

In Vitro CYP450 Enzyme Downregulation by GLP-1/Glucagon Coagonist Does Not Translate to Observed Drug-Drug Interactions in the Clinic^S

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ABSTRACT

NN1177 is a glucagon/glucagon-like peptide 1 receptor coagonist investigated for chronic weight management and treatment of non-alcoholic steatohepatitis. Here, we show concentration-dependent downregulation of cytochrome P450 (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. Physiologic-based pharmacokinetic modeling 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* P450 downregulation, a phase 1 clinical cocktail study was initiated to assess the DDI potential. The study enrolled 45 study participants (body mass index 23.0–29.9 kg/m²) to receive a DDI 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 coadministration of NN1177 on AUC_{0-inf} for midazolam or S-warfarin. Omeprazole, caffeine, and dextromethorphan generally displayed decreases in AUC_{0-inf} and C_{max} following NN1177 coadministration. 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 preclinical 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 preclinical 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. Utilizing *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 (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf; <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions>). In contrast, the US Food and Drug Administration (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 (<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/drug-drug-interaction-assessment-therapeutic-proteins-guidance-industry>). Therapeutic peptides (defined by the FDA as ≤40 amino acids) (D'Aloisio et al., 2021; <https://www.fda.gov/drugs/regulatory-science-action/impact-story-developing-tools-evaluate-complex-drug-products-peptides>) 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

europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf; <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions>). In contrast, the US Food and Drug Administration (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 (<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/drug-drug-interaction-assessment-therapeutic-proteins-guidance-industry>). Therapeutic peptides (defined by the FDA as ≤40 amino acids) (D'Aloisio et al., 2021; <https://www.fda.gov/drugs/regulatory-science-action/impact-story-developing-tools-evaluate-complex-drug-products-peptides>) 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

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ABBREVIATIONS: AUC, area under the curve; BMI, body mass index; CI, confidence interval; DDI, drug-drug interaction; E_{max} , maximum effect; FDA, Food and Drug Administration; f_u , fraction unbound; GLP-1, glucagon-like peptide 1; IL-6, interleukin-6; K_d , equilibrium dissociation constant; NASH, nonalcoholic steatohepatitis; NN1177, NNC9204-1177; P450, cytochrome P450; PBPK, physiological-based pharmacokinetic; PCR, polymerase chain reaction; PK, pharmacokinetic; $t_{1/2}$, half-life; t_{max} , time of maximum observed drug concentration.

to identify potential DDI risks for small molecule drugs are appropriate for assessing similar risks with therapeutic peptides.

Administering concomitant drugs may alter the metabolizing capacity of cytochrome P450 (P450) enzymes, and such DDIs can cause adverse reactions or therapy failure. *In vitro* P450 downregulation 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* P450 downregulation (Hariparsad et al., 2017; Steverson et al., 2019; Turner et al., 2020). For biologics, the native hormone FGF-21 is reported to downregulate 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 P450 downregulation in these cases is currently unknown. Details related to P450 downregulation mechanisms in guidelines from health authorities are presently limited. European authorities recommend further studies if P450 mRNA is decreased by more than 50% without any associated cell toxicity (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf). Likewise, the FDA is proposing to consider additional studies to assess the potential clinical consequences when concentration-dependent downregulation has been observed without any associated cell toxicity (<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions>).

NNC9204-1177 (NN1177) is a linear 29-amino acid synthetic peptide (~4.57 kDa) investigated for chronic weight management and treatment of nonalcoholic steatohepatitis (NASH), a severe form of nonalcoholic fatty liver disease. The chemical structure of NN1177 has been reported elsewhere (Simonsen et al., 2022). Mechanistically, NN1177 acts as a coagonist 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 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 comorbidities and complications such as hypertension, hyperglycemia (prediabetes 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 downregulation of major P450s in standard *in vitro* assays following exposure of clinically relevant NN1177 concentrations. The mechanism of this downregulation is unknown. Physiologic-based pharmacokinetic (PBPK) modeling 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 subcutaneous doses influences the exposure of representative index substrates for multiple P450 enzymes (using the validated DDI cocktail). However, pharmacokinetic (PK) profiles of index substrates in the clinical setting were inconsistent with P450 downregulation. These findings emphasize the remaining challenges and uncertainties associated with commonly used *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 1. These primary human hepatocytes were cultured in collagen-sandwich configuration and supplemented with modified Eagle's medium (MCM+), including 1.23 mg/ml bovine serum albumin, 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), interleukin-6 (IL-6) was from EMD Millipore (Burlington, Massachusetts), and midazolam was from USP (North Bethesda, Maryland). Reagents for RNA isolation and quantitative polymerase chain reaction (PCR) were from Ambion and Applied Biosystems, respectively.

Prior to this study, NN1177 stability was evaluated by incubating 100 and 1000 nM radiolabeled 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 3 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 C_{max} values. Quantitative evaluation of cytotoxicity was measured using a lactate dehydrogenase release assay. Lactate dehydrogenase 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 glyceraldehyde-3-phosphate dehydrogenase, 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 glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1). Catalytic activities for P450s were analyzed in triplicate by liquid chromatography–tandem mass spectrometry 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),

TABLE 1
Downregulation and fraction unbound parameters applied in PBPK simulations

	CYP1A2	CYP3A4
EC ₅₀ (nM) (total NN1177 conc. in hepatocyte incubation approximated to fit observed mRNA data ^a)	40	40
Scenario 1: EC ₅₀ (nM unbound NN1177 ^b), <i>in vitro</i> f _u assumed 0.0023	0.092	0.092
Scenario 2: EC ₅₀ (nM unbound NN1177 ^b) <i>in vitro</i> f _u assumed 0.064	2.58	2.58
Scenario 3: EC ₅₀ (nM unbound NN1177 ^b) <i>in vitro</i> f _u assumed 0.01	0.40	0.40
E _{max}	0.60	0.85

conc., concentration.

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

^bEC₅₀ values for unbound NN1177 for the different scenarios of *in vitro* f_u were calculated as EC₅₀ for the total NN1177 multiplied by the *in vitro* f_u.

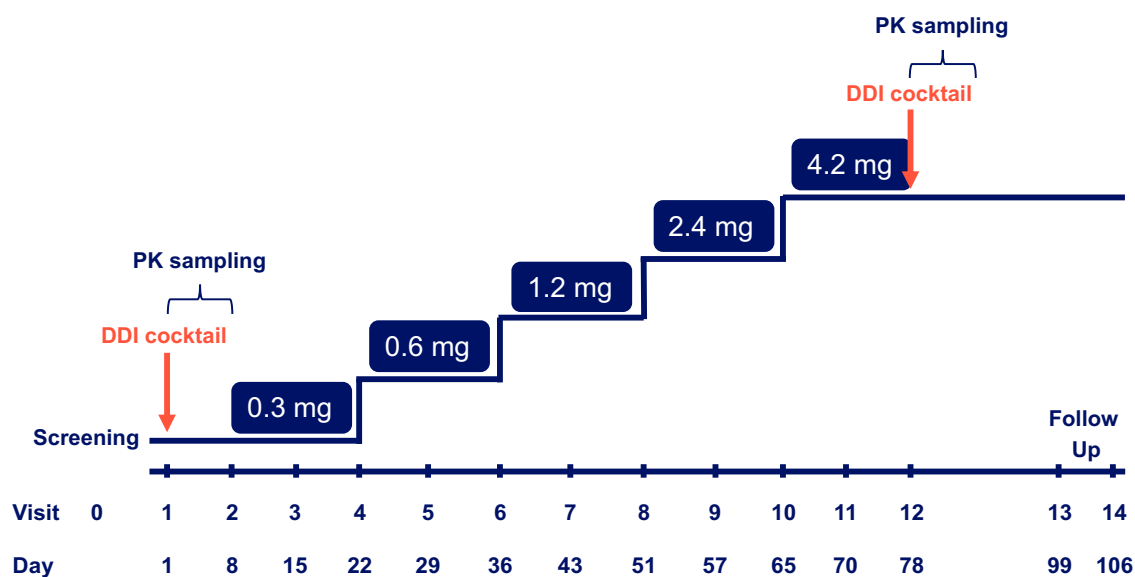


Fig. 1. Trial design. The trial duration was ~5 months and consisted of 14 visits. Screening was followed by enrolment in the trial, administration of index substrates (DDI cocktail), 8–168 hour pharmacokinetic (PK) assessment for different substrates and their metabolites, and an 8-week dose escalation 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 110–148 days from screening to follow-up.

and rifampin (a PXR agonist and inducer of CYP3A4) (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf; <https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers>).

PBPK Modeling

PBPK modeling was used to simulate the theoretical clinical outcome of CYP3A4 and CYP1A2 downregulation indicated by *in vitro* studies using the Open Systems Pharmacology Suite v. 8.0 with PK-Sim and MoBi (<http://www.open-systems-pharmacology.org>). Standard settings were used except where optimizations 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 2. P450 expression levels in hepatocytes using reverse-transcription 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 ($t_{1/2}$) were used: CYP3A4 liver concentration 4.32 μM , $t_{1/2} = 36$ hours in liver; CYP1A2 liver concentration 1.80 μM , $t_{1/2} = 39$ hours in liver. A default subject [30-year-old male, European, mean body weight of 73 kg, height of 176 cm, and body mass index (BMI) of 23.57 kg/m^2] 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 P450 gene regulation likely take place after membrane-bound receptor stimulation since the size and physicochemical properties of the peptide limit 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 nonphysiologic solution was applied. In PK-Sim, P450 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 EC_{50} for intracellular regulation of P450 expression. For more details, see Supplemental Table 3.

Implementation of *In Vitro* P450 Downregulation in PBPK Model

As per guideline recommendations, mRNA data were selected as the endpoint for evaluation of P450 regulation (<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions>). The degree of mRNA downregulation used here represents a worst-case scenario. Parameters describing P450 downregulation were determined so as to best describe the observed hepatocyte mRNA data according to the following equation:

$$\frac{\text{mRNA}}{\text{baseline mRNA}} = (1 - E_{\text{max}}) \times \frac{\text{Conc.}}{EC_{50 \text{ in vitro}} + \text{Conc.}} \quad (1)$$

Where $E_{\text{max, in vitro}}$ is the maximum fraction downregulated, EC_{50} is the NN1177 concentration giving 50% of maximum downregulation, and Conc. is the concentration of NN1177 in the incubation. For CYP3A4, average mRNA data for all three hepatocyte donors (H1401, H1403, and H1407) was used, whereas only the data from H1401 was used for CYP1A2 since the observed downregulation represents a worst-case scenario.

In PK-Sim, the technical implementation of an enzyme suppression is a negative induction, with E_{max} ranging from 0 (no suppression) to -1 (full suppression) since the time course of a P450 enzyme, E , is per default simulated by the differential equation:

$$\frac{dE}{dt} = k_{\text{deg}} \times E_0 \times \left(1 + \frac{E_{\text{max}} \times \text{Conc.}}{(EC_{50} + \text{Conc.})} \right) - k_{\text{deg}} \times E \quad (2)$$

Where k_{deg} is the rate of enzyme degradation, E_0 is the amount of enzyme in the absence of induction/suppression, EC_{50} is the concentration of NN1177 giving 50% of maximum suppression, and E_{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_{max} for CYP3A4 downregulation 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 P450 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 P450s. 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

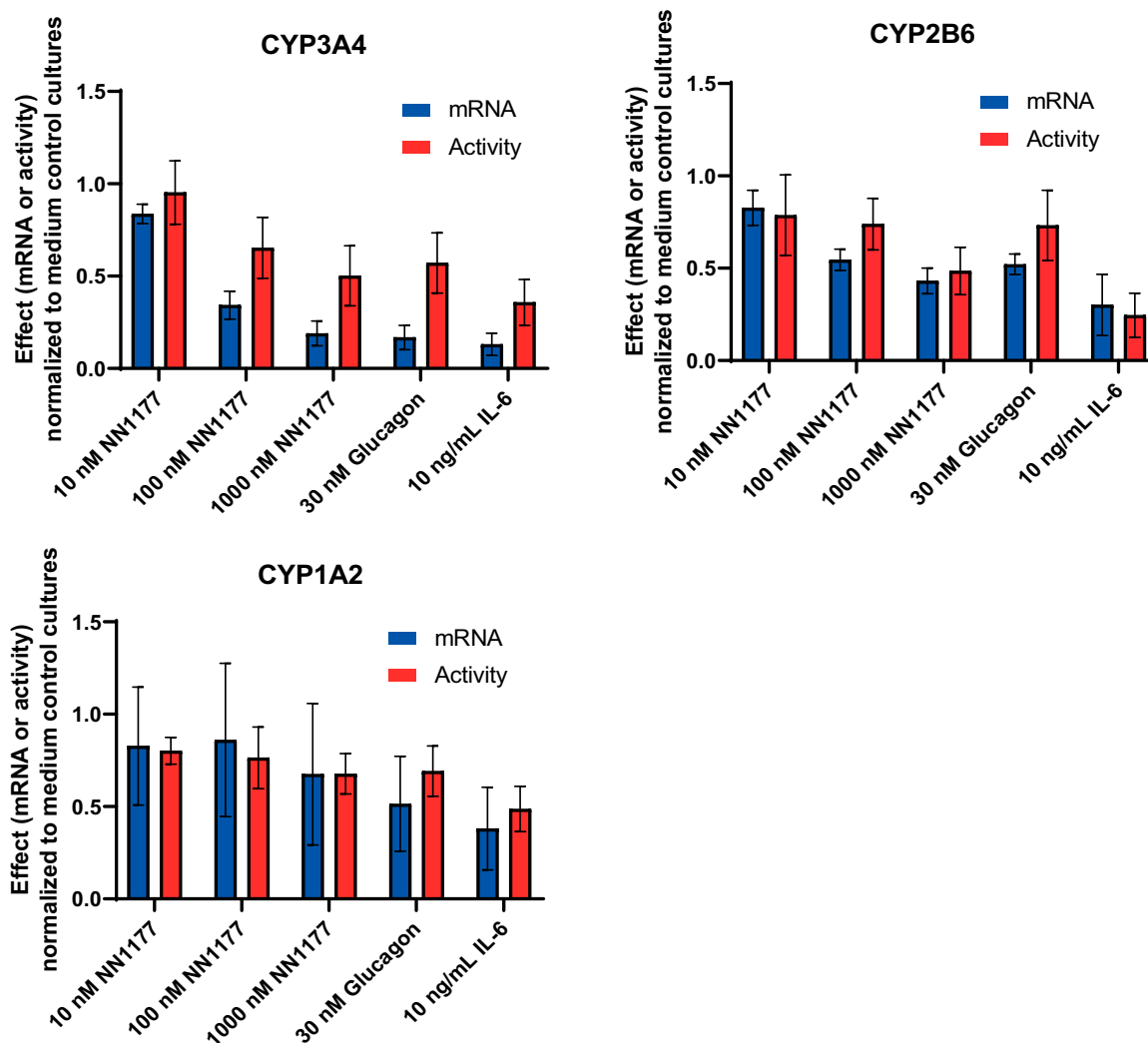


Fig. 2. In vitro P450 mRNA and activity changes in human hepatocytes treated with NN1177. Bar graphs depict fold change in mRNA and enzyme activity in three freshly isolated human hepatocytes relative to medium controls following exposure to NN1177, native glucagon, or IL-6 for CYP3A4, CYP2B6, and CYP1A2. 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.

corresponding equilibrium dissociation constant (K_d) for binding to human serum albumin 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:

$$f_u = \frac{1}{1 + \left(\frac{1}{K_d}\right) \times [A]} \quad (3)$$

Where $[A]$ is the albumin concentration (4.23 mg/ml bovine serum albumin 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 EC_{50} values calculated above using the total NN1177 concentration in the incubations were multiplied by each *in vitro* f_u tested to convert the EC_{50} to the unbound NN1177 concentration.

Clinical Trial Design and Trial Population

The clinical study (clinicaltrials.gov identifier #NCT04059367) was a single-sequence, drug-drug interaction study enrolling 45 healthy male and female subjects between 18 and 60 years of age (BMI 23.0–29.9 kg/m^2). 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 (https://database.ich.org/sites/default/files/E6_R2_Addendum.pdf).

Treatments

All participants followed the same course of treatment. NN1177 was administered subcutaneously 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 3 weeks at the target dose of 4.2 mg (Fig. 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 P450 substrates was selected based on the validated DDI 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 4. The impact on CYP2B6 is not assessed by utilizing the DDI 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 P450 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. Use of any herbal medicines

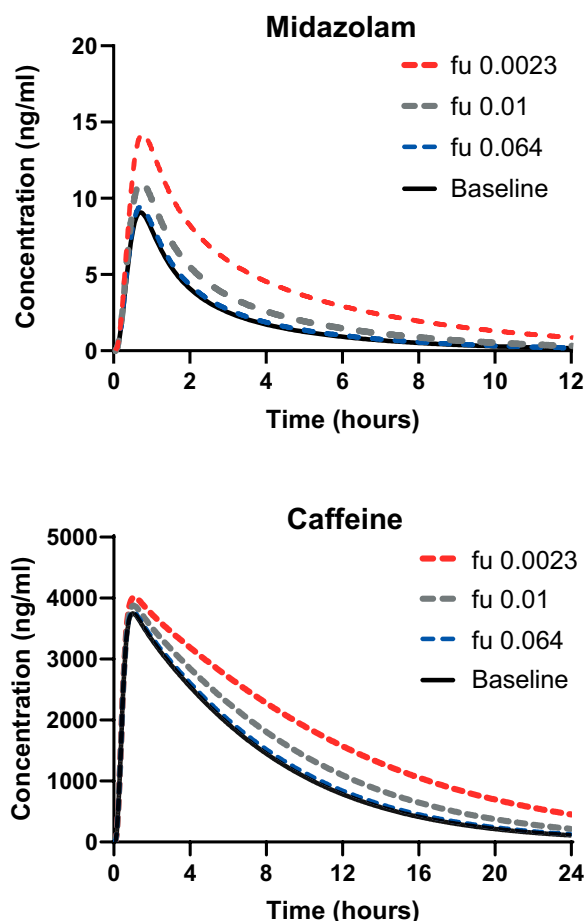


Fig. 3. PBPK simulations for effect of NN1177 on CYP3A4 and CYP1A2 substrates. Predicted plasma concentration versus time (hours) profiles for midazolam and caffeine following exposure to different estimated unbound NN1177 concentrations. Detailed information on PBPK models available in Supplemental Tables 2 and 3.

known to interfere with the metabolic P450 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 channeling 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 (<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry>) and the European Medicines Agency Guideline on Bioanalytical Method Validation (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf). Index substrates and metabolites were measured in plasma using validated liquid chromatography–tandem mass spectrometry methods. Reference-standard materials midazolam, caffeine, omeprazole, and dextromethorphan were obtained from USP (North Bethesda, Maryland); 1-hydroxymidazolam and dextropran 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 predose and postdose at the following time intervals for index substrates and metabolites: midazolam and 1-hydroxymidazolam (predose, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 hours postdose); omeprazole and 5-hydroxyomeprazole (predose, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, and 96 hours postdose); dextromethorphan and dextropran (predose, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, and 72 hours postdose, as well as dextropran alone at 36, 96, 120, 144, and 168 hours postdose); S-warfarin and 7-hydroxy-S-warfarin (predose, 3, 6, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours postdose); caffeine and paraxanthine (predose, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours postdose, as well as paraxanthine alone at 36, 48, 72, 96, 120, 144, and 168 hours postdose). Samples for NN1177 were collected on day 8, 22, 36, 50, and 64, as well as on day 78 predose and postdose at the following time intervals: predose, 2, 5, 8, 12, 16, 20, 24, 30, 36, 42, 48, 72, 120, and 168 hours postdose.

PK parameters [i.e., AUC_{0-inf} , C_{max} , $t_{1/2}$, and time of maximum observed drug concentration (t_{max})] for midazolam, caffeine, omeprazole, dextromethorphan, S-warfarin, and their main metabolites (1-Hydroxymidazolam, hydroxyomeprazole, paraxanthine, dextropran, and 7-Hydroxy-S-warfarin) were calculated by standard noncompartmental methods (Version 8.1; Phoenix WinNonlin, Certara, Princeton New Jersey). For AUC_{0-inf} , datapoints where the extrapolated AUC_{t-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 C_{max} , respectively) were excluded from descriptive statistics. The ratios between the main metabolite and index substrate exposure were calculated for AUC_{0-24h} . Using the Phoenix WinNonlin bioequivalence workflow, log-transformed AUC_{0-inf} , C_{max} , and t_{max} 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 analyzed 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 used, 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 observed by the investigator or reported spontaneously by the subjects were collected. The investigator assessed the adverse event 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 nonserious).

Results

In Vitro Studies

To assess the effect of NN1177 on P450 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 P450 downregulation as it is known to result in pan-suppression of P450 mRNA via a transcriptional mechanism (Abdel-Razzak et al., 1993; Dickmann et al., 2011; Czerwiński et al., 2018;

TABLE 2

PBPK simulated AUC_{0-inf} and C_{max} values and ratios with and without NN1177 for caffeine and midazolam

Scenarios	Midazolam				Caffeine				
	AUC_{0-inf} (ng*h/ml)	C_{max} (ng/ml)	AUC ratio	C_{max} ratio	AUC_{0-inf} (ng*h/ml)	C_{max} (ng/ml)	AUC ratio	C_{max} ratio	
Before NN1177	Baseline	22.6	9.1	1.0	1.0	28,500	3750	1.0	1.0
	f_u 0.0023	53.3	14.2	2.36	1.57	46,700	4000	1.6	1.1
After NN1177	f_u 0.01	31.7	11.1	1.40	1.2	33,310	3880	1.17	1.03
	f_u 0.064	24.1	9.5	1.07	1.0	29,810	3780	1.05	1.0

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 P450 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 P450 downregulation in human hepatocytes treated with both IL-6 and glucagon (Fig. 2). A tabular summary of the interpreted data is available in Supplemental Table 5 (downregulation data presented as percent decrease) and Supplemental Table 6 (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 (Fig. 2; Supplemental Tables 1 and 5). At the clinically relevant concentration of 100 nM, CYP3A4 mRNA decreased between 57.2% and 71.7% compared with control, whereas CYP2B6 mRNA decreased between 39.3% and 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 the FDA and European Medicines Agency (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf; <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions>), additional studies are recommended if substantial concentration-dependent P450 downregulation is observed with *in vitro* assays to explore potential clinical consequences. Therefore, potent P450 downregulation observed for clinically relevant NN1177 concentrations prompted a DDI cocktail study, where several P450 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 modeling was performed to predict upfront the potential clinical impact of P450 downregulation indicated by *in vitro* data. EC_{50} and E_{max} values based on *in vitro* mRNA data in hepatocytes, as well as three scenarios for f_u in the hepatocyte culture, were used for the PBPK modeling 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* EC_{50} in terms of unbound NN1177) with and without NN1177 treatment predicted an increase in the exposure to both compounds following coadministration with NN1177 (Fig. 3). For caffeine, the ratios for AUC_{0-inf} and C_{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-inf} and C_{max} were 2.4 and 1.6, respectively. For larger *in vitro* f_u values and thus higher EC_{50} values in terms of unbound NN1177, AUC_{0-inf} and C_{max} ratios were predicted between 1.0 and 1.4 (Fig. 3; Table 2). 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 (S.D.) age of 43.3 (8.4) years and BMI from 23.0 to 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 Fig. 4. Observed changes in AUC_{0-inf} and C_{max} are shown in Fig. 5. PK analysis of index substrates are available in Table 4, metabolites are available in Supplemental Figure 2, and metabolite/parent ratios are available in Supplemental Table 7.

TABLE 3
Subject demographics

Age, mean (S.D.)	43.3 (8.4)
Sex, n (%)	
Female	26 (57.8)
Male	19 (42.2)
Body mass index, kg/m ² , mean (S.D.)	26.5 (1.9)
Ethnicity, n (%)	
Hispanic or Latino	23 (51.1)
Not Hispanic or Latino	22 (48.9)
Race, n (%)	
Black or African American	11 (24.4)
White	32 (71.1)
Other	2 (4.4)
Genotyping, n (%)	
CYP2C9	45 (100.0)
Poor metabolizer	1 (2.2)
Intermediate metabolizer	7 (15.6)
Normal metabolizer	37 (82.2)
CYP2D6	45 (100.0)
Poor metabolizer	3 (6.7)
Intermediate metabolizer	2 (4.4)
Normal metabolizer	37 (82.2)
Ultra-rapid metabolizer	3 (6.7)
CYP2C19	45 (100.0)
Poor metabolizer	3 (6.7)
Intermediate metabolizer	8 (17.8)
Normal metabolizer	18 (40.0)
Rapid metabolizer	13 (28.9)
Ultra-rapid metabolizer	3 (6.7)

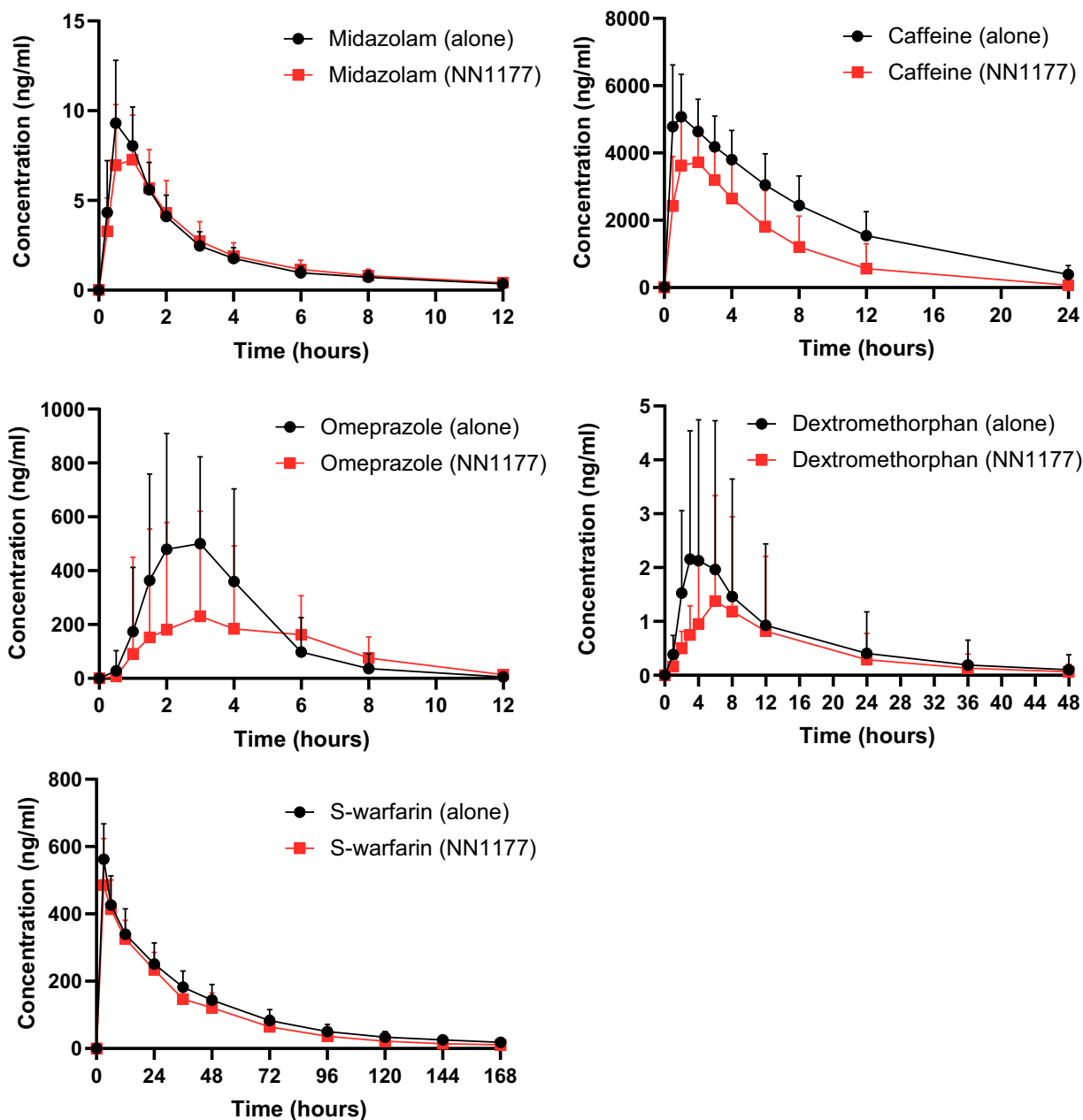


Fig. 4. Effect of NN1177 on Cooperstown 5 + 1 in clinic. Mean PK plasma concentration (ng/ml) and time profiles for midazolam, caffeine, omeprazole, dextromethorphan, and S-warfarin after administration of the Cooperstown 5 + 1 cocktail either alone (black) or following an 8-week dose escalation and 2 weeks of steady-state NN1177 exposure at 4.2 mg (red).

PBPK Modeling. The PBPK model adequately reproduced the mean observed NN1177 plasma concentrations at day 78 (Supplemental Figure 1). Similarly, the PBPK models predicted PK profiles for baseline midazolam area under curve (AUC) and C_{max} and caffeine AUC (within $\pm 30\%$ of the observed median); however, caffeine C_{max} was overpredicted by about threefold. 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 3). At steady-state NN1177 exposure, the geometric mean of AUC_{0-168h} and C_{max} were 16,715 nmol·h/L and 140 nmol/L, respectively. Median of t_{max} was 24 hours (range, 12–72 hours).

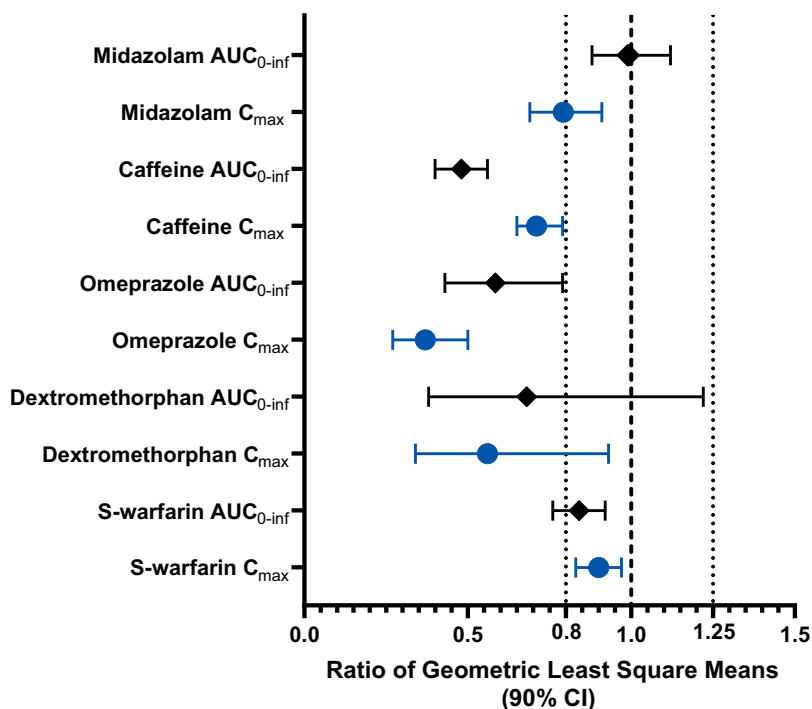
Midazolam. No substantial differences in the geometric least-squares mean midazolam AUC_{0-inf} were observed between

midazolam administered alone or in combination with NN1177 (Fig. 5; Table 4). Midazolam C_{max} decreased by $\sim 20\%$ and $t_{1/2}$ was unchanged, whereas t_{max} effectively doubled when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

Caffeine. Decreased caffeine AUC_{0-inf} was observed with NN1177 coadministration (Fig. 5; Table 4). Caffeine C_{max} decreased by $\sim 30\%$ and $t_{1/2}$ reduced by approximately half, whereas t_{max} increased only modestly (10%) when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

Omeprazole. Decreased omeprazole AUC_{0-inf} was observed with NN1177 coadministration (Fig. 5; Table 4). Omeprazole C_{max} decreased by $\sim 64\%$ and $t_{1/2}$ increased by nearly 60%, whereas t_{max} effectively

Fig. 5. Forest plot. Forest plot depicting the change in AUC_{0-inf} and C_{max} (including 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.



tripled when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

Dextromethorphan. Decreased dextromethorphan AUC_{0-inf} was observed with NN1177 coadministration (Fig. 5; Table 4). Dextromethorphan C_{max} decreased by ~40% and $t_{1/2}$ was unchanged, whereas t_{max} increased by about 33% when the cocktail was administered after NN1177 dosing compared with cocktail administration alone.

S-warfarin. S-warfarin AUC_{0-inf} between cocktail administered alone or in combination with NN1177 decreased modestly by ~16% (Fig. 5; Table 4). S-warfarin C_{max} showed a slight decrease (~10%), whereas $t_{1/2}$ was reduced by nearly 20%, and t_{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 8).

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 diarrhea. The adverse events led to discontinuation from the study for 8 participants.

TABLE 4
PK parameter estimates of P450 substrates

		Geometric Mean (CV%)				Ratio of Geometric LS Means (90% CI)
		N	Alone	N	Plus NN1177	
Midazolam 2 mg	AUC_{0-inf} , ng [*] h/ml	44	25.3 (31.1)	33	25 (38.9)	0.99 (0.88, 1.12)
	C_{max} , ng/ml	44	9.51 (31.1)	33	7.53 (43.2)	0.792 (0.69, 0.91)
	t_{max} , h	44	0.5	33	1	1.35 (1.15, 1.58)
	$t_{1/2}$, h	44	3.68 (27.7)	33	3.67 (22.2)	—
Caffeine 200 mg	AUC_{0-inf} , ng [*] h/ml	42	49600 (36)	33	23500 (54.4)	0.48 (0.40, 0.56)
	C_{max} , ng/ml	42	5370 (23.5)	33	3830 (28.5)	0.71 (0.65, 0.79)
	t_{max} , h	42	1	33	1.1	1.61 (1.34, 1.92)
	$t_{1/2}$, h	42	5.46 (32)	33	2.68 (36.5)	—
Omeprazole 40 mg	AUC_{0-inf} , ng [*] h/ml	39	1690 (69.9)	25	986 (95.2)	0.584 (0.43, 0.79)
	C_{max} , ng/ml	39	707 (57)	25	261 (121)	0.37 (0.27, 0.50)
	t_{max} , h	39	2	25	6	1.93 (1.60, 2.32)
	$t_{1/2}$, h	39	0.951 (40.8)	25	1.51 (64.4)	—
Dex ^a 30 mg	AUC_{0-inf} , ng [*] h/ml	37	16.1 (170)	15	11 (161)	0.72 (0.40, 1.31)
	C_{max} , ng/ml	37	1.58 (122)	15	0.889 (125)	0.59 (0.36, 0.97)
	t_{max} , h	37	3	15	4	1.29 (1.07, 1.56)
	$t_{1/2}$, h	37	7.3 (43.4)	15	7.81	—
S-warfarin 10 mg	AUC_{0-inf} , ng [*] h/ml	44	19700 (26.4)	32	16500 (25.3)	0.84 (0.76, 0.92)
	C_{max} , ng/ml	44	552 (19.6)	32	495 (21.2)	0.90 (0.83, 0.97)
	t_{max} , h	44	3	32	3	1.12 (1.03, 1.22)
	$t_{1/2}$, h	44	41.3 (18.6)	32	33.5 (24.6)	—

^aCV, percent coefficient of variation; Dex, dextromethorphan.

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* downregulation of CYP3A4, CYP2B6, and CYP1A2 at clinically relevant concentrations of the glucagon/GLP-1 coagonist NN1177. We observed similar effects for 30 nM native glucagon, consistent with earlier studies reporting P450 enzyme suppression by native glucagon (Morgan et al., 1998; Iber et al., 2001). In contrast, GLP-1 analogs like semaglutide have been reported to show no relevant effects on P450 enzymes *in vitro* or in the clinic (Bækdal et al., 2019; https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/213051Orig1s000ClinPharmR.pdf; Jordy et al., 2021), likely due to the absence of GLP-1 receptor expression in the human liver (Simonsen et al., 2022). Therefore, the *in vitro* effect on P450 enzymes observed for NN1177 is likely related to its action on glucagon receptors expressed on the hepatocytes. Despite these intriguing *in vitro* findings, all P450 index substrate profiles from a follow-up clinical trial showed PK properties inconsistent with P450 downregulation following NN1177 exposure. Omeprazole, caffeine, and dextromethorphan all displayed decreases in AUC_{0-inf} and C_{max} following NN1177 administration compared with cocktail alone, whereas midazolam and S-warfarin showed no relevant 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 P450s *in vitro* (Iber et al., 2001; Woolsey et al., 2016), prompting us to explore the potential that NN1177 regulates major P450 enzymes. We observed downregulation for all three P450 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 P450 enzymes by binding to their respective receptor to mediate intracellular downstream signaling that regulates the nuclear receptors relevant for P450 expression (e.g., PXR) (Iber et al., 2001; Woolsey et al., 2016). Experiments delineating the molecular mechanism by which NN1177 regulates P450 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 upregulation) during the incubation remains unexplored.

The obtained *in vitro* data were implemented into a PBPK model, and safety concerns were identified for comedications, particularly those metabolized by CYP3A4. Several statins are known to be sensitive CYP3A4 substrate in the clinic (<https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers>), 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 coadministration 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* P450 downregulation to a clinical setting by a therapeutic peptide is not clear; and finally, this study design enables the simultaneous assessment of multiple P450 enzymes. Five different P450 enzymes were evaluated using the Coopers-town 5 + 1 cocktail (Tran et al., 2016), a well established and validated tool to assess the effects on major P450 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 downregulation 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 twofold 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_{max}). Understanding the discrepancy between *in vitro* observations, PBPK modeling, and clinical findings reported here is challenging since there are likely multiple factors involved. For example, trends observed in PK profiles (decreased C_{max} and later T_{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 coadministration. Omeprazole is acid labile and therefore administered in a delayed-release capsule that contains enteric-coated gelatin 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/m²). 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-metabolizing enzymes (Lammers et al., 2020). It is unknown if the reported short-term starvation effect on drug-metabolizing 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 coagonists. Moreover, the PBPK model built here only included one impacting factor (P450 downregulation) 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 Preclinical 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 P450 enzymes (Kim and Novak, 2007), but traditional *in vitro* models do not account for such responsive feedback mechanisms that take place in whole organisms. Utilizing 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 P450s have been deleted and replaced with major human drug-metabolizing P450 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 preclinical 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* P450 downregulation by a peptide with PBPK modeling 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 preclinical studies with established *in vitro* systems that are gold standards for small molecules. These assessments and subsequent conclusions drawn from PBPK modeling 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 preclinical 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

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Performed data analysis: Säll, Alifrangis, Dahl, Friedrichsen, Nygård, Kristensen.

Wrote or contributed to the writing of the manuscript: Säll, Alifrangis, Kristensen.

References

- Abdel-Razzak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin B, Beaune P, and Guillouzo A (1993) Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* **44**:707–715.
- Bækdal TA, Borregaard J, Hansen CW, Thomsen M, and Anderson TW (2019) Effect of Oral Semaglutide on the Pharmacokinetics of Lisinopril, Warfarin, Digoxin, and Metformin in Healthy Subjects. *Clin Pharmacokinet* **58**:1193–1203.
- Czerwinski M, Amunom I, Piryatinsky V, Hallak H, Sahly Y, Bar-Ilan O, Bolliger P, and Bas-san M (2018) Direct and cytokine-mediated effects of albumin-fused growth hormone, TV-1106, on CYP enzyme expression in human hepatocytes *in vitro*. *Pharmacol Res Perspect* **6**:e00397.
- D'Aloisio V, Dognini P, Hutcheon GA, and Coxon CR (2021) PepTherDia: database and structural composition analysis of approved peptide therapeutics and diagnostics. *Drug Discov Today* **26**:1409–1419.
- Dickmann LJ, Patel SK, Rock DA, Wienkers LC, and Slatter JG (2011) Effects of interleukin-6 (IL-6) and an anti-IL-6 monoclonal antibody on drug-metabolizing enzymes in human hepatocyte culture. *Drug Metab Dispos* **39**:1415–1422.
- Dunvald AD, Järvinen E, Mortensen C, and Stage TB (2021) Clinical and Molecular Perspectives on Inflammation-Mediated Regulation of Drug Metabolism and Transport. *Clin Pharmacol Ther*. DOI: 10.1002/cpt.2432 [published ahead of print].
- Food and Drug Administration CaC (2020b) Draft Guidance for Industry. Drug-Drug Interaction Assessment for Therapeutic Proteins: Guidance for Industry.
- Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Böttger J, et al. (2013) Recent advances in 2D and 3D *in vitro* systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol* **87**:1315–1530.
- Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, and Anis AH (2009) The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. *BMC Public Health* **9**:88.
- Hariprasad N, Ramsden D, Palamanda J, Dekeyser JG, Fahmi OA, Kenny JR, Einolf H, Mohutsky M, Pardon M, Siu YA, et al. (2017) Considerations from the IQ Induction Working Group in Response to Drug-Drug Interaction Guidance from Regulatory Agencies: Focus on Downregulation, CYP2C Induction, and CYP2B6 Positive Control. *Drug Metab Dispos* **45**:1049–1059.
- Henderson CJ, Kapelyukh Y, Scheer N, Rode A, McLaren AW, MacLeod AK, Lin D, Wright J, Stanley LA, and Wolf CR (2019) An Extensively Humanized Mouse Model to Predict Pathways of Drug Disposition and Drug/Drug Interactions, and to Facilitate Design of Clinical Trials. *Drug Metab Dispos* **47**:601–615.
- Hendriks DFG, Vorrink SU, Smutny T, Sim SC, Nordling Å, Ullah S, Kumondai M, Jones BC, Johansson I, Andersson TB, et al. (2020) Clinically Relevant Cytochrome P450 3A4 Induction Mechanisms and Drug Screening in Three-Dimensional Spheroid Cultures of Primary Human Hepatocytes. *Clin Pharmacol Ther* **108**:844–855.
- Iber H, Li-Masters T, Chen Q, Yu S, and Morgan ET (2001) Regulation of hepatic cytochrome P450 2C11 via cAMP: implications for down-regulation in diabetes, fasting, and inflammation. *J Pharmacol Exp Ther* **297**:174–180.
- Jonderko G, Golab T, and Jonderko K (1988) A pharmacological dose of glucagon suppresses gastric emptying of a radiolabelled solid meal in humans. *Scand J Clin Lab Invest* **48**:743–746.
- Jordy AB, Albayaty M, Breitschaft A, Anderson TW, Christiansen E, Houshmand-Oregaard A, Manigandan E, and Bækdal TA (2021) Effect of Oral Semaglutide on the Pharmacokinetics of Levonorgestrel and Ethinylestradiol in Healthy Postmenopausal Women and Furosemide and Rosuvastatin in Healthy Subjects. *Clin Pharmacokinet* **60**:1171–1185.
- Kelly T, Yang W, Chen CS, Reynolds K, and He J (2008) Global burden of obesity in 2005 and projections to 2030. *Int J Obes* **32**:1431–1437.
- Kim SK and Novak RF (2007) The role of intracellular signaling in insulin-mediated regulation of drug metabolizing enzyme gene and protein expression. *Pharmacol Ther* **113**: 88–120.
- Krogstad V, Peric A, Robertsen I, Kringen MK, Vistnes M, Hjelmsæth J, Sandbu R, Johnson LK, Angeles PC, Jansson-Löfmark R, et al. (2021) Correlation of Body Weight and Composition With Hepatic Activities of Cytochrome P450 Enzymes. *J Pharm Sci* **110**:432–437.
- Lammers LA, Achterbergh R, Mathôt RAA, and Romijn JA (2020) The effects of fasting on drug metabolism. *Expert Opin Drug Metab Toxicol* **16**:79–85.
- Lammers LA, Achterbergh R, van Schaik RHN, Romijn JA, and Mathôt RAA (2017) Effect of Short-Term Fasting on Systemic Cytochrome P450-Mediated Drug Metabolism in Healthy Subjects: A Randomized, Controlled, Crossover Study Using a Cocktail Approach. *Clin Pharmacokinet* **56**:1231–1244.
- Morgan ET, Sewer MB, Iber H, Gonzalez FJ, Lee YH, Tukey RH, Okino S, Vu T, Chen YH, Sidhu JS, et al. (1998) Physiological and pathophysiological regulation of cytochrome P450. *Drug Metab Dispos* **26**:1232–1240.
- Simonsen L, Lau J, Kruse T, Guo T, McGuire J, Jeppesen JF, Niss K, Sauerberg P, Raun K, and Dornonville de la Cour C (2022) Preclinical evaluation of a protracted GLP-1/glucagon receptor co-agonist: Translational difficulties and pitfalls. *PLoS One* **17**:e0264974.
- Stevison F, Kosaka M, Kenny JR, Wong S, Hogarth C, Amory JK, and Isoherranen N (2019) Does *In Vitro* Cytochrome P450 Downregulation Translate to *In Vivo* Drug-Drug Interactions? Preclinical and Clinical Studies With 13-cis-Retinoic Acid. *Clin Transl Sci* **12**:350–360.
- Than NN and Newsome PN (2015) A concise review of non-alcoholic fatty liver disease. *Atherosclerosis* **239**:192–202.
- Tran JQ, Othman AA, Wolstencroft P, and Elkins J (2016) Therapeutic protein-drug interaction assessment for daclizumab high-yield process in patients with multiple sclerosis using a cocktail approach. *Br J Clin Pharmacol* **82**:160–167.
- Turner PK, Hall SD, Chapman SC, Rehmel JL, Royalty JE, Guo Y, and Kulanthaivel P (2020) Abemaciclib Does Not Have a Clinically Meaningful Effect on Pharmacokinetics of CYP1A2, CYP2C9, CYP2D6, and CYP3A4 Substrates in Patients with Cancer. *Drug Metab Dispos* **48**:796–803.
- Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, and Nauck MA (1996) Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients. *J Clin Endocrinol Metab* **81**:327–332.

Woolsey SJ, Beaton MD, Mansell SE, Leon-Ponte M, Yu J, Pin CL, Adams PC, Kim RB, and Tirona RG (2016) A Fibroblast Growth Factor 21-Pregnane X Receptor Pathway Downregulates Hepatic CYP3A4 in Nonalcoholic Fatty Liver Disease. *Mol Pharmacol* **90**:437-446.

World Medical Association (2013) WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects. Last amended by the 64th WMA General Assembly, Fortaleza, Brazil.

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