Serum protein binding study of biologically active β-carbolines employing spectroscopic, chromatographic and in silico methods

Theses of a PhD dissertation

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Budapest, 2015.
1. Introduction

In the last few years, the emerging resistance to the conventionally applied drugs and the growing number of unresolved problems in the field of medical science have eventuated to a revival of interest in the natural product for healing. Due to the broad range of their pharmacological activity and their structural diversity, they are believed to be promising supplementary compounds of the conventional medical therapy, to serve as new starting compounds in the drug research or for synthesis of combinatorial chemical compound libraries or for exploration of previously unknown molecular mechanisms.

Nowadays, a promising area of the researches is represented by the β-carbolines belonging to the family of indole alkaloids. Their most extensively studied group is the analogues having tri- (norharman (NH), harmane (HH), harmine) and pentacyclic (evodiamine (EVD), rutaecarpine (RTK)) ring systems.

![Chemical structure of the β-carbolines having tri- and pentacyclic ring systems](image)

In the nature, they are presented as constituents of herbs traditionally used Far Eastern Medicine, but due to their formation from indole derivatives, some β-carbolines can also be found in foodstuffs, beverages or as endogenous ligands of the human body (NH, HH). It is worth mentioning that EVD has already been available in Western countries as the main ingredient of several kinds of the dietary supplements. They possess diverse biological activity which makes them an important implements of self-healing, medicinal science and starting compound in numerous drug developments, despite the fact the exact mechanism of their action and structural and pharmacological properties are not entirely understood. The reason for this lies in that the researches mostly focus on revealing their interactions with the specific molecular target (nucleic acid, enzymes) and only a narrow part of the studies pay attention to recognize their behaviour with other important macromolecules. Furthermore, the prognostication of their actions in the living body is rendered more difficult, since some of them...
occur in the nature in racemic form. However, in the pharmacological studies, beyond that they scarcely ever identify the biologically active enantiomer, the interests have never been extended to find out the properties of the other ineffective or toxic effect-bearing enantiomer.

The fate of the active molecules in the living body is defined by the combination of kinetic processes including absorption, distribution, metabolism and elimination (ADME). During the distribution, the active substance bound reversibly to the blood macromolecules reaches the target organs from the circulation. In this process, a significant role is attributed to the two main protein components of the serum, the human serum albumin (HSA) and alpha1-acid glycoprotein (AGP). HSA is the most abundant plasma protein (~ 600 µM) having helical structure, which can transport chemically diverse exogenous (drugs) and endogenous (fatty acids, hormones, bile acids, toxic metabolites) substances. It possesses several binding sites for fatty acids, organic compounds and metal ions. For small apolaric organic molecules, three, clearly separated binding pockets are available. HSA appears to have high binding affinity typically for the acidic and neutral compounds. Albumin binding of some natural β-carbolines has already been studied, but the results are contradictory both in the context of the affinity constant as well as the binding location of these compounds. Thus, by elucidating their HSA binding, not only the knowledge about the factors affecting the distribution process of the β-carbolines can be expanded and clarified, but by revealing the molecular background of the interactions, detailed insight into the poorly understood alkaloid-binding properties of albumin can also be gained.

The other important drug binding protein in the serum is the AGP, the binding cavity of which is represented by a deep, hydrophobic beta-barrel accommodating a wide variety of basic, aromatic compounds. The concentration of AGP in the blood is significantly lower than the albumin (~ 20 µM), but in acute phase reactions (inflammation, infections, cancer) its level considerably rises (~ 60-100 µM) while the concentration of HSA decreases (~ 250- 300 µM). Hence, in certain pathological conditions, the AGP become a significant carrier protein of the body. It is to be noted that AGP exists as a mixture of its three main genetic variants (F1, S and A), which show different drug binding properties. In various diseases, the relative contribution of the variants to the elevated plasma level of AGP is not equal, thus the polymorphism of AGP has to be taken into consideration in the pharmacokinetic studies of the biologically active substances.

According to the free-drug theory, the drug-plasma protein complexes can not pass through the cell membranes, so only the unbound fraction of drugs is capable of achieving the therapeutic target and of inducing the desired pharmacological activity. Furthermore,
depending on the strength of the interaction, the protein binding can influence the metabolism and toxicity of substances, as well as it may cause unexpected drug interactions. Thus, by the detailed examination of the β-carboline’s behaviour against transporter proteins, not only the pharmacologically active free concentration of the compound can be estimated, but we also have opportunity to gain information about the structural factors of the binding.

2. Goals

During the course of my doctoral studies, I endeavoured to give a comprehensive account of the behaviour of natural and synthetic β-carbolines developed by Servier Pharmaceutical Research Institute against plasma proteins. By combination of chromatographic, spectroscopic and in silico methods, I aimed to study the interactions of these alkaloids with HSA and AGP to:

- estimate the binding parameters (association constant, stoichiometry),
- identify and characterize their specific albumin binding sites,
- establish structure-binding affinity relationships,
- study how fatty acids affect the albumin binding of β-carbolines,
- reveal and assign the inherent structural and spectral features of EVD and RTK,
- determine and propose the binding conformation of EVD enantiomers in the presence of plasma proteins,
- perform structure-based protein-ligand docking using the crystal structure of HSA and AGP, the results of which enable us to expose the pharmacophore groups that are responsible for the binding, as well as to understand the molecular background of the interactions.
3. Materials and Methods

3.1. Materials

HSA, AAG, NH, (±)-EVD were Sigma Aldrich products. RTK was obtained from the AK Scientific Company. The pure genetic variants of AAG were separated by following a well-known metal-affinity chromatographic method. HH, harmine and their synthetic analogues were provided by András Kotschy, from the Servier Research Institute of Medicinal Chemistry: [b1]:7-isopropoxy-1-methyl-9H-pyrido[3,4-b]indole, [b2]:1-methyl-7-(1-phenylethoxy)-9H-pyrido[3,4-b]indole, [b3]:1-methyl-7-(2-pyridylmethoxy)-9H-pyrido[3,4-b]indole, [b4]:1-methyl-7-[2-(2-pyridyl)ethoxy]-9H-pyrido[3,4-b]indole, [b5]:9-benzyl-7-methoxy-1-methyl-pyrido[3,4-b]indole, [b6]:7-methoxy-9-[(3-methoxyphenyl)methyl]-1-methyl-pyrido[3,4-b]indole, [b7]:7-methoxy-1-methyl-9-[[4-(trifluoromethyl)phenyl]methyl]-pyrido[3,4-b]indole, [b8]:7-methoxy-1-methyl-9-[1-[4-(trifluoromethyl)phenyl]ethyl]pyrido[3,4-b]indole, [b9]:7-methoxy-1-methyl-9-[2-[4-(trifluoromethyl)phenyl]ethyl]pyrido[3,4-b]indole.

3.2. Immobilized HSA- affinity chromatography

To estimate the values of the association constants and to show relative binding differences such as substituent effects and stereoselective interaction, the albumin-affinity chromatography was applied.

3.3. Ultrafiltration

It was used to identify the stereoselective serum protein binding of the (±)-EVD. The enantiomeric composition of the ultrafiltrate was established with chiral HPLC technique.

3.4. Chiral HPLC analysis

A method known from the scientific literature was applied to separate the EVD enantiomers, to determine the enantiomeric ratio of EVD in the ultrafiltrate and to identify the enantiomers separated by affinity chromatography.

3.5. Circular dichroism spectroscopy

It was conducted to establish the binding parameters of plasma protein-ß-carboline interaction, to ascertain their specific albumin binding site and to analyse the chiroptical properties of EVD enantiomers. In the former case, the induced CD activity of the alkaloids in the presence of proteins was measured, while in ligand displacement studies, the ICD signal changes of the studied or reference compounds were monitored. The non-linear regression
analysis of the ICD activity recorded at increasing molecule/ HSA molar ratio was performed by Microcal Origin 8.6 program.

3.6. Quantum chemical calculation of the CD spectra of (R)- EVD

To identify low-energy conformers of the molecule, conformation analysis was carried out based on the known crystal structure of EVD using Marvin 6.3.0. (Conformer plugin, ChemAxon) program. Theoretical CD spectra calculations of the low-energy conformers obtained as the results of the calculation were performed at DFT level of theory combined with Conductor-like Screening Model (COSMO) as implemented in Turbomole v6.5. program.

3.7. Fluorescence Spectroscopy

To establish the values of the association constants, the inherent fluorescence changes of the β-cabolines in the presence of protein were monitored, as well as in the case of EVD and RTK the protein binding induced emission signals were detected. To identify their binding site, quenching of the intrinsic protein fluorescence or the specific marker ligands was measured in the function of the concentration of the studied compounds. The non-linear regression analysis of the data was accomplished by Microcal Origin 8.6 program.

3.8. Molecular modelling studies

Docking calculations were carried out using DockingServer, where the crystal structure of the HSA and AAG obtained from the RSC Protein Data Bank was used. The outputs of the docking calculations were rendered with the PyMOL program.
4. New scientific results and their relevance

1.) Specific complexes of serum proteins and natural β-carbolines (NH, HH) in a molar ratio of 1:1 have been established. In competitive ligand displacement studies, their specific albumin binding site within the subdomain IIIA has been identified. By performing docking calculation, the molecular background of their interaction has been revealed. The results showed that the alkaloids are stabilized by π-π stacking (Tyr411, Phe488) and H-bonding interactions at site IIIA. It has been found that in this binding position they completely overlap with one of the high-affinity fatty acid sites of albumin. We have showed that NH forms moderately strong (K_a ~ 1.7 × 10^5 M^-1) and HH weak HSA complexes (K_a ~ 2.4 × 10^4 M^-1) while their AGP binding is negligible. The results suggest that HSA constituting the largest part of the blood plasma may be accountable for their distribution in the human body.

2.) The impact of the saturated and unsaturated fatty acid (FA), considered to be primary endogenous ligands of HSA, on the β-carbolin-HSA binding has been studied. By applying CD and fluorescence spectroscopy, the formation of β-carbolin-FA-albumin ternary complexes has been demonstrated. Between the HSA and the β-carbolins, such previously unknown lipid modulated albumin binding interactions have been revealed, where the FAs via cooperative allosteric mechanism brought about the significantly enhanced albumin affinity of NH and HH (~ 3-10 times higher). In addition, the inversion of the induced CD signal, as well as the increase in the emission intensity of the low quantum yield band associated to the bound form of the alkaloids were also observed upon addition of FAs to β-carbolin-HSA complexes referring to their cobinding at site IIIA. Using molecular modelling calculation, we have showed that this phenomenon could be attributed to the altered binding mode of the alkaloids. In view of the results obtained, it can be concluded that the pharmacologically active free serum level of these compounds bearing β-carbolin skeleton strongly depends on the number and chemical constitution of albumin bound to FAs.

3.) In cooperation with Servier Research Institute of Medicinal Chemistry, the behaviour of a series of novel harmine derivatives synthesised by bioisosteric replacement against serum proteins was investigated. AGP and albumin bindings of these analogues were found to be substituent-dependent. We have recognized that modification of the basic β-carboline skeleton in position 7 and 9 by aromatic pharmacophore group resulted in considerably enhanced affinity to both HSA and AGP (K_a ~ 10^5-10^6 M^-1) compared to harmine (K_a ~ 3.0 × 10^4 M^-1), as well as selective AGP genetic variant binding. The outcomes imply that the substitution aimed at
improving the biological activity of these potential therapeutic agents can cause the reduction of their pharmacologically active serum fractions, which findings indicate a parameter to be optimised during the drug development processes.

4.) For the synthetic harmine derivative possessing pyridine side chain in the position 7 (b4), selective, dimeric AGP genetic variant binding was exhibited. By uncovering the molecular background of this interaction, it has been found that the different architecture of the internal drug binding cavity of the two variants is responsible for the π-stacked, dimeric binding of the (b4) on AGP-A variant as opposed to the AGP-F1*S form.

5.) In the course of our endeavours to investigate the ligands having indoloquinazoline-skeleton, strong binding for EVD and RTK was showed on both components of the serum. The strength of the interaction was one order of magnitude higher for EVD on albumin than on AGP. The results suggest that while in healthy bodies the distribution of EVD is regulated mostly by the HSA, in pathological state the fluctuation of the plasma concentration of AGP may also influence the free blood fractions of these alkaloids.

6.) In the case of (±)-EVD enantioselective serum protein binding has been demonstrated, where the stereoselectivity value was higher on HSA than on AGP. The binding preference of the (R) enantiomer both on albumin and AGP has also been verified. Considering that the stereoselective binding may eventuate different ADME profile for the enantiomers, these observations can provide important information to achieve desired therapeutic effect of the biologically active form, as well as to reduce its dose-dependent side effects.

7.) The resolution of the racemic EVD was carried out applying HSA-affinity chromatography. This method may provide a new, promising drug-analytical technique regarding the practical point of view to identify and separate the stereoisomers of EVD and related substances that may appear as concomitant components.

8.) By qualitative assessment and quantum chemical calculation of the CD spectra, the background of the spectral changes of EVD has been revealed, and the structural and chiroptical features of its low energy conformers have been assigned to each other. It has been demonstrated that by following the changes of the intramolecular exciton signals, information can be gained about the conformational equilibrium and the geometry of the predominant conformer of EVD. In ligand displacement studies, the specific albumin binding site of EVD was identified, and the preferred binding conformation of (R)-EVD located in the subdomain...
IIA binding pocket of albumin has been assessed. The findings obtained about the inherent stereochemical features of EVD can help to understand the molecular background of the structure-manifold biological activity relationships of EVD in the future and also highlight the pharmacologically significant conformation adaptation abilities of the alkaloid to target proteins.

9.) In comparative evaluation study, some relationships between the structural and plasma protein binding properties of EVD and the congener RTK were established. It has been shown that its specific HSA binding room similarly to EVD is located in the IIA subdomain of albumin. The results indicated that its rigid, planar skeleton compared to EVD allows a tighter fitting, stronger interaction (K_a ~ 1.4 × 10^6 M^{-1}), and forming of 2:1 (ligand: protein) stoichiometric complex with serum albumin. These outcomes not only enable us to predict the distribution process affecting plasma protein binding of RTK in the body, but they may also serve as basis for the prognosis of the pharmacologically active serum level of drug candidates disposed of similar structure.
Publications contributing directly to this work

