Supplementary Methods

Part A

Estimation of Michaelis-Menten constant (J_{max} and K_m) of milademetan for P-gp transport in human Caco-2 cell monolayer systems

The Michaelis-Menten constant (J_{max} and K_m) of milademetan for P-gp transport was determined using P-gp transport assay performed in human Caco-2 cell monolayers. In the bidirectional transport assay of [^{14}C]milademetan, the culture medium on both the donor and receiver sides was replaced with Hanks’ Balanced Salt Solution (HBSS) buffer and preincubated for 15 minutes at 37°C. The HBSS buffer on the receiver side was then replaced with fresh HBSS buffer, while the HBSS buffer on the donor side was replaced with HBSS buffer containing 1, 3, 10, 30, and 100 µM of [^{14}C]milademetan. A typical potent P-gp/BCRP inhibitor, GF120918 (Elacridar) 5 µM, was also added to both donor and receiver sides to confirm the effect on P-gp in the bidirectional transport assay. After incubation for 120 minutes at 37°C, aliquots of the solution from the receiver side were collected, and radioactivity was measured with a liquid scintillation counter (LSC), 2300TR (PerkinElmer, Inc).

The permeability coefficient (P_{app}) and P_{app} ratio of milademetan were calculated according to the publication by Mikkaichi et al.\(^1\) and were summarized in the Table below.

\[
P_{app} = \frac{1}{C_0 \cdot A} \cdot \frac{dQ}{dt}
\]

\[
P_{appRatio} = \frac{P_{app,B to A}}{P_{app,A to B}}
\]

where \(dQ/dt\) is the steady-state appearance rate of the substrate on the receiver side (dpm/s), \(C_0\) is concentration of the test compound on the donor side (dpm/mL), and \(A\) is the surface area of the monolayer (0.33 cm\(^2\)); and \(P_{app,A to B}\) is the \(P_{app}\) value from the apical-to-basal direction and \(P_{app,B to A}\) is the \(P_{app}\) value from the basal-to-apical direction.

| Milademetan (µM) | Inhibitor         | P_{app,A to B} (10^{-6} cm/s) | P_{app,B to A} (10^{-6} cm/s) | P_{app} ratio (B to A/A to B) |
|------------------|-------------------|-------------------------------|-------------------------------|-------------------------------|
| 1                | –                 | 1.13                          | 18.2                          | 16.11                         |
| 3                | –                 | 1.24                          | 17.5                          | 14.11                         |
| 10               | –                 | 1.86                          | 17.3                          | 9.30                          |
| 30               | –                 | 3.66                          | 9.95                          | 2.72                          |
| 100              | –                 | 4.42                          | 5.79                          | 1.31                          |
| 1                | GF120918 (5 µM)   | 5.07                          | 3.44                          | 0.68                          |

To obtain a single set of parameter estimates for K_m value, maximum velocity (J_{max}), and passive diffusion (CLPD), the kinetic analysis using a 3-compartment model reported by Tachibana et al.\(^2\) was performed. The 3-compartment model consisted of apical, cellular, and basal compartments under the assumption that the passive permeability across apical and basal membranes is identical, and the model was fitted simultaneously to the \(P_{app}\) values both in apical-to-basal and basal-to-apical directions. In the
case where the data were obtained in the presence of the P-gp inhibitor (GF120918 5 µM), the $J_{\text{max}}$ value was set as zero. As shown in the table below, $K_m$, $J_{\text{max}}$, and passive diffusion were calculated to be 0.324 µM, 13.4 pmol/min/cm$^2$, and $17.0 \times 10^{-6}$ cm/s, respectively. The $P_{\text{app}}$ and $P_{\text{app}}$ ratios are reasonably represented by the 3-compartment model shown in the figure below.

### Milademetan P-gp transport kinetic parameter estimates in Caco-2 cell monolayers

| Parameter | Unit | Value | CV (%) |
|-----------|------|-------|--------|
| $K_m$     | µM   | 0.324 | 1.3    |
| $V_{\text{max}}$ | pmol/min/cm$^2$ | 13.4 | 7.8 |
| $P_{\text{AC}}=P_{\text{CA}}=P_{\text{BC}}=P_{\text{CB}}$ | $10^{-6}$ cm/s | 17.0 | 3.9 |

Model-fitting results of $P_{\text{app,A to B}}$ (A), $P_{\text{app,B to A}}$ (B), and $P_{\text{app}}$ ratio (C) of milademetan in Caco-2 cells

References

1. Mikkaichi T, Yoshigae Y, Masumoto Y, et al. Edoxaban transport via p-glycoprotein is a key factor for the drug’s disposition. *Drug Metab Dispos.* 2014;42:520-528.
2. Tachibana T, Kitamura S, Kato M, et al. Model analysis of the concentration-dependent permeability of p-gp substrates. *Pharm Res.* 2010;27:442-446.
Supplementary Methods

Part B

Estimation of inhibitory parameters ($K_i$, $K_{app}$, and $K_{inact}$) of milademetan on CYP450 isoenzymes using human hepatic microsomes

The inhibitory potential of milademetan on the activities of human hepatic CYP isoenzymes were characterized *in vitro* using pooled human hepatic microsomes. To determine the direct inhibitory effect of milademetan on CYP3A, the *in vitro* assays were conducted at 5 concentrations of midazolam (1.25, 2.5, 5, 10, and 25 µM) and 6 concentrations of milademetan (0, 5, 10, 20, 30, and 38 µM). Four modified Michaelis-Menten equations (competitive inhibition, mixed inhibition, noncompetitive inhibition, and uncompetitive inhibition) were fitted to CYP3A activity. The inhibition type was determined to be the combination of competitive and uncompetitive inhibition, and the potency of inhibition ($K_i$) was calculated to be 4.2 µM, shown in the Eadie-Hofstee plot below.

**Eadie-Hofstee plot of midazolam metabolism at milademetan concentrations of 0, 5, 10, 20, 30, and 38 µM**

![Eadie-Hofstee plot](image)

$V_{max} = 1560$ pmol/min/mg

$K_m = 2$ µM

$K_i = 4.2$ µM

$\alpha = 4.7$

To determine the kinetics of metabolism-dependent inhibition, milademetan 0, 2.5, 5, 10, 20, 30, and 38 µM was preincubated in the absence and presence of nicotinamide adenine dinucleotide phosphate (NADPH) (1 mM) for 0, 5, 10, 15, 20, and 30 minutes. The data were processed for calculation of $K_{app}$ and $K_{inact}$ based on the method of Maurer and Fung (2000). The preincubation time for each concentration of milademetan was plotted against the remaining CYP3A activity as percent of control normalized with those obtained in the absence of NADPH in logarithmic scale. The equation was then obtained for each linear curve. The slope of each curve (the exponent of e) representing the observed inactivation
constant ($K_{\text{obs}}$) was recorded. $K_{\text{app}}$ 60.5 µM and $K_{\text{inact}}$ 3.71 h$^{-1}$ were derived based on the linear correlation of the reciprocal of $K_{\text{obs}}$ and the reciprocal of milademetan concentration, shown in the figures below.

**Relationship between observed rates of inactivation and milademetan concentrations for CYP3A4/5 activity (midazolam 1'-hydroxylase)**

![Graph showing calculation of $K_i$ and $K_{\text{inact}}$](image)

$K_i = 60.5$ µM and $k_{\text{rel}} = 0.0619$ minute$^{-1}$

**Reference**

1. Maurer TS, Fung HL. Evaluation of nitric oxide synthase activity and inhibition kinetics by chemiluminescence. *Nitric Oxide*. 2000;4:372-378.
### Supplementary Tables

**Table S1 Model parameter input for the internally modified itraconazole model (SV-Itraconazole_Fasted Soln, V17)**

| Parameter (units)                  | Definition                              | Values       |
|-----------------------------------|-----------------------------------------|--------------|
| Molecular weight (g/mol)          |                                         | 705.6        |
| logP                              | Octanol to water partition coefficient  | 4.47         |
| Compound type                     |                                         | Monoprotic base |
| pKa                               | Ionization coefficient                  | 4.28         |
| B/P                               | Blood to plasma ratio                   | 0.58         |
| $f_u$                             | Unbound fraction                        | 0.016        |
| Main plasma-binding protein       |                                         | Human serum albumin |
| $f_u$ in the gut (fu,gut)          | Unbound fraction in the gut             | 0.016        |
| Distribution model                |                                         | Minimal PBPK model |
| $V_{ss}$ (L/kg)                   | Volume of distribution at steady state  | 2.520463     |
| Enzyme                            |                                         | CYP1A2       |
| Pathway                           |                                         | OH           |
| $CL_{int}$ (µL/min/pmol)          | Intrinsic clearance                     | 1            |
| Enzyme                            |                                         | CYP3A4       |
| Pathway                           |                                         | OH           |
| $V_{max}$ (pmol/min/pmol)         | Maximal metabolism rate                 | 0.065        |
| $K_m$ (µM)                        | Michaelis-Menten constant               | 0.0039       |
| $CL_R$ (L/h)                      | Renal clearance                         | 0            |
| Enzyme                            |                                         | CYP3A4       |
| $K_i$ (µM)                        | CYP3A4 inhibition constant              | 0.0013       |
| Transporter                       |                                         | ABCB1 (P-gp/MDR1) |
| Organ                             |                                         | Gut          |
| $K_i$ (µM)                        | P-gp inhibition constant                 | 0.03         |
| Transporter                       |                                         | ABCB1 (P-gp/MDR1) |
| Organ                             |                                         | Liver        |
| \( K_i (\mu M) \) | P-gp inhibition constant | 0.03 |
|------------------|-------------------------|------|

CYP3A4, cytochrome P450 3A4; PBPK, physiologically based pharmacokinetic; P-gp, P-glycoprotein.
| Parameter (units)                        | Definition                               | Values       |
|-----------------------------------------|------------------------------------------|--------------|
| Molecular weight (g/mol)                |                                          | 721.7        |
| logP                                    | Octanol to water partition coefficient   | 4.47         |
| Compound type                           |                                          | Monoprotic base |
| pKa                                     | Ionization coefficient                   | 4.28         |
| B/P                                     | Blood to plasma ratio                    | 0.58         |
| $f_u$                                   | Unbound fraction                         | 0.016        |
| Main plasma-binding protein             |                                          | Human serum albumin |
| $f_{u,gut}$                             | Unbound fraction in the gut              | 0.016        |
| Distribution model                      |                                          | Minimal PBPK model |
| $V_{ss}$ (L/kg)                         | Volume of distribution at steady state   | 1.03532      |
| Enzyme                                  |                                          | CYP3A4       |
| Pathway                                 |                                          | Pathway 1    |
| $V_{max}$ (pmol/min/pmol)               | Maximal metabolism rate                  | 0.13         |
| $K_m$ (µM)                              | Michaelis-Menten constant                | 0.027        |
| $CL_R$ (L/h)                            | Renal clearance                          | 1.39         |
| Enzyme                                  |                                          | CYP3A4       |
| $K_i$ (µM)                              | CYP3A4 inhibition constant               | 0.0023       |

CYP3A4, cytochrome P450 3A4; PBPK, physiologically based pharmacokinetic.
| Parameter (units) | Definition | Values |
|------------------|------------|--------|
| Molecular weight (g/mol) | | 700.792 |
| logP | Octanol to water partition coefficient | 4 |
| Compound type | Diprotic base |
| pKa | Ionization coefficient | 2.88, 4.11 |
| B/P | Blood to plasma ratio | 0.62 |
| $f_u$ | Unbound fraction | 0.01 |
| Main plasma-binding protein | Human serum albumin |
| $f_a$ | Fraction absorbed | 0.85 |
| $k_a$ (1/h) | Absorption rate constant | 0.55 |
| Lag time (h) | | 0.8 |
| $f_{u,gut}$ | Unbound fraction in the gut | 1 |
| $Q_{gut}$ (L/h) | Nominal flow through the gut | 16.992 |
| Distribution model | Minimal PBPK model |
| $V_{ss}$ (L/kg) | Volume of distribution at steady state | 2.96 |
| $CL_{iv}$ (L/h) | Systemic clearance | 7.32 |
| $CL_R$ (L/h) | Renal clearance | 0 |
| Enzyme | CYP3A4 |
| $K_i$ (µM) | CYP3A4 reversible inhibition constant | 0.005 |

CYP3A4, cytochrome P450 3A4; PBPK, physiologically based pharmacokinetic.
Table S4 Trial design for simulation of DDI effects of moderate CYP3A inhibitors

| Sample size and population | Milademetan | Interacting drugs |
|----------------------------|-------------|-------------------|
| Sim-Healthy Volunteers: 10 trials, 10 subjects in each trial | 100 mg SD on day 7, simultaneous administration with fluconazole | Fluconazole 400 mg on day 1, 200 mg QD on days 2–13 |
| Age: 18–55 years | Fasted conditions | 100 mg SD on day 7, 2 hours after the dose of erythromycin | Erythromycin 500 mg TID on days 1–13 |
| Female: 0.5 | Fasted conditions | 100 mg SD on day 7, simultaneous administration with verapamil | Verapamil 100 mg TID on days 1–13 |

CYP3A, cytochrome P450 3A; DDI, drug-drug interaction; QD, once daily; SD, single dose; TID, 3 times daily.
Table S5 Trial design for simulation of washout period after discontinuation of strong CYP3A inhibitors itraconazole and posaconazole

| Sample size and population | Scenario | Milademetan | Interacting drugs |
|---------------------------|----------|-------------|-------------------|
| Sim-Healthy Volunteers    | 1        | 80 mg QD on days 1–8  
| Age: 20 years Male        |          | 160 mg QD on days 9–14  
|                           |          | Fasted conditions      | Itraconazole 200 mg BID on day 1,  
|                           |          |                          | 200 mg QD on days 2–8  
|                           | 2        | 80 mg QD on days 1–10  
|                           |          | 160 mg QD on days 11–14  
|                           |          | Fasted conditions      | 1 hour before milademetan dose  
|                           | 3        | 80 mg QD on days 1–14  
|                           |          | Fasted conditions      |                  |
|                           | 4        | 80 mg QD on days 1–8  
|                           |          | 160 mg QD on days 9–14  
|                           |          | Fasted conditions      | Posaconazole 200 mg TID on days 1–8  
|                           | 5        | 80 mg QD on days 1–10  
|                           |          | 160 mg QD on days 11–14  
|                           |          | Fasted conditions      | 2 hours before milademetan dose  
|                           | 6        | 80 mg QD on days 1–14  
|                           |          | Fasted conditions      |                  |
|                           | 7        | 160 mg QD on days 1–14  
|                           |          | Fasted conditions      | None |

BID, twice daily; CYP3A4, cytochrome P450 3A4; TID, 3 times daily; QD, once daily.
|                          | \( \text{AUC}_{\text{inf}} \) (ng/mL•h) | \( \text{C}_{\text{max}} \) (ng/mL) | AUCR | \( \text{C}_{\text{max}}^R \) |
|--------------------------|----------------------------------------|------------------------------------|------|------------------------|
| **Inhibitory effect of fluconazole on PK of milademetan** |                                        |                                    |      |                        |
| Control                  | 14,015                                 | 711                                |      |                        |
| With fluconazole         | 24,143                                 | 804                                | 1.72 (1.69–1.76) | 1.13 (1.12–1.14) |
| **Inhibitory effect of erythromycin on PK of milademetan** |                                        |                                    |      |                        |
| Control                  | 14,411                                 | 723                                |      |                        |
| With erythromycin        | 27,537                                 | 805                                | 1.91 (1.83–1.99) | 1.11 (1.10–1.12) |
| **Inhibitory effect of verapamil on PK of milademetan**  |                                        |                                    |      |                        |
| Control                  | 14,384                                 | 766                                |      |                        |
| With verapamil           | 29,011                                 | 893                                | 2.02 (1.93–2.11) | 1.17 (1.15–1.18) |

\( \text{AUC}_{\text{inf}} \) and \( \text{C}_{\text{max}} \) are presented as geometric mean. AUCR and \( \text{C}_{\text{max}}^R \) are presented as geometric mean (90% CI).

\( \text{AUC}_{\text{inf}} \), concentration-time curve from zero to infinity; AUCR, area under the concentration-time curve ratio; \( \text{C}_{\text{max}} \), maximum serum concentration; \( \text{C}_{\text{max}}^R \), maximum serum concentration ratio; PK, pharmacokinetics.
### Table S7 Simulated milademetan AUC\textsubscript{tau} before and after discontinuation of strong CYP3A inhibitors itraconazole and posaconazole

| Scenario | Day 8 | Day 9 | Day 10 | Day 11 | Day 12 | Day 13 | Day 14 |
|----------|-------|-------|--------|--------|--------|--------|--------|
| 1        | 23,172| 32,126\textsuperscript{a} | 34,959 | 28,614 | 24,452 | 22,652 | 21,735 |
|          | (1.12)| (1.56) | (1.69) | (1.39) | (1.18) | (1.10) | (1.05) |
| 2        | 23,172| 23,364 | 22,093 | 22,929\textsuperscript{a} | 21,638 | 21,163 | 20,923 |
|          | (1.12)| (1.13) | (1.07) | (1.11) | (1.05) | (1.03) | (1.01) |
| 3        | 23,172| 23,364 | 22,093 | 16,378 | 13,084 | 11,614 | 10,879 |
|          | (1.12)| (1.13) | (1.07) | (0.79) | (0.63) | (0.56) | (0.53) |
| 4        | 20,323| 28,513\textsuperscript{a} | 30,919 | 30,368 | 28,276 | 25,959 | 24,077 |
|          | (0.98)| (1.38) | (1.50) | (1.47) | (1.37) | (1.26) | (1.17) |
| 5        | 20,323| 20,207 | 19,044 | 24,624\textsuperscript{a} | 25,096 | 24,182 | 23,097 |
|          | (0.98)| (0.98) | (0.92) | (1.19) | (1.22) | (1.17) | (1.12) |
| 6        | 20,323| 20,207 | 19,044 | 17,146 | 15,073 | 13,311 | 12,056 |
|          | (0.98)| (0.98) | (0.92) | (0.83) | (0.73) | (0.64) | (0.58) |
| 7        | 20,495| 20,553 | 20,591 | 20,613 | 20,627 | 20,636 | 20,641 |

Simulation scenario is defined in Table S5. Values in parentheses are fold of change of AUC\textsubscript{tau} to AUC\textsubscript{tau} on day 14 of scenario 7.

AUC\textsubscript{tau}, area under the plasma concentration-time curve over dosing interval; CYP3A, cytochrome P450 3A.

\textsuperscript{a}Day when milademetan dose was returned to 160 mg QD.