SUPPLEMENTAL MATERIALS

Title: In vitro CYP450 enzyme down-regulation by GLP1/glucagon co-agonist does not translate to observed drug-drug interactions in the clinic

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TABLE OF CONTENTS

Supplemental Table S1. Lot information for freshly isolated human hepatocytes used in this study

	H1401	H1403	H1407
Lot information for hepatocytes	XenoTech Lot No. 1401; African American male, age 48 years	XenoTech Lot No. 1403; Caucasian male, age 51 years	XenoTech Lot No. 1407; Caucasian female, age 54 years
Cell viability and morphology	Viability of 88.4%. Treatment with up to 1000 nM NNC9204-1177 exhibited normal hepatocyte morphology.	Viability of 85.0%. Treatment with up to 1000 nM NNC9204-1177 exhibited normal hepatocyte morphology.	Viability of 85.0%. Treatment with up to 1000 nM NNC9204-1177 exhibited normal hepatocyte morphology.
Cytotoxicity by LDH release assay	Treatment with up to 1000 nM NNC9204-1177 had little or no effect (i.e., < 25% increase) on LDH release.	Treatment with up to 1000 nM NNC9204-1177 had little or no effect (i.e., < 25% increase) on LDH release.	Treatment with up to 1000 nM NNC9204-1177 had little or no effect (i.e., < 25% increase) on LDH release.

Supplemental Table S2. Settings for midazolam and caffeine PBPK simulations*

Parameter	Unit	Value Midazolam	Source	Value Caffeine	Source
Is small molecule		Yes	PK-Sim® built-in template	Yes	PK-Sim® built-in template
Molecular Weight	g/mol	325.77		194.20	
Plasma protein binding partner		Albumin		Albumin	
Lipophilicity	Log Units	3.13		-0.07	
Solubility at reference pH	mg/l	0.05		21600.00	
Reference pH		7.00		7.00	
Specific intestinal permeability	dm/min	2.00E-6		3.03E-6	
Permeability	cm/min	0.07		-	
Fraction unbound	%	0.2		0.70	
CYP3A4 k_{cat}	1/min	13.00		-	
CYP3A4 K_m	µmol/l	2.73		-	
CYP3A4 V_{max}	µmol/l/min	0		-	
Volume (kidney)	l	-	-	0.44	-
Blood flow rate (kidney)	l/min	-	-	1.33	-
Plasma clearance	ml/min/kg	-	-	0.01	-

CYP1A2 in vitro V_{max}	pmol/min/mg	-	-	109.00	-
CYP1A2 K_m	µmol/l	-	-	14.70	-
Volume of water/body weight	ml/kg	3.50	-	3.50	-
Dissolution shape for Weibull function	-	Not relevant. Midazolam is given as a solution	-	0.92	Optimized to resemble caffeine PK profile from Machavaram et al. 2019
Dissolution time in Weibull function (50% dissolved)	min		-	20.00	
Use as suspension	-	No	-	Yes	-

*The simulations for PK of NN1177 and for the small molecules were done with the Large molecule simulation environment. As midazolam and caffeine were defined as small molecules but run in Large Molecule simulation setting when co-simulating with NN1177, the Advanced settings in “Compounds” setup were changed to: Diameter 10^{-10} nm, 0 in FcRn affinities, and 0 for Kass for FcRn binding. This way, the small molecules will maintain their distribution according to physicochemical properties without additional contribution from the Large Molecule two-pore formalism governed by molecular size and rescue by FcRn.

Midazolam: The PK-Sim built-in compound template for midazolam was used as the starting point. For the dose administration, a Formulation with properties Dissolved was used as midazolam was administered in a syrup in trial NN9277-4555. The oral dose was 2 mg.

In the built-in template, CYP3A4 was defined as the only CYP that metabolises midazolam with a k_{cat} of 13.00*1/min, and a K_m of 2.73 umol/l.

Caffeine: The PK-Sim built-in compound template for caffeine was used as the starting point. For the dose administration, a Formulation with properties *Tablet* and dissolution via a Weibull function (settings in Table S2) The oral dose was 200 mg. In the built-in template, CYP1A2 was defined as the only CYP that metabolises caffeine with a k_{cat} of 1.01*1/min, and a K_m of 14.70 umol/l.

Supplemental Table S3. Compound specific parameters for NN1177 for PBPK model**

Parameter	Unit	Value NN1177	Source	Organ
Is small molecule		No	Known to be a peptide	-
Molecular Weight	g/mol	4571.07	Calculated	-
Lipophilicity	Log Units	-5.00	Set to be very hydrophilic	-
Solubility at reference pH	mg/l	10000.00	Set to be very soluble	-
P* (interstitial-> intracellular)	cm/min	100.00	Optimized to allow for free transport	Liver Periportal
P* (intracellular-> interstitial)	cm/min	80.00		Liver Periportal
P* (plasma<-> interstitial)	cm/min	100.00		Liver Periportal
P* (interstitial-> intracellular)	cm/min	100.00		Liver Pericentral
P* (intracellular-> interstitial)	cm/min	80.00		Liver Pericentral
P* (plasma<-> interstitial)	cm/min	100.00		Liver Pericentral
K_d (FcRn) endosomal space	µmol/l	6.38	Optimized to NN1177 PK data by PK-Sim optimizer functionality	-
K_d (FcRn) plasma/interstitial	µmol/l	0.21		-
$K_{ass}^{\#}$ (FcRn)	l/µmol/min	0.67	-	-

P*= permeability coefficient; kass[#]= rate constant for association with FcRn

**Being a peptide, NNC9204-1177 (NN1177 above) does not bind the FcRn receptor in a physiological context. However, optimising Kd for FcRn to match the elimination rate observed in trial NN9277-4555 served as a tuning for the endosomal clearance. In the Large molecule implementation in PK-Sim®, FcRn binding serves to protect proteins (e.g., antibodies) from endosomal degradation. For NN1177, the Kd for endosome FcRn was optimised to 6.38 µM (original 6.9 µM). In plasma/interstitial, the optimised value was 0.21 µM (original 6.9 µM). Other settings are listed in the Table S3 above.

For s.c. NN1177 dosing, a PK-Sim *Administration protocol* with the following settings were used: *Administration type*: User Defined, *Target organ*: Skin, *Target compartment*: Interstitial. The doses given were multiplied by 0.67 to account for 67% bioavailability (optimised to PKPD data from trial NN9277-4555). The $t_{½}$ for the first order release rate from the s.c. interstitium was optimised to 0.65 hours (NN9277-4555 trial PK data)

Supplemental Table S4. Cooperstown 5+1 index substrates ("DDI cocktail")

Trial Product	Brand	Dose	Route of Administration	Delivery Device	Instructions
Caffeine	Vivarin	200 mg	Oral	Tablet	One tablet of 200 mg
Warfarin	Coumadin	10 mg	Oral	Tablet	One tablet of 10 mg
Vitamin K	Mephyton	10 mg	Oral	Tablet	Two tablets of 5 mg
Omeprazole	Prilosec	40 mg	Oral	Tablet	Two tablets of 20 mg
Dextro-methorphan	Robitussin	30 mg	Oral	Capsules	Two capsules of 15 mg
Midazolam	Midazolam	2 mg	Oral	Syrup	1 ml of 2 mg/ml syrup

Supplemental Table S5. Mean (n=3) *in vitro* CYP enzyme mRNA and activity following NN1177 exposure

	% Decrease in CYP3A4/5 mRNA / Activity			% Decrease in CYP1A2 mRNA / Activity			% Decrease in CYP2B6 mRNA / Activity		
Test compound	**H1401**	**H1403**	**H1407**	**H1401**	**H1403**	**H1407**	**H1401**	**H1403**	**H1407**
0.1% DMSO	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA
10 nM NN1177	13.9 / NA	12.7 / 0.498	22.4 / 23.6	53.4 / 27.8	5.17 / 13.7	NA / 17.8	6.53 / NA	20.8 / 27.7	24.5 / 39.1
100 nM NN1177	68.3 / 18.5	57.2 / 34.1	71.7 / 51.5	61.8 / 38.2	NA / 5.38	NA / 27.2	50.7 / 27.3	39.3 / 11.6	46.3 / 39.4
1000 nM NNC1177	85.3 / 33.1	73.3 / 50.6	84.5 / 65.6	76.6 / 35.4	7.53 / 20.1	13.3 / 41.3	64.3 / 53.2	50.6 / 37.9	55.7 / 63.4
30 nM Glucagon	86.3 / 27.2	75.7 / 41.6	87.7 / 59.8	78.1 / 38.7	35.8 / 15.1	31.7 / 38.6	45.2 / 32.6	44.2 / 5.57	54.3 / 42.2
25 µM Flumazenil	NA / 6.37	11.0 / 1.55	NA / NA	10.9 / NA	1.57 / 11.2	NA / 7.98	NA / NA	NA / NA	NA / NA
0.1 mM Acetic acid	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA
10 ng/mL IL-6	81.7 / 50.7	85.6 / 66.7	93.5 / 75.2	57.7 / 41.8	42.0 / 46.9	86.2 / 65.1	72.8 / 77.6	52.0 / 62.6	84.7 / 86.3

Supplemental Table S6. Mean (n=3) fold-increase *in vitro* CYP enzyme mRNA and activity for controls

	Fold-Increase in CYP3A4/5 mRNA / Activity			Fold-Increase in CYP1A2 mRNA / Activity			Fold-Increase in CYP2B6 mRNA / Activity		
Test compound	**H1401**	**H1403**	**H1407**	**H1401**	**H1403**	**H1407**	**H1401**	**H1403**	**H1407**
50 µM Omeprazole	NA / NA	NA / NA	NA / NA	42.6 / 48	46.5 / 25.9	135 / 35.5	NA / NA	NA / NA	NA / NA
750 µM Phenobarbital	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	11.8 / 8.04	3.53 / 13.4	13.8 / 20.1
20 µM Rifampin	10.3 / 3.81	12.2 / 8.49	19.4 / 7.68	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA	NA / NA

Supplemental Table S7. AUC_{inf} metabolite/parent ratios before and after NN1177 exposure

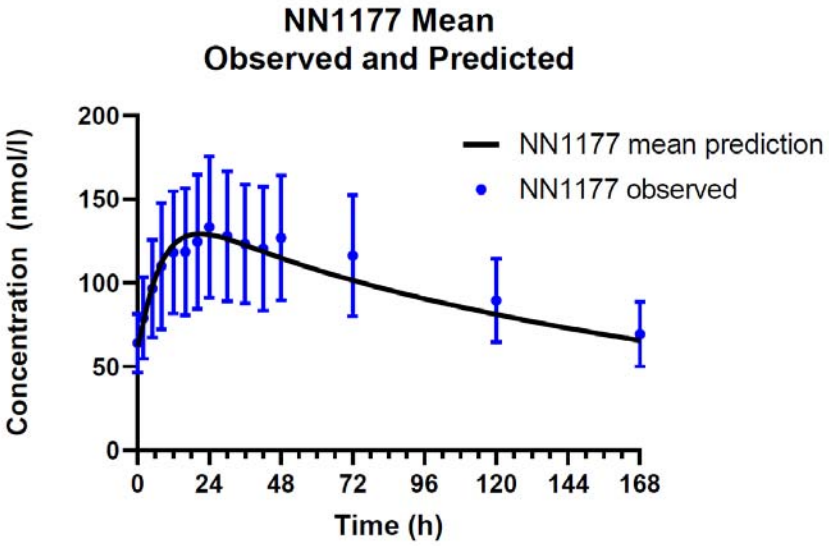
	Alone			+ NN1177		
Metabolite/Parent	**N**	**Arithmetic Mean (%CV)**	**Range**	**N**	**Arithmetic Mean (%CV)**	**Range**
1-hydroxymidazolam/ Midazolam	44	0.42 (37.3)	0.22-0.92	32	0.349 (28.2)	0.22-0.63
Paraxanthine/ Caffeine	42	0.64 (19.5)	0.4-0.84	31	0.84 (23.0)	0.31-1.24
5-hydroxyomeprazole/ Omeprazole	39	0.73 (67.8)	0.14-2.40	25	0.832 (72.2)	0.13-3.14
Dextrorphan/ Dextromethorphan	14	2.94 (87.8)	0.40-9.94	2	4.24 (-)	3.72-4.75
7-S-hydroxywarfarin/ S-Warfarin	28	0.14 (27.5)	0.08-0.21	25	0.35 (68.5)	0.07-1.02

%CV, percent coefficient of variation

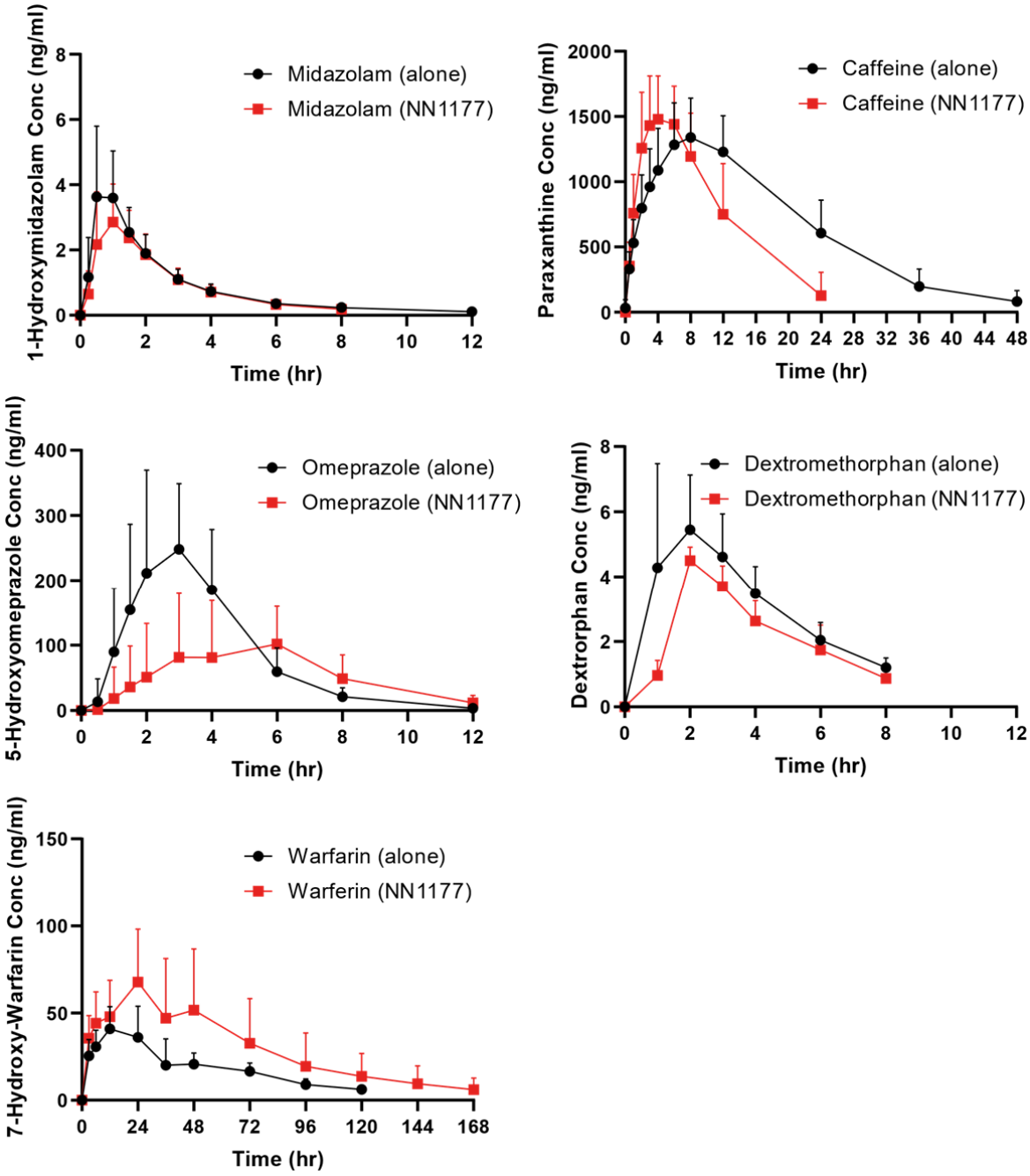
Supplemental Table S8. Change in subject body weight during trial

	Change from baseline to end of NN1177 treatment Mean (SD)	Change from baseline to Follow Up (completers) Mean (SD)
Difference (kg)	-7.8 (3.4)	-4.7 (3.7)
Percent (%)	-10.1 (4.4)	-6.2 (4.8)

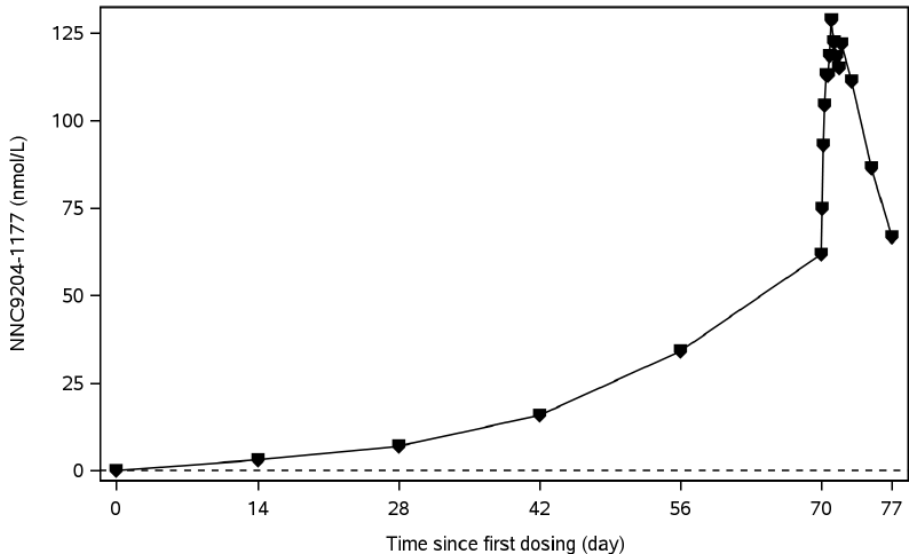
SD, standard deviation; kg, kilograms



Supplemental Figure S1. Observed and predicted NN1177 PK on day 78. Steady state observed and PBPK model predicted concentrations vs time (h; hours) of NN1177 on day 78 (mean; error bars represent standard deviation).



Supplemental Figure S2. Effect of NN1177 on Cooperstown 5+1 metabolite profiles in clinic. Mean PK plasma concentration (Conc) and time profiles for index substrate metabolites 1-hydroxymidazolam, paraxanthine, 5-hydroxyomeprazole, dextrorphan, and 7-hydroxy-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).



Supplemental Figure S3. NN1177 plasma concentration over the course of the trial. Geometric mean plot depicting mean NN1177 (NNC9204-1177) plasma concentrations (nmol/l) from subjects from the start of the trial until 77 days after first dosing (Visit 12; Day 78). Before 70 days after dosing, the plasma concentrations represent trough (pre-dose) concentrations. After this, a PK profile with serial sampling is shown.