In vitro CYP450 enzyme down-regulation by GLP-1/glucagon co-agonist does not translate to observed drug-drug interactions in the clinic

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**List of Abbreviations:** AUC (area under the curve), BMI (body mass index), CI (confidence interval), CV (coefficient of variation), CYP (Cytochrome P450), DDI (drug-drug interaction), EC50, half-maximal effect concentration; $E_{\text{max}}$ maximum effect, $f_u$ (fraction unbound), GLP-1 (glucagon-like peptide 1), mRNA (messenger RNA), PBPK (Physiological-based pharmacokinetic), PCR (polymerase chain reaction), PK (pharmacokinetics), s.c. (subcutaneous), SD (standard deviation), $t_{1/2}$ (half-life), $t_{\text{max}}$ (time of maximum observed drug concentration)
ABSTRACT

NN1177 is a glucagon/glucagon-like peptide 1 receptor co-agonist investigated for chronic weight management and treatment of non-alcoholic steatohepatitis. Here, we show concentration-dependent down-regulation of cytochrome P450 enzymes using freshly isolated human hepatocytes treated with this linear 29-amino acid peptide. Notably, reductions in CYP3A4 mRNA expression (57.2-71.7%) and activity (18.5-51.5%) were observed with a clinically-relevant concentration of 100 nM NN1177. CYP1A2 and CYP2B6 were also affected, but to a lesser extent. Physiological-based pharmacokinetic modelling simulated effects on CYP3A4 and CYP1A2 probe substrates (midazolam and caffeine, respectively) and revealed potential safety concerns related to drug-drug interactions (DDIs). To investigate the clinical relevance of observed in vitro CYP down-regulation, a phase 1 clinical cocktail study was initiated to assess the DDI potential. The study enrolled 45 study participants (BMI 23.0-29.9 kg/m²) to receive a Cooperstown 5+1 cocktail (midazolam, caffeine, omeprazole, dextromethorphan, and S-warfarin/vitamin K) alone and following steady state NN1177 exposure. The analysis of pharmacokinetic profiles for the cocktail drugs showed no significant effect from the co-administration of NN1177 on AUC₀⁻inf for midazolam or S-warfarin. Omeprazole, caffeine, and dextromethorphan generally displayed decreases in AUC₀⁻inf and C_max following NN1177 co-administration. Thus, the in vitro observations were not reflected in the clinic. These findings highlight remaining challenges associated with standard in vitro systems used to predict DDIs for peptide-based drugs as well as the complexity of DDI trial design for these modalities. Overall, there is an urgent need for better pre-clinical models to assess potential drug-drug interaction risks associated with therapeutic peptides during drug development.
SIGNIFICANCE STATEMENT

This study highlights significant challenges associated with assessing drug-drug interaction risks for therapeutic peptides using in vitro systems, since potential concerns identified by standard assays did not translate to the clinical setting. Further research is required to guide investigators involved in peptide-based drug development towards better pre-clinical models in order to more accurately evaluate potential drug-drug interactions.
INTRODUCTION

To ensure the safety and efficacy of new medicinal products, it is of high importance to assess drug-drug interaction (DDI) risk during drug development. Utilising in vitro systems to predict clinical DDI risk is well-established for small molecule drugs following decades of research. Gold standards and recommended procedures are described in published regulatory guidelines (European Medicines Agency, 2012; PMDA, 2019; Food and Drug Administration and CDER, 2020b). In contrast, FDA has recently published a draft guidance on therapeutic protein DDIs, which states that the translation from in vitro studies to the clinic for protein-based drugs is limited (Food and Drug Administration, 2020b). Therapeutic peptides (defined by FDA as ≤40 amino acids (Food and Drug Administration, 2019a; D’Aloisio et al., 2021) represent challenging moieties from a regulatory perspective since this modality is not explicitly covered by available regulatory guidelines for DDI risk assessments. Overall, it is unclear whether recommended in vitro assays used to identify potential DDI risks for small molecule drugs are appropriate for assessing similar risks with therapeutic peptides.

Administering concomitant drugs may alter the metabolising capacity of cytochrome P450 (CYP) enzymes and such DDIs can cause adverse reactions or therapy failure. In vitro CYP down-regulation is commonly detected during drug development (Hariparsad et al., 2017). However, the clinical implications of this mechanism remain questionable as most accessible reports or publications fail to demonstrate clinical effects related to in vitro CYP down-regulation (Hariparsad et al., 2017; Stevison et al., 2019; Turner et al., 2020). For biologics, the native hormone FGF-21 is reported to down-regulate hepatic CYP3A4 in cultured mouse and human cells (Woolsey et al., 2016) and the native glucagon peptide was shown to decrease CYP2C11 mRNA in rat hepatocytes (Iber et al., 2001), yet the clinical impact of observed CYP down-regulation in these cases is currently unknown. Details related to CYP down-regulation mechanisms in guidelines from health authorities are presently limited. European authorities recommend further studies if CYP
mRNA is decreased by more than 50% without any associated cell toxicity (European Medicines Agency, 2012). Likewise, FDA is proposing to consider additional studies to assess the potential clinical consequences when concentration-dependent down-regulation has been observed without any associated cell toxicity (Food and Drug Administration and CDER, 2020b).

NNC9204-1177 (NN1177 for short) is a linear 29-amino acid synthetic peptide (~4.57 kilodaltons) investigated for chronic weight management and treatment of non-alcoholic steatohepatitis (NASH), a severe form of non-alcoholic fatty liver disease. The chemical structure of NN1177 has been reported elsewhere (Simonsen et al., 2022). Mechanistically, NN1177 acts as a co-agonist for the glucagon and glucagon-like peptide 1 (GLP-1) receptors with an anticipated potency ratio of approximately 1:3. Its molecular design ensures chemical and physical stability, as well as strong albumin binding, resulting in a half-life in humans that is expected to be suitable for once-weekly dosing by subcutaneous (s.c.) administration for treating obesity and NASH. Obesity represents a significant public health challenge in all developed countries, an emerging epidemic in many developing countries (Kelly et al., 2008), and is associated with increased risk of death and major co-morbidities and complications such as hypertension, hyperglycaemia (pre-diabetes and type 2 diabetes), dyslipidaemia, certain types of cancer, and cardiovascular diseases (Guh et al., 2009). The growing prevalence of obesity is also associated with an increased incidence of chronic liver disease, including NASH (Than and Newsome, 2015).

Here, we show concentration-dependent down-regulation of major CYPs in standard in vitro assays following exposure of clinically relevant NN1177 concentrations. The mechanism of this down-regulation is unknown. Physiological-based pharmacokinetic (PBPK) modelling revealed potential safety concerns for the simulated effects on CYP3A4 and CYP1A2 activity. This prompted an open-label, single-sequence, cross-over phase 1 trial to address whether steady state NN1177 exposure after once-weekly s.c. doses influence the exposure of representative index substrates for multiple CYP enzymes (using the validated Cooperstown 5+1 cocktail). However, pharmacokinetic (PK) profiles of index substrates in the clinical setting were inconsistent with CYP
down-regulation. These findings emphasize the remaining challenges and uncertainties associated with commonly utilized in vitro systems for predicting DDIs for peptide-based drugs.
MATERIALS AND METHODS

In vitro studies

Three freshly isolated human hepatocyte cultures (H1401, H1403 and H1407) were isolated and characterized at Sekisui XenoTech, LLC (Kansas City, Kansas), with lot-specific details available in Supplemental Table S1. These primary human hepatocytes were cultured in collagen-sandwich configuration and supplemented with modified Eagle’s medium (MCM+), including 1.23 mg/ml BSA, in a humidified chamber (37°C at 95% relative humidity, 95/5% air/CO2). NN1177 was supplied by Novo Nordisk A/S (Bagsværd, Denmark). Glucagon, rifampin, DMSO, flumazenil, omeprazole, phenacetin, and phenobarbital were obtained from Sigma Aldrich (St. Louis, Missouri), bupropion HCl was from Toronto Research Chemicals (Toronto, Canada), acetic acid was from Thermo Fisher Scientific (Waltham, Massachusetts), IL-6 was from EMD Millipore (Burlington, Massachusetts), and midazolam was from USP (North Bethesda, Maryland). Reagents for RNA isolation and quantitative PCR were from Ambion and Applied Biosystems, respectively.

Prior to this study, NN1177 stability was evaluated by incubating 100 and 1000 nM radiolabelled test substance ([3H] NN1177) in MCM+ medium for 0 and 24 hours at 37°C. After 24 hours, >93% of NN1177 remained confirming stability for in vitro experiments performed here where primary human hepatocytes in culture were treated once daily for three consecutive days with either vehicle control (0.1% DMSO or 0.1 mM acetic acid), negative control flumazenil (25 μM), omeprazole (50 μM), phenobarbital (750 μM), rifampin (20 μM), IL-6 (10 ng/mL), glucagon (30 nM), or NN1177 (10, 100 or 1000 nM). Each condition was prepared in triplicate wells. The three NN1177 concentrations span the clinical range, with the middle concentration selected based on simulated clinical Cmax values. Quantitative evaluation of cytotoxicity was measured using a lactate dehydrogenase (LDH) release assay. LDH activity for each sample was determined with a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). mRNA levels for CYP1A2, CYP2B6, and CYP3A4, as well as the endogenous control GAPDH, were measured in triplicate
using reverse-transcription quantitative PCR assays available from Applied Biosystems (catalog # 4351368): CYP1A2 (Hs00167927_m1), CYP2B6 (Hs03044634_m1), CYP3A4 (Hs00604506_m1), and GAPDH (Hs99999905_m1). Catalytic activities for CYPs were analyzed in triplicate by LC-MS/MS and measured using selective catalytic assays for CYP1A2 (phenacetin O-dealkylation), CYP2B6 (bupropion hydroxylation), and CYP3A4 (midazolam 1'-hydroxylation). Positive controls included: omeprazole (an AhR activator and CYP1A2 inducer), phenobarbital (a CAR activator and CYP2B6 inducer), and rifampin (a PXR agonist and inducer of CYP3A4) (European Medicines Agency, 2012; Food and Drug Administration, 2020a).

**PBPK modelling**

PBPK modelling was used to simulate the theoretical clinical outcome of CYP3A4 and CYP1A2 down-regulation indicated by in vitro studies using the Open Systems Pharmacology Suite (OSP) v. 8.0 with PK-Sim® and MoBi (http://www.open-systems-pharmacology.org). Standard settings were used except where optimisations were done as described below. The PK-Sim built-in compound templates for midazolam and caffeine were used as starting points. For dose administrations and other simulations settings, see Supplemental Table S2. CYP expression levels in hepatocytes using RT-PCR-derived gene expression estimates were selected from the PK-Sim v. 8.0 human gene expression database (https://github.com/Open-Systems-Pharmacology/Gene-Expression-Databases/releases/download/v2.0.0/GENEDB_human.expressionDb) and default liver half-lives (t½) were used: CYP3A4 liver concentration 4.32 µM, t½ = 36 hours in liver; CYP1A2 liver concentration 1.80 µM, t½ = 39 hours in liver. A default subject (30-year-old male, European, mean body weight of 73 kg, height of 176 cm, and BMI of 23.57 kg/m²) was used during model development.

A full-scale PBPK model was not necessary for NN1177 as only simulated plasma concentrations and corresponding intracellular concentrations of NN1177 in hepatocytes were needed. Potential
effects on intracellular CYP gene regulation likely take place after membrane-bound receptor stimulation since the size and physicochemical properties of the peptide limits its distribution into cells. Furthermore, NN1177 is not known to bind to any transporters mediating transmembrane transport. Since PK-Sim® does not allow for a mechanistic representation of gene regulation downstream from receptor stimulation, a technical non-physiological solution was applied. In PK-Sim®, CYP regulation and metabolic activity take place in the intracellular hepatocyte compartment. Therefore, the settings in PK-Sim® for plasma-interstitial and interstitial-intracellular permeability were adapted so that the unbound plasma and intracellular concentrations of NN1177 in hepatocytes became equal. This way, the unbound plasma concentration of NN1177 is linked to the EC50 for intracellular regulation of CYP expression. For more details, see Supplemental Table S3.

Implementation of in vitro CYP down-regulation in PBPK model

As per guideline recommendations, mRNA data was selected as the endpoint for evaluation of CYP regulation (Food and Drug Administration and CDER, 2020b). The degree of mRNA downregulation used here represents a worst-case scenario. Parameters describing CYP down-regulation were determined so as to best describe the observed hepatocyte mRNA data according to the following equation:

\[
\frac{mRNA_{\text{baseline}}}{mRNA} = (1 - E_{\text{max, in vitro}}) \frac{\text{Conc.}}{\text{EC50}_{\text{in vitro}} + \text{Conc.}}
\]

where \(E_{\text{max, in vitro}}\) is the maximum fraction down-regulated, EC50 is the NN1177 concentration giving 50% of maximum down-regulation, and Conc. is the concentration of NN1177 in the incubation. For CYP3A4, average mRNA data for all three hepatocyte donors (H1401, H1403, H1407) was used, while only the data from H1401 was used for CYP1A2, since the observed down-regulation represents a worst-case scenario.
In PK-Sim®, the technical implementation of an enzyme suppression is a negative induction, with $E_{\text{max}}$ ranging from 0 (no suppression) to -1 (full suppression) since the time course of a CYP enzyme, $E$, is per default simulated by the differential equation:

$$\frac{dE}{dt} = k_{\text{deg}} \times E_0 \times \left(1 + \frac{E_{\text{max}} \times \text{Conc.}}{(EC50 + \text{Conc.})}\right) - k_{\text{deg}} \times \frac{dE}{dt}$$

where $k_{\text{deg}}$ is the rate of enzyme degradation, $E_0$ is the amount of enzyme in the absence of induction/suppression, EC50 is the concentration of NN1177 giving 50% of maximum suppression, and $E_{\text{max}}$ is the maximum fraction change from baseline. Only the parameter values and not the equation itself may be changed via user input. Therefore, this was implemented as “negative” induction (e.g., the $I_{\text{max}}$ for CYP3A4 down-regulation was 85%, so the corresponding induction in PK-Sim was set to -0.85). Binding to albumin has been shown to decrease the apparent in vitro binding affinity of NN1177 to the glucagon and GLP-1 receptors, and since the CYP downregulation is assumed to be mediated via receptor binding, it is assumed that only the fraction unbound ($f_u$) of NN1177 can act to downregulate the CYPs. The $f_u$ in the in vitro hepatocyte incubation is unknown and hence different scenarios for in vitro $f_u$ values were explored (0.0023, 0.01 and 0.064). The lower $f_u$ of 0.0023 was based on in vitro surface plasmon resonance studies showing that the fraction unbound of NN1177 in human plasma is 0.0023 and that the corresponding equilibrium dissociation constant, $K_d$, for binding to human serum albumin (HSA) is 1.24 µM (unpublished data). An upper value of 0.064 (6.4%) was based on a theoretical calculation of the free fraction in the hepatocyte incubation using the following equation:

$$f_u = \frac{1}{1 + (\frac{1}{K_d})^2[A]}$$

where [A] is the albumin concentration (1.23 mg/ml BSA corresponding to 18 µM albumin) and $K_d$ is the equilibrium dissociation constant. The 0.01 value represents the approximate geometric mean of these two extreme values. For the PBPK simulations, the EC50 values calculated above using the total NN1177 concentration in the incubations were multiplied by each in vitro $f_u$ tested to convert the EC50 to the unbound NN1177 concentration.
Clinical trial design and trial population

The clinical study (ClinicalTrials.gov identifier no. NCT04059367) was a single-sequence, drugdrug interaction study enrolling 45 healthy male and female subjects between 18-60 years of age (BMI 23.0-29.9 kg/m²). Of the 37 participants that completed the trial, up to 33 were included in the final analyses. The study was conducted in accordance with the Declaration of Helsinki (World Medical Association, 2013) and the Good Clinical Practice guideline (ICH Harmonised Tripartite Guideline, 2016).

Treatments

All participants followed the same course of treatment. NN1177 was administered s.c. once weekly. To overcome tolerability issues, an escalation regimen with 2-week escalation steps was applied (0.3 mg, 0.6 mg, 1.2 mg, and 2.4 mg) before three weeks at the target dose of 4.2 mg (Figure 1). Cooperstown 5+1 index substrates (hereafter called the DDI cocktail) were administered before NN1177 treatment was initiated (day 1), and again at the same day as the last 4.2 mg dose of NN1177 (day 78). The DDI cocktail containing representative CYP substrates was selected based on the validated Cooperstown 5+1 cocktail: 2 mg midazolam (CYP3A4), 10 mg S-warfarin/10 mg vitamin K (CYP2C9), 40 mg omeprazole (CYP2C19), 30 mg dextromethorphan (CYP2D6), and 200 mg caffeine (CYP1A2) and listed in Supplemental Table S4. The impact on CYP2B6 is not assessed by utilising the Cooperstown 5+1 cocktail. This was considered acceptable as there is currently no sensitive index substrate for this enzyme. Furthermore, there is a limited number of drugs dependent on CYP2B6 metabolism in the clinic. Food consumption was not permitted 6 hours before and 4 hours after administration of the DDI cocktail. Water consumption was not permitted 1 hour before and after administration of the DDI cocktail. To avoid interference with CYP activity, participants were also asked to refrain from consuming certain foods within 7 days.
prior to and during visits 2 and 12, including: cranberries or cranberry juice, grapefruit or grapefruit juice, orange juice, apple juice, vegetables from the mustard green family, and liver or charbroiled meats. In line with FDA guidance (Food and Drug Administration and CDER, 2020a), use of any herbal medicines known to interfere with the metabolic CYP pathways were not allowed within 14 days prior to, or during, the study. Dietary caffeine consumption was not permitted 48 hours prior to DDI cocktail administration. Smoking and nicotine substitutes were also not permitted 48 hours prior to day 2 and until the completion of the follow-up visit.

**Evaluation methods**

**Assays**

Human plasma samples were drawn and analyzed for NN1177 at Novo Nordisk using a validated luminescent oxygen channelling assay developed by Novo Nordisk. Key reagents included monoclonal antibodies Glu-1F120 and NN454-1F31 from Novo Nordisk A/S (Bagsværd, Denmark). The validation was performed according to the FDA Guidance for Industry for Bioanalytical Method Validation (Food and Drug Administration and CDER, 2018) and the EMA Guideline on Bioanalytical Method Validation (European Medicines Agency, 2011). Index substrates and metabolites were measured in plasma using validated liquid chromatography tandem mass spectrometry (LC-MS/MS) methods. Reference standard materials midazolam, caffeine, omeprazole, and dextromethorphan were obtained from USP (North Bethesda, Maryland), 1-hydroxymidazolam and dextrorphan were obtained from Cerilliant (Round Rock, Texas), S-warfarin and 7-hydroxy-S-warfarin were obtained from Sigma Aldrich (St. Louis, Missouri), paraxanthine/1,7-dimethylxanthine was obtained from Toronto Research Chemicals (Toronto, Canada), and 5-hydroxyomeprazole was obtained from TLC Pharma (Newmarket, Canada).
Pharmacokinetics

PK samples were collected on day 1 and day 78 pre-dose and post-dose at the following time intervals for index substrates and metabolites: midazolam and 1-hydroxymidazolam (pre-dose, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 hrs post-dose); omeprazole and 5-hydroxyomeprazole (pre-dose, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96 hrs post-dose); dextromethorphan and dextrorphan (pre-dose, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, 72 hrs post-dose, as well as dextrorphan alone at 36, 96, 120, 144, 168 hrs post-dose); S-warfarin and 7-hydroxy-S-warfarin (pre-dose, 3, 6, 12, 24, 36, 48, 72, 96, 120, 144, 168 hrs post-dose); caffeine and paraxanthine (pre-dose, 0.5, 1, 2, 3, 4, 6, 8, 12, 24 hrs post-dose, as well as paraxanthine alone at 36, 48, 72, 96, 120, 144, 168 hrs post-dose).

Samples for NN1177 were collected on day 8, 22, 36, 50, 64, as well as on day 78 pre-dose and post-dose at the following time intervals: pre-dose, 2, 5, 8, 12, 16, 20, 24, 30, 36, 42, 48, 72, 120, 168 hrs post-dose.

PK parameters (i.e., AUC₀–inf, Cₘ₅₄₅, t₁/₂ and tₘ₅₄₅) for midazolam, caffeine, omeprazole, dextromethorphan, S-warfarin, and their main metabolites (1-Hydroxymidazolam, hydroxyomeprazole, paraxanthine, dextrorphan and 7-Hydroxy-S-warfarin) were calculated by standard non-compartmental methods (Version 8.1; Phoenix WinNonlin, Certara, Princeton New Jersey). For AUC₀–inf, datapoints where the extrapolated AUC₁–inf was more than 20% were excluded from the descriptive statistics. Poor and ultra-rapid metabolizers for CYP2C9, CYP2C19 and CYP2D6 and two outliers for omeprazole with unphysiological PK profiles (likely due to severe gastric emptying and vomiting at Cmax, respectively) were excluded from descriptive statistics.

The ratios between the main metabolite and index substrate exposure were calculated for AUC₀–24h. Using the Phoenix WinNonlin bioequivalence workflow, log-transformed AUC₀–inf, Cₘ₅₄₅, and tₘ₅₄₅ estimates were evaluated in a linear mixed-effects ANOVA model with a fixed effect for treatment (NN1177) and a random effect for subject. The treatment differences were back-transformed to present the ratio of geometric least squares means and corresponding 90% confidence intervals (CIs).
Pharmacogenomics

Blood samples (6 mL whole blood into EDTA tube) were collected at baseline (visit 2, day 1). The blood samples were shipped and analysed at Quest Diagnostics. Genomic DNA was extracted for CYP2C9, CYP2C19, and CYP2D6 and genotyping was performed by validated quantitative PCR assays. The CPIC genotype to phenotype conversions were utilised and the tested alleles are stated below:

Four common genetic variants were detected in the CYP2C9 gene: CYP2C9*2, CYP2C9*3, CYP2C9*5, and CYP2C9*6. In the absence of an identified CYP2C9 variant, the wild type *1 allele was inferred. For CYP2D6, the normal (wild-type) allele is designated CYP2D6*1. This assay detected 7 null (loss of function) alleles: CYP2D6*3, CYP2D6*4, CYP2D6*5 (gene deletion), CYP2D6*6, CYP2D6*7, CYP2D6*8, and CYP2D6*14A. It also detected 5 reduced activity alleles: CYP2D6*9, CYP2D6*10, CYP2D6*14B, CYP2D6*17, and CYP2D6*41. Duplications of the CYP2D6 gene were also detected, but the assay could not determine copy number. For CYP2C19, the normal allele was designated as CYP2C19. The assay detected CYP2C19*2, *3, *4, *5, *6, *7, *8, *9, *12, and *17. In the absence of an identified CYP2C19 variant, the wild type *1 was inferred.

Safety

All events meeting the definition of an adverse event (AE) observed by the investigator or reported spontaneously by the subjects were collected. The investigator assessed the AE with regards to severity (mild, moderate, severe), causality (relation to trial product: probable, possible, or unlikely), outcome (recovered/resolved, recovering/resolving, recovered/resolved with sequelae, not recovered/not resolved, fatal, or unknown), and seriousness (serious or non-serious).
RESULTS

In vitro studies

To assess the effect of NN1177 on CYP enzymes in vitro, freshly isolated human hepatocyte cultures from three donors were exposed to concentrations spanning the clinical exposure range (10, 100, and 1000 nM). Controls behaved as expected and in line with historical data. In addition to standard induction controls, IL-6 was included as a positive control for CYP down-regulation as it is known to result in pan-suppression of CYP mRNA via a transcriptional mechanism (Abdel-Razzak et al., 1993; Dickmann et al., 2011; Czerwiński et al., 2018; Dunvald et al., 2021). Native glucagon was included as explorative control for two reasons: NN1177 is an agonist of the glucagon receptor and glucagon has been reported to regulate rat CYP enzymes in vitro via the cyclic adenosine monophosphate secondary messenger and protein kinase A (Morgan et al., 1998; Iber et al., 2001). Indeed, we observed CYP down-regulation in human hepatocytes treated with both IL-6 and glucagon (Figure 2). A tabular summary of the interpreted data is available in Supplemental Table S5 (down-regulation data presented as % decrease) and Supplemental Table S6 (induction effects presented as fold increase).

Concentration-dependent reductions in CYP3A4 and CYP2B6 mRNA were observed for NN1177 in all three donors with no observed cytotoxic effects on cells (Figure 2; Supplemental Tables S1 and S5). At the clinically relevant concentration of 100 nM, CYP3A4 mRNA decreased between 57.2-71.7% compared to control, while CYP2B6 mRNA decreased between 39.3-50.7%. Greater than two-fold decreases in both CYP3A4 and CYP2B6 mRNA levels were observed for 1000 nM NN1177. The enzymatic activity of CYP3A4 and CYP2B6 was also reduced, but this was less pronounced than mRNA reduction with activity decreasing 18.5-51.5% and 11.6-39.4%, respectively. In one donor for 100 nM, CYP1A2 mRNA and activity decreased by 61.8% and 38.2%, respectively, but little to no effect was observed in the remaining two donors. According to guidelines from FDA and EMA (European Medicines Agency, 2012; Food and Drug Administration
and CDER, 2020b), additional studies are recommended if substantial concentration-dependent CYP down-regulation is observed with in vitro assays in order to explore potential clinical consequences. Therefore, potent CYP down-regulation observed for clinically relevant NN1177 concentrations prompted a DDI cocktail study, where several CYP enzymes are evaluated simultaneously to assess potential clinical consequences.

**PBPK simulation for effects on CYP3A4 and CYP1A2 substrates**

Prior to initiating a clinical trial, PBPK modelling was performed to predict upfront the potential clinical impact of CYP down-regulation indicated by in vitro data. EC$_{50}$ and E$_{\text{max}}$ values based on in vitro mRNA data in hepatocytes, as well as three scenarios for fraction unbound (f$_u$) in the hepatocyte culture were used for the PBPK modelling and are listed in Table 1. PBPK simulations for midazolam and caffeine for an in vitro f$_u$ of 0.0023 (equivalent to a 1:1 correlation between in vitro and in vivo EC50 in terms of unbound NN1177) with and without NN1177 treatment predicted an increase in the exposure to both compounds following co-administration with NN1177 (Figure 3). For caffeine, the ratios for AUC$_{0\text{-}\text{inf}}$ and C$_{\text{max}}$ with and without NN1177 at f$_u$ 0.0023 were 1.6 and 1.1, respectively. These were higher for midazolam, as the ratios for AUC$_{0\text{-}\text{inf}}$ and C$_{\text{max}}$ were 2.4 and 1.6, respectively. For larger in vitro f$_u$ values and thus higher EC50 values in terms of unbound NN1177, AUC$_{0\text{-}\text{inf}}$ and C$_{\text{max}}$ ratios were predicted between 1.0 and 1.4 (Table 2, Figure 3). Based on these simulations, we were unable to rule out safety risks associated with DDIs following NN1177 exposure and prompting the clinical trial.
Clinical Data

Subject demographics and disposition

Trial NCT04059367 enrolled 45 subjects. Most exposed subjects (26 females, 19 males) were White (71.1%) with a mean (SD) age of 43.3 (8.4) years and BMI from 23.0 and 29.9 kg/m2 (Table 3). Of the 45 subjects who entered the study, 37 completed the trial.

Pharmacokinetics

Up to 45 subjects were included in the baseline PK analysis and up to 33 subjects were included in the steady-state PK assessments following steady-state dosing with 4.2 mg NN1177. The mean PK profiles for each analyte assessed at baseline and steady state are shown in Figure 4. Observed changes in AUC$_{0-\infty}$ and C$_{\text{max}}$ are shown in Figure 5. PK analysis of index substrates are available in Table 4, metabolites are available in Supplemental Figure S2 and metabolite/parent ratios are available in Supplemental Table S7.

PBPK modelling

The PBPK model adequately reproduced the mean observed NN1177 plasma concentrations at day 78 (Supplemental Figure S1). Similarly, the PBPK models predicted PK profiles for baseline midazolam AUC and C$_{\text{max}}$ and caffeine AUC (within ±30% of the observed median), however caffeine C$_{\text{max}}$ was over-predicted by about 3-fold. This discrepancy is expected since the PBPK simulation depends heavily on the tablet used in the trial versus the setup for the oral absorption in the model.

NN1177

NN1177 concentration increased according to the dose escalation (Supplemental Figure S3). At steady state NN1177 exposure, the geometric mean of AUC$_{0-168\text{h}}$ and C$_{\text{max}}$ were 16715 nmol*h/L and 140 nmol/L, respectively. Median of t$_{\text{max}}$ was 24 hours (range: 12-72 hours).
**Midazolam**

No substantial differences in the geometric least squares mean midazolam AUC\(_{0–\text{inf}}\) were observed between midazolam administered alone or in combination with NN1177 (Figure 5, Table 4). Midazolam C\(_{\text{max}}\) decreased by ~20\%, \(t_{\frac{1}{2}}\) was unchanged, while \(t_{\text{max}}\) effectively doubled when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

**Caffeine**

Decreased caffeine AUC\(_{0–\text{inf}}\) was observed with NN1177 co-administration (Figure 5, Table 4). Caffeine C\(_{\text{max}}\) decreased by ~30\%, \(t_{\frac{1}{2}}\) reduced by approximately half, while \(t_{\text{max}}\) increased only modestly (10\%) when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

**Omeprazole**

Decreased omeprazole AUC\(_{0–\text{inf}}\) was observed with NN1177 co-administration (Figure 5, Table 4). Omeprazole C\(_{\text{max}}\) decreased by ~64\%, \(t_{\frac{1}{2}}\) increased by nearly 60\%, while \(t_{\text{max}}\) effectively tripled when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

**Dextromethorphan**

Decreased dextromethorphan AUC\(_{0–\text{inf}}\) was observed with NN1177 co-administration (Figure 5, Table 4). Dextromethorphan C\(_{\text{max}}\) decreased by ~40\%, \(t_{\frac{1}{2}}\) was unchanged, while \(t_{\text{max}}\) increased by about 33\% when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.
S-warfarin

S-warfarin AUC\textsubscript{0–inf} between cocktail administered alone or in combination with NN1177 decreased modestly by \(~16\%\) (Figure 5, Table 4). S-warfarin C\textsubscript{max} showed a slight decrease (\(~10\%\)), whereas t\textsubscript{1/2} was reduced by nearly 20\%, and t\textsubscript{max} was unchanged when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

Pharmacodynamics

Over the course of NN1177 treatment, subjects lost on average 7.8 kg (\(~10\%) of their body weight (Supplemental Table S8).

Safety

A total of 239 adverse events related to treatment with NN1177 were reported by the study participants during the 15 weeks from first dose of NN1177 to last safety monitoring visit. The most prevalent adverse events were gastrointestinal disorders (86.7\%), including nausea, vomiting, gastroesophageal reflux disease, and diarrhoea. The adverse events led to discontinuation from the study for 8 participants. Despite highly contraceptive methods required for study participants, one participant experienced a pregnancy and a spontaneous abortion, which was reported as a serious adverse event with possible relation to NN1177. No subject deaths were reported.
DISCUSSION

Here, we report in vitro down-regulation of CYP3A4, CYP2B6, and CYP1A2 at clinically relevant concentrations of the glucagon/GLP-1 co-agonist NN1177. We observed similar effects for 30 nM native glucagon, consistent with earlier studies reporting CYP enzyme suppression by native glucagon (Morgan et al., 1998; Iber et al., 2001). In contrast, GLP-1 analogues like semaglutide have been reported to show no relevant effects on CYP enzymes in vitro or in the clinic (Bækdal et al., 2019; Food and Drug Administration, 2019b; Jordy et al., 2021), likely due to the absence GLP-1 receptor expression in the human liver (Simonsen et al., 2022). Therefore, the in vitro effect on CYP enzymes observed for NN1177 is likely related to its action on glucagon receptors expressed on the hepatocytes. Despite these intriguing in vitro findings, all CYP index substrate profiles from a follow-up clinical trial showed PK properties inconsistent with CYP down-regulation following NN1177 exposure. Omeprazole, caffeine, and dextromethorphan all displayed decreases in AUC0–inf and Cmax following NN1177 administration compared to cocktail alone, while midazolam and S-warfarin showed no changes between conditions. These findings underscore the challenges associated with assessing DDI risk for therapeutic peptides.

Assessing DDI risks for NN1177 peptide

Other peptides have previously been shown to suppress CYPs in vitro (Iber et al., 2001; Woolsey et al., 2016), prompting us to explore the potential that NN1177 regulates major CYP enzymes. We observed down-regulation for all three CYP enzymes investigated (CYP3A4, CYP1A2, and CYP2B6) in freshly isolated human hepatocytes exposed to NN1177, indicating the potential involvement of multiple nuclear receptors (PXR, AhR, and CAR, respectively). It is reported that peptide hormones such as native glucagon and FGF-21 regulate CYP enzymes by binding to their respective receptor to mediate intracellular downstream signalling that regulates the nuclear receptors relevant for CYP expression (e.g., PXR) (Iber et al., 2001; Woolsey et al., 2016).
Experiments delineating the molecular mechanism by which NN1177 regulates CYP enzymes would be of added value. In addition, it would be of interest to understand the significance of hepatic receptor expression. Potential effects of changes in receptor expression (i.e., down- or up-regulation) during the incubation remains unexplored.

The obtained *in vitro* data was implemented into a PBPK model and safety concerns were identified for co-medications, particularly those metabolised by CYP3A4. Several statins are known to be sensitive CYP3A4 substrate in the clinic (Food and Drug Administration, 2020a) and it is expected that these lipid-lowering medications are in chronic use in the intended target population for NN1177. A risk of increased statin exposure following co-administration with NN1177, which could lead to myopathy and rhabdomyolysis, could therefore not be ruled out.

A cocktail approach was selected in the clinic for three reasons: first, the *in vitro* data indicated the potential of involvement of multiple nuclear receptors; second, the translation of *in vitro* CYP downregulation to a clinical setting by a therapeutic peptide is not clear; and finally, this study design enables the simultaneous assessment of multiple CYP enzymes. Five different CYP enzymes were evaluated using the Cooperstown 5+1 cocktail (Tran et al., 2016), a well-established and validated tool to assess the effects on major CYP enzymes. However, potential DDI risks suggested by *in vitro* assays failed to translate to the clinical setting.

**Potential confounding factors impacting clinical data interpretation**

The observed absence of an effect on midazolam in the clinic was most unexpected given the observed *in vitro* CYP3A4 down-regulation results. It remains uncertain if the *in vitro* signal is a false positive or if the observed *in vitro* effect on CYP3A4 takes place in humans but is masked by other potentially confounding factors. Furthermore, it should be noted that the *in vitro* $f_u$ is currently unknown and the potential impact of this parameter was highlighted by PBPK simulations where a value of 0.0023 was required to simulate a greater than 2-fold increase in midazolam AUC.
However, the observations in clinic could not be fully explained by *in vitro* \( f_u \) values (e.g., the observed decrease in caffeine AUC and \( C_{\text{max}} \)). Understanding the discrepancy between *in vitro* observations, PBPK modelling, and clinical findings reported here is challenging since there are likely multiple factors involved. For example, trends observed in PK profiles (decreased \( C_{\text{max}} \) and later \( T_{\text{max}} \)) suggest NN1177 influenced gastric emptying. This is not entirely surprising since glucagon and GLP-1 delay gastric emptying (Jonderko et al., 1988; Willms et al., 1996). A similar delay here could complicate the interpretation of clinical study results, since the physicochemical and formulation properties of the index substrates might impact the observed results. Evidence supporting this hypothesis includes the observation that omeprazole plasma exposure decreased by approximately half with NN1177 co-administration. Omeprazole is acid labile and therefore administered in a delayed-release capsule that contain enteric-coated gelatine granules of the drug. If gastric emptying were delayed, omeprazole would be more susceptible to degradation, decreasing the intact amount available for absorption. This hypothetical scenario is consistent with lower omeprazole bioavailability in subjects following NN1177 treatment.

The current clinical trial included overweight subjects (BMI between 23.0 and 29.9 kg/m\(^2\)). These subjects lost considerable amounts of weight in a short period of time (approximately 10% of their body weight over the trial period). The impact of extensive weight loss observed here is unknown. A recent study has shown that body weight, waist circumference, hip circumference, fat percent, fat mass and BMI are negatively correlated with the metabolic capacity of CYP3A4 (Krogstad et al., 2021). It is possible that the weight and fat loss induced by NN1177 during the study was associated with a change in CYP3A4 activity. Increased CYP3A4 clearance may have counteracted the CYP3A4 suppression effect observed *in vitro*. Finally, short-term fasting is known to alter the activity of drug metabolising enzymes (Lammers et al., 2020). It is unknown if the reported short-term starvation effect on drug metabolising enzymes is the same for longer-term fasting, equivalent to what subjects experienced here (Lammers et al., 2017).
Collectively, this multitude of possible confounding factors likely results in the complex drug-drug interactions observed here and highlights the challenges of designing an optimal clinical trial for glucagon/GLP-1 receptor co-agonists. Moreover, the PBPK model built here only included one impacting factor (CYP down-regulation) to perform a risk analysis and did not aim to simulate the trial. Therefore, future simulations using more a representative population (e.g., BMI and gender) and incorporating additional factors related to the pharmacological effects of NN1177 (e.g., gastric emptying, inflammation, excessive weight loss/starvation, etc.) should be more informative.

**Better pre-clinical models needed to assess potential DDI risks for peptide-based therapies**

Traditional cell culture models do not account for endogenous regulatory mechanisms within the endocrine hormonal system, which might account for observed differences between *in vitro* assays and clinical settings. For example, insulin is reported to counteract the effect of glucagon on CYP enzymes (Kim and Novak, 2007), but traditional *in vitro* models do not account for such responsive feedback mechanisms that take place in whole organisms. Utilising animal models would provide the benefit of testing compounds in whole organisms, but they are typically avoided for DDI predictions due to species-dependent differences in gene expression regulation and metabolism. These limitations have, however, been resolved in a humanized mouse model (8HUM) where 34 murine CYPs have been deleted and replaced with major human drug metabolising CYP enzymes, together with relevant transcription factors CAR and PXR (Henderson et al., 2019). This model has shown promising results for predictions of small molecule DDIs, but the potential for prediction of peptide-based DDIs still remain unexplored.

Advances in culturing techniques have introduced more physiologically-relevant *in vitro* liver systems with prolonged metabolic activity (Godoy et al., 2013). Recently, it has also been demonstrated that 3D spheroid cultures of primary human hepatocytes improve predictions for
small molecule CYP3A4 induction (Hendriks et al., 2020). Together, novel 3D cellular systems and humanized animal models represent promising new tools for pre-clinical DDI assessments for peptide-based drugs.

Conclusions

In sum, this report represents, to the best of our knowledge, the first to follow-up in vitro CYP down-regulation by a peptide with PBPK modelling and a clinical investigation. Since there are currently no in vitro study design recommendations by health authorities to assess potential DDI risks for peptides intended for therapeutic use, we performed pre-clinical studies with established in vitro systems that are gold standards for small molecules. These assessments and subsequent conclusions drawn from PBPK modelling failed to translate to the clinic, highlighting the challenge of assessing DDI risk for peptides during drug development. This study therefore exposes two things: (1) a significant knowledge gap about in vitro study design requiring the urgent need for better pre-clinical models to assess potential DDI risks for peptide-based drugs in development and (2) the challenges associated with designing an optimal clinical DDI study involving peptide drugs like NN1177 where factors such as delayed gastric emptying, potential endogenous feedback mechanisms, and the effect of weight loss/starvation is unknown. Overall, an intensification of research in these areas is required to better guide investigators during drug development for therapeutic peptides.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: CS, LA, KD, MHF, SBN, and KK. Performed data analysis: CS, LA, KD, MHF, SBN, and KK. Wrote or contributed to the writing of the manuscript: CS, LA, and KK.
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FOOTNOTES

Funding

The study was sponsored by Novo Nordisk A/S.

Conflict of interest

The sponsor was involved in the study design, collection, analysis, and interpretation of data, as well as data checking of information provided in the manuscript. CS, LA, KD, MHF, SBN, and KK are employees at Novo Nordisk A/S. LA, MHF, KK, KD own shares in Novo Nordisk A/S.
FIGURE LEGENDS

Figure 1. Trial design. The trial duration was ~5 months and consisted of 14 visits. Screening was followed by enrolment in the trial, administration of index substrates (DDI cocktail), 8–168-hour pharmacokinetic (PK) assessment for different substrates and their metabolites, an 8-week dose escalation with 2-week escalation steps, followed by 3 weeks on target dose level of NN1177. Index substrates (DDI cocktail) were administered simultaneously with the last NN1177 dose, followed by 8–168-hour PK assessment. The follow-up period was 28 days after the last NN1177 dose. Total duration of trial participation for each subject was 110 to 148 days from screening to follow-up.

Figure 2. In vitro CYP mRNA and activity changes in human hepatocytes treated with NN1177. Bar graphs depict fold change in mRNA and enzyme activity in three freshly isolated human hepatocytes relative to medium controls following exposure to NN1177, native glucagon, or IL-6 for CYP3A4, CYP2B6, and CYP1A2. DMSO was used as the medium control for all drugs, except for IL-6, which used acetic acid. The bars display average (mean) values from three independent donors. Error bars represent standard deviation.

Figure 3. PBPK simulations for effect of NN1177 on CYP3A4 and CYP1A2 substrates. Predicted plasma concentration vs. time (hours) profiles for midazolam and caffeine following exposure to different estimated unbound NN1177 concentrations. Detailed information on PBPK models available in Supplemental Table S2 and Supplemental Table S3.
**Figure 4. Effect of NN1177 on Cooperstown 5+1 in clinic.** Mean PK plasma concentration (ng/ml) and time profiles for midazolam, caffeine, omeprazole, dextromethorphan, and S-warfarin after administration of the Cooperstown 5+1 cocktail either alone (black) or following an eight-week dose escalation and two weeks of steady-state NN1177 exposure at 4.2 mg (red).

**Figure 5. Forest plot.** Forest plot depicting the change in AUC_{0-inf} and C_{max} (including 90% confidence interval; 90% CI) from baseline for midazolam, caffeine, omeprazole, dextromethorphan, and S-warfarin to after NN1177 treatment (4.2 mg). The dotted lines at 0.8 and 1.25 indicate NN1177 treatment did not have a clinically significant effect.
## Tables

### Table 1. Down-regulation and fraction unbound parameters applied in PBPK simulations

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Description</th>
<th>CYP1A2</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (nM) (total NN1177 conc. in hepatocyte incubation approximated to fit observed mRNA data*)</td>
<td></td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Scenario 1: EC50 (nM unbound NN117**), in vitro</td>
<td>fu assumed 0.0023</td>
<td>0.092</td>
<td>0.092</td>
</tr>
<tr>
<td>Scenario 2: EC50 (nM unbound NN117**) in vitro</td>
<td>fu assumed 0.064</td>
<td>2.58</td>
<td>2.58</td>
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<tr>
<td>Scenario 3: EC50 (nM) unbound NN117** in vitro</td>
<td>fu assumed 0.01</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>E_{max}</td>
<td></td>
<td>0.60</td>
<td>0.85</td>
</tr>
</tbody>
</table>

conc., concentration; fu, fraction unbound; EC50, half-maximal effect concentration; E_{max}, maximum effect

*Values are approximated based on 3 concentrations. Based on this uncertainty, the results should be interpreted with caution.

**EC50 values for unbound NN1177 for the different scenarios of in vitro fu were calculated as EC50 for the total NN1177 multiplied by the in vitro fu.
Table 2. PBPK simulated $AUC_{0\text{-inf}}$ and $C_{\text{max}}$ values and ratios with and without NN1177 for caffeine and midazolam

<table>
<thead>
<tr>
<th></th>
<th>Midazolam</th>
<th></th>
<th>Caffeine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$AUC_{0\text{-inf}}$ (ng*h/ml)</td>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>$AUC$ ratio</td>
<td>$C_{\text{max}}$ ratio</td>
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<tr>
<td><strong>Scenarios</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Before</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NN1177 Baseline</td>
<td>22.6</td>
<td>9.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>After</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NN1177 $f_u$ 0.0023</td>
<td>53.3</td>
<td>14.2</td>
<td>2.36</td>
<td>1.57</td>
</tr>
<tr>
<td>$f_u$ 0.01</td>
<td>31.7</td>
<td>11.1</td>
<td>1.40</td>
<td>1.2</td>
</tr>
<tr>
<td>$f_u$ 0.064</td>
<td>24.1</td>
<td>9.5</td>
<td>1.07</td>
<td>1.0</td>
</tr>
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</table>
Table 3. Subject demographics

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>43.3 (8.4)</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>26 (57.8)</td>
</tr>
<tr>
<td>Male</td>
<td>19 (42.2)</td>
</tr>
<tr>
<td>Body mass index, kg/m², mean (SD)</td>
<td>26.5 (1.9)</td>
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<tr>
<td>Ethnicity, n (%)</td>
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<tr>
<td>Hispanic or Latino</td>
<td>23 (51.1)</td>
</tr>
<tr>
<td>Not Hispanic or Latino</td>
<td>22 (48.9)</td>
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<td>Race, n (%)</td>
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<td>Black or African American</td>
<td>11 (24.4)</td>
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<tr>
<td>White</td>
<td>32 (71.1)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (4.4)</td>
</tr>
<tr>
<td>Genotyping, n (%)</td>
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<tr>
<td>CYP2C9</td>
<td>45 (100.0)</td>
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<tr>
<td>Poor metaboliser</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>Intermediate metaboliser</td>
<td>7 (15.6)</td>
</tr>
<tr>
<td>Normal metaboliser</td>
<td>37 (82.2)</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>45 (100.0)</td>
</tr>
<tr>
<td>Poor metaboliser</td>
<td>3 (6.7)</td>
</tr>
<tr>
<td>Intermediate metaboliser</td>
<td>2 (4.4)</td>
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<tr>
<td>Normal metaboliser</td>
<td>37 (82.2)</td>
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<tr>
<td>Ultra rapid metaboliser</td>
<td>3 (6.7)</td>
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<tr>
<td>CYP2C19</td>
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<tr>
<td>Poor metaboliser</td>
<td>3 (6.7)</td>
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<tr>
<td>Intermediate metaboliser</td>
<td>8 (17.8)</td>
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<tr>
<td>Metabolism Type</td>
<td>Count (%)</td>
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<tr>
<td>-------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Normal metaboliser</td>
<td>18 (40.0)</td>
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<tr>
<td>Rapid metaboliser</td>
<td>13 (28.9)</td>
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<tr>
<td>Ultra rapid metaboliser</td>
<td>3 (6.7)</td>
</tr>
<tr>
<td>Drug</td>
<td>AUC₀⁻_INF, ng*h/ml</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Midazolam 2 mg</td>
<td>N Alone</td>
</tr>
<tr>
<td>AUC₀⁻_INF, ng*h/ml</td>
<td>25.3 (31.1)</td>
</tr>
<tr>
<td>C_max, ng/ml</td>
<td>9.51 (31.1)</td>
</tr>
<tr>
<td>t_max, h</td>
<td>0.5</td>
</tr>
<tr>
<td>t½, h</td>
<td>3.68 (27.7)</td>
</tr>
<tr>
<td>Caffeine 200 mg</td>
<td>N Alone</td>
</tr>
<tr>
<td>AUC₀⁻_INF, ng*h/ml</td>
<td>49600 (36)</td>
</tr>
<tr>
<td>C_max, ng/ml</td>
<td>5370 (23.5)</td>
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<tr>
<td>t_max, h</td>
<td>1</td>
</tr>
<tr>
<td>t½, h</td>
<td>5.46 (32)</td>
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<tr>
<td>Omeprazole 40 mg</td>
<td>N Alone</td>
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<tr>
<td>AUC₀⁻_INF, ng*h/ml</td>
<td>1690 (69.9)</td>
</tr>
<tr>
<td>C_max, ng/ml</td>
<td>707 (57)</td>
</tr>
<tr>
<td>t_max, h</td>
<td>2</td>
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<tr>
<td>t½, h</td>
<td>0.951 (40.8)</td>
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<tr>
<td>Dex* 30 mg</td>
<td>N Alone</td>
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<tr>
<td>AUC₀⁻_INF, ng*h/ml</td>
<td>16.1 (170)</td>
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<td>C_max, ng/ml</td>
<td>1.58 (122)</td>
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<td>t_max, h</td>
<td>3</td>
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<tr>
<td>t½, h</td>
<td>7.3 (43.4)</td>
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<td>S-warfarin 10 mg</td>
<td>N Alone</td>
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<td>AUC₀⁻_INF, ng*h/ml</td>
<td>19700 (26.4)</td>
</tr>
<tr>
<td>C_max, ng/ml</td>
<td>552 (19.6)</td>
</tr>
<tr>
<td>t_max, h</td>
<td>3</td>
</tr>
<tr>
<td>t½, h</td>
<td>41.3 (18.6)</td>
</tr>
</tbody>
</table>

*Dex, dextromethorphan; %CV, percent coefficient of variation; CI, confidence interval
Figure 1
Figure 3

Midazolam
- fu 0.0023
- fu 0.01
- fu 0.064
- Baseline

Caffeine
- fu 0.0023
- fu 0.01
- fu 0.064
- Baseline