Binding of an Oxindole Alkaloid from *Uncaria tomentosa* to Amyloid Protein (Aβ1-40)

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The primary aim of this work was to determine the interactions of an oxindole alkaloid (mitraphylline) isolated from *Uncaria tomentosa* with β-amyloid 1-40 (Aβ1-40 protein) applying the capillary electrophoresis (CE) method. Specifically the Hummel-Dreyer method and Scatchard analysis were performed to study the binding of oxindole alkaloids with Aβ1-40 protein. Prior to these studies extraction of the alkaloid of interest was carried out. Identification of the isolated alkaloid was performed by the use of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) combined with electrospray ionization mass spectrometry (ESI-MS). The proposed approach was proved to be an efficient and accurate method for specific compound isolation and identification purposes. Moreover, analytical information from the CE approach can be considered as the valuable tool for binding constant determination. The binding constant of mitraphylline with Aβ1-40 protein determined by the Hummel-Dreyer method and Scatchard analysis equals $K = 9.95 \times 10^5$ M^{-1}. The results obtained showed the significant binding of the tested compound with Aβ1-40 protein. These results are discussed and interpreted in the view of developing a strategy for identification of novel compounds of great importance in Alzheimer disease therapy.

Key words: Binding Constant, Capillary Electrophoresis (CE), Hummel-Dreyer Method, Scatchard Analysis

Introduction

One of the principal features of Alzheimer disease (AD) is the extracellular deposition of fibrous protein aggregates in the form of amyloid plaques (diffuse, primitive or neuritic). The major component of these deposits is the β-amyloid (Aβ) protein, that is a proteolytic fragment of the integral membrane amyloid precursor protein (APP) (Martin, 1999). Hence, neurodegenerative changes in the brains of patients suffering from AD are characterized by Aβ protein aggregation. β-Amyloid 1-40 (Aβ1-40 protein) is the main component of the pathological cerebrovascular plaques observed *post mortem* in the Alzheimer patients. It is a 4.2 kDa protein composed of 39–42(43) amino acids. The potential challenge of AD therapy is to find the way to reduce Aβ protein aggregation and then its toxicity. Among several experimental trials, binding of the Aβ protein to different natural and synthetic compounds appears as a promising possibility. Identification of such compounds would be of great importance in AD therapy (Honig, 2000).

It has been reported previously that oxindole alkaloids from the root bark of *Uncaria tomentosa* prevent Aβ protein aggregation and hence its toxicity. Because of the wide range of pharmacological and biological activities of these compounds, their separations and determinations are of considerable interest. The Alzheimer disease state is characterized by the abnormal accumulation of a normal degradative peptide which becomes resistant to further proteolysis due to conformational changes and then aggregation (Wiśniewski *et al.*, 1993). Conformational changes of β-amyloid from α to β structure expose its hydrophobic surface, which allows the β-fibrils formation. This process is not spontaneous and depends on pH value, tem-

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perature, concentration, specific tissue factors, presence of metal ions, and length of the peptide (Uversky and Fink, 2004; Wolozin and Behl, 2000). Abnormal forms of Aβ protein lead to the neuronal impairment and neuronal death following the neurodegeneration of AD patients. Development of the brain neurodegeneration could be prevented by compounds which inhibit β-protein formation, *e.g.*, neurotrofins, non-steroidal anti-inflammatories (NSAD), and alumina chelatic compounds. It has been reported previously that nicotine also prevents Aβ aggregation due to specific binding to the 1–28 region of the peptide (Salomon *et al*., 1996; Moore *et al*., 2004). Cotinin, the main metabolite of nicotine in human body, also decreases this process (Salomon *et al*., 1996; Wang *et al*., 2000). Among compounds tested, the indole alkaloids that exist in the bark of *Uncaria tomentosa* appear potentially active agents to prevent β-amyloid aggregation. In the course of many pharmacological researches, the interaction of alkaloids from *Uncaria tomentosa* with Aβ protein has been proved by *in vitro* and *in vivo* tests. Also the amyloid inhibitory activity of *Uncaria tomentosa* alkaloids was reported (Castillo and Snow, 2003; Castillo *et al*., 2005). Potential therapeutic strategies could explore interactions such as indole alkaloids-Aβ protein in preventing or reversing the initial conformational change of Aβ protein to its pathological β-pleated sheet form (Ohara *et al*., 1995). A number of very different methods have been developed to measure the ligand-protein parameters. They are mostly based on the bound and free ligand separation in the condition of equilibrium. Among them there are methods like filtration, precipitation, chromatography which may disturb the mutual equilibrium of the constituents (Busch *et al*., 1997a). Capillary electrophoresis (CE) has become an important technique for studies of the nature and strength of biological interactions (Šoltés, 2004). It can be considered as an ideal technique with the prior use of HPLC-MS during the isolation and identification of the compound of interest.

The aim of this research was to examine the binding of a newly identified oxindole alkaloid (mitraphylline) isolated from *Uncaria tomentosa* to Aβ1-40 protein followed by the analysis with TLC, HPLC-MS and CE methods, respectively. An established binding measurement method, namely the Hummel-Dreyer approach, was applied to estimate the association constant of the mitraphylline-Aβ protein complex.

Experimental

Materials

Synthetic Aβ protein fragment 1-40 was purchased from Research Biochemical International (Natick, MA, USA). Oxindole alkaloid mitraphylline was isolated from the root bark of *Uncaria tomentosa* obtained from Peruvian Institute of Andean Phytotherapy (Lima, Peru).

Potassium phosphate monobasic (KH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were from Fluka (Buchs, Switzerland). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were from POCH (Gliwice, Poland). Acetonitrile of chromatographic quality was from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) was from Sigma-Aldrich (St. Louis, MO, USA). Water was prepared with a Mili-Q system (Millipore, Bedford, MA, USA).

Plant material extraction

1 kg of powdered bark was moistened with distilled water and left for 3 h in an encapsulated vessel. Afterwards, such a moistened bark was put into a mixing rotary extractor. The extraction was carried out three times during 3 h with the use of 1,1,2-trichloroethylene at a temperature of 35–40 °C, where 9 L of 1,1,2-trichloroethylene were needed for each extraction. The obtained extracts were then combined and distilled at reduced pressure. The dry remains were dissolved in methanol at 40–45 °C. A precipitate appeared after cooling the solution to room temperature and was then filtered. Next it was rinsed through a filter with methanol. The filtrate, now lacking lipophilic substances, was condensed and the obtained precipitate was recrystallized in boiling methanol. The compound gained in the form of white crystal needles (50 mg) was left for further identification studies.

Sample preparation prior to binding measurements with the use of capillary electrophoresis method

Lyophilized Aβ protein was kept frozen at –20 °C. Before the experiments the sample of Aβ protein was warmed up to room temperature. 1 mg of Aβ protein was dissolved in 1 mL of dis-

tilled and deionized water. Then the solution of Aβ protein was divided into 0.1 mL portions at a concentration of 230 μM and frozen. For the binding procedure Aβ protein at a concentration of 46 μM was prepared using 0.022 M phosphate buffer, pH 5. 6.

The sample of 2.3 mg mitraphylline was dissolved in 3 mL of methanol. Then several dilutions of mitraphylline within the concentration range from 10 to 62.5 μM were prepared in 0.022 M phosphate buffer, pH 5.6.

Thin-layer chromatography experiments

TLC experiments were performed on precoated silica gel Si 60 F_{254} glass plates, ready-made by Merck. Chromatograms on $10 \times 10 \times 0.2$ cm plates were developed to a distance of 9.0 cm in a horizontal DS chamber (Chromdes, Lublin, Poland) at room temperature. The plates were spotted with 5–15 μL of 0.5% solutions of extract and examined fraction in methanol. The mobile phases used for normal-phase chromatography were chloroform/acetone 5:4 (v/v) (mobile phase I), chloroform/ethanol 95:5 (v/v) (mobile phase II) and ethyl acetate/isopropanol/ammonia 100:2:1 (v/v) (mobile phase III). The developed plates were dried in the air at room temperature. Chromatograms were visualized by illumination with UV light (λ = 254 nm) and after treatment with Dragendorff reagent they gave orange or brown, usually stable bands in visible light.

HPLC-MS analysis

Chromatographic separation was done on a Shimadzu HPLC (Shimadzu, Kyoto, Japan) system consisting of two pumps (LC-10 AD and LC-10 AD_{VP}), UV detector (SPD-10 A), autosampler (SIL-10 AD_{VP}), column oven (CTD-10 AD_{VP}) and system controller (SCL-10 AD_{VP}) with a 35 μL injection loop. The alkaloid was separated on a LiChrospher RP (5 μm) column, 250×4 mm I. D. (Merck) prior to MS analysis. The separation was performed with the use of isocratic elution with a mobile phase comprised of water with the addition of 0.1% TFA and acetonitrile with the addition of 0.1% TFA in the proportion of 90:10 (v/v). The elution was carried out at room temperature with a flow rate of 1 mL/min and UV detection set at 254 nm.

Confirmation of the identity of the alkaloid of interest was done with the use of electrospray ionization mass spectrometry (ESI-MS). Mass spectra in ESI-MS method were recorded on a Finningan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray (ESI) ion source. The ion spray needle was maintained at 4.5 kV. Nitrogen gas was used to evaporate the solvent from the charged droplets.

Capillary electrophoresis analysis

Capillary electrophoresis was performed on a Waters Quanta 4000E capillary electrophoresis system (Millipore Corp., Milford, MA, USA). Uncoated fused-silica capillaries from J&W Scientific Inc. (Folsom, CA, USA) of 50 μm I. D., 375 μm O. D. and 61 cm total length were used. All runs were at a fixed temperature of 20 °C in a 0.022 M phosphate buffer, pH 5.6, with an applied voltage of 20 kV. Samples were introduced by means of the 10 s hydrostatic injection method. The detection was at the cathodic end. The wavelength of the UV detector was adjusted to 254 nm.

Before each electrophoretic experiment the capillary was flushed for 2 min with 0.1 M HCl and 0.1 M NaOH, following for another 4–6 min with 0.022 M phosphate buffer, pH 5.6. The buffer solution was filtered through Minisart 0.2 μm filters from Sartorius AG (Göttingen, Germany).

Procedures and calculations

The 0.022 M phosphate buffer, pH 5.6, was prepared daily by mixing appropriate amounts of KH_2PO_4 and Na_2HPO_4 in 250 mL distilled water. Aβ protein sample was stored at –20 °C. The Aβ protein and mitraphylline samples were diluted with 0.022 M phosphate buffer, pH 5.6, prior to the analysis.

To calculate properly the binding parameters of mitraphylline alkaloid, 5–6 data points of the mitraphylline-Aβ isotherm are required. In all studies the Aβ concentration was kept constant at the level of 46 μM. The mitraphylline concentration was varied according to the following scheme: 10, 15, 20, 30, 50, 62.5×10^{-6} M.

The data obtained experimentally were subjected to the analysis by the Scatchard method (Table I). Scatchard method is a commonly used strategy to determine binding parameters in various drug-protein systems. It is based on the measurement of the total fraction of drug bound by a fixed amount of protein, *r*, as a function of the concentration of the drug that remains free in the

Table I. Experimental data along with the binding parameters of the mitraphylline-Aβ protein complex used in the studies.

C_d	A_p	A_c	$A_p - A_c$	D_b	D_f	r	r/D_f
15	127	81	46	8.52E-06	6.48E-06	0.1852	28571
20	387	252	135	1.07E-05	9.29E-06	0.2329	25084
25	178	103	75	1.82E-05	6.80E-06	0.3957	58230
30	494	267	227	2.55E-05	4.49E-06	0.5545	123370
50	801	501	300	2.99E-05	2.01E-05	0.6509	32446
62	1115	751	364	3.01E-05	3.19E-05	0.6533	20447

C_d, total ligand concentration [$\times 10^{-6}$ M].
A_p, peak area after the injection of protein.
A_c, peak area after the injection of buffer.
D_b, concentration of the bound ligand to the protein [$\times 10^{-6}$ M].
D_f, concentration of the free ligand [$\times 10^{-6}$ M].
r, mean number of drug molecules bound per protein molecule.
r/D_f, binding measurement parameter.

solutions, D_f. Taking into consideration the descending, linear part of the Scatchard curve, the apparent association constant, K (M^{-1}), of mitraphylline-Aβ complexes was determined (Fig. 2).

Results and Discussion

Alkaloid identification

Basing on TLC chromatography and HPLC-ESI-MS analysis, it has been stated that the compound isolated from *Uncaria tomentosa* is mitraphylline, which is a pentacyclic oxindole alkaloid. After the HPLC-ESI-MS experiment, MS spectrum analysis allowed to identify unambiguously the molecular ion signal at m/z = 368.9. An intensive fragmented ion occurs during the disintegration of the alicyclic part of the alkaloid. Consequently loosing a methyl group at C-19, it forms a succeeding ion.

Binding measurements

The description of reversible drug-protein binding was based on the multiple equilibrium theory (Klotz and Hunston, 1971; Feldman, 1972) expressed by the following equation:

$$r = \frac{[D_b]}{[P_{total}]} = \sum_{i=1}^{m} n_i \frac{K_i[d_f]}{1 + K_i[D_f]}, \quad (1)$$

where r is the mean number of drug molecules bound per protein molecule, m is the number of classes of independent binding sites, $[D_b]$, $[D_f]$ and $[P_{total}]$ are the concentration of bound drug, free drug and total protein, respectively, n_i is the number of binding sites of class i and K_i is their corresponding association (binding) constant.

Binding constant measurements were based on the Hummel-Dreyer method (Hummel and Dreyer, 1962). In electrophoretic applications of that method (Busch *et al*., 1997b), the capillary was filled with a buffer containing the drug. Then a small amount of sample, which contained the buffer, the drug and the protein, was injected into the capillary. When the electrophoresis started, the components of sample plug migrated with their own mobility in the direction of the detector. On the electropherogram (Fig. 1b) a positive peak appeared which corresponded to the ligand-protein complex and to the free protein. The negative peak emerged at the migration time of the ligand and the area of that peak was directly related to the amount of ligand bound to the protein, $[D_b]$ (Busch *et al*., 1997b). The amount of bound ligand was quantified from an external calibration (Sebille *et al*., 1978; Kraak *et al*., 1992).

In the external calibration method, calculations were based on differences of the total area obtained upon injecting the protein sample and the total area obtained when plane buffer was injected. The concentration of the free ligand, $[D_f]$, could be set as equal to the concentration of the ligand in the buffer, C_d ($C_d = [D_f]$) (Busch *et al*., 1997b; Kraak *et al*., 1992). Hence, the following relationship holds:

$$[D_b] = \left(\frac{A_p - A_e}{A_e}\right) C_d, \quad (2)$$

where A_p is the area of the sample peak, A_e is the area of the buffer peak.

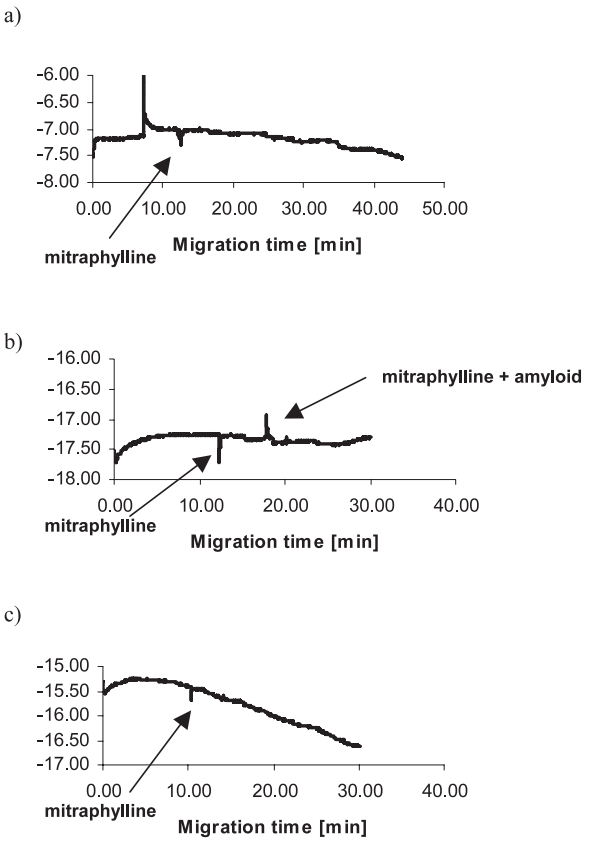

Fig. 1. Electropherograms: a) mitraphylline before the application of the Hummel-Dreyer method (buffer without mitraphylline; mitraphylline injected); b) mitraphylline and mitraphylline-Aβ protein complex after the application of the Hummel-Dreyer method (buffer with mitraphylline at the certain concentration; amyloid at the fixed concentration injected); c) mitraphylline after the application of the Hummel-Dreyer method (buffer with mitraphylline at the certain concentration).

Adsorption of the proteins onto the fused-silica capillary wall may influence the determination of the binding parameters. Adsorption of the proteins resulted from the attractive electrostatic interactions between positively charged regions of the proteins and negative charges present on the fused-silica capillary wall. This led to poor peak shapes, low resolution, incomplete recoveries and irreproducible separations. The deformed protein peaks presented in electropherograms (Fig. 1) could not be attributed only to adsorption and arose due to the slow kinetics of the complexation as was also confirmed by Busch *et al.* (1997b) and Avila *et al.*, (1993). Especially in the Hummel-Dreyer method, the multiple or deformed positive peaks due to protein and protein-ligand complex were not of interest because in calculations only the areas of negative peaks were considered (Sebille *et al.*, 1978).

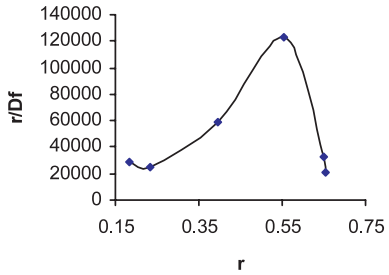

Fig. 2. Scatchard plot of the experimental data for the mitraphylline-Aβ protein complex obtained by the Hummel-Dreyer method.

The Scatchard plot (r/D_f *vs.* r) derived from the experimental data for mitraphylline and Aβ protein binding complex measured by the Hummel-Dreyer method is given in Fig. 2. The shape of that Scatchard plot suggests one class binding character of the analyzed interaction. Binding constants of mitraphylline and alkaloid-Aβ protein have been estimated based on the slopes of linear regression equations derived from the descending three data points of the Scatchard plot. Hence, the binding constant for the mitraphylline-Aβ protein complex is $K = 9.95 \times 10^{-5}$ M^{-1}.

Fig. 3 shows a typical adsorption isotherm for the corresponding Scatchard plot as measured by the Hummel-Dreyer method for the interaction between the mitraphylline alkaloid and the Aβ protein. As it can be seen, the isotherm looks accept-

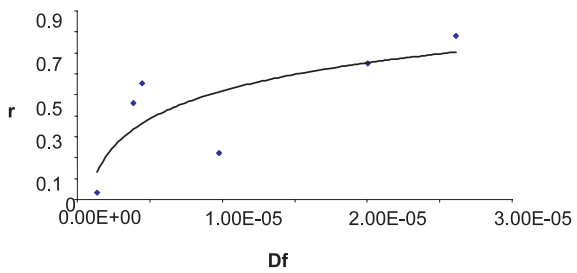

Fig. 3. Binding isotherm of the mitraphylline-Aβ protein complex obtained by the Hummel-Dreyer method.

able but the points, especially at higher *r* values, do not fit the non-linear regression line. It can be supposed that this feature reflects non-specific interactions between mitraphylline and the Aβ protein complex.

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