Research article

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The Journal of Biological and Chemical Luminescence

Received: 02 December 2010, Revised: 28 February 2011, Accepted: 05 March 2011, Published online in Wiley Online Library: 2 May 2011

(wileyonlinelibrary.com) DOI 10.1002/bio.1302

Study of the interaction between ofloxacin and human serum albumin by spectroscopic methods

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ABSTRACT: The binding of ofloxacin (OFLX) to human serum albumin (HSA) was investigated by fluorescence and circular dichroism (CD) techniques. The binding parameters have been evaluated by a fluorescence quenching method. Competitive binding measurements were performed in the presence of warfarin and ibuprofen and suggest binding to the warfarin site I of HSA. The distance *r* between donor (HSA) and acceptor (OFLX) was estimated according to the Forster's theory of non-radiatiative energy transfer. CD spectra revealed that the binding of OFLX to HSA induced conformational changes in HSA. Molecular docking was performed and shows that for the lowest energy complex OFLX is located in site I of HSA, which correlate to the competitive binding experiments. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: human serum albumin; ofloxacin; fluorescence quenching; circular dichroism; molecular modelling

Introduction

Serum albumins are found abundantly in blood plasma and are often termed 'transport proteins' (1–3). They are circulated in the body several times and act as carriers for numerous exogenous and endogenous compounds, mostly through the formation of non-covalent complexes at specific binding sites (4,5). The most popularly studied albumin is human serum albumin (HSA), which has a very high conformational adaptability to a great variety of ligands (6).

Ofloxacin (OFLX; Fig. 1) is a member of the fluoroquinolones family, a class of synthetic antimicrobial agents. The fluoroquinolones are gaining interest because they provide activity against both Gram-positive (fluorine atom) and Gram-negative (piperazine group) organisms, showing few side-effects (7,8).

OFLX is used for the treatment of urinary tract infections. Pharmacological research has shown that the antibacterial activity of the L-enantiomer of ofloxacin (*S*-(–)-ofloxacin or levofloxacin) is higher than that of *R*-(+)-ofloxacin and approximately two-fold higher than that of the racemate (10).

Binding of several ligands can be studied by means of fluorescence spectroscopy, monitoring the changes in the intrinsic fluorescence of albumins, which results from tryptophan and tyrosine residues. This kind of experiment reveals the accessibility of quenchers to albumin's fluorophore groups, help us to understand the albumin's binding mechanisms to these substances and provide clues to the nature of the binding phenomenon (11–13).

Previous studies of fluoroquinolones binding to albumins (14–18) show a 1:1 interaction between ligands and serum albumins, with moderate affinity and inducing conformational changes in the secondary structure of albumins, as revealed by CD spectra. OFLX forms 1:1 complexes with bovine serum albumin (BSA), with binding constants of the order of 10^4–10^5 mol/L (17,18).

The aim of this study was two-fold: to characterize by competitive fluorescence measurements the binding site of ofloxacin, and to correlate the experimental data with the results of molecular modelling of the supramolecular system, HSA–OFLX, considering both possibilities of binding, to site I or II.

Due to the two pKa values, 6 and 8, for the carboxy group and methyl-substituted nitrogen atom from the piperazinic group, it can be considered that at pH 7.4 OFLX exists predominantly in the zwitterionic form.

Experimental

Materials

HSA, fatty acid-free <0.05%, was purchased from Sigma Chemical Company (St Louis, MO, USA). Solutions of OFLX and HSA were prepared in pH 7.4 phosphate buffer and the protein solutions were equilibrated overnight and kept in the dark.

Apparatus and methods

Fluorescence spectra were measured with a Jasco FP-6300 spectrofluorimeter, using a 1.00 cm quartz cell. Both the excitation and emission slits were set at 5.0 nm. Fluorescence spectra were recorded at room temperature (25°C) in the wavelength range 300–550 nm (excitation wavelength 295 nm).

CD measurements were carried out on a Jasco J-815 CD spectrometer, using a 1.00 cm cell at 0.2 nm intervals, with three scans averaged for each CD spectrum in the range 200–260 nm, and the results were expressed as ellipticity (θ) in millidegrees.

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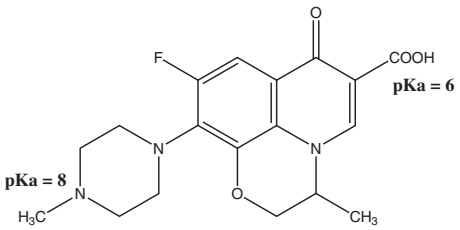

Figure 1. The molecular structure of OFLX with the approximate pKa values (9).

On the basis of preliminary experiments, HSA concentrations were kept fixed at 2.07×10^{-6} mol/L and checked by absorption spectroscopy, with $\varepsilon_{280} = 3.05 \times 10^4$ L/mol cm (19). The drug concentrations were varied to ensure a drug:protein (*d*:*p*) ratio in the range 0–10.

In order to have an insight on the possible location of OFLX, the experiments were also conducted in the presence of warfarin and ibuprofen, known markers for sites I and II, respectively; aliquots of the ligand were added to a solution containing equimolar concentrations (2.07×10^{-6} mol/L) of albumin and markers.

The interaction mode and the binding site of OFLX on HSA were studied using the program Autodock 4.2 (20), in which a Lamarckian genetic algorithm was used to search for the optimum binding site. The three-dimensional 3D structure of HSA (entry code 1AO6) was obtained from Brookhaven Protein Data Bank (http://www.rcsb.org/pdb) (21). Sites I and II were used as the potential sites for target docking simulations. Autodock tools were used to prepare the protein and the ligand. All hydrogen atoms were added, and simultaneously Gasteiger charges were assigned to the protein and the ligand. The zwitterionic form of OFLX was optimized by the DFT method, using the GAMESS-US program (22). Two boxes were considered, $40 \times 40 \times 40$ grid size, with a spacing of 0.375 Å, including the two possible binding sites. The molecular modelling was carried out based on the following parameters: the energy evaluations of 2 500 000, the maximum number of 27 000 iterations for an initial population of 150 randomly placed individuals with a mutation rate of 0.02, a crossover rate of 0.80, and an elitism value of 1.0. The other parameters were defaults.

Results and discussion

Fluorescence quenching and binding parameters

The effect of OFLX on HSA were evaluated by measuring the intrinsic fluorescence intensity of protein before and after addition of OFLX. The quenching of the HSA band is observed, together with an increase of the free OFLX band. As can be seen in Fig. 2, an iso-emissive point appears at 391 nm. It can also be observed that the fluorescence emission of OFLX at the protein maximum (346 nm) is insignificant, even at higher concentrations of the ligand, compared with those used in this study.

The fluorescence quenching data are analysed by the Stern–Volmer equation, as in (23):

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

where F_0 and F are the fluorescence intensities of protein in the absence and presence of the quencher, respectively, k_q is the quenching rate constant of the biomolecule, K_{sv} is the Stern–Volmer constant, τ_0 is the average lifetime of the molecule without quencher and [*Q*] is the quencher concentration.

The graph plotted according to the Stern–Volmer equation is shown in Fig. 3. The K_{SV} value is presented in Table 1 and,

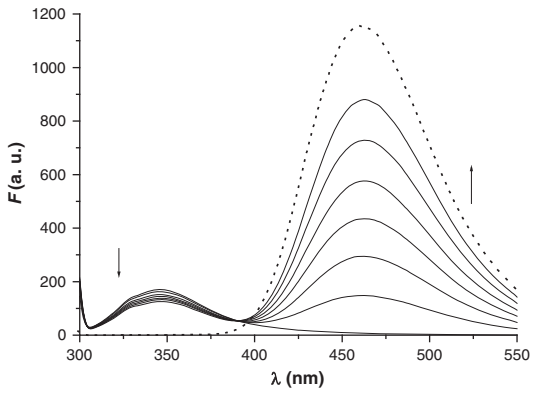

Figure 2. Fluorescence emission spectra of HSA ($C_{HSA} = 2.07 \times 10^{-6}$ mol/L) at pH 7.40 and 25°C in the absence and presence of OFLX, $\lambda_{ex} = 295$ nm, *d*:*p* = 0–1.62. Dotted line, fluorescence spectrum of OFLX, $\lambda_{ex} = 295$ nm, $C_{OFLX} = 6.38 \times 10^{-5}$ mol/L.

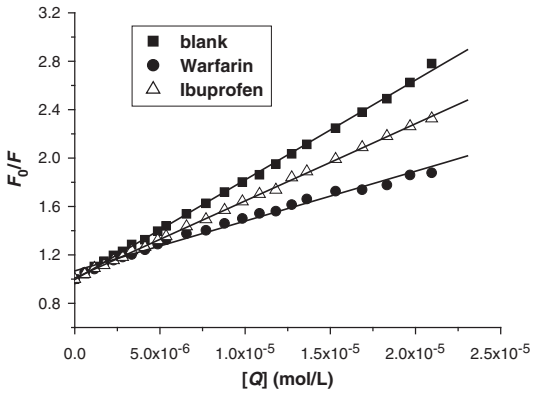

Figure 3. Stern–Volmer curves for the binding of OFLX to HSA in the absence and presence of warfarin and ibuprofen. $C_{HSA} = 2.07 \times 10^{-6}$ mol/L, *d*:*p* = 0 – 10, $\lambda_{ex} = 295$ nm, 25°C, pH 7.4.

considering the fluorescence lifetime of the biopolymer of the order of 10^{-8} s (24), led to the quenching rate constant k_q of 8.20×10^{12} L/mol s. This value, which largely overrides the accepted limit of the rate constant of the diffusional quenching implying biopolymers, 2×10^{10} L/mol s, supports the proposal that the experimental quenching of albumin fluorescence is predominantly due to a static process.

Assuming the predominance of static quenching, the observed change in the fluorescence of HSA with increasing concentrations of OFLX was analysed in terms of the following relationship [equation (2)]:

$$\log\frac{F_0 - F}{F} = n \times \log K - n \times \log\frac{1}{[L_t] - \frac{F_0 - F}{F_0} \times [P_t]} \quad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, and $[L_t]$ and $[P_t]$ are the total quencher concentration and the total protein concentration, respectively (25). By the plot of $\log(F_0 - F)/F$ versus $\log\{1/([L_t] - (F_0 - F)[P_t]/F_0)\}$ (Fig. 4), the number of binding sites *n* and the binding constant *K* can be obtained. Results in Table 1 indicate moderate binding and a single binding site.

This is in agreement with previous experimental data on the interaction of OFLX with BSA, which is similar in structure to HSA. Using the modified SV treatment and the Scatchard model, Hu *et al.* (18) also reported a 1:1 interaction process with somewhat lower binding constants, but of the same order of magnitude.

Binding of ofloxacin in the presence of site markers

The characterization of OFLX binding site to HSA was done by competitive experiments in the presence of site markers, using drugs that specifically bind to a known site or region on HSA. Warfarin and ibuprofen were used as site markers for sites I and II, respectively (26–28).

In order to compare the influence of warfarin and ibuprofen on the binding of OFLX to HSA, the binding process in their presence was analysed as previously for the blank, using the Stern–Volmer method and equation (2).

As can be seen in Figs 3 and 4 and Table 1, the binding constant for warfarin is smaller than that for ibuprofen or the blank. It can be concluded that OFLX can bind with a higher probability to the warfarin site I of HSA.

The energy transfer between ofloxacin and HSA

The distance from the tryptophan residue (donor) to the bound drug (acceptor) in HSA can be calculated according to Forster's theory (29). The efficiency of energy transfer, E, is given by:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (3)$$

where r is the distance between donor and acceptor and R_0 is the distance at 50% transfer efficiency.

$$R_0^6 = 8.8 \times 10^{-25} K^2 n^{-4} \Phi J \quad (4)$$

where K^2 is the orientation factor related to the geometry of the donor–acceptor of dipole and $K^2 = 2/3$ for random orientation as in fluid solution, $n = 1.36$ is the refractive index of the medium, Φ the fluorescence quantum yield of the donor and J is the spectral overlap of the donor emission and the acceptor absorption. J is given by:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (5)$$

where $F(\lambda)$ is the fluorescence intensity of the fluorophore when the wavelength is λ and $\varepsilon(\lambda)$ is the molar absorbance coefficient of the acceptor at the wavelength of λ. From these relationships, J, E and R_0 can be calculated; so the value of r can also be calculated.

From Fig. 5, the overlap integral calculated according to the above relationship is 8.71×10^{-15} cm^3 L/mol . Thus, the value of R_0 is 2.27 nm. The transfer efficiency between the donor (HSA) and acceptor (OFLX) in the complex was determined as follows. The spectra of 1:1 solutions of HSA:OFLX of different concentrations were recorded, together with the spectra of solutions of HSA of the same concentrations. In order to determine the efficiency in our working conditions, E was plotted against the ligand:protein ratio and the value for $d{:}p = 1$ was determined to be 0.144, from which the value of r is 3.05 nm.

Determination of the transfer efficiency in the ligand–HSA complex is not straightforward (30) and was done as follows. Spectra of solutions of HSA of different concentrations were recorded, alone (F_0) and in presence of an equimolar ligand concentration (F), such as to maintain $d{:}p = 1$ in all cases, up to 5×10^{-5} mol/L, the maximum concentration possible due to self-quenching and solubility problems. Plotting the values of efficiency against the ligand concentration (Fig. 6a), the efficiency in the complex can be considered as the value

Table 1. Effects of site markers on the binding of OFLX to HSA

			Equation (2)		
	$K_{SV} \times 10^{-4}$ (L/mol)	R*	n	$K \times 10^{-4}$ (L/mol)	R
Blank	8.20 ± 0.05	0.999	0.94	8.90	0.999
Ibuprofen	6.40 ± 0.03	0.999	0.93	6.80	0.999
Warfarin	4.12 ± 0.01	0.993	0.77	4.40	0.998

*Correlation coefficient.

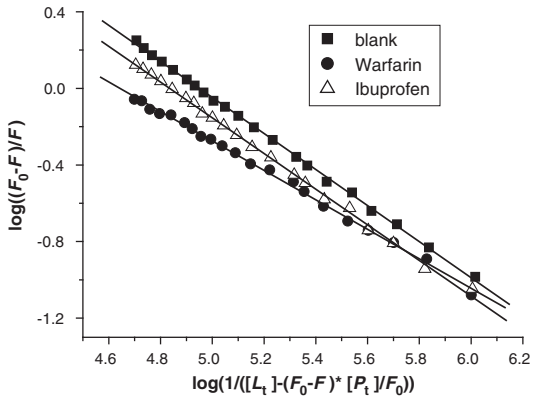

Figure 4. Equation (2) plots of the blank and site markers warfarin and ibuprofen competitive experiments. $C_{HSA} = 2.07 \times 10^{-6}$ mol/L, $d{:}p = 0 - 10$, $\lambda_{ex} = 295$ nm, 25°C, pH 7.4.

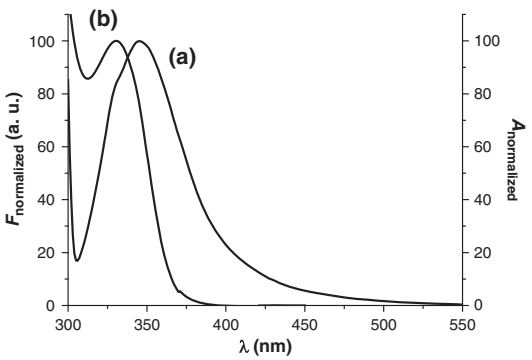

Figure 5. The overlap of the fluorescence spectrum of HSA (a) and the absorbance spectrum of OFLX (b); $C_{HSA}/C_{OFLX} = 1{:}1$.

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corresponding to complete saturation. As this could not be achieved, it can be considered that the value of 0.83, corresponding to the maximum concentration used, is the lower limit for the efficiency in the complex and thus the donor–acceptor distance could be overestimated. By plotting $1/E$ vs. $1/[Q]$ (31) and extrapolating to zero (infinite quencher concentration), the efficiency obtained has the same value, i.e. 0.83, and the corresponding r value is 1.74 nm.

Conformation investigation

To explore the structural change of HSA by addition of OFLX, we measured synchronous fluorescence spectra of HSA (Fig. 7) with various amounts of OFLX. The synchronous fluorescence spectra give information about the molecular enviroment in the vicinity of the chromophore molecules. When $\Delta\lambda$ between excitation wavelength and emission wavelength are 15 and 60 nm, respectively, the synchronous fluorescence gives the characteristic information for tyrosine or tryptophan residues (32).

It is apparent from Fig. 7 that the emission maximum of tryptophan and tyrosine residues have a small blue shift (from 341 to 338 nm for Trp and from 299 to 297 nm for Tyr), which indicates that the polarity around these residues was decreased and the hydrophobicity was increased (33). The same effect for tryptophan in synchronous fluorescence spectra was observed for the interaction between ofloxacin and BSA (18).

CD measurements

To ascertain the possible influence of drug binding on the secondary structure of HSA, we have performed CD studies in the presence of different concentrations of ofloxacin. As shown in Fig. 8, the CD spectra of HSA had two negative bands in the far-UV region at 208 and 220 nm, which are typical characterizations of α-helix structure in protein (34,35). These negative peaks between 208–209 and 222–223 nm were assigned to $n-\pi^*$ transition for the peptide bond of α-helical structure (36). Addition of OFLX to HSA caused only a decrease in the negative ellipticity in the region of far-UV CD without any significant shift of peaks.

The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2/dmol (37), according to equation (6):

$$MRE = \frac{ObservedCD(mdeg)}{C_p n l \times 10} \quad (6)$$

where C_p is the molar concentration of the protein, n is the number of amino acid residues and l is the path length. The α-helical contents of free and combined HSA were calculated from MRE values at 208 nm (38), using equation (7):

$$\alpha\text{–}helix(\%) = \frac{-MRE_{208} - 4000}{33000 - 4000} \times 100 \quad (7)$$

where MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE of the α-form and random coil conformation at 208 nm, and 33 000 is the MRE value of a pure α-helix at 208 nm. From

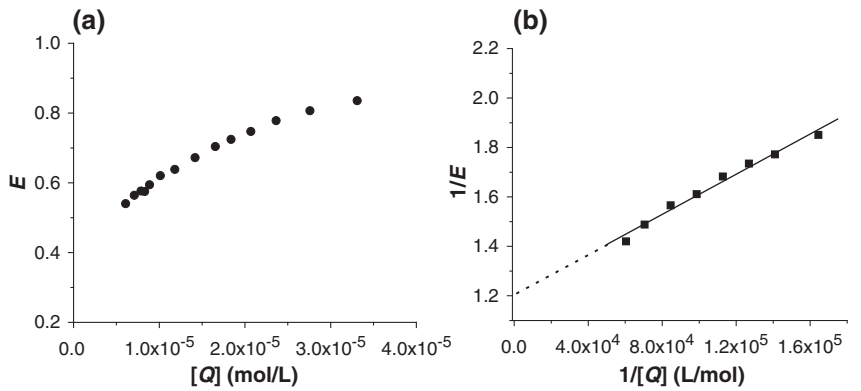

Figure 6. Determination of the efficiency (E) of energy transfer from the Trp residue of HSA to OFLX: (a) E vs. [Q]; (b) $1/E$ vs. $1/[Q]$.

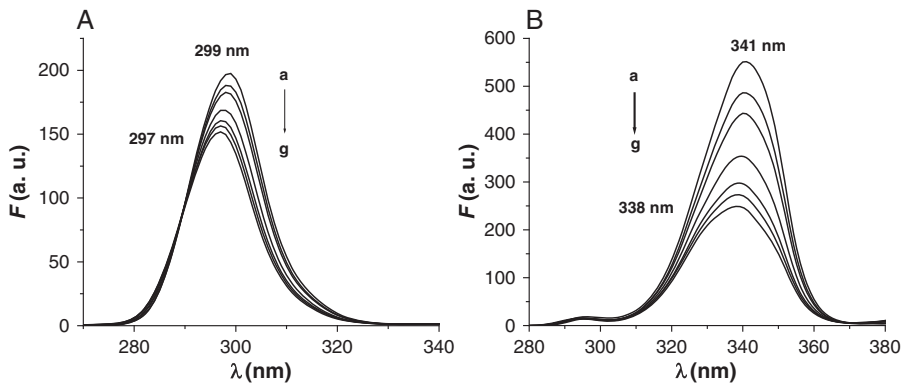

Figure 7. Synchronous fluorescence spectra of HSA in the absence and presence of OFLX (pH 7.4, 25°C): (A) $\Delta\lambda = 15$ nm; (B) $\Delta\lambda = 60$ nm; $C_{HSA} = 3$ μ mol/L; $C_{OFLX} = 0.3$ μ mol/L; from a to g: 0, 1.43, 2.73, 6.00, 8.57, 10.00, 11.25.

equation (7), the *α*-helicity in the secondary structure of HSA was determined. It differed from that of 63.09% in free HSA to 58.74% in the HSA–OFLX complex (Table 2), which was indicative of the loss of *α*-helicity upon interaction.

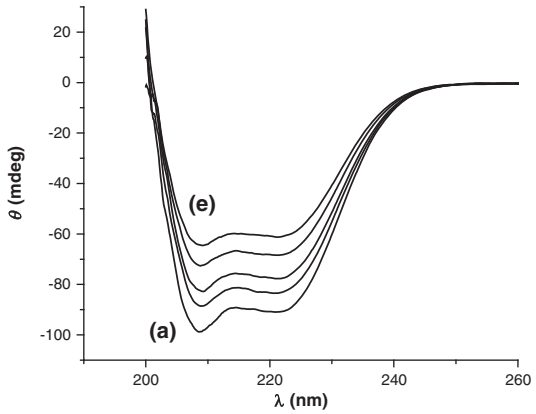

Figure 8. The CD spectra of the OFLX–HSA system at pH 7.4, 25°C. C_{HSA} = 0.75 μ mol/L; C_{OFLX} = 4.98 μ mol/L; *d:p* (a)–(e) = 0; 0.5; 1.0; 2.0; 3.0.

Table 2. Effect of OFLX on the *α*-helix content of HSA (pH 7.4, 25°C)

d:p	*α*-Helix (%)
0	63.09
0.5	60.07
1	59.66
2	59.41
3	58.74

The percentage of protein *α*-helix structure decreased indicated that OFLX bound with the amino acid residue of the main polypeptide chain of the protein.

Molecular modelling study of interaction between OFLX and HSA

The molecular modelling method has been employed to promote understanding of the interaction of OFLX and HSA. The binding sites of most drugs to HSA are located in subdomains IIA and IIIA, i.e. sites I and II.

During the docking process, a maximum of 10 conformers were generated for each binding site. For site I, the conformer with the lowest binding energy, estimated as −5.81 kcal/mol, is represented in Fig. 9. The ligand is located in the proximity of residues GLN 196, LYS 199, CYS 200, ARG 197, ALA 291, GLU 292, LEU 238, TRP 214, ALA 215, ARG 216 and ARG 257. A hydrogen bond with a distance of 2.075 Å was evidenced between OFLX and ARG 257. The distance to TRP 214 is about 8.4 Å, lower than that obtained from experimental data. However, it seems that the second method for determining the energy transfer efficiency estimates this distance better. The energy obtained for the binding of OFLX to site II of HSA is positive, which excludes binding to subdomain IIIA.

Conclusions

The interactions between ofloxacin and HSA have been investigated in this study, using fluorescence, CD spectroscopies and molecular modelling. Quenching experiments revealed 1:1 moderate binding, with an equilibrium constant of about 10^4 L/mol. The characterization of the binding site by competitive experiments using warfarin and ibuprofen showed preferential binding to site I, and synchronous fluorescence analysis indicated changes in the local environment of the Trp and Tyr residues, with an increase in hydrophobicity.

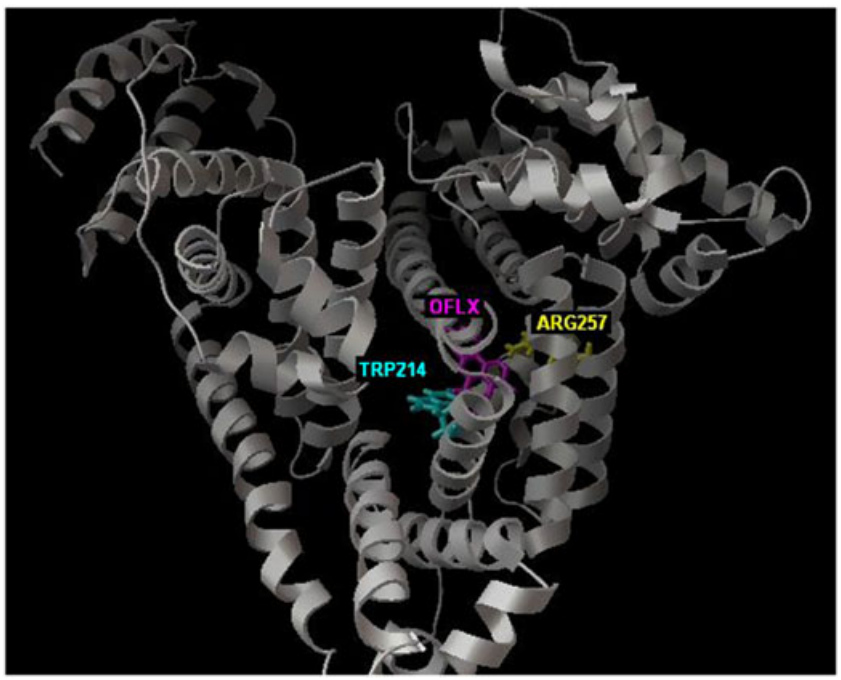

Figure 9. Binding modelling of ofloxacin to site I of HSA.

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The results of molecular modelling support the experimental data for the binding site and allow an estimation of the OFLX-TRP214 distance of about 8.4 Å.

Acknowledgement

Financial support from CNCSIS, Romania (Grant No. PNII ID_1914,494/2008), is gratefully acknowledged.

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