

Interaction of SZV 1287, a novel oxime analgesic drug candidate, and its metabolites with serum albumin

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ABSTRACT

SZV 1287 is our novel multi-target analgesic, which seems to be a promising drug candidate for the treatment of neuropathic pain. Therefore, the drug development process has been started in 2016. Since the pharmacokinetic characterization of a drug candidate is essential and albumin binding of drugs can strongly affect their pharmacokinetic properties, herein we provided the detailed investigation and characterization of the interaction of SZV 1287 and its known metabolites with serum albumin. In a preliminary animal study, we demonstrated the appearance of SZV 1287, oxaprozin, L 2799, L 2805, and L 2811 in the circulation after the *per os* administration of the parent compound to rats. Then albumin-ligand interactions were examined employing fluorescence spectroscopy, affinity chromatography, ultrafiltration, ultracentrifugation, and molecular modeling. Finally, we tested the potential species dependent differences in the albumin binding of SZV 1287, employing human, bovine, porcine, and rat serum albumins. Our results demonstrated that SZV 1287 and its metabolites form highly stable complexes with albumin ($K_a = 10^5$ to 10^6 L/mol). Furthermore, SZV 1287 occupies Sudlow's Site II on human serum albumin. Therefore, it is reasonable to hypothesize that SZV 1287-albumin interaction is an important issue regarding the pharmacokinetics of this drug candidate.

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1. Introduction

SZV 1287 (3-(4,5-diphenyl-1,3-oxazol-2-yl)propanal oxime; Fig. 1), our novel multi-targeting analgesic candidate, is currently being developed for the treatment of neuropathic pain [1]. SZV 1287 is the oxime analog of oxaprozin, a non-steroidal anti-inflammatory drug which has been used in the therapy for a long time [2]. Originally, the compound was designed on the bases of

the metabolism-activated multi-targeting (MAMUT) concept, where the synergistic pharmacological effects of the parent drug and its active metabolite(s) are utilized [3,4]. The parent drug directly blocks amine oxidase copper containing 3 (AOC3), also known as vascular adhesion protein-1. AOC3 is a semicarbazide-sensitive amine oxidase, it produces noxious mediators (e.g., formaldehyde, methylglyoxal, and hydrogen peroxide) during the oxidative deamination of primary amines (e.g. methylamine and aminoacetone) [2]. An active metabolite of SZV 1287, the cyclooxygenase (COX) inhibitor oxaprozin is formed at acidic pH in the gastric juice or as a result of biotransformation [3]. Based on our concept, the inhibition of both AOC3 and COX (by SZV 1287 and

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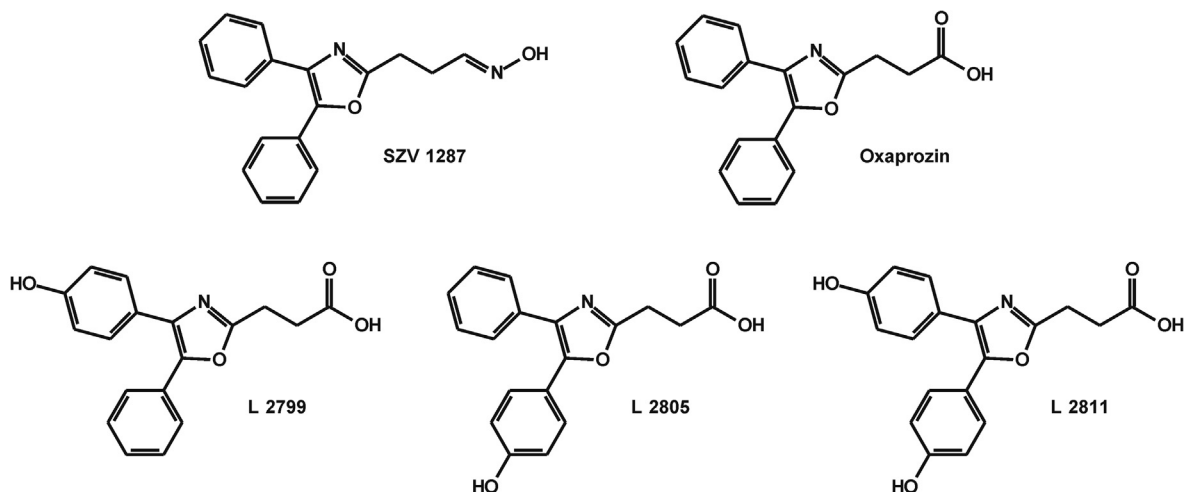


Fig. 1. Chemical structures of SZV 1287 and its metabolites (oxaprozin, L 2799, L 2805, and L 2811).

oxaprozin, respectively) may help to develop a new type of broad-spectrum anti-inflammatory drugs. Furthermore, the direct antagonistic effects of SZV 1287 on the pain-sensing transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) receptors on capsaicin-sensitive peptidergic sensory neurons provide analgesic effect in addition to its anti-inflammatory action [5]. Moreover, we also discovered the analgesic effects of SZV 1287 in different pain models, particularly with neuropathic mechanisms [6,7]; therefore, the drug development process has been started in 2016. *In vitro* and *in vivo* pharmacokinetic characterization of a drug candidate is essential for preclinical drug development. As a part of pharmacokinetic studies of SZV 1287, here we provided the detailed investigation and characterization regarding the interaction of SZV 1287 and its known metabolites with serum albumin.

Human serum albumin (HSA) forms highly stable complexes with numerous endogenous compounds and xenobiotics in the circulation [8]. The tight binding of drugs to HSA can strongly affect their tissue distribution and the speed of their elimination [8,9]; therefore, albumin binding can be an important parameter among the pharmacological properties of medications. The two most relevant drug binding sites in HSA are Sudlow's Site I (SSI) and Sudlow's Site II (SSII), located in subdomain IIA and IIIA, respectively [8]. The interaction of SZV 1287 with albumin has not been investigated yet. However, some data are available regarding the complex formation of its metabolite oxaprozin with HSA. Previous studies suggest the formation of highly stable complex of oxaprozin with the SSII region of the protein [10–12]. Clinical data also support the high relevance of oxaprozin-HSA interaction: At therapeutic concentrations, approximately 99.5% of oxaprozin is albumin-bound, resulting in the low volume of distribution (0.07–0.33 L/kg, which approximates plasma volume) and long elimination half-life ($t_{1/2\beta}$ = 50–60 h) of the drug [13]. Nevertheless, based on our current knowledge, the association constant of oxaprozin-HSA complex has not been determined.

In this study, the interactions of SZV 1287 and its metabolites identified in rats (oxaprozin, L 2799, L 2805, and L 2811; Fig. 1) with HSA were investigated, employing fluorescence spectroscopy, affinity chromatography, ultrafiltration, ultracentrifugation, and molecular modeling. Since SZV 1287 and its metabolites exert fluorescence, the association constants of albumin-ligand complexes were determined based on the albumin-induced increase in their emission signals. Thereafter, to confirm these results, HSA-ligand interactions were examined by high-performance affinity chromatography (HPAC). Binding sites were evaluated based on the displacing ability of SZV 1287 and its metabolites vs. SSI and SSII

markers (warfarin and naproxen, respectively). Furthermore, molecular modeling studies were applied to identify the possible binding positions of SZV 1287 and oxaprozin on HSA as well as to specify amino acids that participate in the interaction. The free fractions of SZV 1287 and oxaprozin in spiked human plasma samples were tested employing ultracentrifugation, after which the concentrations of the ligands in the protein-free fraction were examined employing HPLC-FLD. Finally, the species-dependent alternations regarding the albumin binding of SZV 1287 were examined using human, bovine (BSA), porcine (PSA), and rat (RSA) serum albumins. Our results demonstrate that SZV 1287 and its metabolites form stable complexes with albumin, and likely occupy the SSII region as their high-affinity binding site on HSA. Plasma protein binding is a highly important parameter in drug development because it can strongly affect the pharmacokinetic and pharmacodynamic properties. Therefore, our study contributes to the early development phase of the novel analgesic drug SZV 1287, and include the detailed investigation of its albumin binding.

2. Materials and methods

2.1. Reagents

SZV 1287 [14], oxaprozin [15], as well as L 2799 and L 2805 [16] were synthesized as described previously. The synthesis of L 2811 is described in the Supplementary (Suppl. 1). Human serum (product code: P2918), racemic naproxen (NAP), racemic warfarin (WAR), and serum albumins (human, bovine, porcine, and rat) were purchased from Sigma-Aldrich (St. Louis, MO, US). Other chemicals used were analytical or HPLC grade. Phosphate-buffered saline (PBS, pH 7.4) contained NaCl (137 mmol/L), KCl (2.7 mmol/L), NaH_2PO_4 (8 mmol/L), and K_2HPO_4 (1.5 mmol/L) dissolved in ultrapure water (conductivity < 0.1 $\mu\text{S}/\text{cm}$).

2.2. Animal studies

The experiment was performed on 10–11 week-old male Wistar rats. Rats were purchased from Toxi-Coop Ltd. (Balatonfüred, Hungary) and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy (Medical School, University of Pécs) at 24–25 °C, under 12-h light–dark cycle and provided with standard rodent chow and water *ad libitum*. The experiment was carried out in accordance to the European legislation (Directive 2010/63/EU) and Hungarian Government regulation

(40/2013., II. 14.) regarding the protection of animals used for scientific purposes. The study was approved by the Ethics Committee on Animal Research of University of Pécs (license No: BA 02/2000–67/2017).

To verify the appearance of the parent compound and its metabolites (oxaprozin, L 2799, L 2805, and L 2811) in the circulation, three rats were orally treated with 20 mg/kg of SZV 1287, which was administered in enteric capsules (Torpac, Fairfield, NJ, USA) to prevent the degradation of the compound under the acidic environment of the gastric juice. Rats were starved for 16 h before the experiment, but the food was returned 1 h after the single administration of the capsule. Blood samples were collected from the tail vein into ice-cold tubes containing 8 µL of 50 mg/mL pH 7.5 EDTA (Reanal, Budapest, Hungary) before and after (1, 2, 4, 8, and 24 h) the treatment. Then blood samples were centrifuged for 5 min at 1,000 rpm and for 10 min at 10,000 rpm, and plasma samples were stored at –80 °C until HPLC analyses.

In an Eppendorf tube, 150 µL acetonitrile was added to 50 µL rat plasma and vortexed for 1 min. After centrifugation (10 min, 14,000 g, 4 °C), the supernatant was removed and diluted with water, then SZV 1287, oxaprozin, L 2799, L 2805, and L 2811 were quantified by HPLC-FLD (see details in 2.3).

2.3. HPLC analyses

Samples were analyzed employing an integrated HPLC system (Jasco, Tokyo, Japan) which was built up from an autosampler (AS-4050), a binary pump (PU-4180), and a fluorescence detector (FP-920). Chromatograms were evaluated with ChromNAV software. Samples (20 µL) were driven through a Phenomenex Security Guard™ (C₁₈, 4.0 × 3.0 mm) guard column coupled to a Gemini NX-C₁₈ (150 × 4.6 mm, 3 µm; Phenomenex, Torrance, CA, US) analytical column. The elution was performed with 1.0 mL/min flow rate at room temperature, using acetonitrile and 10 mM sodium phosphate buffer pH 7.0 (30:70 v/v% in eluent A, and 55:45 v/v% in eluent B) as mobile phases. The following gradient program was applied: 0–4 min 100% eluent A, 4–4.5 min linear change to 100% eluent B, 4.5–14.9 min 100% eluent B. SZV 1287 and its metabolites were detected at 368 nm ($\lambda_{\text{ex}} = 320 \text{ nm}$).

Limit of detection (LOD) and limit of quantification (LOQ) values were determined as the lowest concentration where the signal-to-noise ratio were 3 and 10, respectively. The LOD and LOQ values of hydroxylated metabolites (L 2799, L 2805, and L 2811) were 2 nM and 5 nM, respectively. Furthermore, LOD and LOQ of SZV 1287 and oxaprozin were 20 nM and 50 nM, respectively. The linearity was examined in the 0–500 nM concentration range, R^2 was 0.999 for oxaprozin, L 2799, L 2805, and L 2811; while it was 0.992 for SZV 1287. Intra-day precision values (coefficient of variation from seven replicates) were in the 0.6–2.9% range.

2.4. Spectroscopic studies

UV-vis and fluorescence emission spectra were recorded employing Specord Plus 210 spectrophotometer (Analytik Jena, Jena, Germany) and Fluorolog τ3 spectrofluorimeter (Jobin-Yvon/SPEX, Longjumeau, France), respectively. Measurements were carried out at 25 °C in the presence of air. The inner filter effect was corrected using the following equation [17,18]:

$$I_{\text{cor}} = I_{\text{obs}} \times e^{(A_{\text{ex}} + A_{\text{em}})/2} \quad (1)$$

where I_{cor} is the corrected fluorescence emission intensity, I_{obs} is the observed emission signal, while A_{ex} and A_{em} denote the absorbance of test compounds at the excitation and emission wavelengths used, respectively.

Fluorescence emission spectra of SZV 1287 and its metabolites (each 2 µM) were recorded in the absence and presence of albumins (0–10 µM), using 305 nm excitation wavelength. Data were evaluated applying the Hyperquad2006 program package [19], the association constants (K_a) were determined as described previously [18,20].

2.5. Ultracentrifugation studies

To investigate the free fraction of SZV 1287 and oxaprozin, human serum and 40 g/L HSA solution (in PBS, pH 7.4) were spiked with SZV 1287 or oxaprozin (both 2 µM). These samples (3 mL) were transferred to Ultra-Clear™ tubes (14 mL, 14 × 95 mm, open end, thin wall; Beckman Coulter, Brea, CA, US) and centrifuged for 16 h at 170,000 g (20 °C) using a Beckman Optima LE-80 K Ultracentrifuge with swinging bucket rotor (SW40Ti, Beckman Coulter, Brea, CA, US), similarly to the previously reported study [21]. Thereafter, the protein free fraction was carefully removed and directly analyzed by HPLC-FLD (see details in 2.3).

2.6. High-performance affinity chromatography measurements

Interaction of SZV 1287 and its metabolites with HSA was also examined employing HPAC. The HPLC system used was described in 2.3. Samples (5 µL) were driven through the affinity column coated with immobilized HSA (Chiralpak HSA, 50 × 3.0 mm, 5 µm; West Chester, PA, US) with 0.5 mL/min flow rate at room temperature. The mobile phase contained sodium phosphate buffer (0.01 M, pH 7.0) and isopropanol (80:20 v/v%). The isocratically eluted analytes were detected at 368 nm ($\lambda_{\text{ex}} = 320 \text{ nm}$).

2.7. Ultrafiltration studies

Displacing ability of SZV 1287 and its metabolites vs. SSI and SSII markers (warfarin and naproxen, respectively) were examined with ultrafiltration, using the methods described previously [22,23]. Before ultrafiltration, the test compounds (10 and/or 20 µM) were added to warfarin-HSA (1 and 5 µM, respectively) or naproxen-HSA (1 and 1.5 µM, respectively) mixtures in PBS (pH 7.4). Then samples were filtered (10 min, 7500 g, 25 °C, fixed angle rotor) using Pall Microsep Advance centrifugal devices (30 kDa molecular weight cut-off value; VWR, Budapest, Hungary). The concentrations of site markers in the filtrates were quantified by HPLC as it has been reported [22,23], without modifications.

2.8. Modeling studies

Ligand preparation. Ligand structures of SZV 1287 and oxaprozin were built in Maestro (Schrödinger Release 2019–3: Maestro, Schrödinger, LLC, New York, NY, 2019) and energy-minimized with a quantum chemistry program package, MOPAC [24] with a PM7 parametrization [25]. Force calculations were also performed using MOPAC with the gradient norm set to 0.001, the force constant matrices were positive definite. Gasteiger-Marsilli partial charges were assigned in AutoDock Tools [26]. Flexibility was allowed on the ligand at all active torsions. These prepared structures were used for docking.

Target preparation. Atomic coordinates of HSA were obtained from the Protein Data Bank (PDB) with PDB code 1ao6, according to previous studies [18,20,22,27]. The target molecule was equipped with polar hydrogen atoms and Gasteiger-Marsilli partial charges in AutoDock Tools.

Docking. Ligands were docked to the SSII (subdomain IIIA) [28] using AutoDock 4.2.6 [26]. The number of grid points was set to 80 × 80 × 80 at a 0.375 Å grid spacing. The center coordinates of the grid box were the following: 16.000, 28.000, and 22.000.

Lamarckian genetic algorithm was used for global search, with the flexibility of all active torsions allowed on the ligand. Ten docking runs were performed, and the resulted ligand conformations were ranked by their free binding energy values. Representative docked ligand conformations were used for subsequent evaluations and collection of the interacting target amino acid residues with a 3.500 Å cut-off distance calculated for heavy atoms. Blind docking was also performed, the center coordinates of the grid box were 29.535, 31.826, and 23.500. The number of grid points was set to $126 \times 126 \times 126$ at a 0.575 Å grid spacing. 100 docking runs were performed, similarly to the focused docking, with an exception to the cut-off distance, which was set to 5.000 Å.

2.9. Statistics

Each experiment was performed at least in triplicates. Data represent mean and standard error of the mean ($\pm$ SEM) values. Statistical significance was evaluated applying one-way ANOVA and Tukey's post-hoc test ($p < 0.05$ and $p < 0.01$), employing SPSS Statistics software (IBM, Armonk, NY, US).

3. Results and discussion

3.1. Testing the presence of SZV 1287 and its metabolites in the circulation after the per os administration of SZV 1287 to rats

In this preliminary experiment, we aimed to investigate the appearance of SZV 1287 and its suspected metabolites (oxaprozin, L 2799, L 2805, and L 2811) in the circulation. Therefore, three rats were treated with 20 mg/kg SZV 1287, then blood samples were collected before and after (1, 2, 4, 8, and 24 h) the administration. Before the treatment, we did not find interfering peaks with the detection of SZV 1287 or its metabolites in the blood of the animals. However, after administration, SZV 1287, oxaprozin, L 2799, L 2805, and L 2811 appeared in the circulation, showing high variations regarding their plasma concentrations in the three rats. The highest plasma concentrations of SZV 1287 as well as its metabolites were observed 8 h after the administration: 0.063 (± 0.050) μ M SZV 1287, 1.371 (± 0.828) μ M oxaprozin, 0.314 (± 0.188) μ M L 2799, 1.939 (± 1.136) μ M L 2805, and 0.126 (± 0.094) μ M L 2811. Only lower concentrations of SZV 1287 and L 2811 appeared in the blood, while oxaprozin and L 2805 proved to be the major circulating metabolites with micromolar plasma concentrations. Thus, the preliminary animal study demonstrated that, after the oral administration of SZV 1287, the parent compound as well as oxaprozin, L 2799, L 2805, and L 2811 appear at detectable concentrations in the circulation.

3.2. Association constants of ligand-HSA complexes based on fluorescence spectroscopic studies

First, we examined the interactions of SZV 1287 and its metabolites with HSA by fluorescence quenching studies [18,22]. Emission spectrum of HSA (2 μ M) was recorded in the presence of the ligand molecules (0–10 μ M) in PBS (pH 7.4), using 295 nm excitation wavelength. However, under the applied conditions, each compound tested exerted significant fluorescence (Suppl. 2), which strongly interfered with the evaluation of the data at the emission wavelength maximum of HSA (340 nm).

Since the precise determination of K_a values with quenching studies seemed to be highly questionable, we examined the albumin-induced increase in the fluorescence of SZV 1287 and its metabolites. Using 305 nm excitation wavelength, HSA exerted only minor fluorescence at the emission wavelength maxima of the ligand molecules (370–395 nm; data not shown). Therefore,

after the correction of its background fluorescence, the albumin-induced enhancement in the fluorescence of SZV 1287 and its metabolites was evaluated. Emission spectra are demonstrated in Fig. 2. HSA caused a considerable, concentration dependent elevation in the emission signal of each compound tested, showing the largest relative increase in the fluorescence of oxaprozin (5.5-fold) followed by SZV 1287 (3.7-fold) then L 2811, L 2799, and L 2805 (1.8- to 2.2-fold) in the presence of 5-fold excess of HSA (10 μ M) vs. the ligands (Fig. 2F). This phenomenon can be likely explained by the known quenching effects of water molecules on the fluorescence of aromatic fluorophores [29]. Since the interaction with albumin disrupts the hydration shell of the fluorophore, the quenching effects of water molecules decrease, and consequently the emission signal of the albumin-bound form is higher [30,31].

Based on the HSA-induced changes in the emission signal of the compounds tested, K_a values of ligand-HSA complexes were determined by non-linear fitting, employing the Hyperquad software [18]. With a 3×10^6 L/mol association constant, oxaprozin formed the most stable complex with HSA, other compounds showed 2.1- (L 2805), 3.3- (SZV 1287), 3.7- (L 2799), and 6.9-fold (L 2811) lower affinity toward HSA than oxaprozin (Table 1). Our observation that K_a values are in the 10^5 to 10^6 L/mol range (Table 1) suggests that these compounds form highly stable complexes with HSA similarly to phenylbutazone, ibuprofen, or naproxen [9]. Therefore, it is reasonable to hypothesize their very low free fraction in the circulation. Furthermore, it is important to note that the extent of the fluorescence enhancement (e.g. in the presence of the highest albumin concentration used) does not directly correlate with the binding affinity of the ligand toward the protein, as it has also been demonstrated in previous studies [20,31]. Since the steepness of the concentration-intensity curve determines the affinity, even a stronger enhancer can bind to the protein with lower binding constant.

3.3. Interactions of SZV 1287 and oxaprozin with HSA based on ultracentrifugation

To determine the free fractions of SZV 1287 and oxaprozin, undiluted human serum samples and 40 g/L HSA solutions were spiked with these ligands (2 μ M). After ultracentrifugation (see in 2.5), the concentrations of SZV 1287 or oxaprozin were analyzed in the protein-free fraction. The concentration of oxaprozin was below the detection limit (20 nM) suggesting that its free fraction is lower than 1%, which is in agreement with the previously reported data [13]. However, we detected SZV 1287 in the protein-free fraction of both spiked serum samples and HSA solutions. Since its concentration only slightly exceeded the detection limit (20 nM), we cannot give precise data. Nevertheless, we can estimate the very low fraction (1.0 to 1.5%) of free SZV 1287 in the circulation. These observations are in accordance with the high K_a values (10^5 – 10^6 L/mol) of SZV 1287-HSA and oxaprozin-HSA complexes determined using fluorescence spectroscopy (Table 1). Furthermore, the free fractions of SZV 1287 in human serum and HSA solution (with 40 g/L albumin content, approximating the HSA concentration of the blood) were same, demonstrating that albumin is responsible for the plasma protein binding of SZV 1287.

3.4. Interaction of SZV 1287 and its metabolites with HSA based on high-performance affinity chromatography studies

To confirm the results of fluorescence spectroscopic studies, the interactions of SZV 1287 and its metabolites with HSA were also examined by affinity chromatography. Since the formation of ligand-HSA complexes delays the elution of ligand molecules from the HSA-coated column, the higher affinity of the ligand molecule toward HSA leads to its longer retention time. We observed the fol-

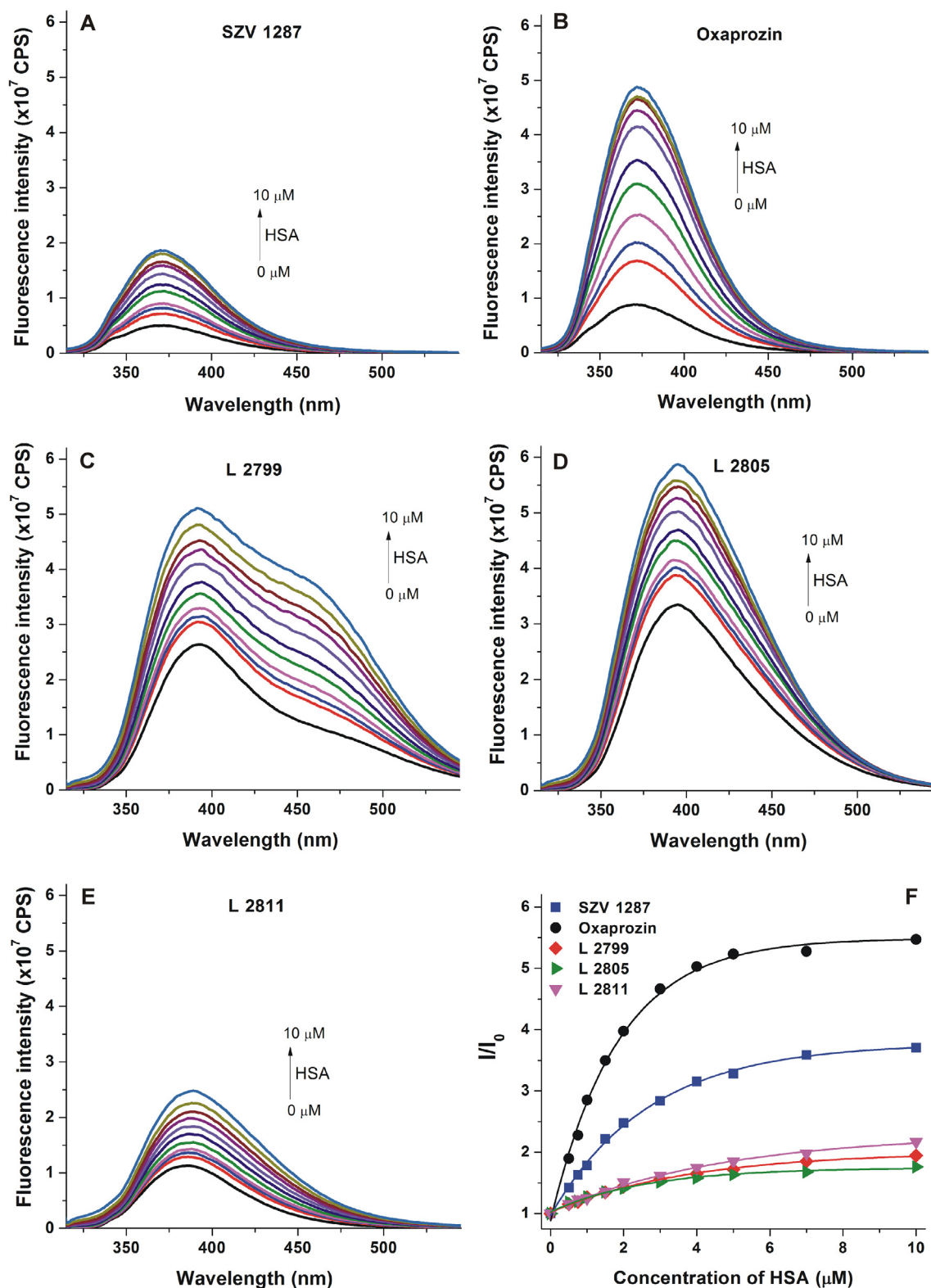


Fig. 2. Effects of HSA on the fluorescence signal of SZV 1287 and its metabolites. Emission spectra of SZV 1287 (A), oxaprozin (B), L 2799 (C), L 2805 (D), and L 2811 (E; each 2 μ M) in the presence of increasing concentrations of HSA (0.0, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.0 and 10.0 μ M) in PBS (pH 7.4; λ_{exc} = 305 nm). HSA-induced increase in the fluorescence of SZV 1287 and its metabolites (F; λ_{em} = 370 nm for SZV 1287 and oxaprozin, 390 nm for L 2799, 385 nm for L 2805, and 395 nm for L 2811).

Table 1

Association constants ($K_a \pm \text{SEM}$) of HSA-ligand complexes.

	SZV 1287	Oxaprozin	L 2799	L 2805	L 2811
K_a [$\times 10^6$ L/mol]	0.91 ± 0.07	3.03 ± 0.13	0.82 ± 0.10	1.47 ± 0.03	0.44 ± 0.02

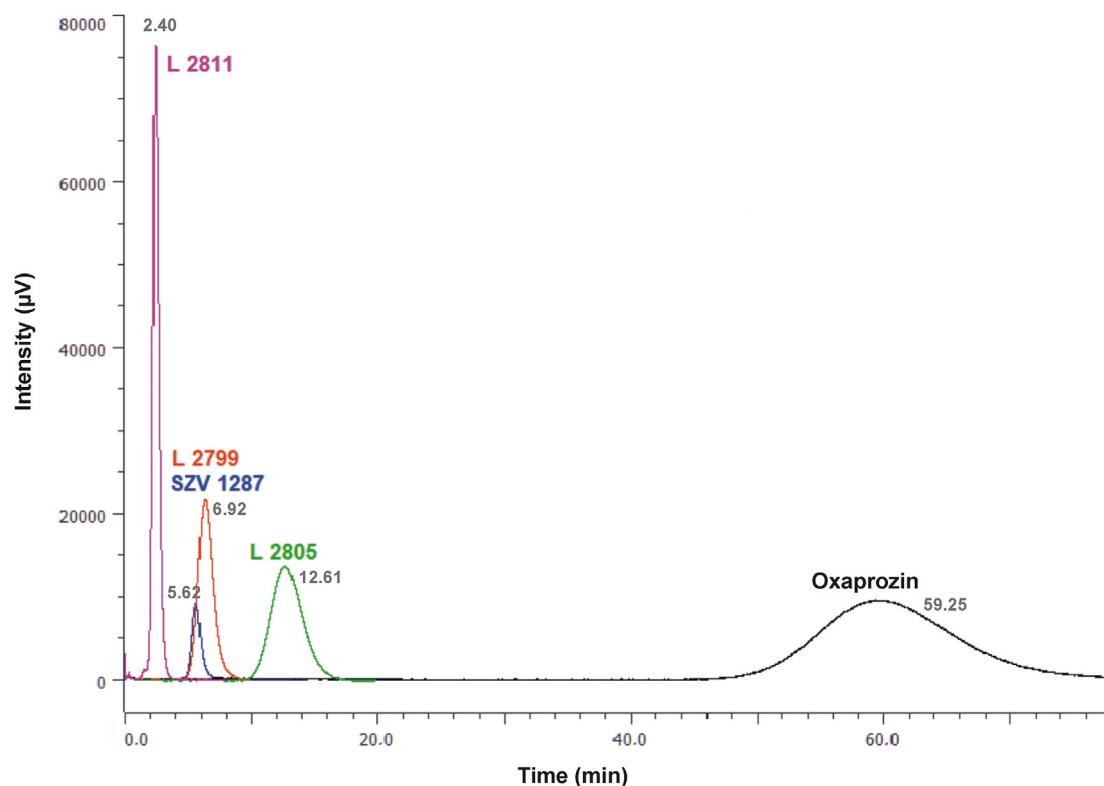


Fig. 3. HPLC chromatograms of SZV 1287, oxaprozin, L 2799, L 2805, and L 2811 with affinity column coated with immobilized HSA (Chiralpak HSA, 50 × 3.0 mm, 5 μm; see experimental details in 2.6).

lowing order of compounds based on their retention times: oxaprozin > L 2805 > L 2799 ≈ SZV 1287 > L 2811 (Fig. 3). In agreement with these observations, spectroscopic studies (Table 1) also demonstrated that oxaprozin forms the most stable complex with HSA followed by L 2805, then SZV 1287 and L 2799 bind with similar affinity, and L 2811-HSA complex is the least stable.

3.5. Testing the binding site of SZV 1287 and its metabolites with ultrafiltration

Molecular displacement of a ligand molecule from albumin can influence the pharmacokinetic and pharmacodynamic properties of strongly plasma protein-bound drugs. Ultrafiltration is a powerful tool to examine the displacement of site markers from serum albumin [22,23]. It is based on the separation of free and albumin-bound molecules [32]. Since HSA is a large (66.5 kDa) macromolecule, albumin and albumin-bound compounds are unable to pass through the filter unit with 30 kDa molecular weight cut-off value. Therefore, the displacement of site markers from HSA results in their increased concentration in the filtrate. Using these principles, the displacement of warfarin (SSI) and naproxen (SSII) from HSA by SZV 1287 and its metabolites was examined. The filtered fractions of site markers were quantified in the absence and presence of 10 and/or 20 μM concentrations of compounds tested. As it is demonstrated in Fig. 4A, each ligand caused significant increase in the filtered fraction of naproxen, in a concentration dependent fashion. The displacement of naproxen from HSA suggests that the high-affinity binding site of SZV 1287 and its metabolites is located in SSII. In agreement with this hypothesis, earlier reports also identified SSII as the binding site of oxaprozin on albumin [10–12]. Some non-steroidal anti-inflammatory drugs (NSAIDs), including diclofenac, ibuprofen, and flurbiprofen also occupy SSII; while other NSAIDs (e.g. aspirin,

phenylbutazone, and indomethacin) preferentially bind to SSI [12]. Compared to our previous studies performed using the same experimental model [22,23], oxaprozin caused strong displacement of naproxen, which is in agreement with its high affinity toward HSA (Table 1).

Only SZV 1287 and oxaprozin modified the filtered fraction of warfarin (Fig. 4B). Interestingly, oxaprozin decreased the bound fraction of the SSI marker while SZV 1287 induced the opposite effect. The latter result suggests that SZV 1287 increased the bound fraction of warfarin, similarly to mycotoxin zearalenone and some of its metabolites [18,33]. Furthermore, it is important to note that oxaprozin displaced much lower amount of warfarin compared to its displacing ability vs. naproxen (Fig. 4). Considering these observations, it is reasonable to hypothesize that SZV 1287 and its metabolites occupy another binding site (likely SSII) and some of these compounds can influence the binding affinity of the SSI ligand warfarin through allosteric modulation.

3.6. Binding sites of SZV 1287 and oxaprozin on HSA based on modeling studies

Molecular modeling is a suitable tool to gain a deeper insight into the albumin-ligand interactions at the molecular level. Thus, it helps to characterize which amino acids are involved in the complex formation, and to understand the differences in binding positions and binding affinities of the ligand molecules, including the parent compound and its metabolites [18,20,22]. SZV 1287 and oxaprozin were docked to SSII on HSA. The binding modes were *de novo* described in the present study. Even if oxaprozin glucuronide is a known [28] ligand to SSII, there is no atomic resolution image of its binding. In the top 1st rank, oxaprozin binds to HSA with an affinity of −7.25 kcal/mol. Furthermore, in the top 3rd rank, SZV 1287 binds with great resemblance to the position

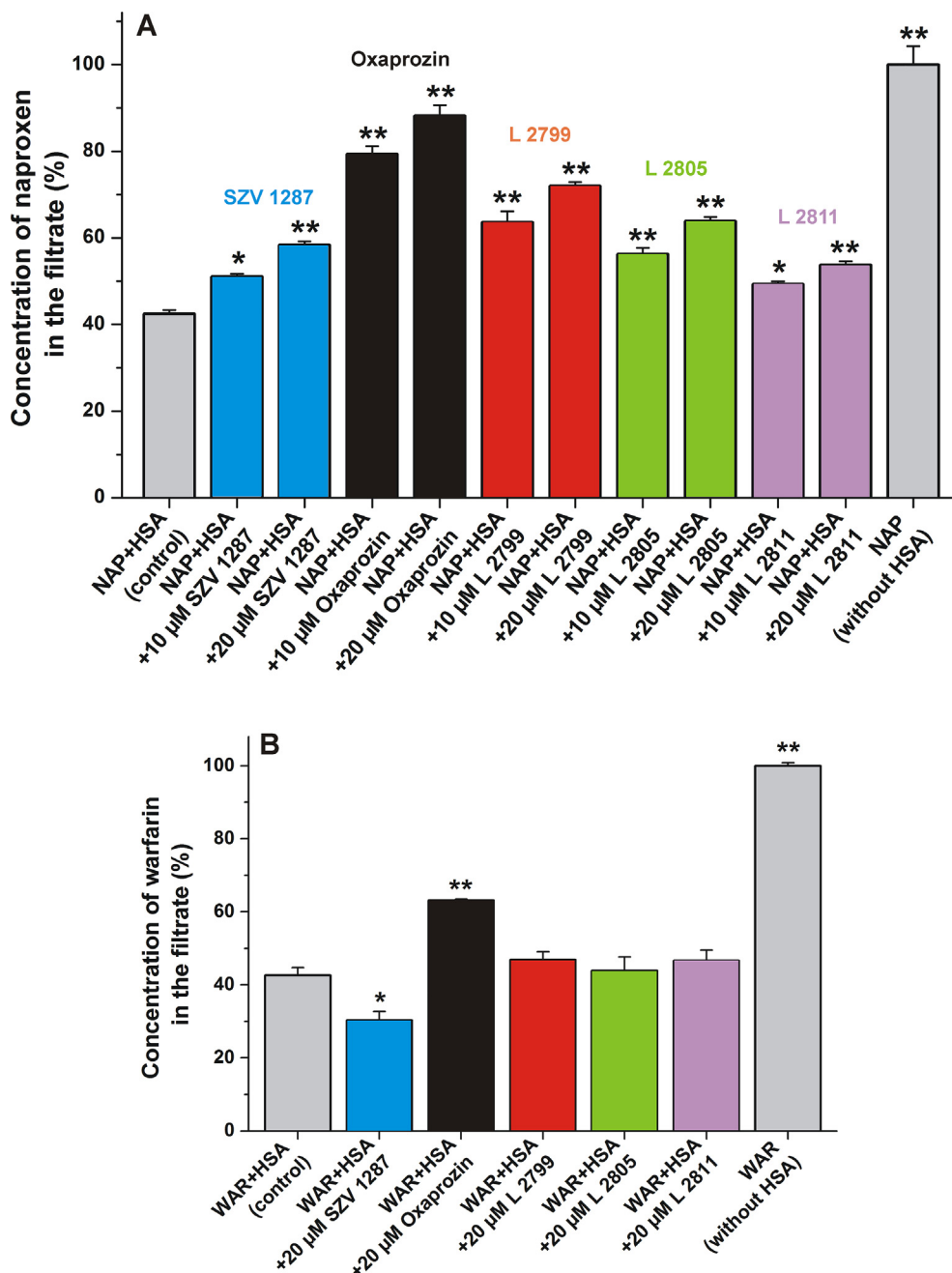


Fig. 4. (A) Concentration of naproxen in the filtrate: Before ultrafiltration, samples contained naproxen and HSA (1.0 and 1.7 μ M, respectively) in the absence and presence of SZV 1287 or its metabolites (10 or 20 μ M) in PBS (pH 7.4). (B) Concentration of warfarin in the filtrate: Before ultrafiltration, samples contained warfarin and HSA (1.0 and 5.0 μ M, respectively) in the absence and presence of SZV 1287 or its metabolites (20 μ M) in PBS (pH 7.4; * $p < 0.05$, ** $p < 0.01$).

of oxaprozin with weaker affinity of -6.72 kcal/mol, which is in agreement with the results of spectroscopic studies, where higher binding constant was determined for the oxaprozin-HSA vs. SZV 1287-HSA complex (Table 1). SZV 1287 found its final binding position through two prerequisite steps around the SSII. We hypothesized the top 3rd rank binding position to be the final of SZV 1287, since its accurate match to that of the top 1st rank binding position of oxaprozin (Fig. 5), which known to be a ligand of SSII [10–12]. The interacting residues were L398, N405, A406, V409, L529, T540, K541, L544, and K545 for oxaprozin (Fig. 5C). Furthermore, L398, K402, N405, A406, V409, K413, L529, T540, K541, L544, K545, and M548 were involved in SZV 1287-HSA interaction (Fig. 5B). Our results suggest that lysine residues (K413 and

K545; Fig. 5B) might stabilize the binding of SZV 1287 through cation- π interactions with the benzene rings of the ligand, and the carboxyl group of oxaprozin might participate in an ionic interaction with the K545 lysine positive charge (Fig. 5C).

According to Kosa et al. [34], the Site II in HSA is a hydrophobic cleft, approximately 16 Å deep and 8 Å wide, with its hydrophobic amino acid residues being well conserved among the species. It was also demonstrated that the negatively charged carboxyl group of ibuprofen is separated from its hydrophobic part, both structures play important role in the complex formation with albumin. Similarly, oxaprozin has also a carboxyl group separated from its heterocyclic core (by two C atoms). Whereas, SZV 1287 contains a C = N-OH head group, and its protonated form does not contain

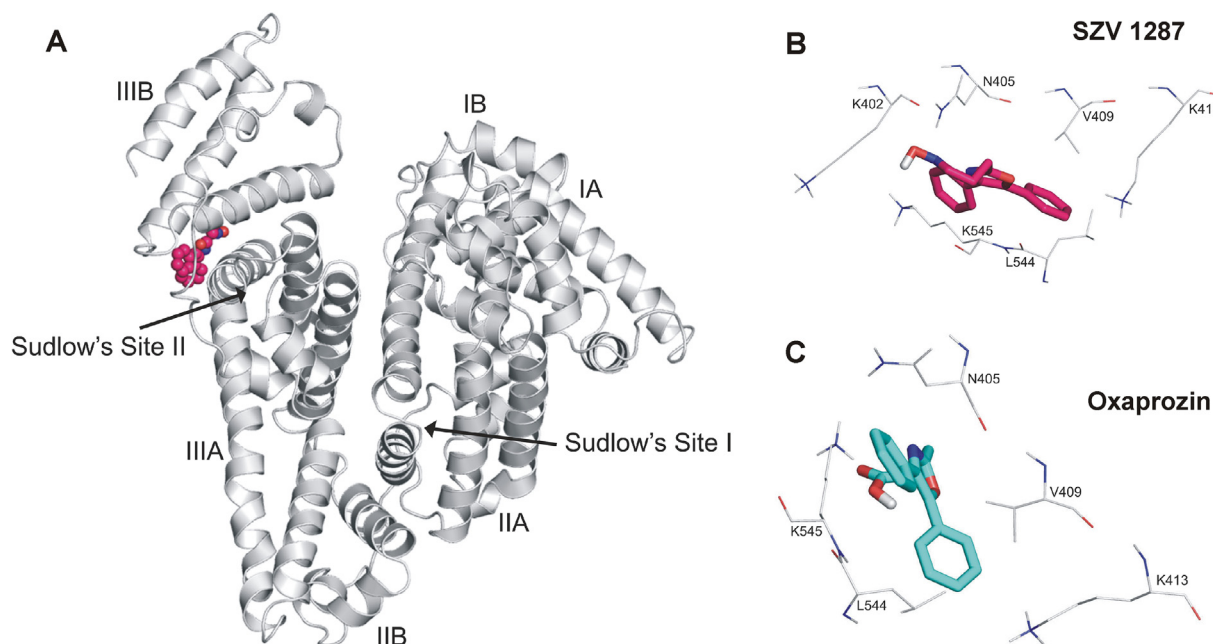


Fig. 5. The binding mode of SZV 1287 (A; space-filling, pink) as docked to the SSII on HSA (teal cartoon). The close-up of binding mode of SZV 1287 (B; pink sticks) and oxaprozin (C; blue sticks) as docked to the SSII on HSA. Most important interacting amino acids are labeled and shown as gray sticks.

a negative charge. However, the complex formation between albumin and diazepam (and other similar compounds) is mainly stabilized by hydrophobic interactions [34]. Based on X-ray crystallographic studies, the binding of ibuprofen and diazepam are accessible under the PDB codes: 2bxg and 2bxf, respectively [35]. Even in the wider binding cavity of the holo structure, the C atom of L387 and the O atom of S489 are 4.7 Å apart. In the apo structure, the amino acid sidechain of R410 protrudes into the entrance of the cavity, further narrowing the binding site. Ibuprofen and diazepam contain 15 and 20 heavy atoms, respectively; thus, they are smaller molecules than SZV 1287 or oxaprozin (both containing 22 heavy atoms). Furthermore, oxaprozin and SZV 1287 have elongated and branching shape, while diazepam is a compact molecule with fused rings. Our docking calculations suggest that the position of oxaprozin and SZV 1287 is located nearby to the entrance of the tight cavity which accommodates ibuprofen and diazepam [35]. Given the size and shape differences of the above-listed molecules, it is fairly rational that oxaprozin and SZV 1287 cannot enter and/or fill this cavity.

3.7. Interactions of SZV 1287 with albumins from different species

Finally, to test the potential species dependent alternations regarding the albumin binding of SZV 1287, its interactions with albumins from different species (bovine, porcine, and rat) were compared to the SZV 1287-HSA complexation. Inter-species differences regarding the albumin binding of SSI and SSII ligand drugs can be resulted from the subtle alterations in the microenvironment (e.g. the differences in the interacting amino acid residues or the size of the hydrophobic cleft) [34]. Moreover, the presence and the location of the binding sites may also vary, which can explain certain differences in binding properties of the ligands [34]. Each albumin induced a considerable increase in the emission signal of SZV 1287: BSA and HSA caused almost four-fold, while PSA (2.5-fold) and RSA (2.8-fold) resulted in lower elevation (Fig. 6). Similarly to section 3.2, K_a values of SZV 1287-albumin complexes were determined based on the albumin-induced increase in the fluorescence of ligand molecules. As it is demon-

strated in Table 2, SZV 1287 formed the most stable complex with HSA, followed by PSA, BSA, and RSA. Nevertheless, the largest difference between K_a values (SZV 1287-HSA vs. SZV 1287-RSA complexes) was only three-fold, which does not mean a large species difference. For example, mycotoxins ochratoxin A and zearalenone show one order of magnitude differences between the stability of some of their albumin complexes [18,20]. Therefore, we can consider only minor species dependent alternations regarding SZV 1287-albumin interactions.

4. Conclusions

In this study, the interactions of SZV 1287 and its metabolites (oxaprozin, L 2799, L 2805, and L 2811) with serum albumin were examined. Fluorescence spectroscopic and HPAC studies demonstrated the formation of stable ligand-HSA complexes ($K_a = 10^5$ to 10^6 L/mol). These findings were also supported by ultracentrifugation experiments, showing the very low free fraction of SZV 1287 and oxaprozin in human plasma and in 40 g/L HSA solution. Ultrafiltration studies with SSI and SSII markers and molecular modeling suggest that the high-affinity binding site of SZV 1287 and its metabolites is located in SSII. Furthermore, experiments with human, bovine, porcine, and rat albumins represented only minor species-dependent differences regarding the albumin binding of SZV 1287. Considering the above-listed observations, albumin binding seems to be a highly relevant parameter in the pharmacokinetics of SZV 1287 and its metabolites. In addition, we also provided a new insight into the pharmacokinetics of oxaprozin by the detailed characterization of its interaction with HSA.

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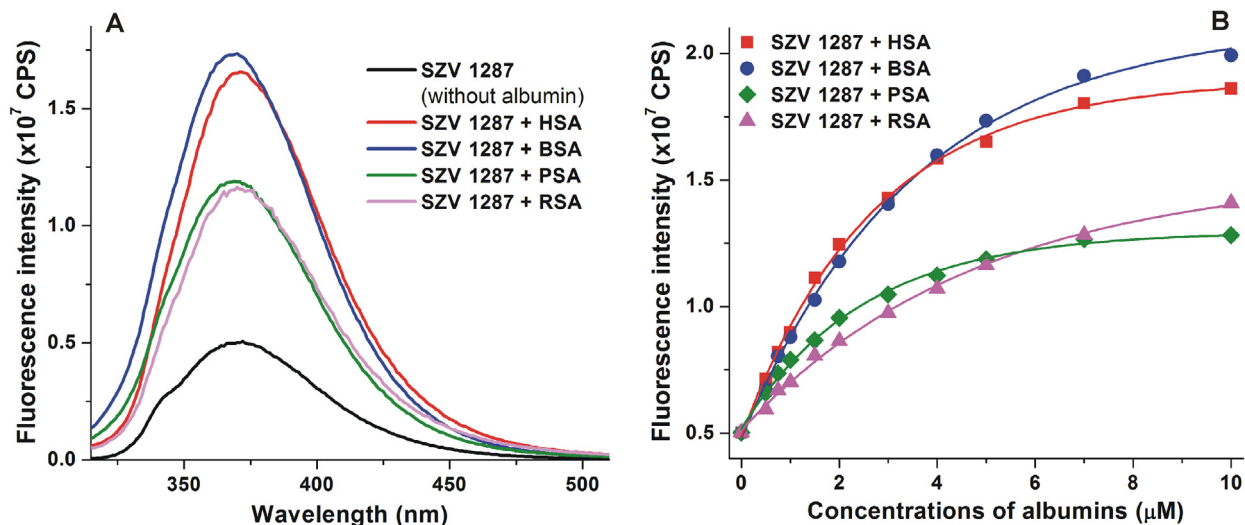


Fig. 6. (A) Fluorescence emission spectrum of SZV 1287 (2 μM) in the presence of human (HSA), bovine (BSA), porcine (PSA), and rat (RSA) serum albumins (each 5 μM) in PBS (pH 7.4; λ_{ex} = 305 nm). (B) Albumin-induced (0.0, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.0 and 10.0 μM) increase in the fluorescence emission signal of SZV 1287 (λ_{ex} = 305 nm; λ_{em} = 370).

Table 2

Association constants ($K_a \pm \text{SEM}$) of SZV 1287-albumin complexes regarding human, bovine, porcine, and rat albumins.

	HSA	BSA	PSA	RSA
$K_a [\times 10^5 \text{ L/mol}]$	9.09 ± 0.77	3.50 ± 0.19	6.83 ± 0.22	2.87 ± 0.07

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CRediT authorship contribution statement

Eszter Fliszár-Nyúl: Methodology, Formal analysis. **Zelma Faisa:** Methodology, Formal analysis. **Violetta Mohos:** Methodology, Formal analysis. **Diána Derdák:** Methodology, Formal analysis. **Beáta Lemli:** Methodology, Formal analysis. **Tamás Kálai:** Resources. **Cecília Sár:** Resources. **Balázs Z. Zsidó:** Methodology, Formal analysis. **Csaba Hetényi:** Methodology, Formal analysis, Funding acquisition. **Ádám I. Horváth:** Methodology, Formal analysis. **Zsuzsanna Helyes:** Conceptualization, Funding acquisition. **Ruth Deme:** Resources. **Dóra Bogdán:** Resources. **Andrea Czompa:** Resources. **Péter Mátyus:** Conceptualization, Resources. **Miklós Poór:** Conceptualization, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2021.115945>.

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