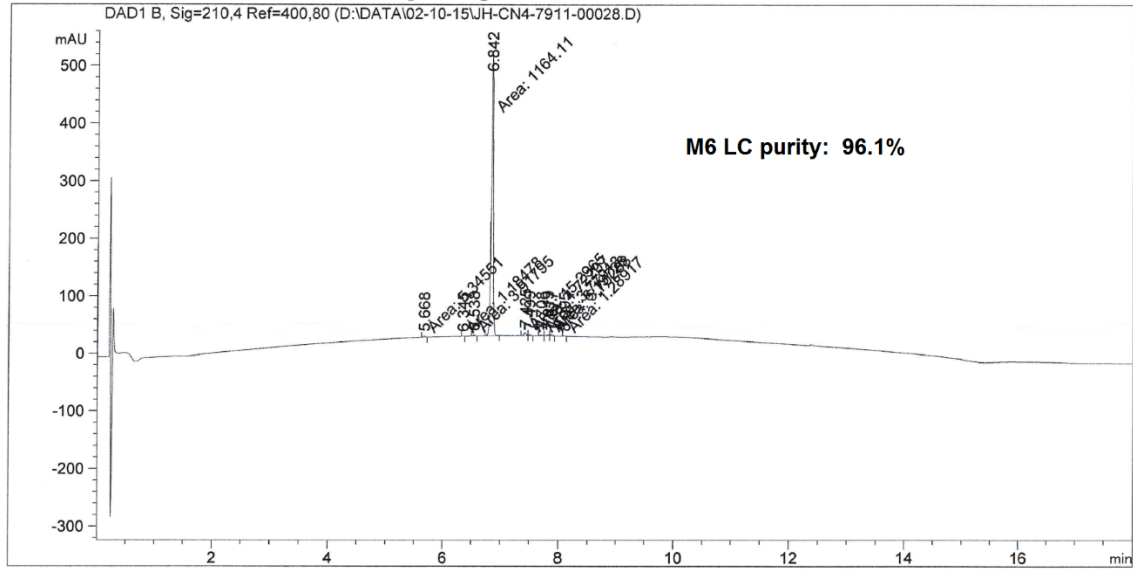
**4-(tert-Butyl)-2-(1-((methoxycarbonyl)oxy)-2-methylpropan-2-yl)-5-(4-oxo-1,4-dihydroquinoline-3-carboxamido)phenyl methyl carbonate (XXXVIII)** – A 50% solution of propylphosphonic anhydride in ethyl acetate (6.5 mL, 11.0 mmol, 2.5 equiv) and pyridine (0.70 mL, 8.8 mmol, 2 equiv) were added to a suspension 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**

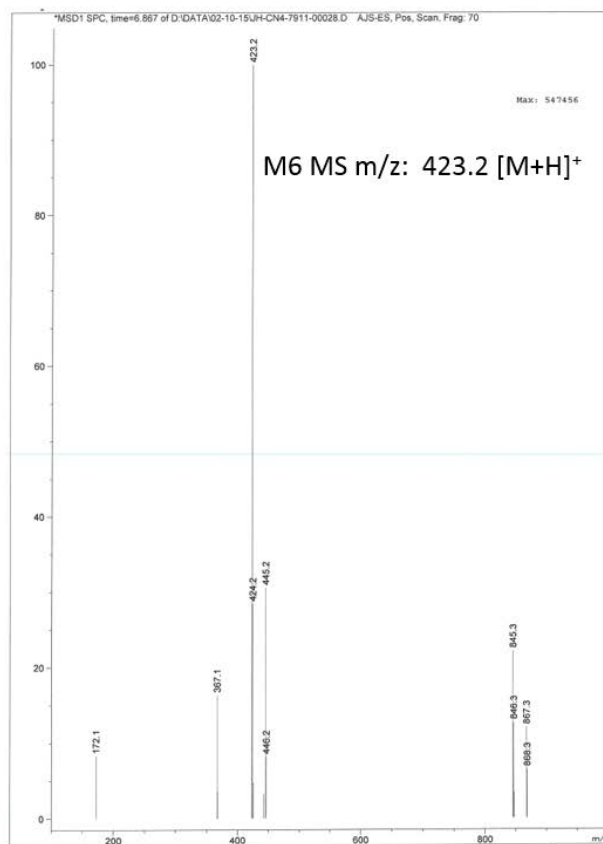
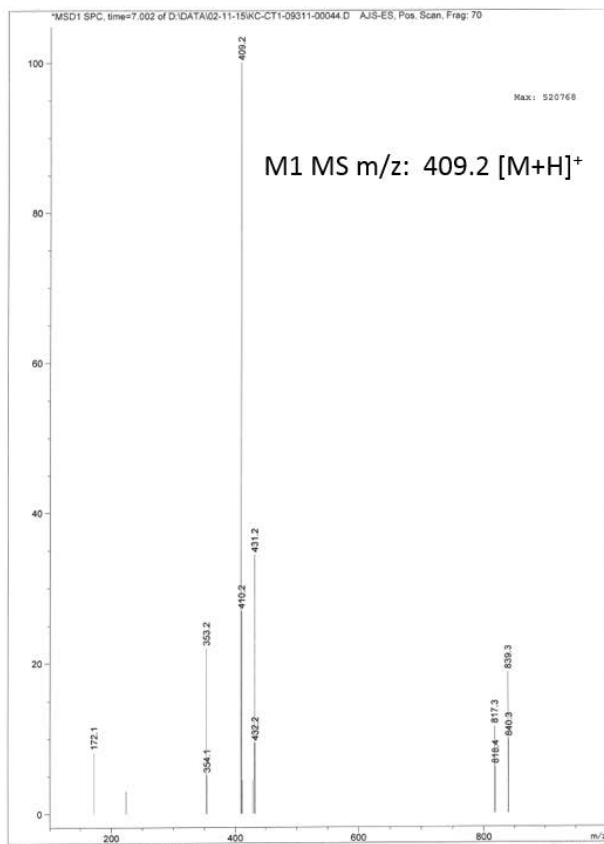


| Elution Time (min) | Peak Area   | Percentage of Total |
|--------------------|-------------|---------------------|
| 3.008              | 3.30583     | 0.5195              |
| 3.573              | 5.34411 e-1 | 0.0840              |
| 3.729              | 2.56416     | 0.4030              |
| 4.207              | 7.60152 e-1 | 0.1195              |
| 4.322              | 1.68791     | 0.2653              |
| 4.507              | 7.88510 e-1 | 0.1239              |
| 4.841              | 6.80801     | 1.0699              |
| 6.896              | 1.82124     | 0.2862              |
| 6.993              | 610.90692   | 96.0049 (M1)        |
| 8.052              | 4.88529     | 0.7677              |
| 8.250              | 1.71433     | 0.2694              |
| 8.518              | 5.51842 e-1 | 0.0867              |

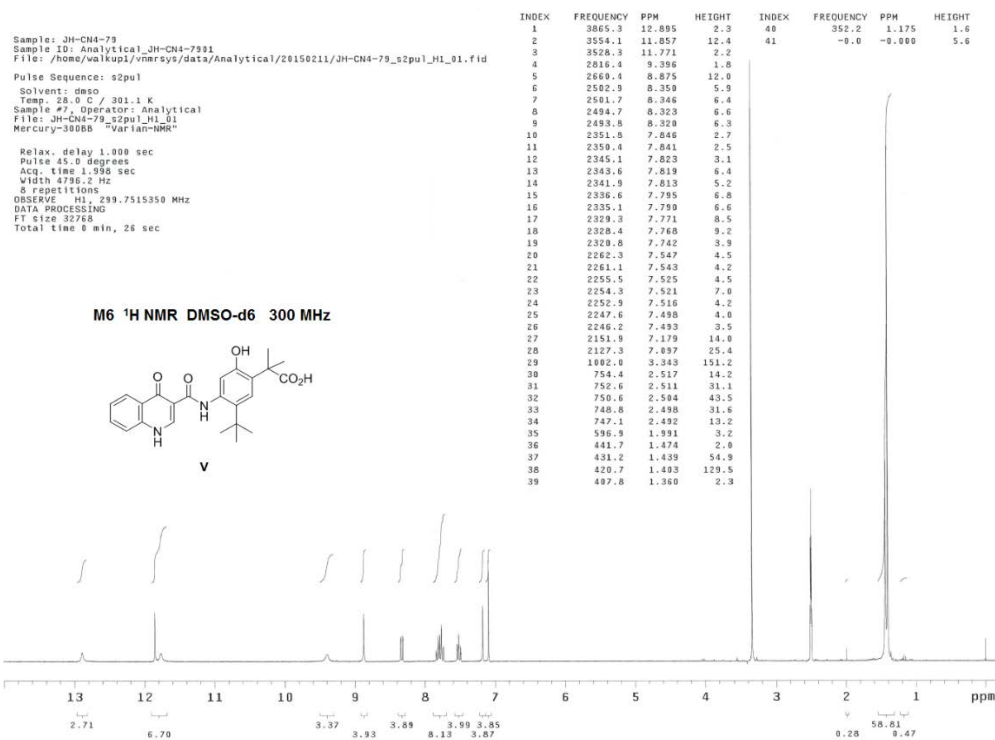
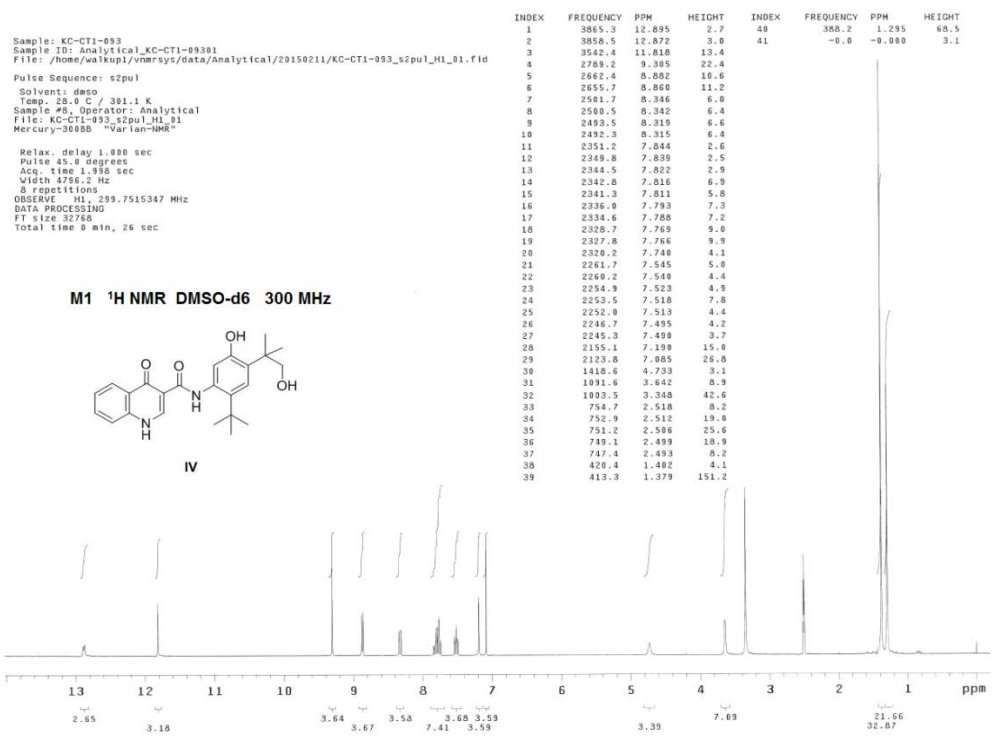


| Elution Time (min) | Peak Area  | Percentage of Total |
|--------------------|------------|---------------------|
| 5.668              | 5.34551    | 0.4412%             |
| 6.345              | 1.18478    | 0.0978%             |
| 6.538              | 3.91795    | 0.3234%             |
| 6.842              | 1164.10718 | 96.0847% (M6)       |
| 7.425              | 15.29650   | 1.2626%             |
| 7.499              | 4.72207    | 0.3898%             |
| 7.708              | 3.77913    | 0.3119%             |
| 7.819              | 6.19726    | 0.5115%             |
| 7.877              | 5.70288    | 0.4707%             |
| 8.095              | 1.28917    | 0.1064%             |

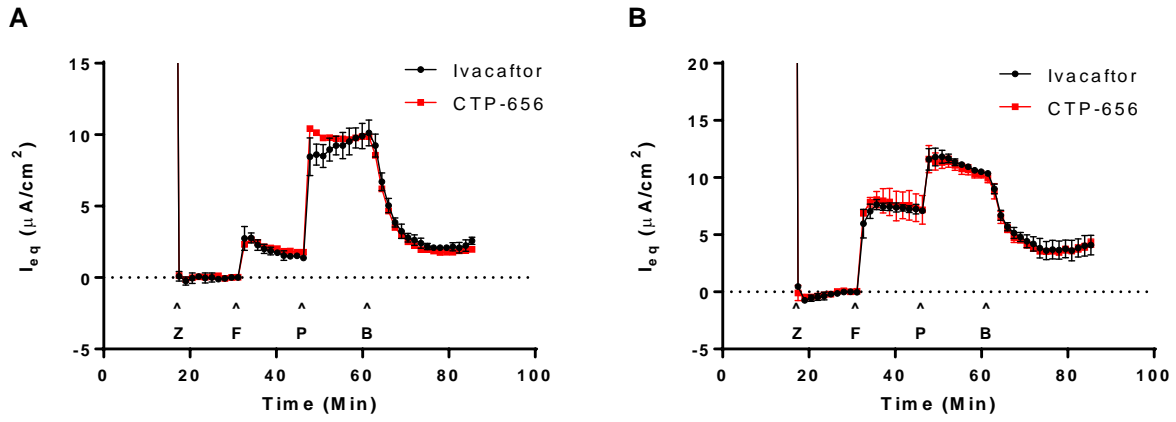
**Supplemental Figure 14. M1 and M6 MS identity assessments**



# Supplemental Figure 15. M1 and M6 <sup>1</sup>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  $\pm$  SD, N = 3 replicates. Z = Benzamil (1  $\mu\text{M}$ ), F = Forskolin (10  $\mu\text{M}$ ); P = Potentiator (300 nM); B = Bumetanide (20  $\mu\text{M}$ ). Data for potentiator addition at 300 nM are shown, which is the approximate  $\text{EC}_{50}$  for VX-770 and CTP-656. Figures are representative of all dose-response comparisons.