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Atypical antipsychotic clozapine binds fibrinogen and affects fibrin formation

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ABSTRACT

Clozapine is an atypical antipsychotic used for the treatment of schizophrenia. The prescribed target daily doses may reach 900 mg. Literature studies report a connection between clozapine usage and thrombosis development. Our *in vitro* study aimed to provide insight into molecular bases of this observation, investigating clozapine binding to fibrinogen, the main plasma protein involved in hemostasis. Fibrinogen/clozapine interaction was confirmed by protein fluorescence quenching, with an affinity constant of 1.7×10^5 M^{-1}. Direct interactions did not affect the structure of fibrinogen, nor fibrinogen melting temperature. Clozapine binding affected fibrin formation by reducing coagulation speed and thickness of fibrin fibers suggesting that in the presence of clozapine, fibrinogen may acquire thrombogenic characteristics. Although no difference in fibrin gel porosity was detected, other factors present in the blood may act

synergistically with altered fibrin formation to modify fibrin clot, thus increasing the risk for development of thrombosis in patients on clozapine treatment. ORAC and HORAC assays showed that clozapine reduced free radical-induced oxidation of fibrinogen. All observed effects of clozapine on fibrinogen are dose-dependent, with the effect on fibrin formation being more pronounced.

Keywords: fibrinogen; clozapine; binding; thrombosis; coagulation; hemostasis.

1. INTRODUCTION

Nearly half a century after launch, the first "atypical" antipsychotic drug clozapine (**Fig. 1A**) is still the only medication licensed for treatment-resistant schizophrenia. Usually prescribed in the target doses of 300–900 mg per day, clozapine is useful in the treatment of schizophrenia symptoms partially or fully resistant to treatment with other antipsychotic drugs, or accompanied by persistent suicidal or self-injurious behavior, by mechanisms that go beyond dopamine receptor (D2) blockade [**1**]. However, considerable effort should be made to balance superior clozapine antipsychotic efficacy with detection, monitoring, and reduction of drug side effects [**2**].

Fibrinogen, one of the most abundant plasma proteins, is a sizeable fibrillar glycoprotein with $(A\alpha B\beta\gamma)_2$ structure and molecular mass of 340 kDa [**3**]. Also known as coagulation factor I, the primary biological function of fibrinogen is in the process of hemostasis. This protein is involved in both primary hemostasis, by its interaction with blood platelets and secondary hemostasis, by fibrin clot formation [**4**]. Fibrinogen interacts with proteins, ions, and small ligands, which all affect its functional properties [**4-7**].

Literature reports suggest that patients who are on chronic clozapine treatment have an increased risk for the development of thrombotic complications. However, the exact mechanism

that may lead to this pathology is not yet known [**1**, **8-10**]. One potential reason for the development of thrombosis may be the capability of clozapine to induce higher platelet activation and aggregation in the presence of agonists [**11**]. Venous thromboembolism may be a consequence of several risk factors for this pathology already present, such as metabolic syndrome and inactivity, besides the direct effect of clozapine on coagulation [**12**]. Sedation and body weight increase with the use of clozapine and can also lead to deep vein thrombosis [**13**]. It seems that the risk for the development of venous thromboembolism is higher in older patients and in women who are taking high doses of this drug [**14-15**]. Comparing concentrations of fibrinogen, plasminogen activator inhibitor-1 and anticoagulant factor antithrombin III, no significant differences were observed between patients receiving clozapine and their relatives [**16**].

It is unknown on a molecular level how clozapine directly affects the development of thrombosis. In our study, we examined interactions of clozapine with fibrinogen and its influence on the functional aspect of fibrinogen. Fibrinogen is very susceptible to oxidation [**17**], and these modifications harm its function [**18**]. Since clozapine has undoubted antioxidant properties [**19**], *in vitro* potential to protect fibrinogen from free radical-induced oxidation was also investigated.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals used were purchased from Sigma (Germany). Fibrinogen was purified (**Section 2.2**) from a pool of human plasma obtained from 10 healthy adult donors, after receiving informed consent and INEP institutional review board approval. Human thrombin was purchased from Human (Germany) and used according to the instruction manual. Clozapine was

provided by Remedica Ltd (Cyprus). A stock solution of the drug (4 mM) was prepared in DMSO. In all experiments performed, DMSO concentration was not higher than 1%.

2.2 Isolation and quantification of human fibrinogen

The double ammonium sulfate precipitation method was used for fibrinogen preparation [**6**]. A saturated solution of ammonium sulfate was added to a final concentration of 20%, and plasma was left at 4°C. After 30 min, plasma was centrifuged for 1 min at 10000 rpm at room temperature, and the obtained protein pellet was dissolved in 50 mM phosphate buffer, pH 7.2 (this buffer was used in all experiments unless otherwise stated). Precipitation step was repeated as described, and after the dissolution of fibrinogen pellet in the same buffer, this solution was used as a protein source for all experiments. A product was homogeneous on SDS-PAGE. The concentration of the purified fibrinogen preparation was calculated using an extinction coefficient at 280 nm: $E^{1\%} = 15.1$.

2.3 Characterization of fibrinogen/clozapine binding by spectrofluorimetry

For the determination of affinity constant and number of the binding site(s), the standard intrinsic protein fluorescence quenching method was used. The experiment was performed on FluoroMax®-4 spectrofluorometer (Horiba Scientific, Japan) at 37°C in triplicate. Fibrinogen (40 nM) was titrated with an increasing concentration of clozapine (0.5 to 4 µM) and allowed to interact for one minute before each measurement. The excitation wavelength was 280 nm, and emission spectra were recorded in a range from 290 to 500 nm, with two accumulations, and slits set at 5 nm. The obtained average emission spectra (for each ligand bolus) were corrected by subtracting emission spectra originating from clozapine itself. Values of emission maxima at 345 nm were corrected for inner filter effect using the following equation [**20**]:

$$F_c = F_0 \times 10^{(Aex + Aem)/2}$$

where F_c is the corrected fluorescence, F_0 is the measured fluorescence, while Aex and Aem correspond to absorbances at excitation (280 nm) and emission (345 nm) clozapine peaks.

The estimation of the binding (association) constant (K_a), and the number of binding sites (n) between fibrinogen and clozapine was done using equation [**21**]:

$$\log\frac{F_0 - F}{F} = -n\log\frac{1}{[L] - [P]\frac{F_0 - F}{F_0}} + n\log Ka$$

where F_0 and F are the emission signals of fibrinogen in the absence and the presence of clozapine, while [L] and [P] correspond to the total concentrations of the ligand and the protein.

For determination of the type of quenching, Stern-Volmer (SV) plot was obtained using the following equation [**20**]:

$$\frac{F_0}{F} = 1 + k_q\tau_o[Q] = 1 + K_{SV}[Q]$$

where F_0 and F are fluorescence intensities of fibrinogen at 345 nm without and with clozapine, k_q is the bimolecular quenching rate constant, τ_o is the average lifetime of the protein without quencher (10^{-8} s), [Q] is the total quencher (clozapine) concentration, and K_{SV} is the SV quenching constant. The obtained slope of the SV plot represents the SV quenching constant.

Synchronous fluorescence spectra of fibrinogen/clozapine complex, using the same device and experimental setup, were obtained at two different scanning intervals: $\Delta\lambda$=15 nm, Tyr residues excitation with emission spectra recorded from 300 to 400 nm, and $\Delta\lambda$=60 nm, Trp residues excitation with emission spectra recorded from 275 to 350 nm, where $\Delta\lambda = \Delta\lambda_{em} - \Delta\lambda_{ex}$.

2.4 Analysis of fibrinogen/clozapine interaction using UV spectrometry

UV spectrometric analysis of clozapine binding to fibrinogen was performed in triplicate on the UV-1800 spectrophotometer (Shimadzu, Japan) at 37°C. Spectra were obtained in the

range of 260–360 nm for fibrinogen alone (5 μM), and in the presence of 10, 25, 50 and 100 μM clozapine. The obtained spectra were then corrected by subtracting the spectra for clozapine alone.

2.5 Fourier-transformation infrared spectroscopy (FT-IR) analysis of fibrinogen/clozapine interaction

FT-IR analysis was performed on IRAffinity-1 (Shimadzu, Japan) to investigate possible changes in fibrinogen secondary structure due to clozapine binding. Portions of 50 μL of samples (4 μM fibrinogen alone or in the presence of 8, 20, and 40 μM of clozapine) were placed on ZnSe windows (32x2 mm; Thermo, Madison, USA) and dried using a stream of nitrogen. The measurements were performed in duplicate, using transmission mode with 100 scans per sample, while the resolution was 4 cm^{-1}. Before further analyses, baselines of spectra were corrected using Spectragryph software [**22**]. Smoothing and Amide I peak deconvolution was performed using Origine Pro 8.6 software (OrigineLab, USA).

2.6 Analysis of clozapine influence on fibrinogen melting temperature

The influence of clozapine binding on fibrinogen temperature stability was determined on FluoroMax®-4 spectrofluorometer (Horiba Scientific, Japan) with the Peltier element. Thermal stability of fibrinogen alone (40 nM) or in the presence of clozapine (40 μM) was studied in the temperature range from 37 to 81°C by measuring fibrinogen intrinsic fluorescence reduction at its emission maximum (345 nm). The rate of temperature increase was 2°C/min, with the equilibration time set to 1 min, and both slits were 5 nm. Results are presented as sigmoidal function with the inflection point representing the melting temperature (T_m) of fibrinogen.

2.7 Analysis of clozapine influence on fibrin formation

Fibrinogen coagulation assay [**23**] was performed in tetraplicate at 37°C, with isolated fibrinogen at a final concentration of 0.34 μM, alone or in the presence of clozapine at final concentrations of 0.34 μM and 0.68 μM. The reaction mixture also contained a physiological concentration of $CaCl_2$ (2.2 mM) and human thrombin (1 IU/mL). Immediately after thrombin addition, the cuvette was placed in the UV-1800 spectrophotometer (Shimadzu, Japan), and a rise in absorbance at 405 nm was continually followed for 8 min. The obtained results were analyzed according to the published procedure [**23**].

2.8 Analysis of clozapine influence on fibrin gel porosity

The porosity of fibrin gel, obtained from 3 μM fibrinogen alone or in the presence of 3 and 6 μM clozapine, was determined in tetraplicate by measuring Darcy constant, which is directly proportional to the porosity of the gel [**24**]. The reaction mixture for the formation of fibrin gel contained fibrinogen, clozapine, 2.2 mM $CaCl_2$, and 1 IU/mL of thrombin. Immediately after thrombin addition, the reaction mixture was transferred to silicon tubes attached to 5 mL syringes and left to polymerase overnight in a humid chamber at room temperature. The next day, syringes were carefully mounted vertically on the holder and filled with phosphate buffer. Buffer was allowed to flow through fibrin gel for 30 min in pre-weighted microcentrifuge tubes, and the effluent was measured using an analytical balance. Darcy constant was calculated using the following equation:

$$Ks = \frac{Q \times L \times \eta}{t \times A \times \Delta P}$$

where Ks represents Darcy constant (cm^2), Q is the volume of a liquid (mL) having viscosity η (P), flowing through a fibrin gel of the length L (cm) and cross-section A (cm^2), in a given time t (s), under a differential pressure (dyn/cm^2).

2.9 ORAC antioxidant assay

The ORAC assay [**25**] was performed in duplicate by measuring the reduction of intrinsic fluorescence of 100 nM fibrinogen, alone or in the presence of 3.4 and 6.8 µM clozapine. The reduction of protein fluorescence was achieved by incubating the samples with 52.5 mM AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride), a free (dominantly peroxyl) radical inducer. Immediately after AAPH addition, samples were mixed and placed in a spectrofluorimeter. Emission maximum of fibrinogen at 345 nm was monitored (FluoroMax®-4, Horiba Scientific, Japan) upon excitation at 280 nm for 25 min at 37°C with slits of 5 nm. Protective effect (PE) of clozapine, expressed in arbitrary units (A.U.), was calculated using the equation [**26**]:

$$PE\ (A.U.)\ [x\ 10^6] = AUC_{FIB+CLZ} - AUC_{FIB}$$

where $AUC_{FIB+CLZ}$ represents the area under the curve obtained for fibrinogen/clozapine mixture and AUC_{FIB} represents the area under the curve obtained for fibrinogen alone.

2.10 HORAC antioxidant assay

In the HORAC assay, the reduction of fluorescence of 100 nM fibrinogen, alone or in the presence of 6.8 and 13.6 µM clozapine, was achieved by incubating samples with the substances [**25**] inducing hydroxyl radical production (31 mM H_2O_2 and 260 µM CoF_2 x $4H_2O$/ 460 µM picolinic acid). Immediately after the addition of all components, mixtures were placed in FluoroMax®-4 spectrofluorimeter (Horiba Scientific, Japan). The further procedure and quantification of the protective drug effect against free radical-induced protein oxidation were identical to that described in **Section 2.9**.

3. RESULTS AND DISCUSSION

3.1 Detection and characterization of fibrinogen/clozapine binding

The protein fluorescence quenching method is widely used for the characterization of small ligand binding. The presence of clozapine efficiently quenches the intrinsic fluorescence of fibrinogen when protein is excited at 280 nm (**Fig. 1B**). Increasing concentrations of clozapine do not cause either red or blue shift of the emission maximum of the protein, suggesting that the environment around Trp and Tyr amino acid residues is not altered as a consequence of binding. From the equation obtained from the double log plot (**Fig. 1C**), affinity constant was calculated to be $1.7 \pm 0.38 \times 10^5$ M^{-1}, with the number of binding sites of 1.2 ± 0.08.

Of all plasma proteins, so far, it was known that clozapine specifically binds only to human serum albumin (HSA). HSA is a transport protein with multiple ligand binding sites, primarily for acidic and neutral drugs [**27**]. Indeed, about 95% of clozapine in blood was found to be bound for HSA [**28**]. What is interesting is that the estimated affinity constant of fibrinogen for clozapine is an order of magnitude higher compared to HSA/clozapine complex [**29**]. The referent level of fibrinogen in plasma (2–4 g/L; i.e., 5.9–11 μM) is much lower than for albumin (35–50 g/L; i.e., 0.53–0.75 mM), as well as its half-life (4 *vs.* 20 days, respectively). The target clozapine plasma levels for the vast majority of patients are 350–450 μg/L [**30**], i.e., 1.1–1.4 μM. Therefore, it seems that preferential clozapine binding to fibrinogen (as assessed by K_a) over HSA will not significantly affect the distribution of the free (active) drug in the circulation and thus its pharmacological properties. However, in the situations when the concentration of fibrinogen is increased (for example due to inflammation), or the concentration of albumin decreased (for example due to impaired kidney function) or if the structure of albumin is modified (e.g., glycated), the equilibrium between two proteins and, thus, clozapine binding may be altered. All named conditions can be seen in patients with diabetes mellitus [**31**].

From the Stern-Volmer plot (**Fig. 1D**), K_{sv} was determined to be 1.52 x 10^5 M^{-1}, and, from this value, the bimolecular quenching rate constant was calculated to be 1.52 x 10^{13} M^{-1}. Since the bimolecular quenching rate constant is about two orders of magnitude higher than the diffusion rates of biomolecules (10^{10} $M^{-1}s^{-1}$), the obtained results suggest that in the presence of clozapine static quenching of fibrinogen takes place [**20**]. Based on synchronous fluorescence data (**Figs. 1E** and **1F**), it follows that both Trp and Tyr residues of fibrinogen notably contribute to the quenching of signal induced by clozapine binding. While HSA has only one, human fibrinogen contains as many as 41 Trp residues [**32**], which jointly with 67 Tyr residues imply that binding of clozapine occurs in an area with both of these amino acids in proximity.

Our results show for the first time that fibrinogen specifically binds clozapine under (partially) simulated pathophysiological conditions.

3.2 Examination of clozapine binding effects on fibrinogen structure and stability

Although generally very small, alterations in the absorption spectra are beneficial for the detection of conformational changes of a protein and ligand binding. UV spectral analysis of clozapine binding to fibrinogen (**Section 2.4**) indicates no changes in protein structure (**Fig. 2A**). Since differences in the absorbance can result as a consequence of environment alterations close to aromatic amino acid side chains [**33**], it seems that this was not the case here, confirming the results obtained from the fluorescence quenching study.

Fourier-transform infrared spectroscopy is one of the most versatile analytical tools in life sciences. FT-IR analysis was performed in our work (**Section 2.5**) to examine if changes in the secondary structure of fibrinogen occur upon clozapine binding. The deconvolution of Amide I region from the obtained spectra resulted in 5 peaks (**Fig. 2B**), revealing a secondary structure composition of 33% α-helix, 21% β-sheet, 24% coils, and 22% random (unassigned) structure,

which is following the published FT-IR fibrinogen data [**34**]. Differences in the proportion of the secondary structure components are insignificant in the presence of even a high concentration of clozapine (**Fig. 2C**).

Another confirmation that the binding of clozapine has no significant effect on the structure and stability of native fibrinogen came from the results of thermal stability analysis (**Section 2.6**). As can be seen from the obtained graphs (**Fig. 2D**), the melting temperature (inflection point on graphs) of fibrinogen is only slightly (0.8°C) higher in the presence of clozapine.

Summing-up all experimental data, it can be said that the formation of complexes between clozapine and fibrinogen has a negligible effect on protein structure and thermal stability. These findings are not surprising. Fibrinogen is a large, fibrillar type of protein, whose structure/stability is not expected to change easily by small ligand binding, as was previously shown in the case of bilirubin [**6**]. On the other hand, clozapine binding induces a small but significant reduction of the α-helix content in HSA [**29**].

3.3 Examination of clozapine binding effects on fibrinogen function

Fibrinogen is involved in both primary and secondary hemostasis, playing an essential role in platelet aggregation and the formation of a fibrin network. While some direct/indirect mechanisms that may lead to thrombotic complications in patients on clozapine therapy are known [**11-13**], exact mechanisms at the molecular level are not elucidated. Therefore, we examined differences in the functional properties between fibrinogen and fibrinogen/clozapine complexes (**Sections 2.7/2.8**).

In the presence of an increased clozapine concentration, fibrinogen formed fibrin clot at reduced speed (log phase of fibrin formation), but with thinner fibers (results based on the

maximal absorbance at 405 nm at the end of fibrin formation) (**Fig. 3A**). These functional characteristics of fibrinogen are similar to those found when fibrinogen was incubated with high (≥10 mM) concentrations of glucose [**35**], and for fibrinogen isolated from patients with post-acute myocardial infarction [**36**], where the protein was extensively post-translationally modified. While fibrin clot with thinner fibers is usually more compact and less porous, this was not the case in our study: similar porosity was obtained with native fibrin and fibrin in the presence of all tested concentrations of clozapine (**Fig. 3B**).

Many factors in the blood affect the outcome of fibrin clot formation and its characteristics [**37**]. Further, some pathologies are characterized by fibrin clots that have thicker fibers compared to healthy controls, but these clots also have lower porosity [**38**]. Fibrin formation is a very complex process involving many factors. Altered fibrin formation as a consequence of clozapine binding may not be sufficient enough to enable the detection of significant alterations in fibrin porosity and to lead to thrombotic behavior directly. However, the detected impact of clozapine on fibrin formation may act synergistically with other factors and contribute to the impairment of the coagulation process, which may progress to the development of thrombosis. Many studies reported on the reduced porosity of fibrin clots that also have thinner fibrin fibers [**39-42**]. It was also reported that patients with schizophrenia are in the hypercoagulable and hypofibrinolytic state, both of which may aid in their increased risk of venous thromboembolism [**43**]. Further controlled studies involving whole plasma from patients on long-term clozapine therapy are needed to determine the extent to which coagulation characteristics of fibrinogen are altered.

The results of our study unambiguously document a clozapine dose-dependent influence on functional characteristics of fibrinogen, an essential protein in the coagulation process.

3.4 Examination of clozapine binding effects on fibrinogen stability against oxidation

Fibrinogen is one of the plasma proteins most susceptible to oxidative modifications. An important factor that usually leads to reduced fibrin porosity is precisely the oxidation of fibrinogen. This modification creates new hydrophobic regions in fibrin clots that are less permeable for plasmin, the main protein involved in fibrinolysis [**44**]. For these reasons, small molecules with antioxidant properties that bind fibrinogen may have a beneficial effect on the coagulation process by protecting protein from free radical-induced oxidation.

Results obtained in ORAC and HORAC tests, where protein, not fluorescein was a target of free radical-induced oxidation (**Sections 2.9/2.10**), suggest that clozapine is capable of protecting fibrinogen from oxidation, in a dose-dependent manner (**Figs. 4A** and **4B**). Calculated protective effect (corresponding to the difference in areas under the curves in the presence and absence of drug, expressed in arbitrary units) in ORAC assay was 71 in the presence of 3.4 μM clozapine and even 128 in the presence of 6.8 μM clozapine. The same trend was evident in the HORAC assay as well, where the protective effect was 102 in the case of 6.8 μM clozapine and 373 in the case of 13.2 μM clozapine. The chemical structure-related antioxidant capacity of clozapine points to its possible neuroprotective potential on the top of its complex antipsychotic mechanism of action [**45**]. Our results showed that clozapine binding may reduce the oxidation of fibrinogen. Based on the molar ratios of fibrinogen and clozapine used, our *in vitro* experimental results suggest that clozapine has a more pronounced effect on fibrin formation than on oxidative protection of fibrinogen.

4. CONCLUSION

Clozapine interacts with isolated fibrinogen, under simulated pathophysiological conditions, with the affinity higher than the one reported for HSA, a conventional blood

transporter of drugs. Although this binding has a negligible effect on the structure and thermal stability of fibrinogen, it influences fibrin formation and may contribute to its observed thrombogenic characteristics in patients on chronic clozapine treatment. On the other hand, clozapine can reduce harmful (free radical-induced) oxidation of fibrinogen. Both detected effects of clozapine are dose-dependent, with the effect on fibrin formation being more pronounced.

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5. LITERATURE

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FIGURE CAPTIONS

Fig. 1. The quenching of fibrinogen fluorescence by clozapine. **A)** Chemical structure of clozapine (8-Chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[*b*,e][1,4]diazepine). **B)** Representative emission spectra (excitation at 280 nm) of fibrinogen (40 nM) in the presence of increasing concentrations of clozapine (0–4 μM). **C)** Double log plot used for the calculation of the binding parameters: affinity constant and number of binding sites; Error bars represent the standard deviation (n=3). **D)** Stern-Volmer (SV) plot used for the calculation of SV quenching constant (K_{SV}); Error bars represent the standard deviation (n=3). Representative synchronous fluorescence spectra of fibrinogen (40 nM) with **E)** Δλ=60 nm (Trp) and with **F)** Δλ=15 nm (Tyr) in the presence of increasing concentrations of clozapine (0–4 μM).

Fig. 2. The influence of clozapine binding on fibrinogen structure and stability. **A)** UV spectra of fibrinogen (5 μM) in the presence of increased concentrations of clozapine (0–100 μM). FT-IR absorption spectra (deconvolution results) of **B)** fibrinogen alone (4 μM) and **C)** in the presence of 40 μM clozapine. **D)** Thermal stability of fibrinogen alone (40 nM) and in the presence of clozapine (40 μM).

Fig. 3. Effects of fibrinogen/clozapine binding on protein function. **A)** Fibrin formation from isolated fibrinogen (0.34 μM) in the presence of an increasing clozapine concentration (0, 0.34, and 0.68 μM). **B)** The porosity of fibrin gel formed from the isolated fibrinogen (3 μM) in the presence of an increasing clozapine concentration (0, 3, and 6 μM).

Fig. 4. Effects of fibrinogen/clozapine binding on the free radical-induced protein oxidation**.** Fibrinogen (0.1 μM) fluorescence decay in **A)** ORAC and **B)** HORAC antioxidant assays in the presence of increasing concentrations of clozapine (0, 3.4, and 6.8 μM in ORAC and 0, 6.8, and 13.6 μM in HORAC assay).

CRediT authorship contribution statement

Nikola Gligorijević: Conceptualization; Methodology; Investigation; Data curation; Vizualization; Formal analysis; Writing - original draft. **Tamara Vasović:** Investigation; Data curation; Visualization; Formal analysis. **Steva Lević:** Methodology; Investigation; Data curation; Software. **Čedo Miljević:** Conceptualization; Resources; Methodology; Writing - review & editing. **Olgica Nedić:** Funding acquisition; Methodology; Writing - review & editing; Supervision. **Milan Nikolić:** Conceptualization, Investigation; Data curation; Writing - original draft; Writing - review & editing; Funding acquisition; Supervision.

Highlights

Clozapine binds fibrinogen with an affinity constant of 1.7×10^5 M^{-1}.

Clozapine binding has a negligible influence of fibrinogen structure and stability.

Clozapine binding unfavorably changes fibrinogen functional properties.

Clozapine binding reduces free-radical induced fibrinogen oxidation.

The effects of clozapine binding to fibrinogen are dose-dependent.

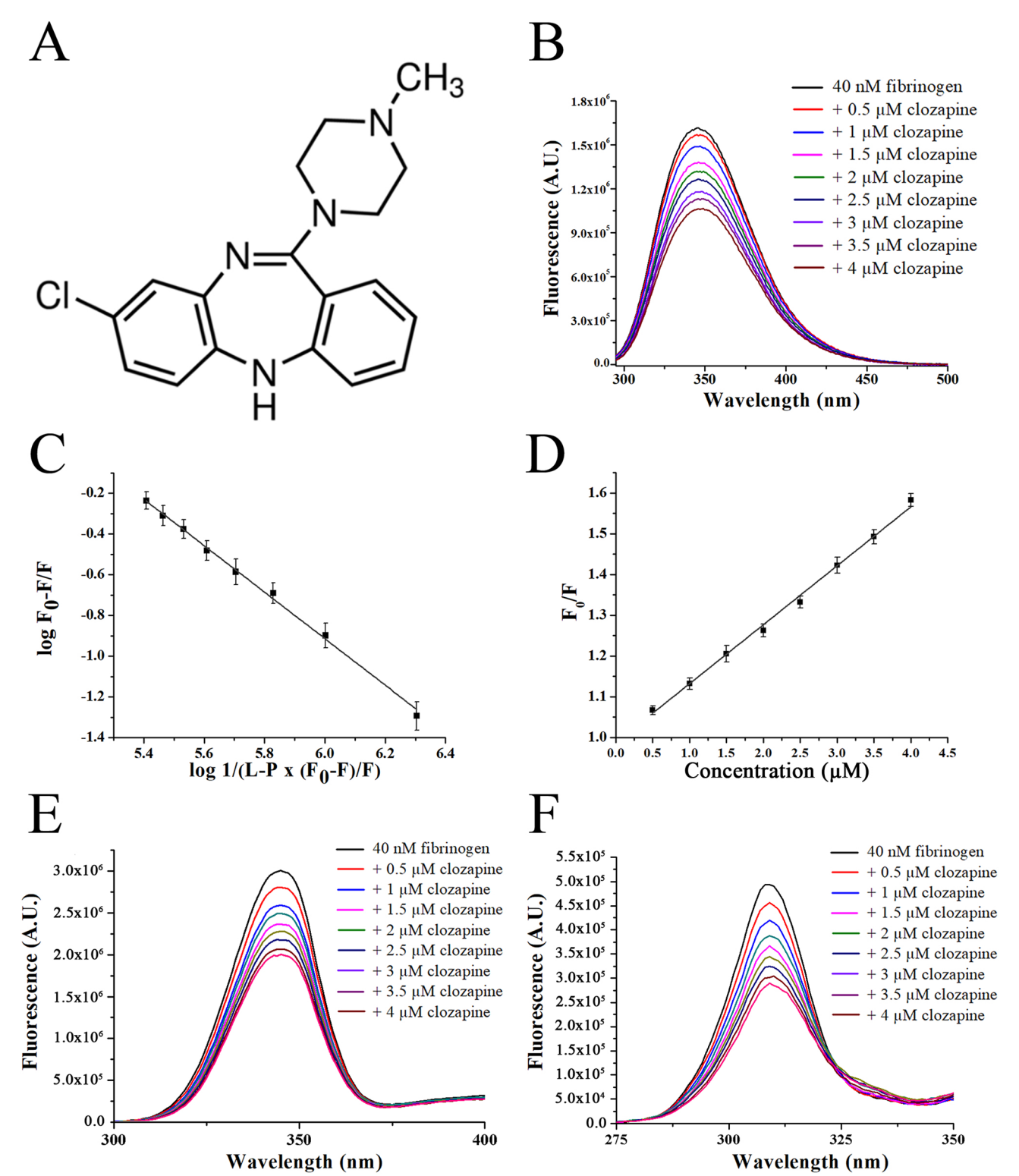
A
CH3
N
N
N
Cl
N
H
B
40 nM fibrinogen
+ 0.5 μM clozapine
+ 1 μM clozapine
+ 1.5 μM clozapine
+ 2 μM clozapine
+ 2.5 μM clozapine
+ 3 μM clozapine
+ 3.5 μM clozapine
+ 4 μM clozapine
Fluorescence (A.U.)
Wavelength (nm)
C
log F0-F/F
log 1/(L-P x (F0-F)/F)
D
F0/F
Concentration (μM)
E
40 nM fibrinogen
+ 0.5 μM clozapine
+ 1 μM clozapine
+ 1.5 μM clozapine
+ 2 μM clozapine
+ 2.5 μM clozapine
+ 3 μM clozapine
+ 3.5 μM clozapine
+ 4 μM clozapine
Fluorescence (A.U.)
Wavelength (nm)
F
40 nM fibrinogen
+ 0.5 μM clozapine
+ 1 μM clozapine
+ 1.5 μM clozapine
+ 2 μM clozapine
+ 2.5 μM clozapine
+ 3 μM clozapine
+ 3.5 μM clozapine
+ 4 μM clozapine
Fluorescence (A.U.)
Wavelength (nm)

Figure 1

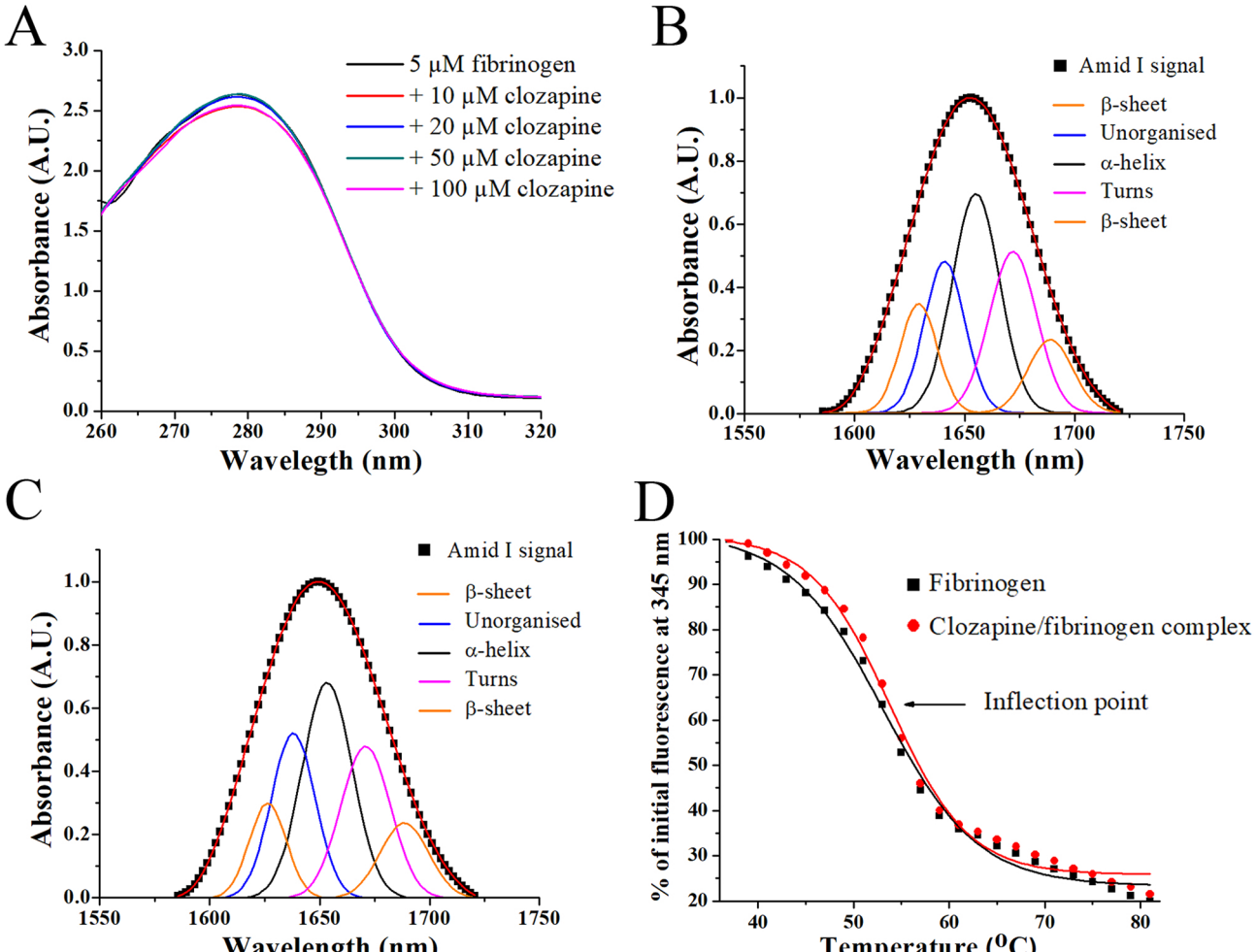
A
Absorbance (A.U.)
Wavelegth (nm)
5 μM fibrinogen
+ 10 μM clozapine
+ 20 μM clozapine
+ 50 μM clozapine
+ 100 μM clozapine
B
Amid I signal
β-sheet
Unorganised
α-helix
Turns
β-sheet
Absorbance (A.U.)
Wavelength (nm)
C
Amid I signal
β-sheet
Unorganised
α-helix
Turns
β-sheet
Absorbance (A.U.)
Wavelength (nm)
D
Fibrinogen
Clozapine/fibrinogen complex
Inflection point
% of initial fluorescence at 345 nm
Temperature (°C)

Figure 2

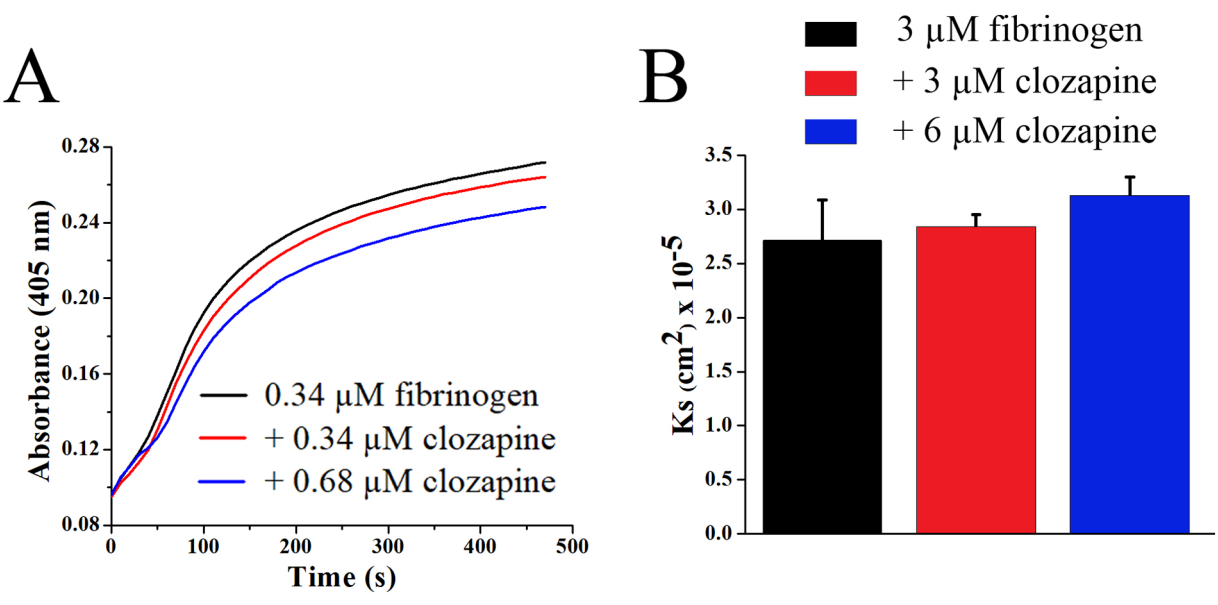
A
Absorbance (405 nm)
Time (s)
0.34 μM fibrinogen
+ 0.34 μM clozapine
+ 0.68 μM clozapine
B
3 μM fibrinogen
+ 3 μM clozapine
+ 6 μM clozapine
Ks (cm2) x 10-5

Figure 3

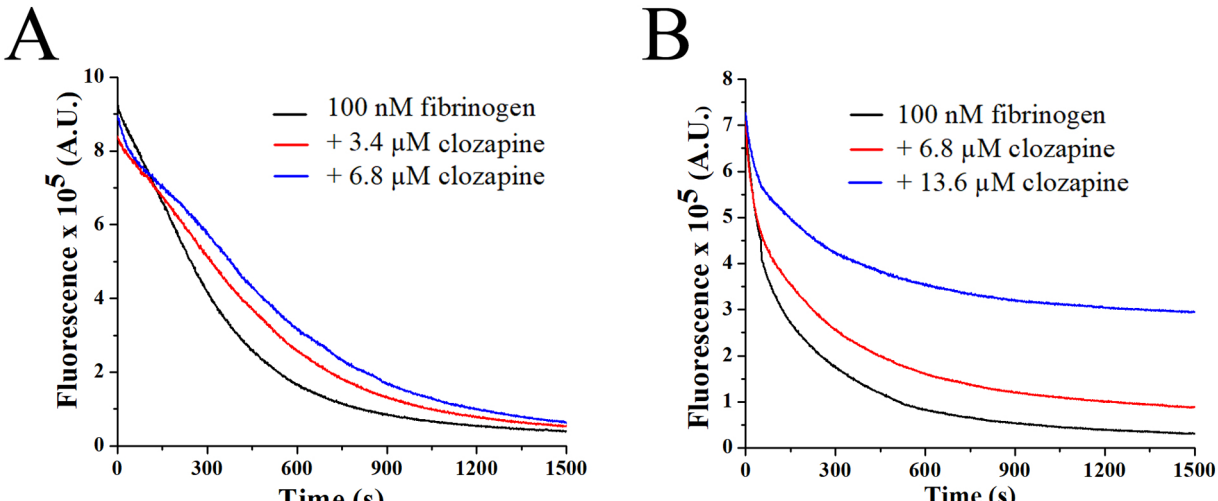
A
B
100 nM fibrinogen
+ 3.4 μM clozapine
+ 6.8 μM clozapine
+ 13.6 μM clozapine
Fluorescence x 10^5 (A.U.)
Time (s)
0
300
600
900
1200
1500

Figure 4