Journal of Chromatography B, 879 (2011) 1781–1788

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Online magnetic bead based dynamic protein affinity selection coupled to LC–MS for the screening of acetylcholine binding protein ligands

Lionel Pochet[a,1], Ferry Heus[a], Niels Jonker[a], Henk Lingeman[a], August B. Smit[b], Wilfried M.A. Niessen[a], Jeroen Kool[a,*]

[a] BioMolecular Analysis, Department of Chemistry and Pharmaceutical Sciences, Faculty of Sciences, VU University Amsterdam, De Boelelaan 1083, 1081HV Amsterdam, The Netherlands
[b] Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, Neuroscience Campus Amsterdam, VU University, The Netherlands

ARTICLE INFO

Article history:
Received 1 March 2011
Accepted 19 April 2011
Available online 28 April 2011

Keywords:
Acetylcholine binding protein (AChBP)
Affinity selection mass spectrometry
Screening assay
Ligand chromatography
Magnetic beads

ABSTRACT

A magnetic beads based affinity-selection methodology towards the screening of acetylcholine binding protein (AChBP) binders in mixtures and pure compound libraries was developed. The methodology works as follows: after in solution incubation of His-tagged AChBP with potential ligands, and subsequent addition of cobalt (II)-coated paramagnetic beads, the formed bead-AChBP-ligand complexes are fetched out of solution by injection and trapping in LC tubing with an external adjustable magnet. Non binders are then washed to the waste followed by elution of ligands to a SPE cartridge by flushing with denaturing solution. Finally, SPE-LC–MS analysis is performed to identify the ligands. The advantage of the current methodology is the in solution incubation followed by immobilized AChBP ligand trapping and the capability of using the magnetic beads system as mobile/online transportable affinity SPE material. The system was optimized and then successfully demonstrated for the identification of AChBP ligands injected as pure compounds and for the fishing of ligands in mixtures. The results obtained with AChBP as target protein demonstrated reliable discrimination between binders with pK_i values ranging from at least 6.26 to 8.46 and non-binders.

1. Introduction

Over the last decade, mass spectrometry (MS) has proved to be a valid technique for the screening of receptor ligands [1–3]. Despite the lower throughput when compared to platereader methodologies traditionally used in high throughput screening (HTS), MS offers several advantages: direct structure elucidation of unknown hits and analysis of bioactive mixtures. Furthermore, the ability to develop "label free" assays makes MS assays widely applicable in screening approaches. Indeed, ligand–receptor complexes can be detected directly, or indirectly after disruption of the ligand–receptor complexes, followed by analysis of the ligands released.

Several distinct MS approaches towards screening of bioactives have been developed allowing the evaluation of protein–ligand binding by MS [4]. (Pulsed) ultrafiltration technologies utilize a target protein in an ultrafiltration chamber [5]. Injection or infusion of ligands results in binding to the target. Subsequent washing, disruption and direction to MS allows analysis of the binders [6]. Separation of ligands bound to a target protein can also be accomplished by rapid size exclusion chromatography [7–9]. After the separation step, a disruption step follows, allowing LC–MS analysis of the released ligands [10]. In direct methods, distinction between ligands and non-binders is monitored directly in the MS by measurement of the protein–ligand complex in the gas phase [11]. Powerful approaches are based on immobilization of the target protein onto a solid support, e.g., affinity chromatography or affinity selection MS. In affinity chromatography, the target protein is immobilized onto a chromatographic column to allow retention of ligands based on their affinity [12–14]. Detection of eluting ligands usually occurs with MS. In affinity selection MS screening techniques, the binding of ligands occurs to immobilized protein targets on a solid support. After washing away non binders, the protein–ligand complex is disrupted and the ligands

Abbreviations: AChBP, acetylcholine binding protein; nAChRs, nicotinic acetylcholine receptors; IMAC, immobilized metal affinity chromatography; HTS, high throughput screening; PBS, phosphate buffered saline; BR, ELISA blocking reagent; ESI, electrospray ionisation; HOAc, acetic acid; TFA, trifluoroacetic acid; EIC, extracted ion chromatogram; LC, liquid chromatography; HPLC, high performance liquid chromatography; SPE, solid phase extraction; NMR, nuclear magnetic resonance; MS, mass spectrometry.

* Corresponding author. Tel.: +31 205987542; fax: +31 205987543.
E-mail addresses: lionel.pochet@fundp.ac.be (L. Pochet), f.a.h.heus@vu.nl (F. Heus), n.jonker@isala.nl (N. Jonker), l.lingeman@vu.nl (H. Lingeman), guus.smit@cncr.vu.nl (A.B. Smit), w.m.a.niessen@vu.nl (W.M.A. Niessen), j.kool@vu.nl (J. Kool).
[1] Visiting scientist from Drug Design and Discovery Center, FUNDP (University of Namur), 61, rue de Bruxelles, 5000 Namur, Belgium.

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.04.023

are analyzed by LC–MS analysis [15]. Modified affinity selection MS approaches developed in our group include dynamic affinity selection MS where the target-ligand incubation occurs in solution followed by capturing of the His-tagged target protein by an immobilized metal affinity column (IMAC) [16]. After immobilization, the procedure is similar to traditional affinity selection MS methodologies. The advantages here are that the incubation occurs under native target conditions in solution and that new target protein is used for every measurement. In recently published work, we utilized the last mentioned approach with immobilized metal affinity chromatographic (IMAC) paramagnetic beads (referred to as 'magnetic beads') as solid support material and the His-tagged estrogen receptor (ligand binding domain) as the target protein [4]. The methodology employs in vial and solution based formation of estrogen receptor–ligand complexes followed by addition of magnetic beads. This in turn results in binding of the (His-tagged) receptor–ligand complexes to the beads. The resulting suspension is injected into and transported online through the analytical system for analysis. The use of a strong movable magnet allows retention and washing of the beads in the tubing on demand and eluting the bound ligands by a disruption step.

In the present study, we applied the magnetic beads based approach to the acetylcholine binding protein (AChBP), a structural analog of the extracellular ligand binding domain of the α7 nicotinic acetylcholine receptor (nAChRs) [17,18] originating from the snail *Lymnaea Stagnalis*. After its crystal structure was published [19], it has become a model for nAChRs to efficiently screen compound libraries for potential ligands and for structure based synthetic approaches in medicinal chemistry [20]. The nAChR family is intensively studied in relation to its potential as pharmaceutical target against epilepsy, Alzheimer's disease, pain relief, Parkinson's disease, anxiety and cognitive and attention deficits [17,18,21–23].

For our ligand fishing approach towards the AChBP, ligands were first incubated with His-tagged AChBP. Cobalt (II) coated paramagnetic (IMAC) affinity beads were then added and allowed to bind to the AChBP. The samples were subsequently injected in an integrated solid phase extraction liquid chromatography mass spectrometry (SPE LC–MS) system in order to perform the online isolation of the AChBP-ligand complex, removal of non-binders, and the elution of the ligands from the magnetic beads for analysis by SPE LC–MS. Finally, the magnetic beads were released to the waste prior to analysis of the next sample.

2. Experimental

2.1. Chemical and biological reagents

AChBP (from species *Lymnaea stagnalis*) was expressed from baculovirus using the pFastbac I vector in Sf9 insect cells and purified from the medium as described by Celie et al. [18]. ELISA blocking reagent (BR) was obtained from Hoffmann-La Roche (Mannheim, D). Glycine·HCL, dextromethorphan hydrobromide, desipramine hydrochloride, cetirizine dihydrochloride, sulfamethoxypyridazine, phenacetin, (±)epibatidine dihydrochloride hydrate, diclofenac sodium salt, potassium dihydrogen phosphate, disodium monohydrogen phosphate, acetic acid (HOAc), trifluoro acetic acid (TFA) and trizma base were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Sodium chloride and ammonium hydrogen carbonate were from Riedel de Haen (Seelze, Germany). LC–MS grade methanol (MeOH; 99.95%) was purchased from Biosolve (Valkenswaard, The Netherlands). respectively. HPLC grade water was produced using a Milli-Q purification system from Millipore (Amsterdam, The Netherlands). 1 μm Cobalt loaded Dynabeads TALON (His-Tag Isolation & Pulldown) were purchased from Dyanal, Invitrogen (Breda, The Netherlands) and supplied in an ethanol/water (20:80) solution. All ligands were synthesized in house by the VU University Medicinal Chemistry department [20] and are detailed in the Supporting Information. All in house synthesized ligands used are depicted as compound (cmpd) **1**–**7** while the non-binders are depicted by their trivial name. Only, the high affinity ligand epibatidine used for the displacement reactions is also named by its trivial name.

2.2. Instrumentation

2.2.1. SPE symbiosis

All SPE experiments were carried out on a Symbiosys Pharma (Spark Holland, Emmen, The Netherlands) sample pre-treatment system. The SPE cartridge used was a Hysphere C18HD (Silica based C18 with high density end-capping, 2 mm × 10 mm; particle size 7.5 μm, adsorbent mass 18.5 mg, carbon content 14.5%) from Spark Holland. The cartrigde was systematically and preventively replaced after 30 injections. In these conditions, no change in the recovery was observed.

2.2.2. LC–MS

The LC–MS system consisted of a high-pressure gradient LC system (Shimadzu LC20, 's Hertogenbosch, the Netherlands) coupled to a Thermo Electron LCQ Deca ion trap MS (Breda, The Netherlands) equipped with an electrospray ionization (ESI) probe. The LC–MS system was operated at 200 μL/min. Solvent A consisted of water/MeOH 99:1 and 0.1:0.02% HOAc/TFA. Solvent B consisted of water/MeOH 1:99 and 0.1:0.02% HOAc/TFA. For separation, an XBridge 3.5 μm particle C18 analytical column (100 mm × 2.1 mm i.d.; Waters, Milford, MA, USA) was used. Gradient LC elution was applied by running a 1.5 min isocratic elution at 30% B, then rising to 95% B in 10 min. The autosampler was set to 4 °C. The MS was operated in positive electrospray ionization mode. N_2 was used as a sheath gas (60 psi) and auxiliary gas (20 psi), the needle voltage was 5000 V and the heated capillary was at 250 °C with the capillary voltage set at 17 V.

2.3. Procedures

2.3.1. Receptor ligand incubation

The composition of the binding buffer used for receptor ligand reaction was 1 mM KH_2PO_4, 3 mM Na_2HPO_4, 0.16 mM NaCl and 20 mM Trizma base pH 7.5 with 0.5 mg/mL ELISA BR. The binding experiments were performed by incubating 20 μL of test compound (10^{-6} M) with 15 μL of AChBP (321 ng/μL, ∼12 μM) in 185 μL of binding buffer for 15 min at room temperature. A 10 μL magnetic bead suspension (20 mg/mL) was added to the mixture. After 5 min, the resulting suspension was placed in the autosampler of the symbiosis at 4 °C for further analysis. The same procedure was followed for the competitive experiments, except that the 15 min incubations were done with 20 μL of the potent AChBP ligand epibatidine (10^{-5} M; pK_i ∼ 9.00), 20 μL of test compound (10^{-6} M) and 15 μL of AChBP (321 ng/μL; ∼12 μM) in 185 μL of binding buffer.

2.3.2. Magnetic beads handling

A 20 mg/mL magnetic bead suspension was prepared according to the recent paper of Jonker et al. [4]. In brief, 120 μL of bead suspension was washed three times with 400 μL binding buffer and finally resuspended in 240 μL of binding buffer resulting in a concentration of 20 mg/mL. The trapping of the magnetic beads was achieved in PEEK tubing (0.25 mm internal diameter) by a 50 N 1.4 T permanent neodymium magnet with dimension of 7 cm × 4 cm × 3.5 cm. In order to trap and release the beads, the PEEK tubing was attached to an in house built, pneumatically driven aluminum arm. By moving the arm either in close proximity of or 2.6 cm away from the magnet, the setup allowed to trap and