BINDING OF 1-[3-(4-TERT-BUTYL-PHENOXY)PROPYL]PIPERIDINE, A NEW NON IMIDAZOLE HISTAMINE H3 RECEPTOR ANTAGONIST TO BOVINE SERUM ALBUMIN

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Abstract: The degree of binding of a drug to plasma proteins has a significant effect on its distribution, elimination, and pharmacological effect since only the unbound fraction is available for distribution into extra-vascular space. The binding of DL76 (1-[3-(4-*tert*-butyl-phenoxy)propyl]piperidine) to bovine serum albumin (BSA) was studied *in vitro* by equilibrium dialysis at 37°C and pH 7.4 over the concentration range of 0.32–317.18 µM and at a physiological protein concentration of 602 µM. Drug concentrations were determined by validated LC/MS/MS method. Nonlinear regression analyses of the data pointed to a single class of binding sites (m = 1) with a dissociation constant of DL76 equal 49.20 µM. Scatchard plot concave-down curve might indicate positive cooperativity, which was confirmed by the Hill plot with the slope higher than one.

Keywords: protein binding, equilibrium dialysis, DL76, bovine serum albumin

Binding to plasma proteins, playing a pivotal role in distribution, elimination and therapeutic effectiveness of drugs, has long been considered as one of their most important physicochemical characteristics. This is because only free (unbound) drug can pass through cell membranes and reach the target site in the body such as an appropriate receptor, transporter or enzyme. Moreover, a knowledge of drugs protein binding properties has become an important issue for understanding pharmacokinetically relevant binding phenomena such as displacement reaction between different drugs or the significant alteration of plasma protein binding of some drugs during certain pathophysiological states (1). For these reasons, the plasma protein binding parameters should be estimated at the early stage of drug discovery as one of the selection criterion for new drug candidates (2).

Compound 1-[3-(4-tert-butyl-phenoxy)propyl]piperidine (DL76) is an example of new non-imidazole histamine H3 antagonist. In preliminary pharmacological studies it shows good affinity for H3 receptors (hK$_i$ equal 22 ± 3 nM; iodoproxyfan binding assay at human H3 receptor stably expressed in CHO-K1 cells) and ED$_{50}$ equals 2.8 ± 0.4 mg/kg (central histamine H3 receptor assay *in vivo* after *p.o.* administration to mice) (3).

Blockade of the H3 autoreceptors by antagonists interrupts the negative feedback mechanism and leads to increased levels of histamine and other neurotransmitters. These effects suggest a potential therapeutic role of H3 receptor antagonists in the treatment of several diseases and neurological disorders, such as schizophrenia, depression, sleep-wake disorders, dementia or epilepsy (4). A lack of the imidazole moiety in the structure of DL76 may improve its pharmacokinetic profile and increase safety of administration comparing to the imidazole H3 antagonist (5).

Although there are many plasma components capable of binding drugs (e.g., acid α$_1$-glycoprotein, lipoproteins, globulins, erythrocytes), albumin is present in the highest quantities and is able to bind to a broad variety of drugs with an affinity sufficient

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to have a significant effect on their pharmacokinetic profile and action (6, 7).

The aim of this study was to evaluate the binding rate of DL76 to bovine serum albumin and to determine its binding parameters such as number of binding sites and affinity constants.

EXPERIMENTAL

Chemicals

1-[3-(4-*tert*-Butyl-phenoxy)propyl]piperidine as a monobasic oxalate was supplied from The Department of Technology and Biotechnology of Drugs (Faculty of Pharmacy, Jagiellonian University Medical College, Kraków, Poland). Pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydro-1*H*-purine-2,6-dione, PTX), used as an internal standard and bovine serum albumin (BSA) BioChemika (fraction V, = 96%) were obtained from Sigma Aldrich (St. Louis, MO, USA) and a molecular weight of 66430 g/mol for the BSA was assumed. HPLC grade acetonitrile, water, formic acid and ethyl acetate were purchased from Merck (Darmstadt, Germany). The phosphate buffer solution 0.067 M (PBS) was adjusted to the desired pH (7.4 ± 0.05) by an addition of hydrochloric acid or sodium hydroxide.

DL76 concentration assay

The quantitative measurement of DL76 in albumin and buffer solution was made by validated LC/MS/MS method, according to the procedure described previously (8). The method was adopted to the modified matrices. LC/MS/MS system consisted of high performance liquid chromatograph Agilent 1100 (Agilent Technologies, Waldbronn, Germany) and triple quadrupole mass spectrometer API 2000 (Applied Biosystems MDX Sciex, Concorde, Ontario, Canada) equipped with an electrospray ionization interface (ESI). ESI ionization was performed in the positive ion mode. The mass spectrometer was operated in selected reactions monitoring mode (SRM) monitoring the transition of the protonated molecular ions m/z 276 to 98 for DL76 and 279 to 181 for internal standard (PTX). Chromatographic separation was carried out with a XBridge™C18 (2.1 × 30 mm, 3.5 µm, Waters, Ireland) analytical column. The mobile phase consisted of acetonitrile/water (v/v) with an addition of 0.1% of formic acid, was set at the flow rate of 300 µL/min and the gradient elution was used. A 100 µL aliquot of phosphate buffered saline containing BSA and unknown concentration of DL76 was briefly mixed with the 10 µL of IS and then 1 mL of ethyl

acetate was added. The mixture was shaken on a mechanical shaker for 20 min and centrifuged at 3,000 rpm for 15 min. The organic layer (0.5 mL) was transferred into conical tubes and evaporated to dryness at 37°C under the stream of nitrogen gas in the TurboVap evaporator (Zymark, Hopkinton, MA, USA). The dry residue was reconstituted with 100 µL of acetonitrile/water (50/50, v/v) and aliquot of 10 µL was injected onto the LC/MS/MS system. Extraction recovery of DL76 from buffered protein solutions was high (100%). The limit of detection in the SRM mode was found to be 18.15 nM and the limit of quantification was 36.3 nM. The precision and accuracy for both intra- and inter-day determination of DL76 ranged from 1.65 to 15.09% and from 88.74 to 113.43%, respectively. The linear detection response was observed in the range 36.3 nM – 7.27 µM and therefore, the samples with expected higher concentrations were diluted. The concentration of DL76 in the PBS was measured by direct injection into an analytical system without prior purification of the sample.

Binding study

The binding of DL76 to BSA was determined by equilibrium dialysis method using Fast Micro-Equilibrium Dialyzer consisting of two chambers with compartment volume of 500 µL each, separated by semi permeable regenerated cellulose dialysis membrane with a molecular weight cut-off of 10,000 Da (Harvard Apparatus, Holliston, MA, USA). One chamber contained various initial concentrations of DL76 (range 0.32–317.18 mM) dissolved in isotonic phosphate buffer (pH 7.4) and the other buffered isotonic solution of physiological concentration of BSA (602 µM). The dialysis was performed for 3 h (time to equilibrium) at 37°C under a constant stirring at 100 rpm. After completion of dialysis, the concentrations of DL76 were measured in both chambers using analytical method described above. Time to achieve the equilibrium state was determined experimentally during preliminary studies by analyzing the concentration of DL76 in both chambers of the dialyzer (DL76 in buffer against BSA in buffer) at increasing time periods. When no change in the concentration of DL76 in both chambers was detected between several time points, an equilibrium state was assumed to be reached. Assays were repeated three to five times, and the average values of concentrations were used to calculate binding data.

In order to eliminate some of the possible experimental errors, drug stability and its adsorption to the dialysis system were tested. The stability of

DL76 was tested for 6 h at temperature of 37°C at the low (0.79 µM) and high (237 µM) concentrations. The study was based on the determination of the concentration of DL76 in the freshly prepared samples and after 6 h of incubation at 37°C. The stability was expressed as a percentage ratio of the concentrations of incubated sample/freshly prepared sample. The DL76 was considered to be stable if less than 5% difference in concentration was observed. The possibility of adsorption of the investigated compound to the dialysis system and membrane was determined by comparing the amount of DL76 added to one chamber before dialysis to the one calculated based on the concentrations measured post-dialysis (buffer with DL76 against buffer) on both sides of the dialysis membrane .

Data analysis

The fraction of DL76 bound to BSA (f_b) was determined by the following equation:

$$f_b = (C_t - C_u)/C_t \qquad (1)$$

where C_t = total concentration of drug in the albumin solution at equilibrium and C_u = unbound drug concentration in the chamber without albumin at equilibrium.

The bound drug concentration was calculated using a formula:

$$C_b = C_t - C_u \qquad (2)$$

Binding parameters were determined by non-linear regression method using Wolfram Mathematica 7 to fit the data to equation:

$$r = \sum_{i=1}^{m} \frac{n_i \cdot C_u}{K_{di} + C_u} \qquad (3)$$

where r = number of moles of drug bound per mole of protein (C_b/P_t; P_t is a total protein concentration); m = number of independent classes of binding sites; K_{di} = dissociation constant for the i-th class and n_i = number of binding sites in the i-th class.

In the regression analysis several factors were taken into consideration such as possible presence of one or two classes of binding sites as well as non-specific binding (NSB). The final model used for description of albumin binding characteristics of DL76 was selected based on goodness-of-fit criteria such as Akaike Information Criterion (AIC) value.

The binding data of DL76 were also analyzed using Scatchard transformation where a curve was produced by plotting r/C_u *versus* r.

RESULTS AND DISCUSSION

DL76 is a new compound, therefore, its protein binding parameters have not been reported previously. To evaluate the binding characteristics of this compound to the main plasma protein i.e., albumin, the equilibrium dialysis method was used. This method is considered to be the reference method from among all the other techniques used for the determination of protein binding (9). Time to reach the equilibrium state is one of the most important variables in the equilibrium dialysis and in this experiment it was quite short and equals 3 h at the
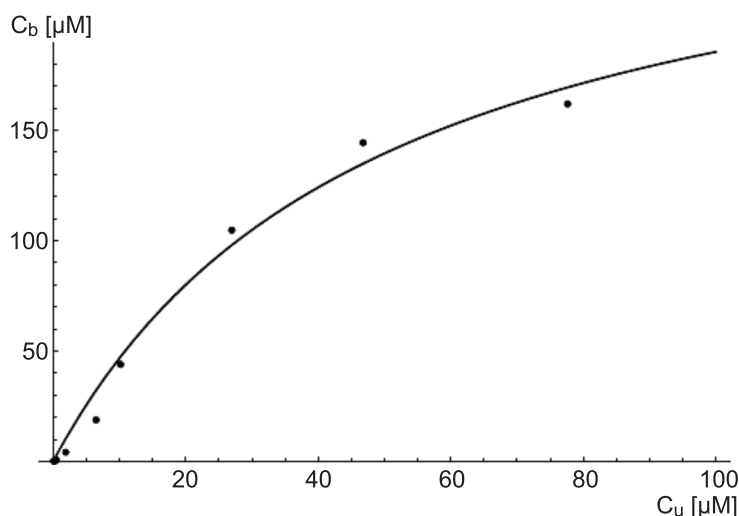

Figure 1. Binding plot of mean (n = 3–5) bound (C_b) *versus* mean free (C_u) concentration values of DL76 to bovine serum albumin (physiological concentration of 602 µM) estimated at 37°C by equilibrium dialysis technique. The experiment was carried out over the DL76 concentration range of 0.32–317.18 µM at pH 7.4

temperature of 37°C. DL76 was determined to be stable during the whole experiment and its adsorption to the dialysis system was below 3%.

The binding plot of DL76 to BSA (602 μM) is presented in Figure 1. The plot was obtained by subsequent iterations using one class of binding sites with or without nonspecific binding site or two classes of binding sites. The AIC values for these three models of DL76 albumin binding were -70.66; -68.66 and -66.66, respectively. The lowest value of AIC indicates the best fitting of data to the one class of binding sites (m = 1) with K_d value equal 49.20 μM and n equal 0.459.

The binding association constant of DL76 (K_a) with BSA being a reciprocal of K_d equals 2.03×10^4 M^{-1}. It was found that the highest percentage of albu-
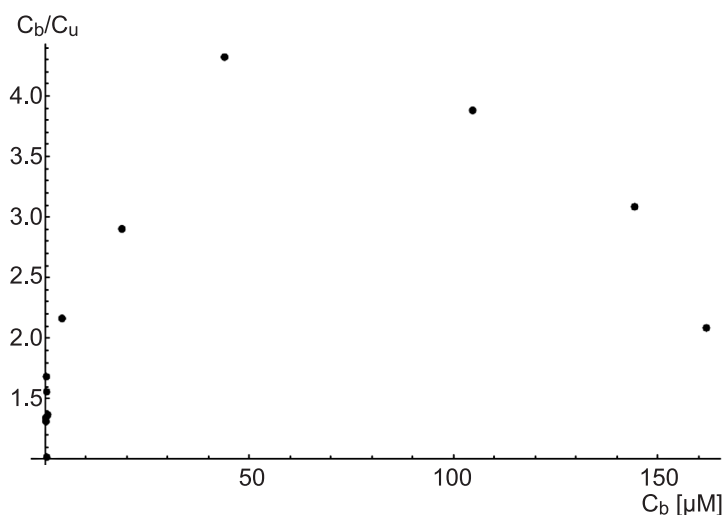

Figure 2. Scatchard plot for binding of DL76 to BSA (602 μM). The results are the mean of 3–5 measurements. The concave downwards curve is characteristic of positive cooperativity binding. Abbreviations and symbols: C_u – unbound compound concentration, C_b – bound compound concentration
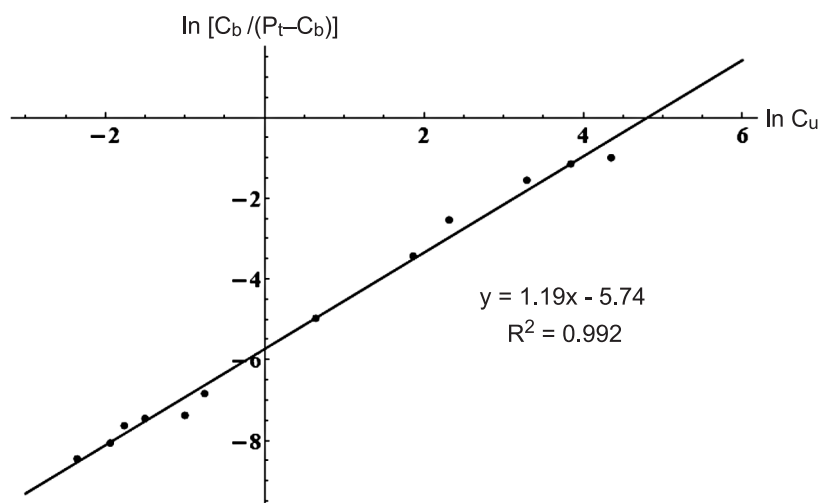

Figure 3. Hill plot for binding between DL76 and BSA. A value of Hill coefficient (slope) above 1 indicates the presence of positive cooperativity in the binding of DL76 to BSA. Abbreviations and symbols: C_u – unbound compound concentration, C_b – bound compound concentration, P_t – protein concentration (602 μM)

min protein binding of DL76 near the saturation level calculated from the equation (1) was about 80%. These data may indicate that DL76 with its physicochemical characteristics (basic with pKa = 9.15 and lipophilic with logP = 4.97) belongs to the group of compounds that highly bind to the BSA with moderate affinity in a typical range of 0^2–10^4 M^{-1} (10).

The results of Scatchard analysis of the binding data are presented in Figure 2. A concave-down curve may indicate the presence of positive cooperativity, which takes place when the binding of a ligand at one site increases the affinity of a separate ligand molecules for binding at another site (11). One the mechanisms of this phenomenon may be an allosteric communication between binding sites (12).

One of the methods used for the differentiation of cooperativity (positive or negative) is the Hill slope analysis presented in Figure 3. The Hill curve was produced by plotting ln $(C_b/(P_t - C_b))$ *versus* ln C_u. A value of Hill coefficient above 1 confirms the presence of positive cooperativity (13). This coefficient also expresses the average number of interacting sites. In presented study, value of the Hill slope was determined to be 1.19, which verifies the positive cooperativity and, in addition, indicates the existence of two binding sites interacting in the process of binding DL76 to BSA.

The classic example of positive cooperativity is oxygen binding to hemoglobin (14). This kind of reciprocal action was proven to exist also in other biological systems. It is suggested in the estrogen receptor α-estradiol interaction at high level of receptor concentration or for interaction of γ-hydroxybutyric acid with the membrane of rat brain (15, 16). Moreover, positive and negative cooperativity was found in an analysis of binding of nicotin-amide-adenine dinucleotide to yeast glyceraldehyde-3-phosphate dehydrogenase (17).

Based on these examples is can be concluded that phenomenon of positive cooperativity may play an important role not only in the plasma protein binding of different compounds but also in their interaction with other proteins e.g., receptors and enzymes.

CONCLUSIONS

The presented study reports the plasma albumin binding characteristics of DL76 compound. From the obtained data it can be concluded that investigated compound binds to the plasma albumin

in approximately 80%, and that there is a positive cooperativity (Hill slope above 1) in the binding process between DL76 and BSA. Using non linear regression analysis, the lowest value of AIC indicates the best fitting of data to the one class of binding sites (m = 1) with moderate protein binding of DL76 (K_d value equal 49.20 μM and n equal 0.459).

The received results give an important information about DL76 compound and can help in the interpretation of some pharmacological and pharmacokinetic data. They also may be a source of useful information for scientists seeking new compounds in this chemical group.

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