

TITLE PAGE

Title: (-)-N-3-Benzylphenobarbital is superior to omeprazole and (+)-N-3-benzylirvanol as a CYP2C19 inhibitor in suspended human hepatocytes

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Running title: CYP2C19 inhibition by (-)-N-3-benzylphenobarbital in SHH

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Non-Standard abbreviations: AO: aldehyde oxidase, CYP: cytochrome P450, CYP2C19:

cytochrome P450 2C19, DDI: drug-drug interaction, fm: fraction metabolized, FDA: U.S. Food and

Drug Administration, FMO: flavine-containing monooxygenase, IVIVC: *in vitro* – *in vivo* correlation,

NAT: N-acetyltransferase, NCE: new chemical entity, SHH: suspended human hepatocytes, UGT:

uridine diphosphate glucuronosyltransferase, WGH: William's medium E, containing 2 mM of glutamine and 15 mM of HEPES

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ABSTRACT

Early assessment of metabolism pathways of new chemical entities (NCEs) guides the understanding of drug-drug interactions (DDIs). Selective enzyme inhibitors are indispensable in cytochrome P450 (CYP) reaction phenotyping. The most commonly applied CYP2C19 inhibitor, omeprazole, lacks selectivity. Two promising alternatives, (+)-N-3-benzylrivanol and (-)-N-3-benzylphenobarbital, are already used as CYP2C19 inhibitors in some *in vitro* studies with suspended human hepatocytes. However, a full validation proving their suitability in terms of CYP and non-CYP selectivity has not been presented in literature. The present study provides a thorough comparison between omeprazole, (+)-N-3-benzylrivanol, and (-)-N-3-benzylphenobarbital in terms of potency and selectivity, and shows the superiority of (-)-N-3-benzylphenobarbital as a CYP2C19 inhibitor in suspended human hepatocytes. Furthermore, we evaluated the application of (-)-N-3-benzylphenobarbital to predict the *in vivo* contribution of CYP2C19 to drug metabolism ($f_{mCYP2C19}$). A set of ten clinically used CYP2C19 substrates with reported *in vivo* $f_{mCYP2C19}$ data was evaluated. $F_{mCYP2C19}$, which was predicted using data from suspended human hepatocyte incubations, underestimated the *in vivo* $f_{mCYP2C19}$. The use of a different hepatocyte batch with a different CYP3A4/CYP2C19 activity ratio, showed the impact of intrinsic CYP activities on the determination of $f_{mCYP2C19}$. Overall, this study confirms the selective CYP2C19 inhibition by (-)-N-3-benzylphenobarbital over other CYP isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4) and clinically relevant non-CYP enzymes (AO, FMO3, NAT2, UGT1A1, UGT1A4, UGT2B7, UGT2B15) in suspended human hepatocytes. (-)-N-3-benzylphenobarbital is therefore the preferred CYP2C19 inhibitor to assess $f_{mCYP2C19}$ in suspended human hepatocytes in comparison to omeprazole and (+)-N-3-benzylrivanol.

SIGNIFICANCE STATEMENT

(-)-N-3-benzylphenobarbital is a more potent and selective inhibitor of CYP2C19 in suspended human hepatocytes than omeprazole and (+)-N-3-benzylirinanol. (-)-N-3-benzylphenobarbital can be used to predict the fraction metabolized by CYP2C19 in suspended human hepatocytes.

1. INTRODUCTION

CYP enzymes have been thoroughly investigated as they are responsible for the biotransformation of almost 80% of all commercially available drugs (Zanger and Schwab, 2013). CYP2C19 is one of the major enzymes in the human liver as it metabolizes ~7% of the clinically used drugs, mostly anticonvulsants and antidepressants (Zanger and Schwab, 2013; FDA, 2020). Inhibitors, inducers, and genetic polymorphisms of CYP2C19 can influence the plasma levels of CYP2C19 substrates, resulting in a risk for toxic or subtherapeutic plasma levels. U.S. Food and Drug Administration (FDA) guidelines recommend the assessment of these possible DDIs in the early stages of drug development (FDA, 2020). The evaluation of the fraction metabolized by a specific CYP enzyme (fm_{CYP}) in drug metabolism, CYP reaction phenotyping, plays an important role in predicting these DDIs. After all, fm_{CYP} influences the susceptibility of victim drugs towards inhibition or induction. Two methods are widely used for the quantitative determination of CYP enzyme involvement: (1) the use of chemicals, drugs or antibodies as specific enzyme inhibitors in pooled human liver microsomes and (2) the use of individual human recombinant CYP enzymes (FDA, 2020).

A limitation of the systems suggested by the FDA is the use of human liver microsomes and recombinant enzymes. These *in vitro* systems are useful to obtain high levels of rare isoforms or to determine the role of one single enzyme in the metabolic profile (Parmentier et al., 2017). However, there are several disadvantages to using recombinant enzymes, namely, (i) the evaluation of the cooperation between enzymes is impossible, (ii) a relative activity factor is needed for scaling to *in vivo*, (iii) enzymes are removed from their natural intracellular environment and (iv) the addition of cofactors is required (Brandon et al., 2003). Human liver microsomes are frequently used because of their low cost, high availability, ease of use, and high throughput (Lindmark et al., 2018). They represent a more advanced experimental model than recombinant enzymes, despite the fact that they also require the addition of cofactors and that they lack transporters or cell membranes as well as several key drug-metabolizing enzymes (e.g. sulfotransferase, aldehyde oxidase (AO), and xanthine oxidase). This could result in higher relative contributions of CYP and uridine diphosphate glucuronosyltransferase (UGT) enzymes to drug metabolism as compared to the human *in vivo*

situation (Brandon et al., 2003). The latter may result in an underestimation of the *in vivo* clearance of a drug product (Brown et al., 2007). Another problem that may arise when using human liver microsomes is the potential dependency of CYP enzyme activity on microsomal isolation (Nelson et al., 2001). Therefore, human hepatocytes are preferred for *in vitro* metabolic clearance experiments because they possess the full complement of phase I and phase II drug-metabolizing enzymes like sulfotransferase enzymes, AO, and xanthine oxidase (Brown et al., 2007). Human hepatocytes are co-factor self-sufficient, allow a simultaneous assessment of multiple enzymes/transporters and better resemble the *in vivo* situation (Jiang et al., 2015; Lindmark et al., 2018). The ability to cryopreserve has helped to overcome the limited availability of human hepatocytes, contributing to the increasing popularity of human hepatocytes as an *in vitro* system to evaluate drug metabolism (Smith et al., 2012).

The availability of selective inhibitors is essential for predicting fm_{CYP} when using human hepatocytes. The most frequently used chemical inhibitor for CYP2C19 is omeprazole. However, omeprazole also inhibits CYP3A4, CYP2C9 (Ko et al., 1997), and UGT1A1 (Liu et al., 2011) in human liver microsomes and hepatocytes. In rare cases, this could result in an overprediction of the CYP2C19 contribution, potentially leading to unnecessary attrition of promising NCEs from the drug pipeline (Cai et al., 2004). Hence, researchers have looked for alternative CYP2C19 inhibitors to omeprazole. Several studies showed the CYP2C19 inhibitory potential of (+)-N-3-benzyl nirvanol and (-)-N-3-benzylphenobarbital in human liver microsomes, and their superiority over omeprazole (Suzuki et al., 2002; Cai et al., 2004). (-)-N-3-Benzylphenobarbital is already used in some *in vitro* suspended human hepatocyte studies (Kazmi et al., 2019), although a full validation in suspended human hepatocytes has presently not been published. Therefore, the current study investigated the potential of (-)-N-3-benzylphenobarbital and (+)-N-3-benzyl nirvanol as CYP and non-CYP selective inhibitors of CYP2C19 in suspended human hepatocytes as alternatives to the non-selective CYP2C19 inhibitor omeprazole. Compounds with a wide range of $fm_{CYP2C19}$ were subsequently applied to validate the selected CYP2C19 inhibitor in suspended human hepatocytes to predict the *in vivo* $fm_{CYP2C19}$.

2. MATERIALS AND METHODS

2.1. Materials

Cryopreserved human hepatocytes (mixed gender pool of 20 donors, batch BSU) were obtained from BioreclamationIVT (Brussels, Belgium). Cryopreserved human hepatocytes with low CYP3A4 activity (mixed gender pool of 3 donors, batch HUP182981) were obtained from Lonza (Walkersville, Maryland, US). (S)-(+)-Mephenytoin was purchased from Sigma-Aldrich (Saint Louis, Missouri, US) and Cayman Chemicals (Ann Arbor, Michigan, US). (-)-N-3-benzylphenobarbital was synthesized at UCB Biopharma SRL (Braine l'Alleud, Belgium). Midazolam was purchased from Apin Chemicals (Compton, Berkshire, UK). Omeprazole (racemic mixture), (+)-N-3-benzylnirvanol, glacial acetic acid, HEPES, dimethyl sulfoxide (DMSO), β -estradiol, sulfamethazine, citalopram, rabeprazole, lansoprazole, diazepam, phenytoin, naloxone, cimetidine, phenacetin, acetaminophen, bupropion, hydroxybupropion, diclofenac, 4-hydroxydiclofenac, dextromethorphan, dextrorphan, rosiglitazone, 5-hydroxyrosiglitazone, 1-hydroxymidazolam, carbazepan, 4-hydroxycarbazepan, 4-hydroxymephenytoin, and internal standards used in the bio-analysis (acetaminophen-D₄, hydroxybupropion-D₆, 1-hydroxymidazolam-D₄, 4-hydroxydiclofenac-¹³C₆, 4-hydroxymephenytoin-D₃, dextrorphan-D₃, and 5-hydroxyrosiglitazone-D₄) were obtained from Sigma-Aldrich (Saint Louis, Missouri, US). Trifluoperazine, pantoprazole, clopidogrel, labetalol, and moclobemide were acquired from Cayman Chemicals (Ann Arbor, Michigan, US). Clobazam, norclobazam, and oxazepam were provided by LGC (Teddington, Middlesex, UK). William's medium E was purchased from Lonza (Verviers, Belgium). Glutamine, cryopreserved hepatocyte recovery medium, and trypan blue 0.4% were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, US). Glucuronidase/arylsulfatase was bought from Roche (Vilvoorde, Belgium). Ammonium acetate was purchased from Merck (Darmstadt, Germany). Ultra-performance liquid chromatography (UPLC) grade water, acetonitrile, methanol, ethanol, formic acid, and trifluoroacetic acid were obtained from Biosolve (Valkenswaard, The Netherlands).

2.2.Methods

2.2.1. Thawing of cryopreserved human hepatocytes

On the day of the experiment, vials of human hepatocytes were taken out of the cryoconservator. The vials were submerged in a water bath (37°C) and gently shaken until the ice was almost completely thawed (1-2 min). The content of the vials was transferred into a falcon tube containing 50 mL of cryopreserved hepatocyte recovery medium and the vial was rinsed with cryopreserved hepatocyte recovery medium (37°C). The falcon tube containing the hepatocytes was centrifuged for 10 min (21°C, 100 g) (4-16KS, Sigma, Osterode am Harz, Germany). The supernatant was removed, and the pellet of hepatocytes was gently loosened by tapping. Two mL/vial of William's medium E, containing 2 mM of glutamine and 15 mM of HEPES (WGH) was added to the falcon tube. A 100 µL aliquot of the hepatocyte suspension was added to 100 µL trypan blue (0.4%), and cells were counted using a hemocytometer. Viability was determined based on trypan blue exclusion (always > 84.4%). The hepatocytes were diluted with WGH to the required concentration and were transferred into the wells of a 48-well plate.

2.2.2. Incubations with suspended human hepatocytes

The hepatocytes (2×10^6 cells/mL) were preincubated for 30 min in a humidified incubator (5% CO₂, 37°C). During the experiment the hepatocytes were kept in suspension using agitation (Titramax 100, 450 rpm). The reaction was initiated by adding one volume of prewarmed (37°C) WGH containing substrates and inhibitors, or inhibitors alone, when a 3 h pre-incubation period with only inhibitors was required. Final concentrations of DMSO and acetonitrile in incubates were $\leq 0.05\%$ and $\leq 1\%$, respectively. After a 30 min incubation time, the reaction was stopped by adding one volume of sample to two volumes of ice-cold acetonitrile in a 96-deep well plate. The plate was centrifuged (15 min, 4°C, at 2908 g) (4-16KS, Sigma, Osterode am Harz, Germany) and the clear supernatant was analyzed by UPLC-mass spectrometry (MS)/MS. For rosiglitazone samples, the obtained supernatant was evaporated under a stream of nitrogen gas and the residue re-dissolved in 100 µL acetate buffer (50 mM, pH 5.0) containing glucuronidase (0.358 U/mL) and arylsulfatase (0.48 U/mL). An extra one-hour incubation (37°C) was carried out to convert the glucuronide and sulfate metabolites back to

5-hydroxyrosiglitazone. The reaction was stopped by adding one volume of sample to two volumes of ice-cold acetonitrile in a 96-deep well plate. The plate was centrifuged (15 min, 4°C, at 2908 g) (4-16KS, Sigma, Osterode am Harz, Germany), and the clear supernatant was used for UPLC-MS/MS analysis. The final concentration of (S)-(+)-mephenytoin, the CYP2C19 substrate, was 5 µM. A range of final inhibitor concentrations was used to assess their CYP2C19 inhibition potential: omeprazole (0, 0.06, 0.2, 2, 6, 20, and 60 µM), (+)-N-3-benzylrivanol (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 µM), and (-)-N-3-benzylphenobarbital (0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µM).

The effects of omeprazole (final concentration: 20 µM), (+)-N-3-benzylrivanol (final concentration: 10 µM), and (-)-N-3-benzylphenobarbital (final concentration: 1 µM) on the activity of major CYP enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4) and AO were evaluated without and with a 3 h pre-incubation period in presence of the inhibitors. Two in-house validated CYP substrate cocktails were used for the incubation of probe substrates, except rosiglitazone and carbazepan (Gerin et al., 2013). Probe substrates were incubated at concentrations close to the K_m value of the probe substrate reaction of interest. Supplemental Table 1 provides an overview of the different probe substrate reactions, the final substrate concentrations, and the cocktail compositions.

For (-)-N-3-benzylphenobarbital, selectivity against clinically relevant non-CYP enzymes was also evaluated. Specific metabolite formation was followed for estradiol (UGT1A1), trifluoperazine (UGT1A4), naloxone (UGT2B7), oxazepam (UGT2B15), sulfamethazine (N-acetyltransferase 2 (NAT2)), cimetidine (Flavin-containing monooxygenase 3 (FMO3)), and carbazepan (AO, described above) in presence and absence of (-)-N-3-benzylphenobarbital (1 µM) in suspended human hepatocytes (1×10^6 cells/mL). Probe substrates were incubated at a final concentration of 0.5 µM, except 2 µM for cimetidine. Reactions were stopped at six different time points (0, 10, 30, 60, 120, and 240 min) by adding one volume of sample to one volume of ice-cold acetonitrile. Plates were centrifuged (15 min, 4°C, at 2908 g) (4-16KS, Sigma, Osterode am Harz, Germany), and the clear supernatant was diluted with two volumes of UPLC grade water before analysis by UPLC-HRMS.

An *in vitro* – *in vivo* correlation (IVIVC) of $f_{mCYP2C19}$ was based on incubations of a set of clinically used known CYP2C19 substrates (phenytoin, diazepam, clobazam, noreclobazam, citalopram,

moclobemide, rabeprazole, lansoprazole, pantoprazole, omeprazole, labetalol, and (S)-(+)-mephenytoin), incubated at a final concentration of 0.5 μ M, except 10 μ M for clobazam and norclobazam, and 0.005 μ M for omeprazole. Incubations were carried out in the presence and absence of (-)-N-3-benzylphenobarbital (1 μ M). Reactions were stopped at six different time points (0, 30, 60, 120, 180, and 240 min) by adding one volume of sample to one volume of ice-cold acetonitrile. Parent depletion was followed in six-fold diluted supernatant (UPLC grade water) by UPLC-MS/MS for rabeprazole, lansoprazole, pantoprazole, omeprazole, labetalol, and (S)-(+)-mephenytoin samples. Three-fold diluted supernatant (UPLC grade water) of phenytoin, diazepam, clobazam, norclobazam, citalopram, and moclobemide was analyzed by UPLC-high resolution mass spectrometry (HRMS) to monitor metabolite formation.

2.2.3. Analytical Methods

2.2.3.1. UPLC-MS/MS methods to monitor metabolite formation

Two CYP substrate cocktail methods were used to analyze the metabolites of CYP probe substrates. Cocktail one refers to the analytical method used to quantify acetaminophen, 4-hydroxybupropion, 4-hydroxydiclofenac, 1-hydroxymidazolam (Gerin et al., 2013), and cocktail two refers to the analytical method used to quantify 4-hydroxymephenytoin and dextropropranolol.

Cocktail 1

One volume of internal standard solution (acetaminophen-D₄, hydroxybupropion-D₆, 1-hydroxymidazolam-D₄, 4-hydroxydiclofenac-¹³C₆) was added to five volumes of the three-fold diluted supernatant. Samples were analyzed on an Agilent 1290 UPLC system (Agilent Technologies Santa Clara, California, USA) coupled to a Sciex API 5000 mass spectrometer (Applied Biosystems, Mississauga, Canada), operated in positive ion mode. Chromatographic separation was obtained using gradient elution, and a Zorbax Eclipse plus XDB C18 (50 x 2.1 mm, 1.8 μ m) column operated at 40°C. The injection volume was 4 μ L and the flow rate 0.350 mL/min. Gradient elution with mobile phase A (H₂O with 0.1% trifluoroacetic acid at pH 2.4) and mobile phase B (acetonitrile) started at 95% A (2.16 min), and decreased respectively to 75% A in 0.24 min, 60% A in 1.46 min, 50% A in

0.01 min, 20% (0.49 min) in 0.47 min, 10% A (0.72 min) in 0.01 min, and finally returned to its initial condition 95% A (0.93 min). Data acquisition and system control were performed by Analyst® 6.1 software. Mass-to-charge (m/z) transitions are listed in Supplemental Table 2. Concentrations were calculated from peak area ratios based on a calibration curve with ten different concentrations. Three quality control samples were analyzed for the qualification of the analysis.

Cocktail 2 & rosiglitazone/carbazeran

Cocktail 2 ((S)-(+)-mephenytoin and dextromethorphan), rosiglitazone and carbazeran samples were incubated separately and analyzed by the same UPLC method. For cocktail 2 and rosiglitazone samples, one volume of internal standard solution (4-hydroxymephenytoin- D_3 , dextrophan- D_3 , 5-hydroxyrosiglitazone- D_4) was added to one volume of supernatant. The plate was evaporated under a stream of nitrogen gas at 50°C. The residue was re-dissolved in 100 μ L H₂O:acetonitrile:formic acid (90:10:0.1, v/v/v). For carbazeran samples, two volumes of internal standard solution (dextromethorphan) were added to one volume of supernatant, no evaporation step was needed. All samples were analyzed on a Shimadzu Nexera X2 UPLC system (Shimadzu Kyoto, Japan) coupled to a Sciex API 5000 mass spectrometer (Applied Biosystems, Mississauga, Canada), operated in positive ion mode. Chromatographic separation was obtained using gradient elution, and a Zorbax Eclipse plus XDB C18 (50 x 2.1 mm, 1.8 μ m) column operated at 30°C. The injection volume was 5 μ L and the flow rate 0.350 mL/min. Gradient elution with mobile phase A (H₂O with 0.1% formic acid) and mobile phase B (methanol with 0.1% formic acid) started at 95% A (1 min), and respectively decreased to 80% A (0.5 min) in 1.5 min, 20% A in 3 min, 10% A (0.98 min) in 0.01 min, and 5% A (1 min) in 0.01 min. Data acquisition and system control were performed by Analyst® 6.1 software. The m/z transitions are listed in Supplemental Table 2. Concentrations were calculated using peak area ratios and a calibration curve with eight different concentrations. Three quality control samples were analyzed for the qualification of the analysis. The cocktail was validated internally, showing no interaction between substrates (data not shown).

2.2.3.2.UPLC-MS/MS method to monitor parent drug disappearance

Parent drug disappearance was measured for six CYP2C19 substrates (rabeprazole, lansoprazole, pantoprazole, omeprazole, labetalol, and (S)-(+)-mephenytoin) to determine the $f_{mCYP2C19}$ in suspended human hepatocytes. The six-fold diluted samples were analyzed on a Shimadzu Nexera X2 UPLC system (Shimadzu Kyoto, Japan) coupled to a Sciex API 5000 mass spectrometer (Applied Biosystems, Mississauga, Canada), operated in positive ion mode, except negative ion mode for (S)-(+)-mephenytoin samples. Chromatographic separation was obtained using gradient elution, and an Acquity HSS T3™ C18 (50 x 2.1 mm, 1.8 μ m) column operated at 40°C. The injection volume and the flow rate were 5 μ L and 0.7 mL/min, respectively. Gradient elution with mobile phase A (ammonium acetate 10 mM with 0.1% acetic acid) and mobile phase B (acetonitrile) started at 90% A (0.30 min), and decreased respectively to 30% A in 3.70 min, 5% A in 1 min, and finally returned to its initial condition 90% A. Data acquisition and system control were performed by Analyst® 6.1 software. The m/z transitions are listed in Supplemental Table 2.

2.2.3.3.UPLC-HRMS method to monitor metabolite formation

Two-fold diluted supernatant of incubations performed with citalopram, clobazam, diazepam, moclobemide, norclobazam, phenytoin, β -estradiol, trifluoperazine, naloxone, oxazepam, sulfamethazine, and cimetidine samples were analyzed by UPLC-HRMS to detect specific metabolites. Samples were analyzed on an Acquity UPLC instrument (Waters, Manchester, UK) coupled to a XevoG2S Qtof high-resolution mass spectrometer (Waters, Manchester, UK), operated in positive ion mode, except negative ion mode for β -estradiol samples, or coupled to a VION ion mobility quadrupole time-of-flight high-resolution mass spectrometer for sulfamethazine samples. Citalopram, clobazam, clopidogrel, diazepam, moclobemide, norclobazam, trifluoperazine, and oxazepam samples were analyzed on a Waters CSH column (100 x 2.1 mm, 1.7 μ m). Injection volume and flow rate were 2 μ L and 3 μ L/min, respectively. A waters HSS C18 (100 x 2.1 mm, 1.7 μ m) column was used for analyzing β -estradiol, naloxone, and cimetidine samples. Injection volume and flow rate were 7 μ L and 400 μ L/min, respectively. Chromatographic separation was obtained using gradient elution with mobile phase A (ammonium acetate 10 mM:acetic acid 0.1%) and mobile phase B (acetonitrile) starting at 90% A (1 min), decreased to 10% A (10.5 min) in 9 min, and

returned to its initial conditions in 10.51 min. Chromatographic separation for sulfamethazine samples was obtained using gradient elution, and a Waters HSS C18 column (100 x 0.3 mm, 1.7 μm). Injection volume and flow rate were 2 μL and 7.5 μL/min, respectively. Gradient elution with mobile phase A (H₂O:formic acid 0,1%) and mobile phase B (acetonitrile:formic acid 0,1%) started at 95% (1 min) A, decreased to 10% A in 8 min (1.5 min), and finally increased until 90% A in 0.01 min. Full scan MS-MS data were acquired using Masslynx® V4.1 SCN884.

2.2.4. Data analysis

The rate of metabolite formation (pmol/min/million cells) was calculated using Equation 1. Where [metabolite] is the concentration of metabolite formed in μM, t is the time of incubation in minutes and [hepatocytes] is the hepatocyte concentration in the incubate in million cells/mL.

$$v = \frac{[\text{metabolite}] \cdot 1000}{t \cdot [\text{hepatocytes}]}$$

[Equation 1]

IC₅₀ values were determined with GraphPad Prism® 7.04 from GraphPad software (San Diego, California, US) using nonlinear regression four-parameter variable slope analysis (Equation 2) (GraphPad, 2020). All results were based on the least square fit.

$$\text{Metabolite formation (\% of control)} = \frac{100}{1 + 10^{((\log IC_{50} - \log [I]) \cdot \text{HillSlope})}}$$

[Equation 2]

Where IC₅₀ (μM) is the concentration of inhibitor resulting in 50% inhibition of the metabolite formation (% of control), the logarithm of the inhibitor concentration (log[I]) is the x variable and the hillslope describes the steepness of the curves.

The *in vitro* intrinsic clearance (Cl_{int}) (μL/min/million cells) was calculated using the *in vitro* half-life (t_{1/2}) (min) of compound disappearance (Equation 3) (Obach et al., 1997).

$$Cl_{int} = -\frac{0.693 \cdot 1000}{t_{1/2} \cdot [\text{hepatocytes}]}$$

[Equation 3]

For low turnover compounds, the intrinsic clearance ($\mu\text{L}/\text{min}/\text{million cells}$) was estimated based on metabolite formation rate. The metabolite formation rate was obtained by measuring the slope of the sum of the normalized peak areas (normalized for parent peak area) of the detected metabolites over time (Equation 4).

$$Cl_{int} = \frac{\text{Slope (normalized peak area over time)}}{[\text{Hepatocytes}]}$$

[Equation 4]

The percentage inhibition (% inhibition) of metabolite formation, used for assessing inhibitor selectivity in suspended human hepatocytes, was measured by comparing the metabolite concentrations (μM) or peak areas in samples with inhibitor ($[\text{metabolite}]_{\text{inhibitor}}$, $\text{Peak area}_{\text{inhibitor}}$) and control ($[\text{metabolite}]_{\text{control}}$, $\text{Peak area}_{\text{control}}$) samples (Equation 5). For the IVIVC, the predicted fm_{CYP} values in suspended human hepatocytes were calculated using Equation 6. Cl_{int} values applied in Equation 6 were obtained using the ratio of parent drug clearance (Equation 3) or metabolite formation (Equation 4) in the absence ($Cl_{\text{int_control}}$) and presence ($Cl_{\text{int_inhibitor}}$) of (-)-N-3-benzylphenobarbital.

$$\% \text{ inhibition} = \left(1 - \frac{[\text{metabolite}]_{\text{inhibitor}}}{[\text{metabolite}]_{\text{control}}}\right) \cdot 100\% = \left(1 - \frac{\text{Peak area}_{\text{inhibitor}}}{\text{Peak area}_{\text{control}}}\right) \cdot 100\%$$

[Equation 5]

$$\text{Fm}_{\text{CYP2C19}} = 1 - \frac{Cl_{\text{int_inhibitor}}}{Cl_{\text{int_control}}}$$

[Equation 6]

Statistical analysis

To study the relationship between the *in vitro* and *in vivo* $\text{fm}_{\text{CYP2C19}}$, a simple linear regression was performed. The linear correlation was evaluated using the RMSE (Equation 7) (Chai and Draxler, 2014). Where \hat{y}_i is the predicted value, y_i is the observed value and n is the number of datapoints.

$$RMSE = \sqrt{\frac{1}{n} \sum_i (\hat{y} - y_i)^2}$$

[Equation 7]

3. RESULTS

3.1. Assessment of the inhibitory potential of omeprazole, (+)-N-3-benzyl nirvanol, and (-)-N-3-benzylphenobarbital against CYP2C19

Optimal conditions were selected in terms of incubation time and hepatocyte concentration to ensure that metabolite formation was determined in the linear range and a probe substrate concentration below the K_m was selected to avoid saturation conditions (data not shown). Inhibitory potentials of omeprazole, (+)-N-3-benzyl nirvanol, and (-)-N-3-benzylphenobarbital against CYP2C19-mediated formation of 4-hydroxymephenytoin from S-(+)-mephenytoin (5 μ M) were evaluated by comparing IC_{50} values without and with a 3 h pre-incubation in presence of the inhibitor in suspended human hepatocytes (Figure 1, Table 1). The IC_{50} of omeprazole was 1.7 μ M. After increasing the pre-incubation time, an IC_{50} -shift was observed from 1.7 to 0.2 μ M. (+). The IC_{50} of N-3-benzyl nirvanol valued 0.2 μ M, and increased to 1.6 μ M after a 3 h preincubation. The most potent inhibitor of CYP2C19 was (-)-N-3-benzylphenobarbital with an IC_{50} value of 0.09 μ M without pre-incubation, and 0.2 μ M after a 3 h inhibitor pre-incubation. Based on the IC_{90} , concentrations of 20 μ M (omeprazole), 10 μ M ((+)-N-3-benzyl nirvanol), and 1 μ M ((-)-N-3-benzylphenobarbital) were selected for further evaluation.

3.2. Evaluation of the selectivity of omeprazole, (+)-N-3-benzyl nirvanol, and (-)-N-3-benzylphenobarbital towards CYP2C19

To evaluate the selectivity of omeprazole, (+)-N-3-benzyl nirvanol, and (-)-N-3-benzylphenobarbital towards CYP2C19, their inhibitory effect on major CYP enzymes and AO was assessed following probe substrate reactions: phenacetin O-deethylation (CYP1A2), bupropion 4-hydroxylation (CYP2B6), rosiglitazone 5-hydroxylation (CYP2C8), diclofenac 4-hydroxylation (CYP2C9), (S)-(+)-mephenytoin 4-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), midazolam 1-hydroxylation (CYP3A4), and carbazepine 4-hydroxylation (AO) in suspended human hepatocytes (1 x 10⁶ cells/mL). Selectivity against other clinically relevant non-CYP enzymes was evaluated for (-)-N-3-benzylphenobarbital. For that purpose, specific metabolite formation was

followed for estradiol (UGT1A1), trifluoperazine (UGT1A4), naloxone (UGT2B7), oxazepam (UGT2B15), sulfamethazine (NAT2), and cimetidine (FMO3).

The data showed that (-)-N-3-benzylphenobarbital (1 μ M) was the most selective inhibitor of CYP2C19. For all CYP enzymes tested, inhibition remained below 20%, with and without inhibitor pre-incubation (Figure 2, Table 2). In contrast, omeprazole (20 μ M) was not as selective since it also affected CYP1A2 (26% inhibition) and CYP2C9 (34% inhibition) (Figure 2). (+)-N-3-benzylrivanol (10 μ M) also showed not to be selective towards CYP2C19. It significantly inhibited CYP1A2 (49%), and after inhibitor pre-incubation it also impacted CYP2B6 (43%), CYP2C8 (31%), and CYP3A4 (47%) activity (Figure 2).

As (-)-N-3-benzylphenobarbital was the most promising CYP2C19 inhibitor based on CYP selectivity, its selectivity against some important non-CYP enzymes was evaluated. (-)-N-3-benzylphenobarbital (1 μ M) caused less than 10% inhibition of the evaluated non-CYP enzymes, AO (0.0%), UGT1A1 (7.3%), UGT1A4 (-6.7%), UGT2B7 (-8.2%), UGT2B15 (1.1%), NAT2 (0.8%), and FMO3 (-11.7%) (Figure 3, Table 2).

3.3.IVIVC of $fm_{CYP2C19}$

To evaluate the use of (-)-N-3-benzylphenobarbital as an *in vitro* tool to predict $fm_{CYP2C19}$, the effect of (-)-N-3-benzylphenobarbital (1 μ M) on the intrinsic clearance of a set of clinically used CYP2C19 substrates, with reported *in vivo* $fm_{CYP2C19}$ data, was evaluated in suspended human hepatocytes.

The use of the BSU hepatocyte batch demonstrated some trend of $fm_{CYP2C19}$ underprediction (RMSE = 0.12) (Table 3). A potential hypothesis is that the intrinsic activity ratio of CYP2C19 against CYP3A4 in the BSU batch is too low to be relevant. This hypothesis was tested by using a hepatocyte batch (HUP182981) with a ten-fold lower CYP3A4 activity and similar activities for other main CYP enzymes (Supplemental Table 3). Results showed the impact of intrinsic CYP activities on the fm determination (RMSE = 0.11) (Figure 4). Additional work is warranted to further identify an optimal human hepatocyte batch for phenotyping purposes. But overall, (-)-N-3-benzylphenobarbital is the most appropriate CYP2C19 inhibitor to determine CYP2C19 contribution in the metabolism of NCEs.

Table 3 provides an overview of the Cl_{int} (HUP182981) together with the predicted and observed $fm_{CYP2C19}$ for the tested compounds.

4. DISCUSSION

FDA guidelines on drug interactions have aided in routinizing the *in vitro* evaluation of the DDI potential of NCEs, decreasing late-stage attrition due to unacceptable pharmacokinetics in the presence of co-administered drugs. These guidelines cover (1) the understanding of the major elimination pathways of NCEs, (2) evaluation of the effect of NCEs on different metabolic enzymes, and (3) estimation of the involvement of different enzymes and transporters in the disposition of the NCEs (FDA, 2020). The FDA recommends to apply at least two of the following methods for reaction phenotyping to avoid false positive/negative prediction of DDIs: (1) the use of chemicals, drugs or antibodies as specific enzyme inhibitors in pooled human liver microsomes or (2) the use of individual human recombinant CYP enzymes (FDA, 2020). The fraction of metabolic clearance (part of the total drug clearance mediated by drug metabolism, $f_{CL_metabolism}$) and the fraction metabolized by a specific enzyme (fraction of the drug metabolism mediated by a specific enzyme, f_{m_CYP}) are key parameters in DDI prediction. An exponential increase of the victim drug's AUC ratio with f_{m_CYP} is observed when $f_{CL_metabolism} \times f_{m_CYP}$ exceeds 0.5. This is associated with an increased risk for toxic drug exposure after co-administration of the drug of interest with a perpetrator for DDI (Bohnert et al., 2016).

Selective and potent chemical inhibitors are available for most major CYP enzymes apart from CYP2C19. Omeprazole, the most commonly used *in vitro* CYP2C19 inhibitor, also inhibits CYP3A4, CYP2C9 (Cai et al., 2004), and UGT1A1 (Liu et al., 2011). Another commonly used and time-dependent inhibitor of CYP2C19, ticlopidine, is also a potent inhibitor of CYP2D6 and CYP2B6 (Khojasteh et al., 2011). In addition to omeprazole and ticlopidine, there are several other non-selective CYP2C19 inhibitors (nootkatone, tranlylcypromine, norfluoxetine) (Suzuki et al., 2002; Cai et al., 2004; Khojasteh et al., 2011). The discovery of (+)-N-3-benzylrivanol and (-)-N-3-benzylphenobarbital brought two promising alternative CYP2C19 inhibitors forward. Recombinant enzyme and microsomal data indicated that (-)-N-3-benzylphenobarbital and (+)-N-3-benzylrivanol are potent CYP2C19 inhibitors with (-)-N-3-benzylphenobarbital showing greater selectivity towards CYP2C19 as compared to omeprazole (Suzuki et al., 2002; Cai et al., 2004). However, a full

validation on the use of these novel CYP2C19 inhibitors in suspended human hepatocytes had not been reported. Therefore, the present study compared omeprazole, (+)-N-3-benzylrivanol and (-)-N-3-benzylphenobarbital as potent and selective inhibitors of CYP2C19, and aimed to characterize (-)-N-3-benzylphenobarbital as a tool for $f_{mCYP2C19}$ estimation in suspended human hepatocytes.

We found that in comparison to omeprazole and (+)-N-3-benzylrivanol, (-)-N-3-benzylphenobarbital showed to be the most potent inhibitor of CYP2C19 in suspended human hepatocytes. The observed IC_{50} value for (-)-N-3-benzylphenobarbital was two-fold lower than reported for human liver microsomes. Potentially this is related to differences in protein binding in these in vitro systems (Cai et al., 2004). Since suspended human hepatocyte experiments can last up to 4 h, the inhibition of CYP2C19 over the entire time-course of the experiment has to be ensured (Smith et al., 2012). Therefore, additional inhibition experiments including a 3 h inhibitor pre-incubation were carried out to select the appropriate inhibitor concentrations. Pre-incubation of the inhibitors resulted in a decrease of the IC_{50} of omeprazole for CYP2C19, pointing towards time-dependent inhibition (Shirasaka et al., 2013). An increase of the IC_{50} of (+)-N-3-benzylrivanol was potentially caused by metabolic instability of the inhibitor itself. The IC_{50} of (-)-N-3-benzylphenobarbital towards CYP2C19 remained within two-fold after a 3 h pre-incubation of the inhibitor.

Selectivity of the currently tested inhibitors towards CYP2C19 was evaluated in comparison to major CYP enzymes, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4. (-)-N-3-benzylphenobarbital (1 μ M) did not cause more than 20% inhibition of these enzymes (without and after a 3 h pre-incubation). These human hepatocytes data confirm the findings by Cai et al. (2004), which advocated the great selectivity of (-)-N-3-benzylphenobarbital in human liver microsomes (Cai et al., 2004). Their study did show a 25% inhibition of CYP2C9 at a (-)-N-3-benzylphenobarbital concentration of 6.25 μ M. However, this is not relevant to the current study as such high concentrations are not needed to achieve over 90% inhibition of CYP2C19 in suspended human hepatocytes. In contrast, omeprazole (20 μ M) inhibited CYP1A2 (26%) and CYP2C9 (34%), and to a lesser extent CYP2C8 (19%), CYP2D6 (8%), and CYP3A4 (15%). A previous study by Ko et al. (1997) demonstrated the inhibition potential of omeprazole for CYP2C9. Yet no inhibition against

CYP1A2 was observed by Ko et al. (1997), which could be explained by the lower concentrations of omeprazole applied in their study (1-5 μM) (Ko et al., 1997). Also, (+)-N-3-benzylrivanol (10 μM) inhibited all the evaluated CYP enzymes, except CYP2C9 and CYP2D6, over 20% without (CYP1A2: 49%) or after a 3 h pre-incubation (CYP1A2: 46%, CYP2B6: 43%, CYP2C8: 31%, CYP3A4: 47%). CYP3A4 inhibition by (+)-N-3-benzylrivanol was in line with previously reported data by Suzuki et al. (2002). Inhibition against CYP1A2, CYP2B6, and CYP2C8 was not observed in that study, possibly due to the use of a (+)-N-3-benzylrivanol concentration which was ten-fold lower (Suzuki et al., 2002). Based on the current comparison, we recommend (-)-N-3-benzylphenobarbital as a selective chemical inhibitor for CYP2C19 in suspended human hepatocytes over omeprazole and (+)-N-3-benzylrivanol.

As the main advantage of using human hepatocytes is the presence of the whole complement of phase I and II drug-metabolizing enzymes, the impact of (-)-N-3-benzylphenobarbital (1 μM) on AO, FMO3, NAT2, UGT1A1, UGT1A4, UGT2B15, and UGT2B7 was evaluated. (-)-N-3-benzylphenobarbital demonstrated less than 10% inhibition of these enzymes, supporting the use of (-)-N-3-benzylphenobarbital as a selective CYP2C19 inhibitor in suspended human hepatocytes. In literature, no data are available about (-)-N-3-benzylphenobarbital's selectivity against non-CYP enzymes. However, these data are important to support the use of (-)-N-3-benzylphenobarbital as a CYP2C19 inhibitor in suspended human hepatocytes.

A set of clinically applied CYP2C19 substrates was used to validate the use of (-)-N-3-benzylphenobarbital for the prediction of $\text{fm}_{\text{CYP2C19}}$. Cl_{int} values were calculated either by following parent depletion or by monitoring metabolite formation. Two tested CYP2C19 substrates, citalopram and moclobemide, were left out of the correlation since turnover was too low to follow parent depletion and not all of the metabolites could be detected by HRMS. For omeprazole, a lower concentration (0.005 μM) was incubated as omeprazole inhibits its metabolism by 20% at a concentration of 0.5 μM , resulting in an underprediction of the $\text{fm}_{\text{CYP2C19}}$ (data not shown).

When the in-house batch of human hepatocytes (BSU) was applied to predict $\text{fm}_{\text{CYP2C19}}$, it provided a trend towards $\text{fm}_{\text{CYP2C19}}$ underprediction. As CYP3A4 is involved in the metabolism of almost all of

the tested substrates, a potential hypothesis is that the BSU batch may have an impaired CYP3A4/CYP2C19 activity ratio. Another experiment was performed using a batch of human hepatocytes with lower CYP3A4 activity and similar activity for other major CYP enzymes as compared to the BSU batch (Supplemental Table 3). The results showed the impact of intrinsic CYP activities on fm determination. As expected, the use of the other hepatocyte batch resulted in an increase of the fm_{CYP2C19} for most compounds. Studies indicating that the activities of several CYP enzymes, including CYP3A4, are increased after cryopreservation of human hepatocytes, could support the choice for hepatocyte lots with lower CYP3A4 activity (Smith et al., 2012).

Another possible explanation for the underestimation of fm_{CYP2C19} is that the inhibition potency of (-)-N-3-benzylphenobarbital could depend on the substrate used to optimize the experimental conditions. A study by Foti et al. (2008) states that the use of different probe substrates for CYP2C19 may result in variable inhibition profiles. They showed that (S)-(+)-mephenytoin was the probe substrate which was most sensitive to inhibition (Foti and Wahlstrom, 2008). Therefore, it is possible that some of the clinical substrates are less sensitive to inhibition, resulting in less than 90% inhibition of the CYP2C19 metabolism and a subsequent underprediction of the fm_{CYP2C19}.

Besides the *in vitro* data, the reported *in vivo* data should also be scrutinized. Most *in vivo* fm_{CYP2C19} values are based on poor metabolizer/extensive metabolizer pharmacokinetics. To our knowledge, no literature data is available on the activity of enzymes other than CYP2C19 in CYP2C19 poor metabolizers. It is possible that the activities of these other enzymes are altered in CYP2C19 poor metabolizers, resulting in a false readout of the true fm_{CYP2C19}. However, an increase in the activity of other CYP enzymes would lead to even lower predicted fm_{CYP2C19} values. Furthermore, the *in vivo* data for some compounds are based on a limited number of subjects, implying that the data could be biased by interindividual variability.

The currently presented *in vitro* – *in vivo* fm_{CYP2C19} correlations open the floor for discussion on relevant CYP activities in human hepatocyte batches for phenotyping studies. In the future, additional research is needed to further identify the optimal human hepatocyte batch for fm_{CYP} estimation. In conclusion, this study confirms the selective CYP2C19 inhibition by (-)-N-3-benzylphenobarbital in

suspended human hepatocytes, already demonstrated using liver microsomes, supplemented with proof of selectivity against clinically relevant non-CYP enzymes (AO, FMO3, NAT2, UGT1A1, UGT1A4, UGT2B7, and UGT2B15). (-)-N-3-benzylphenobarbital is therefore the preferred CYP2C19 inhibitor to assess $fm_{CYP2C19}$ in suspended human hepatocytes over omeprazole and (+)-N-3-benzylrivanol. In an optimized batch of human hepatocytes, (-)-N-3-benzylphenobarbital (1 μ M) can be used to accurately assess the *in vivo* contribution of CYP2C19 to hepatic drug metabolism.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Inhibition profiles for the effect of omeprazole (A), (+)-N-3-benzylrivanol (B), and (-)-N-3-benzylphenobarbital (C) on the formation of 4-hydroxymephenytoin from (S)-(+)-mephenytoin (5 μ M) in suspended human hepatocytes (1×10^6 cells/mL) (BSU) without (●) and after a 3 h (▲) inhibitor pre-incubation. Points represent mean experimental data from two experiments in singlicate \pm variation (n=2). Lines represent nonlinear regression results for four-parameter fits calculated with GraphPad Prism 7.04 from GraphPad software (San Diego, California, US).

Figure 2. Effect of omeprazole (20 μ M), (+)-N-3-benzylrivanol (10 μ M), and (-)-N-3-benzylphenobarbital (1 μ M) on major CYP enzymes (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4) and AO in suspended human hepatocytes (1×10^6 cells/mL) (BSU) without (A) and after a 3 h (B) inhibitor pre-incubation. Bars represent mean values \pm SD. (two experiments in triplicate, n=6) The dotted line represents 20% inhibition.

Figure 3. Effect of (-)-N-3-benzylphenobarbital (1 μ M) on major non-CYP enzymes (FMO3, NAT2, UGT1A1, UGT1A4, UGT2B7, UGT2B15) in suspended human hepatocytes (1×10^6 cells/mL) (BSU). Bars represent values from one single experiment performed in singlicate. The dotted line represents 20% inhibition.

Figure 4. IVIVC between observed *in vivo* (literature) and $fm_{CYP2C19}$ predicted from *in vitro* incubations. Points represent data from one experiment and two different hepatocyte batches: BSU (▲), HUP182981 (●). Dotted lines indicate 10% (green) and dashed line 20% (red) absolute error. (RAB: rabeprazole; PHE: phenytoin; CLO: clobazam; DIA: diazepam; LAB: labetalol; NOR: norclobazam; LAN: lansoprazole; PAN: pantoprazole; OME: omeprazole; MEP: (S)-(+)-mephenytoin)

TABLES

Table 1. Experimentally determined mean IC_{50} values (two experiments in singlicate, $n=2$) of omeprazole, (+)-N-3-benzylirvanol, and (-)-N-3-benzylphenobarbital on 4-hydroxymephenytoin formation from (S)-(+)-mephenytoin (5 μ M) in suspended human hepatocytes (1×10^6 cells/mL) (BSU) without and after 3 h inhibitor pre-incubation.

	IC_{50} (μ M)	IC_{50}
	Without pre-incubation	With pre-incubation
Omeprazole	1.67	0.209
(+)-N-3-Benzylirvanol	0.166	1.592
(-)-N-3-Benzylphenobarbital	0.0892	0.169

Table 2. Mean percentage inhibition (two experiments performed in triplicate, n=6) \pm SD of enzyme activity by (-)-N-3-benzylphenobarbital (1 μ M) in suspended human hepatocytes (1 x 10⁶ cells/mL) (BSU) without and after a 3 h inhibitor pre-incubation. *Mean percentage inhibition (results from a single experiment performed in singlicate, n=1)

Substrate	Enzyme	% inhibition	% inhibition
		30 min pre-incubation	3 h pre-incubation
Carbazeran	AO	-3.97 \pm 7.09	3.05 \pm 6.73
Phenacetin	CYP1A2	-0.567 \pm 2.72	-9.41 \pm 3.53
Bupropion	CYP2B6	-2.40 \pm 6.43	-6.86 \pm 12.2
Rosiglitazone	CYP2C8	0.502 \pm 10.4	10.9 \pm 8.3
Diclofenac	CYP2C9	-0.596 \pm 6.87	4.37 \pm 4.70
(S)-(+)-Mephénytoin	CYP2C19	89.9 \pm 0.8	94.7 \pm 0.5
Dextromethorphan	CYP2D6	-2.49 \pm 3.81	2.57 \pm 3.31
Midazolam	CYP3A4	3.50 \pm 6.60	-11.0 \pm 10.0
Cimetidine	FMO3	-11.7*	/
Sulfamethazine	NAT2	0.757*	/
β -Estradiol	UGT1A1	7.26*	/
Trifluoperazine	UGT1A4	-6.76*	/
Oxazepam	UGT2B15	1.08*	/
Naloxone	UGT2B7	-8.24*	/

Table 3. Determination of CYP2C19 contribution to the metabolic clearance, based on parent depletion or metabolite formation (*), obtained in suspended human hepatocytes (1×10^6 cells/mL) (BSU and HUP182981) for ten marketed drugs using (-)-N-3-benzylphenobarbital ($1 \mu\text{M}$). *In vitro* $\text{fm}_{\text{CYP2C19}}$ values from a single experiment calculated using Equation 6.

Compound	Fm_{CYP2C19} (%)			Reference
	<i>In vitro</i>	<i>In vitro</i>	<i>In vivo</i>	
	BSU	HUP182981	(observed)	
Rabeprazole	27.9	24.9	16	(Sakai et al., 2001)
Phenytoin*	6.45	10.9	20	(Patsalos, 2013)
Clobazam*	18.1	31.1	21	(Walzer et al., 2012)
Diazepam*	25.7	44.2	43	(Sohn et al., 1992)
Labetalol	36.6	39.0	66	(Chan et al., 2013)
Norclobazam*	67.5	74.9	68	(Walzer et al., 2012)
Lansoprazole	47.2	61.2	73	(Sakai et al., 2001)
Pantoprazole	74.6	71.9	83	(Tanaka et al., 1997)
Omeprazole	71.0	76.8	86	(Sakai et al., 2001)
(S)-(+)-Mephenytoin	88.9	77.9	90	(Yao et al., 2003)

Figure 1

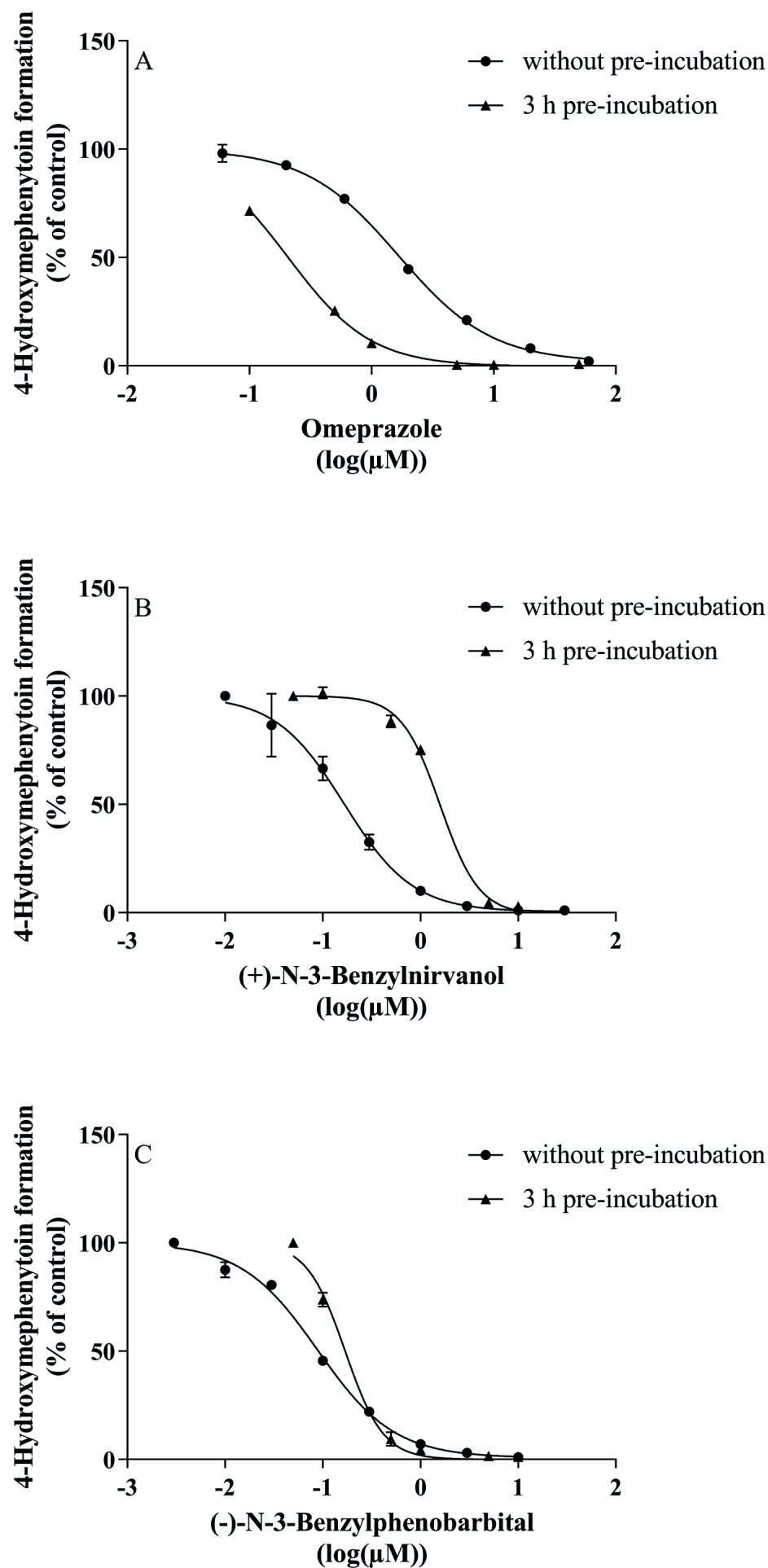


Figure 2

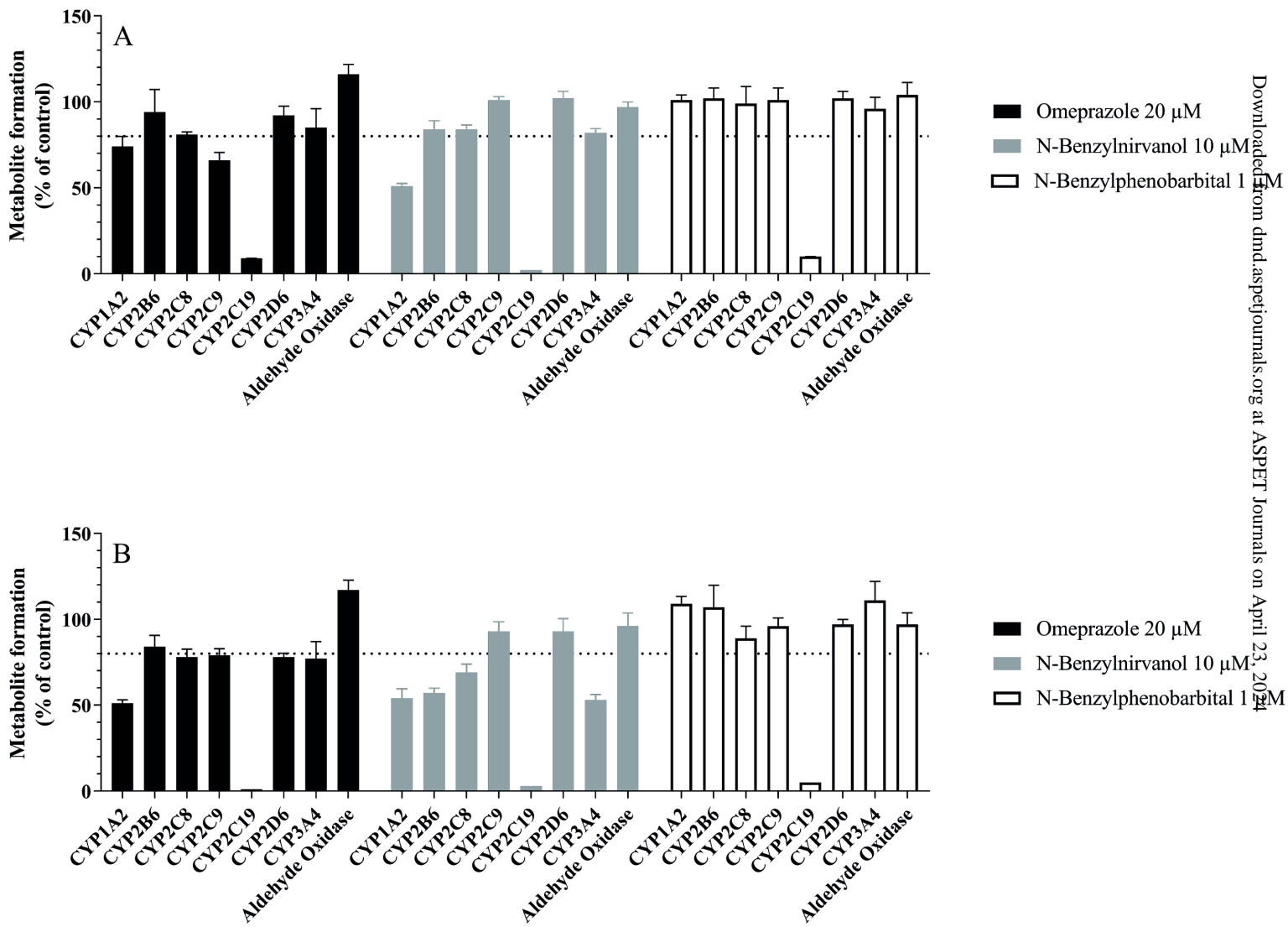


Figure 3

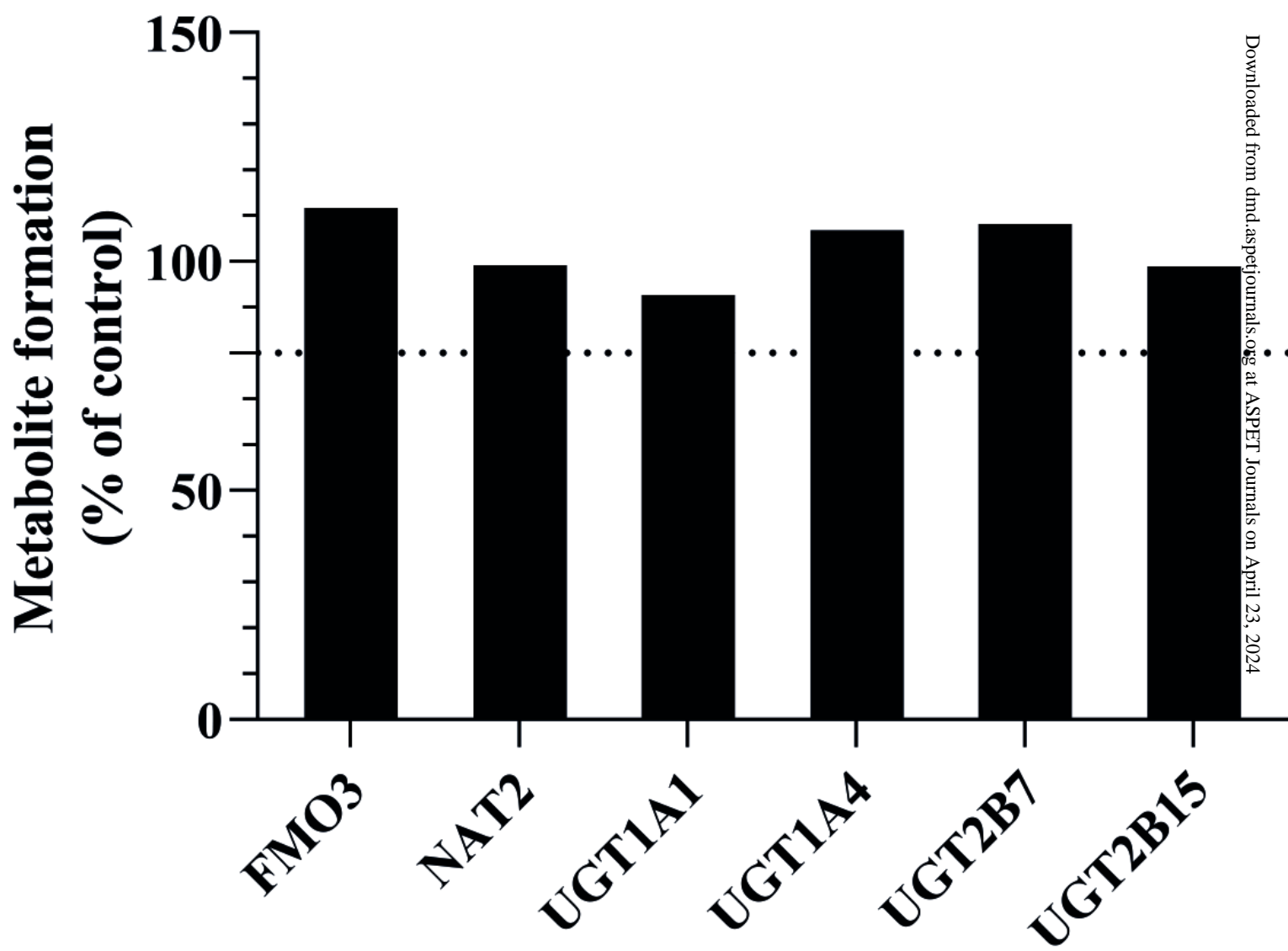


Figure 4

