

Supplementary document

ORIGINAL RESEARCH ARTICLE

Unexpected clinical drug–drug interaction of fexinidazole on midazolam pharmacokinetics: Insights into underlying mechanisms from clinical phase I study results and supporting *in vivo/vitro* evidence

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Short title: Unexpected clinical drug interaction of fexinidazole on midazolam

Supplementary Materials

I. Supplementary Figures

Fig. S1. Chemical structures of a) fexinidazole, b) M1 (sulfoxide), and c) M2 (sulfone).
Structures adapted from FDA prescribing information for fexinidazole tablets

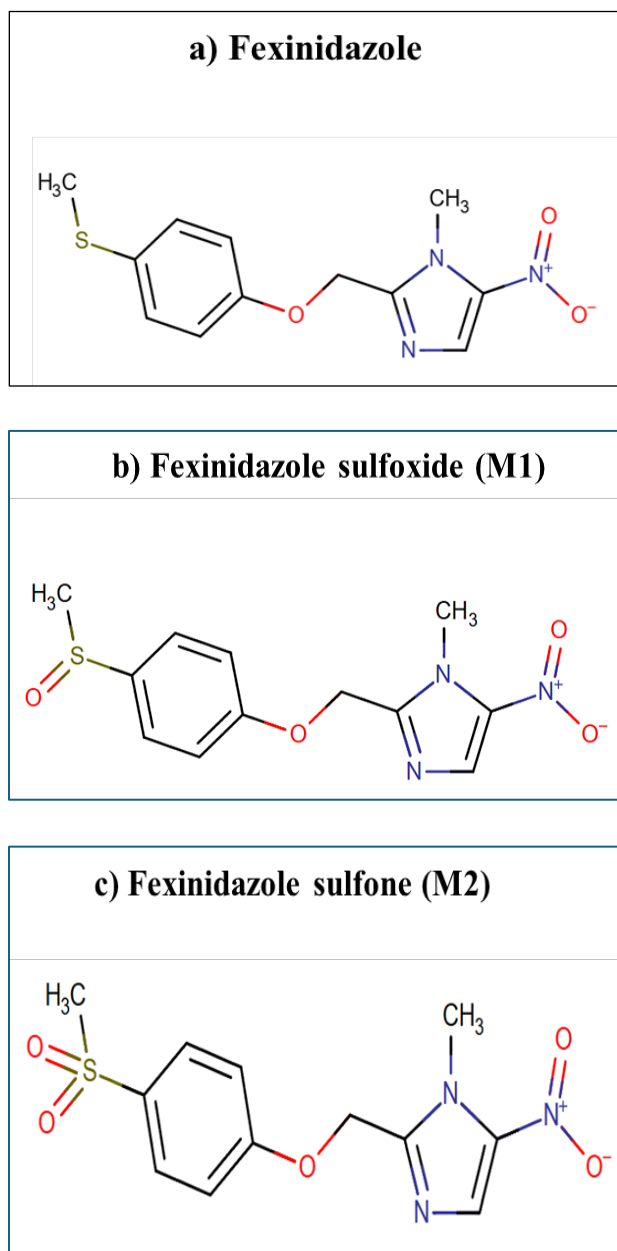


Fig. S2. Metabolic pathways of midazolam in human

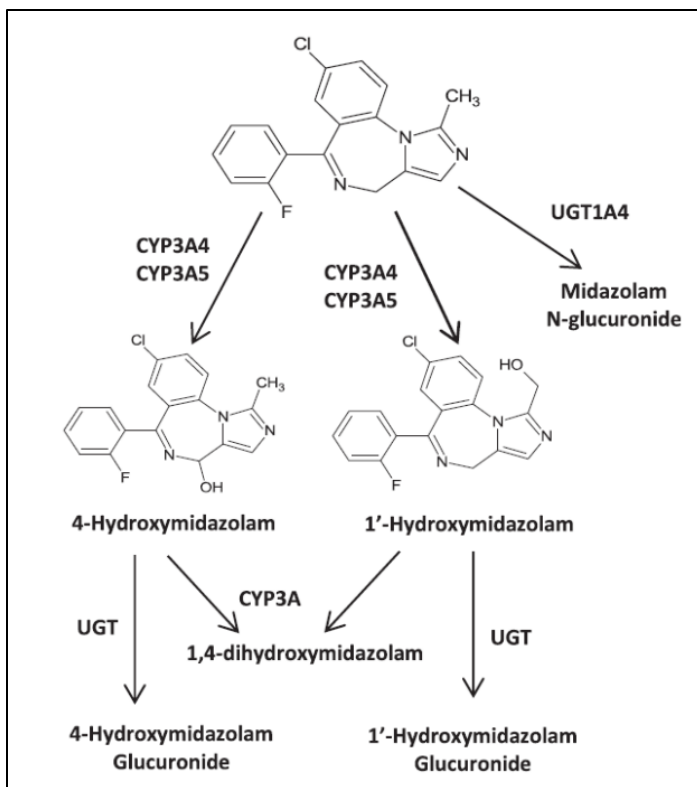


Fig. S3: Metabolic ratio for a) C_{max} and b) AUC of 1-hydroxy midazolam without and with fexinidazole and with treatment effect

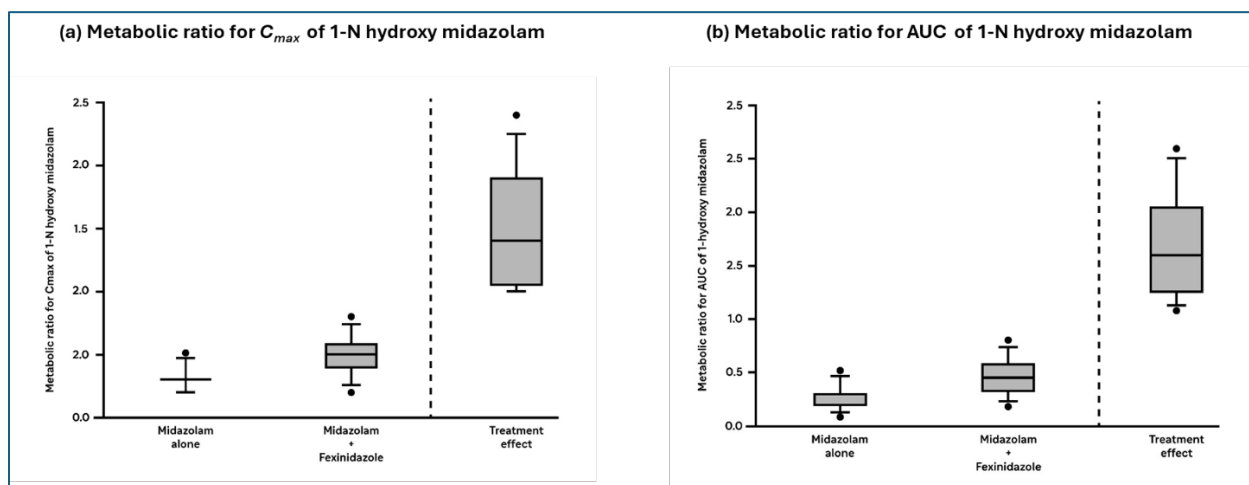
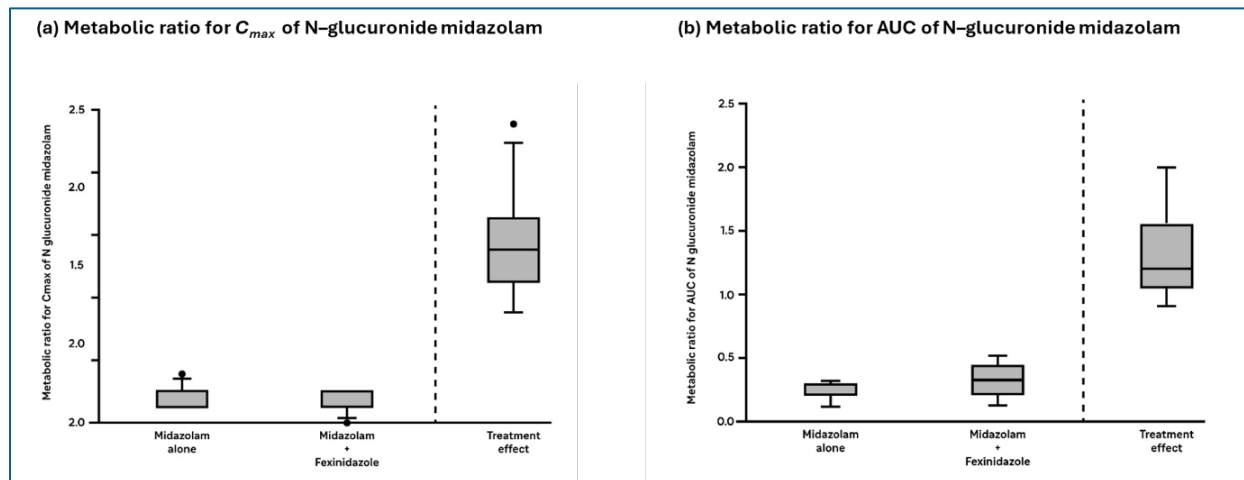


Fig. S4: Metabolic ratio for a) C_{max} and b) AUC of N-glucuronide midazolam without and with fexinidazole, and with treatment effect



CYP2B6 Induction Curves (with EC_{50} and E_{max})

Fig. S5. CYP2B6 mRNA expression as fold changes in hepatocytes from donor 1 after treatment with NC, PC, Fexinidazole, Fexinidazole Sulfoxide (M1), Fexinidazole Sulfone (M2) or with a given mix of the three test items.

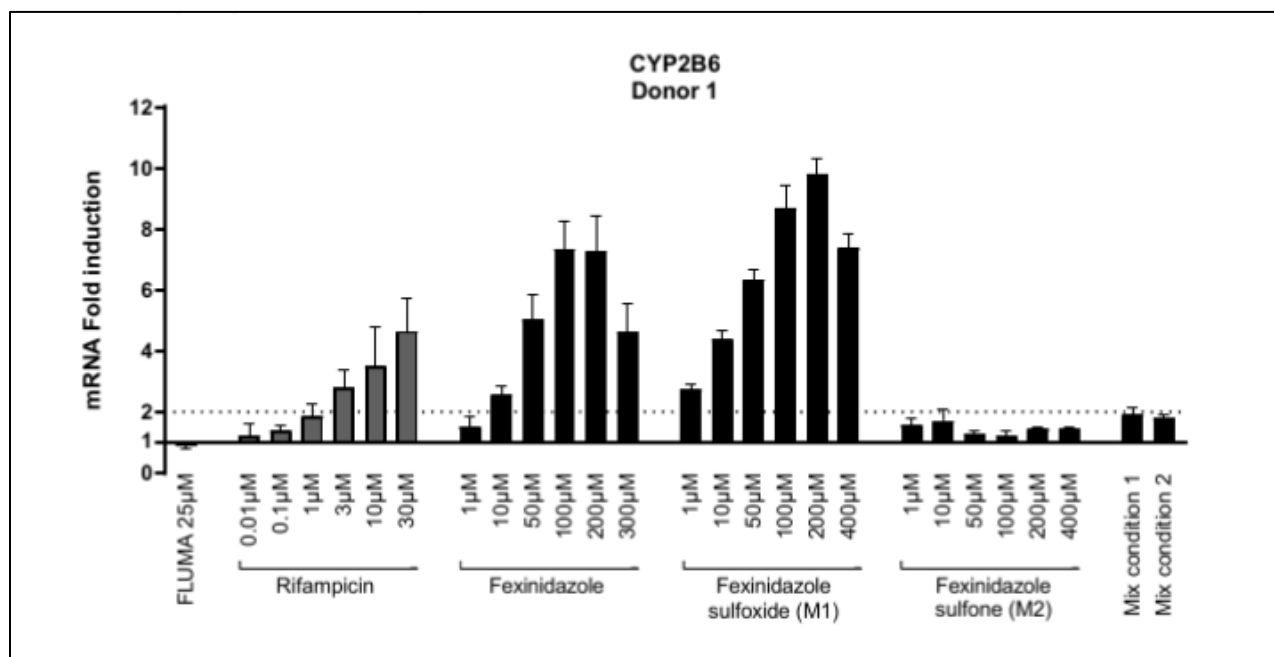


Fig. S6. CYP1A2 mRNA expression as fold changes in hepatocytes from donor 1 after treatment with positive controls or Fexinidazole, Fexinidazole Sulfoxide (M1) and

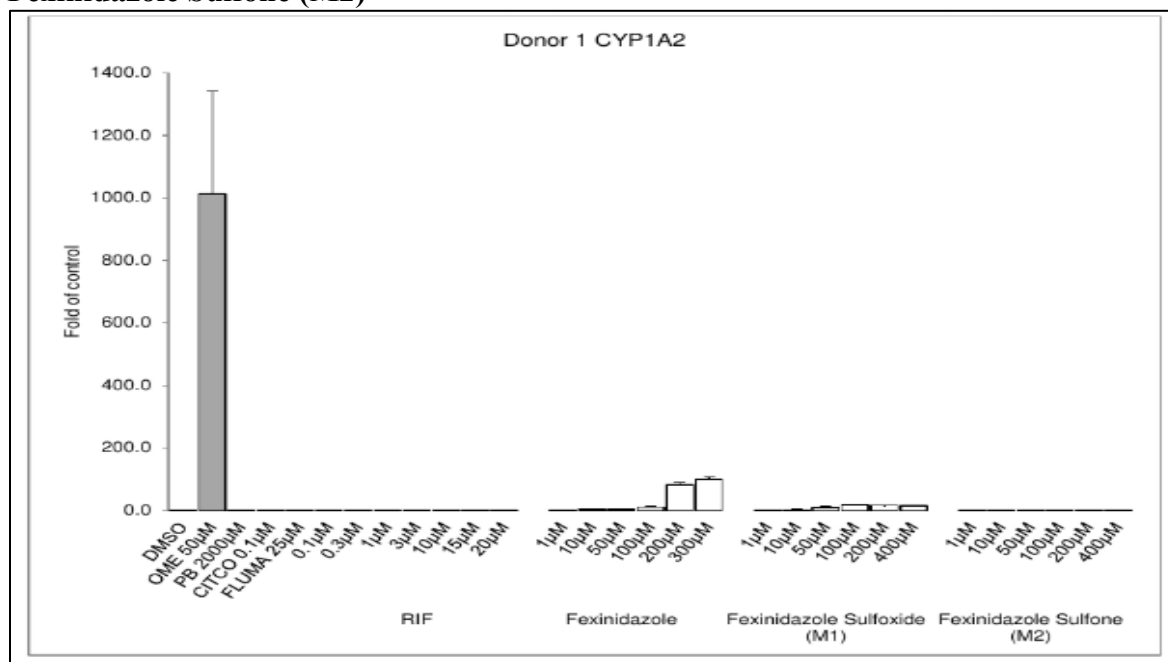
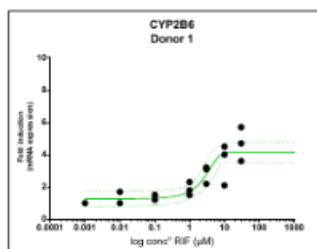
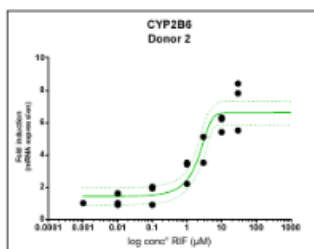
Fexinidazole Sulfone (M2)

Fig. S7. Modeling of E_{max} and EC_{50} of RIF, Fexinidazole and Fexinidazole Sulfoxide (M1) on CYP2B6 mRNA expression

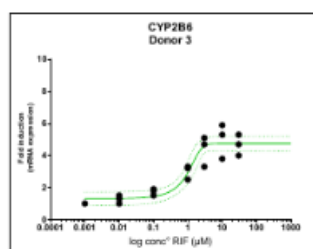
- Rifampicin



	Donor 1
	Best-fit values $\pm$ SD (95% CI)
E_{max}	4.2 ± 0.3 (3.5 to 5.4)
EC_{50}	1.65 ± 0.59 (0.599 to 6.83)

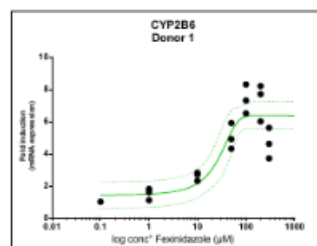


	Donor 2
	Best-fit values $\pm$ SD (95% CI)
E_{max}	6.6 ± 0.4 (5.8 to 7.3)
EC_{50}	1.71 ± 0.33 (1.01 to 2.68)

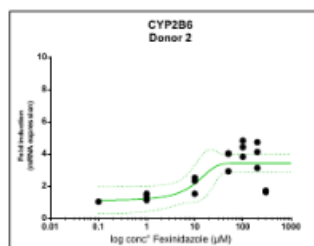


	Donor 3
	Best-fit values $\pm$ SD (95% CI)
E_{max}	4.7 ± 0.2 (4.3 to 5.2)
EC_{50}	0.666 ± 0.166 (0.361 to 1.18)

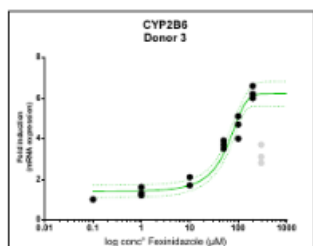
- Fexinidazole



	Donor 1
	Best-fit values $\pm$ SD (95% CI)
E_{max}	6.4 ± 0.4 (5.6 to 7.2)
EC_{50}	21.7 ± 7.0 (7.50 to 36.8)



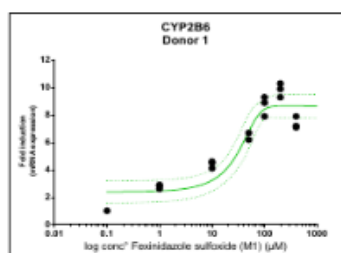
	Donor 2
	Best-fit values $\pm$ SD (95% CI)
E_{max}	3.4 ± 0.3 (2.8 to 4.0)
EC_{50}	NR



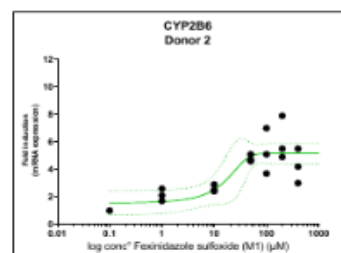
	Donor 3
	Best-fit values $\pm$ SD (95% CI)
E_{max}	6.2 ± 0.3 (5.6 to 7.0)
EC_{50}	46.5 ± 6.1 (32.7 to 64.0)

NR*: Not Relevant, $E_{max}/2 < 2$ -fold of controls. ●: values excluded from modeling.

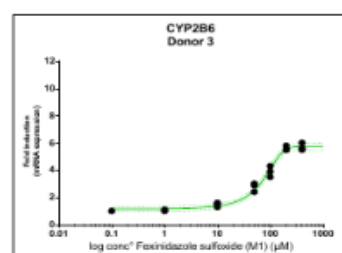
- Fexinidazole sulfoxide (M1)



	Donor 1
	Best-fit values $\pm$ SD (95% CI)
E_{max}	8.7 ± 0.4 (7.8 to 9.5)
EC_{50}	21.5 ± 5.4 (7.01 to 34.4)



	Donor 2
	Best-fit values $\pm$ SD (95% CI)
E_{max}	5.2 ± 0.3 (4.5 to 6.0)
EC_{50}	10.6 ± 5.9 (1.74 to 28.1)



	Donor 3
	Best-fit values $\pm$ SD (95% CI)
E_{max}	5.7 ± 0.1 (5.5 to 6.0)
EC_{50}	60.5 ± 3.9 (52.4 to 69.3)

II. Supplementary Tables

Rifampicin Effect on Midazolam PK

Table S1 Summary of the rifampicin effect on midazolam and 1-hydroxy-midazolam PK (adapted from Link et al., 2008 [13])

Parameters	Midazolam alone	Midazolam + rifampicin	Ratio of median
Midazolam [median (min–max)]			
$C_{\max}$ (ng/mL)	63.1 (25.9–80.2)	2.2 (0.64–10.4)	0.03
AUC (ng*h/mL)	102.9 (64.0–163.7)	1.6 (1.0–7.2)	0.016
$t_{1/2z}$ (h)	3.2 (2.1–4.8)	1.8 (0.98–8.21)	0.56
1-hydroxy-midazolam [median (min–max)]			
$C_{\max}$ (ng/mL)	36.9 (15.0–52.6)	4.4 (2.0–11.7)	0.12
AUC (ng*h/mL)	56.6 (30.7–91.9)	5.4 (3.9–13.1)	0.1
$t_{1/2z}$ (h)	5.8 (1.8–21.8)	3.2 (1.8–6.3)	0.55
R_{met} AUC (1-hydroxy-MDZ/MDZ)	0.55	3.4	-

AUC, area under the plasma concentration versus time curve; $C_{\max}$, maximum plasma concentration observed; MDZ, midazolam; PK, pharmacokinetics; R_{met} , metabolic ratio; $t_{1/2z}$, terminal half-life.

Table S2. Summary of the rifampicin effect on midazolam and 1-hydroxy-midazolam PK (adapted from Dooley et al., 2012 [12])

Parameters	Midazolam alone	Midazolam + rifampicin	Ratio of median
Midazolam [median (IQR)]			

Parameters	Midazolam alone	Midazolam + rifampicin	Ratio of median
$C_{\max}$ (ng/mL)	40.8 (34.8–48.7)	9.8 (7.2–13.6)	0.24
AUC_{0-12h} (ng*h/mL)	151 (116–197)	26.9 (10.0–55.8)	0.17
$t_{1/2z}$ (h)	4.1 (3.5–5.7)	1.7 (1.1–4.4)	0.41
	1-hydroxy-midazolam [median (IQR)]		
$C_{\max}$ (ng/mL)	20.3 (13.8–23.4)	7.1 (2.7–12.3)	0.35
$AUC_{0-12 h}$ (ng*h/mL)	64.7 (54.9–88.2)	30.1 (9.7–38.0)	0.46
R_{met} $AUC_{0-12 h}$ (1-hydroxy-MDZ/MDZ)	0.43	1.12	

AUC, area under the plasma concentration versus time curve; $C_{\max}$, maximum plasma concentration observed; IQR, interquartile range; MDZ, midazolam; PK, pharmacokinetics; R_{met} , metabolic ratio; $t_{1/2z}$, terminal half-life.

III. Supplementary Methods

S1. Selection of study population

Each volunteer had to sign and date the informed consent form before any procedures specified in this protocol were performed.

Inclusion criteria

- **Demography**
 - Male and female participants, between 18 and 55 years of age, inclusive.
 - Body weight between 50.0 and 100.0 kg, inclusive, body mass index (BMI) between 18.00 and 30.00 kg/m², inclusive.

- **Health status**

- Certified as healthy by a comprehensive clinical assessment (a detailed medical history and a complete physical examination).
- Normal vital signs after 10 min resting in a supine position in the following range (if out of the range, it was considered non-clinically significant by the investigator):
 - ✓ 95 mmHg < systolic blood pressure (SBP) < 140 mmHg
 - ✓ 45 mmHg < diastolic blood pressure (DBP) < 90 mmHg
 - ✓ 40 bpm < heart rate (HR) < 100 bpm
- Standard 12-lead ECG parameters after 10 min resting in a supine position in the following ranges: 120 ms < PR < 220 ms, QRS < 120 ms, QTc ≤ 430 ms for male and ≤ 450 ms for female participants and a normal ECG tracing unless the investigator considered an ECG tracing abnormality not clinically relevant.
- Laboratory parameters within the normal range (or the defined screening threshold for the investigator site), unless the investigator considered an abnormality clinically irrelevant for healthy participants; however, serum creatinine, alkaline phosphatase, hepatic enzymes (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]), and total bilirubin (unless the participant had a documented Gilbert syndrome) could not exceed the upper laboratory norm.
- Female participants had to use a double contraception method, including a highly effective method of birth control for at least 2 months before inclusion and up to the end of the study visit (P2, D11 to D15), except if they had undergone sterilization at least 3 months earlier or were postmenopausal. The accepted double contraception methods included the use of an intrauterine device or hormonal contraception in addition to one of the following contraceptive options: (1) condom; (2) diaphragm or cervical/vault cap; (3) spermicide. Menopause was defined as being amenorrhoeic for at least 2 years with plasma follicle-stimulating hormone level >30 UI/L if not treated with hormone replacement therapy (HRT).

- **Regulations**

- Providing written informed consent before undertaking any study-related procedures.

- Being covered by a health insurance system where applicable, and/or in compliance with the recommendations of the national laws in force relating to biomedical research.
- Not under any administrative or legal supervision.
- **Specific to the study**
 - Male participants whose partners were of childbearing potential (including lactating women) had to accept to use, during sexual intercourse, a double contraception method according to the following algorithm: (condom) plus (intrauterine device or hormonal contraceptive) from inclusion up to 3 months after the last fexinidazole dosing. A woman was considered of childbearing potential (WOCBP), i.e., fertile, following menarche, and until becoming postmenopausal unless permanently sterile. Permanent sterilization methods included hysterectomy, bilateral salpingectomy, and bilateral oophorectomy. A postmenopausal state was defined as no menses for 12 months without an alternative medical cause.
 - Male participants whose partners were pregnant had to use, during sexual intercourse, a condom from inclusion up to 3 months after the last fexinidazole dosing.
 - Male participants had to agree not to donate sperm from inclusion up to 3 months after the last fexinidazole dosing.

Exclusion criteria

- **Medical history and clinical status**
 - Any history or presence of clinically relevant cardiovascular, pulmonary, gastrointestinal, hepatic, renal, metabolic, hematologic, neurologic, osteomuscular, articular, psychiatric, systemic, ocular, gynecological (if female), or infectious disease or signs of acute illness.
 - Participant had clinical signs and symptoms consistent with coronavirus disease 2019 (COVID-19), e.g., fever, dry cough, dyspnea, loss of taste and smell, sore throat, fatigue, or confirmed infection by appropriate laboratory test within the last 4 weeks before screening. Participants who had had a severe course of COVID-19 (i.e., hospitalization, extracorporeal membrane oxygenation, and mechanically ventilated).
 - Positive test for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

- Frequent headaches and/or migraine and recurrent nausea and/or vomiting (more than twice a month).
 - Blood donation within 2 months before inclusion.
 - Symptomatic postural hypotension, irrespective of the decrease in blood pressure, or asymptomatic postural hypotension defined as a decrease in SBP ≥ 20 mmHg within 3 min when changing from a supine to a standing position.
 - Presence or a history of drug hypersensitivity, or allergic disease diagnosed and treated by a physician.
 - A history or presence of regular use of recreational drugs or alcohol abuse (alcohol consumption of more than 40 g per day regularly).
 - Smoking more than five cigarettes or equivalent per day, unable to stop smoking from P1D2 and throughout the entire institutionalization (i.e., up to P2D6).
 - Excessive consumption of beverages containing xanthine bases (more than four cups or glasses per day).
 - If female, pregnancy (defined as positive beta-human choriogonadotropin [β -HCG] blood test) or breastfeeding.
 - Participants with congenital prolongation of QT interval, uncorrected electrolyte abnormalities (e.g., hypokalemia or hypomagnesemia), a history of symptomatic cardiac arrhythmia, clinically relevant bradycardia, and severe congestive cardiac failure, a family history of sudden death, or concomitant use of medicinal products that prolong QT interval and induce bradycardia or hypokalemia.
- **Interfering substance**
 - Any medication (including St John's wort, omeprazole) within 14 days before inclusion or within five times the elimination half-life or pharmacodynamic half-life of the medication (except for oral contraception and HRT), any vaccination within the last 28 days (except for COVID-19), and any biologics (antibody or its derivatives) given within 4 months before inclusion.
 - Body weight between 50.0 and 100.0 kg, inclusive, BMI between 18.00 and 30.00 kg/m², inclusive.
 - COVID-19 vaccination: last administration of a vaccine within 1 week (symptom-free) to 2 weeks before inclusion.

- **General conditions**

- Any participant who, in the judgment of the investigator, was likely to be noncompliant during the study or unable to cooperate because of a language problem or poor mental development.
- Any participant in the exclusion period of a previous study according to applicable regulations.
- Any participant who could not be contacted in case of emergency.
- Any participant who was the investigator or co-investigator, research assistant, pharmacist, study coordinator, or other staff thereof, directly involved in conducting the study.

- **Biological status**

- A positive result on any of the following tests: hepatitis B surface antigen (HBsAg), anti-hepatitis B core antibodies (anti-HBcAb), anti-hepatitis C virus (anti-HCV) antibodies, and anti-human immunodeficiency virus 1 and 2 antibodies (anti-HIV1 and anti-HIV2 Ab).
- A positive result on urine drug screen (amphetamines/methamphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, or opiates).
- A positive alcohol breath test.

- **Specific to the study**

- Any contraindications to oral midazolam, according to the applicable labeling.
- Any consumption of citrus (grapefruit, orange, etc.) or their juices within 5 days before inclusion.
- Unable or not agreeing to self-complete the Hospital Anxiety and Depression Scale.

S2. Study conducts and procedures

- The participants were fasted overnight for at least 10 h and 30 min. In both periods (P1 and P2), midazolam/fexinidazole were administered to the participants 30 min after breakfast. The standard breakfast consisted of 150 mL of fruit juice (except for orange and grapefruit), a non-caffeinated beverage with 10 g of sugar, and with or without 200 mL of semi-skimmed milk, 80 g of bread, 10 g of butter, and 30 g of jam.

- Midazolam was prepared extemporaneously by drawing into an oral syringe directly from the ampoule. The midazolam solution was dosed directly into the participant's mouth immediately thereafter.
- After midazolam/fexinidazole administration, the participants were requested to stay in a semi-recumbent position for the next 2 h without any drink. Food was not allowed for at least 4 h after drug administration. Water intake was at least 1,500 mL for each 24-h period.
- Throughout the institutionalization, the participants were not allowed to drink any coffee- or caffeine-containing beverages (e.g., tea and chocolate), alcohol, or quinine or consume nicotine-containing products. The consumption of citrus and their juices was prohibited during the study, starting 5 days before midazolam/fexinidazole administration until P2D6. The participants were also requested to follow a stable lifestyle, avoiding intensive physical activity for the duration of the study until the EOS visit.
- Midazolam could cause sedation, amnesia, impaired attention, and muscle function and affect the ability to drive or operate machinery. Therefore, the participants were informed not to drive or operate machinery. Even though discharge occurred 48 h after a single low dose (2 mg) of midazolam, the participants were accompanied while returning to their homes.
- Safety protocols included advisories against driving or operating machinery because of the effects of midazolam, with the participants escorted home post-study.

S3. Bioanalytical Method

S3.1. Fexinidazole and Metabolites: Fexinidazole-M1-M2 (validated method)

The determination of fexinidazole and its metabolites, fexinidazole sulfoxide (M1) and fexinidazole sulfone (M2) was accomplished using a liquid chromatography method with tandem mass spectrometric detection (UHPLC/MS-MS method).

Fexinidazole and its metabolites, fexinidazole sulfoxide (M1) and fexinidazole sulfone (M2) and its internal standards were isolated from lithium heparinised human plasma by protein precipitation using acetonitrile. The supernatant was evaporated and the residue reconstituted in the injection solvent before injection in the UHPLC/MS-MS system. The compounds of interest were detected

via tandem mass spectrometry. Separation was achieved on a Supelco Ascentis Express C18, 2.7 μm particle size, 50 x 4.6 mm I.D. column using mobile phase made of a mixture of mobile phase A: 10mM pH 3.5 ammonium formate solution and mobile phase B: methanol. Acquisition was performed using the Analyst™ software of MDS Sciex Analyst® (Ontario, Canada).

S3.2. Midazolam – 1 hydroxymidazolam (validated method)

The determination of midazolam and 1-hydroxymidazolam was accomplished using a liquid chromatography method with tandem mass spectrometric detection (LC/MMS-MS method). Midazolam, 1-hydroxymidazolam and its internal standard were isolated from sodium heparinised human plasma by liquid/liquid extraction using hexane/terbutylmethylether. Briefly, after shaking, the tubes were centrifuged and the organic phase was evaporated to dryness. The residue was then reconstituted with acetonitrile and ammonium acetate buffer (pH=9) before injection in the LC/MS-MS system. The compounds of interest were detected via tandem mass spectrometry. Separation was achieved on a Supelco Ascentis Express F5, 2.7 μm , 50x2.1 mm column using mobile phase made of a mixture of mobile phase A: 5mM ammonium acetate containing 0.1% of acetic acid and mobile phase B: acetonitrile containing 0.1% of acetic acid. Acquisition was performed using the Analyst™ software of MDS Sciex Analyst® (Ontario, Canada).

S3.3. Midazolam-N-b-D-glucuronide (qualified method)

The determination of midazolam-N-b-D-glucuronide was accomplished using a liquid chromatography method with tandem mass spectrometric detection (LC/MMS-MS method). Midazolam-N-b-D-glucuronide and its internal standard were isolated from lithium heparinised human plasma by protein precipitation using acetonitrile containing 0.1% formic acid. The supernatant was evaporated and the residue reconstituted in the injection solvent (acetonitrile/5mM ammonium acetate (15/85; v/v) containing 0.1 acetic acid) before injection in the LC/MS-MS system. The compound of interest was detected via tandem mass spectrometry. Separation was achieved on a Phenomenex Kinetex 2.6 μm F5 100A 50 x 2.1 mm column using mobile phase made of a mixture of mobile phase A: 5mM ammonium acetate containing 0.1% of acetic acid and mobile phase B: acetonitrile containing 0.1% of acetic acid. Acquisition was performed using the Analyst™ software of MDS Sciex Analyst® (Ontario, Canada).

S4. In vitro assessment

S 4.1. Protein Binding Study: Methodology and Analytical Details

Plasma free fractions were determined using the Single-Use Thermo Scientific RED System consisting of a disposable plate made of high-density polypropylene that is preloaded with 48 equilibrium dialysis inserts. Each insert is made of two side-by-side chambers separated by a vertical cylinder of 8K MWCO (molecular weight cut off) dialysis membrane validated for minimal non-specific binding. Donor chamber was filled with 300 μ L of human plasma containing Midazolam at different concentrations. The receiver chamber was filled with 550 μ L of PBS, pH 7.4. Reaction mixture was incubated at 37°C under agitation (250 rpm) for 2, 6, 24 and 26 hours. After incubation, at least 100 μ L of dialysate and at least 100 μ L of matrix were sampled. Midazolam concentration in samples was determined by LC-MS/MS. Same analysis on midazolam at the same concentrations was done with human plasma preincubated with Fexinidazole, Fexinidazole sulfoxide and Fexinidazole sulfone during 24h. Quantifications of midazolam and warfarin were performed after the protein precipitation step with solution containing the internal standard (d4-1-OH-Midazolam), compounds were quantified using an electrospray UPLC-MS/MS method developed in our laboratory.

The methods were adapted on an Acquity UPLC pump in tandem with a Xevo TQ-S mass spectrometer (Column ACQUITY UPLC HSS C18, 100Å, 1.8 μ m, 2.1 mm x 100 mm) and was under electrospray positive ion mode with desolvation temperature of 580°C and source temperature of 150°C

Table: Retention Times and MRM Transitions for Protein Binding Assay Test Items

Test Item		MRM Transition	Retention Time (min)
Midazolam	Quantified	326.1 $\rightarrow$ 291.1	2.52
d4-1-OH-Midazolam	Internal Standard	346.2 $\rightarrow$ 328.2	2.56
Warfarin	Quantified	309.3 $\rightarrow$ 163.2	2.73

LC-MS/MS chromatographic data were collected and quantified using the MassLynx software (version 4.2). Calibration standards were used to create a regression line for quantification.

S 4.2. qRT-PCR and LC-MS/MS Analytical Methodology

The effect of PC, NC, Fexinidazole, Fexinidazole Sulfoxide (M1), Fexinidazole Sulfone (M2) or of a given mix of Fexinidazole, M1 and M2, on CYP2B6, CYP3A4, CYP3A5, UGT1A4, UGT2B4 and UGT2B7 mRNA expression was assessed in human hepatocytes upon 96h of treatment. Total RNA was extracted from hepatocytes using the King Fisher™ Flex Purification System with the MagMAX mirVana Total RNA Isolation kit (Thermo Fisher Scientific, USA). RNA was quantified by fluorimetry using the Quantifluor RNA System (Promega, USA) and cDNAs were synthesized from 0.1 µg total RNA using the iScript Reverse Transcription Supermix for RT- qPCR from Biorad (France) at 42°C for 30 min. cDNA samples were then diluted in water (1:5) and 5 µL of each sample was used for real-time PCR amplification by using the SYBR Green kit from Biorad (France). The following program was used: a denaturation step at 95°C for 30 sec, and 40 cycles of PCR (denaturation, 95°C, 10 sec and annealing and extension, 58°C, 1 min). In all cases, the quality of the PCR product was assessed by monitoring a fusion step at the end of the run.

Listed below human **forward and reverse primer** sequences were used:

5'-CCT-GGC-ACC-CAG-CAC-AAT-3' and 5'-GGG-CCG GAC-TCG-TCA-TAC-3' for actin,

5'-GGC-CAT-ACG-GGA-GGC-CCT-TG-3' and 5'-AGG-GCC-CCT-TGG-ATT-TCC-G-3' for CYP2B6

5'- CAC-AAA-CG-GAG-GCC-TTT-TG-3' and 5'- ATC-CAT-GCT-GTA-GGC-CCC-AA-3' for CYP3A4

5'- TGA-TTC-CAA-CTT-ATG-CTC-TTC-A-3' and 5'- ATC-TAT-GCT-GTC-CTT-CTT-CTT-AC-3' for CYP3A5

5'- GTG-GCT-TCT-GCT-GAG-ATG-3' and 5'- CTG-GAC-ACT-GAG-GAG-GAG-3' for UGT1A4

5'- CAG-TTC-TAC-AGT-GAA-GTT-CTA-GG-3' and 5'- GAG-TGG-GTG-AGG-AAA-TTG-AAA-3' for UGT2B4

5'- ATC-TAC-GAG-GCA-ATC-TAC-CA-3' and 5'- TGT-GTT-GAA-GTC-CAC-TCT-AAC-3' for UGT2B7

Quantification cycle (Cq) values were determined using a multi-variable, non-linear regression model to individual well traces and then using this model an optimal Cq value was computed. Cq values are inversely proportional to the amount of target nucleic acid in the sample (i.e., lowest Cq level corresponds to the greatest amount of target nucleic acid in the sample). Cq of reference gene (in this study human actin) was also used as positive control of quantitative PCR.

Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Surveyor MS Plus HPLC (Thermo Electron) system connected to a TSQ Quantum Discovery Max (Thermo Electron) triple quadrupole mass spectrometer equipped with an electrospray (ESI) (Thermo Fisher Scientific, USA) and connected to a PC running the standard software Xcalibur 2.0.7 were used. The HPLC pump flow rate was set to 600 μ L/min and the analytes were separated on an analytical column (Kinetex Phenyl-Hexyl 2.6 μ m 50x2.1mm, Phenomenex, Germany) with pre-column (C6-Phenyl 4x2.0mm, Phenomenex, Germany).

Table: A HPLC gradient was applied with acetonitrile, 0.1% formic acid as organic phase (A) and water with 0.1% formic acid as aqueous phase (B) as shown in table below:

Mobile phase	0 min	0.1 min	0.4 min	1.7 min	1.8 min	2.5 min
A (%)	5	5	97	97	5	6
B (%)	95	95	3	3	95	95

Full scan mass spectra were acquired in the positive mode using syringe pump infusion to identify the protonated quasimolecular ions $[M+H]^+$. Auto-tuning was carried out for maximizing ion abundance followed by the identification of characteristic fragment ions using a generic parameter set: ion-transfer-capillary temperature 350°C, capillary voltage 3.8 kV, collision gas 0.8 mbar argon, sheath gas, ion sweep gas and auxiliary gas pressure were 20, 2 and 8 (arbitrary units), respectively. Ions with the highest S/N ratio were used to quantify the item in the selected reaction monitoring mode (SRM) and as qualifier, respectively.

Table: Overview of the mass spectrometry (MS) conditions and retention time for 1-OH-Midazolam and Midazolam-N-beta-D-glucuronide

Analytes	Molecular weight	[M+H] ⁺ (m/z)	Monitoring ion (m/z)	Scan time (s)	Collision energy (V)	Retention time (min)
1-OH-Midazolam	341.8	342.0	203.0	0.01	25	0.99
Midazolam-N-beta-D-glucuronide	503.1	502.0	326.0	0.01	30	0.97
Griseofulvin (ISTD)	352.8	353.0	215.0	0.01	20	1.10

ISTD: internal standards