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Electronic supplementary information

Recreational drugs 25I-NBOH and 25I-NBOMe bind to both Sudlow's sites I and II of Human Serum Albumin (HSA): Biophysical and molecular modeling studies

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Evaluation of binding and thermodynamic parameters

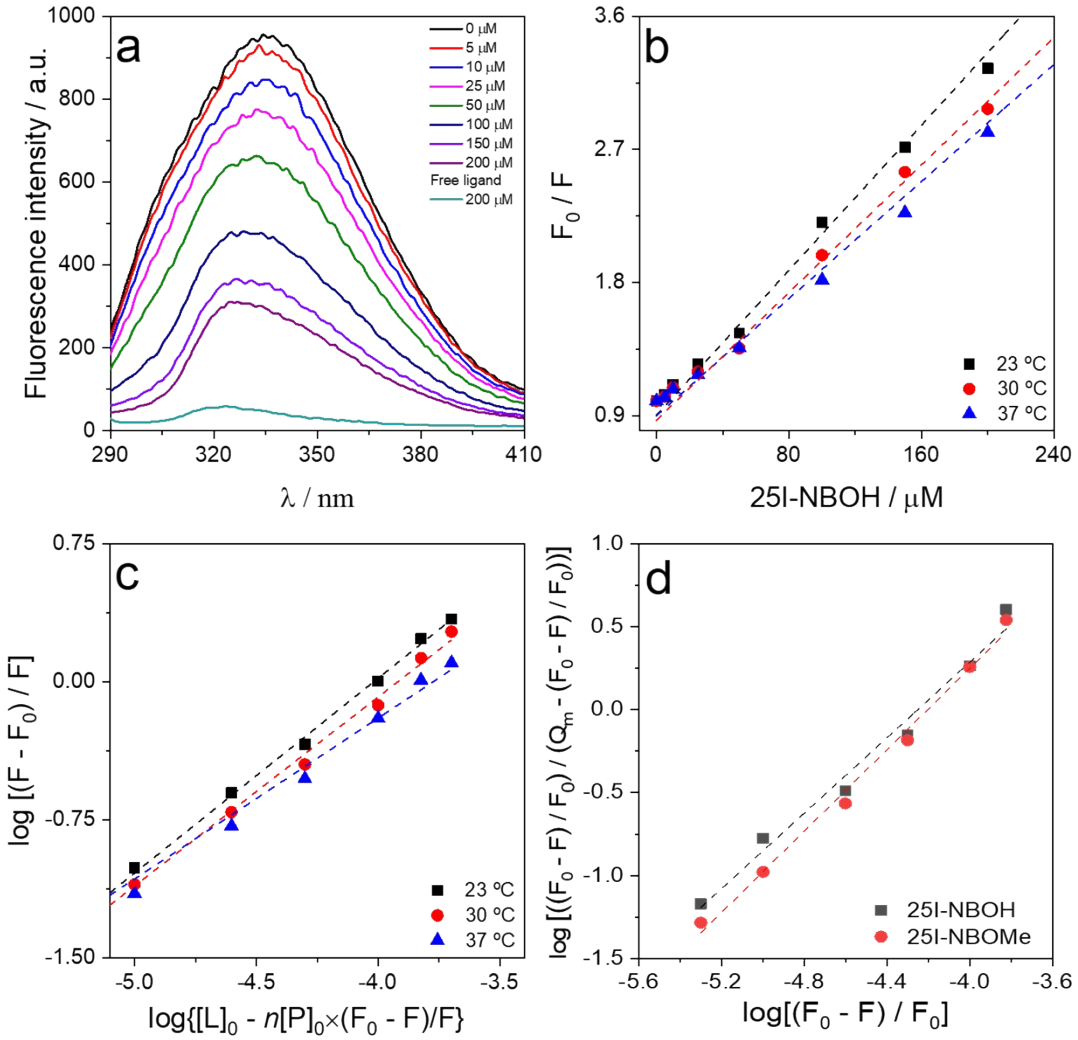

Figure S1. (**a**) Fluorescence emission spectral profile for HSA (4 μM) without and in the presence of different concentrations of **25I-NBOH** (5 - 200 μM) at 30ºC; (**b**) Stern-Volmer quenching plots, (**c**) Double logarithmic curve to the binding constant calculation for **25I-NBOH**, and **(d)** Curve for calculating the Hill coefficient (*n*H) of the HSA-ligands system at 30ºC.

Table S1. Binding parameters of human serum (total protein concentration expressed as HSA content) interaction with **25I-NBOH** and **25I-NBOMe** (both from 5 to 200 μM) at 30°C (303 K) and pH 7.4.

Ligand	Temperature (K)	Stern-Volmer constant		Binding parameters		
		K_{SV} (10^3 M^{-1})	*r*	K_b (10^3 M^{-1})	*n*	*r*
25I-NBOH	30 ºC (303 K)	4.08 ± 0.04	0.9972	4.24 ± 0.04	1.04	0.9964
25I-NBOMe	30 ºC (303 K)	5.14 ± 0.06	0.9956	7.09 ± 0.05	0.97	0.9945

Effect of ionic strength (NaCl)

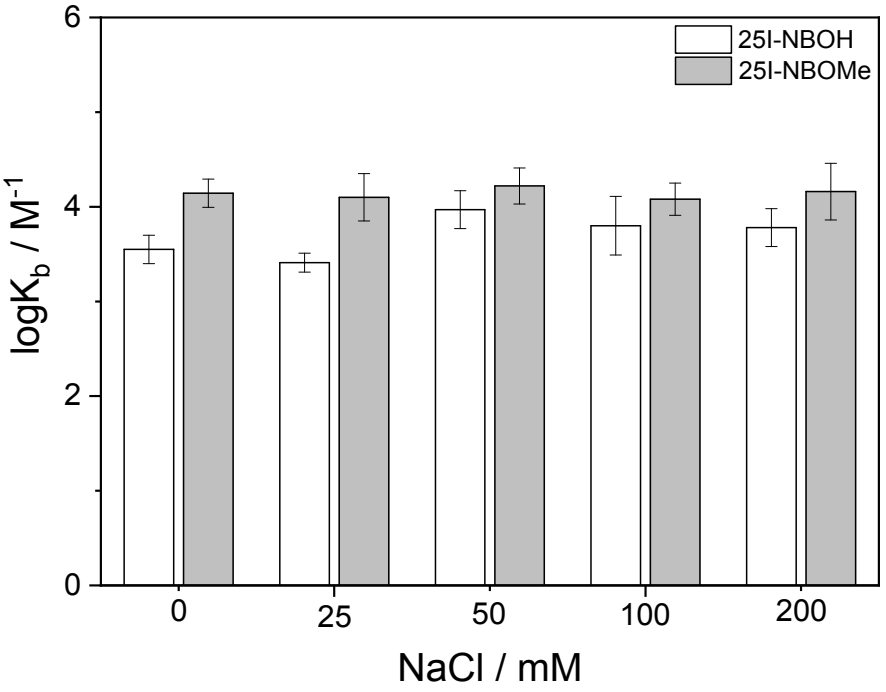

Figure S2. Evaluation of the effect of ionic strength on the interaction process of HSA with **25I-NBOH** and **25I-NBOMe**. Conditions: pH of 7.4 at 30°C, ligands (5 - 200 μM).

Table S2. Evaluation of the effect of ionic strength (NaCl) on the interaction process of HSA with **25I-NBOH** and **25I-NBOMe**. Conditions: pH of 7.4 at 30°C, ligands (5 - 200 μM).

NaCl (mM)	Ligands			
	25I-NBOH		**25I-NBOMe**	
	$\log K_b$	*r*	$\log k_b$	*r*
0	3.54 ± 0.15	0.9986	4.14 ± 0.15	0.9948
25	3.41 ± 0.10	0.9920	4.09 ± 0.25	0.9982
50	3.97 ± 0.20	0.9974	4.22 ± 0.19	0.9974
100	3.80 ± 0.31	0.9951	3.08 ± 0.17	0.9984
200	3.78 ± 0.20	0.9985	4.16 ± 0.30	0.9974

Comments: The variation in ionic strength did not lead to significant changes in the values of $\log K_b$ (Fig. S3, Table S2), so the interaction process between **25I-NBOH** and **25I-NBOMe** with HSA should preferably not have an electrostatic component.

UV-vis interaction studies

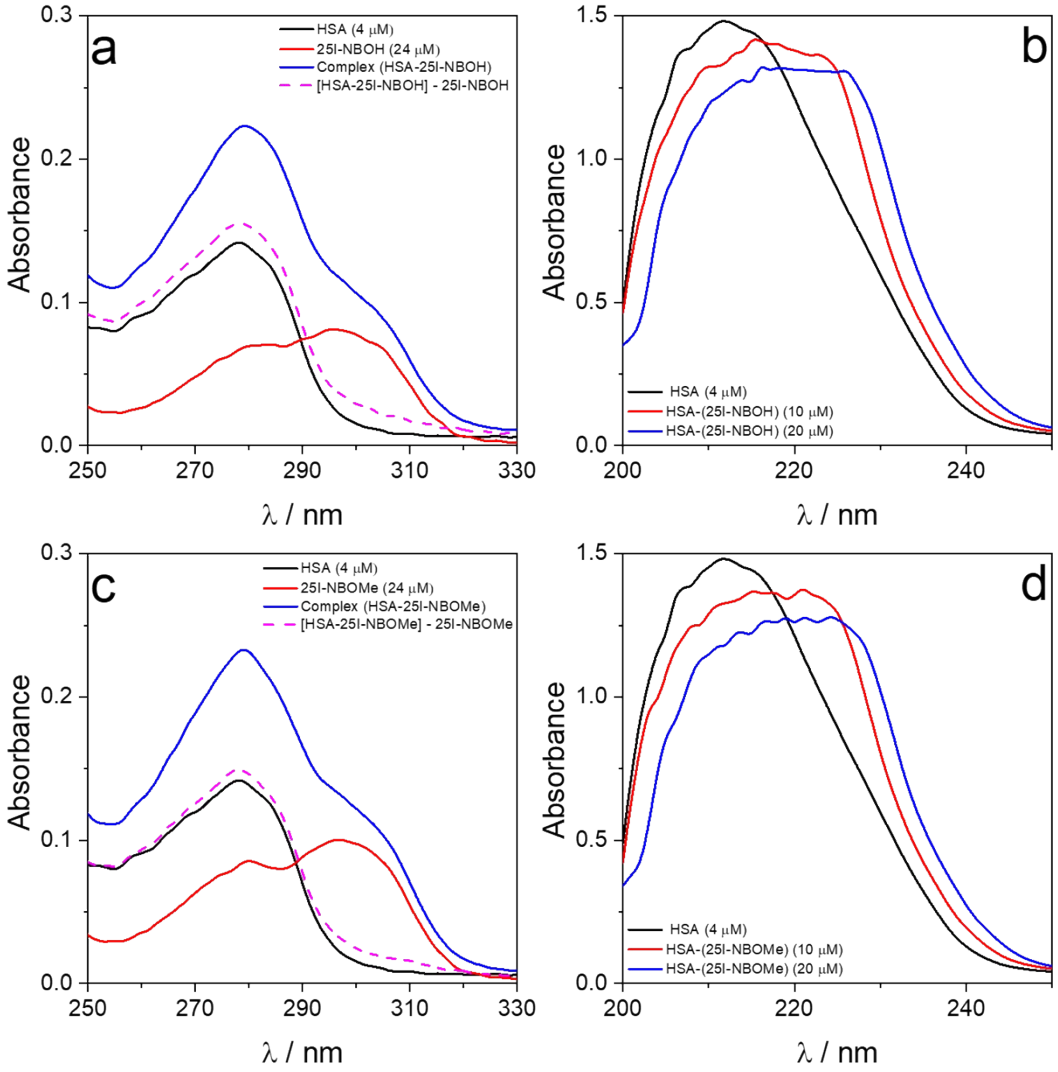

Figure S3. (a, c) UV-vis absorption spectra for HSA (4 μM), **ligand** (24 μM), HSA-**ligand** mixture (complex), and the spectral difference (HSA + **ligand**) – **ligand**. (b, d) UV-vis absorption spectra for HAS in different concentrations of the ligands. Ligand: **a** and **b** = **25I-NBOH** // **c** and **d** = **25I-NBOMe**. Conditions: pH of 7.4 at 30°C.

Table S3. Absorbance values of the evaluated compounds (**25I-NBOH** and **25I-NBOMe**) at 24 μM, HSA (4 μM), and the respective complexes. Conditions: pH of 7.4 at 30°C.

Compounds	A_{HSA}	A_{ligand}	$A_{complex}$	$A_{(HSA + ligand)}$	ΔA^*
25I-NBOH	0.142	0.067	0.222	0.209	-0.013
25I-NBOMe	0.142	0.082	0.232	0.224	-0.008

*$\Delta A = A_{(HSA + ligand)} - A_{complex}$

Comments: Based on the variation of the absorbance signals in the experiments using UV-vis spectroscopy (Fig. S4, Table S3), the formation of the complex in the ground state is confirmed, which also characterizes the static quenching process.

Evaluation of preferential binding site in HSA

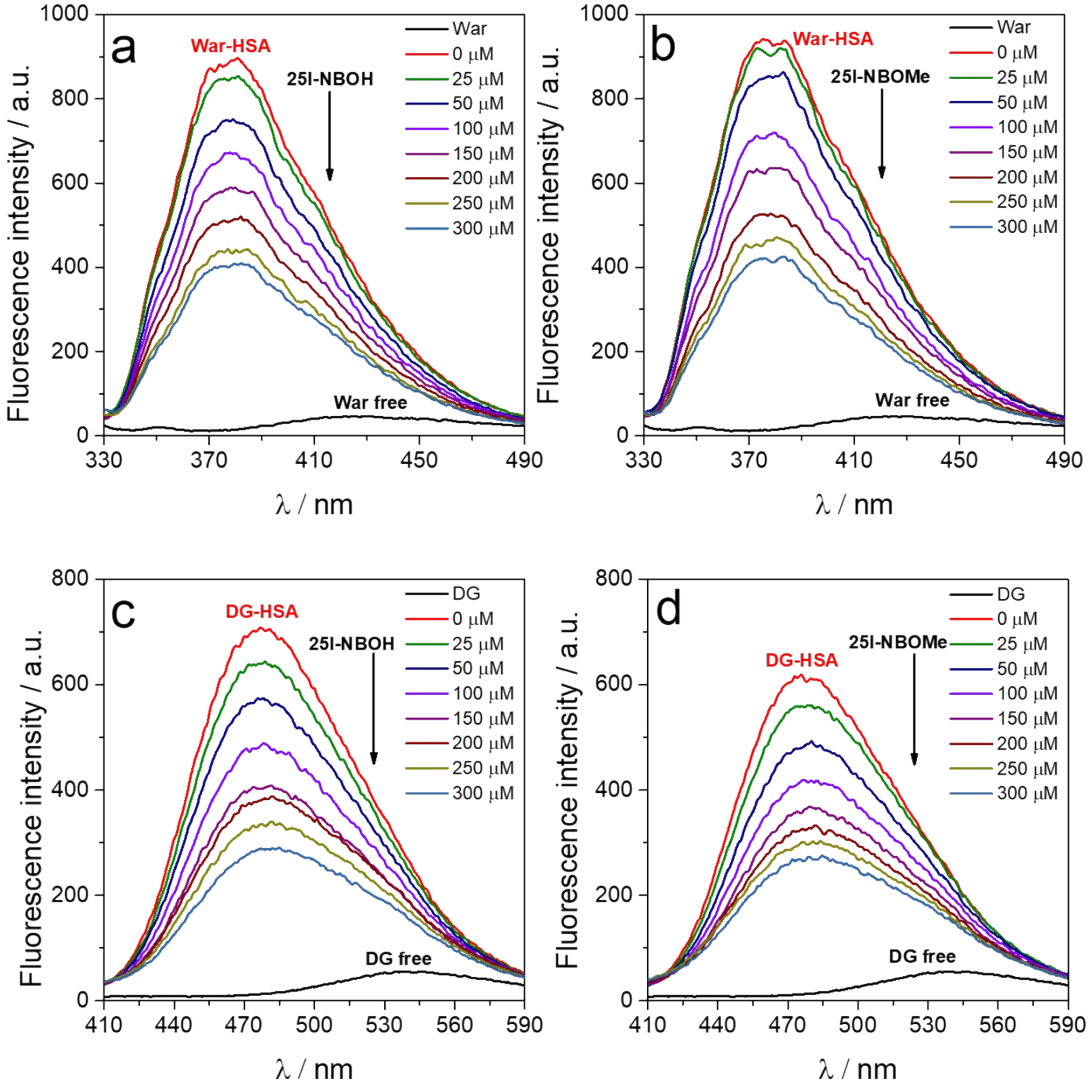

Figure S4. Spectrofluorimetric titration for determining the preferential binding site by War-HSA system titrated with (a) **25I-NBOH** (b) **25I-NBOMe** and DG-HSA system titrated with (c) **25I-NBOH** and (d) **25I-NBOMe**. War = warfarin and DG = dansylglycine. Conditions: pH of 7.4 at 30°C.

Table S4. Determination of the preferential binding site in HSA. Conditions: pH of 7.4 at 30°C.

Site marker	Ligand			
	25I-NBOH		**25I-NBOMe**	
	K_{sv} (10^3 M^{-1})	*r*	K_{sv} (10^3 M^{-1})	*r*
DG	4.52 ± 0.17	0.9963	4.24 ± 0.16	0.9969
War	4.34 ± 0.22	0.9959	4.30 ± 0.13	0.9948

Comments: The warfarin (War, a site I) and dansylglycine (DG, site II) are probes that show reduced fluorescence in the free form; however, when complexed with HSA, there is a significant increase in fluorescence intensity (Fig. S5, Table S4). Besides, these probes are selective for some regions of the HSA, called sites I and II. Therefore, the displacement of both probes (reduction of the fluorescence signal) is substantial, indicating that the ligands (**25I-NBOH** and **25I-NBOMe**) bind to both sites.

Tridimensional fluorescence: structural changes

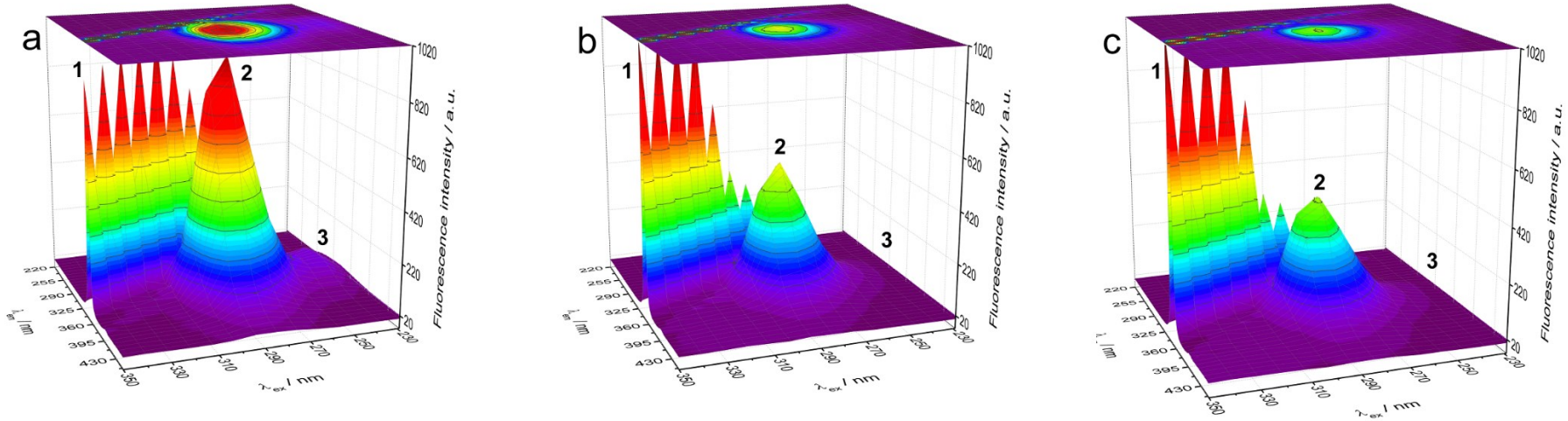

Figure S5. Tridimensional fluorescence spectra of (a) HSA, (b) HSA-**25I-NBOH**, and (c) HSA-**25I-NBOMe** complexes. Protein at 4 μM, **25I-NBOH** 100 μM, and **25I-NBOH** 150 μM. Conditions: pH of 7.4 at 30°C.

Table S5. Tridimensional fluorescence parameters of HSA, HSA-**25I-NBOH**, and HSA-**25I-NBOMe** complexes. Protein at 4 μM, **25I-NBOH** 100 μM, and **25I-NBOH** 150 μM. Conditions: pH of 7.4 at 30°C.

Peak	HSA			HSA-**25I-NBOH**			HSA-**25I-NBOMe**		
	Position (λ_{ex} / λ_{em})	[1]Stokes shift (nm)	[2]I_F	Position (λ_{ex} / λ_{em})	Stokes shift (nm)	I_F	Position (λ_{ex} / λ_{em})	Stokes shift (nm)	I_F
1	$\lambda_{ex} = \lambda_{em}$	0	1015	$\lambda_{ex} = \lambda_{em}$	0	1015	$\lambda_{ex} = \lambda_{em}$	0	1015
2	284 / 334	50	870 (100%)	284 / 331	47	468 (53.8%)	284 / 334	48	404 (46.4%)
3	237 / 330	93	75 (100%)	237 / 332	95	15 (20.0%)	237 / 320	83	14 (18.7%)

[1]Stokes shift is the $\Delta\lambda$ (nm) = $\lambda_{em} - \lambda_{ex}$ / [2]I_F = fluorescence intensity.

Comments: Due to the variations in fluorescence intensity observed in the different systems (peak 2 and 3, mainly) and Stokes shifted (Fig. S6, Table S5), it can be concluded that there was a change in the native structure of the HSA in the presence of the ligands.

Synchronous fluorescence

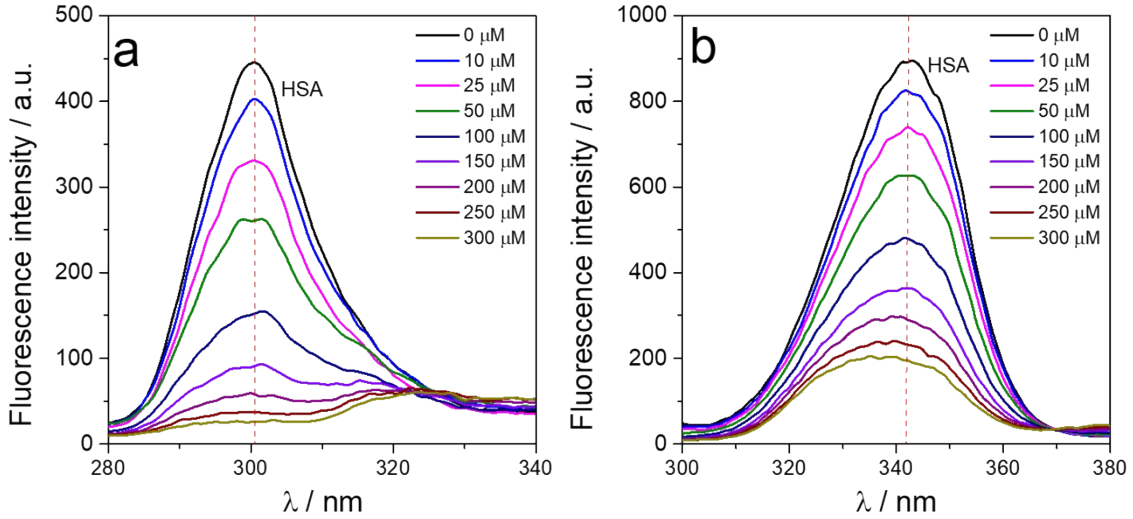

Figure S6. Synchronous fluorescence spectra for HSA without and upon successive additions of **25I-NBOH** at (a) tyrosine residue ($\Delta\lambda$ = 15 nm) and (b) tryptophan residue ($\Delta\lambda$ = 60 nm). Conditions: pH of 7.4 at 30°C.

Comments: Based on the results by synchronous fluorescence, it can be concluded that there was a change in the microenvironment of the Tyr and Trp amino acid residues (Fig. S7). Therefore, their exposure/protection to the solvent due to changes in the HSA structure. Positive variations concerning the shift of the maximum wavelength indicate that the microenvironment of the Tyr residue became more polar due to exposure to water, possibly. In contrast, for the Trp residue (negative variation), there was a protection, due to protein conformational changes. The ligands concentration used to calculate K_{SV} varied from 5 to 200 μM.

Surface HSA hydrophobicity - ANS assay

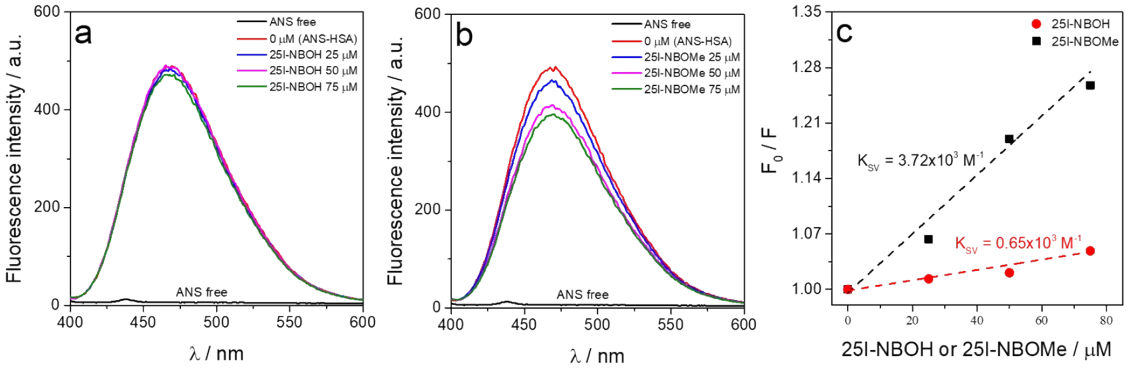

Figure S7. Spectrofluorometric titrations of the complex HSA-ANS with (a) **25I-NBOH** and (b) **25I-NBOMe**, and (c) Stern-Volmer quenching plot. Conditions: pH of 7.4 at 30°C.

Comments: ANS is a probe that, in free form, has reduced fluorescence; however, when binding to hydrophobic sites of HSA, there is an increase in fluorescence intensity (HSA-ANS complex). Compounds capable of displacing ANS from protein binding sites have the same hydrophobic character as the probe. The results indicated that the **25I-NBOH** did not change the fluorescence of the HSA-ANS system (Fig. S8), while the **25I-NBOMe** significantly displaced the ANS and change protein structure; thus, as expected, this ligand has majority hydrophobic character, which justifies its high K_b with this protein.

Förster resonance energy transfer (FRET)

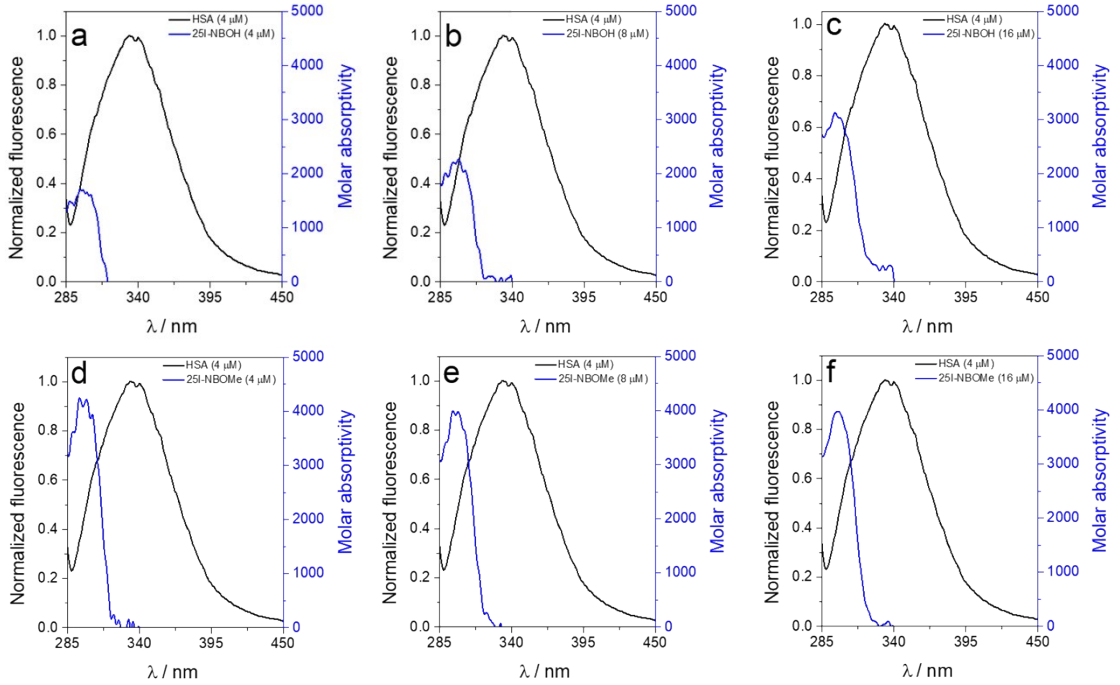

Figure S8. Overlap of the emission spectra of HSA (4 μM) and absorption spectra of (a, b and c) **25I-NBOH** (at 4, 8 and 16 μM, respectively) and (d, e, and f) **25I-NBOMe** (at 4, 8 and 16 μM, respectively). Condition: pH of 7.4 at 30°C.

Table S6. FRET parameters of the interaction between **25I-NBOH** and **25I-NBOMe** with HSA. Condition: pH of 7.4 at 30°C.

Ligand	Concentration (μM)	J (10^{-16} cm^3 M^{-1})	R_0 (nm)	E (%)	r_0 (nm)
25I-NBOH	4	2.27	1.51	5.34	2.24
	8	3.16	1.59	9.14	2.14
	16	4.45	1.65	20.8	2.04
25I-NBOMe	4	6.13	1.76	5.24	2.75
	8	6.73	1.82	9.16	2.58
	16	5.94	1.89	14.4	2.49

Comments: FRET experiments allow calculating the critical distance between HSA donor groups and ligands in different concentrations (Fig. S9, Table S6). It is observed that with increased concentration of ligands, the critical distance (r_0) decreased (Table S8).

NMR ^{1}H: determination of ligand's epitope

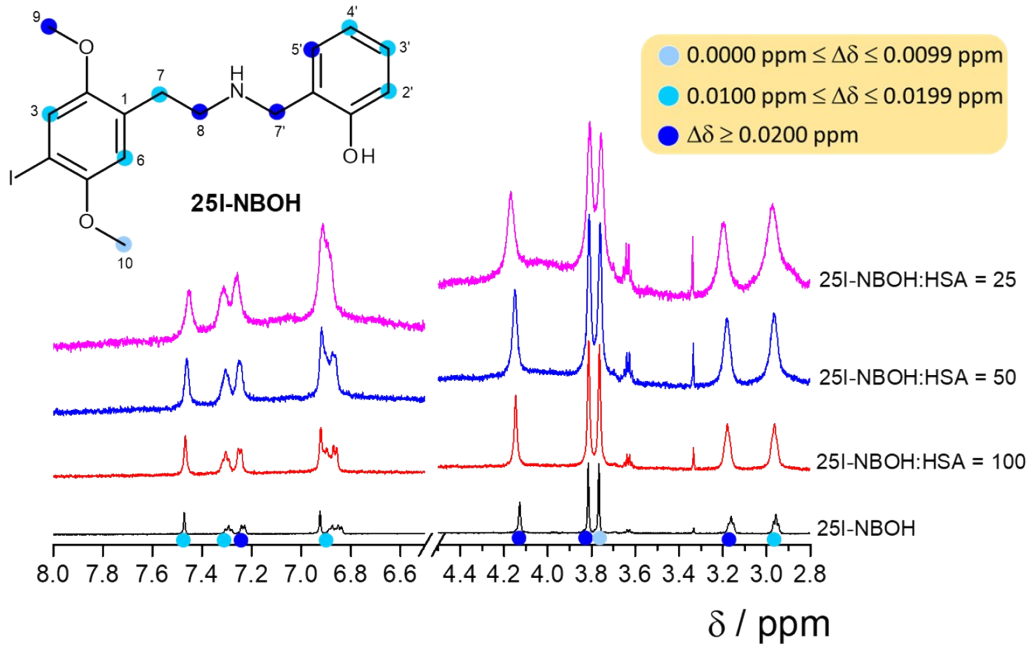

Figure S9. ^{1}H NMR spectrum of **25I-NBOH** (1 mM) in the absence and presence of HAS at different proportions (600 MHz, D_2O, pH 7.40).

Table S7. ^{1}H NMR results for **25I-NBOH** in the absence and presence of HSA (600 MHz, D_2O, pH 7.40).

Hydrogen	δ (ppm)				Δδ[1]
	25I-NBOH	HSA-**25I-NBOH** proportion			
		100	50	25	
3	7.4711	7.4667	4.4617	7.4530	0.0181
6	6.9240	6.9210	6.9170	6.9132	0.0108
7	2.9578	2.9621	2.9660	2.9724	-0.0146
8	3.1622	3.1770	3.1811	3.1959	-0.0337
7'	4.1279	4.1469	4.1503	4.1695	-0.0416
2'	6.8444	6.8640	6.8681	6.8910	-0.0466
3'	7.2936	7.3053	7.3072	7.3117	-0.0181
4'	6.8755	6.8980	6.8681	6.8919	-0.0164
5'	7.2345	7.2481	7.2490	7.2574	-0.0229
9	3.6670	3.7630	3.7610	3.7563	-0.0893
10	3.8148	3.8129	3.8104	3.8086	0.0062

[1]The Δδ was calculated based on the difference between δ(free ligand) and δ(HSA-**25I-NBOH** = 25).

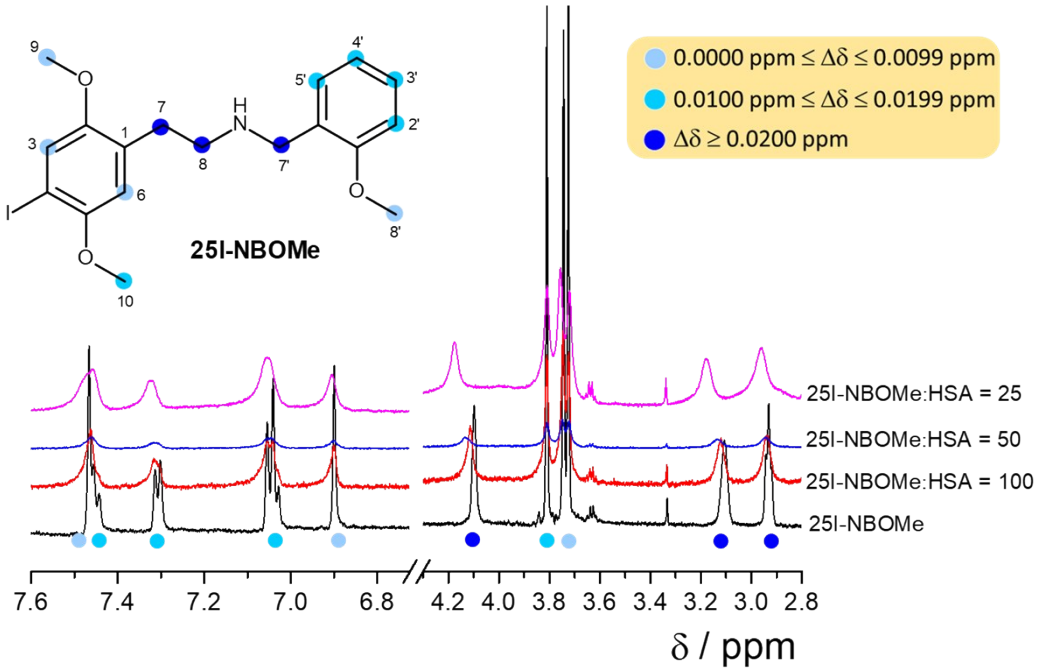

Figure S10. ^{1}H NMR spectrum of **25I-NBOMe** (1 mM) in the absence and presence of HSA at different proportions (600 MHz, D_2O, pH 7.40).

Table S8. ^{1}H NMR results for **25I-NBOMe** in the absence and presence of HSA (600 MHz, D_2O, pH 7.40).

Hydrogen	δ (ppm)				Δδ[1]
	25I-NBOMe	HSA-**25I-NBOMe** proportion			
		100	50	25	
3	7.4659	7.4623	7.4607	7.4571	0.0088
6	6.8994	6.9001	6.9009	6.9056	-0.0062
7	2.9315	2.9389	2.9446	2.9622	-0.0307
8	3.1069	3.1210	3.1352	3.1789	-0.0720
7'	4.0976	4.1143	4.1340	4.1753	-0.0777
2'	7.0407	7.0487	7.0507	7.0545	-0.0138
3'	7.4565	7.4630	7.4607	7.4715	-0.0150
4'	7.0407	7.0487	7.0507	7.0545	-0.0138
5'	7.3077	7.3115	7.3136	7.3236	-0.0159
8'	3.7248	3.7235	3.7214	3.7202	0.0046
9	3.7440	3.7471	3.7490	3.7563	-0.0123
10	3.8100	3.8099	3.8091	3.8096	0.0000

[1]The Δδ was calculated based on the difference between δ(free ligand) and δ(HSA-**25I-NBOMe** = 25).

Comments: The most significant chemical shift variations of the system in the presence and absence of HSA are indicative of the region that corresponds to the epitome of the molecule, that is, those with preferential interactions, which mainly correspond to the alkyl chain between the aromatic rings (Fig. S10 and S11). Considering the data in the Tables S9 and S10, it is clear that other hydrogens participate in the interaction process; however, those with more influence (more intense blue) were highlighted.

Theoretical studies: molecular docking

Table S9. Molecular interactions observed for HSA-**25I-NBOH** and HSA-**25I-NBOMe** complexes obtained by molecular docking (ChemPLP function) in the Sudlow's sites I and II.

Ligand / Site	Amino acid residue	Type of interaction	Distance (Å)
25I-NBOH (site I)	Lys-199	Hydrogen bonding	2.90
	Phe-211	Van der Waals	3.40
	Trp-214	Van der Waals	2.70
	Ala-216	Van der Waals	2.70
	Arg-218	Hydrogen bonding	2.10
	Leu-238	Van der Waals	3.50
	Val-241	Van der Waals	3.20
	His-242	Hydrogen bonding	1.80
	Arg-257	Van der Waals	2.80
	Lys-199	Hydrogen bonding	2.30
	Phe-211	Van der Waals	1.50
	Trp-214	Van der Waals	3.40
25I-NBOMe (site I)	Arg-218	Hydrogen bonding	3.50
	Leu-238	Van der Waals	2.50
	Val-241	Van der Waals	3.10
	His-242	Van der Waals	1.30
	Arg-257	Van der Waals	3.70
	Leu-387	Van der Waals	3.20
	Arg-410	Van der Waals	3.30
	Tyr-411	Van der Waals	3.60
25I-NBOH (site II)	Lys-414	Hydrogen bonding	1.60
	Leu-430	Van der Waals	3.40
	Ala-449	Van der Waals	2.50
	Leu-453	Van der Waals	3.60
	Ser-489	Hydrogen bonding	3.50
	Leu-387	Van der Waals	3.60
	Asn-391	Hydrogen bonding	3.80
	Arg-410	Van der Waals	3.70
25I-NBOMe (site II)	Tyr-411	Van der Waals	3.60
	Lys-414	Van der Waals	1.90
	Leu-430	Van der Waals	2.10
	Leu-453	Van der Waals	3.30
	Leu-457	Van der Waals	3.70

Comments: Molecular docking calculations suggested van der Waals and hydrogen bonding as the main intermolecular forces involved in the interaction between each NPS under study and the amino acid residues inside the subdomains IIA and IIIA (Sudlow's sites I and II, respectively), being in good agreement with the experimental data.