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SHORT COMMUNICATION

Detoxification of the tricyclic antidepressant opipramol and its analog – IS-noh by UGT enzymes before and after activation by phase I enzymes in rat liver microsomes

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Abstract
The present studies were carried out to evaluate the simultaneous one-pot metabolism of opipramol (IS-opi) and analog (IS-noh) by phase I and phase II enzymes present in rat liver microsomes (RLM) as an alternative to separate testing with recombinant enzymes. This approach allows for more time-saving and cost-effective screening of the metabolism of newly discovered drugs. We also considered that the lack of results for phase II, including UGT, often creates problems in correct selection of valuable compounds. Moreover, microsomes data set is richer in the contest and provides medical scientist to determine also the susceptibility of drugs to undergo phase I and then phase II. In the present work, we have shown that IS-noh was metabolized in vitro by phase I enzymes to the oxidation product, which was next transformed with UGTs to glucuronide. The results showed also that the previously known oxidation product of opipramol was changed to previously no reported glucuronidation product by UDP-glucuronosyltransferases. In addition, unlike IS-noh, opipramol did not prove to be the substrate for UGTs. Therefore, tricyclic antidepressants depending on the structure can trigger a different response after contact with UGT enzymes. Some will metabolize directly with UGTs, others only after activation by phase I enzymes.

Keywords TCAs · Drug metabolism · UDP-glucuronosyltransferases · IS-noh · Opipramol

Introduction

Microsomal fraction contains both phase I enzymes: cytochrome P450 (P450s), flavin monooxygenases (FMO), esterases, amidases and epoxide hydrolases and phase II enzymes—UDP-glucuronosyltransferases (UGTs). Therefore, it allows determining the susceptibility of drugs to undergo both phase I and II simultaneously. The use of a cocktail of two activating cofactors stimulates phase I (NADPH for P450s and FMO) and phase II (UDPGA for UGT) metabolism. This method can provide more metabolic information than experiments with only recombinants enzymes, because we obtain in this case metabolites not only directly with UGTs, but also after activation by enzymes phase I (Richardson et al. 2016).

Cytochrome P450 and flavin monooxygenases are a group of enzymes involved in phase I metabolic transformations of drugs (Phillips and Shephard 2008, Shobhana 2019). However, for many therapeutics, e.g., zidovudine, prior oxidative transformation by P450s is not always required before glucuronidation by UGTs (Barbier 2000). UGTs belong to the family of drug-metabolizing enzymes (DME) (Rowland et al. 2013) and are estimated to be involved in about 35% of all reactions in phase II (Evans and Relling 1999). UGT enzymes catalyze the glucuronidation reaction, i.e., the conjugation substrate with glucuronic acid, which leads to the formation of glucuronide (Shobhana 2019). The role of UGTs in drug metabolism for a long time was wrongly overlooked.

Opipramol (IS-opi) is a member of tricyclic antidepressants (TCAs) [Korobkova et al. 2010] and was developed in the 1960s (Gahr et al. 2017). Typically used in the treatment of generalized anxiety disorder (GAD) and somatoform

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disorders. Most TCAs act as monoamine reuptake inhibitors, but opipramol does not, and instead acts primarily as a sigma receptor agonist. It was reported that opipramol was metabolized by P450 2D6 (Gillman 2007; Gahr et al. 2017). In plasma and urine was identified oxidation product of the hydroxyethyl moiety to an acetic acid group at the piperazine side chain, decarboxylation products, opipramol-N-oxide, deshydroxyethyl metabolite, and dibenzoazepine. One of the major metabolites of opipramol is also acetic acid (Lappenberg-Pelzer and Tenczer 1998). Excessive hepatic metabolism should be considered, particularly in patients with impaired hepatic function and polypharmacy (Rudorfer and Potter 1997). Opipramol was chosen for study because the metabolism of IS-opi with UGTs has not yet been described in detail. Compound IS-noh is an analog of opipramol synthesized in the Gdansk University of Technology (Hemine et al. 2020). The metabolic pathway of IS-noh has not been known yet.

Experimental

Chemicals were obtained from Merck KGaA (Darmstadt, Germany): Opipramol (IS-opi), methanol (gradient grade for liquid chromatography), HEPES, alamethicin, NADPH and UDPGA. IS-noh was synthesized according to the method described in Ref (Hemine et al. 2020). Ammonium formate was from Fisher Scientific (Loughborough, UK). Rat pooled liver microsomes, RLMs (20 mg of microsomal protein per ml) were purchased from Tebu-Bio (Le Perray-En-Yvelines, France). All reagents were analytical grade and were purchased from commercial sources and used without further purification.

Screening of metabolism with Rat Liver Microsomes was performed as follows: RLM (2 mg/ml of protein) was assayed for activity toward tested compound as follows. The proteins were incubated in a buffer containing 0,1 M HEPES (pH 7,4), 2 mM MgCl2 with either 0,1 mM substrate in total volume 70 µl. Substrates were added also in buffer HEPES, pH 7.4. Reactions were started by the addition of NADPH (2 mM)/UDPGA (5 mM) or cocktail of NADPH (2 mM) and UGPGA (5 mM) and were incubated for specified time at 37 °C. The reactions were stopped by the addition of 8,75 µl of 1 M HCl, followed by centrifugation at 13 400 rpm for 10 min to pellet the denatured protein. The supernatant fractions were used for high-performance liquid chromatography (HPLC analysis). Control reactions omitting substrate were run with each assay. All incubations were performed in two repetitions.

HPLC analyses of the supernatants were performed using an LC-2040C 3D HPLC system and the LabSolution software package (Shimadzu, Kioto, Japan). Samples were separated using a reversed-phase 5 µm Suplex pKb-100 analytical column (0.46 × 25 cm, C18) (Supelco, Bellefonte, PA) warmed to 25 °C. The analyses were performed at a flow rate of 1 ml/min with the following mobile-phase system: a linear gradient from 15 to 80% methanol in ammonium formate buffer (0.05 M, pH 3.4) for 25 min, followed by linear gradient from 80 to 100% methanol in ammonium formate for 3 min. The column was then re-equilibrated at initial conditions for 10 min between runs. The elution of each metabolite was monitored at 254 nm. HPLC-tandem mass spectrometry analyses of the products were performed by electrospray ionization (ESI) with positive ion detection and a LCMS-2020 mass spectrometer (Shimadzu, Kioto, Japan) in the range m/z 200–800. Samples were separated according to the procedure described under HPLC UV–visible analysis (Fig. 1).

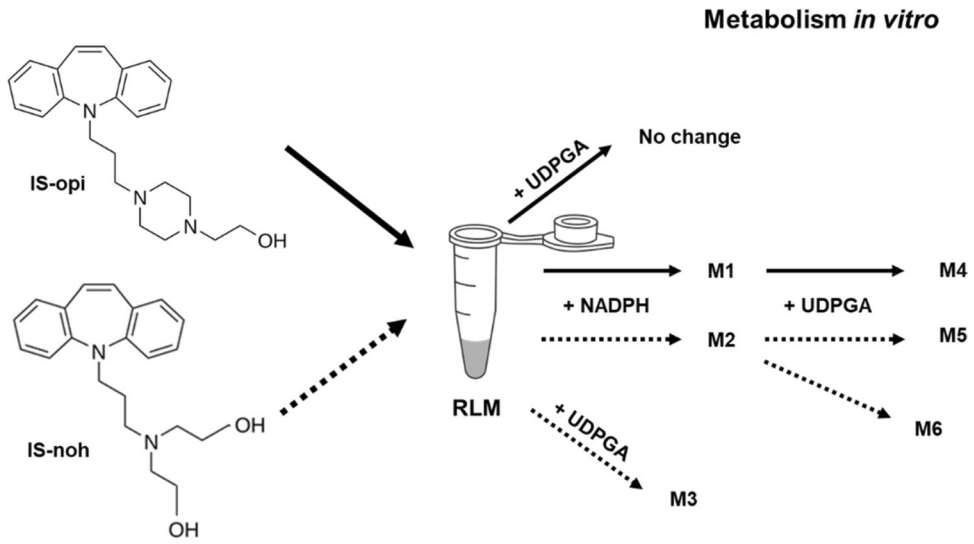

Fig. 1 Structures of IS-opi, IS-noh and scheme of the research. Solid lines – metabolic pathway for IS-opi; dotted lines—metabolic pathway for IS-noh

Results and discussion

The studies on IS-opi and IS-noh transformations were started with the analysis of phase I oxidation metabolism. Each compound was incubated with rat liver microsomes (RLM) and NADPH as a cofactor and the reaction mixtures were monitored by LC/MS analysis with UV–Vis and ESI–MS detection (Table 1). Chromatograms of the products formed after 0, 60, 120 min of incubation of IS-opi and IS-noh with RLM and NADPH are shown in Fig. 2a and b, respectively. After 60 min of incubation with IS-noh, multiple peaks of various intensities were observed. ESI–MS analysis showed that IS-noh underwent metabolic changes, giving one product, M2 with m/z equal 355.2 [mass ion, 339.2 (IS-noh) + 16 (O) + 1]. Also, after 60 min of incubation with IS-opi were observed one metabolite peak, M1 with m/z equal 380.2 [mass ion, 364.2 (IS-opi) + 16 (O) + 1]. This result suggested that IS-opi and IS-noh were transformed by phase I enzyme to the oxidation product, which agrees with the literature data in the case of IS-opi. There are previously known metabolites of opipramol, such as oxidation product of the hydroxyethyl moiety to an acetic acid group at the piperazine side chain and opipramol-N-oxide (Lappenberg-Pelzer M and Tenczer J 1998).

The results also indicated that the compound IS-noh demonstrated significantly lower susceptibility to metabolism compared with IS-opi. As the incubation time progresses, the amount of both compounds in the samples does not change and the amount of biotransformation products is not increased. The UV–Vis spectra of metabolites of IS-opi and IS-noh and those obtained with RLM and NADPH are presented in Fig. 3a and b, respectively. The spectral data of the metabolites were combined with the UV–Vis spectra of the substrate (IS-opi/IS-noh).

Phase II metabolites (glucuronides) were identified using LC/MS analysis. The chromatographic peaks observed above were analyzed using their UV–Vis and ESI–MS spectra. The incubation with rat liver microsomes was performed in the presence of glucuronyltransferase (UGT) and cofactor, UDPGA. Chromatograms of the products formed after 120 min of incubation of IS-opi and IS-noh with rat liver microsomes (RLM) and UDPGA are shown in Fig. 2c and d, respectively. However, only IS-noh was metabolized by UGTs. One metabolite peak was observed—M3. The UV–vis spectra of metabolite M3 were very different from that of the substrate, IS-noh (Fig. 3c). This result indicates the significant changes in the structure of the heterocyclic moiety of this metabolite. Analysis of ESI–MS also showed the M3 (tR = 16,5 min) with m/z equal to 515.2 is a product of 176 m/z units higher than that of the substrate [mass ion, 339.2 (IS-noh) + 175 (free glucuronic acid residue) + 1]. These results suggested that a glucuronic acid residue should be present in this metabolite (Table 1).

Finally, compounds IS-opi and IS-noh were incubated with rat liver microsomes (RLM) together with two activating cofactors NADPH and UDPGA to stimulate both phase I and phase II metabolism. Chromatograms of the products formed after 60 min of incubation are shown in Fig. 2e and f, respectively. Analysis of UV–vis and ESI–MS of the reaction mixtures indicated that the phase I metabolites of IS-opi and IS-noh was transformed by UGT into glucuronide (Table 1). After 60 min of incubation with IS-opi, one metabolite peak (M4) at tR equal 10 min was observed with m/z equal 556 [mass ion, 364.2 (IS-opi) + 16 (O) + 175 (free glucuronic acid residue) + 1]. Three metabolite peaks, M3 (of glucuronidation product), M5 and M6 of various intensities were observed after incubation with IS-noh. The results of ESI–MS analysis, giving two metabolites M5, and M6 with different retention times, but the same m/z equal to 531.2 [mass ion, 339.2 (IS-opi) + 16 (O) + 175 (free glucuronic acid residue) + 1]. Also, analysis of their UV–vis spectra indicated the strong changes in comparison to that of the substrate. The obtained results indicated that an additional oxygen atom and a glucuronic acid residue should be present in these metabolites. The difference in the retention time may result from the substitution of another hydroxyl group in the IS-noh molecule. The UV–Vis spectra of metabolites

Table 1 ESI–MS m/z obtained after IS-opi/IS-noh metabolism with rat liver microsomes (RLM) and NADPH/UDPGA or NADPH and UDPGA as cofactors

Compound	Retention time [min]	ESI–MS m/z		
IS-opi	18.268	364.2	[IS-opi + H]	
M1	14.788	380.2	[IS-opi + 16 + H]	**+O**
M4	10.428	556.2	[IS-noh + 16 + 175 + H]	**+O + free glucuronic aside residue**
IS-noh	17.724	339.2	[IS-noh + H]	
M2	14.204	355.2	[IS-noh + 16 + H]	**+O**
M3	16.884	515.2	[IS-noh + 175 + H]	**+free glucuronic aside residue**
M5	10.228	531.2	[IS-noh + 16 + 175 + H]	**+O + free glucuronic aside residue**
M6	14.972			

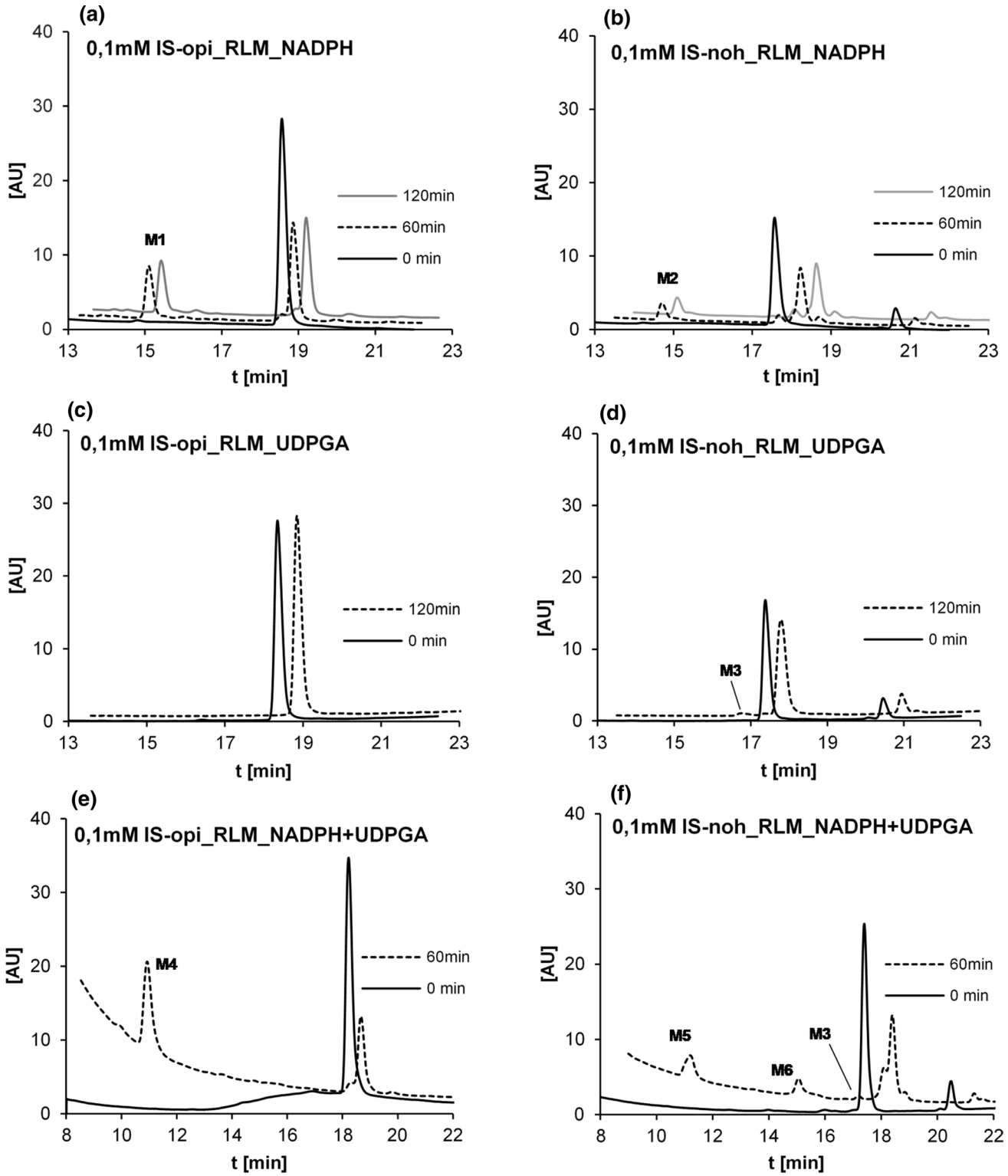

Fig. 2 Metabolism of IS-opi/IS-noh with rat liver microsomes (RLM) and, respectively, NADPH (**a/b**) UDPGA (**c/d**) or NADPH and UDPGA (**e/f**) as cofactors. Representative HPLC chromatograms are shown from incubations in 37 °C of 2 mg/mL microsomal fraction with 0.1 mM IS-opi/IS-noh and 2 mM NADPH and 5 mM UDPGA. The solid, dotted, and gray lines representing the same retention times are off-set for better visualization

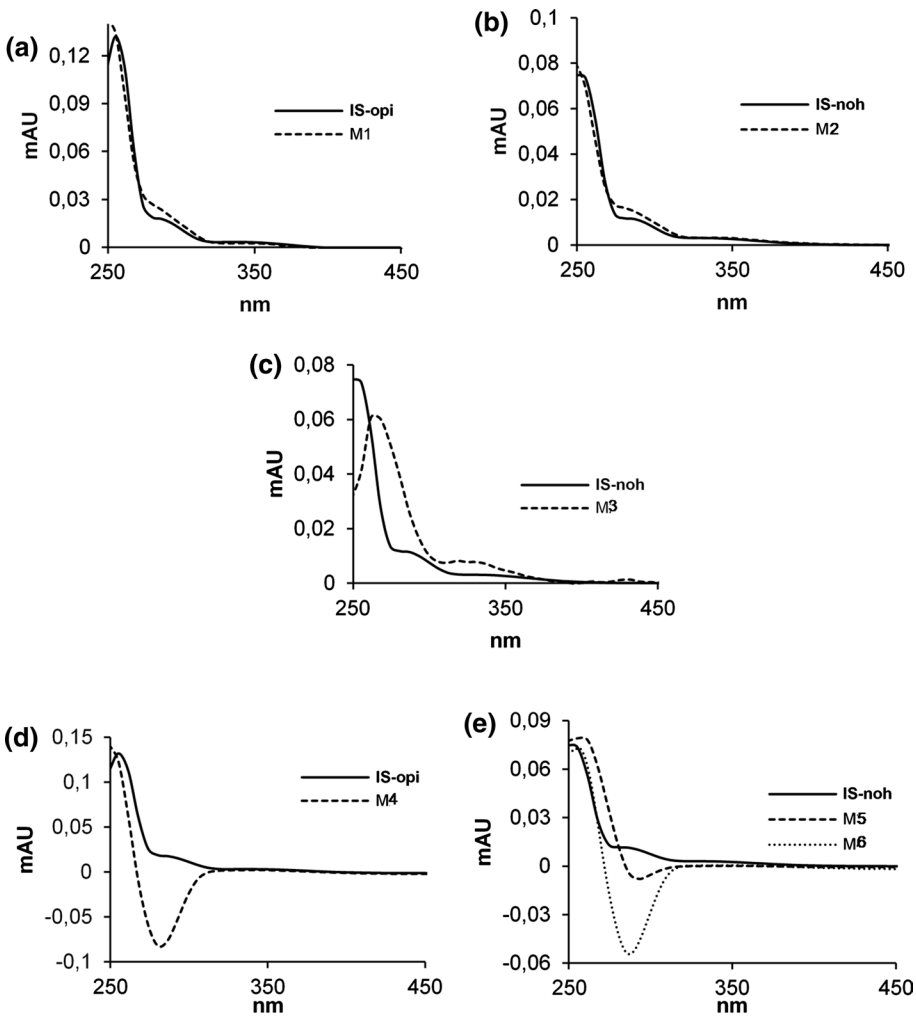

Fig. 3 UV–vis spectra of metabolites of IS-opi /IS-noh formed with RLM and NADPH (**a**/**b**), UDPGA (**c**) or NADPH and UDPGA (**d**/**e**), respectively. Spectra were recorded directly during HPLC analysis by means of a multidiode array detector in an ammonium formate pH 3.4/methanol mobile phase. The symbols of metabolites correspond to those in Fig. 2

of IS-opi, and IS-noh formed with RLM, NADPH and UDPGA are presented in Fig. 3d and e, respectively. The spectral data of the metabolite were combined with UV–Vis spectra of the substrate (IS-opi/IS-noh).

Conclusions

In conclusion, the present study shows that the tested compounds, IS-opi, and IS-noh are sensitive to the metabolic transformation with phase I enzymes which lead to the oxidized metabolites. Phase II transformation of IS-opi was not demonstrated either directly by UGTs, but upon the activation with phase I enzymes we observed the glucuronide of an oxidation product of IS-opi. It seems that glucuronidation does not occur directly for opipramol due to the presence of a piperazine ring in the structure (Pattanawongsa et al. 2020). Compound IS-noh was metabolized with phase I to oxidation product and directly with phase II enzymes (UGT) to glucuronide of IS-noh or upon the activation with phase I to glucuronide of oxidation product of IS-noh. The results showed that detoxification of IS-opi in contrast to IS-noh is much less possible in a living organism, especially in the case of reduced I phase metabolism. This may affect the therapeutic effect of the IS-opi and IS-noh. The result also suggested that not only hepatic metabolism disorders, particularly in patients treated by TACs should be considered. In multi-drug therapies for example when the patient used, e.g., the IS-opi simultaneously with other drugs, which are potential inhibitors or inductors of UGTs, we can observe, respectively, accumulation of IS-opi in the living organism, which may lead to an increase in the toxicity of this drug or increased detoxification process, which can reduce the effectiveness of the therapy" -->. It should also be considered that although glucuronidation is usually associated with inactivation, preserved or elevated biological activity of glucuronide can be observed for some compounds. A spectacular example is a morphine, whose 6-O-glucuronide has a much stronger analgesic effect than the initial aglycone (De Gregori et al. 2012; Mazerska et al. 2016). Therefore, both factors, especially often used multi-drug therapies or/and hepatic metabolism disorders influence an accumulation

of IS-opi in human body, which leads to an increase in the toxicity of this drug or increased detoxification process.

Studies have shown also that to evaluate the simultaneous one-pot metabolism of drugs in microsomal fraction is a cheaper alternative to separate testing with recombinant enzymes. It provides more metabolic information and can prevent the high cost of screening tests of the metabolism new potential drugs.

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Declarations

Conflict of interest The authors declare no conflict of interest associated with this manuscript.

Ethical approval We state that this material has not been published and is not under active consideration by another journal.

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