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Short communication

A protein-coated magnetic beads as a tool for the rapid drug-protein binding study

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ABSTRACT

A simple and fast method for the determination of the association constant (K_a) of ligand to human serum albumin (HSA) has been developed by using human serum albumin-coated magnetic beads (HSA-MB). To date, magnetic beads (MB) have been increasingly used as a bioseparation tool, especially for DNA, RNA, protein, enzyme and cell isolation or purification. In this study, HSA-MB were used as a new tool to determine the affinity of known ligands to HSA. The K_a for L-tryptofan, fenoprofen, ketoprofen, tolbutamide and warfarin obtained from Schathard analysis are consistent with previously reported values. The different K_a values for ketoprofen after the acetylation of HSA-MB by preincubation with acetylosalicylic acid indicate that these beads can be successfully adapted in combined experiment. In addition, the HSA-MB experiment with phenytoin and valproic acid proved to be a simple method to examine drug displacement effect.

1. Introduction

Serum albumin, as a principal component of blood, has been one of the most extensively studied and applied protein in biochemistry [1]. One of the major properties of albumin is its ability to bind reversibly an incredible variety of ligands/drugs. Therefore, an extensive understanding of the albumin protein binding behavior of a particular therapeutic agents is important for its rational use. There are a number of established methods that are used in evaluating the binding of ligands/drugs to proteins, e.g. equilibrium dialysis, ultrafiltration and ultracentrifugation which are limited due to the long periods of time to establish an equilibrium during the dialysis process and analyte adsorption onto the dialysis and ultrafiltration membrane [2]. Recently, affinity chromatography became most popular technique that offers good precision and reproducibility [3–5]. This technique, based on the analysis of single ligand binding to chemically bounded protein supports, does not involve separation steps but is basically limited to the characterization of an immobilized protein with a set of compounds. The other approaches involve the use of optical biosensor technique and spectroscopic techniques, such a UV, fluorescence and circular dichroism (CD) spectroscopy that usually allows selective determination of the binding parameters related to the highest affinity binding site, being the only stereoselective one in most cases [6–9]. Also, BIAcore system and surface plasmon resonance (SPR) are the most recently popular technologies for real-time biomolecule interaction analysis. These are powerful techniques for measuring binding between all types of molecular interactions [10].

Recently, increasing attention has been paid to apply magnetic beads (MB) in different fields of biochemical and biotechnology science. They are also called superparamagnetic iron oxide micro- or nanoparticles, meaning small size magnetic "beads" that makes them particularly suitable for protein and cell isolation [11–14], selection of affinity binders [15], immuno- and electrochemical assay [16–18]. Application and multifunctional properties of MB are determined by the modification of their surface [19]. Different types of organic and inorganic chemicals including natural macromolecules and synthetic polymers have been applied to prepare and modify magnetic support [20,21]. However, insufficient capacity and slow mass transfer kinetics are the limiting factors that restrict the applications of some supports to laboratory scale [22].

To date, no one has reported the use of protein-coated magnetic beads to determine binding kinetics data. In this study, the newly developed HSA-MB were adapted to measure the fraction of free drug (fraction unbound) and evaluate the K_a for the binding of ligand (drug) to HSA. In addition, we performed the study with acetylation of HSA-MB by preincubation with acetylosalicylic acid that indicates different affinities of ketoprofen to unmodified and acetylated HSA. We also demonstrated the simplicity and ready application of the method using HSA-MB in competitive binding experiments including phenytoin and valproic acid.

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2. Materials and methods

2.1. Chemicals

Acetylsalicylic acid, chlorpromazine, fenoprofen, fluphenazine, glutaraldehyde, glycine, imipramine, indomethacin, ketoprofen, lidocaine, L-tryptofan, phenylbutazon, phenytoin, pyridine, quinidine, quinine, sodium azide, sulindac, tolbutamide, trifluopromazine, valproic acid and warfarin were purchased from Sigma–Aldrich (Stainhaim, Germany).

The water used in the study was prepared using a Milli-Q Water Purification System. (Millipore Corporation, Bedford, MA, USA). BcMag, amine-terminated magnetic beads (50 mg/mL, 1 μm) were purchased from Bioclone Inc. (San Diego, CA, USA). All supernatants were separated from MB using a magnetic separator Dynal MPC-S (Invitrogen Corporation, Carlsbad, CA, USA).

2.2. Methods

2.2.1. Preparation of human serum albumin magnetic beads (HSA-MB)

The immobilization of HSA onto the amine-terminated magnetic beads (MB) was performed using the previously described method [15]. Briefly, 25 mg of MB were washed three times (for 1 min) with 1 mL coupling buffer (10 mM pyridine, pH 5.5) in 2 mL microcentrifuge tube. After the wash procedure, MB were suspended in 1 mL of 5% gluteraldehyde and shaken for 3 h. Next, the supernatant was removed and MB were washed three times (for 1 min) in 1 mL of coupling buffer. A solution of 10 mg HSA in 1 mL of coupling buffer was added to activated MB and vigorously shaken. Next, the microcentrifuge tube was shaken at room temperature for seven days with a gentle rotation. After coupling procedure the supernatant was removed and 1 mL of the reaction stop buffer (1 M glycine, pH 8.0) was added and gently rotated for 30 min. The supernatant was removed and the MB were washed three times and stored in 1 mL 10 mM ammonium acetate buffer, pH 7.4 with 0.1% sodium azide. The Micro BCA™ protein assay kit (Pierce, IL, USA) was used to measure the amount of HSA immobilized on the magnetic beads. The control magnetic beads (control-MB) were made in the same manner but without the addition of HSA.

2.2.2. Ligand binding studies to HSA

The binding of representative solutes was studied by HSA-MB. The following ligands were subjected to this study: chlorpromazine, fenoprofen, fluphenazine, imipramine, indomethacin, ketoprofen, lidocaine, L-tryptofan, phenylbutazon, phenytoin, quinidine, quinine, sulindac, tolbutamide, trifluopromazine, valproic acid and warfarin. An amount of 2.5 mg of HSA-MB was placed into microcentrifuge tube. To the beads, 300 μL solution of 100 nM ligand in 10 mM ammonium acetate buffer (pH 7.4) was added and vortexed for 10 min. Next, the microcentrifuge tube was placed into the magnetic separator for 1 min. The supernatant was removed and saved. The next step was the washing procedure of HSA-MB consisting of the three times wash with 500 μL of 15% MeOH in 10 mM ammonium acetate buffer (pH 7.4) and last single wash in 10 mM ammonium acetate buffer (pH 7.4) for 2 min.

The fraction unbound (*fu*) of single ligand in first supernatant was measured by ESI-MS-HPLC and can be expressed as:

$$fu = \frac{[D]}{[D]_{TOT}} \quad (1)$$

where [D] is free drug concentration and $[D]_{TOT}$ is total drug concentration. The association binding constant, K_a, was determined by Scatchard equation:

$$\frac{r}{[D_B]} = nK_a - rK_a \quad (2)$$

where r is the ratio of the bound drug to the protein in molar concentration, $[D_B]$ is bound drug concentration, n is number of binding sites in one protein molecule. The graph is based on 7–9 experiments where different concentrations of a known ligand were incubated with HSA-MB. The ratio $r/[D_B]$ vs. r gives straight line with slope $-K_a$.

The data from the saturation experiments were determined by Rosenthal equation:

$$\frac{[D_B]}{[D]} = \frac{B_{max}}{K_d} - \frac{[D_B]}{K_d} \quad (3)$$

where B_{max} is the number of the active binding site of the immobilized protein and K_d denotes the ligand equilibrium dissociation constant. Single site binding data were analyzed by linear regression and gave straight line where $-1/K_d$ is the slope of the line and X-intercept is B_{max}. The achieved K_a values from Rosenthal plots (expressed as $K_a = 1/K_d$) were compared with K_a values from Scatchard analysis.

Using the Micro BCA assay kit, 172 ± 112 μg of HSA per mL of MB was determined (3.44 μg of HSA per mg of MB). Binding kinetics data of L-tryptofan, tolbutamide and warfarin to HSA were studied at 2.5 mg HSA-MB with 250 μL solution of each ligand in 10 mM ammonium acetate buffer (pH 7.4). The final total concentration of HSA immobilized onto the MB was 517.46 nM. At this protein concentration, the serial concentrations of L-tryptofan (100, 125, 150, 250, 500, 750, 1000, 1250 and 1500 nM), tolbutamide (100, 125, 250, 500, 750 1000, 1250 and 1500 nM), warfarin (100, 125, 250, 500, 750 1000, 1250 and 1500 nM), fenoprofen (100, 125, 250, 500, 1000, 3750 and 4500 nM) and ketoprofen (100, 125, 500, 1250, 1750, 2250 and 3250 nM) were studied. The serial concentrations of ketoprofen (100, 125, 450, 500, 750, 1250 and 1750 nM) were used in experiment with the preincubation of HSA-MB with 150 μL of 10 μM acetylsalicylic acid (ASA).

All measurements of supernatants were done after 10 min incubation (vortex) in triplicate. The supernatant was analyzed by Shimadzu LCMS 2010EV (Kyoto, Japan) composed of vacuum degasser (DGU-2A3), two solvent pump (LC-20AD), autosampler (SIL-20AC), diode array detector (SPD-M20A) and column oven (CTO-20AC). The experiments were performed using positive capillary voltage (3 kV), drying gas flow 1.5/min, temperature 250 °C and the mobile phase composed of 10 mM ammonium acetate buffer (pH 7.4)/acetonitrile (20/80 v/v) with the flow rate of 0.4 mL/min. 25 μL of each collected supernatant was injected.

3. Results

In our preliminary investigation of HSA-MB, an optimization of incubation procedure was the aim. As shown in Fig. 1, the differences in collected supernatant were observed in the comparison between control-MB and HSA-MB. Initially, the first wash (10 mM ammonium acetate pH 7.4) and the second wash (10 mM ammonium acetate pH 7.4, containing 15% of MeOH) were used. It has been clearly demonstrated that HSA ligand (warfarin) is retained until the second wash. That observation confirmed the specific interaction between warfarin and albumin immobilized on the surface of magnetic beads. In further study we discarded the wash with aqueous buffer and used 15% of MeOH in 10 mM ammonium acetate pH 7.4 to elute the ligand from albumin complex.

After immobilization of HSA onto the MB, a series of single test ligands were incubated in ammonium acetate buffer (10 mM, pH 7.4). The total amount (25 mg) of HSA-MB was divided into 10 sets (2.5 mg of HSA-MB each). One set of the beads was used for fraction unbound study. The supernatants which contain the unbound ligands were then analyzed by LC-MS. The values of fraction unbound (*fu*) were calculated using the Eq. (1) and are presented in Table 1. For most binders the results are consistent

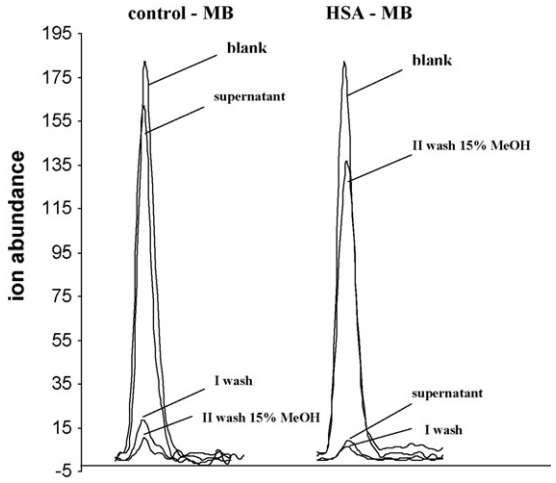

Fig. 1. Chromatographic traces of warfarin produced by the mass spectrometric detection of collected supernatant from control-MB and HSA-MB.

with previously reported *fu* values [23–27]. Although the significant differences between calculated and reported *fu* value for phenytoin (0.46) and valproic acid (0.49) were observed, the *fu* obtained are still consistent with 0.10 and 0.15, respectively. The reported values [24] concern parameters for plasma protein binding. Therefore, the *fu* for imipramine, lidocaine and quinidine here obtained are significantly different because of the high affinity of the basic drugs to alpha$_1$-acid glycoprotein. The disparities between literature values and those obtained from experiments were observed for fluphenazine and tolbutamide. The differences can be caused by shorter incubation time for HSA-MB than in the reported methods and those compounds did not reach equilibrium in applied time.

The influence of valproic acid as a potential displacer of phenytoin was studied with additional incubation experiment. The same amount of HSA-MB was incubated with the 200 μL mixture of 250 nM phenytoin and valproic acid. Comparing *fu* results for phenytoin before (0.46) and after (0.73) incubation with valproic acid, one will acknowledge that valproic acid reduced the binding of phenytoin. The data from the displacement experiment indicate that HSA-MB can be successfully used in displacement study.

In comparison with the previous study [15] the decreased amount (2.5mg) of MB was used to reduce non-specific binding to both the control and the HSA-MB. Using the Micro BCA assay kit, 172 ± 112 μg of HSA per mL of MB was determined (3.44 μg of HSA per mg of MB). On the control-MB, less than 5% of the tested ligand were retained, with imipramine showing the most 4.90% and lidocaine showing the least extent of interaction, with 1.73% of agents non-specifically bound.

In order to test efficiency of one set of HSA-MB (2.5 mg) the incubation of ketoprofen, fenoprofen and phenylbutazone with the same beads was performed after completing the above described experiment. The *fu* for a set of three ligands were 0.58, 0.86, 0.06 vs. 0.49, 0.62 and 0.01, respectively. The slight change in *fu* values showed that the HSA-MB efficiency was decreased but the beads still kept the activity after 71 incubations (nine weeks).

The K_a were determined using the HSA-MB technique in which serial concentrations of known ligands were incubated at constant conditions. One set on HSA-MB (2.5 mg) was used for one ligand. Binding to HSA on the MB was characterized using the warfarin, ketoprofen, fenoprofen, tolbutamide and L-tryptofan. Scatchard plots of warfarin, tolbutamide and L-tryptofan are found in Fig. 2a. L-tryptofan plot can be fitted two lines, which would indicate to different binding sites. However, r value of n_1 = 0.33 would mean that that the binding was non-specific. Using the Eq. (2), the determined K_a were 6.13×10^5 with the total number of bound molecules n = 2.84 (warfarin), 8.17×10^4 with n = 9.23 (L-tryptofan) and 7.11×10^4 with n = 7.43 (tolbutamide). The measurements were done after 10 min incubation in triplicate. The average equilibrium constants (n = 3) achieved by HSA-MB experiments are presented in Table 2. The results of the HSA-MB study agree well with literature data available on the binding parameters (K_a) of studied ligands

Table 1
Comparison of measured and reported [23–27] fraction unbound (*fu*) for a set of ligands and percent of ligand bound to control magnetic beads (control-MB).

Ligand	Measured *fu*	Reported *fu*	Percent of ligand bound to control-MB
Chlorpromazine	0.82 (±0.01)	0.71 [23]	3.12
Fenoprofen	0.62 (±0.03)	–	2.12
Fluphenazine	0.15 (±0.02)	0.04 [27]	3.98
Imipramine	0.85 (±0.01)	0.04[a] [24]	4.90
Indomethacin	0.68 (±0.04)	–	2.12
Ketoprofen	0.49 (±0.05)	0.52 [25]	2.98
Lidocaine	0.81 (±0.06)	0.30[a] [24]	1.73
L-tryptofan	0.85 (±0.03)	–	2.19
Phenylbutazon	0.01 (±0.02)	–	2.69
Phenytoin	0.46 (±0.04)	0.10 [24]	4.40
Quinidine	0.82 (±0.03)	0.20[a] [24]	3.32
Quinine	0.84 (±0.01)	0.65 [26]	3.17
Sulindac	0.36 (±0.02)	–	3.77
Tolbutamide	0.73 (±0.04)	0.04 [27]	2.75
Trifluopromazine	0.88 (±0.06)	–	2.05
Valproic acid	0.49 (±0.05)	0.15 [24]	3.83
Warfarin	0.01 (±0.01)	0.03 [24]	2.41

[a] Basic drugs that are bound significantly to plasma proteins other than albumin [24].

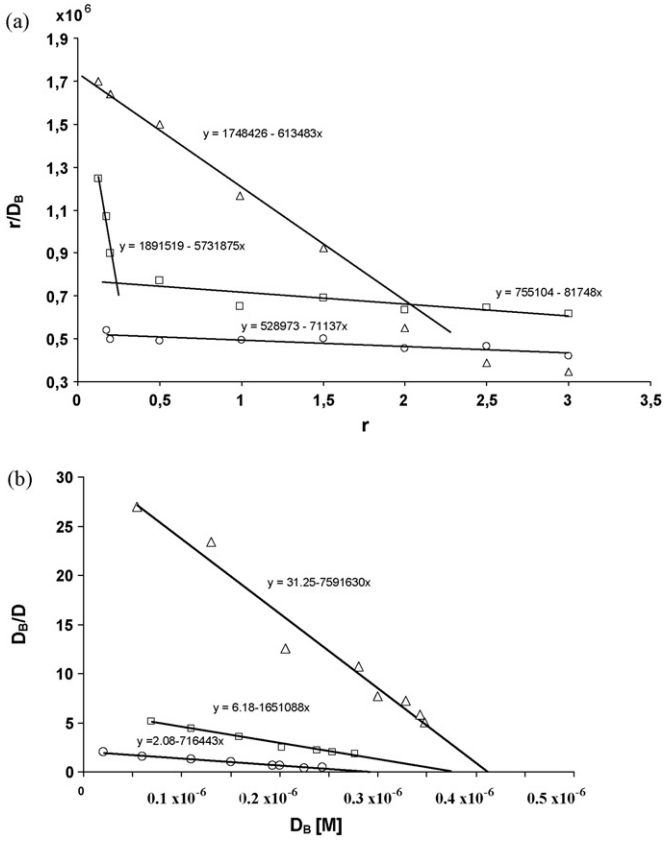

Fig. 2. (a) Scatchard plots obtained from incubation of L-tryptofan (100–1500 nM) (□), tolbutamide (100–1500 nM) (○) and warfarin (100–1500 nM) (△) with HSA-MB, (b) Rosenthal plots obtained from incubation of L-tryptofan (150–1500 nM) (□), tolbutamide (100–500 nM) (○) and warfarin (100–500 nM) (△) with HSA-MB.

Table 2
Comparison of measured and reported [1,28–31] ligand-HSA association constants, K_a.

Ligand	Association constant K_A (M^{-1})	Reported	Reference
L-tryptofan	8,82 (±2.4) × 10^4	1.10 (±0.3) × 10^4	[28]
Fenoprofen	K_{a1} 3.73 (±1.0) × 10^5 K_{a2} 0.97 (±0.8) × 10^4	K_{a1} 3.40 × 10^5 K_{a2} 1.00 × 10^4	[30]
Ketoprofen	K_{a1} 5.82 (±0.9) × 10^5 K_{a2} 5.27 (±1.3) × 10^4	K_{a1} 3.72 × 10^5 K_{a2} 1.14 × 10^5	[31]
Tolbutamide	7.70 (±1.1) × 10^4	2.20 × 10^5	[1]
Warfarin	6.26 (±0.7) × 10^5	2.10 (±0.2) × 10^5	[29]

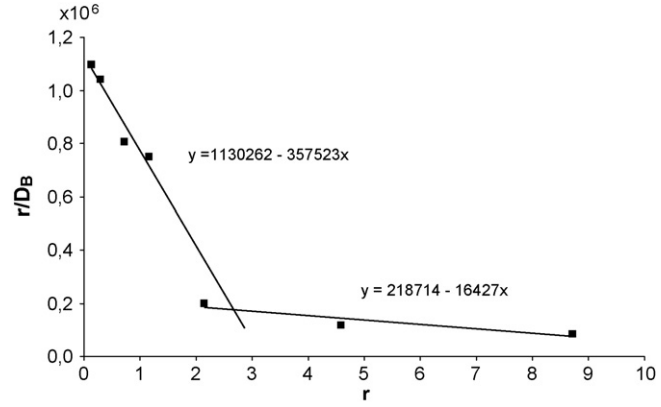

Fig. 3. Scatchard plot of fenoprofen (100–4500 nM) data obtained from incubation with HSA-MB.

measured by equilibrium dialysis, ulrafiltration, zonal and frontal HPAC [28–31].

In order to better evaluate the binding process, the data from saturation experiment were plotted with specific binding on the *x*-axis and specific binding divided by free drug concentration on the *y*-axis (Fig. 2b). These Rosenthal plots allowed to visualize and compare saturation of the single site binding data. The range of concentrations for tolbutamide and warfarin was the same as used in Scatchard analysis. The serial concentrations for L-tryptofan were reduced to the range from 150 to 1500 nM. The relative affinities (K_a) determined using Rosenthal linear regression were 7.59 × 10^6 (warfarin), 1.65 × 10^6 (L-tryptofan) and 7.16 × 10^5 tolbutamide) with average B_{max} value of 87.75 ± 13 pmol (250 μL of 351 nM) (n = 3) and the calculated r^2 values were 0.967 (warfarin), 0.980 (L-tryptofan) and 0.946 (tolbutamide). These affinities are consistent with previously determined values by Scatchard linear regression.

According to the Scatchard analysis for fenoprofen, experimental data could be approximated by two straight lines, suggesting two independent classes of binding sites on HSA reported in the literature [32] as the warfarin-azapropazone binding area (Site I) and the indole-benzodiazepine binding site (Site II) (Fig. 3). Therefore two linear functions that cross *x*-axis were found. For fenoprofen bound to Site I was n_1 = 3.16 with K_{a1} = 3.57 × 10^5 and to Site II was n_2 = 13.41 with K_{a2} = 1.64 × 10^4 M^{-1}. Also, Scatchard plot for ketoprofen implied two binding sites on HSA with different K_a values: K_{a1} 6.67 × 10^5 with n_1 = 2.12 and K_{a2} 4.74 × 10^4 M^{-1} with n_2 = 7.23 (Fig. 4). The measurements were done in triplicate and average equilibrium constants are presented in Table 2.

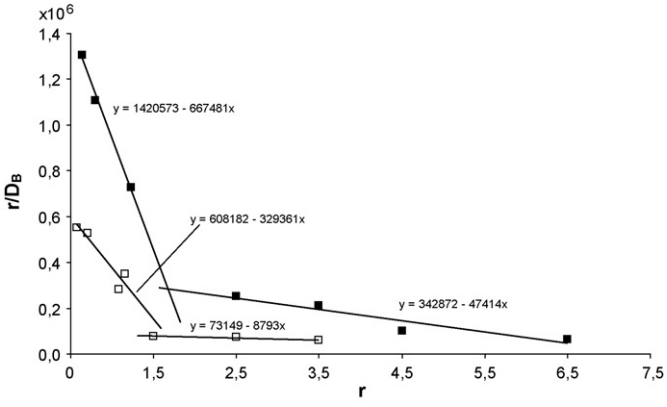

Fig. 4. Scatchard analysis data obtained from incubation of ketoprofen (100–3250 nM) with HSA-MB (■) and (100–1750 nM) with preincubation of the HSA-MB with 10 μM acetylsalicylic acid (□).

The significant effect on the association constant of ketoprofen after the preincubation of HSA-MB with 150 μL of 10 μM acetylsalicylic acid (ASA) was observed (Fig. 4). Scatchard analysis of the data from HSA-MB study showed that ketoprofen still binds at two classes of binding site but with different affinity constants K_{a1} 2.63 (±0.7) × 10^5 and K_{a2} 8.15 (±1.0) × 10^3 M^{-1} vs. K_{a1} 5.82 (±0.9) × 10^5 and K_{a2} 5.27 (±1.3) × 10^4 M^{-1} after pretreatment with ASA. The preincubation with ASA resulted in decrease in the affinity of binding to both, first and second, classes of binding site. This phenomenon is evidently due to the acetylation of amino acid residues of human serum albumin by acetylsalicylic acid which is well described in literature [33,34].

4. Conclusion

The fast and reliable characterization of the drug-human serum albumin binding process has been performed by applying magnetic beads technique. The HSA-MB study showed that the activity of the protein immobilized onto the magnetic beads remains stable for up to nine weeks of continuous use. The results from the initial competition experiment suggest that protein-coated magnetic beads can be employed in drug–drug interaction studies. But the validation of the proposed method for displacement study is essential for reliable information on the competition process. This issue is being investigated and the results will be reported in the future.

From the experimental point of view, presently increasing number of commercially available magnetic beads with different surface groups gives opportunity to extend their application in bioseparation research. As a novel technique, the magnetic beads with appropriate immobilized proteins can be effectively applied not only in isolation and purification study, but also to accurately qualify interaction between ligand and protein. That might be of special importance for modeling of drug action and pharmacokinetics, including possible drug–drug interactions.

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