This is the peer-reviewed version of the following article:

Uzelac, T. N.; Nikolić-Kokić, A. L.; Spasić, S. D.; Mačvanin, M. T.; Nikolić, M. R.; Mandić, L. M.; Jovanović, V. B. Opposite Clozapine and Ziprasidone Effects on the Reactivity of Plasma Albumin SH-Group Are the Consequence of Their Different Binding Properties Dependent on Protein Fatty Acids Content. *Chemico-Biological Interactions* **2019**, *311*. https://doi.org/10.1016/j.cbi.2019.108787.

Opposite clozapine and ziprasidone effects on the reactivity of plasma albumin SH-group are the consequence of their different binding properties dependent on protein fatty acids content

Tamara N. Uzelac [a], Aleksandra L. Nikolić-Kokić [b], Snežana D. Spasić [c], Mirjana T. Mačvanin [a], Milan R. Nikolić [a], Ljuba M. Mandić [a], and Vesna B. Jovanović [a]*

[a] *University of Belgrade - Faculty of Chemistry, Department of Biochemistry, and Center of Excellence for Molecular Food Sciences, Studentski trg 12-16, 11000 Belgrade, Serbia*

[b] *University of Belgrade, Institute for Biological Research "Siniša Stanković", Department of Physiology, Bulevar despota Stefana 142, 11000 Belgrade, Serbia*

[c] *University of Belgrade, Institute of Chemistry, Technology and Metallurgy, Centre for Chemistry, Njegoševa 12, 11000 Belgrade, Serbia*

*Corresponding author: Dr. Vesna B. Jovanović, University of Belgrade - Faculty of Chemistry, Studentski trg 12-16, 11000 Belgrade, Serbia; Phone number: +381 11 333 66 76; E-mail: vjovanovic@chem.bg.ac.rs (V. Jovanović).

Highlights

- Clozapine decreases, while ziprasidone increases free albumin-SH fraction in rats.
- *In vitro* drug effects on HSA-SH level/reactivity depend on stearic acid content.
- Observed effects are associated with differences in drugs binding to (de)fatted HSA.

Abstract

Antipsychotic drugs interfere with the antioxidant defense system provoking complex and often toxicological effects. Here we examined differences in plasma albumin reduced free thiol (SH) group content and its reactivity as a consequence of clozapine (CLZ) and ziprasidone (ZIP) binding. Chronic administration of CLZ reduced, whereas treatment with ZIP increased albumin-SH content in rats. Regardless of the ratio of stearic acid (SA) bound to protein, *in vitro* binding of ZIP to human serum albumin (HSA) increased both the SH group level and reactivity. In contrast, the effect of CLZ on HSA-SH reactivity was dependent on HSA to SA molar ratio. CLZ binding was accompanied by an increase in HSA-SH reactivity in samples with normal, but a reduction of its reactivity level with higher SA/HSA ratio, compared to drug-free samples. We demonstrate by steady-state fluorescence quenching studies that an increase in SA binding to HSA is associated with a significant reduction of binding constant for both antipsychotics. In addition, this is the first report of quantitative characterization of ZIP binding to HSA. Our findings suggest that albumin-SH content and reactivity is modulated by ZIP towards an increased antioxidant defense capacity in circulation, as opposed to CLZ, which can contribute to the safer, more effective treatment of schizophrenia.

Keywords: albumin, clozapine, ziprasidone, thiols.

Abbreviations and acronyms: CLZ, Clozapine; ZIP, Ziprasidone; FA(s), Fatty acid(s); HSA, Human serum albumin; HSA-SH, free thiol in the Cys34 residue of HSA; SA, Stearic acid; HSA/SA, HSA in complex with SA; HSA/CLZ and HSA/ZIP, HSA in complex with CLZ and ZIP, respectively; DMSO, Dimethyl sulfoxide; DTNB, 5,5′-Dithiobis-(2-nitrobenzoic acid).

1. Introduction

Schizophrenia is one of the most common mental disorders that occurs in 1% of the population and can manifest in a variety of ways and with different symptoms [**1**]. Although the disbalance of neurotransmitters turnover plays a central role in the development of schizophrenia [**2**], recent studies emphasize the role of reactive oxygen species, oxidative stress and the accompanying oxidative damage in its pathogenesis [**3,4**]. The content of small non-protein antioxidants such as uric acid, bilirubin, and glutathione is found to be consistently lower in untreated schizophrenic patients compared to healthy controls [**5−8**].

Due to its high concentration in the circulation, human serum albumin (HSA) accounts for a major part of the antioxidant capacity of human plasma by binding and carrying radical scavengers, or by sequestering redox-active transition metal ions [**9**]. In healthy adults, about two-thirds of a free thiol group in the Cys34 residue (HSA-SH) on the surface of the protein exists is a reduced form, constituting the major extracellular source of reactive free thiols [**10**]. HSA serves as the primary binding protein for unesterified fatty acids (FAs) in the blood, with seven asymmetrically arranged binding sites of different affinities for long-chain FAs [**11,12**]. Under physiological conditions, circulating HSA carries only one or two FA molecules and this number increases up to six in the certain disease states [**13**]. There are two canonical high-affinity sites on HSA (Sudlow I and Sudlow II) for binding and transport of lipophilic drugs. Anionic heterocyclic compounds primarily bind to the Sudlow I site located in subdomain IIA of HSA, and aromatic structures preferentially bind to Sudlow II site in subdomain IIIA [**12**]. There is increasing evidence in support of the existence of the third major drug binding region of HSA

within subdomain IB, which accommodates a variety of acidic, neutral, and basic molecules [**14**].

The increased content of plasma FAs is frequently found in schizophrenia [**15,16**], and antipsychotic drugs treatment seems to contribute to a decrease in reactive free thiols antioxidant capacity of plasma **[7]**. The purpose of this study was to establish whether long-term (4 weeks) administration of high doses (corresponding to maximal therapeutic doses used for humans) of two regularly used lipophilic antipsychotic drugs, clozapine (CLZ) and ziprasidone (ZIP) (**Fig. 1**), influences the level of total thiols and the albumin-SH group in rat plasma. In addition, the influence of CLZ and ZIP binding to HSA [defatted or in complexes with stearic acid (SA)] on the HSA-SH content and reactivity were investigated *in vitro*, followed by characterization of CLZ and ZIP binding to HSA under identical, controlled physiological conditions.

Figure 1 here (single column)

2. Materials and methods

2.1. Chemicals and reagents

All chemicals of analytical reagent grade were purchased from Sigma-Aldrich Chemie (Germany) and Merck (Germany) unless otherwise noted. The 20% solution of HSA (96% purity, intended for clinical use, containing 0.42 M of SH-groups per M of HSA) was purchased from Baxter (Austria). Bromocresol green albumin assay kit was purchased from Human (Germany). SA was purchased from Sigma-Aldrich Chemie (Germany). Antipsychotic medications (Zeldox® and Clozapine) and its active ingredients (ZIP and CLZ) were provided by Pfizer (Austria) and Remedica Ltd (Cyprus), respectively.

2.2. Treatment of rats with antipsychotics

Wistar male albino rats (3 months old, weighing 320-350 g) were kept under standard conditions at a temperature of 22°C, at a twelve-hour cycle shift of day and night. Implemented procedures were in accordance with Directive 2010/63/EU concerning the protection of animals for experimental and other scientific purposes. The study design was approved by the Ethical Committee for the use of lab animals at the Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia. Rats were randomly divided into three groups, with 8 animals per group. Two groups of animals were treated with drugs (1 mL/kg/day), the first with ZIP (20 mg/mL) and the second with CLZ (45 mg/mL), and control group with drinking water (1 mL/kg/day) for 4 weeks [**17**]. Applied antipsychotic doses correspond to maximal therapeutic doses for humans [**18**]. Drugs were prepared (water suspension of pulverized tablets) and administered daily in the morning *via* a gastric tube to ensure that no drug loss occurred.

2.3. Isolation of plasma and albumin from rat blood

In order to avoid the influence of anesthetics binding to plasma albumin [**12**], rats were sacrificed by decapitation at 28 days of treatment (following an overnight fast). Blood from the abdominal aorta was collected in a tube with an anticoagulant (disodium EDTA). Erythrocytes and the buffy coat were precipitated by centrifugation at 5000 x g for 10 min at a temperature of 10 °C. The supernatant plasma was immediately separated, frozen and stored at -80 °C until used.

Plasma albumin was isolated by two-step fractional precipitation with saturated ammonium sulfate solution pH 7.4 [**19**]. A stock solution of obtained albumin (94% purity) for further analysis was prepared in 0.1 M sodium phosphate buffer pH 7.4.

104 *2.4 Analysis of biochemical parameters in rat plasma*

105 A biuret method [**20**] was used for the quantification of total plasma proteins. To measure
106 plasma albumin concentration, bromocresol green method was applied [**21**].

107 *2.5. Preparation of defatted HSA and HSA/SA samples*

108 For *in vitro* experiments, the commercial HSA was firstly defatted by charcoal method
109 [**22**], and optionally, its SH-group was then reduced with dithiothreitol [**23**]**.** Working defatted
110 HSA sample with normal physiological content of the free thiol group (about 70%) was prepared
111 by mixing appropriate volumes of defatted HSA and reduced defatted HSA solutions. Protein
112 complexes (HSA/SA samples) with bound SA in molar ratios (protein to FA) of 1:1, 1:2, 1:3 and
113 1:4 were prepared by mixing the appropriate volumes of 50 mM SA in 99% ethanol and 0.25
114 mM defatted HSA in 0.1 M sodium phosphate buffer, pH 7.4.

115 *2.6. Preparation of defatted HSA or HSA/SA samples with antipsychotics*

116 Appropriate volumes of CLZ or ZIP stock solutions (25 mM in DMSO) were added to
117 defatted HSA sample and all HSA/SA samples to get a final protein to drug molar ratio of 1:0.5,
118 1:1.5, and/or 1:2. Resulted mixtures (various HSA/CLZ, HSA/ZIP, HSA/SA/CLZ or
119 HSA/SA/ZIP samples) were incubated at 37°C for one hour before further analysis.

120 *2.7. Thiol(s) quantification*

121 The content of total (protein and non-protein) thiols in rat plasma, thiol (SH-group)
122 content in albumin isolated from rat plasma and in all *in vitro* HSA samples was determined by a
123 modified Ellman method, using DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) reagent [**24**]. The
124 obtained values were expressed in mM (plasma) and M -SH/M protein (albumin or HSA). For

the purpose of relative data comparison, thiol contents in control (defatted) HSA samples were normalized to 100%.

2.8. Determination of HSA-SH group reactivity

For determination of the pseudo-first-order rate constant (*k*') as a measure of HSA-SH reactivity, the amount of DTNB in the reaction HSA mixtures was forty-fold times greater than the total free thiol groups. Reaction kinetics were monitored spectrophotometrically using the protocol described in detail elsewhere [**25**]. Values of *k*′ (s^{-1}) were determined by fitting the natural logarithm of unreacted thiol group concentrations *vs.* time using the linear least squares model.

2.9. Examination of CLZ and ZIP binding to defatted HSA and HSA/SA samples

Fluorescence spectroscopy was used to characterize/compare the binding of SA and/or antipsychotics in various defined ratios to standardize (in terms of reduced thiol Cys34 content) HSA. Working HSA and HSA/SA solutions (**Section 2.5.**) were prepared on a daily basis, by diluting the stock solution of HSA with 0.1 M sodium phosphate buffer pH 7.4, and drugs' stocks with DMSO. Small aliquots of 200 μM CLZ/ZIP solutions were added to 2.5 mL of 1 μM HSA sample solutions, so that the final concentrations of the ligand were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 μM. HSA fluorescence spectra were recorded on the FluoroMax-4 Jobin Yvon (Japan) spectrofluorometer in the 1 cm quartz cell at 37°C. The excitation wavelength was fixed at 295 nm (excitation of the Trp214 residue in the subdomain IIA), and the emission spectra were read at 305 to 420 nm, with 4.5 nm slit widths. The final spectra were presented as the mean value of two accumulations after appropriate blank (buffer and drugs) corrections.

Fluorescence intensities were corrected for the absorption of excited light and the re-absorption of emitted light (the inner-filter effect) according to the equation [**26**]:

$$F_c = F_m\, 10^{(Aex + Aem)/2}$$

where F_m is measured fluorescence, F_c is corrected fluorescence, and A_{ex} and A_{em} are the absorbance values of the quencher measured at the excitation and peak emission (340 nm) wavelength, respectively.

The quenching Stern-Volmer's (SV) constants of HSA complexes containing CLZ/ZIP were determined using equation [**27**]:

$$F_0/F = 1 + k_q\, \tau_0\, [Q] = 1 + K_{SV}\, [Q]$$

where F_0 and F are protein emission fluorescence at 340 nm before and after the addition of the quencher (CLZ/ZIP), respectively, K_{sv} is SV quenching constant, k_q stands for the biomolecule fluorescence quenching rate constant, τ_0 is the average lifetime of the biomolecule without quencher (10^{-8} s) and [Q] is the total quencher concentration.

Estimations of the association (binding) constant (K_a) and a number of binding sites (n) of CLZ/ZIP on HSA samples were done using equation [**28**]:

$$\log (F_0 - F)/F = - n \log(1/([L] - [P] \times (F_0 - F)/F_0) + n \log K_a$$

where [L] and [P] are the total concentration of ligand (CLZ/ZIP) and protein (HSA) samples, respectively.

2.10. Statistical analysis

Each assay was performed in triplicate. The results are expressed as mean ± standard deviation (S.D.). All statistical analysis and graphical representations of data were performed

using the Origin Lab 8.0 (Origin Corporation, USA). A probability level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effects of CLZ and ZIP treatment on the total plasma thiols and albumin-SH levels in rats

After 28 days of treatment of rats with antipsychotics (**Section 2.2.**), total plasma protein and albumin concentrations were significantly lower ($P<0.05$) in comparison with the control group. CLZ produces a significant ($P<0.05$) reduction in both total plasma thiols (42%) and albumin-SH content (21%) compared with controls. ZIP treatment caused a small but significant ($P<0.05$) increase (13%) of the albumin-SH group level and had no significant effect on total plasma thiol levels (**Table 1**).

Table 1 here

3.2. Influence of CLZ or ZIP binding on the HSA-SH content and reactivity in vitro

The influence of CLZ/ZIP binding on the HSA-SH content/reactivity was investigated following one-hour incubation of defatted HSA sample or HSA/SA complexes with CLZ or ZIP (**Section 2.6.)** Regardless of the protein to drug molar ratio and SA content, the binding of CLZ does not affect the HSA-SH level (**Fig. 2**, left panel). However, the binding of ZIP to HSA/SA complexes leads to a small but significant increase ($P<0.05$) (up to 20%) in the HSA-SH level, which correlates with the increasing FA and drug content in HSA-complexes (**Fig. 2**, right panel).

Figure 2 here (2-column fitting image)

The examination of HSA-SH reactivity changes upon CLZ/ZIP binding to defatted HSA sample and HSA/SA complexes was performed by determining the rate constant (*k*') for protein-free thiol group (Cys34) reaction with DTNB (**Section 2.8.**). Graphics obtained after the data linearization show that all reaction samples followed pseudo-first-order reaction kinetics (**Fig. 3**).

Figure 3 here (2-column fitting image)

The effect of CLZ binding on HSA-SH reactivity was found to be dependent on SA content. In comparison with complexes without CLZ, in defatted HSA sample and HSA/SA complexes with normal FA to protein molar ratio (1:1 and 2:1), an increase in the HSA-SH reactivity which correlates with increased CLZ content was observed (**Table 2**). In contrast, a significant decrease ($P<0.05$) in the HSA-SH reactivity towards DTNB was measured in all HSA/SA/CLZ complexes containing an increased FA to protein molar ratio (3:1 and 4:1) (**Table 2**; **Fig. 4**, left panel).

Table 2 here

Figure 4 here (2-column fitting image)

In all examined drug to protein molar ratios, binding of ZIP to either defatted HSA or HSA/SA samples significantly increases (up to 62%) HSA-SH reactivity and this effect is SA-content independent (**Table 2**). Interestingly, the largest single change in reactivity is observed in defatted HSA/ZIP complexes (**Fig. 4**, right panel).

3.3. Characterization of CLZ and ZIP binding to defatted HSA and HSA/SA samples

A steady-state fluorescence study has been performed to gain insight into the differences in binding of antipsychotics to albumin with different SA content. As expected, the binding of

CLZ and ZIP to HSA is followed by a reduction in the intensity of the HSA emission peak in comparison to the corresponding defatted HSA and HSA/SA samples (**Supplementary Fig. S1**). The quenching of intrinsic protein fluorescence (dominantly originating from Trp214 fluorophore) is higher in complexes with CLZ than ZIP (**Fig. 5**).

Figure 5 here (2-column fitting image)

The parameters of CLZ or ZIP binding to defatted HSA and HSA/SA samples (protein to FA molar ratios from 1:1 to 1:4) are calculated (**Section 2.9.**) and the results are shown in **Table 3**. The obtained SV plots were linear and K_{sv} values are calculated from the slope of the curves F_0/F *versus* [Q] (**Supplementary Fig. S2**). Quenching rate constant values ($1.02–2.89\times10^{12}$ $M^{-1}s^{-1}$) are higher than the limiting diffusion rate constant of the biomolecule ($\sim10^{10}$ $M^{-1}s^{-1}$), indicating a static type of quenching [**29**]. The binding site numbers (n) were obtained from the slope of the curves log $(F_0–F)/F$ *vs.* log$(1/([L]–[P] \times (F_0–F)/F_0)$ and K_a values are calculated from the intercept (intercept = n logK_a) (**Supplementary Fig. S3**). The drugs K_a values ranged from 0.25 to 1.90×10^4 M^{-1}. The highest association constants (CLZ: 1.90×10^4 M^{-1} and ZIP: 0.95×10^4 M^{-1}) were obtained for defatted HSA-drug complexes. With an increased content of SA in protein complexes, K_a values for CLZ and ZIP binding decreases, particularly for ZIP (**Table 3**). Following the same trend, the estimated number of HSA-drug binding sites is reduced as well, from 0.792 to 0.592 for CLZ and from 0.786 to 0.545 for ZIP.

4. Discussion

In addition to genetic and environmental factors, the role of oxidative stress in schizophrenia is an important determinant in pathogenesis and progression of the disease [**30,31**].

There is an increasing number of reports concerning the side effects of atypical antipsychotic drugs therapy on body redox and antioxidants homeostasis. Reduction in total proteins and plasma albumin contents observed after 4-week treatment of rats with CLZ and ZIP in this study indicates that adaptive changes during therapy with these drugs are directed more toward fulfilling cellular energy demands at the expense of the maintenance of overall cytosolic composition [**32**]. We found that treatment of rats with high doses of CLZ, but not with ZIP, reduced total plasma thiols compared to the values observed in control animals. In addition, CLZ treatment decreased plasma albumin-SH content. In contrast to CLZ, ZIP treatment increased rat's albumin-SH group content. The total level of reactive thiols, especially from the albumin, is an important determinant of overall plasma antioxidant capacity. Relatively well-preserved total thiol plasma content after the application of ZIP could be a consequence of an increase in albumin-SH group level. Our findings confirm the influence of chronic antipsychotics drug treatment on the plasma antioxidant homeostasis **[7]**, pointing to the adverse effects of CLZ administration in this regard.

An increase in HSA-SH reactivity was detected upon protein interactions with aromatic compounds (enterolactone and enterodiol) and this effect is found to be SA-dependent [**33**]**.** In addition, it was found that polarity changes in the HSA-SH environment during FAs binding increased the reactivity of the HSA-SH group [**25,34**]. We have assumed that binding of CLZ (tricyclic dibenzodiazepine) and ZIP (benzothiazolylpiperazine derivative) to HSA could influence the accessibility/content of the Cys34 thiol group on the protein surface and, therefore, lead to changes in its reactivity/antioxidant capacity. Given the fact that binding sites of antipsychotics and FAs on HSA overlap [**12**] and that FAs themselves modulate the reactivity of

the thiol of HSA [**35**], their competitive/cooperative interactions were investigated *in vitro* using physiologically relevant models with protein to SA molar ratios characteristic for both normal (1:1 and 1:2) and pathological (1:3 and 1:4) conditions. Binding of CLZ to defatted HSA and HSA/SA samples in all protein to drug molar ratios (1:0.5, 1:1.5 and 1:2) did not affect HSA-SH content. On the contrary, binding of ZIP, in general, increased the HSA-SH content. This *in vitro* ZIP effect is more pronounced in HSA samples containing more bound ZIP and SA, and is in agreement with results from *in vivo* study, where chronic ZIP treatment lead to a beneficial increase in the albumin-SH content in rats.

Differences in the HSA-SH content and/or reactivity in HSA/SA samples upon antipsychotics binding likely represent the consequence of their different binding properties. It has been shown that ZIP and CLZ bind to different sites on the HSA molecule. The binding site for ZIP is located in the IB region near to critical Cys34 residue [**14**], while CLZ binds to the Sudlow I binding site near to critical Trp214 [**36**]. Only CLZ binding to HSA/SA samples with physiological FA content led to an increase in the HSA-SH reactivity when compared to drug-free control samples, while increased SA content actually reduced it. In contrast, binding of ZIP consistently increased thiol group reactivity in all HSA/SA samples compared to control ones. It should be noticed that the contribution of ZIP to HSA-SH reactivity increase was somewhat higher in defatted and lower FA content HSA/SA samples. Our finding that HSA-SH content and reactivity (and therefore HSA antioxidant properties) was changed in HSA/antipsychotic complexes (especially with ZIP) and that it can be modulated by FAs and concentration of drugs could be a useful additional factor in the choice and doses of these medicaments in the patients' treatment.

All described variations of the tested parameters are presumably a result of the different defatted HSA or HSA/SA complex conformation perturbations upon CLZ or ZIP binding. Indeed, the decrease of intrinsic protein fluorescence (Trp214 residue excitation) was noticeable in CLZ or ZIP-containing mixtures compared to the defatted HSA and HSA/SA samples without drugs. As expected, the intensity of fluorescence quenching at emission maximum was higher in HSA complexes with CLZ than with ZIP, in view of CLZ binding in the immediate vicinity of the fluorophore. ZIP binds distant from Trp214 fluorophore, therefore producing a lower then CLZ protein fluorescence quenching. On the other hand, the binding of ZIP near Cys34 residue causes a conformational change that positively affects the reactivity of this thiol group.

The finding that plasma FAs content is significantly increased in schizophrenic patients due to the energy metabolic dysfunction [**15,16**] prompted us to quantify the influence of the formation of HSA/SA complexes on CLZ and ZIP binding. The observed binding constants ranged from 0.25 to 1.90×10^4 M^{-1}, suggesting moderate binding of antipsychotics to HSA. The K_a value obtained for defatted HSA/CLZ complex (1.9×10^4 M^{-1}) corresponds to previously reported [**36**]. The observed reduction in binding affinities of CLZ and ZIP to HSA that paralleled increased SA-complex content is presumably the consequence of the overlapping of their binding sites. Primary high-affinity sites of HSA for FAs are numbered as 2, 4 and 5. The secondary sites with moderate affinity for FAs binding correspond to sites 1, 3, 6 and 7 [**37**]. Sudlow I binding site for CLZ in subdomain IIA overlaps with FAs binding site 7, whereas binding site for ZIP located in IB subdomain overlaps with FA site 1 [**12,14**]. Our results suggest that, with increasing concentration of FAs bound to serum albumin, the availability of binding sites and the overall affinity for tested antipsychotics decreases. With increased SA binding, the

295 estimated number of binding sites for CLZ is reduced from 0.792 to 0.592 and from 0.786 to
296 0.545 for ZIP. The observed effect is most likely due to cooperative conformational changes in
297 the flexible native structure of serum albumins induced by competitive binding of FAs and drugs
298 [**38,39**].

299 These results open interesting questions pertinent to pharmacological and toxicological
300 properties of these drugs in clinical settings. One is the actual drug concentration in the
301 circulation of chronically treated patients since higher binding of FAs to HSA could influence
302 their target sites bioavailability. The study presented here is, to the best of our knowledge, the
303 first quantitative characterization of ZIP binding to HSA, with measured K_a value for defatted
304 HSA/ZIP complex of 0.95×10^4 M^{-1} at 37°C.

305 **5. Conclusions**

306 The free plasma albumin-thiol group content plays a particularly important role in the
307 antioxidant defense system. In this study, we report a finding that 4 weeks treatment of rats with
308 CLZ significantly reduced total plasma thiols and albumin-SH content compared to the controls.
309 Surprisingly, treatment of rats with another commonly used antipsychotic, ZIP, leads to an
310 opposite effect on albumin-SH level. We found that both drugs altered the Cys34 thiol group
311 content and reactivity which have been modulated by the SA content in protein complexes.
312 Reactivity is increased upon CLZ, and especially upon ZIP binding to HSA complexes with
313 lower SA levels which are present under physiological condition. This may have a positive effect
314 on an overall antioxidant status, in particular with ZIP which increased plasma albumin-SH
315 content *in vitro* as well. Compensatory increase in the free thiol group protein reactivity is a

possible mechanism for preserving the total thiols plasma content *in vivo*. When the number of SA molecules bound per molecule of HSA is increased to higher values more typical for schizophrenia, CLZ and ZIP binding is reduced. In contrast to CLZ, a positive effect of ZIP on the reactivity of HSA-SH group prevails. The observed differences could be explained by different binding properties of drugs to serum albumin, i.e. specificity of conformational protein changes induced by cooperative FAs and antipsychotics bindings. Taken together, our results suggest that, in contrast to CLZ, ZIP may have an additional beneficial therapeutic effect by contribution to plasma thiols homeostasis.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgment

This work was supported by the Ministry of Education, Science and Technological Development of Serbia (Grants No. 172049 and 173014); and the EU's Horizon 2020 research and innovation program under grant agreement No. 810752. The EC does not share responsibility for the content of the article.

References

[1] K.R. Patel, J. Cherian, K. Gohil, D. Atkinson. Schizophrenia: overview and treatment options. P. T. 39 (2014) 638-645.

[2] G. Remington. Alterations of dopamine and serotonin transmission in schizophrenia. Prog. Brain Res. 172 (2008) 117-140. https://doi.org/10.1016/S0079-6123(08)00906-0

[3] M. Bošković, T. Vovk, B. Kores Plesničar, I. Grabnar. Oxidative Stress in Schizophrenia. Curr. Neuropharmacol. 9 (2011) 301-312. https://doi.org/10.2174/157015911795596595

[4] M. Koga, A.V. Serritella, A. Sawa, T.W. Sedlak. Implications for reactive oxygen species in schizophrenia pathogenesis. Schizophr. Res. 176 (2016) 52-71. https://doi.org/10.1016/j.schres.2015.06.022

[5] R.R. Reddy, M. Keshavan, J.K. Yao. Reduced plasma antioxidants in first-episode patients with schizophrenia. Schizophr. Res. 62 (2003) 205-212. https://doi.org/10.1016/S0920-9964(02)00407-3

[6] H.S. Dahake, S. Ghangle, J. Warade, Y. Pawade, G.S. Kansara. Study of nonenzymatic antioxidants in schizophrenic patients. Intern. J. Res. Med. Sci. 4 (2016) 3768-3772. http://dx.doi.org/10.18203/2320-6012.ijrms20162604

[7] T.L. Huang, C.W. Liou, T.K. Lin. Serum thiobarbituric acid-reactive substances and free thiol levels in schizophrenia patients: Effects of antipsychotic drugs. Psychiatry Res. 177 (2010) 18-21. https://doi.org/10.1016/j.psychres.2009.01.017

[8] A. Ciobica, M. Padurariu, I. Dobrin, C. Stefanescu, R. Dobrin. Oxidative stress in schizophrenia - focusing on the main markers. Psychiatr. Danub. 23 (2011) 237-245.

[9] M. Taverna, A.L. Marie, J.P. Mira, B. Guidet. Specific antioxidant properties of human serum albumin. Ann. Intensive Care 3 (2013) 4. https://doi.org/10.1186/2110-5820-3-4

[10] L. Turell, R. Radi, B. Alvarez. The thiol pool in human plasma: The central contribution of albumin to redox processes. Free Radic. Biol. Med. 65 (2013) 244-253. https://doi.org/10.1016/j.freeradbiomed.2013.05.050

[11] A.A. Bhattacharya, T. Grüne, S. Curry. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. J. Mol. Biol. 303 (2000) 721-732. https://doi.org/10.1006/jmbi.2000.4158

[12] M. Fasano, S. Curry, E. Terreno, M. Galliano, G. Fanali, P. Narciso, S. Notari, P. Ascenzi. The extraordinary ligand binding properties of human serum albumin. IUBMB Life 57 (2005) 787-796. https://doi.org/10.1080/15216540500404093

[13] D.P. Cistola, D.M. Small. Fatty acid distribution in systems modeling the normal and diabetic human circulation. A 13C nuclear magnetic resonance study. J. Clin. Invest. 87 (1991) 1431-1441. https://doi.org/10.1172/JCI115149

[14] F. Zsila. Subdomain IB Is the Third Major Drug Binding Region of Human Serum Albumin: Toward the Three-Sites Model. Mol. Pharm. 10 (2013) 1668-1682. https://doi.org/10.1021/mp400027q

[15] M. Orešič, J. Tang, T. Seppänen-Laakso, I. Mattila, S.E. Saarni, S.I. Saarni, J. Lönnqvist, M. Sysi-Aho, T. Hyötyläinen, J. Perälä, J. Suvisaari. Metabolome in schizophrenia and other psychotic disorders: a general population-based study. Genome Med. 3 (2011) 19. https://doi.org/10.1186/gm233

[16] X. Yang, L. Sun, A. Zhao, X. Hu, Y. Qing, J. Jiang, C. Yang, T. Xu, P. Wang, J. Liu, J. Zhang, L. He, W. Jia, C. Wan. Serum fatty acid patterns in patients with schizophrenia: a targeted metabonomics study. Transl. Psychiatry 7 (2017) e1176. https://doi.org/10.1038/tp.2017.152

[17] A. Nikolić-Kokić, N. Tatalović, J. Nestorov, M. Mijović, A. Mijusković, M. Miler, Z. Oreščanin-Dušić, M. Nikolić, V. Milošević, D. Blagojević, M. Spasić, Č. Miljević.

Clozapine, ziprasidone, and sertindole-induced morphological changes in the rat heart and their relationship to antioxidant enzymes function. J. Toxicol. Environ. Health A 81 (2018) 844-853. https://doi.org/10.1080/15287394.2018.1495587

[18] M.A. Raggi, R. Mandrioli, C. Sabbioni, V. Pucci. Atypical antipsychotics: pharmacokinetics, therapeutic drug monitoring, and pharmacological interactions, Curr. Med. Chem. 11 (2004) 279-96. https://doi.org/10.2174/0929867043456089

[19] V.B. Jovanović, A.Z. Penezić-Romanjuk, I.D. Pavićević, J.M. Aćimović, L.M. Mandić. Improving the reliability of human serum albumin-thiol group determination. Anal. Biochem. 439 (2013) 17-22. https://doi.org/10.1016/j.ab.2013.03.033

[20] H.W. Robinson, C.G. Hogden. The biuret reaction in the determination of serum proteins. 1. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. 135 (1940) 707-725.

[21] B.T. Dumas, W.A. Watson, H.G. Biggs. Albumin standards and the measurement of serum albumin with bromcresol green. Clin. Chim. Acta 258 (1997) 21-30.

[22] R. Chen. Removal of Fatty Acids from Serum Albumin by Charcoal Treatment. J. Biol. Chem. 242 (1967) 173-181.

[23] A.Z. Penezić, V.B. Jovanović, I.D. Pavićević, J.M. Aćimović, L.M. Mandić. HSA carbonylation with methylglyoxal and the binding/release of copper (II) ions. Metallomics 7 (2015) 1431-1438. https://doi.org/10.1039/c5mt00159e

[24] G. Bulaj, T. Kortemme, D.P. Goldenberg. Ionization-reactivity relationships for cysteine thiols in polypeptides. Biochemistry 37 (1998) 8965-8972. https://doi.org/10.1021/bi973101r

401 [25] I.D. Pavićević, V.B. Jovanović, M.M. Takić, A.Z. Penezić, J.M. Aćimović, L.M. Mandić.
402 Fatty acids binding to human serum albumin: Changes of reactivity and glycation level of
403 Cysteine-34 free thiol group with methylglyoxal. Chem. Biol. Interact. 224 (2014) 42-50.
404 https://doi.org/10.1016/j.cbi.2014.10.008
405 [26] M. Van de Weert, L. Stella. Fluorescence quenching and ligand binding: A critical
406 discussion of a popular methodology. J. Mol. Struct. 998 (2011) 144-150.
407 https://doi.org/10.1016/j.molstruc.2011.05.023
408 [27] J.R. Lakowicz. Quenching of Fluorescence. In: J.R. Lakowicz (Ed.), Principles of
409 Fluorescence Spectroscopy, 3rd ed., Springer: New York, 2006, pp. 277-330.
410 [28] S. Bi, L. Ding, Y. Tian, D. Song, X. Zhou, X. Liu, H. Zhang. Investigation of the interaction
411 between flavonoids and human serum albumin. J. Mol. Struct. 703 (2004) 37-45.
412 https://doi.org/10.1016/j.molstruc.2004.05.026
413 [29] Q. Li, W. Yang, L. Qu, H. Qi, Y. Huang, Z. Zhang. Interaction of warfarin with human
414 serum albumin and effect of ferulic acid on the binding. J. Spectr. 2014 (2014) 1-7.
415 http://dx.doi.org/10.1155/2014/834501
416 [30] E. Walker, K. Tessner. Schizophrenia. Perspect. Psychol. Sci. 3 (2008) 30-37.
417 https://doi.org/10.1111%2Fj.1745-6916.2008.00059.x
418 [31] F.E. Emiliani, T.W. Sedlak, A. Sawa. Oxidative stress and schizophrenia: recent
419 breakthroughs from an old story. Curr. Opin. Psychiatry 27 (2014) 185-190.
420 https://doi.org/10.1097/YCO.0000000000000054

[32] N. Todorović, D. Filipović. The antidepressant- and anxiolytic-like effects of fluoxetine and clozapine in chronically isolated rats involve inhibition of hippocampal TNF-α. Pharmacol. Biochem. Behav. 163 (2017) 57-65. https://doi.org/10.1016/j.pbb.2017.10.006

[33] M.M. Takić, V.B. Jovanović, I.D. Pavićević, T.N. Uzelac, J.M. Aćimović, D.K. Ristić-Medić, L.M. Mandić. Binding of enterolactone and enterodiol to human serum albumin: increase of cysteine-34 thiol group reactivity. Food Funct. 7 (2016) 1217-1226. https://doi.org/10.1039/c5fo01346a

[34] V.B. Jovanović, I.D. Pavićević, M.M. Takić, A.Z. Penezić-Romanjuk, J.M. Aćimović, L.M. Mandić. The influence of fatty acids on determination of human serum albumin thiol group. Anal. Biochem. 448 (2014) 50-57. https://doi.org/10.1016/j.ab.2013.11.030

[35] M.J. Torres, L. Turell, H. Botti, L. Antmann, S. Carballal, G. Ferrer-Sueta, R. Radi, B. Alvarez**.** Modulation of the reactivity of the thiol of human serum albumin and its sulfenic derivative by fatty acids. Arch. Biochem. Biophys. 521 (2012) 102-110. https://doi.org/10.1016/j.abb.2012.03.011

[36] X. Wu, J. Liu, Q. Wang, W. Xue, X. Yao, Y. Zhang, J. Jin. Spectroscopic and molecular modeling evidence of clozapine binding to human serum albumin at subdomain IIA. Spectrochim. Acta A Mol. Biomol. Spectrosc. 79 (2011) 1202-1209. https://doi.org/10.1016/j.saa.2011.04.043

[37] G.J. van der Vusse. Albumin as fatty acid transporter. Drug Metab. Pharmacokinet. 24 (2009) 300-307. https://doi.org/10.2133/dmpk.24.300

[38] J. Reichenwallner, D. Hinderberger. Using bound fatty acids to disclose the functional structure of serum albumin. Biochim. Biophys. Acta 1830 (2013) 5382-5393. https://doi.org/10.1016/j.bbagen.2013.04.031

[39] A. Tramarin, D. Tedesco, M. Naldi, M. Baldassarre, C. Bertucci, M. Bartolini. New insights into the altered binding capacity of pharmaceutical-grade human serum albumin: site-specific binding studies by induced circular dichroism spectroscopy. J. Pharm. Biomed. Anal. 162 (2019) 171-178. https://doi.org/10.1016/j.jpba.2018.09.022

Figure Legends

Fig. 1. Chemical structure of clozapine (left) and ziprasidone.

Fig. 2. Changes of HSA-SH group content (%) in the complexes of defatted HSA and HSA/SA samples after CLZ or ZIP binding in comparison to the drug-free complexes. HSA to drug ratios were 1:0.5, 1:1.5 and 1:2. Values are expressed as the mean value (±SD) of three determinations. An asterisk indicates a significant difference ($P<0.05$; *t*-test) in the HSA-SH group content of each HSA/ZIP complexes (colon) in comparison to corresponding HSA/SA complexes. CLZ, Clozapine; ZIP, Ziprasidone; FAs, Fatty acids; HSA, Human serum albumin; HSA-SH, Cys34 free thiol group of HSA; SA, Stearic acid; HSA/SA, HSA in complex with stearic acid.

Fig. 3. Linear models of pseudo-first-order reaction kinetics of the Cys34 thiol group of defatted HSA and HSA/SA complexes (of protein to FA molar ratios 1:1, 1:2, 1:3 and 1:4) with DTNB, obtained after binding of (a) CLZ (in molar ratio HSA/CLZ 1:2) and (b) ZIP (HSA/ZIP 1:2). CLZ, Clozapine; ZIP, Ziprasidone; FAs, Fatty acids; HSA, Human serum albumin; HSA-SH, Cys34 free thiol group of HSA; SA, Stearic acid; HSA/SA, HSA in complex with stearic acid; HSA/CLZ and HSA/ZIP, HSA in complex with clozapine and ziprasidone, respectively; DTNB, 5,5′-Dithiobis-(2-nitrobenzoic acid).

Fig. 4. Singular contributions (Δ k`) of antipsychotics to decrease/increase in reactivity of the Cys34 group of defatted HSA and HSA/SA (molar protein to FA ratios 1:1, 1:2, 1:3 and 1:4) samples compared to the controls. CLZ, Clozapine; ZIP, Ziprasidone; FAs, Fatty acids; HSA, Human serum albumin; SA, Stearic acid; HSA/SA, HSA in complex with stearic acid.

Fig. 5. Fluorescence emission spectra of 1 μM defatted HSA and HSA/SA (protein to FA molar ratio 1:2) samples before and after the addition of CLZ or ZIP (2.0 μM), at 37 °C and pH 7.4, after excitation at 295 nm. CLZ, Clozapine; ZIP, Ziprasidone; FAs, Fatty acids; HSA, Human serum albumin; SA, Stearic acid; HSA/SA, HSA in complex with stearic acid.

Table 1

Plasma biochemical values after 4-weeks treatment of rats with clozapine (CLZ; 45 mg/kg/day) and ziprasidone (ZIP; 20 mg/kg/day), and in the control group. The data are expressed as the mean value (± SD) of three measurements.

Parameter (number of animals)	**Control** (8)	**CLZ** (8)	**ZIP** (8)
Total proteins (g/L)	70.7 ± 5.7	58.1 ± 5.3*	60.5 ± 1.7*
Albumin (g/L)	33.0 ± 5.5	22.0 ± 2.8*	23.3 ± 2.7*
Total thiols (mM)	0.188 ± 0.041	0.109 ± 0.011*	0.157 ± 0.027
Albumin-SH (M –SH/M albumin)	0.215 ± 0.060	0.169 ± 0.035*	0.243 ± 0.045*

* $P<0.05$ (*t*-test) compared to the control group

Table 2

Pseudo-first-order rate constants (*k*'), as the measure of Cys34-SH group reactivity with DTNB in defatted HSA and HSA/SA samples (protein to SA molar ratios: 1:1, 1:2, 1:3 and 1:4), after CLZ and ZIP binding to HSA samples (protein to drug molar ratios of 1:0.5, 1:1.5 and 1:2). *k*' values are expressed as the mean (±SD) of three determinations.

		***k*' x 10^{-3} (s^{-1})**				
		defatted HSA	**HSA/SA 1:1**	**HSA/SA 1:2**	**HSA/SA 1:3**	**HSA/SA 1:4**
Drug free		***18.3 ± 0.6***	***22.8 ± 0.6***	***25.2 ± 0.7***	***28.3 ± 1.1***	***32.7 ± 1.1***
CLZ	**0.5**	19.2 ± 0.9	23.9 ± 0.5	25.5 ± 0.2	23.8 ± 0.5*	27.2 ± 0.4*
	1.5	20.2 ± 0.7*	24.7 ± 0.1*	26.6 ± 0.6	24.3 ± 0.5*	29.6 ± 0.1*
	2.0	20.4 ± 0.3*	25.7 ± 0.2*	27.9 ± 0.3*	25.8 ± 0.4*	30.1 ± 0.7*
ZIP	**0.5**	24.0 ± 0.7*	23.9 ± 0.5	26.2 ± 0.5	29.1 ± 0.5	35.1 ± 0.1*
	1.5	29.1 ± 0.5*	29.3 ± 0.2*	28.7 ± 0.7*	32.1 ± 0.3*	38.4 ± 0.2*
	2.0	29.6 ± 0.3*	30.6 ± 0.6*	30.8 ± 0.5*	33.3 ± 0.5*	38.6 ± 0.7*

* P<0.05 (*t*-test) in comparison to corresponding defatted HSA and HSA/SA samples without drugs. CLZ, Clozapine; ZIP, Ziprasidone; Human serum albumin; SA, Stearic acid; HSA/SA, HSA in complex(es) with stearic acid; DTNB, 5,5′-Dithiobis-(2-nitrobenzoic acid).

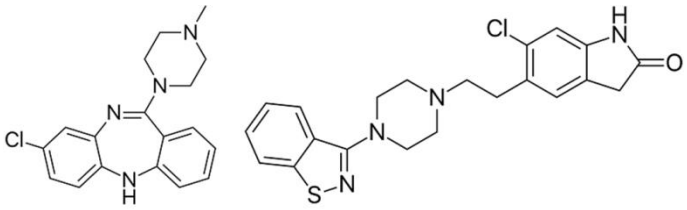
Table 3

Stern-Volmer quenching constants (K_{SV}), fluorescence quenching rate constants (k_q), binding constants (K_a) and number of binding sites (n) for CLZ and ZIP binding to either defatted HSA or HSA/SA samples (molar protein to SA ratios 1:1, 1:2, 1:3 and 1:4), at 37°C and pH 7.4. K_{SV}, K_a and n values are expressed as the mean (± SD) of two independent experiments, each performed in duplicate.

		K_{SV} $(M^{-1})x10^4$	k_q $(M^{-1}s^{-1})x10^{12}$	K_a $(M^{-1})x10^4$	n
CLZ	Defatted HSA	2.89 ± 0.078	2.89	1.90 ± 0.032	0.792 ± 0.013
	HSA/SA 1:1	2.48 ± 0.045	2.48	1.73 ± 0.050	0.789 ± 0.023
	HSA/SA 1:2	2.45 ± 0.064	2.45	1.20 ± 0.048	0.690 ± 0.022
	HSA/SA 1:3	2.36 ± 0.031	2.36	0.99 ± 0.032	0.666 ± 0.021
	HSA/SA 1:4	2.34 ± 0.072	2.34	0.82 ± 0.019	0.592 ± 0.014
ZIP	Defatted HSA	2.18 ± 0.040	2.18	0.95 ± 0.015	0.786 ± 0.013
	HSA/SA 1:1	1.90 ± 0.028	1.90	0.62 ± 0.021	0.661 ± 0.017
	HSA/SA 1:2	1.54 ± 0.034	1.54	0.38 ± 0.016	0.653 ± 0.018
	HSA/SA 1:3	1.12 ± 0.032	1.12	0.29 ± 0.014	0.572 ± 0.024
	HSA/SA 1:4	1.02 ± 0.021	1.02	0.25 ± 0.005	0.545 ± 0.012

CLZ, Clozapine; ZIP, Ziprasidone; HSA, Human serum albumin; SA, Stearic acid; HSA/SA, HSA in complex with stearic acid.

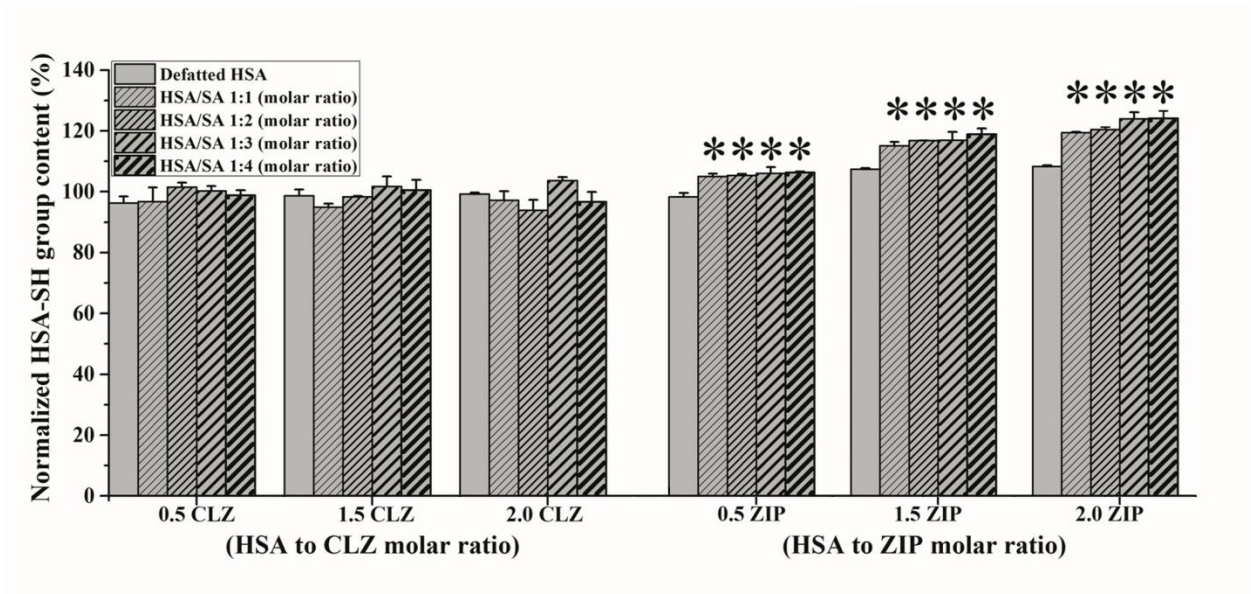
531 Figure 1



532

533

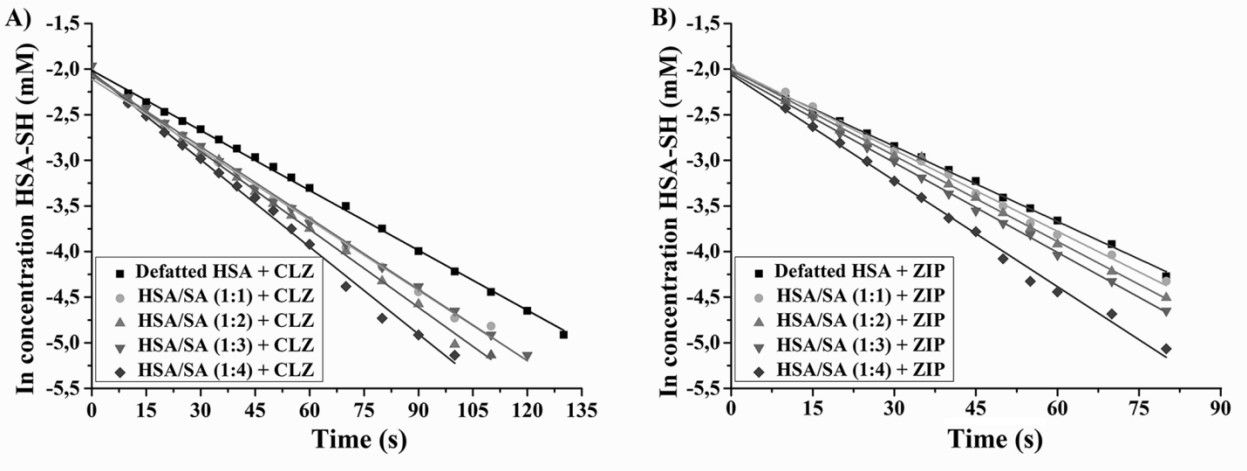
534 Figure 2



535

536

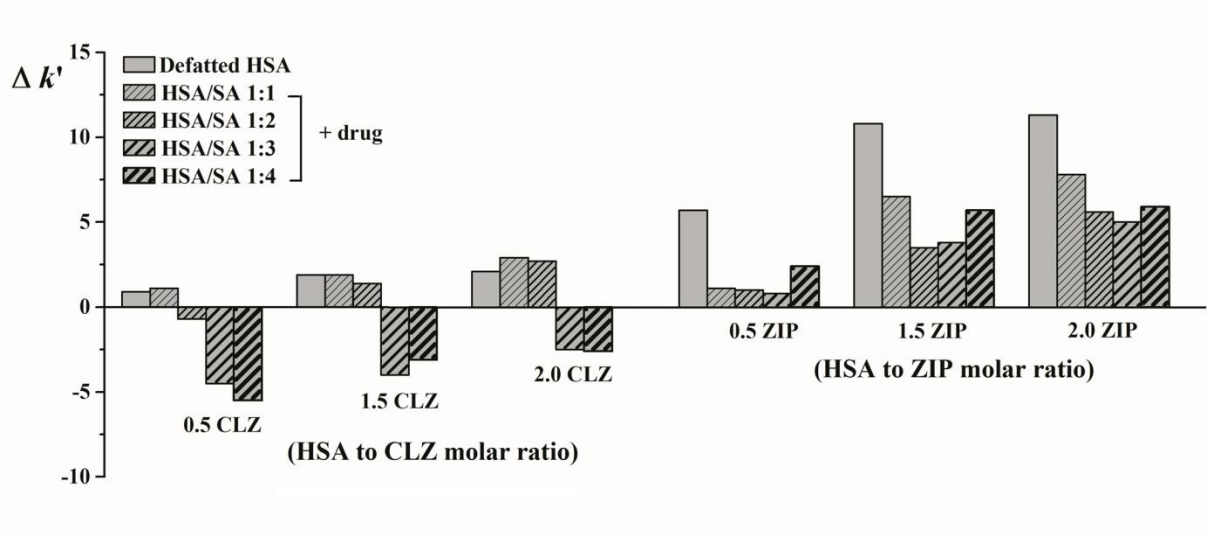
537 Figure 3



538

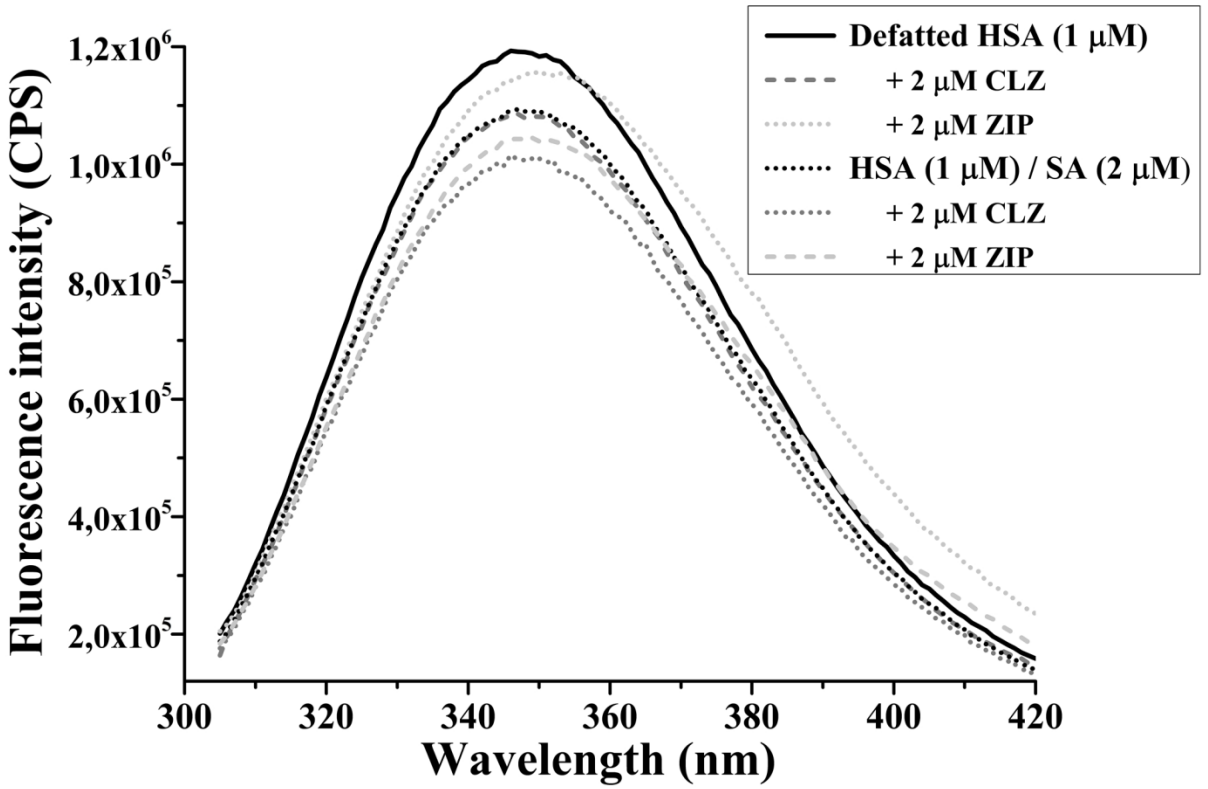
539

540 Figure 4



541

542 Figure 5


Defatted HSA (1 μM)
+ 2 μM CLZ
+ 2 μM ZIP
HSA (1 μM) / SA (2 μM)
+ 2 μM CLZ
+ 2 μM ZIP
Fluorescence intensity (CPS)
1,2x10^6
1,0x10^6
8,0x10^5
6,0x10^5
4,0x10^5
2,0x10^5
300
320
340
360
380
400
420
Wavelength (nm)

543