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Development of surface plasmon resonance biosensor assays for primary and secondary screening of acetylcholine binding protein ligands

Kim Retra [a,*], Matthis Geitmann [b], Jeroen Kool [a], August B. Smit [c], Iwan J.P. de Esch [d], U. Helena Danielson [b,e], Hubertus Irth [a]

[a] Leiden/Amsterdam Center for Drug Research, Division of Biomolecular Analysis, Faculty of Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands
[b] Beactica, 75237 Uppsala, Sweden
[c] Department of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands
[d] Leiden/Amsterdam Center for Drug Research, Division of Medicinal Chemistry, Faculty of Sciences, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands
[e] Department of Biochemistry and Organic chemistry, Uppsala University, Box 576, SE-751 23, Uppsala, Sweden

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ABSTRACT

Surface plasmon resonance (SPR) biosensors recently gained an important place in drug discovery. Here we present a primary and secondary SPR biosensor screening methodology. The primary screening method is based on a direct binding assay with covalent immobilized drug target proteins. For the secondary screening method, a sequential competition assay has been developed where the captured protein is first exposed to an unknown test compound, followed directly by an exposure to a high-molecular-weight reporter ligand. Using the high-molecular-weight reporter ligand to probe the remaining free binding site on the sensor, a significant signal enhancement is obtained. Furthermore, this assay format allows the validation of the primary direct binding assay format, efficiently revealing false positive data. As a model system, acetylcholine binding protein (AChBP), which is a soluble model protein for neuronal nicotinic acetylcholine receptors, has been used. The secondary assay is lower in throughput than the primary assay; however, the signal-to-noise ratio is two times higher compared with the direct assay, and it has a z' factor of 0.96. Using both assays, we identified the compound tacrine as a ligand for AChBP.

Introduction

Surface plasmon resonance (SPR)[1] spectroscopy is a sensitive detection technology that has become a powerful tool for the analysis of biomolecular interactions such as antigen–antibody, ligand–receptor, DNA–protein, and enzyme–inhibitor [1]. The underlying principle is to immobilize one component of a molecular interaction system and thereafter monitor the binding of the complementary partner. SPR systems combined with miniaturized flow systems for efficient sample and buffer delivery to the sensor surface permit continued monitoring of formation and dissociation of complexes. Due to the high sensitivity of modern SPR biosensors, the technique is nowadays implemented during all phases of the drug discovery process. For primary screening, the experimental design generally involves immobilization of the target and the test compounds are used as analytes [2–4]. Alternatively, SPR imaging of sensor surfaces with immobilized test compounds can also be employed [5]. SPR biosensors typically have been used for secondary screening, validating hits from conventional high-throughput screens using fluorescence or radioactive readout techniques [6]. In general, three experimental setups have been used for hit validation: the direct assay [6], the competition assay [7,8], and the inhibition assay [9,10]. The setup used for the direct assay is the same as that used for the primary screen. In a competition assay setup, the test compound is mixed with a known ligand, after which the mixture is injected onto the immobilized protein. The competition assay has two advantages over the direct assay. First, a strong enhancement of the signal is obtained by using a high-molecular-weight reporter molecule [8]. Second, the assay is selective for a certain binding pocket and, therefore, can be used as a follow-up assay after a direct screening [4]. The sensitivity of the competition assay is dependent on the affinity of the reporter ligand. The inhibition assay is common for immunoassays but is also used in receptor–ligand interaction studies. A mixture of the receptor and the test compound is incubated and thereafter flushed over an immobilized high-affinity ligand. One of the

* Corresponding author. Fax: +31 205987543.
E-mail address: k.retra@few.vu.nl (K. Retra).

[1] *Abbreviations used:* SPR, surface plasmon resonance; nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine binding protein; LBD, ligand binding domain; BgTx, α-bungarotoxin; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; Ls, *Lymnaea stagnalis*; Ac, *Aplysia californica*; SDS, sodium dodecyl sulfate; ERα–LBD, estrogen receptor alpha ligand binding domain; EDC, *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide; NHS, *N*-hydroxysuccinimide; RU, resonance units; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; RBS, reporter binding slope; nAChR, nicotinic acetylcholine receptor.

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drawbacks of this method is that both compounds are added simultaneously, and this can be unfavorable for low-affinity compounds.

In this article, we describe two SPR biosensor methodologies that have successively been used for the screening for ligands of nicotinic acetylcholine receptors. Neuronal nicotinic acetylcholine receptors (nAChRs) are a diverse family of pentameric ion channels with a wide distribution throughout cells of the nervous and immune systems. The heterogeneity of the native nAChR populations in the central nervous system presents major possibilities as well as challenges in terms of developing therapeutics targeted at these receptors. The fact that several important physiological processes appear to be regulated by one or more nAChR subtypes makes it possible to target specific functions without affecting other aspects of cholinergic neurotransmission [11,12].

Acetylcholine binding protein (AChBP) is homologous to the ligand binding domains (LBDs) of nAChRs. Pharmacological characterization has demonstrated that their properties most closely resemble those of the α7 nAChRs, which also function as homopentamers. AChBPs have been identified from various snails, including *Lymnaea stagnalis* and *Aplysia californica*; they lack the domain to form a transmembrane ion channel and, therefore, are secreted soluble proteins [13,14]. When AChBP is coupled to the pore domain of an ion channel, a functional chimeric receptor is formed [15].

Here we describe a high-throughput primary screening assay and a follow-up secondary screening assay to identify AChBP ligands. The same SPR biosensor platform was used for both assays. The primary direct binding assay involved the covalent immobilization of AChBP and was designed to be low in cost (relatively fast with low protein consumption), whereas the second assay was designed to be highly sensitive and selective for ligand binding to a defined site. The hit validation assay was based on the reversible capturing of a His-tagged protein on a sensor chip loaded with Ni(II). Subsequently, the test compound was allowed to bind to the receptor, followed by the binding of a reporter ligand. The amount of reporter molecule that binds to the receptor decreases as a function of the amount of test ligand bound in the first step. Consequently, the biosensor signal obtained for the reporter molecule is directly related to the affinity of the test compound for the receptor.

Materials and methods

Materials

Acetylcholine, acetylsalicylic acid, adenosine, ampicillin, epibatidine, epicatechin, estrone, hexestrol, ibuprofen, lidocaine, malic acid, nicotine (racemate), reserpine, sulfamethizole, tacrine, thymidine, α-bungarotoxin (BgTx, batch nos. 017K4062 and 056K4113), Trizma base, Ni(II)–chloride hexahydrate, Hepes, and sodium hydrogen phosphate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride and potassium dihydrogen phosphate were obtained from Riedel–deHaën (Seelze, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from AppliChem (Darmstadt, Germany), and dimethyl sulfoxide (DMSO) was purchased from Fluka (Buchs, Switzerland). HBS-P+, surfactant P20, an amine coupling kit, CM5 chips, and NTA chips were obtained from GE Healthcare (Uppsala, Sweden). Ethanol was purchased from Merck (Darmstadt, Germany).

VUF 10674 was kindly provided by Ewald Edink (Drug Discovery Center, Vrije Universiteit Amsterdam).

Protein expression and purification

HexaHis–*L. stagnalis*–acetylcholine binding protein (Ls–AChBP) and hexaHis–*A. californica*–acetylcholine binding protein (Ac–AChBP) were expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Secreted protein was trapped on a HIS-Select Cartridge (Sigma). The cartridge was washed with 24 ml of 10 mM imidazole, left overnight at 4 °C in 250 mM imidazole, and thereafter eluted with 5 ml of 250 mM imidazole. The concentration of imidazole was decreased by using a Slide-A-Lyzer Dialysis Cassette (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations to strongly decrease. The purity of the protein was checked on a sodium dodecyl sulfate (SDS) gel, and the protein concentration was determined by Bradford analysis.

Estrogen receptor alpha ligand binding domain (ERα–LBD) was obtained as described previously [16].

Primary direct binding assay

All experiments were preformed with a Biacore T100 instrument using CM5 sensor chips. A phosphate buffer with 5% DMSO (1.06 mM KH_2PO_4, 2.97 mM Na_2HPO_4, 0.16 mM NaCl, 0.005% [v/v] surfactant P20, 5% DMSO, pH 7.4, 0.22 μm filtered) was used as running buffer. Ls–AChBP was amine coupled to the sensor chip with *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) using 10 mM NaAc (pH 5.0) as a coupling buffer and phosphate buffer without DMSO as a running buffer. Typical immobilization levels were approximately 3000 resonance units (RU). The first flow channel was left unmodified, and Ls–AChBP was immobilized in the second flow channel. All compounds were dissolved in pure DMSO to form 10-mM stock solutions. The assay was run in a 96-well plate format. Compounds were prepared in running buffer and tested in triplicates at a concentration of 10 μM. The flow rate was 90 μl/min, and the analysis temperature was 25 °C. Test compounds, positive controls, and negative controls were injected in a cycle with a contact time of 60 s and a dissociation time of 90 s, followed by an extra wash of the flow system of 50% DMSO. The assay was preceded by three times priming the system with running buffer and by three start-up cycles with injection of running buffer.

Secondary indirect binding assay

SPR experiments were performed at 20 °C on a BIAcore T100 (GE Healthcare) with a flow rate of 30 μl/min except where indicated differently. A certified nitrilotriacetic acid (NTA) sensor chip was used together with a phosphate-buffered saline (PBS)–Tris buffer as running buffer (1.06 mM KH_2PO_4, 2.97 mM Na_2HPO_4, 0.16 mM NaCl, 20 mM Trizma base, 0.005% [v/v] surfactant P20, pH 7.4, 0.22 μm filtered). The sample compartment temperature was 4 °C, and the system was primed three times, followed by four start-up cycles before the beginning of each experiment. The design of the biosensor assay is shown in Fig. 1. The sensor chip was loaded with Ni^{2+} by passing a 500-μM solution of $NiCl_2$ over the surface during 60 s. Protein was diluted in HBS-P+ buffer (10 mM Hepes, 150 mM NaCl, 0.05% [v/v] surfactant P20, pH 7.4, 0.22 μm filtered) and captured during a 360-s injection with a flow of 5 μl/min, followed by a stabilization period of 120 s. The dual injection command was used to inject the test compound and 5 μM BgTx (both 60 s contact time) directly after each other. The percentage of DMSO in the BgTx injection was adjusted to the percentage of DMSO in the sample injection. The NTA chip was regenerated by an injection of EDTA (0.35 M EDTA, 0.01 M Hepes, 0.15 M NaCl, 0.005% [v/v] P20, pH 8.3, 0.22 μm filtered) for 250 s, followed by a stabilization period of 30 s. The flow system was flushed with two short pulses of 70% ethanol separated by a 60-s buffer injection and followed by a 360-s stabilization period. All injection events were preceded by a pre-dip in running buffer. No Ni^{2+} and protein were injected in the reference channel; all other injections were identical to the analysis channel.

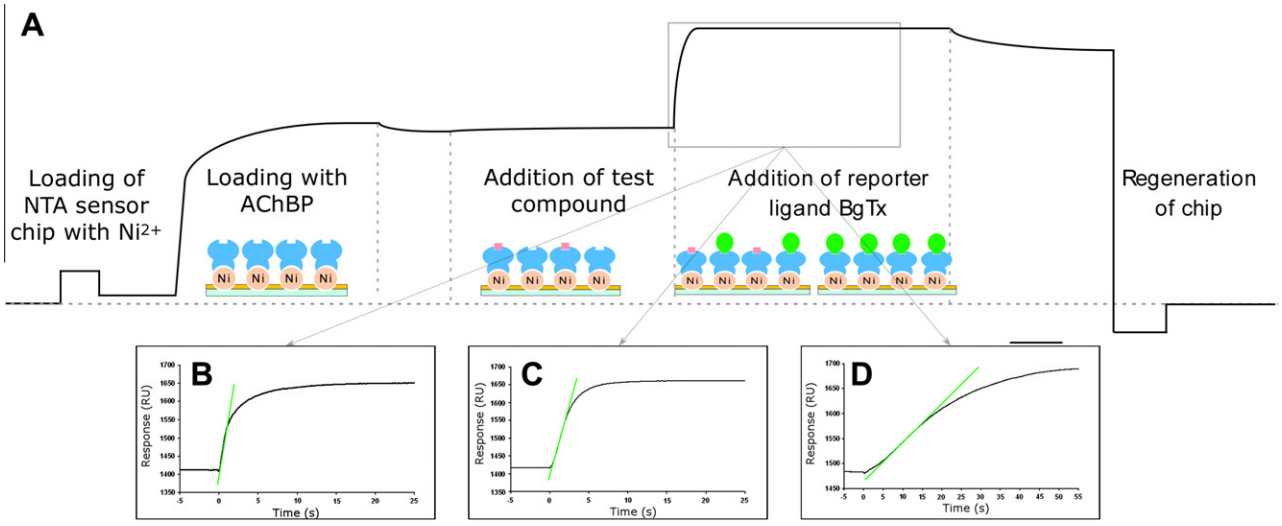

Fig. 1. Setup of the secondary screen and determination of the RBS. First, the NTA chip was loaded with Ni^{2+} (pink circles), after which the AChBP (blue figures) could be captured on the chip. The test compound (pink squares) was injected over the surface and may bind to the AChBP. The reporter ligand (BgTx, green figures) was injected directly after the injection of the test compound. BgTx would first bind to the empty AChBPs and thereafter replace the test compound until all AChBP was bound with BgTx. Finally, the chip surface could be regenerated by injecting EDTA (A). Also shown are determinations of the RBS of estrone (B), acetylcholine (C), and nicotine (D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Determination of the reporter ligand binding slope

The curves of the analysis and the reference channels were subtracted from each other, and the resulting sensorgram was used for the determination of the slope of the binding of the reporter ligand, BgTx (see Fig. 1). The slope was determined for all time points between approximately 10 s before injection of BgTx and 45 s after injection. Slopes were calculated over 5 data points and were assigned to the last time point of the 5 data points. Slopes were approximately 0 before injection of BgTx, but they increased after injection. It was determined where the slope exceeded the value of 5 and remained above 5 for at least 3 data points. The slope of the time point 0.5 s after the slope exceeded 5 was assigned to a reporter binding slope (RBS). Compounds that had a slope value that never exceeded the value of 5 were assigned to an RBS of 5 or else the RBS was determined manually. To manually determine the RBS, this slope was determined in the apparently linear part of the binding curve of BgTx, approximately 0.5 s after the injection of BgTx. The used window was 0.5 s wide (6 data points) for weak affinity ligands and up to 10 s for strong affinity ligands. This slope of the binding of BgTx was strongly influenced by the test ligand and, therefore, was used as a screening parameter. The abbreviation RBS is used in this article to indicate the described screening parameter.

Results

Primary screening assay for AChBP ligands

Ls–AChBP was immobilized on a CM5 chip via amine coupling. A stable surface of approximately 3000 RU was obtained; the same surface could be used for at least 4 days. Ls–AChBP was immobilized in the analysis flow channel, whereas another flow channel was left unmodified to detect nonspecific interactions. The drug-like compound estrone was used as a negative control in the assay. Acetylcholine, a known low-affinity binder for AChBP and the nicotinic acetylcholine receptor (nAChR), was used as a positive control. Both the negative and positive controls were injected every five cycles. A total of 13 drug-like test compounds (acetylsalicylic acid, adenosine, ampicillin, epicatechin, hexestrol, ibuprofen, lidocaine, malic acid, nicotine, reserpine, sulfamethizole, tacrine, and thymidine) were tested in triplicates at 10 μM and randomly injected in triplicates. Among the 13 test compounds, only 1 was a known binder (nicotine); all of the other compounds were unknown to bind to either AChBP or nAChR. The results of the primary screening are shown in Fig. 2.

Hits were detected as compounds giving a signal higher than three times the average of the positive controls plus the standard deviation, as shown by the dotted line in Fig. 2. All compounds below the line were considered as negative in this assay. All injections of the two known binders, acetylcholine and nicotine (B), gave a signal above the hit cutoff. Four other samples were also classified as hits in this assay. Three of them were of the compound tacrine (A), so this compound was considered as a hit and, therefore, was of interest to confirm in a secondary assay. The fourth sample identified as a hit was reserpine (C), but because the other two samples of reserpine were below the hit cutoff, this sample will probably be a false positive due to carryover from nicotine. To validate the hits and negatives, a secondary screening assay was developed.

Secondary screening assay for AChBP ligands

We designed a novel methodology that can be used as a secondary screening assay for hit confirmation using the same SPR biosensor platform as for primary screening. The assay was designed so that the measured binding was directly related to the affinity of the test compound for the receptor, BgTx. The signal is completely mass independent and is selective for the BgTx binding pocket. Promiscuous binders did not influence the following cycles because the protein was refreshed after every cycle. The design of the biosensor assay is shown in Fig. 1 and can be divided into two parts: (i) the capturing of the protein and (ii) the actual two-step sequential assay (binding of the test ligand and subsequent binding of the reporter ligand).

Capturing of hexaHis–Ls–AChBP on NTA chips

Approximately 700–2000 RU of pure hexaHis–Ls–AChBP could be captured on an NTA sensor surface using recommended protocols and the recommended Hepes buffer (HBS-P+). However, Hepes

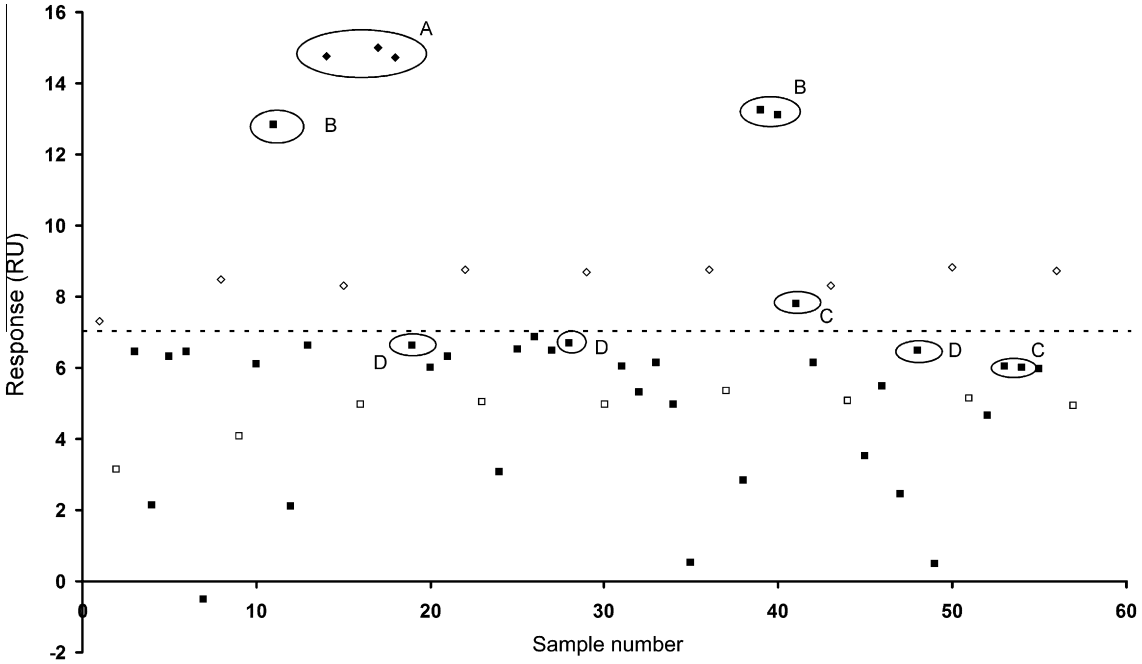

Fig. 2. Identification binders in the primary direct binding screen. Open diamonds: positive controls (acetylcholine); closed diamonds: a known binder (A: nicotine); open squares: negative controls (estrone); closed squares: drug-like molecules (B: tacrine; C: malic acid; D: acetylsalicylic acid).

molecules have been identified in the binding pocket of AChBP [17], which makes buffers containing Hepes less suitable as running buffers. Therefore, PBS–Tris buffer was chosen as an alternative. Unfortunately, protein diluted in this buffer showed approximately 2.5 times lower capturing efficiency (data not shown), making it necessary to dilute the protein in HBS-P+ for capturing, whereas the running buffer was a PBS–Tris buffer. After capturing approximately 1000 RU of AChBP, the drift of the baseline was only between 0 and 15 RU/min.

Binding of BgTx to AChBP

BgTx is an 8-kDa peptide known to bind strongly to AChBP [13]. The interaction was found to have a stoichiometry of 1:1, and the kinetic rate constants were determined as $5.3 \times 10^5\ M^{-1}\ s^{-1}$ (k_{on}) and $4.2 \times 10^{-4}\ s^{-1}$ (k_{off}), resulting in a K_d of 7.9×10^{-9} M, which is in good agreement with the literature [18]. A concentration of 5 μM BgTx was chosen for further experiments due to the very fast occupation of all receptor binding sites. The maximal binding of BgTx on captured AChBP closely approached the theoretical maximal response, which can be calculated via

$$R_{max} = \frac{MW_{analyte}}{MW_{protein}} \cdot R_{protein} \cdot BC,$$

assuming a binding capacity (*BC*) of 1.

Sequential binding of nicotine and BgTx

The two-step sequential approach used to detect the binding of test ligand to Ls–AChBP in an indirect manner was established with nicotine as a positive control. After capturing 650 RU of Ls–AChBP (as described above), 100 μM nicotine or 100 μM estrone (negative control) was injected for 60 s. BgTx was injected immediately thereafter. The RBS (see Materials and methods) was 28 RU/s when a nonbinder was injected and 3 RU/s when nicotine was injected (Fig. 3). The slope of the noise was 0.7 RU/s at most, resulting in a signal-to-noise ratio (*S*/*N*) of 36.

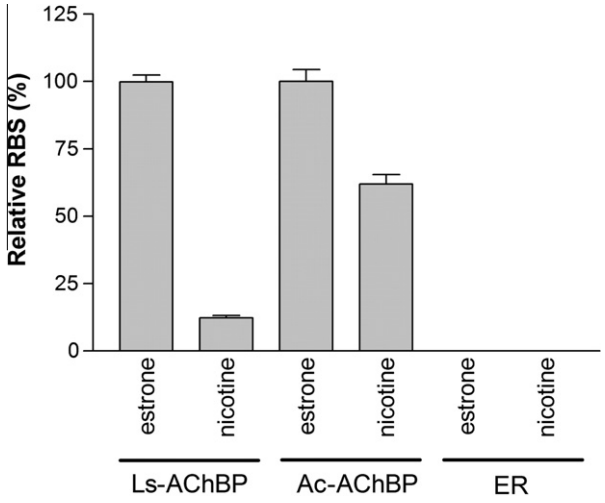

Fig. 3. RBS of BgTx to either Ls–AChBP (650 RU immobilized), Ac–AChBP (350 RU immobilized), or ERα–LBD (3000 RU immobilized) after the binding of 100 μM nicotine or 100 μM estrone. The RBS of BgTx after buffer injection instead of test ligand injection was set at 100% separately for Ls–AChBP and Ac–AChBP.

The same experiment was repeated with hexaHis–Ac–AChBP, resulting in a lower RBS reduction in comparison with Ls–AChBP, in agreement with the literature because it is known that nicotine has a 2.5 times lower affinity for Ac–AChBP than for Ls–AChBP [19]. The experiment was also repeated with hexaHis–ERα–LBD to confirm that BgTx did not bind nonspecifically to the sensor chip surface with a similar protein immobilized. RBS reduction was zero because no binding of BgTx to the protein could be detected (Fig. 3).

Comparison of nicotine detection measured with two SPR assays

The binding of the positive and negative controls nicotine and estrone to AChBP measured with the secondary screen methodology (as shown in Fig. 3) was compared with the binding measured

with the primary screen methodology (Fig. 2). Nicotine (100 μM) and estrone (100 μM) were injected during 60 s over a surface of covalently bound AChBP (see Materials and methods for further details). Signal levels were 1.5 (±0.4) RU after injection of estrone and 9.3 (±0.6) RU after injection of nicotine. The difference in relative signal as compared with the dynamic range of the assay between a binder and a nonbinder was significantly smaller with the direct binding method compared with the coupled method, resulting in a reduced possibility of detecting false negatives. The noise of the direct method was 0.6 RU, resulting in an S/N of 13. Both methods had comparable reproducibilities.

EC_{50} curves of known ligands

EC_{50} curves of three well-known AChBP/nAChR ligands in a broad affinity range were obtained to test the sensitivity and detection range of the method (Fig. 4.). The EC_{50} value of epibatidine could not be determined exactly because the curve showed a sudden drop in RBS at concentrations around 5 nM. Coupled to this drop in RBS was a reduction of the maximum of the total binding of BgTx (data not shown). Therefore, it seems that the sequential assay format caused high-affinity ligands, such as epibatidine, to act as irreversible binders in the used time frame. Nicotine showed an EC_{50} value of 55 nM ($r^2 = 0.96$), and acetylcholine had an EC_{50} value of 7.3 μM in this assay ($r^2 = 0.98$).

Secondary assay with AChBP ligands and drug-like molecules

The developed sequential method was applied for the analysis of the interaction of a small group of compounds for their affinity for Ls–AChBP (Fig. 5): the negative control for the primary assay (estrone), the hit from the primary assay (tacrine), the possibly false positive from the primary assay (reserpine), a drug-like compound that did not give a hit in the primary assay but was very close to the cutoff (acetylsalicylic acid), three well-known AChBP/nAChR ligands (the positive control and a hit from the first assay [acetylcholine, nicotine, and epibatidine] and one relatively new AChBP/nAChR ligand [VUF 10674]). The RBS of BgTx after the binding of acetylsalicylic acid, reserpine, and estrone was indistinguishable from the RBS of BgTx after an injected aliquot of the running buffer. Tacrine, on the contrary, showed a reduction of 23% in RBS. The known AChBP ligands also showed reductions in RBS and were ranked in the same order as they would have been if they had been ranked on the basis of their affinity for AChBP. The new AChBP ligand, VUF 10674, showed a reduction in RBS similar to that of nicotine. This is as expected given that the affinity of VUF 10674 for AChBP measured in a radioligand competition assay is similar to the affinity of nicotine for AChBP (data not shown).

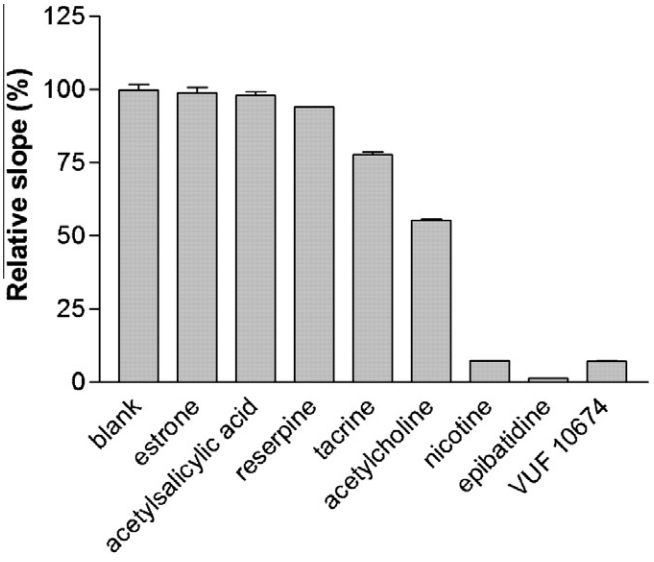

Fig. 5. Secondary screen of eight compounds. Shown are the RBS of BgTx after the binding of 100 μM acetylsalicylic acid, estrone, reserpine, tacrine, acetylcholine, nicotine, epibatidine, and VUF 10674 relative to the RBS of BgTx after injection of an aliquot of running buffer.

Tacrine

Tacrine showed binding to AChBP in both the primary and secondary assays (Figs. 2 and 5). However, tacrine was not previously known as an AChBP ligand. Therefore, it was decided to determine the affinity of this ligand with a classic pharmacological affinity measurement, a radioligand displacement study. In this assay, tacrine showed a pK_i of 4.3, which was lower than the pK_i of acetylcholine, again confirming a lower affinity of tacrine for Ls–AChBP compared with acetylcholine. Acetylsalicylic acid and estrone showed no binding at all to Ls–AChBP in the radioligand competition assay.

We also obtained an EC_{50} curve of tacrine with the presented sequential assay (Fig. 6). The EC_{50} curve of tacrine shows an EC_{50} value of 9.4 μM ($r^2 = 0.99$), which is 2.1 μM higher than the EC_{50}

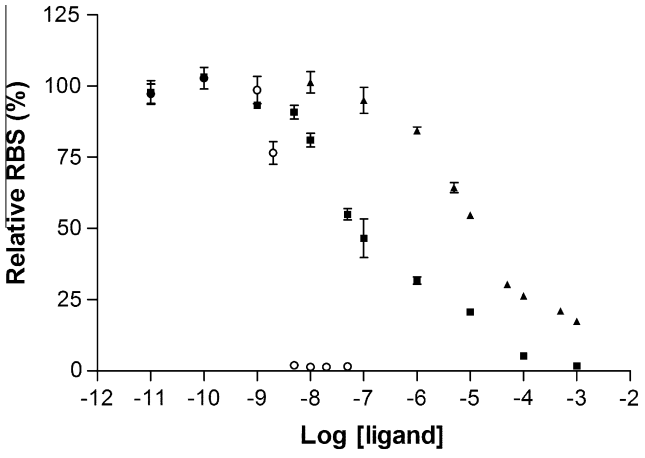

Fig. 4. EC_{50} curves of binding of acetylcholine (triangles), nicotine (squares), and epibatidine (circles) to Ls–AChBP. The RBS obtained after injection of 100 μM estrone is set as 100%.

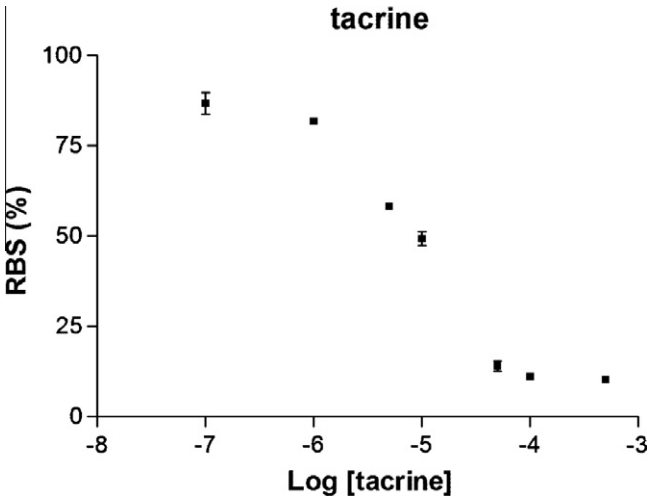

Fig. 6. EC_{50} curve of the binding of tacrine to Ls–AChBP. The RBS obtained after injection of aliquoted buffer is set as 100%.

value of acetylcholine. This lower affinity of tacrine for Ls–AChBP compared with acetylcholine is consistent with the data in Fig. 5 and the data of the radioligand assay.

Discussion

The sensitivity required for detecting interaction with small molecules with low affinities is dependent on the immobilization level, the size of the target molecule (the number of available binding sites per surface area), and the screening concentrations. In the presented primary screening assay, an immobilization of 3000 RU and a compound concentration of 10 μM were sufficient to distinguish between low-affinity binders such as acetylcholine and nonbinding drug-like compounds. Higher concentrations and immobilization levels, however, could easily be applied. One compound, tacrine, was found to be a hit in the primary assay and subsequently was confirmed in the secondary assay, although with a lower affinity than expected from the primary assay.

The method of the secondary assay can be divided into two parts: (i) the capturing of the protein and (ii) the actual sequential assay (binding of the test ligand and the subsequent binding of the reporter ligand). The capturing approach was chosen because of the slow dissociation rate of BgTx. It is less time-consuming to capture new protein each cycle than to wait for the BgTx to completely dissociate from the protein. When the reporter ligand has a faster dissociation rate, the sequential binding assay could also be performed with proteins that are irreversibly bound to the chip. However, refreshing the protein every cycle has the advantage that promiscuous irreversible binders are removed before the next cycle. The covalent coupling of proteins via primary amines is frequently the method of choice for immobilization in SPR assays, generating sensor surfaces with high ligand density and no baseline drift. The affinity of a single histidine tag for the Ni^{2+}–NTA moiety on the chip is relatively weak; however, the amount of histidine residues, the total number of histidine tags (due to, e.g., multimer formation), and the possibility of rebinding (depending on flow rate, protein size, and available Ni^{2+}–NTA sites after capturing) can greatly improve the affinity [20]. Other factors, such as buffer salts and the pH of the buffer, can also influence the overall binding and the dissociation rate.

AChBP was bound very tightly, and low dissociation is detected due to the hexaHis tag, the homopentamer formation, and the optimized capturing conditions.

The second part of the hit validation assay is the sequential binding of the test compound and the subsequent binding of the reporter ligand. Compared with a direct binding assay, the current method gives an improved S/N (36 instead of 16) due to the size of the reporter ligand. Therefore, the method can also be used when only small amounts of protein can be immobilized. The specificity of the sequential assay compared with a direct binding assay is improved as well because test compounds that bind nonspecifically to the protein are not detected in this method, whereas they will be detected in a direct binding assay. A drawback of the presented method is the need for a high-molecular-weight reporter ligand. However, in addition to several nAChRs that have BgTx as a ligand, many of the receptors involved in the immune system also have natural occurring high-molecular-weight ligands. Examples include the toll-like receptor family and the chemokine receptor family; there are, therefore, many possible applications in this emerging drug research field. The key difference with a competition assay and the current assay format is the fact that the test ligands can bind to the receptor without the direct competition of a high-affinity ligand. This will make the assay more sensitive for compounds with a low affinity because these molecules otherwise will not bind at all due to the much higher affinity of the reporter molecule.

The sensitivity of the presented secondary assay is strongly dependent on the amount of protein captured on the chip. Capturing approximately 1000 RU AChBP resulted in a z' factor of 0.96. The obtained z' factor is comparable to, or better than, the values obtained using SPR screening methods described in the literature [2]. The Biacore T100 is capable of data collection at 1 and 10 Hz. The results shown are obtained with 10 Hz, but data collection with a higher frequency would perhaps improve the results.

It should be noted that small differences in affinity can be measured only for low-affinity binders, and no exact affinities can be given. Also, EC_{50} curves showed a better r^2 for low-affinity ligands than for high-affinity ligands. The novel method, therefore, is complementary to the current direct SPR format. The indirect sequential method ensures a strongly enhanced signal for low-affinity ligands, avoiding false negatives during screening. The direct method allows the characterization of the compounds, resulting in exact affinities and also in association and dissociation rates.

Tacrine is known for its inhibition of the acetylcholinesterase and, therefore, is used clinically to treat Alzheimer's disease. Furthermore, it has been shown that tacrine has an inhibitory effect on the human nAChR [21]. The fact that we detected binding of tacrine to AChBP shows that the assay is suitable for the discovery of low-affinity ligands that are typically the starting point for most lead optimization programs. Furthermore, the data support the fact that AChBP indeed might be an appropriate model protein for the screening of nAChRs.

Conclusions

Primary and secondary screening assays for the discovery of AChBP ligands have been developed. The primary assay is based on the conventional experimental design where the target is immobilized and test compounds are used as analytes. For the secondary assay, a novel SPR methodology is used. The presented method shows promising results for the hit validation of ligands of the nAChRs. The use of BgTx as reporter ligand greatly enhances the readout of the assay. The developed method is sensitive and reproducible.

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