UDC 615.015.(2;43)-616.8-009.7

Interaction of propoxazepam with human liver cytochrome P450 2C9 and its therapeutic implications

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Propoxazepam is a novel benzodiazepine-derived analgesic that has completed Phase I clinical trials, demonstrating its safety and appropriate pharmacokinetics. Phase II trials are currently underway to evaluate its effectiveness in treating neuropathic pain. This study aimed to investigate the effect of propoxazepam on CYP2C9 activity in human liver microsomes and to determine the inhibitory mechanisms underlying its action on the enzyme. These findings may contribute to a comprehensive assessment of potential drug-drug interactions involving propoxazepam. To evaluate CYP2C9 activity, we used the reaction of diclofenac hydroxylation and selective inhibitors (as positive controls): sulfaphenazole for reversible and thienylic acid for metabolism-dependent inhibition. Propoxazepam inhibited CYP2C9 activity, with IC_{50} values of 32.7 ± 2.8 μmol/l for reversible and 49.0 ± 12.6 μmol/l for metabolism-dependent inhibition. The highest concentration of free, unbound propoxazepam in plasma that could result in interaction is ≥0.327 μmol/l (0.133 μg/ml). This corresponds, given that only 1.96% of total plasma propoxazepam exists in the free fraction, to a total plasma concentration of 6.8 μg/ml, but the concentrations in the volunteer`s blood are much lower after single oral administration.
Key words: propoxazpepam; CYP2C9; diclofenac; sulphaphenazole; tienilic acid; revercible inhibition; metabolism dependent inhibition; drug-drug interaction.

INTRODUCTION

The cytochrome P450 (CYP) isoenzymes are a group of heme-containing enzymes embedded primarily in the lipid bilayer of the hepatocytes endoplasmic reticulum [1]. The most intensively studied route of exogenous and endogenous substances metabolism is the P450-catalysed mixed function oxidation reaction. Catalytic activity of CYP is associated with redox partner proteins that transfer electrons from NADPH to the hemoprotein heme center.

It is well established that the CYP enzymes are involved in the metabolism of exogenous substances (drugs, alcohols, anti-oxidants, organic solvents, anesthetic agents, dyes, environmental pollutants and chemicals) producing metabolites which may be toxic or carcinogenic. They are also important in the oxidative, peroxidative and reductive metabolism of endogenous physiological compounds such as steroids, bile acids, fatty acids, prostaglandins, biogenic amines and retinoids [2].

Recent evidence [3] suggests that the CYP may have physiological roles in the brain, such as signal transduction by arachidonic acid metabolites which are thought to be involved in the release of peptide hormones from the hypothalamus and pituitary. It has been suggested that the CYP regulates the metabolism and processing of neurotransmitters such as dopamine and serotonin and therefore may have a role in determining the mental state and personality of individuals. CYP2C plays an important role in estrogen biosynthesis in the gonads, brain, placenta and adipose tissue [4, 5]. Metabolites resulting from pathways modulated by CYP may be important in determining ion permeability of membranes and the enzyme activity and turnover

of membranes. In the kidney, metabolites of arachidonic acid produced by renal CYP enhance Na^+/K^+-ATPase and Na^+-K^+-$2Cl^-$ cotransporters resulting in diuresis and natriuresis [6]. These metabolites exhibit both vasoconstrictive and vasodilatatory activity. As a consequence of these effects, the cytochrome P450 system has an important role in the integration of body fluid volume and composition and hence blood pressure regulation.

Altered activity of certain isoenzymes has been implicated in the development of cancer, adrenal hyperplasia and Parkinson's disease [7]. The CYP is increased in patients with breast cancer [8]. As it is involved in the metabolism of estrogens in the breast, CYP is suggested to contribute in the etiology of estrogen-dependent breast tumors. In addition, a deficiency of 21 hydroxylase, caused by defective genetic coding of CYP, is responsible for 90-95% of cases of congenital adrenal hyperplasia [9].

Assessment of the potential of a compound to inhibit a specific CYP enzyme is important, as co-administration of drugs may lead to metabolic inhibition, affecting plasma levels in vivo and potentially causing adverse drug reactions or toxicity. Therefore, research on human CYP enzymes is essential for both optimizing drug therapy and preventing various diseases. CYP inhibition is a significant concern in drug development: The European Medicines Agency (EMA) [10] and the Ministry of Health of Ukraine [11] have issued guidance on drug-drug interactions (DDIs). These documents outline a tiered risk assessment strategy for identifying potential CYP inhibitors, incorporating fundamental evaluation approaches.

CYP enzymes of the 2C subfamily are major isoforms, with CYP2C9 being the most abundantly expressed member in the human liver among the four (CYP2C8, CYP2C9, CYP2C18, and CYP2C19) [12]. CYP2C9 plays a key role in the metabolism of numerous drugs: nonsteroidal anti-inflammatory drugs (including COX-2 selective inhibitors), the hypoglycemic agent tolbutamide, the anticonvulsant phenytoin, and the angiotensin II receptor antagonist losartan.

Propoxazepam is considered a promising drug and is currently undergoing clinical studies in Ukraine. Similar to gabapentinoids (derivatives of the inhibitory neurotransmitter gamma-aminobutyric acid), which are widely used in clinical practice in the treatment of neuropathic pain, propoxazepam also exhibits anticonvulsant effects [13, 14]; these are considered predictive of analgesic action and explain the analgesic component of the compound's pharmacological spectrum. In rats, propoxazepam (2 mg/kg, i.p.), similarly to gabapentin (5 mg/kg, i.p.), reduced hyperglycemia and clinical signs of polyneuropathy after 5 weeks of administration. It also demonstrated an analgesic effect, as indicated by an increased pain sensitivity threshold. Moreover, propoxazepam proved to be more effective than the reference drug, with statistically significant differences observed at 4-6 weeks [15].

Propoxazepam has successfully completed the first stage of clinical studies in healthy volunteers, demonstrating both safety and proper pharmacokinetics [16]. The second phase of clinical research involves studying the drug's analgesic effect in patients with neuropathic pain, either as a monotherapy or as an adjuvant treatment. Evaluation of potential drug–drug interactions (DDIs) is an integral part of drug development and in the overall benefit–risk evaluation of new treatments.

Investigation of CYP isoform inhibition is typically initiated early in drug discovery, with preclinical evaluation using appropriate in vitro human-based systems. Depending on the results of a risk assessment, further formal clinical studies may be required to support regulatory labeling and prescribing recommendations.

The aim of this study was to investigate the effect of propoxazepam on CYP2C9 activity in human liver microsomes (HLMs) and to further explore its inhibitory mechanism on this enzyme. These findings may contribute to a more comprehensive understanding of potential DDIs associated with propoxazepam.

METHODS

Chemical and tissue sources. Propoxazepam and 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). General purpose reagents and solvents were of analytical grade (or a suitable alternative) and were obtained from "VWR International Ltd", "Rathburn Chemicals Ltd", "Sigma Aldrich Chemical Company Ltd" and "Fisher Scientific UK Limited". HLM were obtained from "Corning Ultra Pool HLM 150" (Lot 38292).

Reversible CYP inhibition. Activities of the HLM CYP2C9 were determined according to the standard assay. The formation of metabolite of hydroxylated diclofenac was quantified by UPLC with MS detection. For the inhibitory potential of propoxazepam determination, HLM were incubated, in triplicate, with isoform-selective probe substrates, NADPH and propoxazepam (0.1, 1 and 100 μmol/l). After equilibration, reactions were initiated by the addition of NADPH. Incubations were performed at 37°C and terminated after a relevant time by the addition of the appropriate stop reagent containing an internal standard. The samples were then centrifuged for 5 min to sediment the precipitated protein.

Metabolism dependent CYP inhibition. The metabolism mediated inhibitory potential was assessed using a similar protocol, with the exception that the HLM were pre-incubated for 30 min at 37°C, in triplicate, with propoxazepam and NADPH prior to the addition of the CYP marker substrate diclofenac at a concentration approximating the K_m. The CYP enzyme activity in the presence of propoxazepam was then compared to that in samples incubated without it.

Measurement of drug concentrations. The formation of the metabolites was quantified by LC MS/MS in multiple reaction monitoring mode using electrospray ionization technique. Calibration standard working solutions were used to freshly prepare the calibration standards.

Data analysis. The enzyme activity in the presence of propoaxazepam was expressed as a percentage of its control activity. When possible, IC_{50} values were calculated by non-linear regression using SigmaPlot (v12.5, Systat Software Inc.). For CYP2C9 reversible and metabolism-dependent inhibition, where data fitting was sub-optimal, we used a three-parameter logistic model, describing enzyme activity as a function of propoxazepam concentration, maximum activity, and slope. The extent of microsomal binding, free fraction and recovery, determined using the equilibrium dialysis method, were calculated using appropriate concentration ratios. The enzyme competitive inhibition constant (K_i) was estimated as $IC_{50}/2$. All substrate concentrations used in the current study were adjusted to the K_m.

Statistical analysis was performed using Student`s t-test. Data are presented as the mean ± sem. or standard deviation (SD).

RESULTS

Microsomal binding. The non-specific binding of propoxazepam in HLM have been analyzed due to the need to incorporate the unbound fraction in microsomes to obtain meaningful concentrations for predicting CYP inhibition potential [17]. The results are summarized in Table 1, with post-dialysis recoveries presented in Table 2. These data indicate that microsomal binding was not notably influenced by the concentration of propoxazepam, but was dependent on the microsomal protein concentration. Since the binding of propoxazepam to microsomal proteins under conditions matching those of the IC_{50} experiments was low, no correction for microsomal binding was applied to the reported IC_{50} values.

Propoxazepam protein (HLM) binding interactions resemble drug-receptor interactions in that they are rapid, reversible, and saturable. This does not lead to a pharmacological effect, so this model cannot be used to study the inhibitory effect of the drug on the CYP.

For this purpose, we used the main enzymatic reaction that characterizes the activity of

Table 1. In vitro determination of propoxazepam binding following dialysis of spiked human liver microsomes for 6 h (all concentrations are in mmol/l)

Nominal concentration (μmol/l)	Human liver microsomes (mg/ml)	Actual concentration	Donor concentration	Acceptor concentration	Mean fraction bound (%)	Mean fraction unbound (%)
0.1	0.01	$(4.5\pm0.2)\cdot10^{-2}$	$(1.8\pm0.2)\cdot10^{-2}$	$(1.9\pm0.1)\cdot10^{-2}$	-6.16±12.3	106±12.3
10		8.16±0.25	3.31 ± 0.19	3.55±0.25	-7.21±5.84	107±6
100		85.9±2.5	36.8 ± 3.4	35.7±2.5	2.74±2.55	97.3±0.5
					Mean±SD	103±5
0.1	0.05	$(4.6\pm0.0)\cdot10^{-2}$	$(2.0\pm0.1)\cdot10^{-2}$	1.8±0.0	6.84±3.97	93.2±4.0
10		8.58 ± 0.30	4.19 ± 0.55	3.70±0.55	11.7 ± 6.1	88.3±0.1
100		85.5 ± 1.1	39.5 ± 5.0	32.9±2.2	16.1±5.7	83.9±7
					Mean±SD	88.5 ± 4.7
0.1	1	$(4.9\pm0.1)\cdot10^{-2}$	$(3.5\pm0.9)\cdot10^{-2}$	$(1.0\pm0.2)\cdot10^{-2}$	70.3±4.5	29.7±5
10		8.20 ± 0.12	6.54±0.29	1.62±0.33	75.1±6.0	24.9±0.1
100		98.8 ± 4.1	65.1±11.7	2.3±5.7	63.7±16.5	36.3±5
					Mean±SD	30.3±5.7

2C9. Metabolic activity of CYP enzyme is most often assessed using selective substrate ("marker of metabolic activity"), i.e. a drug (or substance) which is ideally metabolized by a single CYP enzyme [18]. Diclofenac is a commonly used drug substrate for studies of CYP2C9 metabolic activity because diclofenac 4^1-hydroxylation is primarily catalyzed by CYP2C9. Sulphaphenazole and tienilic acid were used as positive controls for reversible and metabolism dependent inhibition correspondingly.

Table 2. Microsomal binding of propoxazepam: post dialysis recoveries

Nominal concentration (μmol/l)	Human liver microsomes (mg/ml)	Recovery (%)
0.1	0.01	83.0 ± 6.6
10		84.1 ± 5.0
100		84.4 ± 6.8
0.1	0.05	82.1 ± 1.7
10		91.8 ± 12.5
100		84.7 ± 8.3
0.1	1	93.6 ± 21.7
10		99.5 ± 0.5
100		88.4 ± 6.5

Results are presented as the mean ± SD from three determinations.

Inactivation of CYP2C9. When drugs interact with CYP, reversible or metabolism-dependent inhibitions are observed, these are significantly different from each other and therefore have different clinical consequences. In order to characterize them, we determined the K_i and IC_{50} of the drug.

Optimal conditions were selected in terms of incubation time and HLM concentration to ensure the metabolite formation was determined in the linear range, and a probe substrate concentration below the K_m was selected to avoid saturation. First, preliminary experiment that involved incubation of diclofenac around its respective K_m value with different propoxazepam concentrations were conducted. Propoxazepam consistently inhibited the activities of CYP2C9 (Table. 3; 4) with IC_{50} = 32.7 ± 2.8 μmol/l for reversible (corresponds to K_i = 16.4 ± 1.4 μmol/l) and 49.0 ± 12.6 μmol/l for metabolism dependent inhibition. Positive controls sulphaphenazole (10 μmol/l) for reversible inhibition and tienilic acid (1.5 μmol/l) for

Table 3. Effect of propoxazepam on CYP2C9-mediated diclofenac 4^1-hydroxylation in HLM (reversible inhibition)

Inhibitor	Nominal concentration, μmol/l	Enzyme activity, pmol/min/mg	Activity (relative to control), %
Control	-	3412 ± 26	-
Propoxazepam solvent (Dimethyl Sulfoxide, 0.5% v/v)	-	3172 ± 41	-
Propoxazepam	0.1	3081 ± 113	97.2 ± 3.6
	0.3	3216 ± 47	101.4 ± 1.7
	1	3208 ± 27	101.1 ± 1.1
	3	3150 ± 42	99.3 ± 1.5
	10	2782 ± 61	87.7 ± 2.1
	30	1632 ± 85	51.5 ± 2.7
	60	676 ± 28	21.3 ± 0.9
	100	230 ± 10	7.3 ± 0.3
Sulphaphenazole solvent control (Acetonitrile, 0.5% v/v)	0	3439 ± 36	-
Sulphaphenaxole	10	214 ± 6	6.8 ± 0.2

metabolism dependent inhibition demonstrated CYP2C9 activity inhibition to 6.29% and to 9,04% in compare to the control.

The concentration of plasma unbound propoxazepam 0.133 μg/ml corresponds to 6.8 μg/ml of the total plasma concentration (assuming that free fraction of prpoxazepam in plasma is 1.96%), this is much higher that maximum of the total propoxazepam plasma concentration after single oral administration.

Table 4. Effect of propoxazepam on CYP2C9-mediated diclofenac 4^1-hydroxylation in HLM (metabolism dependent inhibition)

Inhibitor	Nominal concentration, μmol/l	Enzyme activity, pmol/min/mg	Activity (relative to control), %
Control	-	2743 ± 49	-
Propoxazepam solvent (Dimethyl Sulfoxide, 0.5% v/v)	-	2666 ± 13	-
Propoxazepam	0.1	2605 ± 26	97.7 ± 2
	0.3	2694 ± 47	101 ± 2.5
	1	2610 ± 3	97.9 ± 1.7
	3	2588 ± 82	97.1 ± 3.5
	10	2246 ± 47	84.2 ± 2.3
	30	1594 ± 13	59.8 ± 1.2
	60	653 ± 44	24.5 ± 1.7
	100	216 ± 8	8.1 ± 0.3
Tienilic acid solvent control (Methanol, 0.5% v/v)	-	2504 ± 37	-
Tienilic acid	1.5	226 ± 2	8.5 ± 0.2

DISCUSSION

Assessing the potential of new drugs to cause CYP-mediated drug-drug interactions is crucial, especially given the role of CYP enzymes in brain metabolism, where their expression and function–shaped by disease states–impact drug efficacy, detoxification, and neurotoxicity. Understanding brain P450 complexity can improve treatments for neurological disorders [19]. This study aimed to assess the metabolism-dependent inhibition of CYP2C9 by propoxazepam in HLM, using diclofenac 4'-hydroxylation as a probe reaction.

The interaction between a drug and a CYP enzyme involves binding, often accompanied by changes in the UV–visible absorbance spectrum. A shift of iron from a resting low-spin state to a high-spin state is termed type I change (λ_max~390 nm), while type II change (λ_max~430 nm) [20]. These shifts are commonly used to assess the binding affinity between CYP enzymes and ligands. Earlier we showed [21] that both propoxazepam and 3-hydroxymetabolite produce type II spectral changes upon interaction with rat liver CYPs. Their binding constants differed significantly, suggesting that these compounds may interact with distinct binding sites on CYP.

To better decipher the interaction between propoxazepam and CYP at the molecular level, we used drug docking to CYP2C9 HLM. Propoxazepam has fairly high values (8.15-9.8 cal/mol) of the free energy of interaction with CYP isozymes 1A2, 2B6, 2C9, 2C19, 2D6 and 3A4, though the sets of interacting amino acid residues varied across isozymes [22]. The analysis suggests potential competitive interactions with CYP1A2, 2C19, and 2C8, and to a lesser extent with 2C9, 3A4, and 2B6. Key active site residues of CYP2C9 involved in propoxazepam binding include Phe-100, Leu-102, Ala-103, Phe-114, Asn-217, Leu-366, Pro-367, and Phe-476. A recent model [23] suggests that Phe-114 involved in hydrophobic binding of diclofenac to 2C9. Moreover, the corresponding results are consistent with π–π stacking of Phe 114 with aromatic substrates or inhibitors. These data indicate that Phe 114 plays an important role in recognition of aromatic substrates of CYP 2C9. They also provide the first experimental evidence showing that Phe 476 plays a crucial role in substrate recognition and hydroxylation by CYP 2C9.

In our study, the observed range of $[I]/K_i$ ratios (0.02–0.11) suggests that CYP2C9 is unlikely to be inhibited.

Based on the estimated K_i value (10.3 μmol/l, assuming competitive inhibition) and EMA guidelines [10], propoxazepam would be predicted to cause clinically relevant drug interactions with CYP2C9 substrate at unbound plasma C_{max} concentrations of ≥0.206 μmol/l (84 ng/ml). According to FDA guidance [24], such interactions are likely when $1 + [I]/K_i \geq 1.02$, where [I] is unbound C_{max}. The EMA guidance [10] suggested that an in vivo DDI study is recommended when $[I]/K_i \geq 0.02$, where [I] is the unbound mean C_{max} value obtained during treatment with the highest recommended dose. Thus, the highest predicted unbound C_{max} plasma concentration of propoxazepam, above which the interaction can take place, is 0.327 μmol/l, this gives 133 ng/ml of the unbound plasma concentration and corresponds to 6.8 μg/ml. According to our pharmacokinetics data, after single oral administration these concentrations are much lower. A 30-minute pre-incubation of propoxazepam with microsomes and NADPH prior to substrate addition did not result in statistically significant change in IC_{50} values, and the IC_{50} shift ≤2 suggests that the inhibition mechanism is not metabolism dependent.

According to our data [25], the unbound propoxazepam fraction in human plasma is 1.96%, so it's total concentration, if the inhibition is prognosed, is about 10 μg/ml. Pharmacokinetics study results show that the maximum propoxazepam concentration (22 ng/ml) was reached in blood by 4 h after oral administration on healthy volunteers [16], this is much lower than the estimated prognosed inhibition levels. Thus, propoxazepam is not expected to

be the CYP 2C9 inhibitor in vivo.

IC_{50} > 10 μmol/l suggested weak inhibitory effect for propoxazepam and the IC_{50} shift assay [26], used to differentiate reversible from irreversible inhibition, considers a fold shift >1.5 as significant and the compound is classed as a metabolism dependent inhibitor (0.89 in our study).

CYPs play an important role in psychopharmacology [27]. Recent studies have revealed a bidirectional relationship between the brain and CYPs: while enzymes metabolize endogenous neuroactive substances in the brain, the CNS regulates CYPs in the liver via neuroendocrine and neuroimmune pathways. This functional interplay seems to be implicated in some psychotropic drug effects. Since psychotropics can affect cytochrome P450 in the liver and brain, they may modify their pharmacological effect at both pharmacokinetic and pharmacodynamic levels.

Propoxazepam consistently inhibited CYP2C9 activities, for diclofenac "concentration-activity inhibition" dependence is similar with IC_{50} = 32.7 ± 2.8 μmol/l for reversible and IC_{50} = 49.0 ± 12.6 μmol/l for metabolism dependent inhibition. Used as positive controls, sulphaphenazole (10 μmol/l, reversible inhibition) and tienilic acid (15 μmol/l, metabolism dependent inhibition), as expected, demonstrated CYP2C9 activity inhibition (6.29% and 9,04% compare to control).

Our results have an indirect implication regarding the possible effect of propoxazepam on CYP2C9 activity, a process directly linked to several physiological functions. CYP 2C9 is known to exhibit epoxygenase activity, catalyzing the formation of arachidonic acid or epoxyeicosatrienoic acid epoxides (EETs) [28]. EETs modulate various biological processes, including activation of intracellular protein kinases (e.g., tyrosine kinase and MAP kinase), exert anti-inflammatory effects by inhibiting NF-κB activation and decreasing cytokine-induced expression of vascular cell adhesion molecule-1. Therefore, the minimal inhibition of CYP2C9 by propoxazepam indicates the absence of its effect on arachidonic acid metabolism.

Additionally, our results contribute to the development of a physiologically based pharmacokinetic (PBPK) model, a tool recognized by EMA for drug development and regulatory submissions [10]. PBPK models are validated by comparing predicted and observed pharmacokinetic data. Completion of this model will require data from the ongoing phase II clinical trials of propoxazepam.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ВЗАЄМОДІЯ ПРОПОКСАЗЕПАМУ З ЦИТОХРОМОМ Р450 2С9 ПЕЧІНКИ ЛЮДИНИ ТА ЇЇ ТЕРАПЕВТИЧНЕ ЗНАЧЕННЯ

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Пропоксазепам є новаторським анальгетичним засобом, похідним бензодіазепіну, для якого завершено першу фазу клінічних випробувань, де підтверджено його безпеку та відповідну фармакокінетику. Випробування другої фази тривають для оцінки його ефективності в лікуванні нейропатичного болю. Мета цієї роботи полягала в дослідженні впливу пропоксазепаму на активність CYP2C9 у мікросомах печінки людини і подальшому визначені пригнічувальних механізмів його дії на фермент. Таким чином, це дослідження може бути корисним для комплексної оцінки потенційної взаємодії лікарських засобів пропоксазепаму. В роботі було використано реакцію гідроксилювання диклофенаку та селективних інгібіторів (позитивних контролів): для зворотного - сульфафеназол, для метаболізмзалежного - тієнілову кислоту. При останньому способі пропоксазепам додавали разом з диклофенаком і НАДФН. Пропоксазепам пригнічував активність CYP2C9 з такими значеннями IC_{50}: 32,7 ± 2,8 мкмоль/л для зворотного та 49,0 ± 12,6 мкмоль/л для метаболізмзалежного інгібування. Найвища концентрація вільного, не зв'язаного з протеїнами, пропоксазепаму в плазмі, при якій може відбутися взаємодія, становить ≥0,327 мкмоль/л

(≥133 нг/мл), що відповідає (з огляду на те, що лише 1,96% загальної концентрації пропоксазепаму в плазмі як вільної фракції) 6,8 мкг/мл, проте концентрації сполуки в крові добровольців є значно нижчими при одноразовому пероральному прийомі.

Ключові слова: пропоксазепам; CYP2C9; диклофенак; сульфафеназол; тієнілова кислота; зворотне інгібування; метаболізмзалежне інгібування; міжлікарська взаємодія.

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Received 11.09.2024