УДК 577.117.2+577.151.63+577.112;004.942

ВЗАИМОДЕЙСТВИЕ НИТРОБЕНЗОКСАДИАЗОЛЬНЫХ ПРОИЗВОДНЫХ ПИПЕРАЗИНА И АНИЛИНА С БЫЧЬИМ СЫВОРОТОЧНЫМ АЛЬБУМИНОМ *IN SILICO* И *IN VITRO*

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Рассматривается альбумин как глобулярный белок плазмы крови млекопитающих, который участвует в транспорте гидрофобных метаболитов и некоторых лекарств. Описываются синтез и результаты взаимодействия бычьего сывороточного альбумина *in silico* (докинг) и *in vitro* (спектрофотометрическое и спектрофлуориметрическое титрование) и четырех новых 7-нитробензоксадиазол-4-ил (NBD) производных анилина и пиперазина. Экспериментальная кажущаяся константа диссоциации для NBD-этиниланилина **4** составила приблизительно 10 мкмоль/л. На основании исследований, посвященных связыванию различных соединений с альбумином, проводятся оценки новых потенциальных лекарств или флуоресцентных проб для использования *in vivo*.

Ключевые слова: альбумин; флуоресценция; нитробензоксадиазол; докинг.

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INTERACTION OF NITROBENZOXADIAZOLE DERIVATIVES OF PIPERAZINE AND ANILINE WITH BOVINE SERUM ALBUMINE *IN SILICO* AND *IN VITRO*

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Albumin is a globular protein of plasma of mammalian blood participating in transport of hydrophobic metabolites and drugs. Thus, studies devoted to its binding are valuable as a part of evaluation of new potential drugs or fluorescent probes for *in vivo* usage. Here we describe results concerning synthesis and bovine serum albumin binding assay both *in silico* (docking) and *in vitro* (spectrophotometric and spectrofluorimetric titrations) for four new 7-nitrobenzoxadiazol-4-yl (NBD) derivatives of aniline and piperazine. Experimental dissociation constant for NBD-ethynylaniline **4** was calculated to be about 10 μmol/L.

Keywords: bovine serum albumin; fluorescence; nitrobenzoxadiazoles; docking.

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Introduction

Albumin is the most abundant water-soluble protein found in blood plasma (~60 %) of total blood plasma proteins. It functions are related with transport of small drug molecules, bile-salt, hormones, vitamins, metal ions as well as the maintenance of oncotic pressure between blood vessels and tissues (e. g., see [1; 2]). Thus, it is quite important to evaluate interactions of both new drug-like molecules and potential fluorescent molecular probes pretending to be used *in vivo* with the protein. For this purposes human serum albumin (HSA) and bovine serum albumin (BSA) are commercially available. HSA is very similar with BSA (they are both about 66–68 kDa, HSA and BSA have 585 and 583 amino acid residues, respectively, they share about 76 % homology between their single polypeptide chains and have similar 3D structure) [1; 2]. Thus, BSA is often used initially because it is more available. A lot of various fluorescent compounds have been tested with the albumins and 7-nitrobenzoxadiazol-4-yl (NBD) derivatives are among them. Development of fluorescent probes or labeled bioactive compounds via functionalisation of some bioactive molecules (drugs, metabolites) using derivatisation with commercially available NBD-chloride is a good abundant decision because of NBD small size (for NBD-NH_2, $C_6N_4O_3H_4$, 17 atoms, two cycles, ~0.5 nm^3), microenvironment sensitivity, robust synthetic protocols for amines labeling, good fluorescence properties (blue light excitation, more than 50 nm Stokes shift, moderate quantum yields). NBD derivatives, which were tested with BSA or HSA, include NBD-C12 fatty acid (NBD derivative of ω-aminolauric acid) [3], cholic acid labeled with NBD at positions C3 and C7 [4], NBD-butylamine [2] and homologues C2–C12 as well as benzylamino-NBD [5]. In a pioneering work [1] determined two principle sites of compounds' binding with HSA, Sudlow sites I and II. Sudlow site I is localised in subdomain IIA and its marker drug is warfarin (residues TYR150, LYS195, LYS199, HIS242, HIS288, ARG257 and its single TRP214 close to it; e. g., see structure PDB 2bxd), whereas Sudlow site II is localised in subdomain IIIA and its marker drug is ibuprophen (residues TYR411, LEU430, LEU407, LEU453, ARG410, PHE488, ARG485; e. g., see PDB structure 2bxg) [6]. Analogously, in the BSA case, its Sudlow site I includes ARG194, ARG198, HIS241, TYR149, ARG256, HIS287 and its both TRP213 and TRP134 are close to the site; BSA's Sudlow site II includes LEU452, PHE487, TYR410, LYS413, ARG409, LEU429 [6]. Notably that NBD-C12 fatty acid was reported to have a new binding site to be different from Sudlow sites I and II exhibiting 1 : 1 stoichiometry [3], 7-heptylamino-NBD was reported to compete with ibuprofen (Sudlow site II marker) [5] and a modern paper in «Scientific Reports» states the similar effective competition for buthylamino-NBD [2]. But to the best of our knowledge there are no publications describing interactions of albumins with NBD derivative of N-acylpiperazine or aniline. Being encouraged by the fact and the papers mentioned above we have decided to test NBD derivatives of the type mentioned as potential BSA ligands.

Experimental section

Piperazine, bromoacetyl bromide, pyridine (Py), 7-nitrobenzoxadiazol-4-yl chloride (NBD-Cl), 3-ethynylaniline, hexanoic anhydride and metacryloyl chloride, ninhydrine, silica gel for chromatography, acetone-d_6 were from *Sigma-Aldrich* (USA). $NaHCO_3$ (*Bashkir Soda Company*, Russia), acetonitrile (AcN), methanol (MeOH) (*Merck*, USA) were used. Synthesis of the compounds was done as described below (fig. 1).

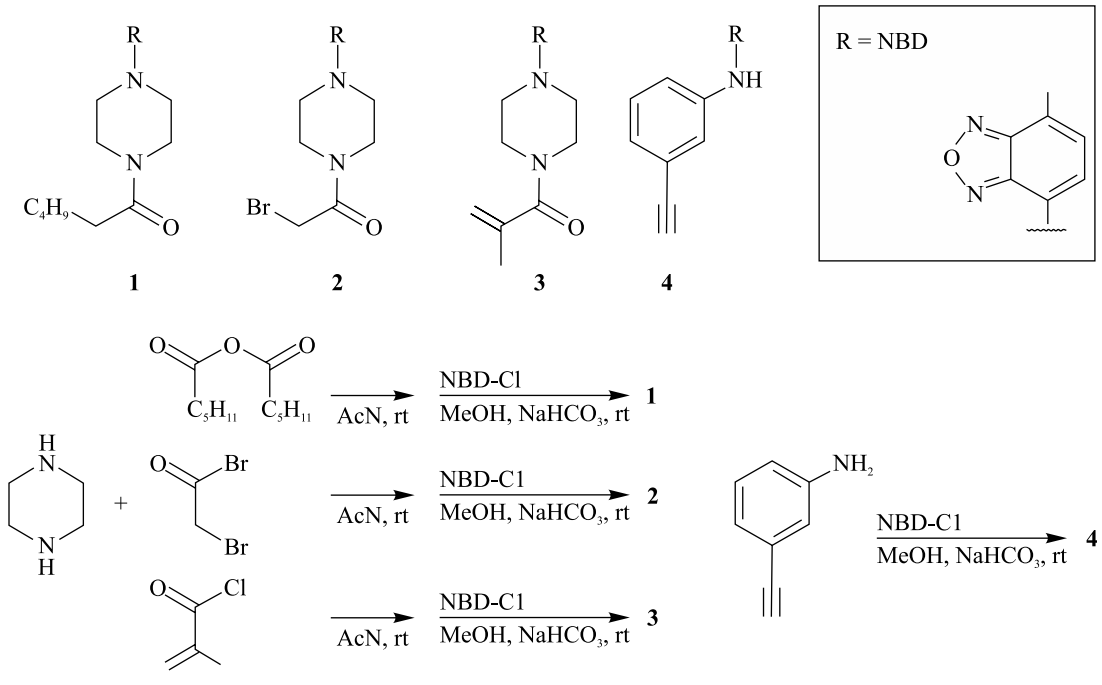

Fig. 1. Structures and schemes of one-pot synthesis of N-acylated NBD-piperazines (**1**–**3**) and NBD-ethynylaniline (**4**); rt – room temperature

Briefly, N-acylated NBD-piperazines (**1**–**3**) were prepared *via* dropwise addition of diluted (2–5 mg/mL, 1.05 eq) acetonitrilic solutions of corresponding anhydrides to acetonitrilic solution of piperazine (20 mg/mL, 1 eq) within 10 min at room temperature. After piperazine consummation according to thin layer chromatography (TLC) with ninhydrine and heat development, $NaHCO_3$ (3 eq) and NBD-Cl in MeOH (5 mg/mL, 1 eq) were added. The mixtures were stirred 15–30 min until NBD-Cl consummation according to TLC. Then solvents were removed from the synthetic mixtures and the products were purified using column chromatography (SiO_2, gradient of benzene and acetone) by collecting orange fractions with green fluorescence followed by solvent removal using rotary evaporator. Yields were within 40–60 % giving the products as brown-reddish solids. NBD-ethynylaniline **4** was synthesised and purified in the similar manner (see fig. 1) with yield 70 % as a brown-reddish solid.

Mass-spectrometric analysis was carried out using LCMS-2020 (*Shimadzu*, Japan) system as described [7] using MeOH both as the eluent and for sample dilution. Nuclear magnetic resonance (NMR) analysis of **4** was performed on Bruker Avance 500 MHz instrument (Germany) as noted below.

Compound **1**, 1-hexanoyl-4-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)piperazine, ESI-MS: m/z $[M + H]^+$ for $C_{16}H_{22}N_5O_4^+$; calculated 348.17. Compound **2**, 1-bromoacetyl-4-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)piperazine, ESI-MS: m/z 449.9 $[M + Br]^-$ for $C_{12}H_{12}N_5O_4Br_2$; calculated 449.92. Compound **3**, 1-methacryloyl-4-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)piperazine, ESI-MS: m/z 318.0 $[M + H]^+$ for $C_{14}H_{16}N_5O_4^+$; calculated 318.12. Compound **4**, N-(3-ethynylphenyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine, ESI-MS: m/z 279 $[M - H]^-$ for $C_{14}H_7N_4O_3^-$; calculated 279.03; ^1H NMR (500 MHz, acetone-d_6) chemical shift 8.48 (d, C**H**═C—NO_2), 7.60 (dd, C**H**═C—NH—), 7.16–7.20 (m, —C**H**— benzene), 5.15 (s, **H**—C≡C alkyne).

Docking was done as described [8] using *AutoDock Vina* [9] software. Grid centers for all calculations were 7 × 7 × 7 nm^3 with their centers at geometrical centers of correspondent proteins, exhaustiveness was set to 20, five structures of BSA (PBD codes 3v03, 4f5s, 4jk4, 4or0, 6qs9) were used. Values of energy of binding (E_{bind}) and the amino acids surrounding of ligands have been tabulated. Phospholipid membrane permeability was also estimated *in silico* using PerMM server (*https://permm.phar.umich.edu/server*); if logarithmic values calculated are more than –4.35 for a compound, it is considered as membrane permeable [10]. Lipophilicity was also evaluated *in silico* as logP values computed using *Hyperchem 7.0*.

Fluorescence and absorbance spectra were recorded using spectrofluorimeter Solar SM2203 (*Solar*, Belarus) operated at default parameters excepting slits were 10 nm; emission spectra were recorded using excitation at both 295 nm (BSA) and 460 nm (NBD). Titrations were performed using 10 μmol/L bicarbonate buffer; compounds were added in ethanol.

Results and discussion

Four new (according to Pubchem database identity search) fluorescent NBD derivatives were synthesised to be evaluated as potential molecular probes or fragments (synthetic blocks) of such compounds. As individual probes, besides fluorescence due to NBD-N< motif, they possess groups which could promote membrane biding (see fig. 1, **1** and **4**), cytochromes P450 inactivation [11; 12] (**4**), reaction with biological thiols *via* S_N2 substitution (**2**) or Michaelis type addition (**3**). To impact on evaluation of biological properties of the compounds we have decided to perform few *in silico* computations and to test *in vitro* their ability to bind with BSA. *In silico* computed logP values and membranes permeability data are shown in table 1.

Table 1

***In silico* computed logP values and membranes permeability data**

Compound, brutto-formula	Free energy of binding (DOPC*), kcal/mol	Log of permiability coefficient			LogP
		BLM*	BBB* (Po)	$CACO_2$ (Po)	
1, $C_{16}H_{21}N_5O_4$	–4.73	–0.44	–3.05	–3.59	2.81
2, $C_{12}H_{12}N_5O_4Br$	–3.88	–1.67	–3.48	–3.91	0.94
3, $C_{14}H_{15}N_5O_4$	–4.05	–1.08	–3.28	–3.76	2.03
4, $C_{14}H_8N_4O_3$	–4.57	0.27	–2.80	–3.41	3.06
NBD-Bu [2]	–5.03	1.03	–2.53	–3.21	2.41

*For detail see [10].

Comparison of logP values and computed free energy of binding (DOPC) allow to guess that **1**, **3** and **4**, but not **2** have good chances to be bound in a hydrophobic protein civility or membrane; phospholipid bilayer permeability parameters are moderate and NBD-Bu [2] together with **4** demonstrate the best values in the structure under discussion.

To rationalise further experimental BSA titrations we performed *in silico* docking computations with five BSA structures. The results are summarised in table 2.

The data obtained demonstrated that all the compounds can likely be bound with BSA in sites different from classical Sudlow sites I and II. According to modelling with two of fifth structures (4f5s and 4or0) **3** and **4** can be bound close to Sudlow sites I TYR149 and the nearest TRP213 allowing fluorescence resonance energy transfer (FRET) from excited TRP to NBD moiety of the molecules. Also the smallest E_{bind} values (–10.9 to –9.1 kcal/mol) were computed for **4** and **3**. It is good in comparison with the value reported for NBD-Bu (–7.32 μmol/L) [2]. It should be noted that the docking poses differ considerably for different BSA structures highlighting multiple options of BSA to bind of the compounds; the computed binding sites for **3** and **4** (BSA structure with PDB code 4f5s) are shown below (fig. 2).

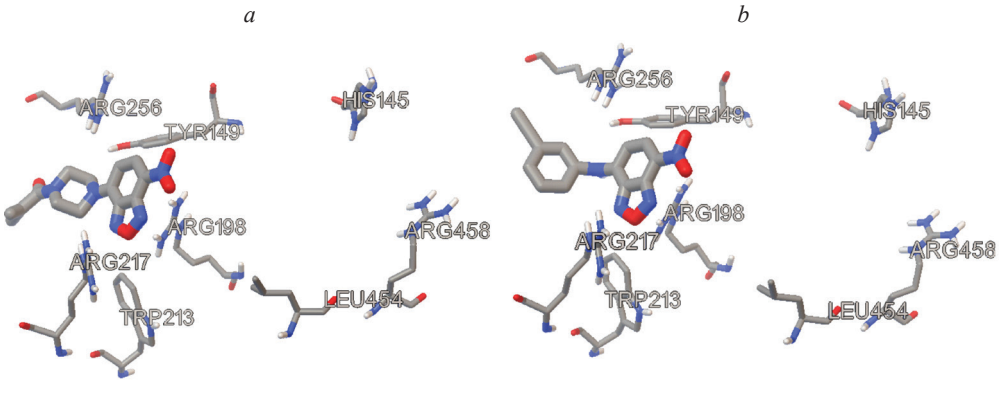

Fig. 2. In silico calculated positions of **3** (*a*) and **4** (*b*) in a site of BSA
carbon, nitrogen and oxygen atoms are depicted with gray, blue and red, respectively.
Additionally, amino acids' residues names and positions are indicated

Table 2

E_{bind} values and computed amino acid surroundings for compounds 1–4 *in silico* interactions with BSA structures

PDB code	Compound	E_{bind}, kcal/mol / amino acids residues
3v03	**1**	–8.5 / ASP108; ARG144; HIS145; ARG458; ARG196; **TYR147**; LEU189; ILE455; **TYR451**; LYS431; VAL432
	2	–8.3 / ASP108; ARG144; HIS145; ARG458; **TYR147**; ARG196; LEU189; ILE455; LYS431
	3	–9.1 / HIS145; ARG458; ASP108; ARG144; **TYR147**; ARG196; PRO146; LEU189; ILE455; LYS431
	4	–9.1 / LEU326; GLU353; LYS350; LEU346; VAL481; LEU330; GLY327; VAL215; LYS211; ASP323; ARG208
4f5s	**1**	–8.9 / ARG144; HIS145; ARG458; PRO146; **TYR147**; ARG196; LEU189; ARG435; ILE455; **TYR451**; LEU454
	2	–8.6 / ARG435; ILE455; LYS431; **TYR451**; ARG458; HIS145; LEU189; ASP108; ARG196
	3	–9.8 / ARG198; ARG256; **TYR149**; ARG217; ARG194; **TRP213**; HIS241; LEU237; LEU259; LEU218; ILE263
	4	–9.6 / LEU237; LEU218; ARG256; LEU259; ILE263; **TYR149**; HIS241; ARG198; ARG194; ARG217; **TRP213**
4jk4	**1**	–8.9 / ASP108; ARG144; HIS145; ARG458; ARG196; **TYR147**; GLU424; LEU189; ILE455; **TYR451**; GLU186
	2	–8 / LYS431; GLU424; LEU189; ALA193; **TYR451**; HIS145; ARG458; ASP108; ARG144; ARG196; **TYR147**
	3	–9.2 / HIS145; ARG458; ASP108; ARG144; ARG196; ALA193; **TYR147**; LEU189; GLU424; **TYR451**
	4	–10 / PHE550; LYS524; LEU528; MET547; LEU531; VAL546; PHE508; PHE506; PHE501; VAL575; LEU574
4or0	**1**	–8.3 / ILE455; LEU454; ARG458; LEU189; ARG435; THR190; HIS145; ASP108; ARG144; ASP111; LEU112
	2	–9.1 / ARG194; ARG198; **TRP213**; **TYR149**; ARG256; HIS241; LEU259; LEU218; LEU237; PHE222; ILE289
	3	–9.8 / LEU189; LYS431; ARG435; THR190; ALA193; GLU186; ARG458; SER192; HIS145; ARG196; ASN457
	4	–10.4 / LEU346; LEU480; ALA209; **TRP213**; VAL481; LYS350; LEU197; ARG198; ARG194; VAL342; ASP450
6qs9	**1**	–8.2 / HIS145; ARG458; ARG144; ASP108; GLU424; ARG196; ASP111; LEU112; LYS114; LEU115; ARG185
	2	–8.4 / ARG144; HIS145; SER192; ARG458; GLU424; PRO110; LEU189; ARG435; ILE455; **TYR451**; LEU454
	3	–10.9 / **TYR149**; ARG256; ARG198; ARG217; ARG194; **TYR156**; HIS241; LEU237; LEU259; LEU218; ILE263
	4	–9.7 / **TYR149**; HIS241; ARG256; LEU237; ILE289; LEU259; ILE263; ARG198; ARG217; **TYR156**; ARG194

Notably, that **2** and **3** can be attached covalently to thiol of surface exposed Cys34 – the single of 35 cysteine of BSA, which do not form intermolecular —S—S— bridge, but it might be in sulfenic acid state [13; 14].

To estimate BSA binding *in vitro* fluorescence titrations were done to determine intrinsic BSA fluorescence (due to two TRP and twenty TYR residues) as well as change in NBD group fluorescence which can differ upon binding to hydrophobic BSA sites. The data obtained indicate on effective intrinsic fluorescence quenching for **4** (fig. 3).

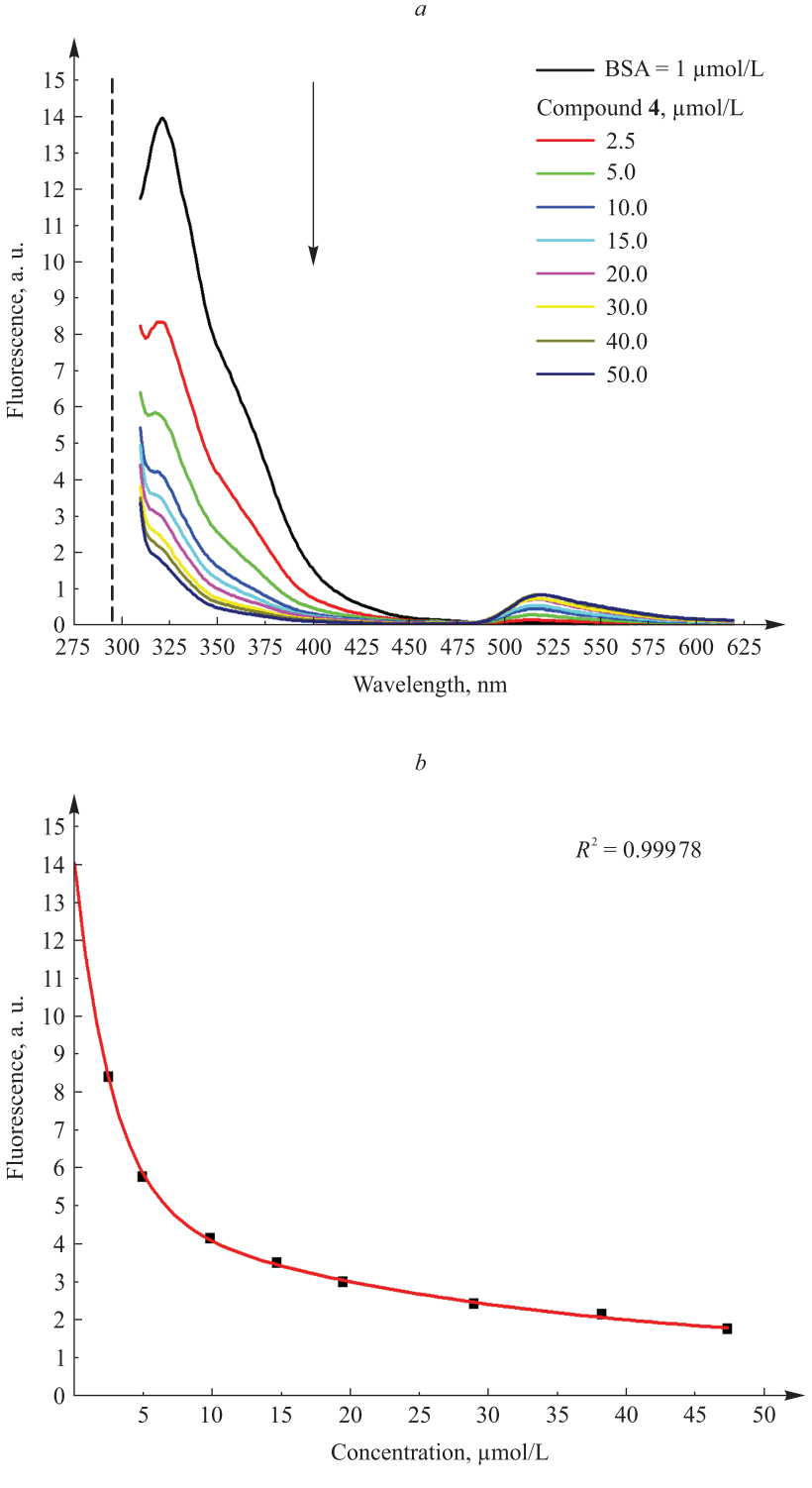

Fig. 3. Decrease of BSA (1 μmol/L) intrinsic fluorescence (excitation at 295 nm, depicted by dashed line) during direct titration by **4** (2.5–50.0 μmol/L): spectra overlaid (*a*); values for emission at 332 nm *vs* concentration of **4** fitted with 1 : 1 binding equation (exponential decay) (*b*)

Direct titration curves demonstrated saturation at 50 μmol/L of **4** at 1 μmol/L of BSA with apparent dissociation constant (K_d) value ~10 μmol/L (data not shown). Moreover, increase of fluorescence of **4** at increasing concentration of BSA was also detected using excitation both at 460 nm (NBD group-dependent) and 295 nm (dependent on TRP and TYR residues) as it is shown in fig. 4.

The results indicate that **4** is a good ligand for BSA which can bind close to Sudlow site I with K_d value ~10 μmol/L, but quite high in comparison with the reported value for NBD-Bu (~1.2 μmol/L, reciprocal to association constant binding constant, $K_a = 8.3 \cdot 10^5$ L/mol) [2].

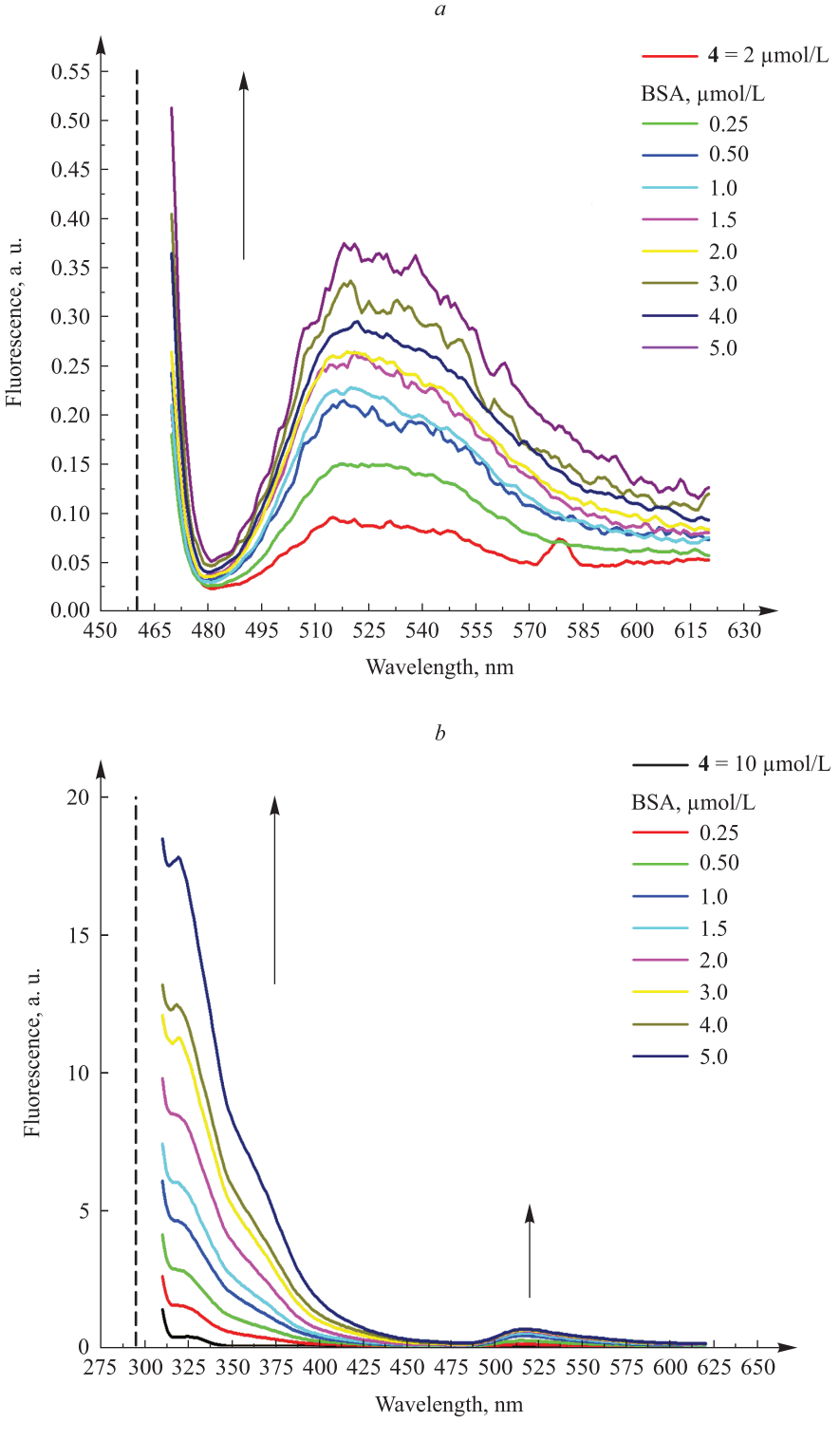

Fig. 4. Increase of **4** fluorescence at 530 nm (excitation at 460 nm (*a*) and 295 nm (*b*), depicted by dashed lines) during inverse titration by BSA (2.5–5.0 μmol/L)

The same tests were done for the rest of compounds and some resulting spectra are depicted in fig. 5 and 6.

Fluorescence was increased during titration of 1 with BSA (see fig. 5, *a*), but without considerable saturation at 5-fold excess of the protein. Also no signal at 530 nm appeared during the inverse titration using excitation at 295 nm (see fig. 5, *b*) reflecting the lack of fluorescence resonance energy transfer from BSA TRP and TYR residues to NBD of **1**. In general, aliphatic piperazine-based **1**, **2** and **3** demonstrated no increase of their fluorescence at BSA addition and excitation at 295 nm indicating worse ability to FRET in comparison with aromatic aniline **4**.

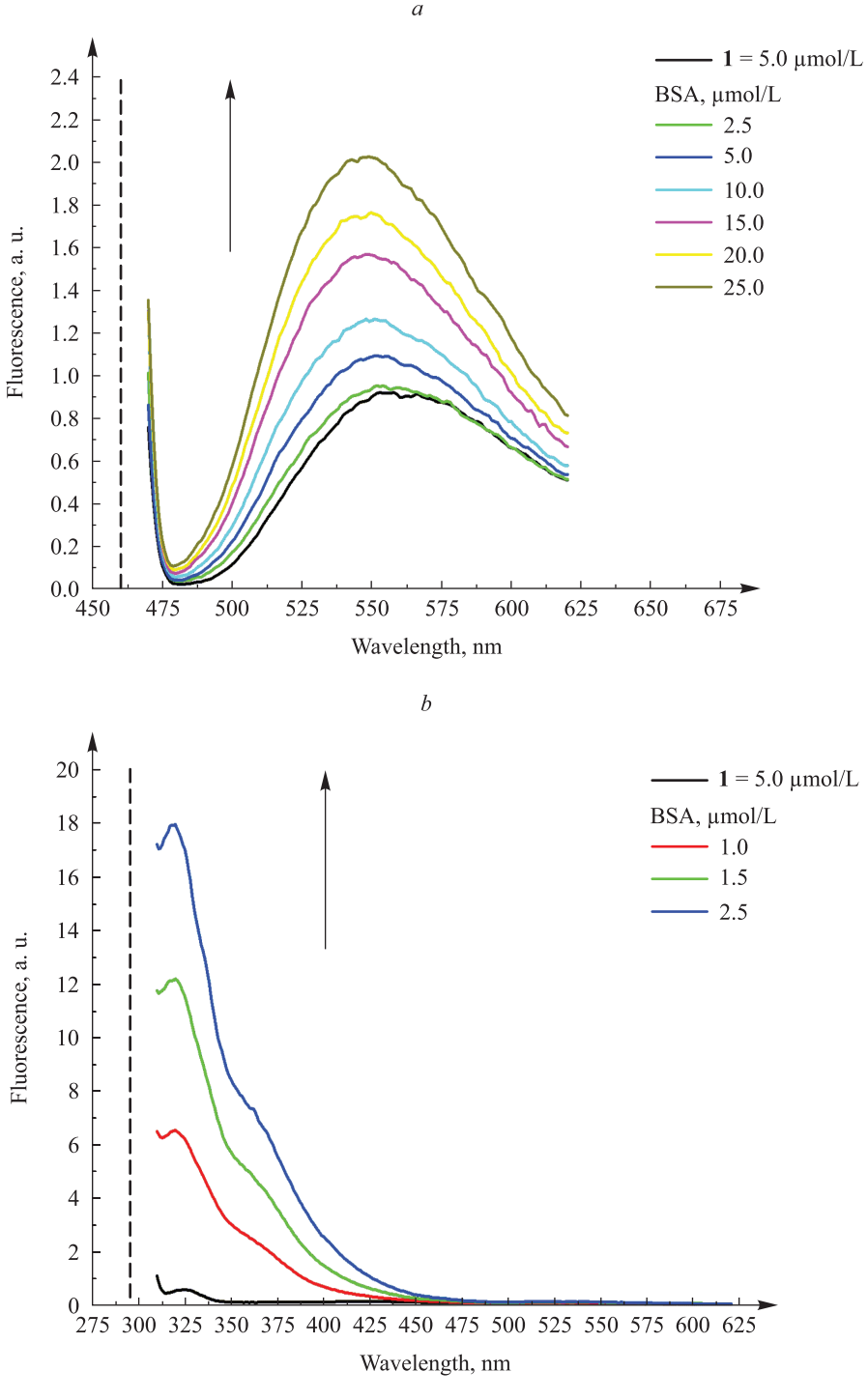

Fig. 5. Fluorescence spectra obtained at BSA interaction with **1** (reverse titration):
increase of fluorescence of **1** (excitation at 460 nm)
during titration of the compound with BSA reflecting binding in a hydrophobic site of the protein (*a*);
negligible increase of **1** fluorescence (excitation at 295 nm)
during titration of the compound with BSA reflecting distant localisation
of its NBD group from TRP and (or) TYR residues (*b*)

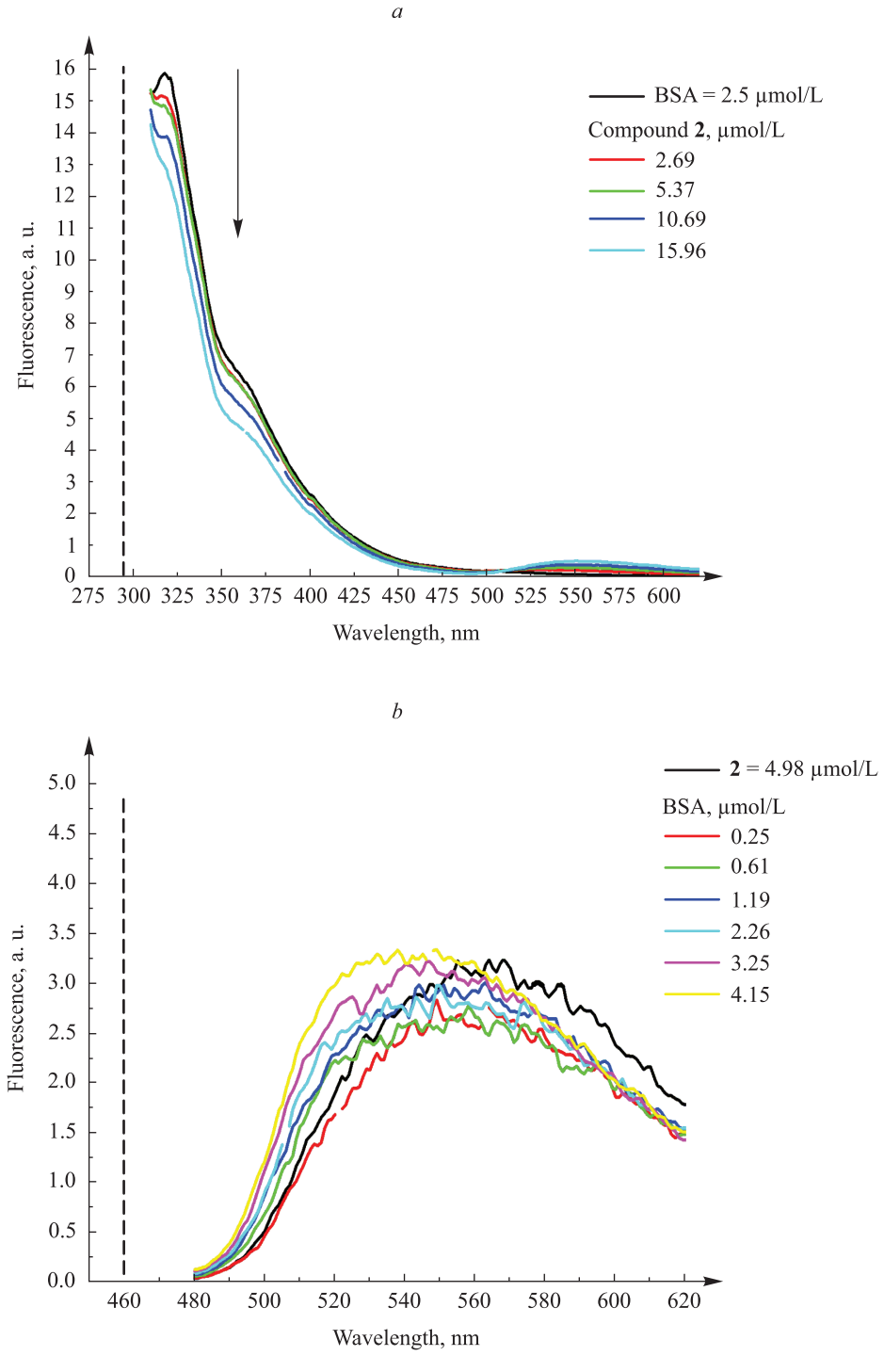

Fig. 6. Fluorescence spectra obtained at BSA interaction with **2**: moderate decrease of BSA fluorescence (excitation at 295 nm) during titration of the protein with **2** (direct titration) (*a*); negligible increase of **2** fluorescence (excitation at 460 nm) during titration of the compound with BSA (inverse titration) (*b*)

For **2** (see fig. 5) and **3** (data not shown) small decrease of BSA fluorescence were monitored at direct titration with the ligands and negligible increase of their own florescence (excitation at 460 nm) during at inverse titration (BSA addition) were monitored. That could be interpreted as poor binding with BSA or binding at site of high polarity. Conversely, direct titration curves analysis demonstrates saturation with $K_d \sim 15$ μmol/L for **3**, $K_d \sim 10$ μmol/L for **2** and $K_d \sim 20$ μmol/L for **1** (data not shown). Thus, either fluorescence change of both **2** and **3** reflect their binding with BSA poorly or they are truly poor ligands for the protein. Additional test are necessary to clarify this taking into consideration potential ability of **2** and **3** to be attached to the Cys34.

Conclusion

Studies devoted to binding of albumins, the main mammalian blood plasma protein with a drug and metabolites transport function and redox-sensitive cysteine with free thiol group, are valuable as a part of evaluation of new potential drugs or fluorescent probes pretending to be used *in vivo*. Four newly synthesised fluorescent 7-nitrobenzoxadiazole-4-yl derivatives, bearing N-acylpiperazine (bromoacetyl, metacryloyl and hexanoyl) and 3-ethynylaniline groups were synthesized as possible fluorescent probes or synthetic blocks for such compounds with yields 40–70 %. Using *in silico* computations the compounds were shown to be able to bind efficiently with bovine serum albumin with energy of binding from –8.3 to –10.9 kcal/mol and NBD-ethynylaniline **4** demonstrated affine binding close to TYR149 and TRP213. Using fluorescence titration approach **4** was found to decrease BSA fluorescence efficiently with apparent $K_d \sim 10$ μmol/L according to direct and inverse titration data. Also **1**, **2** and **3** no increase of their fluorescence at BSA addition and excitation at 295 nm indicating worse ability to FRET in comparison with **4**. Conversely, direct titration curves analysis demonstrates saturation with $K_d \sim 15$ μmol/L for **3**, $K_d \sim 10$ μmol/L for **2** and $K_d \sim 20$ μmol/L for **1**.

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