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Benocyclidine (BTCP) as Non-labelled Reporter Ligand for MS Binding Assays for the PCP Ion Channel Binding Site of the Desensitized Torpedo Nicotinic Acetylcholine Receptor (nAChR)

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In this study we present MS Binding Assays for the PCP ion channel binding site of Torpedo californica nicotinic acetylcholine receptor (nAChR) as an alternative to radioligand binding assays. As MS Marker Benocyclidine (BTCP) was employed, found to be more affine (K_d of 84.2 nM) than the radioligands, e.g. [^{3}H]PCP, used so far in respective binding assays. Based on a highly sensitive and fast LC-ESI-MS/MS method for quantification of BTCP samples, BTCP MS Binding Assays for the PCP ion channel binding site of Torpedo nAChR could be established comprising saturation, kinetic and competition experiments. The affinities obtained in competitive BTCP MS Binding Assays for ligands addressing the PCP ion channel binding site of Torpedo nAChR were in excellent accord with those reported from radioligand experiments. Thus, the new BTCP MS Binding Assays represent a potent and reliable alternative to radioligand binding assays used so far for the characterization of ligand binding to the PCP ion channel binding site of the nAChR.

Introduction

The Torpedo californica nicotinic acetylcholine receptor (nAChR) is a commonly used model for receptors of the ligand-gated ion channel superfamily and furthermore, widely studied due to its similarity to the human muscle nAChR. It consists of five heteromeric but structurally homologous subunits with the composition of t2αβγδ that matches the human embryonic muscle type, while the γ subunit is exchanged with an ε subunit to h2$\alpha_1\beta_1\varepsilon\delta$[1–3] in the human adult muscle type. The nAChR function is associated with general muscular actions, and receptor associated deficits lead to muscular weakness and fatigability as it can be seen in the autoimmune disease myasthenia gravis where muscle nAChRs are destroyed by antibodies.[4] The effect of impaired acetylcholine neurotransmission is also visible in organophosphate poisoning through insecticides or warfare agents. By causing an inhibition of the acetylcholinesterase that is in charge of the decomposition of acetylcholine, organophosphates lead to an uncontrolled increase of acetylcholine and thus to an overstimulation of muscarinic and nicotinic acetylcholine receptors. In case of the nAChRs this results in the desensitization of this receptor, which may lead to respiratory paralysis with lethal consequences[5,6] due to a failure of signal transduction. The nAChR is postulated to have different conformational states.[7] Besides the resting (closed) and the open state there are also an intermediate (fast onset) and a desensitized (slow onset) state, with the latter resulting from a prolonged overstimulation of the receptor, making it far less susceptible to activation.[3,7–9] It is claimed[9] that these conformational states and their transitions can be influenced by a variety of ligands targeting the orthosteric binding site with acetylcholine [Figure 1; **1**] representing the endogenous ligand, or the ion channel pore and other compounds such as positive or negative allosteric modulators (PAMs or NAMs). The affinity of ligands addressing the ion channel distinctly depends on the functional state of the receptor with many ligands showing higher affinity to the desensitized state as shown for antidepressants.[1] One of the most studied ion channel ligands is phencyclidine (PCP; Figure 1; **2**) (PCP binding site), an ion channel blocker and dissociative anesthetic, that is mostly known for its effect on the NMDA receptor.[10,11] The binding sites in the nAChR ion channel have been extensively studied in radioligand binding assays using different tritium labeled reporter ligands such as [^{3}H]PCP,[12–14] [^{3}H]TCP (tenocyclidine; Figure 1; **3**)[1,15,16] and [^{3}H]imipramine[17–19] (Figure 1; **4**). However, the usage of radioligands is associated with various drawbacks resulting from safety issues, governmental restrictions and the generation of radioactive waste, that must be disposed. Such drawbacks may be overcome by employing alternative assay techniques, that do not require radioactive labeling. Thus, fluorescence based assays have been established using *rac*-quinacrine (Figure 1; **5**) or ethidium bromide (Figure 1; **6**) addressing the nAChR ion channel[12,20,21], which however suffer from background radiation and other interfering effects. An interesting alternative to radioligand binding assays are MS Binding Assays, the concept of which has been introduced by our group[22–25] in recent years. In the present study we wanted to probe the applicability of this

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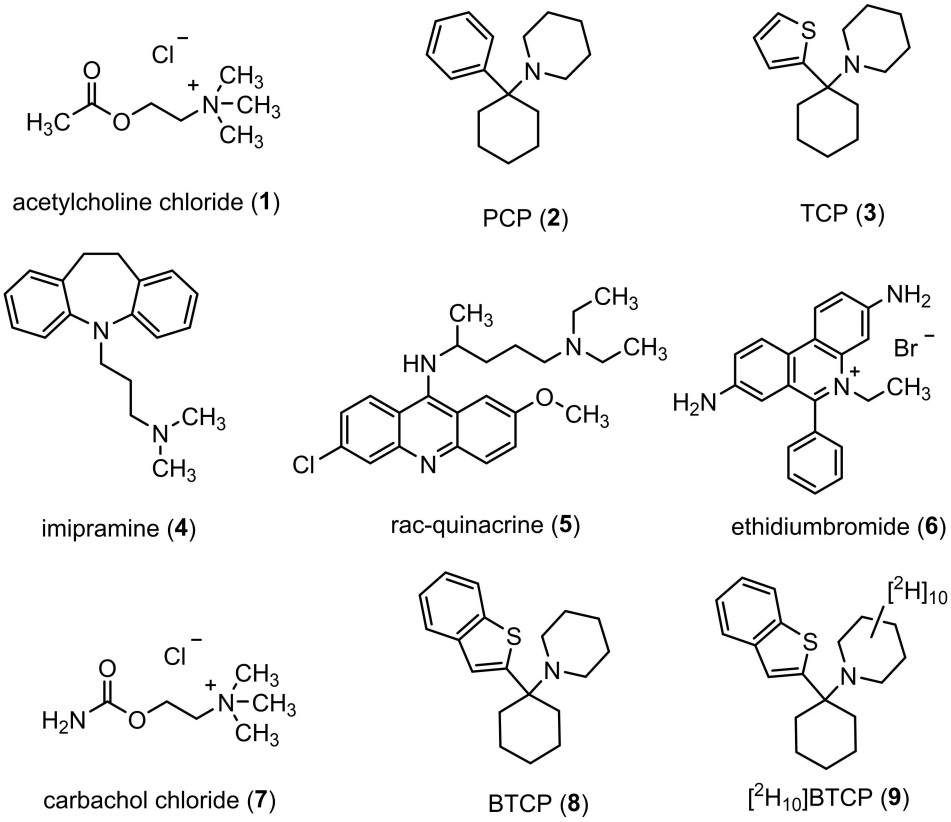

Figure 1. Structures of orthosteric agonists (**1**, **7**) and compounds addressing the ion channel of nAChR (**2** - **6**, **8**, **9**).

concept for the PCP ion channel binding site of desensitized Torpedo nAChR.

In MS Binding Assays unlabeled compounds serve as reporter ligands. These are quantified by means of mass spectrometry (MS). Apart from that, the performance of MS Binding Assays is analogous to that of radioligand binding experiments to which they are also comparable with regard to sample throughput and reliability of the obtained data. Thus, also in MS Binding Assays, for the quantification of reporter ligand bound in the binding experiment, the target with bound reporter ligand has to be separated from the incubation mixture. This can be achieved by filtration or centrifugation with both principles having advantages and disadvantages. Filtration based assays are faster and allow a higher throughput, but for this approach reporter ligands with slow off-kinetics are required to warrant a negligible loss of bound reporter ligand during washing steps. In contrast, centrifugation-based assays can be performed with reporter ligands with faster off-kinetics commonly reflected in lower binding affinities. The centrifugation step is, however, time consuming and thus associated with a reduced sample throughput. In case of filtration based radioligand binding assays, reporter ligand bound to the target can be directly quantified by liquid scintillation counting of the filtration residue. In contrast, in MS Binding Assays reporter ligand bound to the target, which as part of the membrane fraction is retained on the filter, has to be liberated (for example by treatment with organic solvents) prior to its quantification by means of LC–MS/MS.

The present study aimed at the development of MS Binding Assays for ligands addressing the PCP ion channel binding site of the desensitized Torpedo nAChR. For the establishment of such MS Binding Assays, PCP should be chosen as reporter ligand, as radioligand binding assays employing this ligand in its radiolabeled form as reporter ligand are quite common. Furthermore, as found by Sichler et al.[24] during establishment of MS Binding Assays addressing the MB327 binding site of the nAChR, PCP is well quantifiable by LC-ESI-MS/MS under the condition already used for the MB327 MS Binding Assay. The affinity of PCP to its binding site in the ion channel lumen of the nAChR is, however, rather low with a K_d of about 0.3 μM in the desensitized state of the receptor.[15] Textbooks state that compounds with a K_d higher than 30 nM are typically associated with off-kinetics that are critically fast for a filtration based separation of bound from unbound reporter ligand and may lead to a severe loss of specifically bound marker during washing steps.[26] Therefore, we decided to establish binding assays based on centrifugation for the separation similar to the earlier mentioned MB327 MS Binding Assays. For lower affinity reporter ligands this appears to be the more reliable approach. As previously indicated, the detection and quantification of PCP has already been described by Sichler et al.[24] employing the LC–MS/MS method developed for their MB327 MS Binding Assay. However only preliminary binding experiments describ-

ing the influence of carbachol (Figure 1; **7**) on PCP binding[24] had been conducted by these authors. In the present study MS Binding Assays, as will be described in the following, were based on the workflow and quantification of the aforementioned approach of Sichler et al. Yet, the performance was partly modified, as e.g., an internal standard was employed to enhance the reliability of the LC-ESI-MS/MS quantification. The thus modified assay setup was found to be suitable for saturation as well as competition experiments but suffered from drawbacks resulting from the low affinity of the reporter ligand and the use of centrifugation for the separation step (as partly discussed before). The details will be described below.

Interestingly, in the aforementioned preliminary MS binding experiments using non-labelled PCP as reporter ligand, benocyclidine (BTCP) (Figure 1; **8**), a known selective dopamine reuptake-inhibitor, available to us from another study,[27] appeared to possess a distinctly higher binding affinity for the Torpedo californica nAChR than PCP. Hence, we decided to explore the potential of BTCP as reporter ligand, anticipating that this would allow the set-up of filtration based MS Binding Assays for the PCP binding site of the desensitized nAChR.

Results and Discussion

Establishment of centrifugation-based MS Binding Assays using PCP as reporter ligand

Phencyclidine (PCP) is a common reporter ligand for binding assays addressing the ion channel binding site of desensitized Torpedo californica nAChR. While establishing their MS Binding Assays for MB327,[24] Sichler et al. had already demonstrated that the chromatographic conditions developed for the analysis of MB327 could be also used for quantification of PCP as already outlined above. Furthermore, the suitability of this analytical approach for the performance of MS Binding Assays with PCP as reporter ligand could be demonstrated in preliminary binding experiments. Hence, we decided to use this established setup as starting point for the development of fully elaborate MS Binding Assays addressing the PCP binding site of the desensitized nAChR with PCP as reporter ligand. At first, the quantification method that had been developed for MB327, but had also been found suitable for PCP analysis as described by Sichler et al., was optimized for PCP. To this end a PCP focused tuning of the mass spectrometer's source parameters was undertaken, while the chromatographic conditions and compound specific parameters were left unchanged. The assay volume was increased to prevent depletion of the reporter ligand PCP when used at lower concentrations. Besides, commercially available [$^2H_5$]PCP was added as internal standard, to achieve a more robust quantification of PCP. After these optimization steps had been performed, PCP was characterized as reporter ligand in saturation experiments according to the concept of MS Binding Assays. In analogy to the procedure described by Sichler et al.,[24] here as well the heat shock approach (protein denaturation at 60 °C for 1 h) was applied for the determination of non-specific binding. However, the non-specific binding found in saturation experiments, especially at high reporter ligand concentrations, was very high as compared to total binding (see Figure 2A). Considering these results and the situation in established [^{3}H]PCP binding assays, which use exclusively competition for determination of non-specific binding, we thought it worthwhile to compare the results for non-specific binding that are obtained from the heat shock approach to those determined with a competitor. In saturation experiments a striking difference in non-specific binding was obtained for the heat shock as compared to the competition method using tenocyclidine (TCP, Figure 2A) as competitor. The non-specific binding determined by the competitor method was distinctly lower than that observed employing the heat shock approach. Hence, the specific binding utilizing TCP as competitor for the determination of non-specific binding was higher than that based on the heat shock approach (Figure 2B). As a result thereof, for the competitor approach the density of target binding sites (B_{max}) by amounting to 400 ± 57 pmol/mg protein was higher than that found for the heat shock method

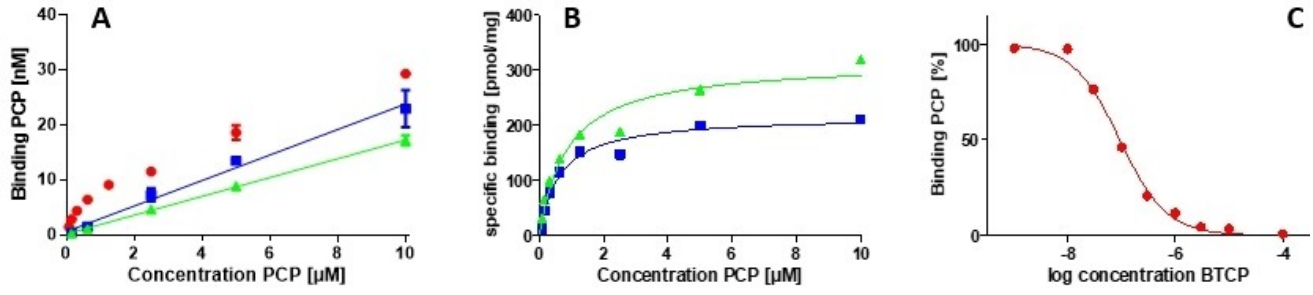

Figure 2. Representative saturation experiments showing total and non-specific **(A)** as well as specific binding **(B)** of PCP to Torpedo californica nAChR in presence of 100 µM carbachol as obtained in the centrifugation-based approach. Non-specific binding was either determined by addition of 1 mM TCP as competitor or by heat shock denaturation (60 °C, 60 min) of the protein prior to reporter ligand addition. **(A)** Experimental data (mean ± SD, n = 3) for total binding (red circles), non-specific binding determined by means of a competitor (green triangle) and non-specific binding determined by heat denaturation (blue square). **(B)** Specific binding (means) calculated from (**A**) by subtraction of non-specific binding (competitor, green triangles; heat denaturation, blue squares) from total binding and fitted by nonlinear regression analysis. **(C)** Representative competition curve in presence of 300 nM PCP, 100 µM carbachol and increasing concentrations of BTCP. Data points represent specific binding (means) of PCP. 100% is defined as specific binding without competitor and 0% as non-specific binding in the presence of 1 mM TCP.

being 206 ± 19 pmol/mg protein. Furthermore, also the K_d value resulting from the saturation isotherm generated by taking into account non-specific binding from the competitor method was distinctly higher (1.26 ± 0.27 μM) than the one obtained by considering non-specific binding determined by the heat shock approach (0.39 ± 0.06 μM). With regard to determination of non-specific binding of PCP in the PCP binding assays we concluded to employ the competition method with a structurally analogous competitor, i.e. TCP, as this is also exclusively done in corresponding radioligand binding assays. That way also a sound basis was obtained for a comparison of the results generated in MS Binding Assays with those reported for radioligand binding assays described in literature.

Identification of BTCP as ligand with enhanced affinity for the PCP binding site

In literature PCP and TCP are frequently found as ligands addressing the ion channel with comparingly high affinities for the PCP binding site at Torpedo californica nAChR. Thus, it seemed likely, that also other related *N*-cyclohexylpiperidine derivatives might exhibit high affinities for this binding domain. Having benocyclidine in stock (benzothiophenylcyclohexylpiperidine, BTCP) (Figure 1; **8**), a known selective dopamine

reuptake-inhibitor, structurally related to PCP and TCP exhibiting a benzothienyl moiety instead of a thienyl and a phenyl moiety as in TCP and in PCP, respectively,[27] we wondered whether BTCP might be a good binder of the PCP binding site of the nAChR as well and possibly even better than PCP and TCP. Interestingly, the K_i determined for BTCP in a competitive PCP MS Binding experiment (Figure 2C) was indeed remarkably low (K_i = 71.8 ± 9.7 nM), i.e. its affinity high. With its K_i < 100 nM it is far more affine for the PCP binding site of desensitized Torpedo californica nAChR than PCP (0.3 μM)[15] and TCP (0.25 μM).[15] Furthermore, BTCP has the advantage that it is not subject to governmental restrictions for drugs of abuse and, in addition, is commercially available as well. As we were not aware of any other compounds with a higher affinity for the nAChR PCP binding site than BTCP, we decided to study the latter with regard to its suitability for filtration based MS Binding Assays. To this end as a first step the analytical conditions for quantification of BTCP as a reporter ligand had to be established, which was realized as described in the following section.

Development of an LC-ESI-MS/MS method for the quantification of BTCP

Prior to the employment of BTCP as a reporter ligand in MS Binding Assays, a LC-ESI-MS/MS method had to be established for the quantification of this compound in samples resulting from the corresponding binding experiments. For this purpose, a triple quadrupole mass spectrometer coupled to a pneumatically assisted electrospray ionization (ESI) source was used in the multiple-reaction monitoring (MRM) mode. The suitability of this approach for the quantification of small molecules regarding selectivity and sensitivity has repeatedly been demonstrated in former studies. Yet, ESI-MS/MS mass spectrometry data for BTCP have not been described so far. When studied in the positive ionization mode, the expected $[M+H]^+$ parent ion with *m/z* 300.4 and the fragment ions *m/z* 215.3, 147.2, 86.2 and 81.2 (Figure 3A) were identified. For the further LC–MS/MS method development the mass transition with the highest intensity (*m/z* 300.4/215.3) was selected in combination with the optimized compound specific parameter settings (see Experimental Section).

As BTCP is structurally closely related to PCP we intended to make use of the chromatographic conditions already developed for quantification of PCP in MS Binding Assays (see above and Sichler et al.[24]). Employing these conditions, i.e. a YMC-Triart Diol-HILIC column (50 mm × 2 mm, 3 μm) in combination with a mobile phase consisting of 20 mM ammonium formate buffer (pH 3.0) and acetonitrile (20:80, *v/v*) at a flowrate of 800 μL min^{-1} a retention time of 0.35 min resulted, which enabled fast run times and therefore a high throughput (see Figure 3C). With the chromatographic conditions being defined, the source-dependent parameters of the mass spectrometer were optimized as described in the Experimental Section.

As for the quantification of analytes in biological matrices also the quantification of reporter ligands in MS Binding

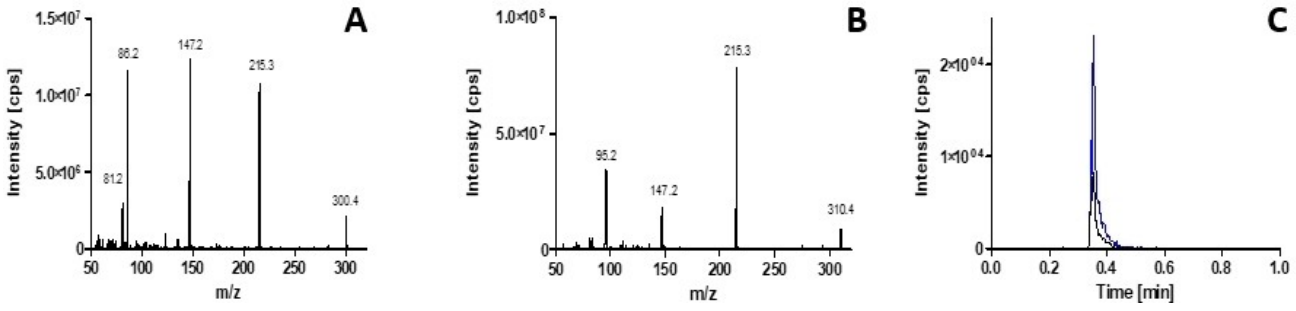

Figure 3. Product ion scan for the $[M+H]^+$ parent ion of **(A)** BTCP (*m/z* 300.4) and **(B)** [$^2H_{10}$]BTCP (*m/z* 310.4); **(C)** MRM chromatogram of a matrix standard with 10 nM BTCP (*m/z* 300.4/215.3, blue) and 5 nM [$^2H_{10}$]BTCP (*m/z* 310.4/215.3, black). For LC, an YMC-Triart Diol-HILIC (50 mm × 2 mm, 3 μm) column as stationary phase in combination with a mobile phase consisting of ammonium formate buffer (20 mM, pH 3.0)/acetonitrile (20:80, *v/v*) at a flow rate of 800 μL min^{-1} was used.

Assays[28,29] by LC-ESI-MS/MS may be accomplished employing an external or internal standard. However, the quantification based on an internal standard can distinctly improve the robustness of a LC-ESI-MS quantification method, in particular when internal standard and analyte are virtually co-eluting. Hence, we decided to use a deuterated analogue, i.e. the ten times deuterated BTCP derivate [$^2H_{10}$]BTCP (Figure 1; **9**) as internal standard. [$^2H_{10}$]BTCP (**9**) was prepared in two steps in analogy to a synthesis of the non-labelled compound[30,31] (see below). Compound **9** was characterized for its mass transitions at a triple quadrupole mass spectrometer under ESI-positive conditions as described before. The *m/z* ratio of the parent ion was found to amount to 310.4 and reflects the increase in mass effected by the deuterium atoms present in the product. On the other hand, two major product ions of [$^2H_{10}$]BTCP (**9**) were found to possess the same *m/z* ratio as those of BTCP (**8**) (fragment ions *m/z* 215.3 and 147.2). This is well in line with identical fragmentation pathways for both parent ions, of **8** and **9**, and is to be attributed to the loss of neutral fragments comprising the piperidine subunit in its non-deuterated form in case of **8** and deuterated form for **9** (compare Figure 3A and Figure 3B).

For quantification of [$^2H_{10}$]BTCP (**9**), the mass transition *m/z* 310.4/215.3 was chosen, which corresponds to the one, used for quantification of unlabeled BTCP. In this context, it is worth mentioning, that preliminary experiments had revealed that a reliable quantification of BTCP can also be achieved employing commercially available [$^2H_5$]PCP as internal standard. Hence, also unlabeled PCP could serve as internal standard for this purpose instead of an isotopically labelled internal standard. In other words, marker quantification in MS Binding Assays may as well be performed employing non-labelled compounds as internal standards, as it is common in most areas of quantitative analytical chemistry.

After the LC-ESI-MS method had been developed, it was validated with regard to robustness and reliability of the data as described in section "method validation".

Syntheses [$^2H_{10}$]BTCP hydrochloride (9·HCl) as internal standard

The synthesis of [$^2H_{10}$]BTCP hydrochloride (**9·HCl**) has been performed in analogy to methods reported for the non-labelled compound[30,31] and is depicted in Figure 4. Reaction of cyclohexanone (**10**) with commercially available [$^2H_{11}$]piperidine (**11**) and NaCN afforded **12**. This product, which was isolated by filtration and used for the next reaction step without prior purification, was subsequently added to a Grignard reagent derived from 2-iodobenzothiophene. This gave, after workup, the desired product [$^2H_{10}$]BTCP (**9**). Finally, upon treatment of the free base with aqueous HCl the hydrochloride **9·HCl** was obtained, the overall yield amounting to 81.5%.

Method validation

The reliability of the established LC-ESI-MS/MS method for the quantification of BTCP in MS Binding Assays was validated regarding selectivity, linearity, lower limit of quantification (LLOQ), precision and accuracy according to the recommendation of the FDA guidance for bioanalytical method validation.[32] For the preparation of calibration samples and quality control samples (QC) matrix blanks were produced applying the procedure for conventional binding samples whereby in contrast, no reporter ligand was added (for details see LC-ESI-MS/MS method validation) and the protein residue on the filter obtained after filtration was eluted with acetonitrile containing the reporter ligand BTCP in nine concentration levels and internal standard [$^2H_{10}$]BTCP at a concentration of 5 nM. A single validation set contained BTCP concentrations in the range of 195 pM to 50 nM, as preliminary binding experiments had shown that a coverage of this concentration range is needed. In addition, it comprised five levels of quality control (QC) samples between 200 pM and 40 nM as well as blanks and zero calibrators. Calibration samples excluding the lowest two concentrations at 195 pM and 390 pM as well as blanks and zero calibrators were employed in triplicates while QC samples and the mentioned two calibration samples with the lowest concentrations were applied in hexaplicates. Validation was performed with five sample sets on different days for the

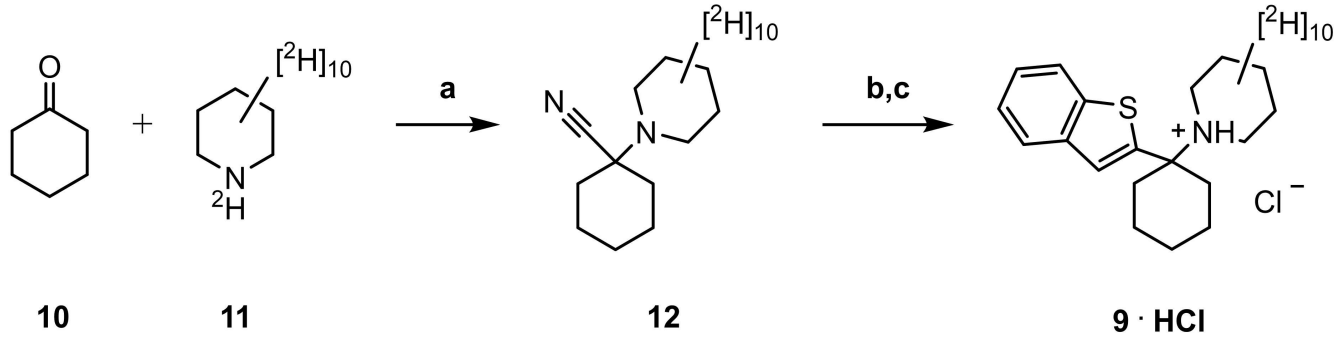

Figure 4. Synthesis of [$^2H_{10}$]BTCP. Reagents and conditions: a) $NaHSO_3$, NaCN, H_2O, rt, 18 h; b) Addition to benzo[h]thiophen-2-yl magnesium iodide prepared from 2-iodobenzothiophene and magnesium turnings in abs. Et_2O, reflux, 17 h; c) 1 M HCl, MeOH, rt.

determination of intra- and inter-batch precision and accuracy. A table summarizing all results from the validation experiments can be found in the Supporting Information, while representative examples for a calibration curve (showing linearity, Figure 5A), a matrix blank (showing selectivity, Figure 5B) and a LLOQ sample (Figure 5C) are depicted in Figure 5. For accuracy, representing the closeness of determined to nominal analyte concentration, the FDA guidance allows a deviation of ±15% (and ±20% at the concentration level of the LLOQ). None of the validation runs exceeded ±7% intra-batch accuracy in any calibration or QC sample concentrations and none ±3% for inter-batch accuracy thus fulfilling these criteria. Also, precision defined as relative standard deviation (RSD) of the measured samples met the FDA criteria requiring RSD of ≤15% (≤ 20% at the concentration level of the LLOQ) with an intra-batch RSD ≤ 8% for any calibration and QC samples and an inter-batch RSD ≤ 4%. Selectivity was demonstrated by the injection of individually prepared matrix blanks. These did not show any interfering signals for the chosen mass transitions of the analyte BTCP (**8**) (Figure 5B). On the basis of the shown validation parameters, it can be stated that the developed LC-ESI-MS/MS method is suitable for a reliable quantification of BTCP as reporter ligand in MS Binding Assays.

Kinetic experiments

After successful validation of the LC-ESI-MS/MS quantification method the association and dissociation rates of BTCP to and from Torpedo californica nAChR, respectively, were studied in kinetic experiments. That way also the information should be obtained, whether dissociation kinetic is slow enough to allow the use of filtration for the separation step (including washing steps), where the loss of bound marker should not exceed 10% due to dissociation during the separation step. With a time period of around 10 s typically needed for filtration based separation, a dissociation rate constant of about $\leq 10^{-2}\ s^{-1}$ for the marker compound is required to achieve this goal.[26] As in case of the saturation and competitive experiments, also kinetic experiments were performed at a temperature of 25 °C and, to ensure the receptor to be in the desensitized state, 100 μM of carbachol was present. Dissociation rates were measured in the time interval from 15 seconds to one hour with the BTCP concentration being set to 100 nM (a concentration close to the expected K_d). These experiments were accomplished by pre-incubation of protein samples for two hours with 100 nM BTCP in a 96-well plate, whereafter dissociation was initiated by addition of PCP (100 μM final concentration) as competitor as described in the section "BTCP kinetic experiments". Dissociation experiments were terminated by separating the Torpedo californica membrane fragments from the incubation mixture using filtration followed by immediate washing with ice-cold 154 mM ammonium acetate buffer (washing buffer) of the residue obtained. Based on the results for the amount of receptor bound BTCP (**8**) in these dissociation experiments, a dissociation curve was established by means of nonlinear regression (see "Data analysis") from which the dissociation rate constant (k_{off}) could be obtained. The analysis of three experiments (representative curve given in Figure 6B) yielded a mean value for k_{off} amounting to $1.3\times10^{-2} \pm 0.2\times10^{-2}\ s^{-1}$. Hence, the k_{off} of BTCP (**8**) is relatively low considering its K_d of 84.2 nM (see next section) for which a k_{off} of around $8.4\times10^{-2}\ s^{-1}$ had to be expected, when, as common, an average on-rate of $10^6\ s^{-1}\ M^{-1}$ is assumed. Whatsoever, an off-rate close to the limit $\leq 10^{-2}\ s^{-1}$ considered to be sufficient for filtration assays was achieved. Yet, to be on the safe side washing steps after filtration were performed at 0–4 °C by employing an ice-cold washing buffer as often done in filtration based ligand binding assays.

In case of the association experiments binding of BTCP to nAChR was monitored for a total time of two hours at a concentration of 100 nM (of BTCP), with the individual binding experiments being terminated as described for the dissociation experiments. From the resulting data an association curve was established by means of nonlinear regression (see "Data analysis"). The observed association rate constant (k_{obs}) obtained therefrom determined in three experiments (a representative curve is given in Figure 6A) amounts to $5.0\times10^{-3} \pm 0.9\times10^{-3}\ s^{-1}$. When the data for k_{obs} and k_{off} were finally used for the

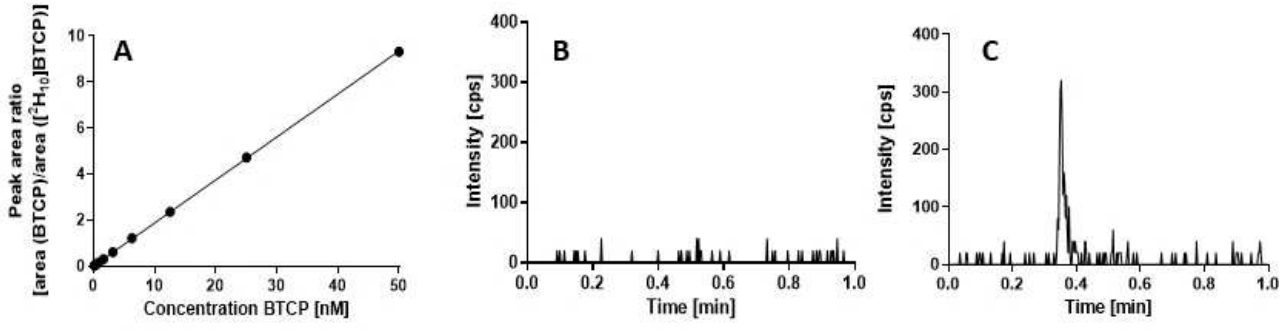

Figure 5. Validation of the BTCP LC-ESI-MS/MS quantification method. **(A)** Representative calibration curve for BTCP in a range from 195 pM to 50 nM employing 5 nM [$^2H_{10}$]BTCP as internal standard. Peak area ratio of reporter ligand vs internal standard (y-axis) was plotted against the nominal concentration of the reporter ligand BTCP (x-axis). The resulting calibration function obtained by linear regression analysis was $y = 0.192\times + 0.00574$ ($r = 0.9993$). **(B)** MRM chromatogram for a matrix blank ($m/z = 300.4/215.3$) (demonstration of selectivity). **(C)** MRM chromatogram for BTCP at the LLOQ at 195 pM ($m/z = 300.4/215.3$).

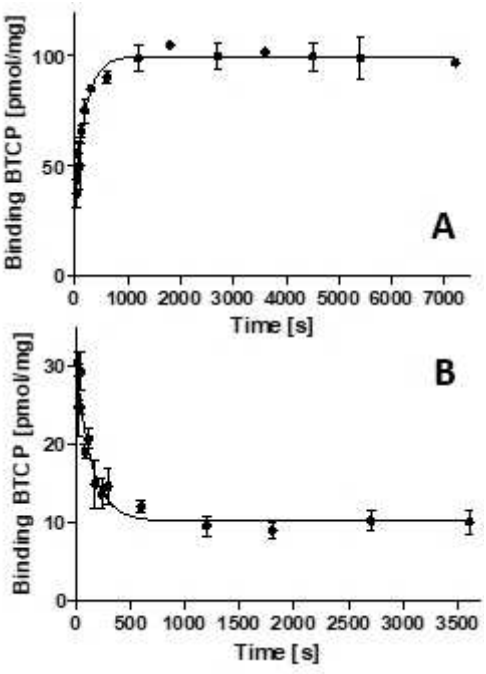

Figure 6. Representative kinetic experiments for the association and dissociation of BTCP at torpedo nAChR in presence 100 μM carbachol. **(A)** Experimental data (means ± SD, n = 3) of the association of 100 nM BTCP at 25 °C to nAChR terminated by filtration after the specified times. **(B)** Experimental data (means ± SD, n = 3) of the dissociation of 100 nM BTCP from nAChR induced through addition of 100 μM PCP and terminated by filtration after the specified times.

calculation of k_{on} according to the equation $k_{obs} = k_{on} \times [L]$ - k_{off} with the marker concentration amounting to 100 nM an association rate constant (k_{on}) of $1.8 \times 10^5 \pm 0.09 \times 10^5$ s^{-1} M^{-1} was found. Finally, when using the data for k_{on} and k_{off} according to the equation $K_d = k_{off} / k_{on}$ a K_d of 73 ± 3 nM was received, which correlates nicely with the affinity obtained for BTCP (K_i = 71.8 ± 9.7 nM) in preliminary competition experiments with PCP as MS marker as well as the K_d value determined in saturation experiments (84.2 ± 11.6 nM; see next section).

Saturation Assays with BTCP as reporter ligand for the PCP binding site of Torpedo nAChR

After it had been demonstrated that the off kinetics of BTCP binding is slow enough to allow the use of filtration as separation technique in binding assays, we started to perform saturation experiments, for which a concentration range for the determination of total and non-specific BTCP of about 0.1 K_d to 10 K_d, following common recommendations, should be covered. For the determination of total binding the membrane fragments were to be incubated with the reporter ligand BTCP. Determination of non-specific binding should be accomplished by the competitor approach, employing a large excess of a compound competing with the marker ligand (BTCP) to block all specific binding sites. That way, a better comparability of our results with literature data, which are based on the competitor approach as well, should be ensured. In addition, the concept of heat shock denaturation (60 °C, 60 min) as described above for the PCP MS Binding Assays for the determination of non-specific binding should be employed here, too. In doing so, information should be gained whether the phenomenon described above for PCP binding, i.e. the difference in non-specific binding between the competitor and the heat shock method, is observed for BTCP, too. All experiments in this study were done in presence of 100 μM carbachol to ensure, that nAChRs are forced into their desensitized state. For the determination of total and non-specific binding of BTCP toward Torpedo nAChR, the respective membrane fragments were incubated with eight different concentration levels of BTCP (8 nM–2 μM) in the absence or presence of 1 mM PCP (the latter for the determination of non-specific binding), respectively. The general workflow that was followed for the performance of these saturation assays is depicted in Figure 7. For the reasons mentioned above, non-specific binding was determined by the heat shock method, as well, i.e. by heating the protein to 60 °C for 60 min prior to reporter ligand addition. As already observed for PCP, non-specific binding was significantly different depending on the mode of determination. Heat shock denaturation of the target protein led to distinctly higher non-specific binding than the competitor method. Figure 8 illustrates the results of a

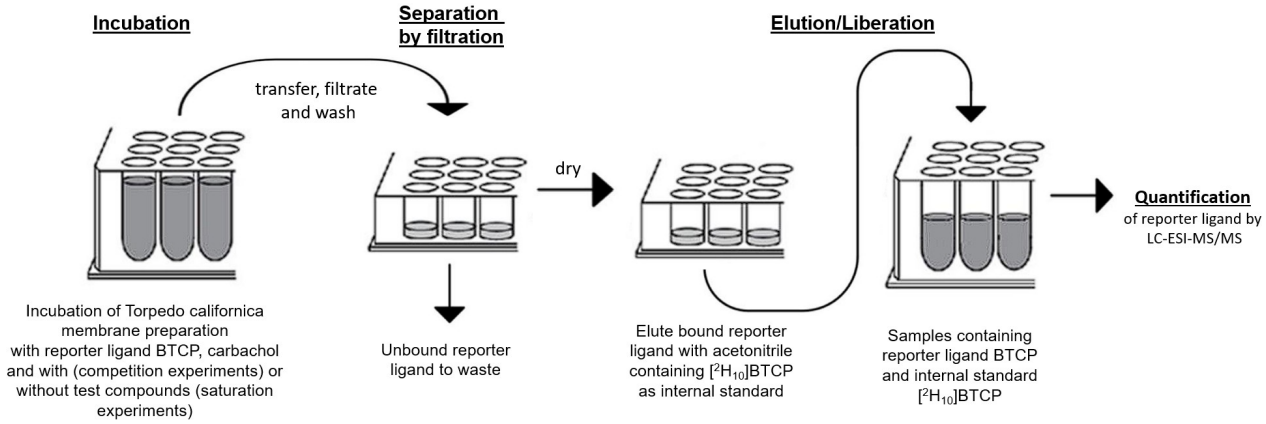

Figure 7. Workflow of the developed BTCP MS Binding Assays for Torpedo nAChR.

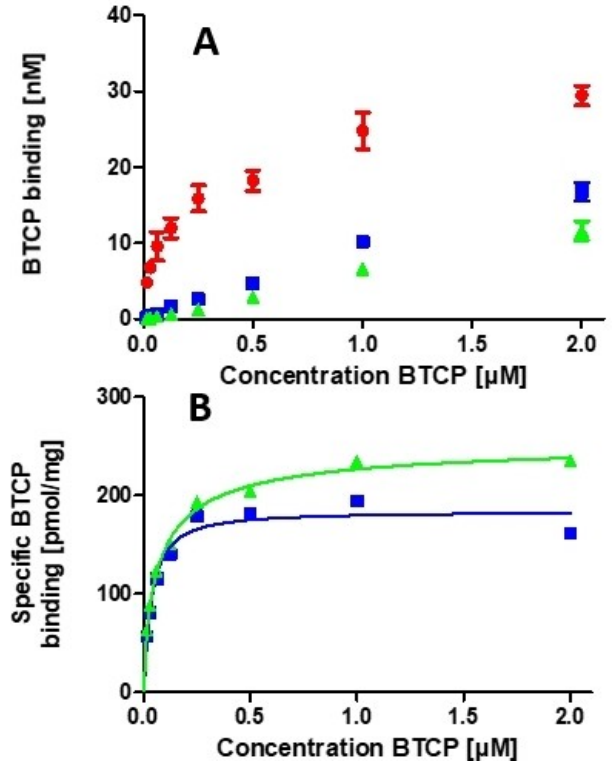

Figure 8. Representative saturation experiment showing total and non-specific (**A**) as well as specific binding (**B**) of BTCP to Torpedo californica nAChR in presence of 100 µM carbachol. Non-specific binding was either determined by addition of 1 mM PCP as competitor or by heat shock denaturation (60 °C, 60 min) of the protein prior to reporter ligand addition. (**A**) Experimental data (means ± SD, n = 3) for total binding (red circles); non-specific binding determined with competitor (green triangles) and non-specific binding determined by means of heat shock denaturation (blue squares). (**B**) Specific binding (means) calculated from (**A**) by subtraction of non-specific binding [competitor (green triangles) or heat shock denaturation (blue squares)] from total binding and fitted by nonlinear regression analysis.

representative saturation experiment, showing total binding as well as non-specific binding (Figure 8A) and specific binding (Figure 8B) for both approaches used for the determination of non-specific binding. For the sake of completeness, it should be mentioned, that in all samples used for the determination of total as well as non-specific binding, BTCP concentration could be quantified in the validated range of the LC–MS quantification method. The specific binding curves yielded slightly different K_d values of 84.2 ± 11.6 nM and 44.1 ± 4.8 nM and B_{max} values of 267 ± 10 pmol/mg protein and 191 ± 5 pmol/mg protein for the competition and heat shock approach, respectively. Remarkably, B_{max} values of saturation curves calculated on the basis of non-specific binding determined by the heat shock approach for the determination of non-specific binding are apparently very similar for saturation experiments with BTCP (191 ± 5 pmol/mg protein) and PCP (206 ± 19 pmol/mg protein) as MS Marker, while B_{max} values of the saturation experiments utilizing the competitor approach for the determination of non-specific binding differ considerably for BTCP (267 ± 10 pmol/mg protein) and PCP (400 ± 57 pmol/mg protein). However, it seems reasonable to speculate, that the latter difference is due to the different separation techniques used for PCP (centrifugation) and BTCP (filtration) saturation experiments. Binding to low affinity binding sites postulated for PCP is likely to be also relevant in the case of BTCP but might be detectable to a lower extent for BTCP, as possibly more of the low affinity binding portion of BTCP in the BTCP assay is lost during washing in the separation step based on filtration (BTCP) as compared to the PCP assay utilizing centrifugation for this purpose.

The K_d value determined via kinetic (73 ± 3 nM) and saturation experiments (84.2 ± 11.6 nM) utilizing the competitor approach for evaluation of non-specific binding match very well with the affinity that was found for BTCP in the PCP MS Binding Assays (K_i = 71.8 ± 9.7 nM) according to which BTCP represents the ligand with the highest affinity for the PCP binding site of desensitized Torpedo californica nAChR reported so far.

Competitive BTCP MS Binding Assays

Finally, based on the so far developed methodology competitive MS Binding Assays addressing the ion channel binding site of desensitized nAChR with BTCP as reporter ligand should be established. These should than be used to characterize the affinity of ligands of the PCP ion channel binding site of the nAChR, that are known from [^{3}H]PCP or [^{3}H]TCP radioligand binding assays, the latter of which is an established substitute of the former[15]

A comparison of the results from the competitive BTCP MS Binding Assays with those from the aforementioned radioligand binding assays might finally allow to assess the suitability of the competitive BTCP MS Binding Assays to serve as a substitute of the above mentioned radioligand binding assays. In this context we decided to determine non-specific binding in the MS Binding Assays by means of the competitor approach to improve the comparability of our data from MS Binding Assays with those from literature from respective radioligand experiments following the same approach.

Characterization experiments could be successfully carried out by incubating the nAChR preparation with BTCP as MS reporter ligand at a fixed concentration close to the K_d value (in this study 100 nM BTCP was used) in presence of 100 µM carbachol and the test compound at eight different concentration levels covering a range of at least four log units (for general workflow see Figure 7). The thus obtained data were used to establish competition curves with the top and bottom level defined by the specific marker binding in absence of any inhibitor and non-specific marker binding in the presence of 1 mM PCP, respectively (see e.g. Figure 9). A set of eleven known ligands addressing the ion channel of the nAChR that are commercially available was characterized according to this procedure. This test set included the well-known ligands of the PCP ion channel binding site phencyclidine (PCP) (Figure 1; **2**) and tenocyclidine (TCP) (Figure 1; **3**), as well as the antidepressants imipramine (Figure 1; **4**), amitriptyline (Figure 10; **13**), bupropion (Figure 10; **14**) and doxepin (Figure 10; **15**) that are

known to also bind to the ion channel of nAChR. In addition, this set contained *rac*-quinacrine (Figure 1; **5**), a former drug for malaria prophylaxis and four adamantane derivates (Figure 10; **16–19**), that were introduced as ligands addressing the ion channel of the nAChR by Arias et al.[16] For all 11 compounds well-fitting sigmoidal competition curves were obtained with all compounds reducing BTCP binding down to the level of non-specific binding (for representative examples see Figure 9A). IC_{50} values determined from these competition curves as well as the K_i values calculated according to the Cheng-Prusoff equation[33] together with the corresponding pK_is are given in Table 1.

The affinities (pK_D, pK_i) determined in radioligand binding assays using [^{3}H]TCP,[1,15–17] and [^{3}H]PCP[20] as reporter ligands that have been published for these compounds are summarized there as well (Table 1). All values determined by the BTCP MS Binding Assays fit very well with those from radioligand binding assays reported in literature. A graphical correlation of the pK_i values from the MS Binding Assays (in Table 1) with pK_i and pK_D (for **2** and **3**) values from radioligand binding experiments is given in Figure 9B. As can be seen from this Figure (Figure 9B), the data from the BTCP MS Binding Assays match very well with those from radioligand binding experiments from literature, clearly indicating that as observed repeatably before for MS Binding Assays, also the new assay addressing the ion channel of desensitized Torpedo nAChRs provides highly reliable results.

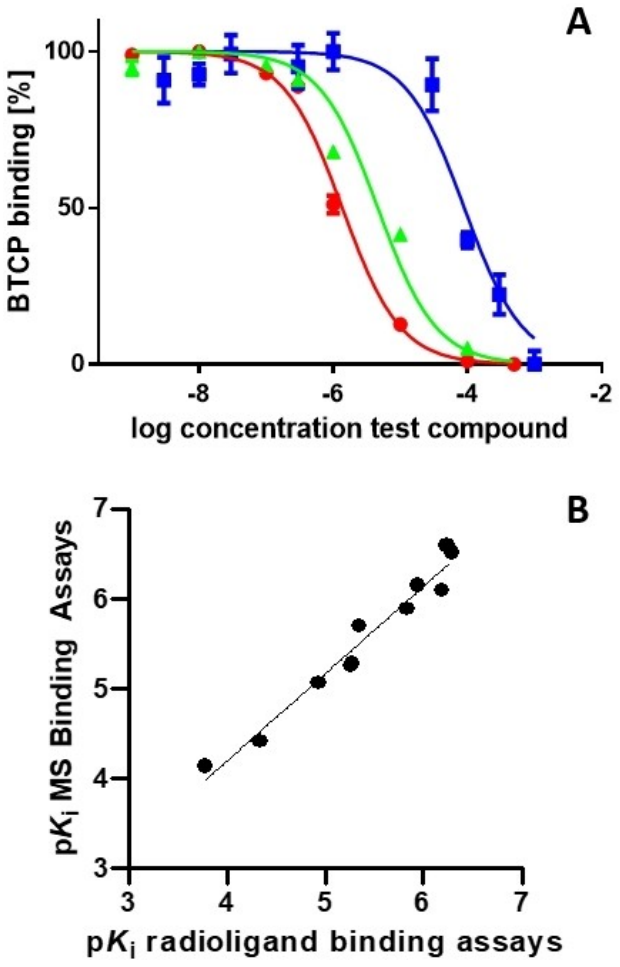

Figure 9. (A) Representative competition curves obtained for PCP (red circles), 1-adamantanemethylamine (blue squares) and doxepin (green triangles) in BTCP MS Binding Assays addressing the Torpedo nAChR. Data points represent specific binding (means ± SD) of BTCP (100 nM) in presence of 100 μM carbachol and varying concentrations of PCP, doxepin and 1-adamantanemethylamine. 100% is defined as specific binding without competitor and 0% as non-specific binding determined with 1 mM PCP. **(B)** Graphical correlation of published pK_D and pK_i values from radioligand binding assays of ligands listed in Table 1 and of pK_i values determined in BTCP MS Binding Assays addressing the desensitized Torpedo californica nAChR (see also Table 1) with the function $y = 0.9727 \times + 0.3031$ ($R^2 = 0.9622$).

Conclusion

This study describes the development of the first MS Binding Assays for the PCP ion channel binding site of desensitized Torpedo californica nicotinic acetylcholine receptor (nAChR). As an alternative to radioligand binding experiments these MS Binding Assays have the advantage to overcome the drawbacks and restrictions connected to the usage of radioisotope labeled reporter ligands. After initially non-labelled PCP had been employed as reporter ligand in centrifugation-based binding assays in analogy to [^{3}H]PCP often used for this purpose in radioligand binding experiments, BTCP was detected as a promising alternative. This compound, BTCP, a structural analog of PCP and TCP, was identified as ligand of the PCP binding site of the Torpedo californica nicotinic acetylcholine receptor (nAChR) and most importantly found to display the highest affinity described so far for the aforementioned binding site. Due to the flexibility of the concept of MS Binding Assays regarding the reporter ligand that is employed a switch from PCP to BTCP as MS marker could be easily performed. For quantification of BTCP by means of LC-ESI-MS/MS a previously developed LC method found suitable for this purpose was used and validated for its application in filtration based MS Binding Assays. These MS Binding Assays were established following already known protocols and were very efficient as they were based on filtration instead of centrifugation in the separation step. For binding of BTCP towards the desensitized Torpedo californica nAChR in saturation experiments a K_d of 84.2 nM was found, well matching with the K_d of 73.0 nM from kinetic experiments. Furthermore, results from kinetic experiments, i.e. the k_{off} rate, further confirmed the prior assumption that the dissociation of the BTCP target complex is slow enough for using filtration in the separation step. The binding affinities (pK_i) found in competition experiments for eleven ion channel ligands known from literature demonstrated an excellent correlation with published results from radioligand binding assays, thus again highlighting the validity of results derived from MS Binding Assays. Overall, the developed MS Binding Assays represent a significant improvement for binding studies regarding the PCP binding site of the nAChR of Torpedo californica and can hence be assumed a powerful substitute for radioligand binding assays primarily used so far for this purpose. Furthermore, as the filtration based setup of the newly developed MS Bindings Assays allows a much higher through-

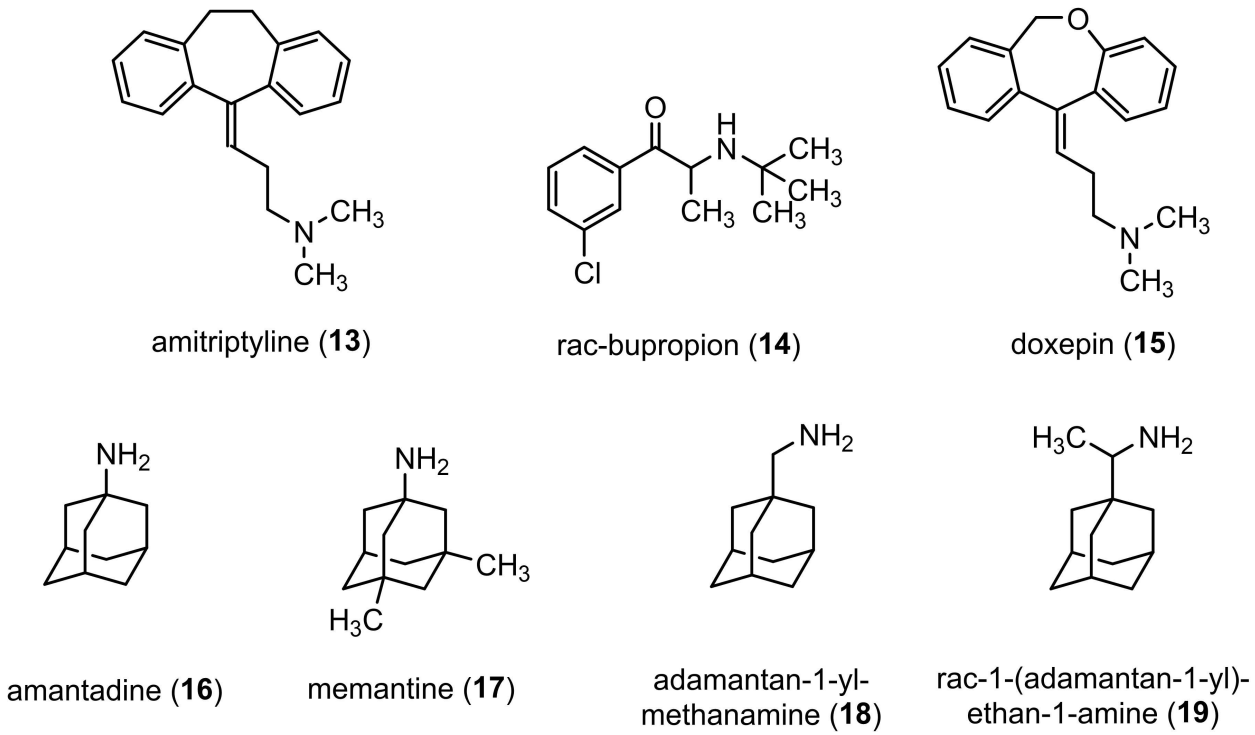

Figure 10. Compounds addressing the ion channel of the Torpedo nAChR.

Table 1. Affinities of ligands addressing the PCP ion channel binding site of nAChR of desensitized Torpedo californica determined in BTCP MS Binding Assays (means ± SEM, n = 3–4) in comparison to affinities from radioligand binding assays published in literature.

	Number of experiments	MS Binding Assays IC_{50}	K_i [μM]	pK_i [a]	Radioligand binding assays K_D [μM]	pK_D [a]
PCP (**2**)	4	0.98 ± 0.17	0.51 ± 0.07	6.29	0.30 ± 10[15] [b]	6.52
TCP (**3**)	4	1.04 ± 0.06	0.57 ± 0.03	6.24	0.25 ± 0.04[15] [b]	6.60
					K_i [μM]	pK_i [a]
imipramine (**4**)	3	1.92 ± 0.48	1.16 ± 0.07	5.94	0.7 ± 0.1[1] [b]	6.15
rac-quinacrine (**5**)	4	2.97 ± 0.25	1.49 ± 0.06	5.83	1.30 ± 0.02[20] [c]	5.89
amitriptyline (**13**)	4	1.06 ± 0.13	0.64 ± 0.09	6.19	0.80 ± 0.1[1] [b]	6.10
rac-bupropion (**14**)	4	9.22 ± 1.29	4.50 ± 0.62	5.35	2.00 ± 0.1[17] [b]	5.70
doxepin (**15**)	3	7.95 ± 1.48	5.41 ± 0.28	5.27	5.30 ± 0.3[1] [b]	5.28
amantadine (**16**)	3	480 ± 96.5	166 ± 21.6	3.78	72.6 ± 12.2[16] [b]	4.14
memantine (**17**)	3	15.6 ± 1.8	5.50 ± 0.3	5.26	5.50 ± 1.6[16] [b]	5.26
adamantan-1-yl-methanamine (**18**)	3	95.9 ± 10.5	45.6 ± 8.4	4.35	37.7 ± 4.1[16] [b]	4.42
rac-1-(adamantan-1-yl)-than-1-amine (**19**)	3	34.0 ± 8.2	11.7 ± 0.3	4.93	8.50 ± 0.7[16] [b]	5.07

[a] Calculated from K_D and K_i values [b] [^{3}H]TCP used as reporter ligand [c] [^{3}H]PCP used as reporter ligand.

put than related centrifugation-based assays, they are far more suitable for the screening of individual compounds and compound libraries than the latter. BTCP itself represents a useful tool compound for studies of the muscular Torpedo californica nAChR and is likely to be a promising starting point for the development of even more affine ligands for the PCP binding site of the desensitized nAChR.

Experimental Section

Material

Phencyclidine (PCP) and Tenocyclidine (TCP) were synthesized in-house according to literature[34] and [$^2H_{10}$]BTCP in analogy to a literature procedure[30,31] (see below). Benocyclidine (BTCP), [$^2H_5$]PCP and carbachol chloride (carbamylcholine chloride) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Imipramine, amitriptyline and doxepin were purchased from Fagron (Barsbüttel, Germany), *rac*-bupropion from Alfa Aesar (Karlsruhe, Germany) and quinacrine from TCI (Tokyo, Japan). Polyethyleneimine (PEI), ammonium hydroxide solution (25% for LC–MS) and ammonium formate (for mass spectrometry) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Torpedo californica electro plaque tissue

was purchased from Aquatic Research Consultants (San Pedra, CA, USA). Water for mobile phase and buffers were obtained in-house from a satorius arium pro water purification system. Acetonitrile for LC–MS (HPLC grade) and formic acid (for LC–MS) were purchased from VWR Prolabo (Darmstadt, Germany). Na_2HPO_4 and $CaCl_2$ were bought from Applichem GmbH (Darmstadt, Germany) NaH_2PO_4, KCl and ammonium acetate were purchased from Grüssing (Filsum, Germany). NaCl was obtained from Bernd Kraft (Duisburg, Germany). All salts had a purity of $\geq$98%. 96-well glass fiber filter plates were supplied by Pall (AcroPrep Advance, glass fiber, 1.0 μm, 350 μL, Pall, Dreieich, Germany). 96-well polypropylene microtiter plates, tubes and tips were purchased from Sarstedt (Nümbrecht, Germany).

LC–MS instrumentation

Experiments for method development and validation as well as binding experiments were performed on an API 3200 triple quadrupole mass spectrometer (Sciex, Darmstadt, Germany) with a pneumatic assisted ESI-probe in a Turbo-V source coupled to an Agilent 1200 HPLC system (vacuum degasser, quaternary pump, column oven; Agilent, Waldbronn, Germany) and a Shimadzu SIL-HTA auto sampler (Shimadzu, Duisburg, Germany). For all kinds of MS-based analyses, Q1 and Q3 were operated with unit resolution. For HPLC, a YMC-Triart Diol-HILIC (50 mm × 2.0 mm, 3 μm; YMC Europe GmbH, Dinslaken, Germany), protected by two inline filters (0.5 and 0.2 μm, IDEX, Wertheim-Mondfeld, Germany) upstream to the column was employed. For all experiments, the column temperature was set to 20 °C. The built-in syringe pump with a 1 mL syringe was used for direct infusion of analyte solutions into the mass spectrometer.

LC–MS/MS method development

Mass spectrometric investigations were performed with ESI-source in positive mode. The compound-dependent parameters (DP, EP, CE, CXP) for the precursor and product ions of BTCP and $[^2H_{10}]$BTCP were determined using the Analyst version 1.6.1 Compound Optimization tool by direct infusion of a 400 nmol L^{-1} solution [in methanol with 0.1% (*v/v*) formic acid] at a flow rate of 5 μL min^{-1}. The optimized parameters were Declustering Potential (DP) 16 V, Entrance Potential (EP) 7.5 V, Collision Cell Exit Potential (CXP) 4 V and Collision Energy (CE) 18 V for both analytes. The source dependent parameters were determined via the flow injection analysis (FIA) mode employing the Compound Optimization Wizard of Analyst version 1.6.1 by repeatedly injecting 10 μL 10 nM BTCP and $[^2H_{10}]$BTCP. The parameters were set as following: Curtain Gas (CUR) 20 psi, Collision Gas (CAD) 4 psi, Ion Spray Voltage (IS) 5000 V, Source Temperature (TEM) 500 °C, Nebulizing Gas (GS1) 55 psi and Auxiliary Gas (GS2) 20 psi.

Quantification of BTCP by LC-ESI-MS/MS

The quantification of BTCP was done under HILIC conditions. The mobile phase consisted of acetonitrile and ammonium formate buffer (20 mM, pH 3.0) in a ratio of 20:80 (*v/v*) and the flowrate was set to 800 μL/min. 40 μL of the sample in 100% acetonitrile was injected to the LC-ESI-MS/MS system at a column temperature of 20 °C. BTCP (reporter ligand) and $[^2H_{10}]$BTCP (internal standard) were monitored at the mass transitions of *m/z* 300.1/215.1 and 310.4/215.0, respectively.

LC-ESI-MS/MS method validation

The method was validated for the aspects of linearity, selectivity, precision, accuracy and lower limit of quantification (LLOQ) with the limits given by the FDA guidance for bioanalytical method validation.[32] Blank matrix for the validation samples was received following the complete sample generation described for the BTCP saturation experiments, except for incubation which was carried out without reporter ligand (and other ligands for the target binding site). A 1 mM BTCP stock solution in water (stored at 4 °C in the fridge) was diluted to ten-fold concentrated working solutions in acetonitrile. Samples for calibration and quality control were generated by spiking of the blank matrix with solutions of BTCP resulting in concentrations from 195 pM to 50 nM in acetonitrile including 5 nM $[^2H_{10}]$BTCP as internal standard during the elution step. Selectivity was investigated by analysis of blank matrix. For linearity, nine concentration levels (195 pM, 390 pM, 781 pM, 1.56 nM, 3.123 nM, 6.250 nM, 12.5 nM, 25 nM, 50 nM) were examined in triplicates, except for 195 pM (LLOQ) and 390 pM, which were prepared in hexaplicates. Calibration was achieved by plotting the obtained area ratios of BTCP/ $[^2H_{10}]$BTCP (y) against the concentration of BTCP (x) and a linear regression analysis with a 1/x weighting over the data set. Precision and accuracy were evaluated with quality control (QC) samples at 5 different concentration levels (200 pM, 400 pM, 1 nM, 20 nM, 40 nM) in hexaplicates. Precision was calculated as RSD of the concentration levels in intra and inter batch analysis. The LLOQ was defined as the lowest concentration level that constantly yields a signal-to-noise (S/N) ratio of at least 5:1 and reached an accuracy of 80–120% and a precision of $\leq$20%. The criteria for all other QC samples were 85–115% (accuracy) and RSD $\leq$15%. For each MS Binding Assay a calibration with eight concentration levels in triplicates in the expected concentration range was performed.

Preparation of nAChR-enriched plasma membrane fragments from Torpedo californica

Preparation of membrane fragments from frozen Torpedo californica electro plaque tissue was carried out following a procedure, that has been described by Niessen et al.[35] and, in addition, been modified as described by Sichler et al.[24] Thus, a twofold volume of extraction buffer was added to the frozen tissue and the mixture was homogenized. After centrifugation, the pellet was washed twice by centrifugation and afterwards homogenized in a Dounce Homogenizer (Wheaton, NJ, USA) with 50 strokes. After a final centrifugation step, the pellets were resuspended in storage buffer (120 mM NaCl, 5 mM KCl, 8.05 mM Na_2HPO_4, 1.95 mM NaH_2PO_4, pH 7.4) and rapidly frozen in liquid nitrogen and stored in a −80 °C fridge. The total protein concentration was determined by the bicinchoninic acid method (Smith et al.[36]) using bovine serum albumin as standard. For the binding assays, aliquots of the membrane fractions were rapidly thawed, diluted and used without further preparation.

PCP binding assays

For saturation experiments aliquots of the Torpedo californica membrane preparation (approx. 100 μg/cap) were incubated with PCP (at least nine concentration levels from 15.9 nM to 5 μM) and 100 μM carbachol (for desensitization of the receptor) in triplicates in incubation buffer containing 1.8 mM NaH_2PO_4, 7.2 mM Na_2HPO_4, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ at pH 7.4, in 1.5 mL Eppendorf-caps with a total volume of 1250 μL for 2 h in a shaking water bath at 25 °C. Samples for the determination of non-specific binding in at least six concentration levels from 15.9 nM to 5 μM in triplicates were either incubated in presence of 1 mM TCP as

competitor or pretreated with heat denaturation at 60 °C for 1 h in a shaking water bath prior to the addition of PCP and carbachol.

For competition experiments Torpedo californica membrane preparations were incubated in triplicates with 300 nM PCP, 100 μM carbachol and increasing concentrations of the competitor BTCP in a range from 1 nM to 100 μM. Non-specific binding (0% PCP binding) was determined incubating triplicates of membrane preparations with 300 nM PCP and 100 μM carbachol in presence of 1 mM TCP as competitor and total binding (100% PCP binding) incubating triplicates of membrane fragments with 300 nM PCP and 100 μM carbachol without addition of competitor. After incubation, samples from saturation and competition experiments were centrifuged for 5 min at 4 °C and 20000 rpm (Rotor 3331, Heraeus Biofuge Stratos, Thermo Scientific) to separate bound from unbound reporter ligand. Supernatant was carefully removed with a vacuum connected Pasteur pipette and the pellet washed twice with 1.5 mL ice-cold washing buffer (154 mM ammonium acetate pH 7.4). The bound reporter ligand was liberated by addition of 500 μL acetonitrile and ultra-sonification for 1 h. After vortexing and recentrifugation, 50 μL of the supernatant were transferred into a 96-well plate and the latter supplemented with 50 μL of a 5 nM solution of [$^2H_5$]PCP in acetonitrile (as internal standard), 50 μL 20 mM ammonium formate buffer pH 3 and 100 μL of acetonitrile to give a total volume of 250 μL. The plate was sealed with aluminum foil and subjected to LC-ESI-MS/MS quantification. Quantification was performed as described for BTCP but with assigned mass transitions of *m/z* 244.0/86.1 and 249.0/86.2 for PCP (reporter ligand) and [$^2H_5$]PCP (internal standard), respectively.

BTCP saturation experiments

BTCP was incubated with aliquots of the Torpedo californica membrane preparation (approx. 10–20 μg/well) and 100 μM carbachol (for desensitization of the receptor) in incubation buffer in polypropylene 96-well plates (1.2 mL) in a total volume of 250 μL for 2 h in a shaking water bath at 25 °C. Samples for the determination of non-specific binding were incubated in presence of 1 mM PCP as competitor. For the determination of non-specific binding via heat shock, the Torpedo californica membrane preparation aliquots were pretreated with heat at 60 °C for 1 h in a shaking water bath prior addition into the wells of a 96-well plate. 96-well filter plates were pretreated for 2 h with 200 μL of a 0.5% (*m/v*) polyethyleneimine solution per well and washed with 200 μL washing buffer (see above) prior use. After incubation, 200 μL of sample volume were transferred to the filter plate on a vacuum manifold (Multi-Well Plate Vacuum Manifold, Pall, Dreieich, Germany) and filtered. Filter-entrapped membrane fragments with bound reporter ligand were washed two times with 100 μL of ice-cold washing buffer and dried for 1 h at 50 °C before cooling to room temperature again. Target bound reporter ligand was eluted three times employing each time 66.6 μL acetonitrile containing 5 nM of [$^2H_{10}$]BTCP as internal standard. The plates were sealed with aluminum foil and subjected to LC-ESI-MS/MS analysis.

BTCP competition experiments

BTCP competition experiments were performed in analogy to the procedure of the BTCP saturation experiments. Aliquots of Torpedo californica membrane preparation (approx. 10–20 μg/well) were incubated in triplicates with 100 nM BTCP in presence of 100 μM carbachol and at least eight different concentration levels of the test compound covering a range of at least four log units in a total volume of 250 μL for 2 h in a shaking water bath at 25 °C. Non-specific binding (0% BTCP binding) was determined incubating triplicates of membrane preparations with 100 nM BTCP and 100 μM carbachol in presence of 1 mM PCP as competitor and total binding (100% BTCP binding) incubating triplicates of membrane fragments with 100 nM BTCP and 100 μM carbachol without addition of competitor. After incubation, 200 μL of sample volume were transferred to the filter plate on a vacuum manifold (Multi-Well Plate Vacuum Manifold, Pall, Dreieich, Germany) and filtered. Filter-entrapped membrane fragments with bound reporter ligand were washed two times with 100 μL of ice-cold washing buffer and dried for 1 h at 50 °C before cooling to room temperature again. Target bound reporter ligand was eluted three times employing each time 66.6 μL acetonitrile containing 5 nM of [$^2H_{10}$]BTCP as internal standard. The plates were sealed with aluminum foil and subjected to LC-ESI-MS/MS analysis.

BTCP kinetic experiments

Kinetic experiments were carried out in analogy to the procedure for the BTCP saturation experiments. For association experiments, samples were prepared in a 96-well plate as a suspension of Torpedo californica membrane preparation (ca. 10–20 μg/well) and 100 μM carbachol in incubation buffer at a volume of 200 μL and placed into a shaking water bath at 25 °C. Kinetic experiments were started by the addition of 50 μL of a 500 nM BTCP solution in incubation buffer resulting in a total volume of 250 μL and an assay concentration of 100 nM. The starting time, as compared to the termination of the association experiment, was varied twenty times with the time intervals ranging from 2 h to 15 s. For the determination of non-specific binding, the samples were incubated in presence of 100 μM PCP as a competitor. To terminate incubation, 200 μL of the samples were transferred to a 96-well filter plate and immediately washed with 200 μL of ice-cold washing buffer.

For dissociation experiments, samples were prepared in a 96-well plate from a suspension of Torpedo californica membrane preparation (ca. 10–20 μg/well) and 100 μM carbachol in incubation buffer at a volume of 150 μL and placed into a shaking water bath at 25 °C. After addition of 50 μL of a 500 nM solution of BTCP the system was allowed to equilibrate for 2 h. Samples for non-specific binding were additionally incubated in presence of 1 mM PCP. After equilibration, 50 μL of a 500 μM solution of PCP were added to each well to start dissociation. Dissociation time was varied sixteen times with the time intervals ranging from 15 s to 1 h. To terminate dissociation, 200 μL of the samples were transferred to a 96-well filter plate and immediately washed with 200 μL of ice-cold washing buffer. In both, association and dissociation experiments, filter plates were dried for 1 h at 50 °C. Liberation of the target bound reporter ligand was performed by three times filtration of 66.6 μL acetonitrile containing 5 nM of [$^2H_{10}$]BTCP as internal standard. The plate was sealed with aluminum foil and subjected to LC-ESI-MS/MS analysis.

Data analysis

Data acquisition and establishment of calibration curves were performed using Analyst v. 1.6.1 (Sciex, Darmstadt, Germany). Binding constants were determined using Prism v. 5.0 (GraphPad Software, La Jolla, *CA*, USA). In saturation experiments, the total number of binding sites (B_{max}) and the equilibrium dissociation constant (K_d) were calculated with "one site – specific binding" regression analysis. Specific binding was defined as the difference between total and non-specific binding. Competition experiments were analyzed with "one site – fit K_i" regression analysis setting the top and bottom level to specific binding in absence of any inhibitor or non-specific binding, respectively.

The dissociation rate constant (k_{off}) was obtained from the dissociation curves by means of a "dissociation – one phase exponential decay" regression analysis and the observed association rate constant (k_{obs}) from the association curves by a "one-phase association" regression analysis. The equilibrium dissociation constant was calculated according to k_{off}/k_{on}. Results are given as mean ± SEM with n ≥ 3.

Synthesis of 1-[1-(Benzothiophen-2-yl)cyclohexyl]-(2,2,3,3,4,4,5,5,6,6-$^2H_{10}$)piperidine hydrochloride ([$^2H_{10}$]BTCP·HCl) (9·HCl)

To a stirred solution of $NaHSO_3$ (590 mg, 5.67 mmol) and cyclohexanone (**10**) (535 µL, 5.15 mmol) in H_2O (1.3 mL) at rt dropwise a solution of NaCN (284 mg, 5.67 mmol in 1.2 mL H_2O) was added followed by [$^2H_{10}$]piperidine (**11**) (510 µL, 5.15 mmol). The reaction mixture was stirred for 18 h at rt. The resulting precipitate was removed by filtration, washed with H_2O (25 mL) and dried *in vacuo* to yield 875 mg (84%) of **12**. The thus obtained product (colorless solid) was directly used without further purification in the next step. A solution of **12** (376 mg, 1.86 mmol) in Et_2O (10 mL) was added over 15 min to a Grignard reagent prepared from 2-iodobenzothiophene (498 mg, 1.86 mmol) and magnesium turnings (48.3 mg, 1.99 mmol) in abs. Et_2O (8 mL) at rt and the resulting mixture was stirred for 17 h under reflux. Then, it was cooled to rt, quenched with saturated NH_4Cl solution (25 mL) and stirred for 30 min. The organic phase was separated and the aqueous phase was additionally extracted with Et_2O (5×25 mL). The combined organic phases were extracted with HCl (1 M, 3×25 mL). The aqueous extracts were brought to pH 9 with NH_3 solution (25%) and extracted with Et_2O (5x25 mL) again. The combined organic phases were washed with H_2O, dried ($MgSO_4$) and concentrated in vacuo to yield 604 mg of crude [$^2H_{10}$]BTCP. Of the free base 186 mg (0.6 mmol) was dissolved in a mixture of MeOH (5 mL) and 1 M HCl (10 mL) and lyophilized to give 199.9 mg (0.58 mmol, 97% with regard to the used fraction of **12**) of [$^2H_{10}$]BTCP•HCl (white powder).

NMR spectra were measured at 25 °C with a Brucker BioSpin Avance III HD (500 MHz) and referenced to the residual solvent signal protons. ^{13}C NMR spectra measured in D_2O were referenced using the unified chemical shift scale with Ξ-values recommended by the IUPAC[37] and provided as automated script by the NMR software MestReNova (Version 12.0.0) with which all further processing and analysis has been performed. Infrared spectra were measured with a Perkin-Elmer FT-IR 1600 employing solid samples as KBr pellets. HRMS data were obtained with a Finnigan LTQ FT.

^1H NMR (500 MHz, D_2O) δ = 1.07–1.30 (m, 3H; CH_2), 1.47 (d_{broad}, J = 11.7 Hz, 1H; CH_2), 1.70–1.86 (m, 4H; CH_2), 2.70 (d_{broad}, J = 12.4 Hz, 2H; CH_2), 7.35–7.45 (m, 2H, Ar–H), 7.58 (s, 1H, Ar–H), 7.80–7.93 ppm (m, 2H, Ar–H); ^{13}C NMR (125 MHz, D_2O) δ = 23.0 (m_{br}, CD_2), 25.0 (m_{br}, CD_2), 25.6 (CCH_2CH_2), 26.6 ($CCH_2CH_2CH_2$), 35.8 (CCH_2), 49.7 (m_{br}, NCD_2), 72.6 (CH_2CCH_2), 125.0 (SCCHCH), 127.5 (SCCCHCH), 128.0 and 128.8 (SCCHCH and SCCCHCH), 131.4 (SCCHC), 139.2 (SCCHC), 142.0 and 142.6 (SCC and SCC) ppm; IR (KBr): ν̃ = 3424, 2936, 2529, 1626, 1458, 755 cm^{-1}; HRMS (ESI^+): *m/z* calcd for $C_{19}H_{16}{}^2H_{10}NS$ $[M+H]^+$: 310.2413; found: 310.2410

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: MS Binding Assays · PCP binding site · liquid chromatography · mass spectrometry · nicotinic acetylcholine receptor

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