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Research Communication

Binding of Anti-Parkinson's Disease Drugs to Human Serum Albumin is Allosterically Modulated

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Summary

Binding of drugs to plasma proteins is an important determinant for their efficacy because it modulates drug availability to the intended target. Co-administered drugs may bind to the same protein site or to different functionally linked clefts following competitive and allosteric mechanisms. Here, we report a thermodynamic and computational characterization of the binding mode of apomorphine and benserazide, two therapeutic agents co-administered in the treatment of Parkinson's disease, to human serum albumin (HSA). Apomorphine binds to HSA with a simple equilibrium ($K_d = 3.1 \times 10^{-6}$ M). Conversely, benserazide binds to HSA with two independent equilibria ($K_{d1} \leq 10^{-6}$ M and $K_{d2} = 5.0 \times 10^{-5}$ M). Values of K_d and K_{d2} increase to 1.5×10^{-5} M and 5.0×10^{-4} M, respectively, in the presence of heme. Accordingly, the K_d value for heme binding to HSA increases from 5.0×10^{-7} M to 4.8×10^{-6} M and 9.2×10^{-7} M, in the presence of saturating amounts of apomorphine and benserazide, respectively. The K_{d1} value for benserazide binding to HSA is not affected by heme binding, whereas apomorphine and benserazide inhibit warfarin binding to HSA, and vice versa. Therefore, apomorphine and the second benserazide molecule bind to the warfarin site, allosterically linked to the heme site. Simulated docking of apomorphine and benserazide into the warfarin site provides favorable values of intermolecular energy (-23.0 kJ mol^{-1} and -15.2 kJ mol^{-1}, respectively). Considering the apomorphine, benserazide, and HSA-heme plasma levels and the possible co-administration of warfarin, these results appear relevant in the management of patients affected by Parkinson's disease. © 2010 IUBMB IUBMB *Life*, 62(5): 371–376, 2010

Keywords human serum albumin; anti-Parkinson drugs; heterotropic interactions; molecular docking simulations; thermodynamics.

Abbreviations HSA, human serum albumin; PD, Parkinson's disease; L-DOPA, L-dihydroxyphenylalanine; MAO-B, monoamine oxidase type B; COMT, catechol-*O*-methyltransferase.

INTRODUCTION

Parkinson's disease (PD), the most common age-related movement disorder, is characterized by severe motor symptoms, including uncontrollable tremor, slowness of movements, and rigidity, that originate from progressive depletion of dopamine-producing nigrostriatal neurons (*1, 2*). In the absence of suitable diagnostic tools that would permit a neuroprotective cure, the only therapeutic approach is to alleviate PD symptoms by administering the dopamine precursor L-dihydroxyphenylalanine (L-DOPA) associated to a peripheral DOPA-decarboxylase inhibitor (*e.g.*, benserazide) (*2–4*). However, the short half-life of L-DOPA, showing negligible binding to plasma proteins, is responsible for plasmatic level fluctuations and in turn for a pulsatile receptor stimulation. This sort of nonphysiologic stimulation induces a change in post-synaptic neuron activation/inhibition, with the consequent upsurge of motor fluctuations that may become disabling more than PD itself (*5*). To manage with these symptoms monoamine oxidase type B (MAO-B) inhibitors, catechol-*O*-methyltransferase (COMT) inhibitors, and dopamine receptor agonists (*e.g.*, apomorphine) are commonly used in the anti-PD therapy (*3, 6*).

Binding of drugs to plasma proteins is an important determinant for their efficacy because it modulates drug availability to the intended target. Drugs may bind to the same protein site or

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to different functionally linked clefts following competitive and allosteric mechanisms. Human serum albumin (HSA) has an extraordinary binding capacity for endogenous and exogenous ligands (*e.g.*, heme, aminoacids, bilirubin, metal ions, fatty acids, and drugs), making it an important determinant for pharmacokinetics (*7–17*).

Drug binding to HSA takes place mainly in two regions: Sudlow's site I, in the subdomain IIA (*i.e.*, the warfarin site), and Sudlow's site II, in the subdomain IIIA (*i.e.*, the ibuprofen site) (*9, 12, 13, 15, 18, 19*). Heme binds to an oblate cavity located in subdomain IB that has been proposed to be evolved to specifically bind the prosthetic group, supporting a role for HSA in heme homeostasis (*20–24*).

HSA is subject to reversible conformational transitions induced by allosteric effectors and pH changes. In particular, warfarin inhibits allosterically heme binding to HSA, and in turn heme impairs warfarin binding to HSA (*23–27*). Similarly, other Sudlow's site I ligands inhibit allosterically heme binding to the same extent as observed for warfarin, and vice versa (*15, 26, 28*).

Here, thermodynamics of anti-PD drugs apomorphine and benserazide binding to HSA, in the absence and in the presence of the stereotypical HSA ligands heme and warfarin, is reported. Furthermore, the binding mode of apomorphine and benserazide to HSA has been investigated by automated docking simulation.

MATERIALS AND METHODS

All reagents (from Sigma-Aldrich, St. Louis, MO) were of the highest purity available and were used without further purification. HSA was essentially fatty acid-free according to the charcoal delipidation protocol (*29–31*). The ferric heme stock solution was prepared by dissolving the heme in 1.0×10^{-1} M NaOH; the actual heme concentration was determined as the bis-imidazolate complex in sodium dodecylsulfate micelles with the extinction coefficient of 14.5 mM^{-1} cm^{-1} at 535 nm (*32*). Apomorphine and benserazide stock solutions (2.0×10^{-1} M) were prepared by dissolving the drugs in 1.0×10^{-1} M phosphate buffer, pH 7.2. Ibuprofen stock solution (5.0×10^{-2} M) was prepared by dissolving the drug in 1.0×10^{-1} M phosphate buffer, pH 7.2. Warfarin stock solution (3.0×10^{-2} M) was prepared by dissolving the drug in the pure water at pH 10.0, then pH was adjusted to 7.2 with HCl.

Binding of apomorphine and benserazide to HSA was followed spectrofluorimetrically using an optical cell with 1.0-cm path length on a Jasco FP-6200 spectrofluorimeter (Jasco, Easton, MD) (*33, 34*). The intrinsic tryptophan-dependent fluorescence of HSA was recorded between 290 nm and 450 nm; the excitation wavelength was 280 nm. Values of the dissociation equilibrium constant(s) for apomorphine and benserazide binding to HSA were determined by adding increasing amounts of apomorphine (final concentration, 1.0×10^{-5} M to 1.0×10^{-3} M) and benserazide (final concentration, 1.0×10^{-4} M to 3.0×10^{-3} M) solutions to a fixed HSA solution (final concentration, 5.0×10^{-6} M). The intrinsic fluorescence of anti-Parkinson's drugs was subtracted to the drug-induced quenching of HSA intrinsic fluorescence to determine values of the dissociation equilibrium constants for drug binding to HSA.

Binding of heme and drugs to HSA, in the absence and in the presence of a third component, was investigated spectrophotometrically using an optical cell with 1.0-cm path length on a Cary 50 Bio spectrophotometer (Varian, Palo Alto, CA). Values of the dissociation equilibrium constant(s) for apomorphine, benserazide, and heme binding to HSA, in the absence of a third component, were determined by adding increasing protein amounts to a fixed ligand concentration in 1.0×10^{-1} M phosphate buffer, 10% DMSO, pH 7.2. Values of the dissociation equilibrium constant(s) for apomorphine, benserazide, warfarin, and heme binding to HSA, in the presence of a third component, were determined by adding increasing ligand amounts to a fixed protein concentration (1.0×10^{-5} M), in the presence of either 1.0×10^{-5} M apomorphine or 1.0×10^{-4} M benserazide or 1.0×10^{-4} M warfarin or 1.0×10^{-5} M heme, in 1.0×10^{-1} M phosphate buffer, 10% DMSO, pH 7.2.

Ligand binding isotherms were analyzed by plotting the fluorescence or absorbance changes as a function of the ligand or the HSA concentration. Data have been analyzed according to Eq. (1):

$$\Delta A = \frac{\Delta A_{max} \times \left[(K_d^{-1} \times [L]_t + N \times [P]_t \times K_d^{-1} + 1) - \sqrt{(K_d^{-1} \times [L]_t + N \times [P]_t \times K_d^{-1} + 1)^2 - 4K_d^{-2} \times [L]_t \times N \times [P]_t} \right]}{2K_d^{-1} \times [L]_t} \quad (1)$$

where ΔA is the difference at the maximum fluorescence or absorbance value, ΔA_{max} is the fluorescence or absorbance difference at saturating ligand concentration, K_d is the dissociation equilibrium constant for the ligand–protein complex formation, $[L]_t$ is the total ligand concentration (*e.g.*, apomorphine, benserazide, warfarin, heme), $[P]_t$ is the total concentration of the protein (*e.g.*, either HSA or HSA-apomorphine or HSA-benserazide or HSA-warfarin or HSA-heme), and N is the number of equivalent binding sites.

In the case of the spectrophotometric analysis of benserazide binding to HSA, where a simple equilibrium is not sufficient to fit the data, they have been analyzed according to Eq. (2):

$$\Delta A = \frac{\Delta_1([L]_t/K_{d1} + [P]_t/K_{d1} + 1) - \sqrt{([L]_t/K_{d1} + [P]_t/K_{d1} + 1)^2 - 4K_{d1}{}^{-2}[L]_t[P]_t}}{2[L]_t/K_{d1}}$$

$$+ \frac{\Delta_2([L]_t/K_{d2} + [P]_t/K_{d2} + 1) - \sqrt{([L]_t/K_{d2} + [P]_t/K_{d2} + 1)^2 - 4K_{d2}{}^{-2}[L]_t[P]_t}}{2[L]_t/K_{d2}} \quad (2)$$

where K_{d1} is the dissociation equilibrium constant for benserazide binding to the high affinity site of HSA, K_{d2} is the dissociation equilibrium constant for benserazide binding to the low affinity site of HSA, $[L]_t$ is the total concentration of benserazide, ΔA_1 and ΔA_2 are the difference at maximum of the absorbance value for the high and low affinity binding site, respectively.

Automatic flexible ligand docking simulation to HSA was performed by using Autodock 4.0 and the graphical user interface AutoDockTools (*35, 36*). The structures of HSA-heme and HSA-warfarin were downloaded from the Protein Data Bank (PDB codes 1O9X and 2BXD) (*11, 13*), corresponding to heme-bound (FA1), myristate-bound (FA2-FA7) and warfarin-bound (FA7), myristate-free HSA, respectively. Ligands in the site being investigated were removed before docking. The HSA-ligand complexes were drawn using UCSF Chimera (*37*). Apomorphine and benserazide structures were calculated using the Dundee PRODRG server (*38*). Single bonds were allowed to rotate freely during the Monte Carlo simulated annealing procedure. The analysis of the conformational space was restricted to a cubic box of 60 Å edge centered on the coordinates of heme or warfarin. Monte Carlo simulated annealing was performed by starting from a temperature of 900 K with a relative cooling factor of 0.95 K/cycle, in order to reach the temperature of 5 K in 100 cycles (*35, 36*).

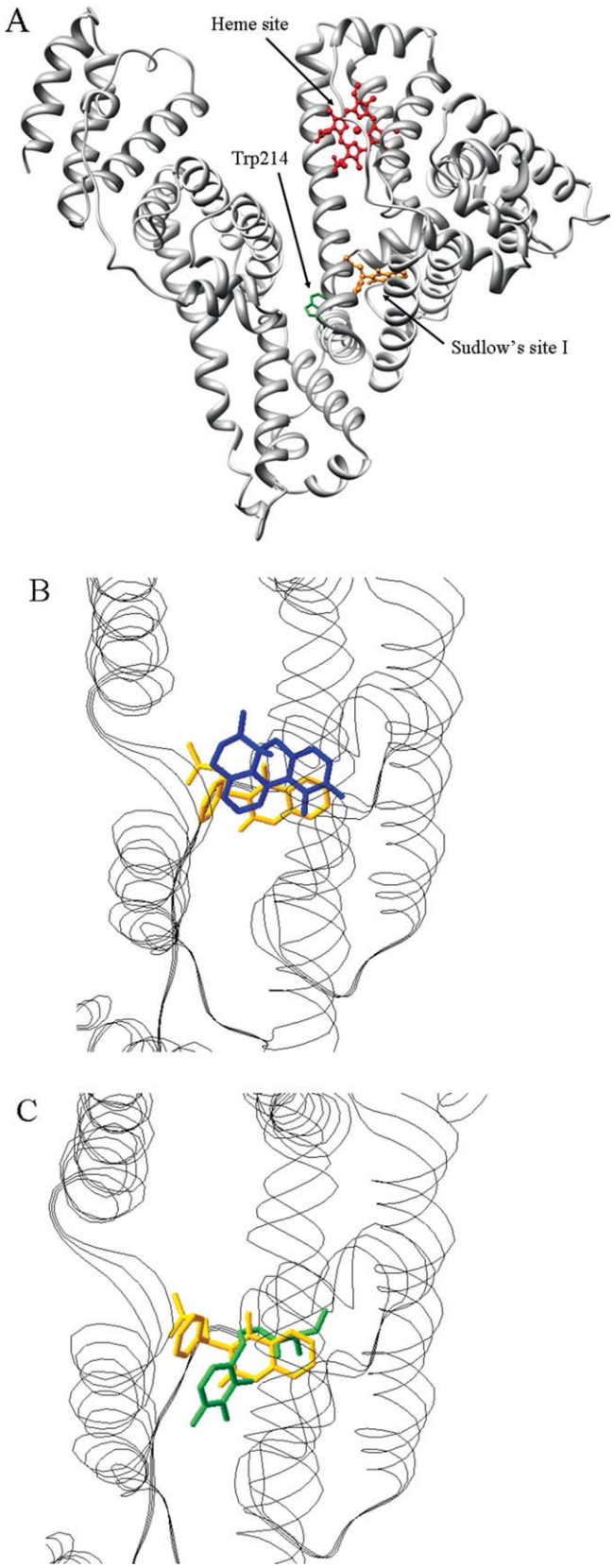
RESULTS

Apomorphine and benserazide binding to HSA was investigated by analyzing the perturbation of the intrinsic HSA fluorescence, mainly due to the unique Trp residue present at position 214 (Fig. 1) close to Sudlow's site I. Data analysis according to Eq. (1) allowed the determination of K_d values for apomorphine and benserazide binding to HSA following a simple equilibrium (3.0×10^{-6} M and 5.0×10^{-5} M, respectively; see Table 1, see Supporting Information Figure S1 (panel A and S1 panel B). In parallel, apomorphine and benserazide binding to HSA was investigated spectrophotometrically. Data analysis according to Eq. 1 showed that apomorphine binds to HSA with a simple equilibrium ($K_d = 3.2 \times 10^{-6}$ M; see Table 1, see Supporting Information Figure S2 panel A and S2 panel B). Conversely, the analysis of benserazide binding to HSA according to Eq. (2) revealed the occurrence of two independent binding equilibria with $K_{d1} \leq 10^{-6}$ M and $K_{d2} = 5.0 \times 10^{-5}$ M (see Table 1). Note that values of K_d and K_{d2} for apomorphine and benserazide binding to HSA, obtained by different spectroscopic techniques, are in excellent agreement (see Table 1).

As reported for warfarin [(*40, 41*); see Table 1], apomorphine and benserazide binding to HSA is inhibited by the heme. Indeed, the K_d value for apomorphine binding to HSA increases from 3.1×10^{-6} M (average value obtained from different techniques), in the absence of the heme, to 1.5×10^{-5} M, in the presence of saturating heme concentration (1.0×10^{-5} M) (see Table 1, see Supporting Information Figure S3 panel A and S3 panel B). Similarly, the K_{d2} value for benserazide binding to HSA increases from 5.0×10^{-5} M, in the absence of the heme, to 5.0×10^{-4} M, in the presence of saturating heme concentration (1.0×10^{-5} M) (see Table 1, see Supporting Information Figure S4). According to linked functions (*42, 43*), the K_d value for heme binding to HSA increases from 5.0×10^{-7} M, in the absence of third components, (*39*) to 4.8×10^{-6} M and 9.2×10^{-7} M, in the presence of 1.0×10^{-5} M apomorphine and 1.0×10^{-4} M benserazide, respectively (see Table 1). Note that the high affinity benserazide binding site ($K_{d1} \leq 10^{-6}$ M) is not functionally coupled to the heme cleft (see Table 1). On the other hand, benserazide binding to the high affinity site is prevented by ibuprofen ($=1.0 \times 10^{-4}$ M), without affecting the K_{d2} value (data not shown).

As shown in Table 1, warfarin inhibits apomorphine binding to HSA, the K_d value for apomorphine binding to HSA increasing from 3.1×10^{-6} M, in the absence of warfarin (average value obtained from different techniques, see Supporting Information Figure 5), to 1.2×10^{-4} M, in the presence of 1.0×10^{-4} M warfarin. According to linked functions (*42, 43*), apomorphine inhibits warfarin binding to HSA, the K_d value for warfarin binding to HSA increasing from 1.4×10^{-6} M, in the absence of apomorphine (*40*), to $\geq 10^{-4}$ M, in the presence of 1.0×10^{-5} M apomorphine (see Table 1). Similarly, benserazide inhibits warfarin binding to HSA, the K_d value for warfarin binding to HSA increasing from 1.4×10^{-6} M, in the absence of benserazide (*40*), to $\geq 10^{-4}$ M in the presence of 1.0×10^{-4} M benserazide (see Table 1).

An automated docking analysis of apomorphine and benserazide into Sudlow's site I has been performed. As shown in Figure 1, both anti-PD drugs are able to enter the warfarin site with favorable values of intermolecular energy, corresponding to -23.0 kJ mol^{-1} and -15.2 kJ mol^{-1} for apomorphine and benserazide interaction, respectively. It is worth to note that these values account for K_d values reported in Table 1. Indeed, the difference of intermolecular energy values for apomorphine



and benserazide docking (*i.e.*, $\Delta\Delta G = 7.8$ kJ mol^{-1}) is in good agreement with that calculated from experimentally determined K_d values for drug binding to HSA (*i.e.*, $\Delta\Delta G = 7.0$ kJ mol^{-1}).

DISCUSSION

Apomorphine and benserazide display "classical" HSA-drug interactions in Sudlow's site I. Indeed, apomorphine and benserazide binding can be followed by measuring the quenching of the emission fluorescence of Trp214. Accordingly, apomorphine and benserazide binding to HSA is prevented by warfarin, which binds to Sudlow's site I (*13*). Furthermore, apomorphine and benserazide binding to HSA are functionally linked to heme association. Values of K_d reported in Table 1 are consistent with the fact that all drugs that bind to Sudlow's site I (including apomorphine and benserazide) are able to lower by about one-order of magnitude the affinity of the heme. Consistently, heme binding lowers by about one-order of magnitude the affinity of all drugs that bind to Sudlow's site I (including apomorphine and benserazide) (*26, 28, 39*). Therefore, the analysis of binding equilibria and docking simulations demonstrates consistently that apomorphine and benserazide bind to Sudlow's site I with dissociation equilibrium constants in the micromolar range (see Table 1).

The therapeutic efficiency of drugs in combination therapy is strictly dependent upon the mutual interaction(s) of binding equilibria with plasma proteins with particular reference to HSA. Indeed, the affinity of drugs for HSA is one of the most important factors affecting their free active concentration (*15, 28, 33, 39, 44*). Despite the relevance of peripheral DOPA-decarboxylase inhibitors and dopamine agonists administered in the management and treatment of PD, little is known about their pharmaco-kinetic and -thermodynamic properties (*45*). Apomorphine was investigated as a prototypical dopamine agonist in terms of binding to serum and tissue proteins by radiolabeled ligand ultrafiltration and dialysis techniques, showing that apomorphine is bound greater than 90% to serum proteins at pharmacological concentration (*46*). On the other hand, a quantitative determination of benserazide binding to HSA was never reported before. This issue is of particular relevance when high doses are administered. Indeed, one single dose contains 100

Figure 1. Panel A: Ribbon representation of the heart-shaped structure of HSA with heme (red) in its primary binding site and warfarin (orange) in Sudlow's site I. The Trp214 residue is rendered with green sticks. Heme and warfarin are rendered as ball and stick. Panel B: Superimposition of warfarin (orange, from X-ray diffraction data) and apomorphine (blue, from ligand docking) in Sudlow's site I. Panel C: Superimposition of warfarin (orange, from X-ray diffraction data) and benserazide (dark green, from ligand docking) in Sudlow's site I. Atomic coordinates were taken from PDB entries 1O9X [HSA-heme; (*11*)] and 2BXD [HSA-warfarin; (*13*)].

Table 1
Values of K_d (M) for ligand binding to HSA in the absence and in the presence of third components, at pH 7.2 and 25°C[a]

	HSA	HSA-warfarin	HSA-heme	HSA-apomorphine	HSA-benserazide
Apomorphine	$\mathbf{3.0 \times 10^{-6}}$	n.d.	n.d.	n.a.	n.d.
	3.2×10^{-6}	1.2×10^{-4}	1.5×10^{-5}	n.a.	n.d.
Benserazide	$\mathbf{5.0 \times 10^{-5}}$	n.d.	n.d.	n.d.	n.a.
	$\leq 10^{-6}$; $\boldsymbol{5.0 \times 10^{-5}}$	n.d.	$\leq 10^{-6}$; $\boldsymbol{5.0 \times 10^{-4}}$	n.d.	n.a.
Heme	5.0×10^{-7}[b]	1.2×10^{-6}	n.a.	4.8×10^{-6}	9.2×10^{-7}
Warfarin	1.4×10^{-6}[c]	n.a.	2.0×10^{-5}[d]	$\geq 10^{-4}$	$\geq 10^{-4}$

n.a., not applicable; n.d., not detectable.
[a]Values of K_d were obtained spectrofluorimetrically (bold) and spectrophotometrically (normal). Values in italic refer to K_{d1} and in bold italic to K_{d2}.
[b]From (*39*).
[c]From (*40*).
[d]From (*41*).

mg L-DOPA associated to 25 mg benserazide (*47*). In the case of a 1 g/day L-DOPA administration, benserazide reaches plasma levels in the micromolar range, competing with Sudlow's site I ligands (*e.g.*, apomorphine and warfarin) and allosterically inhibiting heme binding (*26, 28, 39*). Thus, the anti-PD drug concentration required to achieve the therapeutic plasma level in the presence of plasma proteins appears to be at least one-order of magnitude higher than that required in their absence. Lastly, apomorphine and benserazide binding to HSA is impaired by heme association and vice versa. The increase of the heme level in plasma under pathological conditions (*e.g.*, severe hemolytic anemia, crash syndrome, and postischemic reperfusion) may facilitate drug release with the concomitant intoxication of the patient. Accordingly, the toxic plasma heme concentration may increase in patients under anti-PD drug therapy.

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