HCV antiviral drugs have the potential to adversely perturb the maternal-fetal communication axis through inhibition of CYP3A7 DHEA-S oxidation

Hannah M. Work, John C. Hackett and Jed N. Lampe

1Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Aurora, Colorado 80045, United States.

2Biomolecular Sciences Institute & Department of Chemistry & Biochemistry, School of Integrated Science & Humanity, College of Arts, Sciences, & Education Florida International University, Miami, Florida 33199, United States.

*To whom correspondence should be addressed (jed.lampe@cuanschutz.edu).
Abbreviations:

HCV, hepatitis C virus; CYP3A7, cytochrome P450 3A7; DBF, dibenzylfluorescein; DHEA-S, dehydroepiandrosterone-sulfate; TDI, time-dependent inhibition; MBI, mechanism-based inactivation
The hepatitis C virus (HCV) poses a great risk to pregnant people and their developing fetus, yet no HCV antiviral treatment guidelines have been established. While there has been a substantial increase in the development of HCV antivirals, the effect they have on the developing fetus remains poorly defined. Many of these drugs are metabolized through the cytochrome P450 CYP3A pathway, which is mediated by CYP3A7 in the fetus and developing infant. In this study, we sought to investigate the effect HCV antivirals have on CYP3A7 metabolism, as this CYP enzyme plays a vital role in proper fetal and neonatal development. Of the thirteen HCV antivirals we investigated, eight (~62%) inhibited CYP3A7 metabolic activity by 50% or more at a concentration of 20 µM. Furthermore, paritaprevir, asunaprevir, simeprevir, danoprevir, and glecaprevir all had observed half-maximal inhibitory concentrations between the range of 10-20 µM, which is physiologically relevant in comparison to the $K_m$ of DHEA-S oxidation (reported to be between 5 to 20 µM). We also discovered that paritaprevir is a time-dependent inhibitor of CYP3A7, which shifts the $IC_{50}$ ~2-fold from 11 µM to 5 µM. Upon further characterization, paritaprevir inactivates DHEA-S metabolism by CYP3A7, with $K_i$ and $K_{inact}$ values of 4.66 µM and 0.00954 min$^{-1}$, respectively. Depending on treatment plan and off-label drug use, HCV treatment could adversely affect the fetal-maternal communication axis by blocking fetal CYP3A7 metabolism of important endogenous hormones.
SIGNIFICANCE STATEMENT

The prevalence of HCV in pregnant people is estimated between 1-8% of the global population, yet little-to-no information exists about the risk antiviral treatment poses to the developing fetus. There is a potential risk of drugs adversely affecting mother-fetal communication by inhibiting fetal hepatic CYP3A7, an integral enzyme for estriol production. We discovered that five HCV antivirals inhibited DHEA-S metabolism by CYP3A7, and paritaprevir inactivated the enzyme. Our studies demonstrate the potential threat these drugs pose to proper fetal development.
INTRODUCTION

Hepatitis C virus (HCV) is estimated to infect over 185 million people worldwide, often leading to severe liver damage (Thrift, El-Serag, and Kanwal 2017; Mohd Hanafiah et al. 2013; CDC 2022). The virus can cause both acute and chronic hepatitis, ranging from asymptomatic to serious, lifelong illnesses including liver cirrhosis and cancer. For some, the virus is cleared spontaneously without any intervention, but for the majority of individuals, chronic HCV infection develops over time (Thrift, El-Serag, and Kanwal 2017). Globally, an estimated 58 million people have chronic HCV infections, but this is likely to be an underestimate as studies tend to exclude high-risk groups such as incarcerated persons (Thrift, El-Serag, and Kanwal 2017; Blach et al. 2022). Moreover, while HCV infections have recently decreased in Asia, they are currently on the increase in North America primarily due to an ageing population, illustrating that the disease burden from HCV infection will be with us for some time to come (Yang et al. 2023).

Women account for ~35% of HCV cases, and an estimated 15 million women of childbearing age (15-49 years old) have HCV worldwide according to cohort studies (Pott et al. 2018; Dugan et al. 2021). The prevalence of HCV in pregnant women is estimated between 1-8% of the global population, with prevalence dependent upon demographics (Arshad, El-Kamary, and Jhaveri 2011; Le Campion et al. 2012). The pathogenesis of HCV infection during pregnancy remains poorly understood, and so does the effect it poses on pregnancy outcomes (Yeung et al. 2014; He et al. 2023). However, some studies report an increased rate and/or risk of low birth weight, small for gestational age, preterm birth, low Apgar scores, congenital malformations, use of intensive care, assisted ventilation, and overall perinatal mortality relative to HCV-negative women (Yeung et al. 2014; He et al. 2023).

It is widely accepted that there is a significant risk of mother-to-infant transmission, occurring in about 5% of cases, and the risk of vertical transmission increases ~10-fold if the mother is co-infected with HIV (Le Campion et al. 2012; He et al. 2023). Over the last two decades, a
significant effort has been made to combat HCV with the development of novel antiviral therapies. However, there are currently no guidelines implemented for their use in pregnant people. Thus, the effect of off-label use of these medications on pregnancy outcomes remains undefined.

Recent studies of HIV antiviral use during pregnancy have been correlated with adverse effects on the developing fetus, including increased rate of preterm delivery, adrenal dysfunction, and elevated plasma concentrations of adrenal steroid hormones such as dehydroepiandrosterone 3-sulfate (DHEA-S) (Dulanjalee Kariyawasam et al. 2020; D. Kariyawasam et al. 2014; “Lopinavir/Ritonavir (Kaletra, LPV/r) | NIH” n.d.). It has been hypothesized that some of these effects may stem from inhibition of cytochrome P450 CYP3A7, the predominant enzyme expressed in fetal livers (Kandel and Lampe 2021). CYP3A7 accounts for ~50% of the total hepatic CYP content in fetal livers and is thus the major metabolizing enzyme for xenobiotics (Li and Lampe 2019). It also regulates an important function during development; CYP3A7 metabolizes DHEA-S to 16α-hydroxy-DHEA-S (Kandel and Lampe 2021; Li and Lampe 2019; Kitada et al. 1987; Torimoto et al. 2007), and this metabolite is a precursor molecule to the production of estriol (Kandel and Lampe 2021; Li and Lampe 2019), a critically important hormone in bringing the pregnancy to full term (Chatuphonprasert, Jarukamjorn, and Ellinger 2018). Low estriol levels during the last trimester of pregnancy have been linked to both premature birth and low birth weight, two leading causes of infant mortality (YILMAZ GULEC et al. 2022; Deng et al. 2022).

While HCV antivirals have demonstrated clinical effectiveness, significant questions remain regarding their metabolism and its relationship to reported hepatotoxicity. Whereas some drugs in this class have been reported to be substrates of CYP3A4, no reports have studied the effect they have on fetal CYP3A7. Although CYP3A4 and CYP3A7 share ~88% amino acid sequence similarity and overlapping substrate specificity, the $K_{cat}$’s of these enzymes differ significantly;
CYP3A7 has, on average, a hundredfold lower catalytic rate compared to CYP3A4 (Li and Lampe, 2019). CYP3A7’s lower catalytic activity, along with its broad range of substrates, makes it vulnerable to drug-drug or drug-hormone interactions that may lead to adverse drug reactions.

Herein, we investigated the inhibitory effects of 13 FDA-approved HCV antiviral drugs on CYP3A7 activity. This was initially assessed with a high-throughput fluorescent assay previously developed by our lab that utilizes the probe dibenzylfluorescein (DBF) (Work, Kandel, and Lampe 2021). Drugs that reduced CYP3A7 activity by 50% or more in this assay were further characterized by determining their IC$_{50}$ values using an LC-MS/MS-based method and measuring 16α-hydroxy-DHEA-S formation. We also investigated if any of these compounds were time-dependent (TDI) or mechanism-based inactivators (MBI) of CYP3A7 using a single-point fluorescent assay, and any positive hits were again followed up by further characterization using LC-MS/MS and DHEA-S as an activity probe. Based on our data, HCV antivirals could pose a serious threat to the developing fetus and neonate by blocking CYP3A7 metabolism of DHEA-S and thus affecting the fetal-maternal communication axis.

**MATERIALS AND METHODS**

**Chemicals and enzymes.** Human CYP3A7 coexpressed with human NADPH reductase in *Escherichia coli* cells (Bactosomes) and supplemented with purified human cytochrome b$_{5}$ (catalog #CYP060) was purchased from BiolVT® (Westbury, NY). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate (NADP$^{+}$), and the internal standard estriol-3-sulfate were obtained from Sigma-Aldrich (St Louis, MO). The HCV antiviral compounds were purchased MedChemExpress (Monmouth Junction, NJ) and all compounds were pre-dissolved in DMSO at a stock concentration of 10 mM. The substrate probes, dibenzylfluorescein (DBF) and DHEA-S, were purchased from Cayman Chemical Company (Ann Arbor, MI). The metabolite standard, 16α-hydroxy DHEA-S, was purchased from
Steraloids (Newport, RI). All other chemicals and solvents were obtained from standard suppliers and were of reagent or analytical grade.

**In vitro screening assay of recombinant CYP3A7 inhibition.** Assays were prepared as previously described (Work, Kandel, and Lampe 2021). In brief, Assays were conducted in triplicate in 96-well black polystyrene microtiter plates (Costar®, catalog # 266) in a 100 µL volume and prepared on ice. The reactions contained 10 pmol/mL of CYP3A7 bactosome enzyme, 0.1 M potassium phosphate buffer (pH 7.4), 3.3 mM magnesium chloride, and 0.2 µM DBF (~K_m). All drugs were previously prepared in DMSO and aliquoted to the reaction mixture to a final concentration of 20 µM. DMSO concentrations were kept below 0.2% of the final reaction volume. Enzyme/substrate/drug (E/S/D) mixes (80 µL) were pre-equilibrated at 37 °C for 3 minutes, and reactions were initiated by the addition of the NADPH-regenerating system mix (20 µL) consisting of NADP⁺ (1 mM), glucose-6-phosphate (10 mM), and glucose-6-phosphate dehydrogenase (2 IU/mL). The reactions were incubated for 10 min at 37 °C under agitation and were stopped by the addition of 2 M NaOH (75 µL). Fluorescent signal was measured using the Tecan Infinite® 200 PRO plate reader at an excitation/emission wavelength of 485/538 nm. Reactions without CYP3A7 present and substituted with water were used as a background control, and ketoconazole (20 µM) was used as a positive inhibitory control. Percent activity remaining was calculated based on the fluorescent signal from the solvent control reactions.

**Half-maximal inhibitory concentration determination of drug compounds using DHEA-S as a probe substrate of CYP3A7.** Reactions were similar to those described above and as previously described (Kandel and Lampe 2021), except the reactions were prepared in clear 1.1 mL deep-well polystyrene plates (Sigma, catalog #BR01352) at a volume of 150 µL and contained 5 µM DHEA-S substituted for DBF as a substrate. Reactions contained various concentrations of drug compounds ranging between 0.5 and 100 µM and were dissolved in DMSO (0.3% v/v) to generate a concentration-dependent inhibition curve. After equilibrating the
reactions at 37 °C for 3 min and initiating the reactions by the addition of a NADPH-regenerating system, the reactions were incubated for 5 min at 37 °C under agitation and were stopped by the addition of ice-cold methanol (150 μL) containing 50 ng/mL estriol-3-sulfate internal standard. Precipitated proteins were collected by centrifugation of the stopped reaction samples for 20 min at 2,500 × g and 4 °C, and supernatant aliquots of 5 μL were analyzed by LC-MS/MS. The solvent control (DMSO) was used to indicate 100% activity, and incubations without the NADPH-regenerating system mix present served as negative controls.

**Determination of potential time-dependent inhibition (TDI) with DHEA-S.** Reactions were prepared similarly as those described above for IC₅₀ generation, except, in order to generate potential inactivating metabolites or intermediates, enzyme/drug mixtures were pre-incubated at 37°C for 30 minutes in the presence of NADPH regeneration mixture (120 μL volume mixture). Then, a mixture of probe substrate DHEA-S (5 μM final), NADPH regeneration mixture, and water (30 μL mixture) was added to the reaction. Reactions were further incubated at 37°C for 5 minutes, after which reactions were stopped by the addition of 150 μL methanol containing 50 ng/mL of estriol-3-sulfate internal standard.

**Time-dependent inhibition of CYP3A7.** Recombinant CYP3A7 bactosomes (25 pmol/ml) were preincubated in triplicate with paritaprevir at 2.25, 4.5, 9, 36, and 90 μM in 0.1 M potassium phosphate buffer (pH 7.4) and 3 mM MgCl₂. Paritaprevir was dissolved in DMSO (0.3%, v/v) and solvent control (DMSO) was used as the 100% control activity. The pre-incubation reactions (160 uL) were started by the addition of the NADPH-regeneration mix (40 μL). After 0, 7, 14, and 21 minutes at 37 C, a 20 uL aliquot of the recombinant CYP3A7/paritaprevir preincubation was transferred to a 50 uM DHEA-S (~10x Km) reaction (180 uL) containing 0.1 M potassium phosphate buffer (pH 7.4), 3 mM MgCl₂, and the NADPH-regeneration mix. The DHEA-S incubations were stopped after 4 min at 37C by the addition of methanol (200 uL) containing 50 ng/mL estriol-3-sulfate internal standard. Precipitated proteins
were collected by centrifugation of the stopped reaction samples for 20 min at 2,000 × g and 4°C, and aliquots of 5 μL were analyzed by LC-MS/MS for formation of the 16α-hydroxy DHEA-S metabolite.

**Analytical method for DHEA-S hydroxylation.** The DHEA-S incubations with recombinant CYP3A7 enzyme were analyzed by LC-MS/MS using a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system interfaced by electrospray ionization with a Waters Xevo TQ-S micro tandem quadrupole mass spectrometer (Waters Corp., Milford, MA) in negative ionization mode and with the multiple reaction monitoring (MRM) scan type. The following source conditions were applied: or 0.5 kV for the capillary voltage, 150 °C for the source temperature, 450 °C for the desolvation temperature, 50 L/h for the cone gas flow, and 900 L/h for the desolvation gas flow. The following mass transitions, collision energies (CEs), and cone voltages (CVs) were used to detect the respective analytes: 383 > 97, CE= 26 V, CV = 75 V for the 16α-hydroxy DHEA-S metabolite, 367 > 97, CE = 34 V, CV = 80 V for DHEA-S, and 367 > 287, CE = 28 V, CV = 70 V for the internal standard estriol-3-sulfate. DHEA-S and its hydroxylated metabolite were separated on a Waters BEH C18 column (1.7 μm, 2.1 × 100 mm) by flowing 5 mM ammonium acetate in water and methanol at 0.4 mL/min. The following gradient was used: 10% organic (methanol) held for 0.5 min, increased to 98% over 3.5 min, and held at 98% for 1.2 min. The MS peaks were integrated using the QuanLynx software (version 4.1, Waters Corp., Milford, MA), and the analyte/internal standard peak area ratios were used for relative quantification.

The mean analyte/internal peak area ratio for the 16α-hydroxy DHEA-S metabolite was determined for the solvent control samples and was referred to as 100% control activity to calculate the percent remaining activity in samples containing drug inhibitors. GraphPad Prism software was used for dose–response curve fitting to determine the estimated apparent IC_{50} value for each drug tested. The sum-of-squares F-test was used to compare IC_{50} values.
produced when reactions were pre-incubated in the presence of NADPH for 30 minutes versus those that were not pre-incubated for 30 minutes.

**Molecular docking.** An in silico docking routine was employed in order to structurally elucidate the binding interactions between CYP3A7 and paritaprevir. The recently obtained CYP3A7 crystal structure with the endogenous substrate, DHEA-S, bound (8GK3, Liu et al. 2023) was downloaded from the Protein Data Bank using the Fetch function in Chimera v. 1.17.3, build 42480 (https://www.cgl.ucsf.edu/chimera/). With the exception of chain C, all other protein chains in the asymmetric unit were deleted from the file and all bound DHEA-S substrate ligands were removed. The CYP3A7 receptor was prepared by removing all extraneous water and other solvent molecules using the DockPrep function in Chimera. The file was saved in the .pdbqt format in preparation for docking. The paritaprevir 2D sdf ligand file was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and converted into a 3D pdb file using Chimera. The structure was optimized using the RI-B3-LYP functional and def2-SVP basis set in Turbomole, v. 7.7 (https://www.turbomole.org/). The energy minimized ligand was then further parameterized by adding polar hydrogens and identifying rotatable bonds using MGL AutoDock Tools v. 1.5.7 (UCSD Molecular Graphics Lab and The Scripps Research Institute). The fully optimized ligand file was then saved in the .pdbqt format, suitable for docking. A docking grid was identified by using the GridBox function in AutoDock tools and defined using the following parameters: grid box center - x-center = 32.9, y-center = 56.5, z-center = 32.4, with the total number of grid points in each dimension being: x-dimension = 56, y-dimension = 40, and z-dimension = 40. A docking script configuration file was prepared using a simple text editor with the energy range set to 4 and the exhaustiveness set to 24. AutoDock Vina (v. 1.1.2 for Linux) was executed using the configuration file with both PDBQT.out and log.out file options selected. Output files were analyzed using the ViewDock function of Chimera, and ranked according to individual binding energies ($\Delta G$).
RESULTS

Initial CYP3A7 screen for inhibition with HCV antivirals. Based upon found reports of many of these compounds being CYP3A4 substrates or inhibitors, we expected to obtain a similar pattern when investigating CYP3A7. However, after testing these drugs at a 20 µM concentration, only eight were observed to significantly inhibit recombinant CYP3A7 activity by 50% of more, including the following: danoprevir, glecaprevir, asunaprevir, simeprevir, paritaprevir, velpatasvir, ledipasvir, and dasabuvir (Figure 1). Of those eight, only two had previously reported CYP3A4 effects on their FDA label (velpatasvir and glecaprevir).

Dose-response curves generated by DHEA-S. We further characterized the eight compounds that inhibited CYP3A7 DHEA-S activity significantly at 20 µM (>50%) by determining their half-maximal inhibitory concentration ($IC_{50}$). The results are presented in Figure 2. All HCV inhibitors tested had relatively similar $IC_{50}$ values, ranging between 10 µM and 22 µM. We additionally tested ritonavir (Figure 2F) because it is a common pharmacoenhancer coadministered with HCV antivirals, and it was the most potent inhibitor of CYP3A7 with an $IC_{50}$ value of 0.0719 µM (95% CI: 0.0676-0.0767 µM). Of the HCV antivirals, paritaprevir was observed to be the most potent CYP3A7 inhibitor with an $IC_{50}$ value of 10.9 µM (95% CI: 9.44-12.7 µM). In contrast, danoprevir had the highest $IC_{50}$ value, 21.9 µM (95% CI: 19.5-24.5 µM).

Glecaprevir, simeprevir, and asunaprevir $IC_{50}$ values were between the values of paritaprevir and danoprevir, which were observed to be 12.1 µM (95% CI:10.9-13.5 µM), 16.1 µM (95% CI:14.8-17.4 µM), and 18.3 µM (95% CI:16.9-19.7 µM), respectively. The results match well with our initial inhibition assays using the DBF fluorescent assay: paritaprevir was observed to be the most potent (~25% activity remaining at 20 µM, Figure 1), which our DHEA-S assay results confirm. Similarly, glecaprevir, asunaprevir, and simeprevir all inhibited CYP3A7 to a similar degree (~35% activity remaining at 20 µM, Figure 1), and the DHEA-S assay resulted in similar
IC$_{50}$ values for these three compounds. This gives us confidence in the reliability of both assays and their results regarding CYP3A7 effects.

**Paritaprevir is a time-dependent inhibitor (TDI) of CYP3A7.** We checked to see if any of these HCV antivirals posed a greater risk to the developing fetus by investigating their potential inactivation effects on CYP3A7. Initially testing two concentrations and incubating CYP3A7 with individual HCV drugs for 30 minutes in the presence and absence of NADPH before adding the probe substrate DBF, we observed a significant change in potency only with paritaprevir (Supplemental Figure S1). We followed this observation with our DHEA-S metabolic assay and observed a shift in the IC$_{50}$ due to pre-incubating CYP3A7 with paritaprevir in the presence of NADPH (Figure 3A), indicating that paritaprevir is a mechanism-based inactivator (MBI) of CYP3A7. As a comparison, we performed the same experiment with ritonavir, a known MBI of CYP3A4 and CYP3A7 (Kandel and Lampe 2021). A comparison of these two drugs and their MBI effects on CYP3A7 is presented in Table 2. Ritonavir is a more potent inhibitor of CYP3A7 DHEA-S activity by 2-3 orders of magnitude and has a significantly higher fold-change when pre-incubated in the presence of NADPH for 30 minutes.

We further characterized CYP3A7 inactivation by paritaprevir by determining its $K_I$ and $k_{\text{inact}}$ values. Loss of CYP3A7 activity was measured as a function of time using varying paritaprevir concentrations, and the decrease in activity was both time-dependent and concentration-dependent in the presence of NADPH regeneration system (Figure 4A). Linear regression analysis of the time course data was used to determine the $k_{\text{obs}}$ values at the concentrations of paritaprevir tested (Figure 4B). The resulting $K_I$ and $k_{\text{inact}}$ values were 4.66 µM (95% CI: 0.756 uM-17.8 uM) and 0.00954 min$^{-1}$ (95% CI: 0.00634 min$^{-1}$-0.0136 min$^{-1}$), respectively.

**Molecular docking of paritaprevir.** Docking of paritaprevir into the CYP3A7 active site yielded nine energetically viable poses, with $\Delta G$ scores between -12.7 and -11.6 kcal/mol. The most energetically favorable pose (-12.7 kcal/mol) is represented in Figure 5. This pose puts the
C9 carbon of paritaprevir in the closest proximity to the heme iron (4.5 Å; Figure 5C). While CYP3A7 specific metabolites of parataprevir have not yet been reported in the literature, this suggests that oxidation is most likely to take place on the central extended ring system and not on the peripheral moieties of the drug. Additionally, the large number of rotatable bonds in the ligand structure suggests a high degree of conformational flexibility in the drug that may allow it to sample multiple distinct conformations within the CYP3A7 active site, leading to multiple products, as observed with CYP3A4 (Shen et al. 2016). There were no hydrogen bonds noted in the interaction of the drug with CYP3A7. Nevertheless, the sulfonamide of paritaprevir forms charge paring interactions with the backbone of F304 and F213 in the CYP3A7 structure and these seem to anchor the drug in the most favorable binding pose. Paritaprevir also demonstrates cation-pi interactions with both F479 and Y57, and an edge-on interaction with the aromatic ring of W58. Other important interactions include multiple hydrophobic contacts between F/G loop residues and the phenanthrene ring of paritaprevir.

DISCUSSION

Although the World Health Organization has proposed a strategy to reduce and eliminate Hepatitis C by 2030, HCV remains a global issue today and still affects roughly 100 million people around the world (Dore and Bajis 2021). While there is already a significant burden on the general population, this is exacerbated in pregnant people who have HCV, as there is little-to-no treatment guidelines available, and it is unclear what threat current treatments pose to the developing fetus. The little information we do have on the effect of HCV antivirals on fetal development comes from either a) animal studies, which historically have a poor correlation with human outcomes, or b) previously reported exposure during pregnancy, which is a very limited dataset.

When it comes to determining fetal and neonatal exposure, it must be taken into account that the drug must first pass the placenta of the pregnant individual to get to the fetus or be
transferred by breastmilk to the neonate. To date, placental transfer of some HCV antivirals has been observed in rabbits (grazoprevir) and in rats (sofosbuvir, ledipasvir, velpatasvir, grazoprevir, and glecaprevir) (Freriksen et al. 2019). If the HCV antiviral is passed to the fetus or neonate, one of its main modes of clearance is by cytochrome P450 CYP3A7. This enzyme is highly expressed in fetal and neonatal livers, accounting for up to 50% of the total CYP content and 87-100% of the total CYP3A content (Li and Lampe 2019; Kitada et al. 1987). CYP3A7 also plays a vital role in fetus and neonatal development, as it metabolizes DHEA-S into 16α-hydroxy-DHEA-S, a precursor to estriol production. Thus, in this study we sought to investigate the risk HCV antiviral therapy may pose to developing fetuses and neonates by characterizing CYP3A7 inhibition by these drugs.

The thirteen HCV antiviral drugs investigated in this study are presented in Table 1. The FDA labels most of these compounds as Pregnancy Category B due to their lack of embryotoxicity during animal testing, but human and rodent interspecies differences can be significant, particularly in regards to metabolism. Some of the other drugs examined do not yet have a pregnancy category label since they are investigational drugs in clinical trials, and others are labeled X due to their required co-administration with ribavirin and interferon α-2b, as this medication shows significant teratogenic and embryocidal effects at as low as 1/20th of the recommended human dose (“REBERTRON (Ribavirin and Interferon Alfa-2b) Combination Therapy Label” 1998). Of the thirteen HCV antivirals, eleven were reported to be CYP3A4 substrates or inhibitors. However, of those eleven, only five mention their effect on CYP3A4 on their respective FDA label (these are boceprevir, telaprevir, grazoprevir, velpatasvir, and glecaprevir) (“VICTRELIS Label” 2011; Isakov et al. 2016; “INCIVEK Label” 2011; Smolders et al. 2017; Ahmed, Lutchman, and Kwo 2017; Brennan et al. 2015; Miao et al. 2020; “MAVYRET Label” 2017; Freriksen et al. 2019; Eley et al. 2015; “EPCLUSA Label” 2016). Despite the fact that none of the aforementioned drugs are deemed safe for use during pregnancy and no
clinical trials have been performed in this patient population group, we did find reports of their use during pregnancy. Velpatasvir, ledipasvir, paritaprevir, and sofosbuvir all have been previously used during pregnancy (AbdAllah et al. 2021; Zeng et al. 2022; Curtis and Chappell 2023; Kislovskiy et al. 2021; Chappell et al. 2020).

We began with an initial screen of these antivirals in order to determine their propensity for inhibition of CYP3A7 metabolism of dibenzylfluorescein (DBF). Although this reaction is not unique or specific to CYP3A7, previous findings determined that CYP3A7 demonstrated maximal activity with this fluorescent substrate, producing the lowest $K_m$ and highest signal-to-noise ratio (Work, Kandel, and Lampe 2021). Additionally, these assays utilize only recombinant CYP3A7 and NADPH reductase supplemented with cytochrome $b_5$, without interference from other CYP enzymes or alternative clearance pathways. Using this technique, we discovered that eight of the thirteen tested HCV antivirals inhibited CYP3A7 by 50% or more at a concentration of 20 µM (Figure 1). Of the eight aforementioned drugs, ledipasvir was the only antiviral not reported to be a CYP3A4 inhibitor or substrate (Table 1). However, we were unable to further investigate ledipasvir-mediated inhibition, as well as the other NS5a protease inhibitor velpatasvir, due to solubility issues with each compound in reaction mixtures; at higher concentrations each drug was found to precipitate out of solution. In contrast, of the five drugs that did not significantly inhibit CYP3A7, four of them are reported to be CYP3A4 substrates or inhibitors (boceprevir, narlaprevir, telaprevir, and grazoprevir) (Table 1). While these two enzymes are ~88% identical in amino acid sequence, these results demonstrate the slight, but important, variations in substrate specificity that may alter toxicological outcomes.

We further characterized five of the drugs that showed >50% inhibition of CYP3A7 by determining their half-maximal inhibitory concentrations ($IC_{50}$s) using DHEA-S as an activity probe, as this is a more physiologically relevant marker. Paritaprevir, glecaprevir, asunaprevir, danoprevir, and simeprevir all had $IC_{50}$ values ranging between 10 µM and 21 µM. These five
drugs, along with dasabuvir and grazoprevir, all contain a nitrogen-sulfone bond. Sulfate sidechains are typically favorable to CYP3A7 as the naturally occurring endogenous substrate, DHEA-S, contains a sulfate moiety that anchors it in the active site; CYP3A7 is known to be a more efficient metabolizer of DHEA-S than DHEA (Li and Lampe, 2019). This may help explain these drugs’ higher affinity for CYP3A7 binding and thus inhibition of DHEA-S metabolism. It is probable that dasabuvir is also an inhibitor of DHEA-S metabolism by CYP3A7, but we were unable to test high enough concentrations of dasabuvir due to solubility issues and DMSO constraints in reactions. Similarly, grazoprevir most likely inhibits DHEA-S metabolism but less potently (i.e. higher IC₅₀ value) than the five drugs we followed up on based on our criteria from the DBF assay results.

Upon further investigation, paritaprevir showed indication of being a mechanism-based inactivator of CYP3A7, causing a ~2-fold shift in the CYP3A7 IC₅₀ value from 11 µM to 5.5 µM (Table 2, Figure 3). This drug inactivated CYP3A7 DHEA-S metabolism, with Kᵢ and kᵢnact values of 4.66 µM and 0.00954 min⁻¹, respectively. Although the Kᵢ value is rather moderate compared to the other known CYP3A7 inactivator ritonavir (Kᵢ=0.392 µM, (Kandel and Lampe 2021)), it is still within the range to pose a potential threat to the neonate. The Kₘ of DHEA-S for CYP3A7 has been reported to be between 5 µM and 20 µM, so paritaprevir could compete with DHEA-S for binding to CYP3A7 and inactivate the enzyme, blocking downstream production of estriol and leading to disruption of the fetal-maternal communication axis.

While paritaprevir has been reported to be a CYP3A4 substrate (Smolders et al. 2017; Ahmed, Lutchman, and Kwo 2017; Shebley et al. 2017; Shen et al 2016), there are no reports regarding paritaprevir exhibiting mechanism-based inactivation of CYP3A4. This could be due to the selection of test systems utilized, i.e. human liver microsomes (HLMs) instead of isolated/recombinant CYP3A4. In more complicated testing systems, such as HLMs, other CYPs and Phase II enzymes could be contributing to paritaprevir binding and metabolism,
masking the TDI or MBI effect it may have on CYP3A4 alone (Shebley et al. 2017). This suggests that the TDI may not be significant in regards to CYP3A4. However, in a developing fetus who may be exposed to the drug, it could prove very significant due to the fact that CYP3A7 is the only CYP3A enzyme in the fetal liver and is known to have an average $K_{cat}$ 1/100 that of CYP3A4 for most drug substrates (Li and Lampe, 2019), hence increasing the risk for drug-drug interactions and drug toxicity in the developing fetus and neonate. Post-marketing surveillance revealed instances of sudden alanine aminotransferase elevations and drug-induced hepatotoxicity with paritaprevir in combination with other HCV antiviral inhibitors (“Viekira Pak” 2012; Kumar et al. 2019). While the mechanism of liver injury is currently unknown, it is possible that reactive metabolites produced from one or more of the drugs in the combination treatment may play a role. Additionally, reactive metabolites could function to inhibit the clearance pathways for other CYP3A drug substrates, leading to drug-drug interactions and adverse side effects.

In a study performed by Shen et al (2016), the group identified 18 different oxidized metabolites of paritaprevir in plasma, urine, and feces after healthy male subjects received a 200 mg dose of $[^{14}\text{C}]$paritaprevir co-administered with 100 mg of ritonavir. The group further determined that the two most abundant oxidized metabolites (M2 and M24) were formed by hepatic enzymes, most likely CYP3A4. Both the M2 and M24 metabolites are proposed to be oxidized at or near the C9 carbon of the substrate. The M24 metabolite was postulated to form from an unstable epoxide intermediate produced by CYP3A4 in vivo that readily reacts with GSH to form a glutathione adduct (Shen et al.; Supplemental Figure 1). While the complete characterization of the CYP3A7 metabolite profile of paritaprevir is beyond the scope of our study reported here, we have attempted to provide a plausible mechanism for protein adduction and inactivation based on the reactivity of this known CYP3A metabolite (Supplemental Figure
While this potential inactivation pathway still needs to be confirmed, it may go some way to explain the TDI observed with paritaprevir and CYP3A7.

To gain additional insight into the structural details of the paritaprevir-CYP3A7 interaction, we conducted an in silico docking study. The most stable binding interaction between paritaprevir and CYP3A7 is represented in Figure 5. This binding pose places the C9 carbon of paritaprevir in the closest position to the heme iron (~4.5 Å; Figure 5B), suggesting a possible site of oxidation which corresponds well with what has been previously reported for CYP3A4 (Shen et al., 2016). However, this needs to be validated with future metabolite identification studies. Despite this, the number of interactions between paritaprevir and residues within the CYP3A7 active site suggest a high degree of stability between the ligand and the protein (Figure 5C).

Although there are likely differences between the metabolism of paritaprevir by CYP3A4 and CYP3A7, it is plausible that oxidation at the C9 position could produce the reactive epoxide intermediate identified previously, leading to CYP3A7 alkylation at nucleophilic residues, such as cysteine thiols, e.g. Cys58 (Supplemental Figure S2A), causing CYP3A7 inactivation. The effects of paritaprevir on CYP3A inhibition may have initially been missed in the clinical studies due to the overwhelming inhibitory effect of ritonavir on CYP3A4 (Shebley et al. 2017). Experiments are currently underway to determine if the TDI effects of paritaprevir differ between CYP3A7 and CYP3A4 in recombinant systems and HLMs.

While the IC50s of CYP3A7 by the hepatitis C antivirals tested were all comparatively high (5 µM-20 µM), they may be in a clinically relevant concentration range. The recommendations for HCV treatment range between 8 and 24 weeks of daily antiviral administration depending on stage of infection/disease, with concomitant treatment of 2-4 antivirals at doses between 100 mg and 500 mg daily (Smolders et al. 2019; Talal et al. 2018). The maximal plasma concentrations (Cmax) recorded in non-pregnant, HCV-free adults also has a wide range
depending on treatment plan, drug, and usage of pharmacokinetic enhancers like ritonavir. The results vary widely between 100 ng/mL and 15,000 ng/mL, or roughly 0.1 to 20 µM, as seen in the case of paritaprevir dosed with or without ritonavir (Menon et al. 2016). As both drugs inhibit and inactivate CYP3A7, the high circulating plasma levels in adults makes drug exposure to the fetus highly probable and this could lead to significant adverse effects due to their CYP3A7 interactions. Additionally, the high Cmax values could be further exacerbated if patients have chronic HCV infection, affecting normal liver function and metabolism of these drugs. While no studies exist on fetal exposure to maternal HCV treatment (e.g., umbilical cord concentration, placental concentrations, etc.) due to a plethora of ethical and logistical reasons, it can be speculated that, due to the high plasma levels and evidence of placenta and breastmilk transfer of antivirals, these drugs could pose a serious threat to fetuses and neonates.

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DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

AUTHOR CONTRIBUTIONS

Hannah Work and Jed Lampe participated in research design. Hannah Work conducted all experiments and performed data analysis. John Hackett optimized the paritaprevir ligand for docking into the CYP3A7 active site. Hannah Work and Jed Lampe wrote the manuscript.
REFERENCES


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No author has an actual or perceived conflict of interest with the contents of this article.
FIGURE LEGENDS

Figure 1: CYP3A7 inhibition by HCV antivirals as assessed by the DBF HTS fluorescence assay. All drugs were tested in triplicate, with the black circles representing individual test points and error bars representing the standard deviation. Calculations were based upon the solvent control (representing 100% activity).

Figure 2: Dose-response curves of CYP3A7 activity depletion by A) danoprevir, B) glecaprevir, C) asunaprevir, D) simprevir, E) paritaprevir, and F) ritonavir. Black circles represent the average of triplicate runs (n=3) and error bars represent the standard deviation. Curves were fit to the [Inhibitor] vs response (three parameters) equation in GraphPad Prism (version 10.0.2) to determine IC_{50}s and goodness-of-fits for each drug (R^2).

Figure 3: Dose-response curve shifts of A) paritaprevir and B) ritonavir following 30-min NADPH pre-incubation. Black squares and circles represent the average of triplicate experiments (n=3) in which there was and was not a 30-minute pre-incubation with NADPH, respectively. Error bars represent the standard deviation of each concentration tested. Points were fitted to the [Inhibitor] vs. response (three parameters) curve in GraphPad Prism (version 10.0.2).

Figure 4: Time- and concentration-dependent inhibition of CYP3A7 DHEA-S 16α-hydroxylation by paritaprevir. A.) Pseudo-first-order kinetic plots at five paritaprevir concentrations based on the percent CYP3A7 activity remaining versus the pre-incubation time in the presence of NADPH. All points represent the average of triplicate incubations, and error bars represent the standard deviation. B.) Inactivation rate constants (k_{obs}) at the five paritaprevir concentrations assessed (R^2: 0.864).

Figure 5: Docking of paritaprevir within the CYP3A7 active site. A.) Most energetically favorable pose of paritaprevir (delta G = -12.7 kcal/mol) docked in the CYP3A7 active site.
(Paritaprevir show in cyan, heme prosthetic group shown in red, and the CYP3A7 protein backbone shown in tan) B.) Close up of paritaprevir in the CYP3A7 active site showing the relative distance of the C9 of paritaprevir to the heme iron and interacting amino acid residues (phenylalanines show in green, tyrosine in yellow, and tryptophan in magenta; other colors same as panel A). C.) Cut-away view of paritaprevir in the CYP3A7 active site showing the solvent accessible surface of the protein and distance from C9 to the heme iron (red; other colors same as panel A and B). D.) Space filling model of paritaprevir in the CYP3A7 active site. (color scheme same as above).
### Table 1: HCV antiviral drugs, their corresponding structures, reported use in pregnant persons, and CYP3A4 inhibition characteristics

<table>
<thead>
<tr>
<th>Drug and Structure</th>
<th>HCV Target</th>
<th>FDA Pregnancy Category</th>
<th>Reported Use in Pregnant People? (Y/N)</th>
<th>Reported CYP3A4 Inhibitor or Substrate? (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boceprevir</td>
<td>NS3/4A protease</td>
<td>X (due to use with ribavirin and peginterferon alfa)</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Narlaprevir</td>
<td>NS3/4A protease</td>
<td>NA*</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Telaprevir</td>
<td>NS3/4A protease</td>
<td>X (due to use with ribavirin and peginterferon alfa)</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Grazoprevir</td>
<td>NS3/4A protease</td>
<td>B</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Danoprevir</td>
<td>NS3/4A protease</td>
<td>NA*</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Drug</td>
<td>Enzyme Type/Complex</td>
<td>B</td>
<td>N</td>
<td>Y</td>
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</tr>
<tr>
<td>Glecaprevir</td>
<td>NS3/4a protease</td>
<td>B</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Asunaprevir</td>
<td>NS3/4A protease</td>
<td>NA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Simeprevir</td>
<td>NS3/4A protease</td>
<td>X (due to use with ribavirin and peginterferon alfa)</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Paritaprevir</td>
<td>NS3/4A protease</td>
<td>B</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Velpatasvir</td>
<td>NS5A replication complex</td>
<td>B</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Ledipasvir</td>
<td>NS5A replication complex</td>
<td>B</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Medication</td>
<td>NS5B polymerase</td>
<td></td>
<td></td>
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<tr>
<td>-------------</td>
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</tr>
<tr>
<td>Sofosbuvir</td>
<td>B</td>
<td>Y</td>
<td>N</td>
<td></td>
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<tr>
<td>Dasabuvir</td>
<td>B</td>
<td>N</td>
<td>Y</td>
<td></td>
</tr>
</tbody>
</table>

*approved in other countries

still in clinical trial/investigational

withdrawn
Table 2: IC\textsubscript{50} shift of paritaprevir and ritonavir due to 30-minute pre-incubation in presence of NADPH.

<table>
<thead>
<tr>
<th>Drug Inhibitor</th>
<th>0-min pre-inc IC\textsubscript{50} (µM) (95% CI; R\textsuperscript{2})</th>
<th>30-min pre-incubation +NADPH IC\textsubscript{50} (µM) (95% CI; R\textsuperscript{2})</th>
<th>Fold-change (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paritaprevir</td>
<td>11.6 (10.6-12.6; 0.990)</td>
<td>5.51 (5.20-5.83; 0.996)</td>
<td>2.11 (1.82-2.42)</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.0716 (0.0661-0.0777; 0.986)</td>
<td>0.0153 (0.0119-0.0193; 0.916)</td>
<td>4.68 (3.42-6.53)</td>
</tr>
</tbody>
</table>
A. % Control

[Danoprevir] (μM)

B. % Control

[Glecaprevir] (μM)

C. % Control

[Asunaprevir] (μM)

D. % Control

[Simeprevir] (μM)

E. % Control

[Paritaprevir] (μM)

F. % Control

[Ritonavir] (μM)

IC\textsubscript{50}: 12.1 μM
R\textsuperscript{2}: 0.985

IC\textsubscript{50}: 18.3 μM
R\textsuperscript{2}: 0.992

IC\textsubscript{50}: 16.1 μM
R\textsuperscript{2}: 0.991

IC\textsubscript{50}: 11.6 μM
R\textsuperscript{2}: 0.992

IC\textsubscript{50}: 0.072 μM
R\textsuperscript{2}: 0.995
Figure 3

A. [Paritaprevir] (μM)

0-min pre-incubation
30-min pre-incubation
+NADPH

B. [Ritonavir] (μM)

0-min pre-incubation
30-min pre-incubation
+NADPH

% Control

0 25 50 75 100

0 0.001 0.01 0.1 1

Figure 3
Figure 4

A. Log % Activity Remaining vs. Pre-incubation time (min)

- 90 uM
- 45 uM
- 9 uM
- 4.5 uM
- 2.25 uM

B. Observed rate constant ($k_{\text{obs}}$, min$^{-1}$) vs. Paritaprevir concentration (µM)
Figure 5