Evaluating drug-drug interaction risk associated with peptide analogues using advanced in vitro systems

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**Abbreviations:**

Abstract

Drug-drug interaction (DDI) assessment of therapeutic peptides is an evolving area. The industry generally follows DDI guidelines for small molecules, but the translation of data generated with commonly used in vitro systems to in vivo is sparse. In the current study, we investigated the ability of advanced human hepatocyte in vitro systems namely HepatoPac, spheroids, and Liver-on-a-chip to assess potential changes in regulation of CYP1A2, CYP2B6, CYP3A4, SLCO1B1 and ABCC2 in the presence of selected therapeutic peptides, proteins, and small molecules. The peptide NN1177, a glucagon and GLP-1 receptor co-agonist, did not suppress mRNA expression or activity of CYP1A2, CYP2B6, and CYP3A4 in HepatoPac, spheroids, or Liver-on-a-chip; these findings were in contrast to the data obtained in sandwich cultured hepatocytes. No effect of NN1177 on SLCO1B1 and ABCC2 mRNA was observed in any of the complex systems. The induction magnitude differed across the systems (e.g., rifampicin induction of CYP3A4 mRNA ranged from 2.8-fold in spheroids to 81.2-fold in Liver-on-a-chip). Small molecules, obeticholic acid and abemaciclib, showed varying responses in HepatoPac, spheroids and Liver-on-a-chip, indicating a need for EC₅₀ determinations to fully assess translatability data. HepatoPac, the most extensively investigated in this study (3 donors), showed high potential to investigate DDIs associated with CYP regulation by therapeutic peptides. Spheroids and Liver-on-a-chip were only assessed in one hepatocyte donor and further evaluations are required to confirm their potential. This study establishes an excellent foundation towards the establishment of more clinically-relevant in vitro tools for evaluation of potential DDIs with therapeutic peptides.
Significant statement

At present, there are no guidelines for drug-drug interaction (DDI) assessment of therapeutic peptides. Existing in vitro methods recommended for assessing small molecule DDIs do not appear to translate well for peptide drugs, complicating drug development for these moieties. Here, we establish evidence that complex cellular systems have potential to be used as more clinically-relevant tools for the in vitro DDI evaluation of therapeutic peptides.
Introduction

In vitro studies needed to assess potential drug-drug interactions (DDIs) for therapeutic peptides during drug development remain unclear with no specific guidance currently available from health authorities. In contrast, in vitro to in vivo extrapolation (IVIVE) for small molecule DDI assessments is well-established (European Medicines Agency 2012; United States Food and Drug Administration 2020; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use 2023). Although peptide DDI assessment is not part of the regulatory requirement, many companies in the pharmaceutical industry follow the same procedures used for small molecules (Säll et al., 2023).

Evaluating potential perpetrator effects of therapeutic peptides on cytochrome P450 (CYP) enzymes and transporters expression/activity is important as several biologics have been reported to alter CYP expression/activity in vitro and/or in vivo (Cheung et al., 1996; Liddle et al., 1998; Iber et al., 2001; Jürgens et al., 2002; Woodcroft et al., 2002; Song and Chiang, 2006). For example, the protein FGF-21 suppressed CYP3A4 expression in vitro (Woolsey et al., 2016); glucagon suppressed Cyp2c11 expression in rat hepatocytes in a concentration-dependent manner, with similar trends seen for CYP7A1 in human hepatocytes (Iber et al., 2001; Song and Chiang, 2006); insulin suppressed Cyp2e1 in rat hepatocytes (Woodcroft et al., 2002); growth hormone (GH) induced multiple CYP enzymes in vitro, with a 910% increase in CYP3A4 mRNA in plated human hepatocytes (Liddle et al., 1998). However, treatment with GH has clinically indicated induction of CYPs in GH-deficient adult but in healthy men GH only resulted in induction of CYP1A2 and not CYP3A4 (Cheung et al., 1996; Jürgens et al., 2002). Besides these, several proinflammatory cytokines, such as interleukin-6 (IL-6), are known to suppress CYP expression with clinical implications (Coustant et al., 2022; Dunvald et al., 2022; Gatti and Pea, 2022).
Recently, we published *in vitro* and clinical DDI assessments for the peptide NN1177, a linear 29-amino acid synthetic peptide (~4.57 kDa) co-agonist for the glucagon and the glucagon-like peptide-1 receptors (Säll et al., 2022). This co-agonist suppressed CYP2B6 and CYP3A4 in a dose-dependent manner *in vitro* using freshly isolated primary human hepatocytes from three donors in a sandwich culture (a standard method recommended in DDI guidelines for small molecule drugs). CYP1A2 was also suppressed in one donor. However, a follow-up clinical DDI trial showed no effect on the pharmacokinetic (PK) profile of midazolam (CYP3A4 substrate), while the area under the curve (AUC) of caffeine (CYP1A2 substrate) was decreased after NN1177 treatment (Säll et al., 2022). These findings highlight the disconnect between the clinical outcome and *in vitro* results obtained while following standard DDI guidelines for small molecule drugs and challenges in translation of *in vitro* CYP suppression data to *in vivo*. Similarly, the small molecule abemaciclib suppressed the mRNA for CYP3A4, CYP1A2, and CYP2B6, but no clinically relevant effects were observed (Turner et al., 2020). Moreover, obeticholic acid (OCA), suppressed CYP1A2 and CYP3A4 *in vitro*, but a clinical DDI trial with OCA showed no effect on midazolam (CYP3A4 substrate) (Edwards et al., 2017; Ishida et al., 2019) and while caffeine AUC did increase the AUC of the CYP1A2-mediated major metabolite of caffeine did not (Edwards et al., 2017; Ishida et al., 2019).

Current evidence of IVIVE for CYP suppression data is limited and molecular mechanisms behind suppression are often unclear (European Medicines Agency 2012; Hariparsad et al., 2017; United States Food and Drug Administration 2020). In a survey performed by the IQ consortium, 16/17 respondents had observed downregulation in routine CYP induction studies. However, only three companies performed follow-up clinical studies of *in vitro* down-regulation, and none showed any clinical significance (Hariparsad et al., 2017).
current in vitro systems used for the evaluation of DDIs, such as plated human hepatocytes in a monolayer or in a sandwich culture, clearly do not translate well for CYP suppression (Hariparsad et al., 2017). A potential reason for this lack of translation could be related to low CYP baseline expression levels in sandwich cultured human hepatocytes. Accordingly, human hepatocytes rapidly de-differentiate in sandwich culture, which could affect their potential to correctly detect changes in CYP regulation caused by indirect mechanisms, which are the expected effect of peptides/proteins (Bell et al., 2016). Maintaining native expression profiles is important for in vitro systems to provide data for informative IVIVE (Hariparsad et al., 2017). This was shown by Hendriks et al., where the small molecule AZD1208 was only identified correctly as an CYP3A4 inducer through a complex pathway in 3D spheroids of hepatocytes, but not in 2D sandwich culture of human hepatocytes (SCHH) (Hendriks et al., 2020).

Novel culture formats of human hepatocytes have emerged in recent years. The current study investigated three such systems: (1) HepatoPac, where human hepatocytes are cultured in islands and then co-cultured with stromal cells (Khetani and Bhatia, 2008); (2) 3D spheroids, where hepatocytes in ultra-low adherent plates self-assemble into spheroids (Bell et al., 2016); and (3) a microphysiological Liver-on-a-chip system, where human hepatocytes are seeded in a scaffold with continuous perfusion of the cells (Rubiano et al., 2021; Docci et al., 2022). All these are long-term culture systems that retain hepatocyte identity and more stable CYP expression compared to SCHH throughout culture time (Khetani and Bhatia, 2008; Bell et al., 2016; Rubiano et al., 2021). Similar is expected for transporters, but the experimental evidence is, so far, sparse.

In the current study, nine drugs (2 small molecules, 5 peptides and 2 proteins) were initially screened, as perpetrators, for their potential to regulate the expression CYP enzymes
(CYP3A4, CYP1A2 and CYP2B6) and the transporters SLCO1B1 (OATP1B1) and ABCC2 (MRP2) in SCHH. Subsequently, peptide NN1177, protein rFGF-19 as well as small molecules, OCA and abemaciclib, were selected for further evaluation in HepatoPac, spheroids, and Liver-on-a-chip. This work aims to systematically evaluate different complex and more physiologically-relevant in vitro systems for their ability to investigate CYP and transporter regulation and improve the in vitro prediction of potential clinical DDI risks associated with therapeutic peptides.
Materials and Methods

Cryopreserved primary human hepatocytes were purchased from BioIVT (Westbury, NY) (donor QNT, BGF, and IVL). Donor demographic information (ethnicity, gender, and age) is listed in Table S1. Fetal bovine serum, Glycogen RNA grade, Insulin-Transferrin-Selenium (ITS -G), dexamethasone Nunclon™ Sphera™ 96-Well plates, ATP solution, Hepatocyte culture media (Williams E medium w/o phenol red), plating supplement A kit, maintenance supplement B kit and cryopreserved hepatocyte recovery medium (CHRM Medium) were purchased from Thermo Fisher Scientific (Waltham, MA). Transferrin, hydrocortisone, Lin-oleic acid, D-(-)-Glucose (45%), Penicillin-Streptomycin, 30% BSA solution, Isopropanolol, fatty acid-free BSA, BSA, Sodium Selenite, human recombinant FGF-19 were purchased from sigma-aldrich (St. Louis, MO). GlutaMAX Supplements were purchased from Thermo Fisher Scientific or from Life Technologies (Bleiswijk, Netherlands). Phosphate-buffered saline, Williams E medium w/o phenol red and glucose, Heps,TRIzol reagent, PenStrep, and FBS were acquired from Life technologies. Sterile filtered DMSO was purchased from R&D systems. Acetic acid was purchased from Biosolve Chimie (Dieuze, France). BD Matrigel™ basement membrane matrix was purchased from Corning B.V. (Glendale, AZ). 48-well plate were either coated in-house with rat tail collagen type I (Corning B.V.) or purchased pre-coated from Corning B.V. Phenacetin, acetaminophen, testosterone, 6β-hydroxytestosterone, acetaminophen-(ring-d4), omeprazole, phenobarbital, rifampicin, flumazenil and mercaptoethanol were purchased from Merck (Kenilworth, NJ). (±) Hydroxybupropion, (±)-hydroxybupropion-D6 and 6β-hydroxytestosterone-D3 solutions were obtained from Cerilliant (Merck). Phenobarbital was provided by the Hospital Pharmacy at Odense University Hospital, Odense. Bupropion hydrochloride were from either Merck or Toronto Research Chemical. Recombinant human IL-6 was purchased from Peprotech EC.
Ltd. (London, UK). Human native glucagon, GLP-1 and glucagon co-agonist analogue (NN1177), Gastric inhibitory polypeptide (GIP) analogue (NN0194), amylin analogue (NN1213), FGF-21 analogue (NN0119) and human GH (Norditropin® (somatropin)) were procured internally at Novo Nordisk. OCA and abemaciclib mesylate were purchased from Abcam plc (Cambridge, UK) and SCBT (Dallas, TX), respectively. RNA isolation kits: RNeasy mini kit and RNeasy micro kit, Qiazol, and RNase-Free DNase kit were procured from Qiagen (Hilden, Germany) and NucleoSpin RNA, Mini kit was from Macherey-Nagel (Duren, Germany). iScript cDNA synthesis kit was procured from Bio-Rad (Hercules, CA) and cDNA synthesis kit from Thermo Fisher scientific. TaqMan™ fast advanced master mix and TaqMan™ gene expression assays (Table S2) were procured from ThermoFisher scientific.

**Test compounds and controls**

The compounds OCA (0.1 and 1.0 µM), abemaciclib (0.5 and 5.0 µM), native glucagon (30 nM), NN1177 (100 and 1000 nM), Amylin analogue (0.6 and 6.0 µM), GIP analogue (0.72 and 7.2 µM), recombinant FGF-19 (80 and 800 ng/mL), FGF-21 analogue (0.236 and 2.36 µM), and GH (0.88 and 8.8 µg/mL) were initially tested in SCHH and OCA, abemaciclib, NN1177, and FGF-19 was afterwards tested using HepatoPac, Liver-on-a-chip liver chip, and spheroids of hepatocytes, at the same concentrations. The concentrations of the compounds tested were based on the expected or the reported clinically relevant total human plasma steady state Cmax concentrations and 10-fold Cmax steady state (Rossi et al., 2014; US Food and Drug Administration 2015; Patnaik et al., 2016; Säll et al., 2022).
As positive controls, omeprazole (50 µM) was used for aryl hydrocarbon receptor (AhR)–mediated induction of CYP1A2, phenobarbital (750 µM) constitutively active receptor (CAR)–mediated induction of CYP2B6, and rifampicin (20 µM) was the positive control for pregnane X receptor (PXR)–mediated induction of the CYP3A4. IL-6 at 10 ng/mL was used as a potential positive control for suppression of CYP3A4, CYP1A2 and CYP2B6. Relevant vehicle controls (glucagon, Flumazenil, Rifampicin, Omeprazole, Phenobarbital, Amylin analogue, GIP analogue, OCA, and abemaciclib where dissolved in DMSO, GH and FGF-21 where in formulation buffer, FGF-19 in PBS with 0.1 % BSA, IL-6 in 10 mM acetic acid, and NN1177 where in incubation media with 0.1 % DMSO), were used for each compound and the concentration DMSO in the culture media did not exceed 0.1%.

Sandwich-cultured human hepatocyte preparation and treatment

Hepatocytes (donor IVL and QNT) were thawed quickly and resuspended in CHRM media. Cell suspension was centrifuged at 95g for 15 minutes. Supernatant was discarded carefully, and cell pellet was resuspended in warm (37 °C) hepatocyte platting medium. Before seeding, cell viability was estimated using the Trypan blue dye exclusion test. The human hepatocyte cell density was adjusted to 0.7 to 1 million viable cells per ml of the hepatocyte platting medium (William’s E medium w/o phenol red, 5% FBS, cocktail A (1% PenStrep, 4 µg/mL Insulin, 2mM GlutaMAX™, and 15 mM HEPES) and 1 µM dexamethasone). The hepatocytes were then seeded onto collagen I–coated 48-well plates at ~0.2 million viable cells per well 200 µl of the medium. Cells were cultured in a 37°C incubator with 5% CO₂ and 95% relative humidity. 4-6 h after seeding, plating efficiency was evaluated by visual inspection under microscope and cells were washed once with platting medium to clear dead and unattached cells. Afterwards, cells were overlaid with cold maintenance (William’s E
medium w/o phenol red, cocktail B (0.5% PenStrep, 6.25 µg/mL Insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenous acid, 1.25 mg/mL BSA, 5.35 µg/mL linoleic acid, 2mM GlutaMAX™, and 15 mM HEPES) and 0.1 µM dexamethasone) supplemented with 0.25 mg/mL matrigel and the plate was placed in the incubator immediately. After overnight settling, the cells were treated daily for 3 days (total 72 hours) with test compounds or control compounds. Cell morphology was evaluated visually at each media change. For assessment of viability, a lactate dehydrogenase release assay was used for all conditions, following the procedure of the manufacture (Roche kit supplied by Merck). After 72 hours treatment the activity of CYP1A2, CYP2B6, and CYP3A4 were measured using phenacetin, bupropion, and testosterone, respectively. The cells were initially treated with 100 µM phenacetin for 1 hour and then washed with medium, and then treated with a cocktail of 500 µM bupropion and 200 µM testosterone for 1 hour. After activity assay, the cells were lysed using the Qiagen RNeasy kit with the addition of beta-mercaptoethanol in the lysis buffer and stored at -80 °C. The RNA isolation followed the protocol of the manufacture.

**HepatoPac**

HepatoPac was used in a 24 well format with 21,000 hepatocytes per well, and donor BGF, IVL, and QNT were used, as well as a stromal-only plates as control plates. The cells were seeded at BioIVT (Westbury) and shipped 5-6 days after seeding. The cells arrived 2-4 days after shipment and the medium was change immediately upon arrival with the supplied maintenance medium. The cells were then kept in an incubator at 37°C with 10% CO2 and 95% relative humidity. The medium was changed again 1-2 days after arrival with maintenance medium and the experiment was started at day 12 after seeding of hepatocytes. The experiment was initiated by changing the media with the supplied application medium sup-
plemented with 1.25 mg/mL BSA with an equilibration period of 1-2 hours before treatment. After the equilibration period, the cells were treated daily for 3 days (total 72 hours) with test compounds and control compounds (total volume: 400 µl). The cells were visually inspected upon each media change. After treatment, the activity of CYP1A2, CYP2B6, and CYP3A4 were measured using phenacetin, bupropion, and testosterone, respectively, in application medium supplied with 1.25 mg/mL BSA. The cells were initially treated with 100 µM phenacetin for 1 hour and then washed with medium, and then treated with a cocktail of 500 µM bupropion and 200 µM testosterone for 1 hour. Before the experiments, the linearity of the metabolite formation was ensured. The rate of metabolite formation was measured using LC-MS/MS. After the activity assay, cells were lysed with 350 µl RLT buffer containing 10 µl/ml mercaptoethanol. Lysed cells were immediately kept at -80 °C. RNA isolation using Qiagen RNeasy kit or Macherey-Nagel NucleoSpin RNA purification, and the procedure was according to the manufacture's protocol.

3D spheroids of hepatocytes

Donor BGF was used for formation of hepatocyte 3D spheroids. Hepatocytes were thawed quickly and resuspended in CHRM media. Cell suspension was centrifuged at 100g for 10 minutes. Before seeding, cell viability was estimated using Trypan blue dye. The cells were diluted with plating media medium (William’s E medium w/o phenol red, 5% FBS, cocktail A (1% PenStrep, 4 µg/mL Insulin, 2 mM GlutaMAX™, and 15 mM HEPES) and 1 µM dexamethasone) and for each well 1.500 cells in 100 µL were transferred to an ultra-low attachment 96-well plates. The plates were afterwards shortly centrifuged for 2 min at 200 g and placed in a 37°C incubator with 5% CO2 and 95% relative humidity for 5 days to let the hepatocyte self-assemble into spheroids. At day 5, 6, and 7 the media was changed with
maintenance media (William’s E medium without phenol red, 1.25 mg/mL BSA, 0.1 µM dexamethasone, 100 units of penicillin, 0.1 mg/mL streptomycin, 1.72 µM insulin, 68.75 nM transferrin, 3.87 nM sodium selenite, and 2 mM GlutaMAX). At day 8 after cell seeding, the cells were treated daily for 3 days (total 72 hours) with test compounds and controls (total volume: 100 µl). Triplicate samples of 16 spheroids were treated for each RNA isolation, and triplicate samples of single spheroids were treated for the activity of CYP1A2 and of CYP2B6/CYP3A4, respectively. After treatment, 16 spheroids per replicate were collected and lysed using Qiazol and stored for later RNA isolation at -80 °C. Simultaneous spheroids of each treatment were added an activity solution of either 100 µM phenacetin or of 500 µM bupropion and 200 µM testosterone. The activity assays were then terminated after either 2, 8, and 24 hours, where 8 hours was used for further analysis (Figure S10). RNA isolation from spheroids was performed with phenol-chloroform extraction method (Qiazol) that included glycogen as RNA co-precipitant. The viability of the 3D spheroids was assessed after thawing, during cultivation and after treatment based on the quantification of ATP using the CellTiter-Glo® Cell viability assay following the protocol of the manufacture (Promega).

Liver-on-a-chip

Donor BGF was used for the PhysioMimix Liver-on-a-chip™ MPS-LC12 system (CN Bio Innovations). The plates were, the day before cell seeding, primed with seeding media prepared with William’s E media without glucose and phenol red, supplemented with 15 mM HEPES, 2 mM GlutaMax, 5.5 mM D-(+)-glucose, 200 pM insulin, 100 nM hydrocortisone, 5% FBS, and 1% PenStrep, using the designated priming program in an incubator at 37°C and with 5% CO2 and 95% relative humidity. The day after priming, hepatocytes were thawed quickly and resuspended in CHRM media. Cell suspension was centrifuged at 100g
for 10 minutes. Before seeding, the cell viability was estimated using a NucleoCounter (Chemometec). 0.6 million viable hepatocytes were added to each well with a total volume of 1600 µL and the seeding program was run. 1 day after seeding, the media was changed to maintenance media prepared with William’s E media without glucose and phenol red, supplemented with 15 mM HEPES, 2 mM GlutaMax, 5.5 mM D-(−)-glucose, 200 pM insulin, 100 nM hydrocortisone. 1.25 mg/mL fatty-acid free BSA, 6.25 ng/mL sodium selenite, 6.25 µg/ml transferrin, 20 µM linoleic Acid, 0.5% PenStrep. The media was subsequently changed on day 4, 6, and 8 after seeding using the designated media change program. At day 11 after cell seeding, the cells were treated daily for 3 days (total 72 hours) with test compounds and controls (total volume: 1600 µl). The amount of secreted human albumin present in the media, was used to assess the viability and activity of the hepatocytes. After treatment of compounds for 72 hours the activity of CYP1A2, CYP2B6, and CYP3A4 were measured using phenacetin, bupropion, and testosterone, respectively, in maintenance medium. The cells were initially treated with 100 µM phenacetin for 1 hour and then washed with medium, and then treated with a cocktail of 500 µM bupropion and 200 µM testosterone for 1 hour. Before the experiment the linearity of the metabolite formation was ensured. The rate of metabolite formation was measured using LC-MS/MS. After the activity assay, the cell-containing scaffolds were transferred to tubes with trizol and prefilled with 0.1 mm zirconium beads (Merck). The cells were then homogenized/detached with a Bed Bug microtube homogenizer and the supernatant transferred to -80 °C. RNA isolation was performed using Direct-zol RNA Microprep kit (Zymo Research) following the protocol of the manufacture.

During each media change, media was stored at -80 °C. After the end of the experiment the albumin content in the spent media was analyses. The amount of albumin present was
measured using the Human Serum Albumin DuoSet ELISA and DuoSet ELISA Ancillary Reagent Kit 2, following the protocol of the manufacture (R&D Systems).

RT-qPCR and analysis

Isolated RNA from all the in vitro systems was reverse transcribed, and the prepared cDNA was used for qPCR. TaqMan Fast Universal PCR Master Mix was used for the qPCR reaction together with the TaqMan assays CYP1A2, CYP2B6, CYP3A4, CYP7A1, SLCO1B1, GAPDH, RPLP0, ABCC2, and HPRT1 (Table S2). The PCR reaction was done using QuantStudio 12K Flex system (Applied biosystems) and the data was analysed using R-studio using the delta-delta ct method. For Hepatopac the stromal-only cells did not give a ct-value with the used TaqMan assays.

LC-MS/MS

For each activity assay, after the incubation of phenacetin, and bupropion and testosterone, samples were withdrawn and mixed with 1:1 ice-cold acetonitrile containing the internal standards acetaminophen-(ring-d4) or hydroxybupropion-D6 and 6β-Hydroxytestosterone-D3, respectively. The samples were then stored at -20 °C until analysis. The analysis was performed with a ACQUITY UPLC® HHS T3 1.8 mm 2.1 x 100 mm Column at a column temperature of 40°C. A gradient mobile phase system was used consisting of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). The flow rate was 0.4 mL/min. Analysis was performed on a SYNAPT G2 (Waters), hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer with positive electrospray ionization mode using multiple reaction monitoring (MRM) (Table S3). The response of the
formed metabolites acetaminophen (CYP1A2), hydroxybupropion (CYP2B6), and 6β-hydroxytestosterone (CYP3A4) were compared to a standard curve of each respective metabolite, and the respective control were subtracted. For the Hepatopac samples 90% of the cell were stromal cells, therefore 90% of the formation of the metabolites in stromal-only cells were subtracted for each test compound and each metabolite respectively.
Results

Initial screening in sandwich cultured human hepatocytes

Initial screening of 5 peptides (NN1177, native glucagon, GH, amylin analogue, GIP analogue), 2 proteins (recombinant FGF-19 and FGF-21-analogue) and two small molecules (abemaciclib and OCA) was performed in SCHH (2 donors). The regulation of CYP1A2, CYP2B6, and CYP3A4 by different compounds is shown in Figure 1. Lactate dehydrogenase (LDH) release was measured after treatment with each compound (Figure S1) and indicated no cytotoxicity. Control inducers (e.g., rifampicin for CYP3A4) was noted for all enzymes/donors behaved as expected (Figure S2).

No relevant or concentration-dependent effects on CYP1A2 expression were observed in SCHH treated with NN1177, glucagon, FGF-21 analogue, amylin analogue, GIP analogue, or GH. The exception was FGF-19 which induced CYP1A2 by 2.8-fold at 800 ng/mL (donor IVL), whereas abemaciclib suppressed CYP1A2 at the highest concentration with a decrease of 0.26-fold. OCA also showed slight CYP1A2 suppression in donor IVL at the highest concentration (1.0 µM: 0.41-fold). No relevant effects on CYP2B6 expression were observed for FGF-19, glucagon, OCA, FGF-21 analogue, amylin analogue, GIP analogue, or GH. NN1177 increased the expression of CYP2B6 by 3.4-fold in donor IVL at both concentrations investigated. Abemaciclib slightly suppressed CYP2B6 with 0.46 and 0.51-fold in donor QNT and IVL, respectively. OCA, NN1177, FGF-19, and glucagon attenuated expression of CYP3A4 below 0.5-fold in a concentration-dependent manner in both donors. GH slightly up-regulated CYP3A4 for both tested concentrations, whereas no relevant effect was observed for abemaciclib, amylin analogue, GIP analogue, or FGF-21
analogue. No relevant effects were observed on \textit{SLCO1B1} (OATP1B1) expression by any proteins or small molecules investigated (Figure S3). However, \textit{CYP7A1} expression was attenuated by OCA, glucagon, NN1177, and FGF-19 in a concentration-dependent manner (Figure S3), and the GIP analogue suppressed CYP7A1 at both concentrations in donor QNT. Based on these results and available clinical DDI data (Edwards et al., 2017; Turner et al., 2020; Säll et al., 2022), four compounds (NN1177, FGF-19, OCA, and abemaciclib) were selected for further testing in more advanced \textit{in vitro} systems.

\textit{General evaluation of CYP regulation in complex \textit{in vitro} systems}

Induction and suppression potential were investigated in HepatoPac, 3D spheroids, and Liver-on-a-chip using known inducers of CYP1A2, CYP2B6, and CYP3A4 (omeprazole, phenobarbital, and rifampicin, receptively). IL-6 was used as a potential suppression control. HepatoPac studies were performed using 3 donors (IVL, QNT and BGF), whereas spheroids and Liver-on-a-chip were performed in donor BGF (Figure 2). Due to specific requirements (QNT could not form spheroids) of these advanced systems, it was only possible to have one common hepatocyte donor (BGF) in all three systems.

In all the \textit{in vitro} systems, the induction controls responded with an increase in mRNA and activity of the CYP enzyme induced. However, the magnitude of the response differed substantially across the different systems (Figure 2). The induction of CYP1A2 by omeprazole showed the following rank order Liver-on-a-chip (40.6-fold) > spheroids (13.5-fold) > HepatoPac (8.9-fold) at the mRNA level, whereas at the activity level order was spheroids (18.7-fold) > Liver-on-a-chip (12.1-fold) > HepatoPac (3.6-fold). CYP2B6 was induced by phenobarbital at the mRNA level in the order HepatoPac (11.2-fold) > spheroids...
(5.0-fold) > Liver-on-a-chip (4.0-fold) and for activity was HepatoPac (9.2-fold) > Liver-on-a-chip (3.5-fold) > spheroids (2.5-fold). Rifampicin induced CYP3A4 in the order Liver-on-a-chip (81.2-fold mRNA and 20.0-fold activity) > HepatoPac (4.1-fold mRNA and 6.5-fold activity) > spheroids (2.8-fold mRNA and 2.0-fold activity). IL-6 did not have a relevant suppression effect (>0.5-fold) on the expression of CYP1A2, CYP2B6, or CYP3A4 at the mRNA or activity level in spheroids. Similar observations were made in the Liver-on-a-chip system where only CYP2B6 activity was 0.47-fold suppressed and CYP1A2 mRNA was 2.9-fold induced. In HepatoPac, IL-6 did suppress mRNA and activity of CYP1A2 and CYP2B6 in donor QNT (0.20-fold mRNA and 0.20-fold activity, and 0.13-fold mRNA and 0.27-fold activity, respectively), but not below 0.5-fold in donors IVL or BGF. For CYP3A4, IL-6 suppressed the mRNA in all donors (QNT: 0.07-fold, IVL: 0.42-fold, and BGF: 0.19-fold) but the activity was suppressed below 0.5-fold cut-off only in donor QNT (0.23-fold).

The absolute rate of the metabolite formation in each system, donor and CYP enzyme is depicted in Table 1. The absolute activity is in a similar range for all donors in HepatoPac with one exception: the donor BGF showed approximately half the activity for CYP3A4 and CYP1A2 compared to the other donors. The order of activity is the same for all donors: CYP3A4 > CYP1A2 > CYP2B6. A similar order is observed for spheroids with approximately the same magnitude of difference in activity between the CYP enzymes. For Liver-on-a-chip, a different order of activity was observed: CYP1A2 > CYP3A4 > CYP2B6. Comparing the activity across systems and assuming no loss of cells after seeding, then the metabolite formation rate is in order: HepatoPac > Spheroids > Liver-on-a-chip. The number of cells in Liver-on-a-chip was not determined and it has previously been shown that the seeding efficiency is around 50% (Docci et al., 2022). Considering seeding efficiency, Liv-
er-on-a-chip still had far less activity per million hepatocytes of all investigated CYP enzymes compared to the other systems. Generally, the fold change in activity and mRNA in the presence of NN1177, FGF-19, OCA, and abemaciclib correlated well for all in vitro systems investigated (Figure S9)

The effect of NN1177, FGF-19, obeticholic acid, and abemaciclib on CYP1A2, CYP2B6, and CYP3A4 in complex in vitro systems

The fold change in mRNA and activity data obtained in HepatoPac, spheroids, and Liver-on-a-chip is shown for CYP1A2 (Figure 3), CYP2B6 (Figure 4), and CYP3A4 (Figure 5). Changes in mRNA expression of CYP7A1, SLCO1B1, and ABCC2 are shown in Figure S4, Figure S5, and Figure S6, respectively. Furthermore, the normalized base mRNA expression of the investigated genes is shown in Figure S11. The ATP content in spheroids after each treatment was in the range of the media control for all samples except 5.0 µM abemaciclib (Figure S7). For 5.0 µM abemaciclib, there was no ATP content detected in the spheroids and visual inspection showed poor condition. CYP1A2, CYP2B6, and CYP3A4 activity/mRNA were low / not detected following treatment of 5.0 µM abemaciclib in the spheroids. In HepatoPac, 5.0 µM abemaciclib resulted in a high number of large vacuoles (data not shown). The human albumin secreted by hepatocytes to the media was used as an integrity control for Liver-on-a-chip and showed that all treatments except abemaciclib and FGF-19 had a similar albumin production of approximately 1 µg/day after the last day of treatment (Figure S8). In the case of abemaciclib and FGF-19, a decrease in albumin concentration in the media was observed compared to the respective control.
OCA suppressed in a concentration-dependent manner the mRNA expression and activity of CYP1A2, CYP2B6, and CYP3A4 in HepatoPac (1.0 µM, mRNA: 0.35, 0.30, and 0.17-fold, respectively) and in Liver-on-a-chip (1.0 µM, mRNA: 0.53, 0.38, and 0.46-fold, respectively), but not in spheroids. Notably, the expression of CYP7A1 was highly suppressed by OCA in HepatoPac (0.1 and 1.0 µM: 0.04 and 0.02-fold, respectively) and in Liver-on-a-chip (0.1 and 1.0 µM: 0.41 and 0.03-fold, respectively), in contrast to spheroids (Figure S4). OCA showed no relevant effect on ABCC2 (MRP2) expression and only a minor suppression of SLCO1B1 was observed in HepatoPac at 1.0 µM (0.46-fold).

Overall, abemaciclib caused no relevant effects at 0.5 µM at either the mRNA or activity level for CYP1A2, CYP2B6, CYP3A4, CYP7A1, SLCO1B1, and ABCC2 in HepatoPac, spheroids, or Liver-on-a-chip, except for the activity of CYP2B6 (0.39-fold of activity) and mRNA of CYP1A2 (2.4-fold) and CYP3A4 (3.7-fold) in Liver-on-a-chip. In individual donors, QNT in HepatoPac, abemaciclib did suppress mRNA/activity below 0.5-fold for CYP1A2 (0.35-fold mRNA and 0.37-fold activity), CYP2B6 (0.43-fold mRNA), CYP3A4 (0.33-fold mRNA), SLCO1B1 (0.37-fold mRNA), and CYP7A1 (0.26-fold mRNA) (also mRNA in donor BGF of 0.39-fold), and the activity in donor BGF was suppressed for CYP2B6 (0.35-fold) and CYP3A4 (0.37-fold). 5.0 µM abemaciclib caused more than 0.5-fold suppression of both mRNA and activity of all CYPs in HepatoPac, excepted in donor BGF in HepatoPac with 0.54-fold. In the case of CYP1A2 and CYP2B6, no activity was observed in the spheroids at this high concentration of abemaciclib. This finding agrees with the observed formation of vacuoles in HepatoPac and absence of ATP content in these spheroids. CYPs in Liver-on-a-chip were not suppressed by this high concentration of abemaciclib, except the activity of CYP2B6 (0.43-fold), and induction was found for CYP1A2 (3.0-fold) and CYP3A4 (4.74-fold) mRNA.
The mRNA expression and activity of CYP1A2, CYP2B6, and CYP3A4 were not affected by NN1177 in spheroids and HepatoPac, except for donor QNT in HepatoPac where the expression of CYP2B6 mRNA at 1000 nM was changed 0.36-fold. No suppression was observed in Liver-on-a-chip, but CYP2B6 activity was induced 2.17-fold at 100 nM, and CYP3A4 was also induced at 100 nM (mRNA 2.82-fold) and at 1000 nM (mRNA = 3.80-fold and activity = 2.16-fold). CYP7A1 mRNA was slightly suppressed only in spheroids at 1000 nM (0.39-fold), but no relevant effects were seen in any of the in vitro systems on SLCO1B1 or ABCC2 mRNA expression.

FGF-19 did not have any relevant effect on the mRNA expression or activity of any CYP enzymes investigated in spheroids, except for CYP7A1 (0.40-fold at 800 ng/mL). In HepatoPac, 800 ng/mL FGF-19 suppressed the activity of CYP1A2 (0.28-fold) in donor QNT and the mRNA expression and activity in donor BGF (0.46 and 0.45-fold, respectively) and QNT (0.22 and 0.23-fold, respectively). The expression of CYP3A4 was also suppressed by 800 ng/mL in donor QNT (0.17-fold mRNA and 0.28-fold activity) and IVL (0.34-fold mRNA). In the case of Liver-on-a-chip, CYP1A2 and CYP3A4 mRNA expression was induced by FGF-19 by 2.58 and 2.37-fold, respectively. In contrast, CYP7A1 was suppressed by FGF-19 in a concentration-dependent manner in all in vitro systems with the largest effect seen HepatoPac (0.10-fold across all donors), followed by spheroids (0.40-fold), and Liver-on-a-chip (0.47-fold). No relevant effects of FGF-19 were observed on SLCO1B1 or ABCC2 in any of the advanced in vitro systems.
Discussion

In the current study, four *in vitro* systems were evaluated for their ability to investigate potential CYP/transporter regulation by therapeutic peptides. Nine compounds were initially investigated in SCHH; subsequently, one peptide (NN1177), one protein (FGF-19) and two small molecules (abemaciclib and OCA) were further investigated in HepatoPac, spheroid, and Liver-on-a-chip systems. HepatoPac, the most extensively investigated in this study (3 donors), showed high potential to investigate DDIs associated with CYP regulation by therapeutic peptides. Spheroids and Liver-on-a-chip were only assessed in one hepatocyte donor and further evaluations are required to confirm their potential.

Recent study highlighted difficulties associated with predicting clinical DDIs for peptides such as NN1177. NN1177 suppressed multiple CYPs (CYP3A4, CYP2B6, CYP1A2) following clinically relevant exposure in freshly isolated human hepatocytes (Säll et al., 2022). However, PK-profiles from a follow-up clinical DDI trial showed no indication of down-regulation of CYP enzymes following NN1177 exposure (Säll et al., 2022). Here, we investigated four *in vitro* systems with a range of complexities and their ability to evaluate peptide DDI risks. NN117 suppressed *CYP3A4* and *CYP1A2* mRNA expression in SCHH, in line with data previously reported in freshly-isolated hepatocytes (Säll et al., 2022), but *CYP2B6* was not suppressed in this system. In more advanced cell systems (HepatoPac, spheroids, and Liver-on-a-chip), the mRNA expression and activity of CYP1A2, CYP2B6, and CYP3A4 were not suppressed by NN1177, with the exception that suppression of CYP2B6 was noted in one donor in HepatoPac. Less stable expression of enzymes in SCHH may rationalise these differences in findings between SCHH and the more complex systems (HepatoPac, spheroids, and Liver-on-a-chip). Given available clinical DDI data for NN1177, the absence of an effect on CYP3A4 would have been correctly predicted by
HepatoPac and spheroids if performed before the clinical study, whereas SCHH indicated suppression and Liver-on-a-chip indicated a minor induction (2.2-fold). Notably, the observed decrease in AUC by 52% of caffeine (CYP1A2 probe) after NN1177 administration was not explained by findings from any system investigated here. The results of the clinical DDI trial might be affected by several confounding factors complicating the interpretation of the outcome and a suppression of CYP enzymes cannot definitively be ruled out from PK data only (Säll et al., 2022). However, our data collectively suggests that the use of HepatoPac provides promising in vitro DDI assessment for NN1177. In the case of spheroids and Liver-on-a-chip, more donors need to be investigated to provide a complete evaluation. Due to the lack of available clinical DDI data for a range of therapeutic peptides, NN1177 was the only peptide investigated in the current study. More clinical DDI data for different therapeutic peptides are required to fully assess the potential advantages of using HepatoPac more broadly for in vitro DDI risk assessment of therapeutic peptides.

Suppression is commonly observed in routine induction studies of small molecules (Hariparsad et al., 2017). However, a robust IVIVE is currently not established due to the lack of compounds where the underlying mechanism is understood and the lack of clinical data with relevant CYP suppression observed. Several confounding factors might potentially complicate the interpretation of in vitro data. For example, Stevison et al has showed a disconnect between the in vitro suppression of CYP2D6 and the clinic by retinoids (Stevison et al., 2019). They proposed that the effects of retinoids on CYPs are complex and involve multiple nuclear receptors and that the IVIVE disconnect could be due to endogenous regulators that are not well represented in vitro. Furthermore, time-dependent CYP inhibition and cytotoxicity may attenuate mRNA and activity (Hariparsad et al., 2017). Here, we also investigated the ability of HepatoPac, spheroids, or Liver-on-a-chip to evaluate CYP sup-
pression caused by small molecules OCA and abemaciclib and the protein FGF-19. Abemaciclib and OCA are examples where in vitro suppression data failed to correlate with clinical findings (Edwards et al., 2017; Zhang et al., 2017; Ishida et al., 2019; Turner et al., 2020). OCA has shown to suppress CYP enzymes in a dose-dependent manner in SCHH (Zhang et al., 2017; Ishida et al., 2019). In our study, we found similar suppression of CYP1A2 and CYP3A4 in HepatoPac and Liver-on-a-chip, whereas there was no effect on any of the enzymes investigated in spheroids. Clinically, OCA did not suppress CYP3A4 and potentially also not CYP1A2 (Edwards et al., 2017). To fully assess whether the in vitro systems predict the observed clinical outcome of CYP1A2 and CYP3A4 regulation by OCA more extensive evaluation of the effect across concentration range is necessary (EC$_{50}$ determination), together with integration of such data in mechanistic models. Additionally, it could be relevant to investigate if there are difference in the intracellular concentration across the in vitro systems.

Abemaciclib attenuates the mRNA and activity of CYP1A2, CYP2B6, and CYP3A4 in plated primary human hepatocytes (Turner et al., 2020). Despite this, abemaciclib showed no clinically relevant effect on CYP1A2 and CYP3A4 (Turner et al., 2020). When disregarding the 5.0 µM concentration of abemaciclib, suppression of CYP1A2, CYP2B6 and CYP3A4 were observed in 1 donor for HepatoPac but not in the Liver-on-a-chip or spheroid systems. With the lack of multiple donors for Liver-on-a-chip and spheroids, it is not possible to assess if suppression would be expected. In addition, abemaciclib caused cytotoxicity at higher concentrations and this effect was most pronounced in spheroids. This difference in cytotoxicity observations highlight that the in vitro systems do respond differently to xenobiotics and that cytotoxicity is an important factor in assessing suppression. Formation of vacuoles was also observed by Turner et al. at ≥ 7.5 µM for abemaciclib, but the major
metabolites showed grainy cytoplasm from ≥ 2.5 µM (Turner et al., 2020). The cause of the difference in observed cytotoxicity in our study is not known.

A general assessment of these novel and advanced in vitro systems is important to understand their potential use in a drug development setting. The response to induction control has been shown to vary significantly from lot-to-lot in plated human hepatocytes after treatment with omeprazole (CYP1A2, 8.1-34-fold), phenobarbital (CYP2B6, 4.4-20-fold), or rifampicin (CYP3A4, 3.1-33-fold) (Yajima et al., 2014). In a study using SCHH, the response of CYP3A4 to rifampicin showed up to 134-fold induction and a large donor-to-donor and intra-donor variation (Kenny et al., 2018). They also showed that activity fold-change is both lower and less variable compared to mRNA (Kenny et al., 2018). Whether these observations hold true for more advanced in vitro systems have not yet been investigated. Previous studies with HepatoPac have reported induction of CYP1A2, CYP3A4 and CYP2B6 (Dixit et al., 2016; Rose et al., 2016) and the induction magnitude was generally in agreement with monoculture data (Dixit et al., 2016). However, higher induction potency of rifampicin was noted in HepatoPac compared to plated hepatocytes in the same donors, which might be caused by a higher intracellular rifampicin concentration due to more activity of transporters in HepatoPac (Moore et al., 2016). Docci et al also show that CYP3A4 activity in the control phase is stable during the experiment in contrast to monolayer where the control activity declines which biases the estimation of the fold-induction (Docci et al., 2019). IL-6 has previous shown to supress CYP3A4 in HepatoPac, spheroids, and Liver-on-a-chip (Ramsden et al., 2015; Long et al., 2016; Klöditz et al., 2023). However, in the current study, we have only found a suppression of CYP3A4 (<0.5-fold) by IL-6 in HepatoPac, whereas these trends were not evident in the spheroid and Liver-on-a-Chip system. There are currently no guideline recommendations for positive controls regarding CYP suppression. In
our study, IL-6 was included as a potential positive control for CYP suppression considering in vitro data currently available in the literature (Dunvald et al., 2022). In a recent study in hepatic 3D spheroids, IL-6 was reported to cause suppression of CYP1A2 and CYP2B6, although the effect was not consistent across all experiments (Klöditz et al., 2023). Notably, the variability between individual experiments was higher after 7 days compared to a 3 day in culture (Klöditz et al., 2023). In case of CYP3A4, the response to IL-6 obtained under the conditions comparable to the ones used in the current study was generally below 0.5-fold threshold, with an observed maximum suppression of ~0.2-fold. The inconsistency of IL-6 suppression effect in our experiments in spheroids and Liver-on-a-chip was unexpected and may be attributed to a combination of system and donor specific factors; further investigations with multiple donors are therefore required.

Overall, HepatoPac is a stable and robust in vitro system to evaluate the regulation of CYP1A2, CYP2B6, and CYP3A4. Although rifampicin has been used to induce CYP3A4 to evaluate the attenuation by IL-6 in Liver-on-a-chip (Long et al., 2016), the induction potential of positive controls in Liver-on-a-chip has not been published. Interestingly, we found that the activity of Liver-on-a-chip is low, despite not being normalized to hepatocyte number, and fold-induction (especially for CYP3A4) is much higher than HepatoPac and spheroids (Figure 2). Although only one donor was used in Liver-on-a-chip, the same donor was used across all in vitro systems and rifampicin treatment resulted in a ~10x fold higher activity which is comparable to effects previously reported (Long et al., 2016). The high fold-induction could be associated with a lower basal CYP3A4 activity (Table 1) in Liver-on-a-chip using this donor. Although lower basal activity/ expression may be favourable for induction studies, it would provide less dynamic range to access potential suppression. To fully evaluate reasons for the high induction and the impact on suppression evaluation
further studies are needed. Our results of induction of CYP1A2, CYP2B6, and CYP3A4 in spheroids with controls (omeprazole, phenobarbital, and rifampicin, respectively) are in agreement with recently reported evaluation of spheroids for induction of CYP enzymes and transporters (Järvinen et al., 2023).

The current study provides a comprehensive evaluation of HepatoPac, spheroids, and Liver-on-a-chip cellular systems and their ability to investigate induction and suppression of metabolic enzymes and transporters. The same donor was used across all three novel systems to avoid donor-to-donor variation. However, other experimental factors did vary between the systems (e.g., culture day, insulin concentration in the media). In addition, suppression caused by peptides are expected to take place via receptor interactions that subsequently cause intracellular downstream effects leading to changes in CYP mRNA levels (Iber et al., 2001; Kim and Novak, 2007; Woolsey et al., 2016). The potential difference in receptor expression and their impact on subsequent effects on CYP mRNA expression across the in vitro systems are currently not known. Proteomic assessment of the complex in vitro systems including ADME proteins and relevant receptors is required for improved understanding of capabilities of these systems and to gain more insight to the underlying mechanism of CYP suppression by peptides. HepatoPac and spheroids showed comparable activity of CYP1A2, CYP2B6, and CYP3A4 with HepatoPac having the highest metabolite formation rate, whereas the measured activity of these enzymes was much lower in Liver-on-a-chip using the same donor of hepatocytes. An important note is that any loss of cells has not been accounted for, which may affect direct comparison of this system to HepatoPac and spheroids. Another notable difference is the high fold-induction of CYP3A4 by rifampicin in Liver-on-a-chip. Clinically, rifampicin has shown to induce the mRNA level of CYP3A4 around 4-fold in human liver biopsies (Marschall et al., 2005). Donor suitability is
another consideration when evaluating complex in vitro systems. Not all donors (e.g., IVL and QNT in this study) could form liver spheroids; the factors determining compatibility of donors with spheroid formation are currently not fully understood. Furthermore, the number of cells in each spheroid is limited, and the culture of spheroids is laborious. Liver-on-a-chip requires a specific setup available, and the throughput is currently low compared to the other novel systems. In contrast, HepatoPac is more established, but potential impact/interference coming from stromal cells on the experimental outcome needs to be understood.

In conclusion, our work demonstrates a more clinically relevant evaluation of CYP-mediated DDI risk for peptide NN1177 in advanced cellular systems compared to SSCH. To fully assess the potential of advanced in vitro systems for evaluation and prediction of clinical DDI risk of therapeutic peptides, additional studies and better understanding of inter-donor differences are required. In addition, more clinical DDI data for therapeutic peptides are necessary for robust verification of the in vitro findings. Nevertheless, the current work provides an excellent foundation for exploration of advanced cellular systems for the in vitro DDI evaluation of therapeutic peptides.

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Data Availability Statement:

The authors declare that all the data supporting the findings of this study are contained within the paper.

Author Contributions:

Participated in research design: RAN, DKB, CGJ, EJ, TS, AG, and CS.

Conducted experiments: RAN, DKB, and EJ.

Performed data analysis: RAN.

Interpretation of data: RAN, AG, TS, EJ, and CS.

Wrote or contributed to the writing of the manuscript: RAN, DKB, EJ, TS, AG, and CS.

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: RAN, CS, and CGJ are employed at Novo Nordisk A/S. DKB was employed at Novo Nordisk A/S when work was performed. DKB is employed at AstraZeneca. CS, and CGJ own shares in Novo Nordisk A/S. EJ, AG, and TS: none.

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International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (2023) ICH draft Guideline M12 on drug interaction studies.


US Food and Drug Administration (2015) Clinical pharmacology and biopharmaceutics review(s) for Application number 207999Orig1s000.


Figure Legends

Figure 1. *In vitro* assessment of mRNA expression of *CYP1A2*, *CYP2B6*, and *CYP3A4* in sandwich culture human hepatocytes treated with 9 different compounds in two donor (Blue = IVL and red = QNT). Each sample is relative to the respective control and the ct-values are normalized to *RPLP0* (*ribosomal protein, large, P0*) expression. Data are presented as boxplots with individual dots represent individual experiments (technical replicate) (*n* = 2-9). The horizontal solid line represents no change compared to controls samples and the dotted lines are the cut-off of 2- and 0.5-fold change. GH: Growth Hormone. GIP: Gastric inhibitory polypeptide. FGF: Fibroblast growth factor

Figure 2. *In vitro* fold change in mRNA (white boxplot, left column) and activity (grey boxplot, right column) of *CYP1A2*, *CYP2B6*, and *CYP3A4* after treatment with induction and suppression control compounds in HepatoPac, Liver-on-a-chip and spheroids (Donors: blue = IVL, red = QNT, and green = BGF). Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized to *HPRT1* expression. Activity of *CYP1A2*, *CYP2B6*, and *CYP3A4* was measuring by the conversion of phenacetin to acetaminophen, bupropion to hydroxybupropion and testosterone to 6β-hydroxytestosterone, respectively. For spheroids the activity after 8 hours is depicted. The boxplot represents the overall results for all donors with each dot representing an individual experiment (technical replicate) (*n* = 3 – 6). Omeprazole = 50 µM, phenobarbital = 750 µM, rifampicin = 20 µM, and IL-6 = 10 ng/ml. The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change.
Figure 3. *In vitro* fold change of mRNA (white boxplot, left column) and activity (grey boxplot, right column) of CYP1A2 after treatment with NN1177, FGF-19, obeticholic acid, and abemaciclib in HepatoPac, Liver-on-a-chip and spheroids (Donors: blue = IVL, red = QNT, and green = BGF). Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized to *HPRT1* expression. Activity of CYP1A2 was measuring by the conversion of phenacetin to acetaminophen. For spheroids the activity after 8 hours is depicted. The boxplot represents the overall results for all donors with each dot representing an individual experiment (technical replicate) (*n* = 2 – 6). The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change. FGF: Fibroblast growth factor.

Figure 4. *In vitro* fold change of mRNA (white boxplot, left column) and activity (grey boxplot, right column) of CYP2B6 after treatment with NN1177, FGF-19, obeticholic acid, and abemaciclib in HepatoPac, Liver-on-a-chip and spheroids (Donors: blue = IVL, red = QNT, and green = BGF). Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized to *HPRT1* expression. Activity of CYP2B6 was measuring by the conversion of bupropion to hydroxybupropion. For spheroids the activity after 8 hours is depicted. The boxplot represents the overall results for all donors with each dot representing an individual experiment (technical replicate) (*n* = 2 – 6). The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change. FGF: Fibroblast growth factor.
Figure 5. *In vitro* fold change of mRNA (white boxplot, left column) and activity (grey boxplot, right column) of CYP3A4 after treatment with NN1177, FGF-19, obeticholic acid, and abemaciclib in HepatoPac, Liver-on-a-chip and spheroids (Donors: blue = IVL, red = QNT, and green = BGF). Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized to *HPRT1* expression. Activity of CYP3A4 was measuring by the conversion of testosterone to 6β-hydroxytestosterone. For spheroids the activity after 8 hours is depicted. The boxplot represents the overall results for all donors with each dot representing an individual experiment (technical replicate) (n = 2 – 6). The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change. FGF: Fibroblast growth factor
### Tables

**Table 1.** Absolute activity of metabolite formation of probe substrates of CYP1A2, CYP2B6, and CYP3A4. The Activity is normalized by the number of seeded cells. Cells seeded: HepatoPac ≈ 21,000, Spheroids ≈ 1,500, and Liver-on-a-chip ≈ 600,000. The metabolite formation is depicted as mean ± SD. n = 3 – 6 technical replicates

<table>
<thead>
<tr>
<th>System</th>
<th>Enzyme</th>
<th>Metabolite formation [pmol/min/million cells]</th>
<th>Donor ID</th>
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<tr>
<td>HepatoPac</td>
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<td>96.7 ± 39.2</td>
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<td></td>
<td>112.4 ± 18.9</td>
<td>QNT</td>
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<td></td>
<td></td>
<td>62.5 ± 29.0</td>
<td>BGF</td>
</tr>
<tr>
<td></td>
<td>CYP2B6</td>
<td>5.0 ± 1.7</td>
<td>IVL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0 ± 2.8</td>
<td>QNT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.0 ± 0.6</td>
<td>BGF</td>
</tr>
<tr>
<td></td>
<td>CYP3A4</td>
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<td>460.3 ± 190.9</td>
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<td>Spheroids</td>
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<td>CYP2B6</td>
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<td>CYP3A4</td>
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<td>Liver-on-a-chip</td>
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<td>5.2 ± 0.3</td>
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<td>----------------</td>
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<td>CYP2B6</td>
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<td></td>
<td>CYP3A4</td>
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Supplementary material:

Evaluating drug-drug interaction risk associated with peptide analogues using advanced in vitro systems

Rune Aa. Nørgaard, Deepak K. Bhatt, Erkka Järvinen, Tore B. Stage, Charlotte Gabel-Jensen, Aleksandra Galetin, and Carolina Säll
**Figure S1.** LDH release in sandwich culture of human hepatocytes in response to treatment with various compounds. The LDH release is normalized to the LDH release in media (low control) and 0.1% Triton x is set to 100% LHD release. Donor: IVL.
Figure 2. mRNA fold change of CYP1A2, CYP2B6, and CYP3A4 after treatment with induction and suppression control compounds in sandwich culture of human hepatocytes. Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized by the RPLP0 expression. Each dot represents an individual experiment. Omeprazole = 50 µM, phenobarbital = 750 µM, rifampicin = 20 µM, and IL6 = 10 ng/ml. The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change. n = 8 - 9.
Figure S3. In vitro assessment of mRNA expression of SLCO1B1 and CYP7A1 in sandwich culture human hepatocytes treated with 9 different compounds. Each sample is relative to the respective control and the ct-values are normalized by the RPLP0 expression. Each dot represents an individual experiment. The horizontal solid line represents no change compared to controls samples and the dotted lines are the cut-off of 2 and 0.5 fold change. n = 2-9
Figure S4. Fold change in mRNA of CYP7A1 after treatment with obeticholic acid, abemaciclib, NN1177, and FGF-19 in HepatoPac, Liver-on-a-chip and spheroids. Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized by the HPRT1 expression. Each dot represents an individual experiment. The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change. The boxplot represents the overall results for all donors. n = 1 – 6 (for obeticholic acid the expression was in some samples not at a detectable level)
Figure S5. Fold change in mRNA of *SLCO1B1* after treatment with obeticholic acid, abemaciclib, NN1177, and FGF-19 in HepatoPac, Liver-on-a-chip and spheroids. Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized by the *HPRT1* expression. Each dot represents an individual experiment. The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change. The boxplot represents the overall results for all donors. n = 2 – 6
Figure S6. Fold change in mRNA of ABCC2 after treatment with obeticholic acid, abemaciclib, NN1177, and FGF-19 in HepatoPac, Liver-on-a-chip and spheroids. Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized by the HPRT1 expression. Each dot represents an individual experiment. The horizontal solid line represents no change compared to control samples and the dotted lines are the cut-off of 2- and 0.5-fold change. The boxplot represents the overall results for all donors. n = 2 – 3
Figure S7. ATP content of spheroids after 3 days of treatment with the respective compound. Donor: BGF. n = 5
Figure S8. Human Albumin in media of Liver-on-a-chip after 3 days of treatment with respective compounds. Donor: BGF. n = 3
Figure S9. Correlation plot of activity fold change against mRNA fold change in HepatoPac, Liver-on-a-chip, and spheroids of CYP1A2, CYP2B6, and CYP3A4. Each sample is relative to the respective control and the ct-values for the mRNA levels are normalized by the HPRT1 expression. Each dot represents the mean value of a specific treatment.
Figure S10. Absolute activity of metabolite formation of probe substrates of CYP1A2, CYP2B6, and CYP3A4 in hepatic spheroids after 2, 8, and 24 h incubation. n = 3 (technical replicates). Donor = BGF
Figure S11. Delta CT values of CYP1A2, CYP2B6, CYP3A4, SLC01B1, CYP7A1, and ABCC2 in Sandwich culture, HepatoPac, Spheroids, and Liver-on-a-chip treated with 0.1 % DMSO. The CT values of the respective genes are normalized to GAPDH as this housekeeping gene was measured for all systems. The comparison of the systems use GAPDH as housekeeping gene assumes that GAPDH is similar in the 4 systems. $n = 3 - 9$ (technical replicates)

Tables

Table S1 Donor demographic

<table>
<thead>
<tr>
<th>Donor lot</th>
<th>Sex</th>
<th>Race</th>
<th>Age</th>
<th>Used for in vitro system</th>
</tr>
</thead>
<tbody>
<tr>
<td>QNT</td>
<td>Male</td>
<td>African-American</td>
<td>58</td>
<td>HepatoPac and sandwich culture</td>
</tr>
<tr>
<td>IVL</td>
<td>Female</td>
<td>Asian</td>
<td>57</td>
<td>HepatoPac and sandwich culture</td>
</tr>
<tr>
<td>BGF</td>
<td>Male</td>
<td>Hispanic</td>
<td>56</td>
<td>HepatoPac, spheroids, and Liver-on-a-chip</td>
</tr>
</tbody>
</table>
Table S2. TaqMan Gene Expression Assays used for RT-qPCR of mRNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Used for in vitro systems:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>Hs00604506_m1</td>
<td>All</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Hs00167927_m1</td>
<td>All</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Hs04183483_g1</td>
<td>All</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>Hs00167982_m1</td>
<td>All</td>
</tr>
<tr>
<td>SLC01B1</td>
<td>Hs00272374_m1</td>
<td>All</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs02786624_g1</td>
<td>2D sandwich culture</td>
</tr>
<tr>
<td>RPLP0</td>
<td>Hs00420895_gH</td>
<td>2D sandwich culture</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hs99999909_m1</td>
<td>HepatoPac, 3D spheroids, and Liver-on-a-chip</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs99999905_m1</td>
<td>HepatoPac, 3D spheroids, and Liver-on-a-chip</td>
</tr>
<tr>
<td>MRP2</td>
<td>Hs00166123_m1</td>
<td>HepatoPac, 3D spheroids, and Liver-on-a-chip</td>
</tr>
</tbody>
</table>

Table S3. Mass spectrometry conditions used for in vitro selective activity assays for CYP1A2, CYP2B6, and CYP3A from the in vitro study conducted in primary cultures of human hepatocytes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Metabolite</th>
<th>Internal Standard</th>
<th>Ionization mode</th>
<th>Mass Transitions</th>
<th>Trap CE</th>
<th>Transfer CE</th>
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</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>Acetaminophen-D4</td>
<td>ESI+</td>
<td>152.2/110.1; 156.1/114.1</td>
<td>4;4</td>
<td>8;8</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>Hydroxy-Bupropion</td>
<td>Hydroxy-Bupropion-D6</td>
<td>ESI+</td>
<td>256.2/238.2; 262.1/244.1</td>
<td>4;4</td>
<td>8;7</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>6-beta-hydroxytestosterone</td>
<td>6-beta-hydroxytestosterone-D3</td>
<td>ESI+</td>
<td>305.2/269.2; 308.3/272.2</td>
<td>4;4</td>
<td>8;8</td>
</tr>
</tbody>
</table>