Title Page

Article category: Original articles

Title: Evaluation of the Drug–Drug Interaction Potential of Ensitrelvir Fumaric Acid with Cytochrome P450 3A Substrates in Healthy Japanese Adults

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Journal Name: ***Clinical Drug Investigation***

Running Title: DDI of Ensitrelvir and CYP3A substrates

Supplementary Material

Pharmacokinetics of ensitrelvir in DDI study

The plasma concentration-time profiles until 312 hours from last dose of ensitrelvir at 750/250 mg are shown in **Supplementary Fig. 1**.

Supplementary Methods

All reagents and solvents used in CYP inhibition study were of analytical grade. Human liver microsomes were purchased from Sekisui XenoTech, LLC. Reversible and time-dependent inhibitory effects of ensitrelvir (0.1 to 100 μM) on major human liver CYP enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A [substrates: midazolam and testosterone]) were investigated using human liver microsomes and probe substrates at approximately 37°C in 50 mM potassium phosphate buffer (pH 7.4) with 3 mM MgCl2 and 1 mM EDTA. To investigate the type of TDI, preincubation of S-217622 for 30 minutes in liver microsome was performed with or without reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) (**Supplementary Table 2**). The incubation with probe substrate was conducted for 5 min. The samples were centrifuged at 920 × g for 10 min at 10 °C, and the supernatant fractions were analyzed by LC-MS/MS.

To determine the inactivation parameters, ensitrelvir was preincubated in human liver microsomes and an NADPH-regenerating system for 0, 3, 6, 9, 15 and 30 min (**Supplementary Table 3**). An aliquot of the preincubation mixture was transferred to a second tube containing the probe substrate (30 μM midazolam and 210 μM testosterone), the incubation was continued for 5 min.

Bioanalytical procedure

Chemicals and regents

Ensitrelvir fumaric acid was developed in Shionogi & Co., Ltd. Dexamethasone, prednisolone, and midazolam were purchased from Fuji Film Wako Pure Chemical (Osaka, Japan). The stable isotopes showed in **Supplementary Fig. 2** were used as internal standard (IS). [13C,2H5]-ensitrelvir was developed in Shionogi & Co., Ltd. [2H4]-dexamethasone and [2H6]-prednisolone were purchased from Alsachim (Illkirch Graffenstaden, France). [2H4]-midazolam malate was purchased from Spelco (Darmstadt, Germany).

Plasma sample preparation

Plasma sample (10 μL, 100 μL, 50 μL, and 100 μL) was used for determination of ensitrelvir, dexamethasone, prednisolone, and midazolam, respectively. Ensitrelvir and prednisolone, plasma samples were precipitated with acetonitrile containing the IS. After centrifugation, the supernatant was diluted to mach with the component of the mobile phase of chromatographic separation. As for dexamethasone and midazolam, plasma samples were added the IS and extracted by solid phase extraction using OASIS HLB μElution plate (Waters, Milford, MA, USA) and SOLAμ HRP plate (2mg/1mL; Thermo Fisher Scientific, Waltham, MA, USA), respectively.

Chromatographic and mass spectrometric conditions

Ensitrelvir

Thermo Fisher Scientific TSQ Altis (Waltham, MA, USA) with a Thermo Fisher Scientific Vanquish Binary Flex system was used in Part 1 (dexamethasone cohort and prednisolone cohort) and Sciex API4000 (Framingham, MA, USA) with a Shimadzu LC-20A system (Kyoto, Japan) was used in Part 2 (midazolam cohort) (**Supplementary Table 4**). The LC separation was achieved on a YMC-Triart C18 column (50 mm ×2.1 mm I.D., 3 μm; YMC, Kyoto, Japan). Mobile phases were 0.1% formic acid for A and acetonitrile containing 0.1% formic acid for B (55:45, v/v), which was pumped at a flowrate of 0.5 mL/min..For MS detection, electrospray ionization (ESI) in the positive ion mode was used. The precursor/product ion transitions were m/z 532/145 for ensitrelvir, m/z 538/147 for [13C,2H5]-ensitrelvir.

Dexamethasone

AB Sciex Triple Quad 4500 (Framingham, MA, USA) with a Shimadzu LC-20A and LC-30A system (Kyoto, Japan) was used. The LC separation was achieved on an Inertsil ODS-SP (50 mm ×2.1 mm I.D., 3 μm; GL Sciences, Tokyo, Japan) (**Supplementary Table 4**). Mobile phases were 10 mmol/L

ammonium formate for A and methanol for B (40:60, v/v), which was pumped at a flowrate of 0.3 mL/min. For MS detection, ESI in the positive ion mode was used. The precursor/product ion transitions were m/z 393/355 for dexamethasone, m/z 397/359 for [2H4]-dexamethasone.

Prednisolone

AB Sciex API5000 (Framingham, MA, USA) with a Shimadzu UFLC system (Kyoto, Japan) was used. The LC separation was achieved on a LUNA Phenyl-Hexyl (100 mm ×2.0 mm I.D., 3 μm; Phenomenex, Torrance, CA, USA) (**Supplementary Table 4**). Mobile phases were 10 mmol/L ammonium formate containing 0.1% formic acid for A and acetonitrile containing 0.1% formic acid for B (75:25, v/v), which was pumped at a flowrate of 0.2 mL/min. For MS detection, ESI in the negative ion mode was used. The precursor/product ion transitions were m/z 405/329 for prednisolone, m/z 411/333 for [2H6]-prednisolone.

Midazolam

AB Sciex Triple Quad 6500 (Framingham, MA, USA) with a Shimadzu LC-30A system (Kyoto, Japan) was used. The LC separation was achieved on an InertSustain C18 HP (100 mm ×2.1 mm I.D., 3 μm; GL Sciences, Tokyo, Japan) (**Supplementary Table 4**). Mobile phases were 10 mmol/L ammonium acetate/0.1% acetic acid (1:1, v/v) for A and methanol for B. A linear elution gradient was set at a flow rate of 0.4mL/min with mobile phase consisted of 50% of A and 50% of B at 0min, and to reach 5% of A and 95% of B at 4.5min. For MS detection, ESI in the positive ion mode was used. The precursor/product ion transitions were m/z 326/291 for midazolam, m/z 330/295 for [2H4]-midazolam.

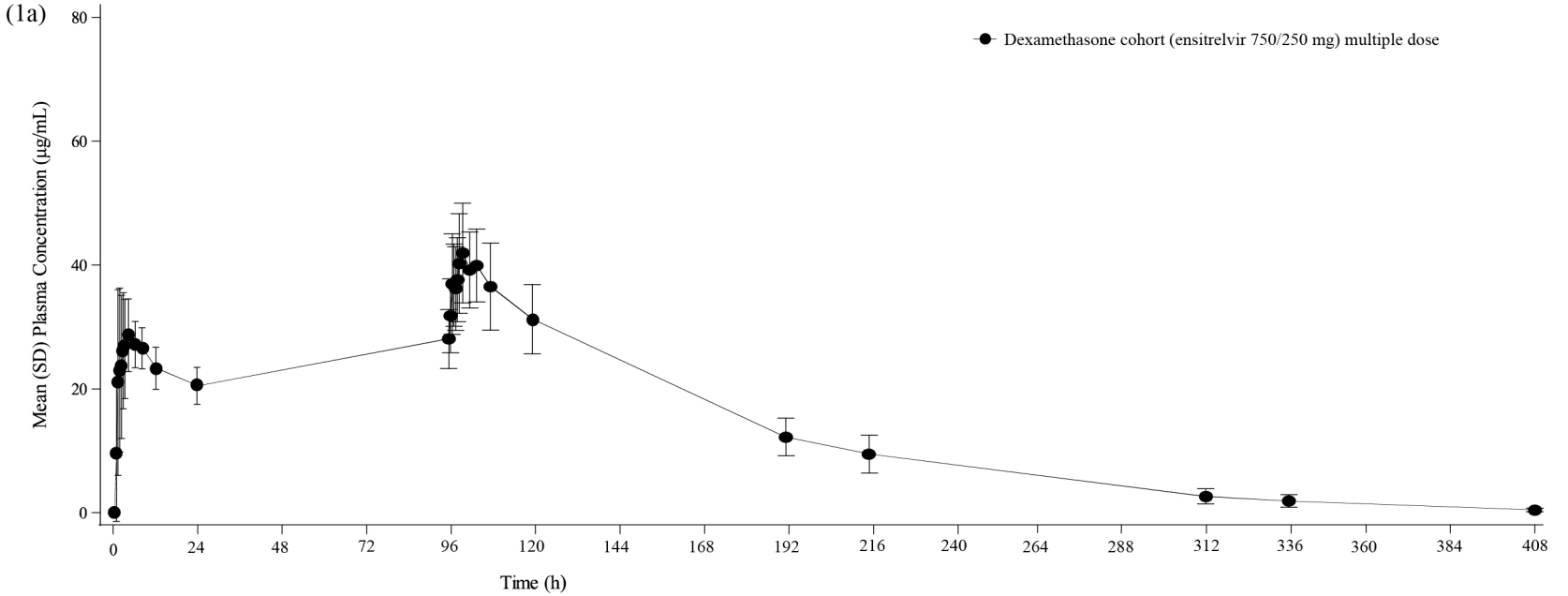
Validation

Validation for each bioanalytical method was carried out according to the Japan MHLW guideline on bioanalytical method validation. The quantification range, the lower limit of quantification (LLOQ), and the values of linearity, accuracy, precision, and recovery are summarized in **Supplemental Table 4**.

Supplementary Figures and Tables

Supplementary Fig. 1: Mean (SD) plasma concentration profile of ensitrelvir following multiple-dose administration of ensitrelvir, once daily for 5 days in the fasted state in Japanese healthy adult participants, until 408 hours after the initial administration of ensitrelvir: (**1a**) 750/250 mg (dexamethasone cohort); (**1b**) 750/250 mg (prednisolone cohort).

Supplementary Fig. 2: Chemical structures of [13C,2H5]-ensitrelvir, [2H4]-dexamethasone, [2H6]-prednisolone, and [2H3]-midazolam malate.


(1a)
Dexamethasone cohort (ensitrelvir 750/250 mg) multiple dose
Mean (SD) Plasma Concentration (µg/mL)
0
20
40
60
80
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360
384
408
Time (h)

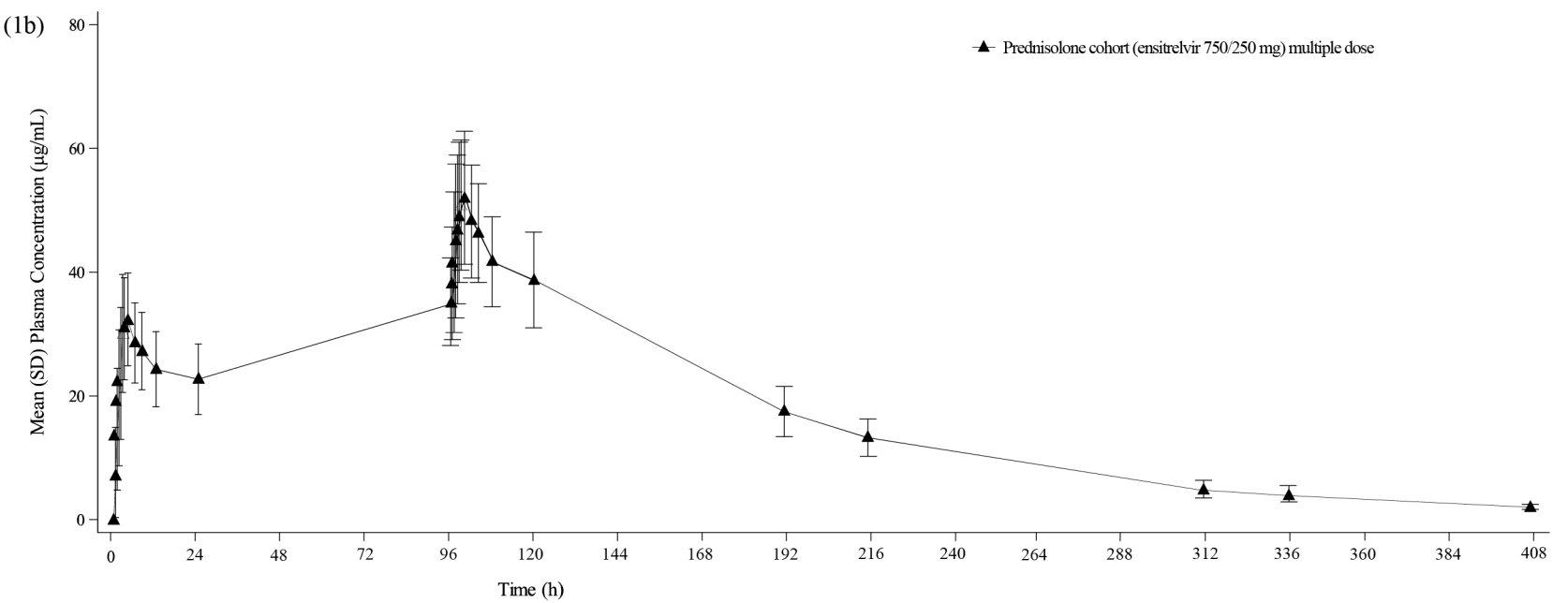
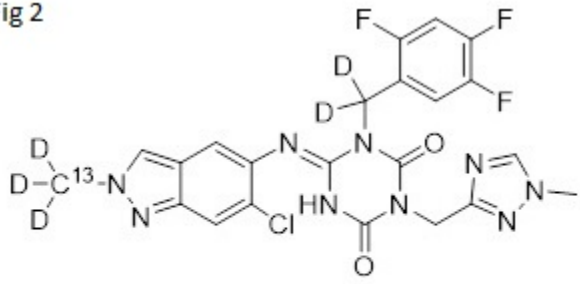
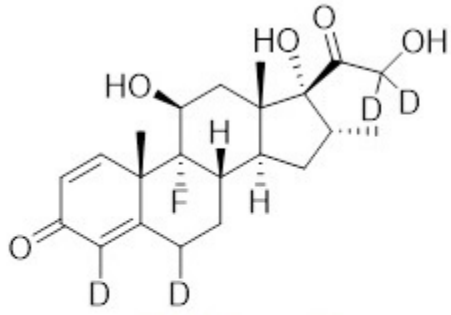

(1b)
Prednisolone cohort (ensitrelvir 750/250 mg) multiple dose
Mean (SD) Plasma Concentration (µg/mL)
0
20
40
60
80
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360
384
408
Time (h)

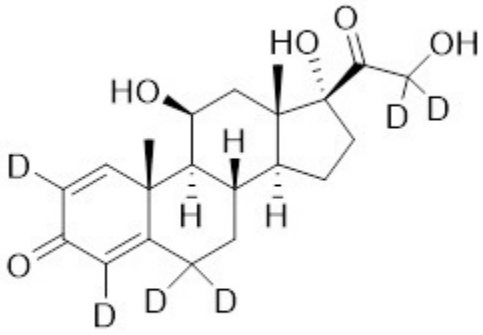
Fig 2



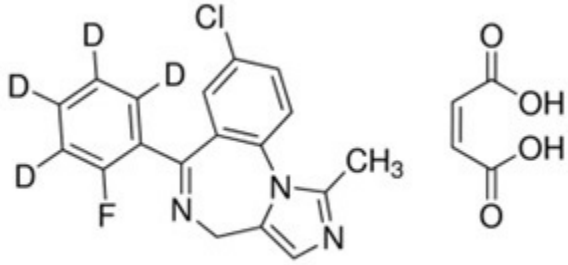
[13C,2H5]-ensitrelvir



[2H4]-dexamethasone



[2H6]-prednisolone



[2H4]-midazolam malate

Supplementary Table 1: Demographic and baseline characteristics of participants in each part.

		Dexamethasone cohort Ensitrelvir 750/250 mg + Dexamethasone N = 14	Prednisolone cohort Ensitrelvir 750/250 mg + Prednisolone N = 14	Midazolam cohort Ensitrelvir 375/125 mg + Midazolam N = 14
Sex, male		14 (100.0)	14 (100.0)	14 (100.0)
Age, years	Mean (SD)	35.0 (5.8)	31.9 (5.1)	36.0 (7.4)
	Min	27	21	23
	Median	35.5	31.0	35.5
	Max	45	39	47
Height, cm	Mean (SD)	170.25 (4.60)	175.46 (4.27)	172.26 (4.91)
	Min	159.9	166.7	164.1
	Median	170.00	174.90	173.30
	Max	179.6	181.2	179.5
Weight, kg	Mean (SD)	68.01 (6.48)	66.21 (7.68)	65.17 (5.71)
	Min	54.1	55.3	57.8
	Median	69.50	64.80	64.00
	Max	77.1	84.8	75.4
BMI, kg/m^2	Mean (SD)	23.29 (1.82)	21.51 (2.24)	22.04 (1.58)
	Min	19.4	18.8	19.7
	Median	23.75	21.25	22.00
	Max	25.5	27.6	24.8
Race, Asian	n (%)	14 (100.0)	14 (100.0)	14 (100.0)
Alcohol usage	n (%)	0	0	0
Tobacco usage	n (%)	0	0	0
Caffeine usage	n (%)	0	3 (21.4)	0
SARS-CoV-2 test, negative	n (%)	14 (100.0)	14 (100.0)	14 (100.0)

The last values taken before the first administration were used if there were multiple results.
BMI, body mass index; Max, maximum; Min, minimum; N, number of participants; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation.

Supplementary Table 2: Time-dependent interaction of ensitrelvir with liver CYP enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A

Enzyme	Probe substrate	Probe substrate (μM)[a]	Metabolite monitored	Internal standard	IC_{50} (μM)			
					0-min preincubation	30-min preincubation without NADPH	30-min preincubation with NADPH	Potential for time dependent inhibition
CYP1A2	Phenacetin	90	Acetaminophen	Acetaminophen-d4	> 100	> 100	> 100	No
CYP2B6	Efavirenz	5	8-Hydroxyefavirenz	8-Hydroxyefavirenz-d4	> 100	> 100	> 100	No
CYP2C8	Amodiaquine	2	N-Desethylamodiaquine	N-Desethylamodiaquine-d5	35	34	34	No
CYP2C9	Diclofenac	12	4′-Hydroxydiclofenac	4′-Hydroxydiclofenac-d4	> 100	> 100	> 100	No
CYP2C19	S-Mephenytoin	60	4′-Hydroxymephenytoin	4′-Hydroxymephenytoin-d3	> 100	> 100	> 100	No
CYP2D6	Dextromethorphan	10	Dextrorphan	Dextrorphan-d3	> 100	> 100	> 100	No
CYP3A	Midazolam	3	1′-Hydroxymidazolam	1′-Hydroxymidazolam-d4	> 100	> 100	39	Yes (NADPH-dependent)
CYP3A	Testosterone	60	6β-Hydroxytestosterone	6β-Hydroxytestosterone-d3	> 100	> 100	81	Yes (NADPH-dependent)

[a]Concentrations based on K_m value in the test system. Ensitrelvir showed a reversible inhibitory effect on CYP2C8 but not the other CYP enzymes including CYP3A, and the time-dependent inhibition with NADPH-dependent manner on CYP3A. According to the DDI guidelines [1-3], a mechanistic static model suggested that ensitrelvir inhibits CYP3A but not CYP2C8 in the clinical dosing regimen of ensitrelvir at 375/125 mg.

Supplementary Table 3: The inactivation parameters of enstirelvir on CYP3A

Enzyme	Substrate	K_I (μM)	K_{inact} (min^{-1})
CYP3A	Midazolam	84	0.046
	Testosterone	86	0.055

K_I half-maximal inactivation concentration
k_{inact} maximal inactivation constant rate

Supplementary Table 4: Summary of bioanalytical methods

Analyte	Ensitrelvir		Dexamethasone	Prednisolone	Midazolam
	Used in Part 1	Used in Part 2			
Quantification range (ng/mL)	10.0 to 300000	200 to 200000	0.500 to 2000	2.00 to 2000	0.0500 to 400
LLOQ (ng/mL)	10.0	200	0.500	2.00	0.0500
Linearity (%)*	93.8 to 105.0	91.9 to 108.4	94.7 to 102.7	96.9 to 102.0	99.0 to 102.2
Between-run Precision (RSD, %)	1.3 to 3.2	6.4 to 10.1	1.3 to 3.0	1.1 to 3.1	2.4 to 7.0
Between-run Accuracy (%)	92.3 to 107.8	99.1 to 104.0	97.5 to 102.0	101.0 to 105.7	100.8 to 108.8
Recovery (%)	98.0 to 105.3	82.6 to 87.2	94.9 to 108.9	100.6 to 102.4	83.8 to 86.8

*Evaluated by mean accuracy of back-calculated concentration of calibration samples at each concentration level

Supplementary References

1. Ministry of Health, Labour and Welfare in Japan. Pharmaceutical Safety and Environmental Health Bureau. Evaluation and Licensing Division. Guideline on drug interaction for drug development and appropriate provision of information. July 2018.

2. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research. Guidance for Industry. In vitro Drug Interaction Studies—Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions. January 2020.

3. European Medicines Agency. Committee for Human Medicinal Products. Guideline on the Investigation of Drug Interactions. January 2013.