ARTICLE | OPEN ACCESS

**Drug Discovery**

**To Cite:**
Golovenko M, Valivodz I, Reder A, Larionov V. Propoxazepam drug-drug interaction, mediated by cytochrome 450 2d6 (preclinical *in vitro* data). *Drug Discovery* 2025; 19: e5dd2047
doi: https://doi.org/10.54905/dissi.v19i43.e5dd2047

**Author Affiliation:**
[1]Doctor of Biological Sciences, Head of Department of Biomedicine, A.V. Bogatsky Physical-Chemical Institute of National Academy of Sciences of Ukraine, 65080, 86 Lyustdorfs`ka road, Odesa, Ukraine
[2]PhD in Biological Sciences, Junior Researcher, Department of Biomedicine, A.V. Bogatsky Physical-Chemical Institute of National Academy of Sciences of Ukraine, 65080, 86 Lyustdorfs`ka road, Odesa, Ukraine
[3]PhD in Chemical Sciences, General Director, SLC "Interchem", 65080, 86 Lyustdorfs`ka road, Odesa, Ukraine
[4]PhD in Biological Sciences, Senior Researcher; Head of Laboratory of Molecular Pharmacology and Medicine, Department of Biomedicine, A.V. Bogatsky Physical-Chemical Institute of National Academy of Sciences of Ukraine, 65080, 86 Lyustdorfs`ka road, Odesa, Ukraine

*Corresponding Author
PhD in Biological Sciences, Senior Researcher; Head of Laboratory of Molecular Pharmacology and Medicine, Department of Biomedicine, A.V. Bogatsky Physical-Chemical Institute of National Academy of Sciences of Ukraine, 65080, 86 Lyustdorfs`ka road, Odesa,
Ukraine
Email: vitaliy.larionov@gmail.com

**ORCID List**
Mykola Golovenko 0000-0003-1485-128X
Iryna Valivodz 0000-0001-7465-7089
Anatoliy Reder 0000-0002-1801-8378
Vitalii Larionov 0000-0003-2678-4264

**Peer-Review History**
Received: 17 October 2024
Reviewed & Revised: 21/October/2024 to 17/February/2025
Accepted: 21 February 2025
Published: 27 February 2025

**Peer-Review Model**
External peer-review was done through double-blind method.

Drug Discovery
pISSN 2278–540X; eISSN 2278–5396

© The Author(s) 2025. Open Access. This article is licensed under a Creative Commons Attribution License 4.0 (CC BY 4.0)., which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

DISCOVERY SCIENTIFIC SOCIETY

# Propoxazepam drug-drug interaction, mediated by cytochrome 450 2d6 (preclinical *in vitro* data)

**Mykola Golovenko**[1], **Iryna Valivodz**[2], **Anatoliy Reder**[3], **Vitalii Larionov**[4*]

## ABSTRACT

The relevance of this study is the need to study potential drug interactions of the new analgesic propoxazepam, which belongs to the benzodiazepine group. Benzodiazepines are widely used for their anxiolytic and analgesic properties, and understanding their metabolic interactions is critical for safe clinical use. This study aimed to examine the effect of propoxazepam on CYP2D6 enzyme activity in human liver microsomes, a key enzyme in the metabolism of various drugs. The impact of propoxazepam on the 1-hydroxylation of bufuralol was analyzed using in vitro methods with human liver microsomes. This study involved adding propoxazepam at varying concentrations (0 to 100 μM) to the microsomal preparations along with the substrate and cofactors, specifically NADPH. It was established that propoxazepam consistently inhibited CYP2D6 activity, with the "concentration-activity dependence" for both reversible and metabolism-dependent inhibition being similar: 67.5 ± 4.2 μM for reversible inhibition and 73.8 ± 3.3 μM for metabolism-dependent inhibition. In addition, pharmacokinetic data showed that the predicted maximum unbound plasma propoxazepam concentration that could lead to significant interactions is ≥0.675 μM (approximately 0.275 μg/mL). Importantly, this concentration would not be reached after a single oral dose, suggesting that significant inhibition of CYP2D6 is unlikely in a clinical setting. The practical value of this work lies in the potential use of the findings by healthcare professionals and pharmacists to assess the safety of propoxazepam application in clinical practice, ultimately aiding in better management of patient therapies involving multiple medications.

**Keywords:** Propoxazepam, bufuralol, quinidine, paroxetine, reversible inhibition, metabolism dependent inhibition

## 1. INTRODUCTION

Pharmacokinetic interactions between drugs, commonly known as drug-drug interactions (DDIs), play a crucial role in combined drug therapy, significantly influencing both therapeutic efficacy and toxicity. Although these interactions are often unpredictable, understanding their mechanisms has advanced significantly over the past few decades. Notably, researchers Pang et al., (2010) have emphasized the importance of molecular and analytical methodologies in elucidating the complex biochemical pathways involved in drug metabolism and transport. Researchers have identified the substances termed 'perpetrators' of pharmacokinetic drug-drug interactions among medications that inhibit or induce the enzymes responsible for drug metabolism, particularly the cytochrome P450 (CYP) enzymes.

These perpetrators can clinically alter the clearance rates of associated object drugs. CYP2D6, which accounts for approximately 2% of total cytochrome P450 in the liver, is involved in the metabolism of nearly 20% of drugs undergoing biotransformation. Studies Paine et al., (2006) highlight the clinical importance of CYP2D6, highlighting its involvement in the metabolism of various drugs, including tricyclic antidepressants and beta-blockers. Polymorphisms in the CYP2D6 gene result in diverse metabolic phenotypes among individuals, with poor metabolizers primarily carrying specific genotypes, such as *3, *4, *5, and *6. In contrast, ultra-rapid metabolizers often have multiple copies of *1, *2, or *35 alleles (Cicali et al., 2020).

Inhibitors of CYP2D6 can substantially elevate the plasma levels of certain drugs, leading to adverse effects. For instance, potent inhibitors like fluoxetine and paroxetine can nearly completely suppress CYP2D6 activity. However, the studies showed that CYP2D6 genetic deficiencies do not significantly alter plasma concentrations of substrates in patients using CYP2D6 inhibitors. It was noted Zhou, (2009) that, poor metabolizers are less likely to experience significant DDIs mediated by CYP2D6 when exposed to potent inhibitors, while individuals with extensive or ultra-rapid metabolism may still be at risk.

In this context, evaluating of the CYP2D6-mediated metabolic profile and the potential inhibitory effects of new chemical entities becomes essential. Propoxazepam, a promising new medication currently undergoing clinical trials in Ukraine, exhibits similarities to gabapentinoids, which are well-known for their analgesic properties due to their effects on the GABAergic system. In the study Golovenko et al., (2017) there have established the involvement of GABAergic pathways in the analgesic and anticonvulsant actions of propoxazepam, emphasizing its therapeutic potential.

Given the need for information on DDIs for subsequent phases of clinical trials, it is critical to predict possible interactions based on the results of earlier studies, especially those assessing the safety and pharmacokinetic profiles of propoxazepam. Despite this need, inhibition of CYP2D6 activity by propoxazepam has not yet been studied. Therefore, this study aims to evaluate the effects of propoxazepam on CYP2D6 activity *in vitro*, utilizing bufuralol as a classical marker of CYP2D6 metabolic activity in human liver microsomes.

## 2. MATERIALS AND METHODS

### Chemical and tissue source

The synthesis of propoxazepam and 3-hydroxy derivative (7-bromo-5-(2chlorophenyl)-3-hydroxy-1,3dihydro-2H-1,4-benzodiazepin-2-one, major metabolite) was made according to the method. The internal standard Propoxazepam-D7 ($C_{18}H_9BrClD_7N_2O_2$) was supplied by SLC Interchem (purity ≥98.0%, MM 414.73 g/mol). The study used all reagents of analytical grade (or suitable alternatives), primarily sourced from VWR International Ltd (United Kingdom), Rathburn Chemicals Ltd, Sigma Aldrich Chemical Company Ltd (Germany), and Fisher Scientific UK Limited (United Kingdom), unless mentioned separately.

Human liver microsomes (HLM, highly characterized for catalytic activity measuring necessary metabolizing CYPs enzymes, 150 donors, 300 alleles for diploid genes) were received from Corning Ultra Pool HLM 150 (Lot 38292 Corning® UltraPool™ Microsome Hu Liver 150 Donor Pool, Merck, Germany). The experimental part of the study took place at the Labcorp Early Development Laboratories Ltd. (Otley Road, Harrogate, UK) from January to June 2021.

### Incubation conditions

Bufuralol hydroxylation was used as a marker for CYP2D6 mediated activity. Stock solutions of propoxazepam were prepared in dimethylsylfoxide (DMSO) and diluted in the incubation mixtures to ensure the final organic solvent concentration did not exceed 0.5%

(v/v). The formation of all metabolites was quantified by ultra-performance liquid chromatography (UPLC) with mass detection. To determinate the inhibitory potential of propoxazepam, HLM were incubated in triplicate with isoform-selective probe substrates, NADPH and propoxazepam at concentrations ranging from 0.1 to 100 μM. After equilibration, reactions were initiated by adding NADPH. The incubations were performed at 37°C and terminated after the appropriate incubation period by adding methanol, containing an internal standard.

The samples were then centrifuged for 5 minutes to sediment the precipitated protein. Substrate concentrations approximated to the Km for CYP2D6. Vehicle samples contained an equivalent volume of the appropriate solvent instead of propoxazepam or the positive control. Metabolism-mediated inhibitory potential was investigated using a similar procedure, except that the HLM were pre-incubated for 30 minutes at ca. 37°C, in triplicate, with propoxazepam (over the same 0.1-100 μM concentration range) and NADPH before the addition of the CYP marker substrate (at a concentration which approximated to the Km). Paroxetine (10 μM), a metabolism dependent inhibitor, and quinidine (1 μM), a probe for reversible inhibition, were used as positive controls to assess the effects on CYP2D6.

CYP enzyme activity for the pre-incubated samples in the presence of propoxazepam was compared to that of samples incubated in its absence. The nonspecific binding of Propoxazepam to human microsomes was also analysed to estimate its potential impact on Propoxazepam concentration determination at nominal concentrations 0.1; 10 and 100 μM. Equilibrium dialysis determined the microsomal binding of propoxazepam. All incubations were carried out on an orbital shaker (200 rpm) placed within an incubator maintained at ca. 37°C and 5% CO2/95% air. HLM were diluted with assay buffer to protein concentrations of 0.01, 0.05, and 1 mg/mL and spiked with propoxazepam at concentrations of 0.1, 10, and 100 μM.

Triplicate microsome samples with added Propoxazepam were then dialyzed against assay buffer at 37°C/5% CO2 for 6 hours. Aliquots of the stock spiked microsomes, along with samples from the protein and buffer chambers, underwent analysis using a qualified liquid chromatography with tandem mass spectrometry (LC-MS/MS) method and the concentration of propoxazepam was calculated. The propoxazepam recovery from the apparatus was also calculated. Quality Control In each analytical run, the following QC samples were analyzed in duplicate as a minimum:

Low range: 0.3 ng/mL (LoQC), 6 ng/mL (Lo-MeQC), 40 ng/mL (MeQC), 200 ng/mL
(Hi-MeQC) and 400 ng/mL (Hi-QC).

High range: 30 ng/mL (LoQC), 600 ng/mL (Lo-MeQC), 4000 ng/mL (MeQC),
20000 ng/mL (Me-HiQC) and 40000 ng/mL (HiQC).

**Measurement of drug concentrations**

The formation of all metabolites was quantified by LC-MS/MS in Multiple Reaction Monitoring (MRM) mode using electrospray ionization (ESI) as the ionisation technique, according to the following steps:

Preparation of Calibration Standards (Calibration standard working solutions were freshly prepared. An aliquot of 50 μL (low range) or 10 μL (high range) of these standards was added to a 2 mL 96-well microplate. A corresponding volume of water was added to the reagent blank sample).

Addition of Internal Standard (An internal standard solution (Propoxazepam-D7) at 10 ng/mL (low range) or 500 ng/mL (high range) in acetonitrile (25 μL) was added to all samples, calibration standards, and QC samples.

Vortex Mixing (All samples were vortex mixed at 2000 × g for 10 minutes to ensure proper mixing of components).

Acetonitrile Addition (Acetonitrile was added (150 μL for low range or 400 μL for high range) to all samples, followed by a second mixing at 2000 × g for 10 minutes).

Centrifugation (Samples were centrifuged at 2000 × g for approximately 5 minutes at 5°C to separate the phases).

Transfer of Supernatant (Aliquots of the supernatant (80 μL for low range or 20 μL for high range) were transferred to a clean 96-well microplate. Acetonitrile (60 μL) was added only to high range samples).

Addition of Ammonium Formate (A solution of 10 mM ammonium formate (aqueous) mixed with formic acid (100:0.2 v/v) was added to all samples (120 μL)).

Final Centrifugation and Storage (Samples were spun in a centrifuge at 2000 × g for approximately 5 minutes at 5°C, then stored at 2-8°C (nominally 4°C) before LC-MS/MS analysis).

Low-range calibration curves (0.1 to 500 ng/mL) and high-range curves in the range (10 to 50,000 ng/mL) were constructed by adding known amounts of Propoxazepam, with the extraction of these standards alongside each batch of study samples. The

calibration curves were calculated by quadratic weighted (1/x2) least squares regression analysis, with the peak area ratio plotted against analyte concentration.

The binding of Propoxazepam to microsomes was assessed using equilibrium dialysis with the HTDialysis system. The dialysis setup consisted of Teflon bars in an aluminum clamp, featuring individual cellulose dialysis membrane strips with a molecular weight cut-off of 12-14 kDa. This design enabled loading and sampling from both sides of the membrane. Incubations were performed on an orbital shaker at approximately 200 rpm within an incubator set to about 37°C with a gas mixture of 5% CO2 and 95% air.

Human liver microsomes were diluted in assay buffer to achieve protein concentrations of 0.01, 0.05, and 1 mg/mL and then spiked with Propoxazepam at concentrations of 0.1, 10, and 100 µM. Triplicate samples of the spiked microsomes underwent dialysis against assay buffer at around 37°C and 5% CO2 for 6 hours. Subsequently, analysts analyzed aliquots from the stock-spiked microsomes, along with samples from both the protein and buffer chambers, using a validated LC-MS/MS method, which allowed for the calculation of propoxazepam concentrations. Additionally, the recovery of Propoxazepam from the dialysis apparatus was determined.

**Data analysis**

The activity of enzyme, in the presence of various concentrations of propoxazepam, was expressed as a percentage of the appropriate control activity. The IC50 (the concentration at which CYP probe substrate activity decreased by 50%) was calculated by non-linear regression using validated Sigma Plot software (Version 12.5, Systat Software Inc).

Due to sub-optimal data fits, the data for the CYP2D6 reversible and metabolism-dependent inhibition assays were fitted to a 3-parameter equation without the background function (1):

$$y = \frac{Range}{1+\left(\frac{x}{IC_{50}}\right)^{2}} \quad (1)$$

Range = the maximum $y$ range, i.e., control conversion rate (no inhibitor), $s$ = slope factor, $y$ = conversion rate of probe substrate to metabolite, $x$ = propoxazepam concentration.

The extent of microsomal binding, determined using the equilibrium dialysis method, was calculated from the following equations (2)-(4):

$$\%\text{Bound} = \frac{Cp-Cb}{Cp} \times 100 \quad (2)$$

$$\%\text{Free fraction} = \frac{Cb}{Cp} \times 100 \quad (3)$$

$$\%\text{ Recovery} = \frac{(CpVp+CbVb)}{CpiVp} \times 100 \quad (4)$$

Where Cp = concentration in protein compartment, Cb = concentration in buffer compartment, Cpi = initial concentration in spiking solution, Vp = volume in protein compartment, Vb = volume in buffer compartment

Assuming enzyme competitive inhibition (Ki) can be estimated as follows:

IC50=Ki (1+ S/Km)

If [S] = Km then IC50 = 2Ki

All substrate concentrations used in the current study approximated the Km.

Results were presented as mean ± standard error of the mean (SEM). Statistical analysis used Student t-test with a significance level of $p \leq 0.05$. This study ensures full compliance with the ethical norms governing human-derived biological research.

## 3. RESULTS

The data demonstrate that propoxazepam at multiple (0 to 100 µM) concentrations consistently inhibited the activities of CYP2D6 (Table 1). To determine the possible effect of Propoxazepam on reversible inhibition of CYP2D6 it was incubated with microsomal fraction and corresponding substrate (bufuralol). The concentration-activity inhibition dependence was found to be similar, with IC50: 67.5 ± 4.2 µM reversible inhibition and IC50: 73.8 ± 3.3 µM for metabolism-dependent inhibition. Positive controls quinidine one µM (for reversible inhibition) and paroxetine (10 µM) (for metabolism-dependent inhibition) demonstrated the expected CYP2D6 activity

inhibition, reducing activity to 22,8 % and 5,6 %, respectively, compared to the control. The data demonstrate that propoxazepam at multiple (0 to 100 μM) concentrations consistently moderately inhibited the activities of CYP2D.

**Table 1** Effect of Propoxazepam on CYP2D6-mediated bufuralol 1-hydroxylation in human liver microsomes: reversible and metabolism-dependent inhibition.

| Propoxazepam concentration, μM | Activity (to control), % | |
|---|---|---|
| | Reversible inhibition | Metabolism-dependent inhibition |
| 0.1 | 112±6.8 | 105.6±3.4 |
| 0.3 | 109.9±7.9 | 108.4±4.2 |
| 1 | 114.6±11.4 | 106.7±5.3 |
| 3 | 106.9±6.1 | 102±3.8 |
| 10 | 100.2±4.5 | 96.2±5.2 |
| 30 | 83.2±3.6 | 81.3±4.1 |
| 60 | 61±3.1 | 61.7±2.5 |
| 100 | 40.8±2.5 | 42.6±3.1 |
| positive control | 22.7±1a | 5.6±0.5b |

Notes: a - Quinidine, 1.0 μM, b - Paroxetine, 10 μM

Source: compiled by the authors

The data were used to estimate the calculated IC50 values and the inhibition constant (Ki). To characterise the potential of Propoxazepam for 2D6 inhibition, the IC50 shift (the ratio of IC50 values for reversible and metabolism-dependent inhibitions was also calculated, along with the unbound plasma concentrations of Propoxazepam above which inhibition is possible (Table 2). The results of the microsomal binding experiment are summarized in (Table 3). The data indicate that microsomal binding was not notably dependent on propoxazepam concentration but on microsomal protein concentration. Mean free fractions at microsomal protein concentrations of 0.01, 0.05, and 1 mg/mL were 103 ± 5%, 88.5 ± 4.7%, and 30.3 ± 5.7%, respectively, across the propoxazepam concentration range used.

**Table 2** Parameters of Propoxazepam reversible and metabolism-dependent inhibitions *in vitro* (the values of IC50, M±m)

| Substrate | Inhibition concentration, IC50, μM | | IC50 shift (IC50 reversible/IC50 metabolism-dependent) | Inhibition constant, Ki, μM | Unbound plasma concentration | |
|---|---|---|---|---|---|---|
| | Reversible inhibition | Metabolism dependent | | | μM | μg/mL |
| Bufuralol | 67.5±4.2 | 73.8±3.3 | 0.91 | 33.75±2.1 | 0.675 | 0.275 |

Source: compiled by the authors

## 4. DISCUSSION

The cytochrome P450 (CYP) superfamily of enzymes, represented by a variety of isoforms, is responsible for the oxidative and reductive metabolic transformation of medications (Zhao et al., 2021). One of these isoenzymes, CYP 2D6, is not abundant in the human liver (its quantity doesn't exceed ~2% of the total CYP content), but it participates in the biotransformation of more than 20% of drugs (Lam and Scott, 2019). Considering that CYP2D6 is the only non-inducible enzyme Lam and Scott, (2019), its inhibition can lead to the accumulation of unmetabolized initial drug and an increased risk of side effects. Propoxazepam (a 1,4-benzodiazepine derivative) is a novel analgesic that simultaneously inhibits acute and chronic pain with anti-inflammatory and anticonvulsant components and currently passes the initial phases of clinical studies.

The initial stage of interaction between a drug and a cytochrome P450 (CYP) involves the binding process. When a ligand binds to a CYP enzyme, it often causes a shift in the UV-visible absorbance spectrum, typically observed in the Soret band. This shift occurs when

the iron transitions from a resting low-spin state to a high-spin state, associated with at least a partial loss of the H2O ligand from the iron; this is referred to as a type I change (λmax ~390 nm). In contrast, type II change involves the formation of a low-spin iron complex bound to a nitrogen atom of a ligand (λmax ~430 nm) (Pelletier et al., 2023). These changes can help characterize the binding affinity between CYP and its ligands.

Previous studies Golovenko et al., (2023) showed that the interaction of both propoxazepam and its 3-hydroxy metabolite with rat liver cytochrome P450 (CYP) resulted in the second type of spectral changes in the hemoprotein. Their binding constants varied significantly, indicating the potential for substrate interaction with distinct regions of the CYP. Quantitative indicators of the inhibitory activities of propoxazepam and metabolite, using the method of differential spectroscopy are only indicative in nature, but still, they can suggest at least a significant possibility of inhibitory interaction in clinical use of drugs. To better understand the interactions between propoxazepam and CYP at the molecular level, docking analysis of the drug with the CYP2C8 isoform was performed.

Propoxazepam exhibits relatively high values (8.15-9.8 cal/mole) for the free energy of interaction with various CYP isoenzymes, including 1A2, 2B6, 2C9, 2C19, 2D6, and 3A4. However, there are differences in the number of standard amino acid residues involved in interactions with individual substrates. Based on the binding energy values, it can be inferred that propoxazepam has the lowest competitive (inhibitory) effect on the 3A4 isoform (with testosterone as the substrate) and 2D6. The analysis of propoxazepam's interactions with different CYP isoenzymes suggests the potential for competitive interactions with 1A2, 2C19, and 2C8, to a lesser extent with 2C9, 3A4, and 2B6. Additionally, it is anticipated that propoxazepam may also act as a substrate for these CYP isoforms (Larionov et al., 2023).

Bufuralol and dextromethorphan are recommended by the Guideline on the investigation of drug interactions (EMA, 2012). Approximately 60% of the regulatory submissions from the pharmaceutical industry use bufuralol hydroxylation as the marker reaction to determine CYP2D6 inhibition potency *in vitro*, whereas approximately 30% use dextromethorphan O-demethylation (Yuan et al., 2002). Bufurulol undergoes metabolism in humans, resulting in the formation of metabolites that have 1-adrenoceptor blocking activity (Pringle et al., 1986). The aliphatic hydroxylation of bufuralol is under polymorphic control, similar to that shown for alicyclic hydroxylation of debrisoquine (Dayer et al., 1986). Thus, Propoxazepam LC-MS/MS-based CYP2D6 inhibition assay using selective substrate (bufuralol) was undertaken.

The potential inhibition of CYP2D6 by propoxazepam was assessed in two variants, allowing for the evaluation of both reversible and metabolism-dependent types of inhibition. Inhibition can occur due to the drug itself (propoxazepam) or due to the metabolite produced during the CYP catalytic cycle. Propoxazepam can inhibit directly, either as a direct or time-dependent inhibitor. In contrast, the metabolite can cause inhibition as mechanism-dependent (either reversible or irreversible) or quasi-irreversible. Optimal conditions for incubation time and HLM concentration were determined to ensure that metabolite formation was measured within the linear range. Additionally, a probe substrate concentration below the Km value was selected to avoid saturation.

Initial experiments involved incubating each substrate probe at a concentration near its respective Km value, while varying propoxazepam concentrations from 0 to 100 μM. Propoxazepam showed similar concentration-dependent 2D6 activity inhibition with IC50 67.5 ± 4.2 μM (reversible) and IC50 73.8 ± 3.3 μM (for metabolism-dependent inhibition) (Table 1). Positive controls, quinidine (1 μM) for reversible inhibition and paroxetine (10 μM) for metabolism-dependent inhibition, demonstrated the expected CYP2D6 activity inhibition to 22,8 % and to 5,6 %, respectively, compared to the control. The calculated indicator of the possible metabolism dependent inhibition, the shift in the IC50 ratio (ratio of reversible and metabolism-dependent) is less than 1, which demonstrate that the metabolism dependent inhibition is unlikely for 2D6.

For the reversible inhibition variant, the Ki was also calculated (Table 1), since it reflects the binding affinity and often used by investigators to characterise the functional strength of the inhibitor (Bachmann and Lewis, 2005). Regulatory guidance indicates EMA, (2012) that drug developers should account for nonspecific binding in microsomes if it is anticipated to impact the analysis of kinetic data. Assessing microsomal protein binding helps clarify the relationship between *in vitro* metabolism and *in vivo* pharmacokinetics. This information is crucial because it provides insight into how the drug is distributed and eliminated in the body, which is essential for developing its drug metabolism and pharmacokinetics (DMPK) profile, as well as assessing its potential for drug-drug interactions.

The non-specific binding of Propoxazepam human microsomes have also been analyzed to incorporate the unbound fraction in microsomes, which is necessary for predicting CYPs inhibition potential and determining meaningful drug concentrations (Rostami-Hodjegan and Tucker, 2007). Propoxazepam binding to microsomal protein (Table 3) was low when incubated under conditions that reflected those in the IC50 experiments. Therefore, no microsomal binding correction factor was applied to the reported IC50 values.

According to the 2013 EMA guidance EMA, (2012), "an *in vivo* drug-drug interaction study with a sensitive probe substrate is recommended when [I]/Ki ≥0.02, where [I] is the unbound mean Cmax value obtained during treatment with the highest recommended dose".

**Table 3** *In vitro* determination of binding of Propoxazepam (0.1, 10 and 100 μM) following dialysis of spiked human liver microsomes for 6 hours

| Group | Nominal concentration, μM | HLM, mg/ml | Actual concentration, μM | Donor concentration, μM | Acceptor concentration, μM | Fraction bound, % | Fraction unbound, % | Fraction unbound, mean value |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.1 | 0.01 | 0.045 ± 0.002 | 0.018 ± 0.002 | 0.019 ± 0.001 | -6.2 ± 2.3 | 106.1 ± 7.1 | 103.6 ± 3.2 |
| | 10 | | 8.2 ± 0.3 | 3.3 ± 0.2 | 3.6 ± 0.6 | -7.2 ± 2.8 | 107.4 ± 3.5 | |
| | 100 | | 85.9 ± 2.5 | 36.8 ± 3.4 | 35.7 ± 2.5 | 2.7 ± 2.6 | 97.3 ± 1.4 | |
| 2 | 0.1 | 0.05 | 0.046 ± 0.001 | 0.021 ± 0.001 | 0.018 ± 0.001 | 6.8 ± 1.9 | 93.2 ± 2.3 | 88.5 ± 2.7* |
| | 10 | | 8.6 ± 0.3 | 4.2 ± 0.6 | 3.7 ± 0.6 | 11.7 ± 3.6 | 88.3 ± 3.5 | |
| | 100 | | 85.5 ± 1.1 | 39.5 ± 5.1 | 32.9 ± 2.2 | 16.1 ± 4.7 | 83.9 ± 3.3 | |
| 3 | 0.1 | 1 | 0.049 ± 0.001 | 0.035 ± 0.009 | 0.011 ± 0.002 | 70.3 ± 4.5 | 29.7 ± 2.6 | 30.3 ± 3.3** |
| | 10 | | 8.2 ± 0.2 | 6.5 ± 0.3 | 1.6 ± 0.3 | 75.1 ± 6.4 | 24.9 ± 3.5 | |
| | 100 | | 98.8 ± 4.1 | 65.1 ± 11.7 | 22.3 ± 5.7 | 63.7 ± 16.5 | 36.3 ± 9.5 | |

Notes: HLM: human liver microsomes; * - statistically significant at p≤0.05 (in compare to group 1); ** - statistically significant at p≤0.01 (in compare to group 1)
Source: compiled by the authors

So, the highest predicted unbound Cmax plasma concentration of Propoxazepam, above which the interaction can take place, is higher than 0.675 μM (or 0.275 μg/mL based on the Propoxazepam molecular weight 414.73 g/mol). As our data show Golovenko et al., (2021), the unbound Propoxazepam fraction in human plasma is 1.96 %, so the total concentration, where the inhibition can be expected, is 14282 ng/mL (or 14.28 μg/mL).

Propoxazepam pharmacokinetic data Golovenko et al., (2023) show that oral single dose administration leads to Cmax 22.276 ng/ml in blood Tmax ~ 4 hours. This is low compared to the predicted level of 14,282 ng/mL for potential inhibition. Even with multiple administration, it is unelickely that the steady-state concentration will reach this level. Although the additional pharmacokinetic studies for multiple administration are needed, the data obtained suggest that propoxazepam inhibition of CYP2D6 is unlikely.

## 5. CONCLUSIONS

This study aimed to evaluate the effects of Propoxazepam on CYP2D6 activity *in vitro*, utilizing bufuralol as a classical marker of CYP2D6 metabolic activity in human liver microsomes. The findings revealed that Propoxazepam exhibits inhibitory effects on CYP2D6, with calculated IC50 values of 67.5 ± 4.2 μM for reversible inhibition and 73.8 ± 3.3 μM for metabolism-dependent inhibition. Notably, the predicted unbound plasma Cmax concentration necessary for clinically relevant drug interactions with co-administered CYP2D6 substrates was established at ≥0.675 μM (approximately 0.275 μg/mL), corresponding to a total blood concentration of 14,282 ng/mL.

These results indicate that while Propoxazepam can inhibit CYP2D6, the observed concentrations in pharmacokinetic studies suggest that clinically significant interactions are unlikely during standard dosing regimens. This finding is particularly relevant for clinicians when considering Propoxazepam in treatment plans that involve other medications metabolized by CYP2D6, thereby potentially reducing the risk of adverse drug interactions. The practical implications of this work highlight the importance of understanding drug metabolism to ensure the safe and effective use of pharmacological agents.

Future research should focus on further pharmacokinetic evaluations, mainly through studies involving multiple administration stusies, to validate these findings and explore any cumulative effects. Additionally, investigating the interactions of Propoxazepam

with other CYP isoforms may provide a more comprehensive understanding of its metabolic profile and overall implications for patient care. Such research is crucial for optimizing therapeutic strategies and enhancing patient safety in clinical settings.

**Acknowledgements**
The authors acknowledge the financial support of SLC "Interchem"

**Authors Contribution**
Work conceptualization was performed by AR and MG in cooperation with VL. Experimental part and data analysis were conducted by IV and VL. MG, VL and AR participated in manuscript writing and conclusions carrying out.

**Ethical approval & declaration**
In this article, the human liver-microsomes regulations followed as per the ethical committee guidelines of Laboratory of Molecular Pharmacology and Medicine, Department of Biomedicine, A.V. Bogatsky Physical-Chemical Institute of National Academy of Sciences of Ukraine; the authors observed the propoxazepam drug-drug interaction, mediated by cytochrome 450 2d6 (preclinical *in vitro* data). Human liver microsomes were received from Corning Ultra Pool HLM 150 (Lot 38292 Corning® UltraPool™ Microsome Hu Liver 150 Donor Pool, Merck, Germany). The experimental part of the study took place at the Labcorp Early Development Laboratories Ltd. (Otley Road, Harrogate, UK). The ethical guidelines are followed in the study for Human liver microsomes observation & experimentation.

**Informed consent**
Not applicable.

**Conflicts of interests**
Author Reder AS is an employee of SLC "Interchem", the financial sponsor of this study. The authors declare that this financial support did not influence the study design, data collection, analysis, interpretation, or manuscript writing.

**Funding**
This study was funded by SLC "Interchem".

**Data and materials availability**
All data associated with this study are present in the paper.

## REFERENCES

1. Bachmann KA, Lewis JD. Predicting inhibitory drug-drug interactions and evaluating drug interaction reports using inhibition constants. Ann Pharmacother 2005; 39(6):1064-72. doi: 10.1345/aph.1E508
2. Cicali EJ, Smith DM, Duong BQ, Kovar LG, Cavallari LH, Johnson JA. A Scoping Review of the Evidence Behind Cytochrome P450 2D6 Isoenzyme Inhibitor Classifications. Clin Pharmacol Ther 2020; 108(1):116-125. doi: 10.1002/cpt.1768
3. Dayer P, Leemann T, Küpfer A, Kronbach T, Meyer UA. Stereo- and regioselectivity of hepatic oxidation in man--effect of the debrisoquine/sparteine phenotype on bufuralol hydroxylation. Eur J Clin Pharmacol 1986; 31(3):313-318. doi: 10.1007/BF00981130
4. EMA. Guideline on the Investigation of drug interactions. Final (CPMP/EWP/560/95/Rev. 1 Corr.2) 2012.
5. Golovenko MY, Reder AS, Larionov VB, Andronati SA, Akisheva AS. Cross-species differential plasma protein binding of propoxazepam, a novel analgesic agent. Biopolym Cell 2021; 37(6):459–468. doi: 10.7124/bc.000A68
6. Golovenko MY, Reder AS, Zupanets I, Bezugla N, Larionov V, Valivodz IP. A Phase I study evaluating the pharmacokinetic profile of a novel oral analgesic propoxazepam. J Pre Clin Clin Res 2023; 17(3):138–144. doi: 10.26444/jpccr/169426

7. Golovenko NY, Larionov VB, Reder AS, Valivodz IP. An effector analysis of the interaction of propoxazepam with antagonists of GABA and glycine receptors. Neurochem J 2017; 11(4):302–308. doi: 10.1134/S1819712417040043.
8. Lam YWF, Scott SS (Eds.). Pharmacogenomics: Challenges and opportunities in therapeutic implementation. 2nd edn. Academic Press, 2019; 428. doi: 10.1016/C2016-0-03878-0
9. Larionov VB, Golovenko MY, Kuzmin VE, Valivodz IP, Nefyodov OO. Propoxazepam interaction with cytochromes CYP450 isoforms on the base of molecular docking-analysis. Dopov Nat Akad Nauk Ukr 2023; 96–102. doi: 10.15407/dopovidi2023.03.096
10. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". Drug Metab Dispos 2006; 34(5):880-886. doi: 10.1124/dmd.105.008672
11. Pang KS, Rodrigues AD, Peter RM. Enzyme- and transporter-based drug-drug interactions: Progress and future challenges. 1st edn. Springer, New York, 2010; 746. doi: 10.1007/978-1-4419-0840-7
12. Pelletier RD, Rettie AE, Kowalski JP. Improved methods for the detection of heme and protoporphyrin IX adducts and quantification of heme B from cytochrome P450 containing systems. J Chromatogr B Analyt Technol Biomed Life Sci 2023; 1231:123921. doi: 10.1016/j.jchromb.2023.123921
13. Pringle TH, Francis RJ, East PB, Shanks RG. Pharmacodynamic and pharmacokinetic studies on bufuralol in man. Br J Clin Pharmacol 1986; 22(5):527-34. doi: 10.1111/j.1365-2125.1986.tb02931.x
14. Rostami-Hodjegan A, Tucker GT. Simulation and prediction of in vivo drug metabolism in human populations from in vitro data. Nat Rev Drug Discov 2007; 6(2):140-148. doi: 10.1038/nrd2173
15. Yuan R, Madani S, Wei XX, Reynolds K, Huang SM. Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. Drug Metab Dispos 2002; 30(12):1311-9. doi: 10.1124/dmd.30.12.1311
16. Zhao M, Ma J, Li M, Zhang Y, Jiang B, Zhao X, Huai C, Shen L, Zhang N, He L, Qin S. Cytochrome P450 Enzymes and Drug Metabolism in Humans. Int J Mol Sci 2021; 22(23):12808. doi: 10.3390/ijms222312808
17. Zhou SF. Polymorphism of human cytochrome P450 2D6 and its clinical significance: Part I. Clin Pharmacokinet 2009; 48 (11):689-723. doi: 10.2165/11318030-000000000-00000