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Disposition of PF-07321332 (Nirmatrelvir), an Orally Bioavailable Inhibitor of SARS-CoV-2 3CL Protease, across Animals and Humans

Heather Eng¹, Alyssa L. Dantonio¹, Eugene P. Kadar¹, R. Scott Obach¹, Li Di¹, Jian Lin¹,
Nandini C. Patel², Britton Boras³, Gregory S. Walker¹, Jonathan J. Novak^{†,1}, Emi Kimoto¹, Ravi
Shankar P. Singh², and Amit S. Kalgutkar²

Affiliations:

¹Pfizer Worldwide Research, Development & Medical; Groton, CT 06340, USA

²Pfizer Worldwide Research, Development & Medical; Cambridge, MA 02139, USA

³Pfizer Worldwide Research, Development & Medical; La Jolla, CA 92121, USA

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Corresponding Author: Amit S. Kalgutkar, Ph.D.; amit.kalgutkar@pfizer.com; Tel. (617)-551-3336

Orcid ID:

Amit Kalgutkar: <https://orcid.org/0000-0001-9701-756X>

Li Di: <https://orcid.org/000-0001-6117-9022>

Heather Eng: <https://orcid.org/0000-0002-2914-3967>

Emi Kimoto: <https://orcid.org/0000-0003-1284-2769>

Eugene Kadar: <https://orcid.org/0000-0003-3724-6517>

R Scott Obach: <https://orcid.org/0000-0002-6604-401X>

Nandini C. Patel <http://orcid.org/0000-0002-2735-7662>

Alyssa Dantonio: <https://orcid.org/0000-0001-9067-3660>

Ravi Shankar P Singh: <https://orcid.org/0000-0001-6298-8219>

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Abbreviations:

SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus, COVID-19, coronavirus disease 2019; 3CL^{pro}, 3C-like protease; PF-00835231, N-((1S)-1-[[[(1S)-3-hydroxy-2-oxo-1-[(3S)-2-oxopyrrolidin-3-yl]-methyl]propyl]amino]carbonyl]-3-methylbutyl)-4-methoxy-1*H*-indole-2-carboxamide; iv, intravenous; PF-07321332, (nirmatrelvir, 1R,2S,5S)-N-[(1S)-1-Cyano-2-[(3S)-2-oxopyrrolidin-3-yl]ethyl]-6,6-dimethyl-3-[3-methyl-N-(trifluoroacetyl)-L-valyl]-3-azabicyclo[3.1.0]hexane-2-carboxamide; K_i, inhibition constant; dNHBE, differentiated normal human bronchial epithelial cells; EC₉₀, 90% effective concentration; po, oral; PK, pharmacokinetics; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; LC-MS/MS, liquid chromatography tandem mass spectrometry; MTBE, methyl-tert-butyl ether; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; HIF, human intestinal fluid; SCHH, sandwich-cultured human hepatocytes; HBSS, Hanks balanced salt solution; CL_{biliary,int,app}, apparent intrinsic biliary clearance; CL_{biliary}, biliary clearance; HLM, human liver microsomes; NADPH, β-Nicotinamide adenine dinucleotide phosphate, reduced form; ROE, rotating Overhauser effect; V_{max}, maximal velocity; K_M, substrate concentration at half-maximal velocity; CL_{int,app}, apparent intrinsic clearance; CL_{int,app,scaled}, scaled apparent intrinsic clearance; f_{u,mic}, fraction unbound in liver microsomes; f_{CL}, fractional clearance; f_m, fraction metabolized; f_a, fraction of the po dose absorbed from the gastrointestinal lumen; CL_{renal}, renal clearance; V_{dss}, steady state distribution volume; C_{max}, maximal plasma concentration; T_{max}, time to reach C_{max}; AUC, area under the plasma concentration-time curve; t_{1/2}, half-life; P_{app}, apparent absorptive permeability; MDCKII-LE, low efflux Madin-Darby canine kidney cells; BCRP, breast cancer resistant protein; MDR1, multidrug resistant gene 1; f_{u,p}, plasma unbound fraction; BPR, blood to plasma ratio; CL_{hep}, in

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vitro hepatic clearance; m/z^+ , protonated molecular ion; CID, collision-induced dissociation; J , coupling constant; HCD, high energy C-trap dissociation; HSQC, multiplicity edited heteronuclear single quantum coherence; CL_p , plasma clearance; OAT, organic anion transporter; MATE, multidrug and toxin extrusion protein; OCT, organic cation transporter; OATP, organic anion transporting polypeptide; oral F, oral bioavailability; $F_a \times F_g$, fraction of the oral dose absorbed; IC_{50} , half-maximal inhibitory concentration; TDI, time-dependent inhibition; k_{inact} , maximal rate of inactivation; K_I , inactivator concentration required to achieve 50% k_{inact} ; MI complex, metabolic-inhibitor complex; mRNA, messenger RNA; IND_{max} , maximum fold induction; EC_{50} , inducer concentration required to achieve 50% IND_{max} ; HEK, human embryonic kidney cells; HBD, hydrogen bond donor count.

Abstract

The SARS-CoV-2 3C-like protease inhibitor PF-07321332 (nirmatrelvir), in combination with ritonavir (PaxlovidTM), was recently granted emergency use authorization by multiple regulatory agencies for the treatment of COVID-19 in adults and pediatric patients. Disposition studies on nirmatrelvir in animals and in human reagents, which were used to support clinical studies are described, herein. Plasma clearance was moderate in rats (27.2 ml/min/kg) and monkeys (17.1 ml/min/kg), resulting in half-lives of 5.1 and 0.8 h, respectively. The corresponding oral bioavailability was moderate in rats (34–50%) and low in monkeys (8.5%), primarily due to oxidative metabolism along the gastrointestinal tract in this species. Nirmatrelvir demonstrated moderate plasma protein binding in rat, monkey, and human with mean unbound fractions ranging from 0.310–0.478. The metabolism of nirmatrelvir was qualitatively similar in liver microsomes and hepatocytes from rat, monkey, and human; prominent metabolites arose via cytochrome P450 (CYP) mediated oxidations on the P1 pyrrolidinone ring, P2 6,6-dimethyl-3-azabicyclo[3.1.0]hexane, and the tertiary-butyl group at the P3 position. Reaction phenotyping studies in human liver microsomes revealed that CYP3A4 was primarily responsible (fraction metabolized=0.99) for the oxidative metabolism of nirmatrelvir. Minor clearance mechanisms involving renal and biliary excretion of unchanged nirmatrelvir were also noted in animals, and in sandwich-cultured human hepatocytes. Nirmatrelvir was a reversible and time-dependent inhibitor as well as inducer of CYP3A activity in vitro. First-in-human pharmacokinetics studies have demonstrated a considerable boost in the oral systemic exposure of nirmatrelvir upon co-administration with the CYP3A4 inhibitor ritonavir, consistent with the predominant role of CYP3A4 in nirmatrelvir metabolism.

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Significance Statement:

The manuscript describes the preclinical disposition, metabolism, and drug-drug interaction potential of PF-07321332 (nirmatrelvir), an orally active peptidomimetic-based inhibitor of the SARS-CoV-2 3CL protease, which has been granted emergency use authorization by multiple regulatory agencies across the globe for the treatment of COVID-19 in COVID-19-positive adults and pediatric patients, who are at high risk for progression to severe COVID-19, including hospitalization or death.

Introduction

Coronaviruses are a assorted family of RNA viruses, which can lead to respiratory, enteric, and neurological diseases in humans and animals (Hu et al., 2021). Emerging coronaviruses of human health concern include two highly infectious coronaviruses, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), which were detected in humans in 2002 and 2012, respectively (Cui et al., 2019). An novel coronavirus termed as SARS-CoV-2 recently appeared in Wuhan, China, and led to an outbreak of uncommon viral pneumonia (Zhu et al., 2020). With high transmission rates, this new disease (known as coronavirus disease 2019 (COVID-19)) has quickly spread globally, resulting in 366 million confirmed COVID-19 cases, and over 5.64 million deaths as of January 2022 ([Coronavirus Death Toll and Trends - Worldometer \(worldometers.info\)](https://www.worldometers.info/coronavirus/)). Isolation of the virus bronchoalveolar fluid from infected patients and genomic RNA sequencing indicated that the causative agent is a betacoronavirus, which was not encountered previously. Despite the novelty, the genomic sequence identity of SARS-CoV-2 is similar to the one coded for SARS-CoV (~79%) and MERS-CoV (~50%) (Lu et al., 2020).

The SARS-CoV-2 genome contains two open reading frames, which encode two large polyproteins (Ziebuhr et al., 1997; Zhou et al., 2020), from which functional proteins are generated through proteolysis. SARS-CoV-2 is known to encode a papain-like cysteine protease and a chymotrypsin-like cysteine protease known as main protease or 3C-like protease (3CL^{pro}) (Jin et al., 2020; Pillaiyar et al., 2016). The coronavirus 3CL^{pro} is a three-domain protease, containing a Cys145-His41 catalytic dyad between domains I and II (Anand et al., 2003; Yang et al., 2003). A common structural feature among 3CL^{pro} substrates is the presence of a P1 Gln residue at the cleavage site, which is not encountered in any known human equivalent cysteine

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protease. This observation presents an unique opportunity in designing small-molecule inhibitors of 3CL^{pro} with relatively high selectivity over the human proteome (Rut et al., 2021).

During the 2002 SARS outbreak, we initiated efforts to identify small-molecule inhibitors of the SARS-CoV-1 3CL^{pro}, which led to the design of N-((1*S*)-1-[[[(1*S*)-3-hydroxy-2-oxo-1-[[[(3*S*)-2-oxopyrrolidin-3-yl]-methyl]propyl]amino]carbonyl]-3-methylbutyl)-4-methoxy-1*H*-indole-2-carboxamide (PF-00835231, Figure 1) as a potent inhibitor of SARS-CoV-1, and more recently SARS-CoV-2 (Boras et al., 2021; Hoffman et al., 2020). The phosphate derivative (PF-07304814) of PF-00835231 is currently being studied as an intravenous (iv) treatment option in hospitalized COVID-19 patients (Boras et al., 2021). In response to the COVID-19 pandemic, we revitalized discovery efforts to identify orally active SARS-CoV-2 3CL^{pro} inhibitors using PF-00835231 as a starting point. A key objective in this exercise was to improve upon the poor oral absorption characteristics of PF-00835231, while retaining the favorable inhibitory effects against SARS-CoV-2 in recombinant enzyme and cellular antiviral assays. This research activity culminated in the design of (1*R*,2*S*,5*S*)-N-[(1*S*)-1-cyano-2-[(3*S*)-2-oxopyrrolidin-3-yl]ethyl]-6,6-dimethyl-3-[3-methyl-N-(trifluoroacetyl)-L-valyl]-3-azabicyclo[3.1.0]hexane-2-carboxamide (PF-07321332, nirmatrelvir) as an orally active antiviral agent (Owen et al., 2021). Nirmatrelvir inhibited (inhibition constant (K_i) = 3.11 nM) recombinant SARS-CoV-2 3CL^{pro} protease activity via a reversible covalent mechanism involving reaction of the cyano group with the catalytic Cys145 residue (see Figure 1). Nirmatrelvir also repressed SARS-CoV-2 replication in physiologically relevant cellular systems such as differentiated normal human bronchial epithelial (dNHBE) cells and human adenocarcinoma-derived alveolar basal epithelial cells with 90% effective unbound concentration (EC_{90,u}) values of 181 nM and 215 nM, respectively. Oral (po) administration of nirmatrelvir to a mouse-adapted SARS-CoV-2 model resulted in reduction

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of lung viral titers in a dose-dependent fashion, and protected infected mice from lung tissue damage and weight loss (Owen et al., 2021). Preclinical disposition mechanisms of nirmatrelvir were characterized in animals and in human reagents to support animal safety and first-in-human clinical studies. The potential for pharmacokinetic (PK) interactions arising from inhibition/induction of major human cytochrome P450 (CYP) enzymes and drug transporters was also examined in vitro systems. The collective results, including the demonstration of the predominant role for CYP3A4 in nirmatrelvir metabolism, which presented an option of boosting nirmatrelvir systemic exposure in humans by utilizing the CYP3A4 inhibitor ritonavir, are described herein.

Materials and Methods

Chemicals and Materials. Nirmatrelvir (chemical purity > 99% by HPLC and NMR) was synthesized at Pfizer Inc. (Groton, CT). Stock solutions of nirmatrelvir were prepared in either 100% dimethyl sulfoxide (DMSO) or acetonitrile. Commercially obtained chemicals and solvents were of high-performance liquid chromatography or analytical grade. In vitro studies were conducted on human tissue, which was acquired from a third party, and was confirmed as compliant with Pfizer policies, including Institutional Review Board/Independent Ethics Committee approval. Animal PK studies were conducted in agreement with federal, state, local and institutional guidelines on the use of laboratory animals in research in AAALAC accredited facilities, and were approved by Pfizer's or Bioduro's (Shanghai, PRC) Institutional Animal Care and Use Committee. Description of experimental procedures for passive permeability, efflux, plasma protein/human liver microsomal binding, blood cell partitioning, liver microsome/hepatocyte stability, preclinical PK, CYP inhibition/induction and transporter

inhibition, and liquid chromatography tandem mass spectrometry (LC-MS/MS) assays for nirmatrelvir quantitation are provided as supplemental information.

Aqueous Solubility and Dissolution Measurement. Equilibrium solubility of crystalline nirmatrelvir in water was measured using an anhydrous form ('form 1') as well as a methyl-tert-butyl ether (MTBE) co-solvate form) in duplicate, utilizing a temperature cycling method (Murdande et al., 2009). Briefly, nirmatrelvir was weighed in a screw top vial and an appropriate amount of water (≥ 0.5 ml) was added. The vial was placed in an Environ-Genie incubator (Scientific Industries, Bohemia, New York) and a temperature cycling program (8 h at 40 °C, 5 h at 15 °C, followed by 12 h at 25 °C) was applied. After a pH measurement, the solutions were centrifuged (5 min, 14000 rpm) for particulate separation, and the supernatants assayed by ultra-performance liquid chromatography for nirmatrelvir concentrations. Dissolution studies (Murdande et al., 2009) were performed with nirmatrelvir (150 mg, MTBE co-solvate or anhydrous 'form 1') in distilled water or 0.1 N HCl (500 ml), using a suspension of well-dispersed nirmatrelvir particles in 0.5% w/v methyl cellulose. A dissolution test was conducted using a USP II dissolution apparatus (rotating at 100 rpm) with in-situ fiber optic UV-visible spectroscopy (Distek, Inc., North Brunswick, NJ).

Metabolic Stability in Intestinal Fluid. Fresh simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared in-house according to the United States Pharmacopeia National Formulary (https://online.uspnf.com/uspnf/document/1_GUID-190BB90A-8350-41D4-82E0-88920705A993_1_en-US; https://online.uspnf.com/uspnf/document/1_GUID-4A435135-EDA6-4309-B967-0CE322595823_1_en-US). SGF was a solution of 3.2 g/l of pepsin and NaCl dissolved in deionized water (pH 1.2). SIF was a suspension of 10 g/l of pancreatin in monobasic potassium phosphate dissolved in deionized water (pH 6.8). Human intestinal fluid (HIF)

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(National BioService LLC, Saint-Petersburg, Russia) was a mixture of pancreatic juice and bile obtained directly from the duodenum of two fasted adult volunteers via a surgical probe. An aliquot of SGF, SIF, or HIF was prewarmed to 37 °C for 5 min followed by the addition of 1 μM nirmatrelvir (0.5% organic in final incubation, 200 μl final incubation volume). Incubations were conducted in duplicate or triplicate. At various time points (0.25, 10, 20, 40, 60, 90, and 120 min), a 20 μl aliquot of the incubation mixture was removed and quenched with acetonitrile (100 μl) containing diclofenac (25 ng/ml) as internal standard. Samples were vortexed, centrifuged (5 min, 2300 x g) and the supernatant (50 μl) was diluted with 200 μl of water. Quantitation of nirmatrelvir in the samples was performed using LC-MS/MS.

Biliary Excretion in Sandwich Cultured Human Hepatocytes (SCHH). Plateable

cryopreserved human hepatocytes lot Hu4165 (female, Caucasian, age 57) were purchased from Invitrogen (Waltham, MA). The SCHH methodology and data analysis applied here were described previously (Bi et al., 2006; Kimoto et al., 2017). Briefly, hepatocytes were plated in collagen-coated 24-well plates on day 1 at a density of 0.375×10^6 cells/well and overlaid with ice-cold matrigel after 18-24 h of incubation. On day 5, SCHH were first rinsed twice with Hanks balanced salt solution (HBSS) containing $\text{Ca}^{2+}/\text{Mg}^{2+}$ (standard) or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS, and then preincubated for 10 min with standard or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS at 37 °C. After aspirating the preincubation buffer, uptake was initiated by adding prewarmed nirmatrelvir (0.5 μM) to 37 °C and terminated at a designated time (10 and 20 min) by adding ice-cold standard HBSS. Hepatocytes were lysed with methanol containing an internal standard for quantification of PF-07321332 by LC-MS/MS. The in vitro apparent intrinsic biliary clearance ($\text{CL}_{\text{biliary,int,app}}$) was scaled up to a whole liver after application of physiological scalars of $(120 \times 10^6 \text{ cells/g})$

liver) $\times$ (21 g liver/kg body weight) to estimate the human biliary clearance (CL_{biliary}) (Kimoto et al., 2017).

Metabolite Identification. Nirmatrelvir (10 μ M) was incubated in Wistar-Han rat (male and female), Cynomolgus monkey (male), and pooled (mixed sex of 50) human liver microsomes (HLM) (protein concentration = 2 mg/ml) (Sekisui XenoTech (Kansas City, KS)) or recombinant human CYP enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP3A7, and CYP4F2 100 pmol/ml each) (Corning, Glendale, AZ) in 100 mM potassium phosphate buffer (pH 7.4) containing β -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (1.3 mM), $MgCl_2$ (3.3 mM) at 37 °C for 1 h. Incubations were quenched with acetonitrile (0.6 ml). Because nirmatrelvir possesses very poor UV absorbance, a second set of incubations were generated where the parent compound was only added after the acetonitrile quench. These incubations mixtures, used to assess metabolite formation by HPLC-UV, ensured an improved delineation of nirmatrelvir-related material from other components in the incubations. The quenched incubation mixtures were centrifuged (5 min, 1800 $\times$ g) and the supernatants were evaporated to dryness. Metabolite identification in hepatocytes was performed in nirmatrelvir (10 μ M) incubations with Wistar-Han rat (male, pool of 24 animals), cynomolgus monkey (male, pool of 3 animals), and human (pool of 13 donors of mixed sex) hepatocytes (BioIVT, Baltimore, MD) at 0.75×10^6 cells/ml. Periodic aliquots were removed at 0, 1, and 4 h, and quenched with 5 volumes of acetonitrile. Samples were centrifuged (5 min, 1800 $\times$ g) and evaporated to dryness using a Genevac evaporative centrifuge.

LC-MS/MS Analysis for Metabolite Identification. The residues were reconstituted in 50–150 μ l of 1% formic acid containing 20% acetonitrile and analyzed by ultra-high performance liquid

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chromatography-UV-high resolution mass spectrometry operated in positive ion mode using an Orbitrap Elite mass spectrometer in line with a Vanquish HPLC-UV with cooled autoinjector (ThermoFisher, Waltham, MA). Injection volumes were 5 to 15 μ l, depending on the experimental run. A Kinetex XB C18 column (Phenomenex, Torrance, California) was used (2.1 x 100 mm, 2.6 μ m) with a flow rate of 0.4 ml/min heated to 45 °C. Mobile phase A was comprised of 0.1% formic acid in water and mobile phase B was comprised of acetonitrile. The mobile phase composition began at 5% B held for 0.5 min followed by a linear gradient to 70% B at 11 min, a second linear gradient to 95% B at 13 min, a 1 min wash at 95% B, and finally a 2 min re-equilibration period at 5% B. UV was monitored between 200 to 400 nm and chromatograms were reconstructed using λ at 200 nm.

Biosynthesis of Nirmatrelvir Metabolites for Structural Elucidation. Nirmatrelvir (25 μ M), HLM (2 mg/ml), $MgCl_2$ (3.3 mM), and NADPH (1.3 mM) were incubated in potassium phosphate buffer (100 mM, pH 7.4) (40 ml) at 37 °C for 55 min followed by the addition of acetonitrile (40 ml). The solution was centrifuged (1800 x g) for 5 min and concentrated in a Genevac vacuum centrifuge. The remaining mixture was treated with acetonitrile (0.5 ml), formic acid (0.5 ml), and water to a volume of 50 ml. This mixture was centrifuged (30 min, 40000 x g) and the supernatant was injected into a Polaris C18 column (Agilent, Santa Clara, CA) (4.6 x 250 mm; 5 μ m) through a Jasco HPLC pump (Jasco Inc., Easton, MD) at 0.8 ml/min. After injection, the column was moved to an Acquity HPLC-UV in line with a CTC Analytics fraction collector (Zwingen, Switzerland) and linear trap quadrupole Velos mass spectrometer (ThermoFisher, Waltham, MA). The material was eluted with a mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) at a flow rate of 0.8 ml/min. The gradient was initiated at 2% mobile phase B with a linear gradient to 15% mobile phase B at 5 min, a second

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linear gradient to 60% mobile phase B at 85 min, and a third linear gradient to 95% mobile phase B at 90 min. This composition was held for 9 min followed by a 10 min reequilibration period to initial conditions. The eluent was passed through the UV detector, then a splitter that directed the flow to the fraction collector and mass spectrometer in an approximate 15:1 ratio. Fractions were collected every 20 seconds and those containing metabolites of interest were evaluated for purity by ultrahigh pressure LC-MS with UV detection described above to facilitate pooling. Pooled fractions were evaporated in a vacuum centrifuge and residues evaluated by NMR spectroscopy for structural elucidation.

NMR Spectroscopy. Samples were dissolved in DMSO- d_6 “100%” (0.045 ml) (Cambridge Isotope Laboratories, Andover, MA) and placed in a NMR tube (1.7 mm) in a dry argon atmosphere. 1H and ^{13}C spectra were referenced using residual DMSO- d_6 (1H δ = 2.50 parts per million relative to tetramethylsilane, δ = 0.00, ^{13}C δ = 39.50 parts per million relative to tetramethylsilane, δ = 0.00). NMR spectra were recorded on a Bruker Avance 600 MHz (Bruker BioSpin Corporation, Billerica, MA) controlled by Topspin V4.0 and equipped with a 1.7 mm TCI Cryo probe. 1D spectra were recorded using an approximate sweep width of 8400 Hz and a total recycle time of approximately 7 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal to noise. For through-space experiments (rotating-frame Overhauser effect (ROE) or nuclear Overhauser effect) the sample temperature was raised to 340 K. Two dimensional NMR data were recorded using the standard pulse sequences provided by Bruker. At minimum, a 1K x 128 data matrix was acquired using a minimum of 2 scans and 16 dummy scans with a spectral width of 10000 Hz in the f2 dimension. The 2D data sets were zero-filled to at least 1k data point. Post-

acquisition data processing was performed with either Topspin V3.2 or MestReNova V9.1. A series of standard 1D and 2D NMR experiments were performed on each sample.

Enzyme Kinetics of Nirmatrelvir Metabolite Formation in HLM. Experimental conditions used in the kinetics study design were chosen to yield linear reaction velocities as determined from preliminary range finding experiments. To determine the rates of metabolite formation, nirmatrelvir (0.02–200 μ M) was incubated with HLM (pool of 50 mixed gender donors; Sekisui XenoTech, Kansas City, KS) diluted to 0.3 mg/ml microsomal protein in 100 mM potassium phosphate buffer (pH 7.4) supplemented with $MgCl_2$ (3.3 mM) and NADPH (1.3 mM) for a final incubation volume of 300 μ l. nirmatrelvir stock solutions were prepared by serial dilution in acetonitrile such that the final concentration of acetonitrile in the incubations was 1.0%. Incubations were conducted at 37 °C in duplicate. After 15 min, aliquots of the incubation mixture (200 μ l) were quenched with acetonitrile (600 μ l) containing internal standard (25 ng/ml diclofenac). Samples were vortexed, centrifuged (5 min, 2300 x g), and the supernatant was diluted with an equal volume of water. Samples were analyzed by LC-MS/MS for quantitation of nirmatrelvir oxidative metabolites M1 (PF-07329265), M2 (PF-07329266), M3 (PF-07329267), and M4 (PF-07329268) for which authentic standards were available through biochemical synthesis.

The LC-MS/MS system consisted of a Sciex Triple Quadrupole 5500 mass spectrometer (Sciex, Framingham, MA), equipped with an electrospray ion source and Agilent 1290 binary pump (Agilent Technologies, Santa Clara, CA). Aqueous mobile phase (A) was composed of water with 0.1% formic acid and organic mobile phase (B) consisted of acetonitrile with 0.1% formic acid. Samples (10 μ l) from various in vitro incubations were injected onto a Kinetex XB-C18 (2.1 x 50 mm, 2.6 μ m) (Phenomenex, Torrance, CA) column maintained at room

temperature with a flow rate of 0.5 ml/min. The gradient program began with 10% mobile phase B held for 0.2 min, followed by a linear gradient to 40% B over 2.3 min, then to 98% over 0.3 min, held at 98% B for 0.6 min followed by re-equilibration to initial conditions for 0.3 min. The mass spectrometer was operated in positive detection mode with the following analyte mass transitions (Q1/Q3): diclofenac 296/250, nirmatrelvir 500/110, PF-07329265 (M1) 516/126, PF-07329266 (M2) 516/289, PF-07329267 (M3) 516/291, and PF-07329268 (M4) 498/110 (optimized Q1 mass representing loss of H₂O in the source). The range of analyte concentration for standard curve was 0.25–250 nM (M1 and M4) and 0.25–100 nM (M2 and M3). Integration and quantitation of metabolite and internal standard peak areas were performed using Analyst version 1.7 (Sciex, Framingham, MA) to derive the analyte to internal standard peak area ratios. Standard curves for the quantitation of metabolite concentration were prepared from plots of concentration versus area ratio and analyzed using a linear regression with 1/x² weighting.

Data Analysis Formation rates (*v*) were calculated by dividing the measured metabolite concentration by incubation time and protein concentration of the incubation. The Michaelis-Menten model was used to derive the apparent enzyme kinetic parameters *V*_{max} (maximal velocity) and *K*_M (substrate concentration at half-maximal velocity) using GraphPad Prism version 8.02 (La Jolla, CA):

$$v = \left(\frac{V_{\max} \times S}{K_M + S} \right)$$

Assessment of the enzyme kinetics was conducted by visual inspection of the Eadie-Hofstee diagrams constructed from the plot of *v* versus the ratio of *v* and substrate concentration (Nagar et al., 2014):

$$v = -K_M \left(\frac{v}{S} \right) + V_{\max}$$

When observations of nonlinear kinetics in Eadie-Hofstee plots for metabolite formation data were suggestive of multi-enzyme contribution, fitting to a two-enzyme non-saturable Michaelis-Menten model was attempted:

$$v = \left(\frac{V_{\max 1} \times S}{K_{M1} + S} \right) + (CL_{\text{int}(2)} \times S)$$

where subscript 1 represents a high-affinity/low-capacity enzyme and $CL_{\text{int}(2)}$ represents the apparent intrinsic clearance rate of the secondary low-affinity/high-capacity enzyme (Nagar et al., 2014).

Alternately, for metabolite formation profiles showing characteristics of allosteric sigmoidal in the Eadie-Hofstee plots, the following equation was used to fit the data:

$$v = \left(\frac{V_{\max} \times S^h}{K_{\text{half}}^h + S^h} \right)$$

where K_{half} is the enzyme concentration that gives a half maximal velocity, and h is the Hill slope. Assessment of best fit was based on a number of criteria, including visual inspection of the data plots (Michaelis-Menten and Eadie-Hofstee), distribution of the residuals, size of the sum of the squared residuals, and the standard error of the estimates. Selection of a model other than Michaelis-Menten was based on the F-test ($p < 0.05$) or the Akaike Information Criterion (Nagar et al., 2014). In vitro apparent intrinsic clearance, ($CL_{\text{int,app}}$) and scaled apparent intrinsic clearance ($CL_{\text{int,app,sc}}$) were calculated for each metabolic pathway using the following equations:

$$CL_{\text{int,app}} = \frac{V_{\max}}{K_M}$$

$$CL_{\text{int,app,sc}} = CL_{\text{int,app}} \times \frac{45 \text{ mg protein}}{\text{g liver}} \times \frac{21 \text{ g liver}}{\text{kg body weight}}$$

Unbound CL_{int} values were calculated by dividing the respective CL_{int} by the unbound liver microsomal free fraction ($f_{u,mic}$) determined in HLM using equilibrium dialysis. When necessary, the measured $f_{u,mic}$ was scaled to reflect the respective microsomal protein concentration according to the method of Austin et al (2002). The fractional contribution by an individual metabolic pathway ($f_{CL(HLM)}$) in HLM to the total in vitro clearance of PF-07321332 was calculated as:

$$f_{CL(HLM)} = \frac{CL_{int,app,x}}{CL_{int,app,all}}$$

where, $CL_{int,app,x}$ is the intrinsic clearance for metabolite x , and $CL_{int,app,all}$ is the sum of in vitro CL_{int} values for all metabolites monitored.

Multi Point Selective Inhibition of CYP3A4/5 Activity in HLM. The effect of CYP3A-selective inhibition by ketoconazole on nirmatrelvir oxidative metabolite formation was assessed in HLM. Incubations (0.3 ml) were conducted in 100 mM potassium phosphate buffer (pH 7.4) containing $MgCl_2$ (3.3 mM), NADPH (1.2 mM), HLM (0.3 mg/ml), and nirmatrelvir (2.5 μM) at 37 °C for 15 min in the absence or presence of varying concentrations of ketoconazole (0.001–10 μM). Chemical inhibition experiments were conducted in triplicate. Sample bioanalysis for analyte quantitation was performed using LC-MS/MS as previously described in the enzyme kinetics protocol.

The maximum contribution of CYP3A to nirmatrelvir metabolism in HLM was determined by the percent activity remaining of each metabolite in the presence of a broad concentration range of ketoconazole (0.001–10 μM). The percent activity remaining was obtained by normalizing concentration data to the averaged solvent controls. Curve fitting of the activity

versus inhibitor concentration data by nonlinear regression to a four-parameter inhibition model was conducted using GraphPad Prism:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + \left(\frac{10^{\text{LogIC}_{50}}}{10^X} \right)^{\text{HillSlope}}}$$

where bottom is the minimum activity, top is the maximal activity, IC_{50} is the inhibitor concentration at half maximal activity, X is the inhibitor concentration, and Y is percent of enzyme activity remaining. The maximal contribution by the CYP isoform was determined as the difference between the maximum and minimum percent activity remaining based on data fitting.

The fraction of nirmatrelvir metabolized (f_m) to a single metabolite x by a specific CYP isoform z ($f_{mMx,CYPz}$) was calculated using the following equation:

$$f_{mMx,CYPz} = f_{INH} \times f_{CL}$$

where f_{INH} is the scaled fraction of metabolite (M) x inhibited by a CYP specific inhibitor z and f_{CL} is the fraction of nirmatrelvir clearance represented by metabolite x determined from enzyme kinetic experiments.

Single Point Selective Inhibition of CYP3A4 vs CYP3A5 Activity in HLM. Nirmatrelvir (2.5 μM) was incubated with HLM pooled from CYP3A5*1/*1 (extensive metabolizers) (Sekisui XenoTech, Kansas City, KS) in the presence of selective chemical inhibitors ketoconazole (1 μM) (CYP3A4/5) and CYP3cide (1 μM) (CYP3A4) for the determination of CYP3A4 and CYP3A5 isoform contribution to the metabolism of nirmatrelvir. Incubations (0.3 ml) were conducted in 100 mM potassium phosphate buffer (pH 7.4) containing MgCl_2 (3.3 mM), NADPH (1.2 mM), 0.3 mg/mL HLM at 37 °C for 15 min in a heat block. Chemical inhibition studies were conducted in triplicate. Stock solutions of ketoconazole and CYP3cide were

prepared in 50/50 methanol/H₂O. The microsome mixtures containing inhibitor and NADPH were warmed for 5 min (keto) or 10 min (CYP3cide) prior to initiating the reaction by addition of nirmatrelvir. Solvent control incubations were performed in the absence of chemical inhibitors. Sample bioanalysis for analyte quantitation was performed using LC-MS/MS as previously described in the enzyme kinetics procedure. To discern the contributions of CYP3A4 and CYP3A5 isoforms to nirmatrelvir metabolism in HLMs, the differential effects of CYP3A and CYP3A4 selective chemical inhibitors on metabolite formation rate was calculated using the following formula:

$$\% \text{Inhibition (unscaled)} = \left(1 - \frac{v_{\text{inh}}}{v_{\text{ctrl}}}\right) \times 100\%$$

where v_{inh} is the formation rate in the presence of inhibitor and v_{ctrl} is the formation rate in the solvent control incubation. Determination of CYP3A5 contribution to each metabolic pathway was calculated as the difference in % inhibition by ketoconazole (CYP3A4/5) versus CYP3cide (CYP3A4) in HLM 3A5 *1/*1. Statistical analyses were conducted using GraphPad v. 8.02 to determine the statistical significance of inhibitor effect on metabolite formation rate versus minus inhibitor solvent control, and between the effects of ketoconazole versus CYP3cide, using a one-way ANOVA analysis with a Bonferonni's Test for multiple comparisons. Statistical significance was set at $\alpha = 0.01$.

Results

Passive Permeability, Efflux Potential, and Solubility Characteristics of Nirmatrelvir.

nirmatrelvir (molecular weight = 499.53) is a moderately lipophilic (LogP = 1.68) neutral compound, which demonstrated low passive permeability (P_{app}) of 1.76×10^{-6} cm/s in the low efflux Madin-Darby canine kidney (MDCKII-LE) cell assay (Di et al., 2011). In Caco-2 cell

monolayers, nirmatrelvir (0.11–231 μM) also exhibited low apical (A) to basolateral (B) permeability with values ranging from 0.66–1.19 $\times 10^{-6}$ cm/s (Supplemental Table 1). Co-incubation of nirmatrelvir (0.32 μM) with an efflux inhibitor cocktail comprised 10 μM Ko143 (breast cancer resistant protein (BCRP) inhibitor), 10 μM PSC833 (multidrug resistant gene 1 (MDR1) inhibitor), and 100 μM MK571 (multidrug resistance protein inhibitor) led to a considerable increase in the apparent permeability of nirmatrelvir from 0.80 ± 0.15 to 4.05 ± 0.26 cm/s (see Supplemental Table 1).

The substrate properties of nirmatrelvir towards human MDR1 and BCRP efflux transporters were examined in transporter-transfected MDCK cells by measuring the bidirectional permeability (P_{app} AB and P_{app} BA) (Supplemental Table 2). In MDCKI-MDR1 cells, nirmatrelvir (0.3–3.0 μM) demonstrated mean P_{app} (AB) and P_{app} (BA) values ranging from 0.12–0.21 ($\times 10^{-6}$ cm/s) and 9.94–10.3 ($\times 10^{-6}$ cm/s), respectively, resulting in efflux ratios of 48.4–83.7 across the concentration range. Inclusion of the MDR1 inhibitor PSC833 (5 μM) in the incubation mixtures led to a dramatic reduction (48–84 $\rightarrow$ 1.7–2.3) in nirmatrelvir efflux ratios. Under these experimental conditions, the prototypic MDR1 substrate quinidine (0.5 μM) demonstrated mean P_{app} (AB), P_{app} (BA), and efflux ratio values of 1.4 ($\times 10^{-6}$ cm/s), 78 ($\times 10^{-6}$ cm/s), and 58, respectively. Inclusion of the MDR1 inhibitor PSC833 (5 μM) decreased the efflux ratio of quinidine from ~ 58 to 1.0. The efflux ratio of nirmatrelvir (0.3–3.0 μM) in MDCKII-BCRP cells ranged from 1.4 to 1.5, suggesting that nirmatrelvir was not a substrate of human BCRP. Under these experimental conditions, the known BCRP substrate pitavastatin demonstrated mean P_{app} (AB) and P_{app} (BA) values of 0.4 ($\times 10^{-6}$ cm/s) and 11 ($\times 10^{-6}$ cm/s), respectively, which resulted in an efflux ratio of ~ 25 . Inclusion of the selective BCRP inhibitor Ko143 (5 μM) led to a significant decrease of the pitavastatin efflux ratio from 25 to 0.2.

The equilibrium solubility of crystalline nirmatrelvir-MTBE co-solvate and anhydrous ‘form 1’ in unbuffered water was 7.21 mg/ml (pH 7.9) and 1.2 mg/ml (pH 7.0), respectively. The dissolution of crystalline nirmatrelvir-MTBE co-solvate in water was ~ 20-times faster than crystalline nirmatrelvir anhydrous ‘form 1’. The likelihood of a peptidase mediated hydrolytic cleavage across the peptide backbone in nirmatrelvir was examined in gastrointestinal fluids, which included SIF and SGF and intestinal fluid from healthy human volunteers (Smart et al., 2014; Wang et al., 2015). nirmatrelvir (1 μ M) was stable ($t_{1/2} > 240$ min) towards hydrolytic cleavage in 2-hour incubations with SIF, SGF, and HIF.

Plasma Protein Binding, Blood-to-Plasma Partitioning, and Non-Specific HLM Binding of Nirmatrelvir. Nirmatrelvir (0.3–10 μ M) demonstrated moderate and concentration-independent plasma protein binding in rat, monkey, and human with mean unbound plasma fractions ($f_{u,p}$) of 0.478, 0.434, and 0.310, respectively (Supplemental Table 3). Nirmatrelvir (0.3–10 μ M) was stable in rat, monkey, and human plasma for ~ 6 h at 37 °C. The mean ($\pm$ SD) blood-to-plasma ratios (BPRs) for nirmatrelvir (1 μ M) in rat, monkey, and human were 0.83 ± 0.049 , 0.68 ± 0.024 , and 0.60 ± 0.024 , respectively, suggesting that nirmatrelvir distributed into plasma preferentially over red blood cells. The mean ($\pm$ SD) of unbound fraction of nirmatrelvir (2 μ M) in HLM (1 mg/ml protein concentration) was 0.824 ± 0.017 .

Metabolic Stability Studies with Nirmatrelvir. The metabolic stability of nirmatrelvir (1 μ M) was examined in liver microsomes (fortified with NADPH) and cryopreserved hepatocytes from rat, monkey, and human using a substrate depletion assay format (Table 1). Nirmatrelvir did not demonstrate metabolic turnover in liver microsomes from animals and human when NADPH was excluded from the incubations. In the presence of NADPH, nirmatrelvir demonstrated low metabolic turnover ($CL_{int,app} = 6.1$ μ l/min/mg) in rat liver microsomes, but underwent extensive

metabolism ($CL_{int,app} = 161 \mu\text{l/min/mg}$) in liver microsomes from monkey. Metabolic turnover in NADPH-supplemented HLM was moderate ($CL_{int,app} = 28.8 \mu\text{l/min/mg}$, unbound $CL_{int,app} = 34.9 \mu\text{l/min/mg}$). In cryopreserved hepatocytes from rat, monkey, and human, nirmatrelvir demonstrated low total $CL_{int,app}$ ($\mu\text{l/min}/10^6$ cells) of 4.64, 10.3, and 5.16, respectively. The total $CL_{int,app}$ in liver microsomes and hepatocytes were scaled to yield the corresponding hepatic clearance (CL_{hep}) values (Table 1) using the well-stirred model (Di et al., 2012).

Metabolite Characterization. Figures 2 and 3 depict HPLC-UV chromatogram of incubation mixtures of nirmatrelvir (10 μM) in NADPH-supplemented liver microsomes and cryopreserved hepatocytes from rats, monkeys, and humans, respectively. Elucidated structures of the metabolites based on mass and ^1H NMR spectral data are depicted in Figure 4. A total of 5 oxidative metabolites were observed in liver microsomes, to varying degrees, across rat, monkey, and human. The metabolites were derived from mono-hydroxylation (metabolites M1–M4) and dehydrogenation (m/z 498). Although metabolism in hepatocytes was apparently slower than in liver microsomes under the conditions examined, a similar metabolic profile was observed in the two matrices. There were no human unique metabolites of nirmatrelvir; all metabolites observed in HLM and human hepatocytes were also characterized in liver microsomes and hepatocytes from rats and monkeys. The regiochemistry of oxidation was tentatively assigned based on comparison of the mass spectral fragmentation data for the parent and its oxidized products. Structures of metabolites M1–M4 were unambiguously identified via NMR characterization of the biosynthesized samples obtained from scaled-up incubations of nirmatrelvir in NADPH-fortified HLM.

Nirmatrelvir. A protonated molecular ion (m/z^+) of 500.2473 was observed for nirmatrelvir, which is consistent with a molecular formula of $\text{C}_{23}\text{H}_{33}\text{N}_5\text{O}_4\text{F}_3$ (-1.23 ppm). Diagnostic fragment

ions in the collision-induced dissociation (CID) spectrum included m/z 319.1625 (cleavage of the acyl moiety on the 2-position of the [3.1.0]azabicyclic ring) and m/z 291.1813 (cleavage of the acyl moiety on the 3-position of the [3.1.0]azabicyclic ring), which yielded opposing portions of nirmatrelvir. Further fragmentation of m/z 319 yielded the [3.1.0]azabicyclic ring (m/z 110.0964) such that modifications of this portion of the molecule were readily observable in the mass spectra (see elucidation of M1 and M2 below).

The ^1H NMR spectra of nirmatrelvir (Supplemental Figure 1) contains three amide resonances assigned to three hydrogens; H14: δ 9.41 (d, coupling constant (J) = 8.1 Hz, 1H), H17: 9.02 (d, J = 8.4 Hz, 1H) and H23: 7.67 (s, 1H). Additionally, the ^1H NMR spectra of nirmatrelvir contains fifteen aliphatic resonances assigned to twenty-nine hydrogens; H18: 4.98 (m, 1H), H13: 4.42 (d, J = 8.3 Hz, 1H), H6: 4.17 (s, 1H), H2'': 3.92 (dd, J = 10.0, 5.4 Hz, 1H), H2': 3.70 (d, J = 10.4 Hz, 1H), H22': 3.15 (t, J = 8.9 Hz, 1H), H22'': 3.05 (q, J = 8.9 Hz, 1H), H20: 2.41 (m, 1H), H19''/21'': 2.12 (m, 2H), H19'/21': 1.72 (m, 2H), H3: 1.58 (t, J = 6.3 Hz, 1H), H5: 1.33 (d, J = 7.5 Hz, 1H), H9: 1.04 (s, 3H), H26/32/33: 0.99 (s, 9H) and H10: 0.86 (s, 3H).

Metabolite M1 (PF-07329265). Metabolite M1 (designated as PF-07329265) possessed a protonated molecular ion of m/z^+ 516.2435, consistent with a molecular formula of $\text{C}_{23}\text{H}_{33}\text{N}_5\text{O}_5\text{F}_3$ (1.3 ppm), which was indicative of a monohydroxylation in nirmatrelvir. The dominant fragment ion (m/z 335.1585) in the CID spectrum of PF-07329265 suggested that the N-((S)-1-cyano-2-((S)-2-oxopyrrolidin-3-yl)ethyl)formyl portion in nirmatrelvir was not modified (Supplemental Figure 2). In the high energy C-trap dissociation (HCD) spectrum, the fragment ion of m/z 126.0917 is diagnostic of the region of hydroxylation being the 6,6-dimethyl-3-azabicyclo[3.1.0]hexane fused ring.

The ^1H NMR spectrum (Supplemental Figure 3) of biosynthesized PF-07329265 revealed that only the resonances of the methyl hydrogens (H9 and H10) of the 6,6-dimethyl-3-azabicyclo[3.1.0]hexane fused ring had changed significantly from those observed in the ^1H NMR spectrum of nirmatrelvir. Notably, the ^1H methyl resonance observed at δ 1.04 in nirmatrelvir was absent in isolated PF-07329265, and a new resonance was observed at δ 3.24 which integrated to two hydrogens. In the ^1H - ^{13}C multiplicity edited heteronuclear single quantum coherence (HSQC) data of the isolate, this new resonance correlated to a carbon with a chemical shift of δ 67.9 (Supplemental Figure 4, panels B and C). The integration, ^1H and ^{13}C chemical shifts of this new resonance are all consistent with the oxidation of one of the geminal-dimethyl groups on the azabicyclohexane moiety.

To determine the configuration of this stereocenter, a series of ROE experiments were performed (Supplemental Figure 4, panels D-F). Irradiation of ^1H methyl resonance at δ 0.86 led to an enhancement of the resonance assigned as the H6 methine and the H2' methylene. Additionally, when the new resonance at δ 3.24 was irradiated, only the remaining methyl of the dimethylazabicyclohexane motif was significantly enhanced. With the stereo configuration of carbon 6 of the dimethylazabicyclohexane fixed in the *S* orientation, an energy minimized three dimensional structure of PF-07329265 (Supplemental Figure 5) placed the remaining methyl at 2.6 Å from the C2-position methine (carbon 6 using Advanced Chemistry Development laboratory nomenclature). In this model, the new methylene is 3.8 Å from the C2-position methine. Based on this data, the structure of M1 (PF-07329265) is assigned as (1*R*,2*S*,5*S*,6*S*)-N-[(1*S*)-1-cyano-2-[(3*S*)-2-oxopyrrolidin-3-yl]ethyl]-6-(hydroxymethyl)-6-methyl-3-[3-methyl-N-(trifluoroacetyl)-L-valyl]-3-azabicyclo[3.1.0]hexane 2-carboxamide.

Metabolite M2. Metabolite M2 (PF-07329266) possessed a protonated molecular ion of m/z^+ 516.2441, which was consistent with a molecular formula of $C_{23}H_{33}N_5O_5F_3$ (2.5 ppm) and also suggested an monohydroxylation in nirmatrelvir. The CID spectrum possessed the same fragment ions as M1 (PF-07329265) but with differing abundances suggesting that, like M1, the hydroxylation has occurred on the 6,6-dimethyl-3-azabicyclo[3.1.0]hexane fused ring (Supplemental Figure 6). The 1H NMR spectrum of biosynthesized PF-07329266 indicated that only the methyl protons in the dimethylazabicyclohexane ring had changed significantly from the ones observed in similarly acquired spectra of nirmatrelvir (Supplemental Figure 7). The 1H methyl resonance observed at δ 0.86 was absent in the isolated material with a new resonance observed at δ 3.26 which integrated to two protons. This data established that M2 (PF-07329266) is a diastereomer of M1 (PF-07329265) at the C6-position whose structure is assigned as (1*R*,2*S*,5*S*,6*R*)-*N*-{(1*S*)-1-cyano-2-[(3*S*)-2-oxopyrrolidin-3-yl]ethyl}-6-(hydroxymethyl)-6-methyl-3-[3-methyl-*N*-(trifluoroacetyl)-*L*-valyl]-3-azabicyclo[3.1.0]hexane-2-carboxamide.

Metabolite M3. Metabolite M3 (PF-07329267) possessed a protonated molecular ion of m/z^+ 516.2268, consistent with a molecular formula of $C_{23}H_{33}N_5O_5F_3$ (3.8 ppm) and a monohydroxylated nirmatrelvir metabolite. The CID spectra of m/z 516 did not provide meaningful fragment ions for structural assignment, however the HCD spectrum afforded m/z 237.0856 and 110.0968 as additional diagnostic fragment ions (Supplemental Figure 8). The latter ion indicates that the azabicyclohexane ring is unmodified. The former, m/z 237.0856, is consistent with azabicyclohexane ring scission yielding an ion possessing the trifluoroacetyl and tert-butyl groups with a dehydrated hydroxyl group. This creates the possibilities of hydroxylation of the amide nitrogen of the trifluoroacetamide, the tert-butyl group, or the carbon connecting these two. The 1H spectrum of biosynthesized PF-07329267 (Supplemental Figure 9)

indicated a change in the proton resonances of the tert-butyl methyl relative to those observed in similarly acquired spectra for nirmatrelvir. The tert-butyl resonance in nirmatrelvir displayed a chemical shift at δ 0.99, which integrated to 9 hydrogen atoms. In the ^1H spectrum of PF-07329267, the resonance was now at δ 0.89 and integrated to a total of 6 hydrogen atoms. Additionally, the HSQC spectral data revealed the presence of a new inequivalent methylene pair of resonances at δ 3.14/3.53 corresponding to a carbon with a chemical shift of 68.5 (Supplemental Figure 10). Based on these data the structure of M3 (PF-07329267) is assigned as (1*R*,2*S*,5*S*)-*N*-{(1*S*)-1-cyano-2-[(3*S*)-2-oxopyrrolidin-3-yl]ethyl}-3-[4-hydroxy-3-methyl-*N*-(trifluoroacetyl)-*L*-valyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide.

Metabolite M4. Metabolite M4 (PF-07329268) was the dominant oxidative metabolite of nirmatrelvir in liver microsomes and hepatocytes from animals and human. PF-07329268 comprised two closely eluting interconverting diastereomers, and possessed a protonated molecular ion of m/z^+ 516.2436, which was consistent with a molecular formula of $\text{C}_{23}\text{H}_{33}\text{N}_5\text{O}_5\text{F}_3$ (1.5 ppm), and indicated yet another monohydroxylation in nirmatrelvir. PF-07329268 underwent facile in-source dehydration to m/z 498.2327, and subsequent fragmentation was conducted on this major ion (Supplemental Figure 11). The CID spectra of m/z 498 yielded a major ion of m/z 319.1636, which indicates that the azabicyclo[3.1.0]hexanyl-dimethyloxobutane-trifluoroacetamide portion was unmodified. The facile loss of water and the equilibrating diastereomers suggest, but do not prove, that the site of hydroxylation is on the C5-position of the pyrrolidinone ring. The ^1H spectrum of biosynthesized PF-07329268 (Supplemental Figure 12) revealed that the resonances of the C22 methylene were absent. Moreover, the ^1H - ^{13}C HSQC spectra of the isolated metabolite revealed the presence of a new ^1H methine resonance at δ 5.01, which correlated with a carbon atom at a chemical shift of δ 77.3

(Supplemental Figure 13). Based on these data, M4 (PF-07329268) was assigned as (1*R*,2*S*,5*S*)-*N*-{(1*S*) 1-cyano-2-[(3*R*)-5-hydroxy-2-oxopyrrolidin-3-yl]ethyl}-6,6-dimethyl-3-[3-methyl-*N*-(trifluoroacetyl)-*L*-valyl]-3-azabicyclo[3.1.0]hexane-2-carboxamide.

Pharmacologic Activity of M4 (PF-07329268). M4 (PF-07329268), the major in vitro oxidative metabolite of nirmatrelvir in animals and human, was active against recombinant SARS-CoV-2 3CL^{pro} with a K_i value (3 nM) comparable to the one noted with nirmatrelvir (Owen et al., 2021). Moreover, PF-07329268 also repressed SARS-CoV-2 replication in epithelial Vero E6 cells (Boras et al., 2021). However, the corresponding EC_{50} for PF-07329268 in the cellular antiviral assay was relatively weaker than the one observed with the parent compound (PF-07329268 EC_{50} = 690 nM, PF-07321332 EC_{50} = 74.5 nM).

Metabolite m/z 498. A protonated molecular ion at m/z^+ 498.2332 was observed, consistent with a molecular formula of $C_{23}H_{31}N_5O_4F_3$ (1.9 ppm). While this might be considered an in-source dehydration product of a hydroxylated metabolite, the presence of a sodiated ion at m/z 520.2151 ($C_{23}H_{30}N_5O_4F_3Na$; 1.7 ppm) precludes this possibility and suggests that it is a dehydrogenated metabolite of nirmatrelvir. The fragments in CID and HCD spectra for this metabolite (Supplemental Figure 14) are identical to those generated from fragmentation of the in-source dehydrated ion of m/z 498 of metabolite M4, therefore it is likely that the dehydrogenation occurred on the pyrrolidinone ring. However, the exact site of the dehydrogenation was not clear.

HPLC-UV profile of nirmatrelvir (10 μ M) incubations with a panel of major human recombinant enzymes revealed that only CYP3A4 (and 3A5) (see Figure 2) were able to recapitulate the metabolic pathways of nirmatrelvir observed in NADPH-supplemented HLM and human hepatocytes.

Enzyme Kinetics and f_{CL} Determination for Nirmatrelvir Metabolism in HLM. Enzyme kinetic parameters and unbound $CL_{int,app}$ values for the formation of M1 (PF-07329265), M2 (PF-07329266), M3 (PF-07329267), and M4 (PF-07329268) in NADPH-supplemented HLM incubations with nirmatrelvir (0.02–200 μ M) are depicted in Table 2, and associated kinetic data are presented graphically in Figure 5 and corresponding Eadie-Hofstee plots are presented in Supplemental Figure 15. Visual inspection of the Eadie-Hofstee plots suggested typical hyperbolic Michaelis-Menten kinetics for M1 and M4. Metabolite formation rates for M1 and M4 were fitted to a Michaelis-Menten model with resulting K_M and V_{max} values of 28.0 μ M and 17.2 pmol/min/mg, and 25.5 μ M and 381 pmol/min/mg, respectively. Formation rates for M2 were best fitted to a 2-enzyme non-saturable model where the low affinity kinetic parameters could not be accurately defined with the nirmatrelvir concentration range tested, and therefore the secondary rate ($CL_{int(2)}$) of the low affinity enzyme(s) was estimated by linear approximation. The K_M , V_{max} , and $CL_{int(2)}$ estimates for M2 were 24.0 μ M, 9.79 pmol/min/mg, and 0.00508 μ L/min/mg, respectively. M3 formation rate data were best fit to an allosteric sigmoidal model where the estimated K_{half} , V_{max} , and Hill values were 31.0 μ M, 15.2 pmol/min/mg, and 0.959, respectively. Based on these kinetic parameter estimates, the unbound $CL_{int,app}$ values for the formation of M1, M2, M3, and M4 were 0.654, 0.439, 0.522, and 15.9 μ L/min/mg, yielding a summed unbound $CL_{int,app}$ of 17.5 μ L/min/mg for the oxidative metabolism of nirmatrelvir to M1–M4. Based on the output of the metabolite identification work, the in vitro hepatic metabolism of nirmatrelvir can be assumed to be predominantly oxidative in HLM, with an overall $f_{m,CYP}$ of 1. The fractions of nirmatrelvir clearance (f_{CL}) representing formation of M1, M2, M3, and M4 were 0.037, 0.025, 0.030, and 0.91, respectively.

Contribution of CYP3A4/5 in the Oxidative Metabolism of Nirmatrelvir. Contributions by CYP3A4/5 to the metabolism of nirmatrelvir were examined using a multiple concentration CYP selective chemical inhibitor study design in HLM (Table 3 and Figure 6). For all four metabolites, inhibition curves were best fit to a four-parameter inhibition model. In the presence of ketoconazole (0.001–10 μ M) the formation of M1, M2, M3, and M4 was inhibited 95%, 96%, 98%, and 99%, respectively.

The relative contribution of CYP3A4 and CYP3A5 towards the oxidative metabolism of nirmatrelvir to M1–M4 was examined in HLM from genotyped individuals expressing high (CYP3A5*1/*1) CYP3A5 enzyme in the presence or absence of non-selective CYP3A4/5 and selective CYP3A4 inhibitors, ketoconazole and CYP3cide (Tseng et al., 2014), respectively (Table 4). Differences between the inhibitory effects of CYP3cide and ketoconazole for the formation of M1 (3.7%), M3 (3.0%), and M4 (12%) were not statistically significant using a one-way ANOVA with Bonferroni's multiple comparison test ($\alpha = 0.01$). Assessment of the differential inhibitory effects on M2 formation rates was not possible due to concentrations below the limit of quantitation in the presence of both inhibitors. Because M2 represents a minor metabolic pathway ($f_{CL} = 0.025$), further studies were not conducted. In summary, no significant CYP3A5 contribution is expected to the overall metabolism of nirmatrelvir in CYP3A5*1/*1 individuals.

Assigned CYP f_m values for the oxidative metabolites of nirmatrelvir in HLM, based on the combined results of nirmatrelvir enzyme kinetics and chemical inhibition studies, are presented in Table 3. After normalizing the contribution of CYP3A to the f_{CL} for each metabolic pathway, the fraction of M1, M2, M3, and M4 generated by CYP3A4 ($f_{m,CYP3A}$) was 0.036, 0.024, 0.029, and 0.90, respectively, with a combined CYP3A4 f_m of 0.99 and f_m related to other unassigned

CYPs of 0.01. Collectively, these results indicated that the major enzyme involved in the metabolism ($f_m = 0.99$) of nirmatrelvir in HLM is CYP3A4.

Biliary Excretion of Unchanged Nirmatrelvir in SCHH. The potential for hepatobiliary excretion of nirmatrelvir in unchanged form was examined in SCHH. Following a 10 min incubation in SCHH, the corresponding $CL_{\text{biliary,int,app}}$ for nirmatrelvir (0.5 μM) in SCHH was 2.1 $\mu\text{L}/\text{min}/\text{mg}$, which was scaled using the methodology described by Kimoto et al (2017) to yield a predicted human CL_{bile} of 2.7 $\text{mL}/\text{min}/\text{kg}$. The corresponding biliary excretion index of nirmatrelvir was $\sim 43\%$. In comparison, positive control rosuvastatin (0.5 μM) (Kimoto et al., 2017) demonstrated $CL_{\text{biliary,int,app}}$ and biliary excretion index of 5.0 $\mu\text{L}/\text{min}/\text{mg}$ and 52%, respectively.

PK of Nirmatrelvir in Animals. The PK parameters of nirmatrelvir after single iv and po dose administration in rats and monkeys is shown in Table 5 and mean plasma concentrations versus time profile are shown in Supplemental Figure 16. Nirmatrelvir (1 mg/kg) demonstrated moderate plasma clearance (CL_p) in rats (27.2 $\text{mL}/\text{min}/\text{kg}$) and monkeys (17.1 $\text{mL}/\text{min}/\text{kg}$) with low (0.33 L/kg – monkeys) to moderate (1.8 L/kg – rats) $V_{d_{ss}}$ values in the two species. The terminal $t_{1/2}$ ranged from 0.79 h (monkeys) to 5.1 h (rats). The percentage of unchanged nirmatrelvir excreted over a 24-h period in rat and monkey urine was 17% and 7.0%, which yielded CL_{renal} values of 4.5 and 1.3 $\text{mL}/\text{min}/\text{kg}$, respectively. The unbound renal blood clearance of nirmatrelvir in rats and monkeys, estimated using the protocol described by Dong et al (2016), was 12 $\text{mL}/\text{min}/\text{kg}$ and 4.0 $\text{mL}/\text{min}/\text{kg}$, respectively, and was within the range of the glomerular filtration rate in the respective species (rats = 8.7 $\text{mL}/\text{min}/\text{kg}$ and monkeys = 4.0 $\text{mL}/\text{min}/\text{kg}$) (Lin, 1995), suggesting little-to-no active tubular secretion. Consistent with these observations were the findings on the lack of substrate properties of nirmatrelvir towards a broad array of the major

human renal transporters, including the organic anion transporters (OAT)1 and 3, multidrug and toxin extrusion protein (MATE)1 and 2K, organic cation transporter 2, and organic anion transporting polypeptide (OATP)4C1 (Pfizer data on file). Following iv administration of nirmatrelvir (1 mg/kg) to bile-duct cannulated rats, $8.9 \pm 4.7\%$ of the administered dose was observed in rat bile in unchanged parent form, which translated to a $CL_{biliary}$ of 2.4 ml/min/kg. Comparison of the predicted CL_{hep} values using NADPH-supplemented liver microsomes and hepatocytes from rats and monkeys (Table 1) with the observed CL_{plasma} (after subtracting for CL_{renal} and CL_{bile}) revealed a reasonably good (within ~ 2.5 – 5.0 -fold in rats and within 2-fold in monkeys) in vitro-in vivo correlation for clearance.

Oral PK studies were conducted with two crystalline versions of nirmatrelvir; nirmatrelvir-MTBE co-solvate and anhydrous nirmatrelvir ‘Form 1’. Following po administration of nirmatrelvir (10 mg/kg) as MTBE co-solvate, nirmatrelvir was rapidly absorbed ($T_{max} = 0.25$ – 1.5 h) in rats and monkeys, with corresponding oral bioavailability (F) values of 50% and 8.5%, respectively. The corresponding fraction of the nirmatrelvir po dose absorbed ($F_a \times F_g$) in rats and monkeys was 82% and 18%, respectively. The relatively lower F of nirmatrelvir in monkeys is likely due to CYP-mediated oxidative metabolism along the gastrointestinal tract, since nirmatrelvir demonstrated a facile metabolic decline ($t_{1/2} = 20.5$ min, $CL_{int,app} = 33.8$ μ l/min/mg) in monkey intestinal microsomes in a NADPH-dependent fashion. This hypothesis is further substantiated by the observation that unchanged nirmatrelvir constituted $\sim 4\%$ of the administered dose in feces collected from monkeys after the po dosing (data not shown). As such, nirmatrelvir did not demonstrate any metabolic turnover ($t_{1/2} > 240$ min, $CL_{int,app} < 2.89$ μ l/min/mg) in rat and human intestinal microsomes in the presence of NADPH. Anhydrous nirmatrelvir ‘Form 1’ orally administered (10 mg/kg) to rats also demonstrated rapid absorption

($T_{\max} = 0.25$ h), albeit with a lower F and lower $F_a \times F_g$ values of $34 \pm 19\%$ and 56% , when compared with the anhydrous nirmatrelvir-MTBE co-solvate.

Interactions with Human CYP Enzymes. CYP Inhibition. Nirmatrelvir exhibited no reversible ($IC_{50} > 100$ μ M) inhibition of CYP1A2 (phenacetin-*O*-dealkylation), CYP2B6 (bupropion hydroxylation), CYP2C8 (amodiaquine *N*-dealkylation), CYP2C9 (diclofenac-4'-hydroxylation), CYP2C19 (*S*-mephenytoin-4'-hydroxylation), and CYP2D6 (dextromethorphan-*O*-demethylation) catalytic activities in NADPH-supplemented HLM. Nirmatrelvir, however, demonstrated weak reversible inhibition of CYP3A4/5 catalyzed testosterone-6 β -hydroxylase, midazolam-1'-hydroxylase, and nifedipine oxidase activities with IC_{50} values of 58.3 μ M, 106 μ M, and 45.1 μ M, respectively (Supplemental Table 4). An IC_{50} shift of 2.13x (midazolam-1'-hydroxylase) and 1.73x (testosterone-6 β -hydroxylase) following a 30-min preincubation of nirmatrelvir in NADPH-supplemented HLM (Supplemental Table 4), suggested that nirmatrelvir is a reversible and time-dependent inhibitor of CYP3A enzyme activities. In the IC_{50} shift assay, nirmatrelvir was devoid of time-dependent inhibition of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 enzyme activities. Under the present experimental conditions, known CYP isozyme-specific reversible (and time-dependent) positive control inhibitors (Walsky and Obach, 2004) demonstrated potent reversible and time-dependent inhibition of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A activity, respectively, in NADPH-supplemented HLM (Pfizer data on file).

Kinetics for the Time-dependent inhibition (TDI) of CYP3A by Nirmatrelvir. Nirmatrelvir demonstrated time (1–40 min)- and concentration (0.1–100 μ M)-dependent inhibition of the midazolam-1'-hydroxylase and testosterone-6 β -hydroxylase activities in NADPH-supplemented HLM (Figure 7). Nirmatrelvir (100 μ M) did not cause TDI of CYP3A activity in HLM

incubations in the absence of NADPH (see Figure 7). The corresponding kinetic constants maximal rate of inactivation (k_{inact}) and inactivator concentration required to achieve 50% k_{inact} (K_I) for TDI of the CYP3A catalyzed midazolam-1'-hydroxylase (and testosterone 6 β -hydroxylase) activity by nirmatrelvir were 0.0142 min⁻¹ (0.0165 min⁻¹) and 15.5 μ M (13.9 μ M), respectively. The ratio of k_{inact}/K_I for the two marker substrates midazolam and testosterone was 0.916 ml/min/ μ mol and 1.23 ml/min/ μ mol, respectively (Supplemental Table 5). The rates of CYP3A inactivation in HLM with positive control verapamil (Walsky and Obach, 2004) were within historical results (Pfizer data on file).

Metabolic Intermediate (MI) Complex Formation. The potential for quasi-irreversible inhibition of CYP3A4 by nirmatrelvir via the formation of a metabolic intermediate complex (Orr et al., 2012) was examined in incubations of nirmatrelvir with NADPH-supplemented recombinant human CYP3A4 supersomes by monitoring the absorption spectra for the signature MI Soret absorbance at λ ~455 nm (Franklin, 1974). As shown in Supplementary Figure 17, the absorbance at 455 nm did not increase following addition of NADPH to incubations of CYP3A4 supersomes and nirmatrelvir. In contrast, an expected increase in absorbance at 455 nm was observed in incubations of CYP3A4 supersomes/NADPH with the positive control troleandomycin, which is known to inactivate CYP3A4 via MI complex formation (Mayhew et al., 2000).

CYP Induction. The potential for induction of CYP3A4 messenger RNA (mRNA) and enzyme (midazolam-1'-hydroxylase) activity was examined in 4 lots (BXM, BNA, FOS, and HH1144) of cryopreserved human hepatocytes treated with nirmatrelvir (0.01-200 μ M) for 48 hours. Nirmatrelvir demonstrated moderate induction of CYP3A mRNA levels and in a dose-dependent fashion in all four donor hepatocytes. Induction of CYP3A mediated midazolam-1'-

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hydroxylase activity was seen in 3 out of the 4 lots. The maximal fold induction (Ind_{max}) and inducer concentration required to achieve 50% Ind_{max} (EC_{50}) values for fold increases in CYP3A mRNA levels and midazolam-1'-hydroxylase activity in the four individual human hepatocytes with nirmatrelvir are shown in Supplemental Table 6. A cytotoxicity assay using Promega's CellTiter 96® aqueous nonradioactive cell proliferation assay kit (Promega, WI) showed no reduction in hepatocyte cell viability after treatment with nirmatrelvir at the tested concentrations. Under these experimental conditions, treatment of the four donor human hepatocyte lots with a single concentration (10 μM) of the positive control rifampicin led to the expected increases in CYP3A4 mRNA and catalytic activity. Moreover, rifampin demonstrated ~ 2–3-fold greater increase in CYP3A mRNA and ~ 2–5-fold greater increase in enzyme activity relative to the effects observed with nirmatrelvir at the higher end of its tested concentration range (50–200 μM).

Interactions with Human Drug Transporters. Inhibitory effects of nirmatrelvir against the major human intestinal, hepatobiliary and renal drug transporters were examined using human embryonic kidney (HEK)293 cells or vesicles (Supplemental Table 7). Nirmatrelvir inhibited N-methylquinidine transport in human MDR1 expressing HEK293 vesicles with an IC_{50} value of 71 μM . Inhibition of rosuvastatin transport ($\text{IC}_{50} = 44 \mu\text{M}$) in OATP1B1 expressing HEK293 cells was also observed with nirmatrelvir. Relatively weaker inhibition (IC_{50} 's $> 100 \mu\text{M}$) by nirmatrelvir was noted against OATP1B3, the organic cation or anion transporters (OCT1, OCT2, OAT1 and OAT3), MATE1, and MATE2K.

Discussion

A major objective of our discovery campaign was to identify a compound(s) that had improved preclinical oral absorption relative to the intravenous agent PF-00835231 (oral F and $F_a \times F_g =$

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1.4% and 3.3% (rat), < 0.05% and < 1% (monkey)), while retaining the antiviral potency towards SARS-CoV-2 (Owen et al., 2021). The low oral absorption of PF-00835231 was largely attributed to a high hydrogen bond donor (HBD) count (Veber et al., 2002), which resulted in poor passive permeability (MDCK II $P_{app} < 0.27 \times 10^{-6}$ cm/s) across cells (Boras et al., 2021). Reduction in HBD count (PF-00835231 HBD = 5 $\rightarrow$ PF-07321332 (nirmatrelvir) HBD = 3) was achieved through the M^{pro} co-crystal structure-guided replacements of the P1' (α -hydroxymethyl ketone $\rightarrow$ nitrile), P2 (leucine $\rightarrow$ 6,6-dimethyl-3-azabicyclo[3.1.0]hexane as a cyclic leucine mimetic), and P3 (methoxyindole $\rightarrow$ trifluoroacetamide capping group) structural elements to yield a broad coronavirus inhibitor PF-07321332 (nirmatrelvir), which retained the potent inhibitory attributes of PF-00835231 towards SARS-CoV-2 3CL pro , and possessed antiviral activity in cells, and in a mouse-adapted SARS-CoV-2 model of COVID-19 (Owen et al., 2021). Nirmatrelvir also demonstrated pan-human coronavirus activity through inhibition of 3CL pro enzymes associated with alpha- and beta-coronaviruses capable of infecting humans (Owen et al., 2021). Lack of inhibitory effects towards several mammalian protease enzymes established the selectivity of nirmatrelvir as a coronavirus inhibitor.

A reduced HBD count also led to considerable improvements in oral absorption. Although nirmatrelvir demonstrated relatively low passive permeability, and was a MDR1 substrate in vitro, oral administration of two crystalline forms (MTBE co-solvate and anhydrous 'form 1') of nirmatrelvir (10 mg/kg) to rats led to reasonable oral F (34-50%), with the MTBE co-solvate demonstrating better absorption relative to the anhydrous 'form 1'. The superior oral absorption profile of the MTBE co-solvate presumably occurred because of its higher thermodynamic aqueous solubility and faster dissolution rate as compared to 'form 1'. The relatively poor bioavailability of MTBE co-solvate in monkeys was attributed to oxidative metabolism by CYP

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enzymes along the gastrointestinal tract, based on the monkey intestinal microsomal stability data. Nirmatrelvir was resistant to CYP oxidations in intestinal microsomal preparations from rats and human, and moreover, complete resistance towards peptide backbone hydrolysis via the putative action of digestive enzymes was also noted in gastrointestinal fluids.

The low oral F (8.5%) of nirmatrelvir in monkeys did not deter toxicological investigations in this species (Owen et al., 2021). Daily dosing of nirmatrelvir-MTBE co-solvate (60–600 mg/kg) in 2-week regulatory toxicity studies led to robust increases in $C_{\max}$ and AUC (Supplemental Table 8), most likely due to saturation and/or autoinhibition of first-pass oxidative metabolism in monkeys with increasing doses. Corresponding unbound systemic concentration ($C_{\max}$ and AUC) margins at the highest dose (600 mg/kg) were > 200-fold over the measured unbound EC_{90} (181 nM) in the dNHBE assay. Rats orally administered with nirmatrelvir (40–1000 mg/kg) yielded unbound concentrations of ~ 273-fold ($C_{\max}$) and 65-fold (AUC) over the dNHBE unbound EC_{90} at the 1000 mg/kg dose (Owen et al., 2021). nirmatrelvir was virtually devoid of any adverse findings at the highest doses tested in animals. It is noteworthy to point out that the SARS-CoV-2 dNHBE EC_{90} was also targeted as a minimally efficacious clinical concentration based on the observation that trough/ $C_{\min}$ concentrations over cellular antiviral EC_{90} values are required for clinical efficacy with protease inhibitors and other antivirals (Food and Drug Administration, 2006; Reddy et al., 2012). Improvements in oral absorption also permitted a thorough evaluation of the antiviral activity of nirmatrelvir in a mouse model of COVID-19. Twice daily administration of nirmatrelvir (300 and 1000 mg/kg) in mice for 4 days protected the animals from weight loss and led to statistically significant reductions in viral load in the lungs of the infected animals with corresponding trough plasma concentrations maintained at ~0.9-fold and 4.0-fold over the dNHBE EC_{90} (Owen et al., 2021).

The anticipated clearance mechanisms of nirmatrelvir in humans include oxidative metabolism, almost exclusively by CYP3A4, and some degree of renal and biliary excretion. In NADPH-supplemented HLM and human hepatocytes, nirmatrelvir demonstrated moderate metabolic turnover, to yield $CL_{int,app}$ values of 28.8 $\mu\text{l}/\text{min}/\text{mg}$ and 5.16 $\mu\text{l}/\text{min}/10^6$ cells, respectively, which were scaled to afford estimated CL_{hep} values of 6.3 and 3.2 $\text{ml}/\text{min}/\text{kg}$, respectively. Metabolic pathways of nirmatrelvir in liver microsomes and hepatocytes from animals and human, comprising exclusively of CYP oxidation products, were virtually identical. The major metabolite M4, derived from pyrrolidinone ring oxidation in nirmatrelvir, demonstrated potent inhibition of recombinant SARS-CoV-2 3CL^{pro} activity, but was relatively weaker as an antiviral agent in cells, potentially due to an impaired permeability, which was driven by the introduction of an additional HBD (OH group). Reaction phenotyping revealed that the selective CYP3A4/5 inhibitor ketoconazole inhibited the formation of the oxidative metabolites M1–M4 in HLM by 95–99%, with minimal contribution from CYP3A5 in pooled HLM with high CYP3A5*1/*1 activity. Overall, this in vitro exercise, which established a preponderance of CYP3A4 mediated metabolism of nirmatrelvir, also created a potential opportunity to enhance the therapeutic concentrations of nirmatrelvir in humans following concomitant administration with the CYP3A4 inhibitor ritonavir. Ritonavir is used as a PK booster of several marketed protease inhibitors such as darunavir and lopinavir that are predominantly metabolized by CYP3A4 (Cvetkovic and Goa, 2003; Mckeage et al., 2009; Larson et al., 2014).

Examination of inhibitory effects of nirmatrelvir towards major human CYP enzymes revealed inhibitory (reversible and time-dependent) and induction effects only against CYP3A. The mechanistic basis for the CYP3A TDI by nirmatrelvir is presently unclear, and rules out a

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quasi-irreversible inhibitory pathway via MI complex formation as previously demonstrated with some peptidomimetics (Ernest et al., 2005). Projection of PK interactions through mechanistic modeling (Food and Drug Administration, 2020) of the predicted human PK parameters and the in vitro CYP3A inhibition/induction data suggested that nirmatrelvir is likely to increase the AUC of drugs metabolized by CYP3A by 3.0–4.0-fold (Pfizer data on file). Moreover, regulatory guidance (European Medicines Agency, 2012; Food and Drug Administration, 2020) for assessing DDIs arising from inhibition of drug transporters also suggested the possibility of interactions between nirmatrelvir and drugs that are substrates for MDR1 and OATP1B1, based on the weak in vitro inhibitory effects noted for these transporters. Overall, the disposition attributes, particularly the dominance of CYP3A-catalyzed oxidative metabolism over non-metabolic elimination pathways, and the CYP3A4 inhibitory/inducing effects of nirmatrelvir is largely reminiscent of the profiles seen with marketed peptidomimetics, particularly ones (e.g. indinavir, darunavir, and lopinavir) used to treat acquired immunodeficiency syndrome (Lin et al., 1996; Kumar et al., 2004; Vermeir et al., 2009).

First-in-human pharmacokinetics of the crystalline anhydrous ‘form 1’ of nirmatrelvir administered as a single agent (150 mg) and in combination with ritonavir (nirmatrelvir 250 mg + ritonavir at -12 h, 0 h, and 12 h) (www.ClinicalTrials.gov NCT04756531) revealed a considerable enhancement in systemic concentrations of nirmatrelvir in the presence of ritonavir (Owen et al., 2021), which was consistent with the predominant role of CYP3A4 in nirmatrelvir metabolic clearance. The plasma concentrations of nirmatrelvir in the presence of ritonavir at 12 h post-dose were considerably greater than the SARS-CoV-2 antiviral EC₉₀ value in dNHBE cells, which bolstered our confidence in attaining antiviral activity against pan coronaviruses (Owen et al., 2021). Detailed data from the safety, tolerability, and clinical pharmacokinetics of

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nirmatrelvir and nirmatrelvir/ritonavir combination across single- and multiple-ascending doses have been recently submitted for publication (Singh et al., 2022). In a recent phase 2/3 trial (Study NCT04960202 on [ClinicalTrials.gov](https://clinicaltrials.gov)), the combination of nirmatrelvir/ritonavir (Paxlovid™) demonstrated robust efficacy at reducing hospitalization and death in high-risk COVID-19 patients by 89% (within three days of symptom onset) and 88% (within five days of symptom onset) compared to placebo. Paxlovid™ has been recently granted emergency use authorization by multiple regulatory agencies across the globe including the United States Food and Drug Administration for the treatment of mild-to-moderate COVID-19 in adults and pediatric patients (12 years of age and older), who are at high risk for progression to severe COVID-19, including hospitalization or death.

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Authorship Contributions

Participated in research design: Kalgutkar, Boras, Di, Obach, Singh

Conducted experiments: Dantonio, Eng, Kadar, Kimoto, Novak, Obach, Walker

Contributed new reagents or analytic tools: Obach, Walker

Performed data analysis: Boras, Dantonio, Di, Lin, Eng, Kadar, Kimoto, Novak, Obach, Patel, Walker, Singh

Wrote or contributed to the writing of the manuscript: Kalgutkar, Boras, Dantonio, Di, Eng, Kadar, Kimoto, Obach, Patel, Walker, Singh

Footnotes

[§]ASK, BB, AD, LD, HE, EPK, EK, JJN, RSO, NCP, GSW, and RSPS are employees of Pfizer and some of the authors are shareholders in Pfizer Inc. JJN was an employee of Pfizer Inc. during part of this study.

[‡] Present address: Praxis Precision Medicines; Cambridge, MA 02142.

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Figure Legends

Figure 1. Chemical structures of PF-00835231 (intravenous COVID-19 clinical candidate) and PF-07321332 (nirmatrelvir, oral COVID-19 clinical candidate) and binding mode of PF-07321332 in the 3CL^{pro} active site of SARS-CoV-2. PF-07321332 inhibits 3CL^{pro} activity via a reversible covalent bond formation between its nitrile group and an active site Cys145 residue.

Figure 2. HPLC-UV chromatograms of NADPH-supplemented liver microsomal incubations and human recombinant CYP3A4 with PF-07321332 (nirmatrelvir, 10 μ M). Drug-related peaks are denoted with P for parent drug and M for metabolites. All unmarked peaks represent non-drug-related interferences present in control incubations (not shown). Metabolite M2 in the human liver microsomal incubation is a shoulder on the larger interfering peak. Metabolite M4 is present as two closely eluting peaks representing the two interconverting diastereomers.

Figure 3. HPLC-UV chromatograms of cryopreserved primary hepatocyte incubation extracts for nirmatrelvir (10 μ M). Drug-related peaks are denoted with P for parent drug and M for metabolites. All unmarked peaks represent non-drug-related interferences present in control incubations (not shown). Metabolite M4 is present as two closely eluting peaks representing the two interconverting diastereomers.

Figure 4. Metabolites of PF-07321332 (nirmatrelvir) in liver microsomes and hepatocytes from rat, monkey, and human.

Figure 5. Substrate saturation plots for the formation of the primary PF-07321332 (nirmatrelvir) metabolites PF-07329265 (M1), PF-07329266 (M2), PF-07329267 (M3), and PF-07329268 (M4) following incubation of PF-07321332 (0.02–200 μ M) with HLM (0.3 mg/ml).

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Figure 6. Inhibition of the formation of M1–M4 metabolites of PF-07321332 (nirmatrelvir, 2.5 μ M) in HLM in the presence of the selective CYP3A inhibitor ketoconazole (0.001–10 μ M). Panel A, PF-07329265 (M1); Panel B, PF-07329266 (M2); Panel C, PF-07329267 (M3); and Panel D, PF-07329268 (M4)

Figure 7. Time-, concentration-, and NADPH-dependent inhibition of CYP3A4/5 catalyzed midazolam-1'-hydroxylase (panels A and C) and testosterone-6 β -hydroxylase (panels B and D) activities in HLM by PF-07321332 (nirmatrelvir). 100-NADPH = PF-07321332 (100 μ M) incubations in HLM in the absence of NADPH co-factor. Panels C and D depict enzyme kinetics parameter fit (k_{obs} versus PF-07321332 concentrations) for midazolam and testosterone hydroxylation, respectively.

Tables

TABLE 1

Metabolic stability of nirmatrelvir in liver microsomes and hepatocytes

Reagent	Co-factor	$t_{1/2}$ (min)	$CL_{int,app}$ (μ l/min/mg or μ l/min/ 10^6 cells)	CL_{hep} (ml/min/kg)	Observed CL_{plasma} (ml/min/kg) ^c
Liver					
Microsomes ^a					
Rat	NADPH	114	6.08	5.8	22.7
Monkey	NADPH	4.31	161	25	15.8
Human	NADPH	24.1	28.8	6.3	---
Hepatocytes ^b					
Rat	NA	300	4.64	11	22.7
Monkey	NA	135	10.3	13	15.8
Human	NA	280	5.16	3.2	---

^aMetabolic stability of nirmatrelvir (0.1 or 1.0 μ M) was examined in NADPH-supplemented liver microsomes from rat, monkey and human (1 mg/ml protein concentration) for 60–80 min at 37 °C. Liver microsomal stability studies were conducted in triplicate.

^bMetabolic stability of nirmatrelvir (1.0 μ M) was examined in hepatocytes from rat, monkey and human (0.5×10^6 cells/ml) for 240 min at 37 °C. Hepatocyte stability studies were done in duplicate.

^cDepicted in vivo CL_{plasma} is after subtracting CL_{renal} in the respective species.

Stability data governed by half-life ($t_{1/2}$) was scaled to apparent intrinsic clearance ($CL_{int,app}$) and hepatic plasma clearance (CL_{hep}) using the well-stirred model (see Supplemental Information).

NA = not applicable.

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TABLE 2

Enzyme kinetics and fractional clearance determination for the metabolism of nirmatrelvir in human liver microsomes

	PF- 07329265 (M1)	PF-07329266 (M2)	PF- 07329267 (M3)	PF- 07329268 (M4)	Total
Model	MM	2-enzyme MM ^b	2-enzyme MM ^c	AS	MM
K _M (μM)	28.0	24.0	--	31.0	25.5
V _{max} (pmol/min/mg)	17.2	9.79	--	15.2	381
h	--	--	--	0.959	--
CL _{int,app,u} ^a (μl/min/mg)	0.654	0.434	0.00541	0.522	15.9
f _{CL}	0.037	0.025	0.030	0.030	0.91
					1.00

AS = Allosteric sigmoidal; f_{CL} = fractional clearance; h = Hill slope; K_M = substrate concentration at half-maximal velocity; MM = Michaelis-Menten; V_{max} = maximum velocity.

Data represent the mean of two replicates.

^aProtein adjusted microsomal free fraction = 0.940 for 0.3 mg/ml incubation protein concentration. ^bHighest affinity parameters reported. ^cLow affinity linear approximation, CL_{int,app,u} reported.

f_{CL} was calculated from:

$$f_{CL(HLM)} = \frac{CL_{int,app,x}}{CL_{int,app,all}}$$

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TABLE 3

Assignment of fractional CYP contribution to the metabolism of nirmatrelvir in pooled HLM

Metabolic Pathway	Fraction of Clearance (f _{CL}) ^a	Fraction Inhibited ^b		Fraction Metabolized (f _m) ^d	
		CYP3A4	Unassigned ^c	CYP3A4	Unassigned
PF-07329265 (M1)	0.037	0.95	0.048	0.036	0.0018
PF-07329266 (M2)	0.025	0.96	0.043	0.024	0.0011
PF-07329267 (M3)	0.030	0.98	0.020	0.029	0.00058
PF-07329268 (M4)	0.91	0.99	0.0084	0.90	0.0077
Total	1.0			0.99	0.01

Data represent mean of three replicates. Nirmatrelvir (2.5 μM) was incubated in pooled HLM in the absence/presence of ketoconazole (0.001–10 μM).

^af_{CL} = fractional clearance (see Table 2).

^bThe maximum contribution was determined by the percent activity remaining compared with a no inhibitor solvent control. Maximum percent inhibition was calculated using the equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + \left(\frac{10^{\text{LogIC}_{50}}}{10^X} \right)^{\text{HillSlope}}}$$

^cOther CYP isoforms unaccounted for, by inhibition with ketoconazole.

^df_m = fraction metabolized calculated from:

$$f_{mMxCYPz} = \frac{\% \text{ inhibition}}{100} \times f_{CL}$$

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TABLE 4

*Inhibition of nirmatrelvir (2.5 μ M) metabolism in pooled HLM with high (CYP3A5*1/*1)*

CYP3A5 activity

HLM Lot	Inhibitor	CYP	PF-07329265 (M1)	PF-07329266 (M2)	PF-07329267 (M3)	PF-07329268 (M4)
			% Inhibition	% Inhibition	% Inhibition	% Inhibition
HLM 3A5*1/*1	Control	-	-	-	-	-
	Ketoconazole (1 μ M)	3A4/5	98	>94	98	97
	CYP3cide (1 μ M)	3A4	94	>94	95	85
	% contribution CYP3A5		NS (3.7)	ND	NS (3.0)	NS (12) ^a

ND = not determined; NS = not significant; Data represent the mean of triplicate experiments. Significance based on one-way ANOVA analysis with Bonferroni's multiple comparison test, $p < 0.01$.

In cases where analyte concentrations were undetectable, the velocity was calculated using the LLOQ value.

^ap-value was 0.0294.

TABLE 5
Pharmacokinetics of nirmatrelvir in rats and monkeys

Species ^a	Dose (mg/kg)	C _{max} (ng/ml)	T _{max} (h)	AUC _{0-∞} (ng·h/ml)	CL _p (ml/min/kg)	Vd _{ss} (l/kg)	t _{1/2} (h)	Oral F (%)	F _a x F _g (%) ^d
Rat	1.0 (iv)	----	----	632 (523, 741)	27.2 (22.5, 31.9)	1.8 (1.4, 2.2)	5.1 (3.3, 7.0)	----	
	10 (po) ^{b,e}	1290 (851, 1730)	1.5 (1.0, 2.0)	3190 (1890, 4480)	----	----	4.0 (2.9, 5.1)	50 (30, 71)	82
	10 (po) ^{b,f}	1450 ± 373	0.25 ± 0.0	2170 ± 1180	----	----	2.8 ± 1.4	34 ± 19	56
Monkey	1.0 (iv)	----	----	977 (965, 988)	17.1 (16.9, 17.3)	0.33 (0.31, 0.34)	0.79 (0.74, 0.83)	----	
	10 (po) ^{b,e}	1450 (1050, 1850)	0.25 (0.25, 0.25)	831 (747, 914) ^c	----	----	----	8.5 (7.7, 9.4)	18

^aPharmacokinetic parameters were calculated from plasma concentration–time data and are reported as mean (± S.D. for n=3 and individual values for n=2). All pharmacokinetics were conducted in males of each species (Wistar-Han rats and cynomolgus monkey). Intravenous (iv) doses for nirmatrelvir were administered as a solution in 10% DMSO/30% PEG400/60% deionized water

(rats) or 5% (v/v) PEG400/95% (v/v) of 23% 2-hydroxypropyl- β -cyclodextrin in aqueous sodium phosphate buffer pH = 6.0 (monkeys).

^bOral (po) pharmacokinetics studies were conducted in the fed state. Oral rat pharmacokinetics studies were conducted with crystalline nirmatrelvir anhydrous MTBE solvate or anhydrous 'Form 1'. Nirmatrelvir-MTBE solvate and anhydrous 'form 1' forms were formulated as solutions in 10% (v/v) ethanol/10% (v/v) Capmul MCM/35% (v/v) PEG400/45% (v/v) Tween80 and 2% (v/v) Tween80/98% of 0.5% (w/v) methyl cellulose in deionized water, respectively. Rats received 8 ml/kg oral of 1% Hydroxypropyl cellulose in deionized water immediately prior to the nirmatrelvir-MTBE solvate po dose. For po pharmacokinetics assessments in monkeys, crystalline MTBE solvate form of nirmatrelvir was administered as a solution in 2% (v/v) Tween80/98% of 0.5% (w/v) methyl cellulose in deionized water.

^cAUC_{0-t}.

^dFraction of the po dose absorbed estimated from CL_{plasma} after subtracting CL_{renal}. CL_{plasma} was converted into CL_{blood} by dividing CL_{plasma} by the blood-to-plasma ratio in the respective species. Q is the hepatic blood flow (70 ml/min/kg (rats) and 44 ml/min/kg (monkeys)).

$$F_a \times F_g = \frac{F}{1 - \frac{CL_{blood}}{Q}}$$

^eNirmatrelvir was dosed as the anhydrous MTBE solvate.

^fNirmatrelvir was dosed as the anhydrous 'form 1'.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7