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On-line screening of conformationally constrained nicotines and anabasines for agonist activity at the α3β4- and α4β2-nicotinic acetylcholine receptors using immobilized receptor-based liquid chromatographic stationary phases

Ruin Moaddel[a], Krzysztof Jozwiak[a,b], Rika Yamaguchi[a,c], Christopher Cobello[a], Kevin Whittington[a], Tarun K. Sarkar[d], Sankar Basak[d], Irving W. Wainer[a,*]

[a] *Bioanalytical and Drug Discovery Unit, National Institute on Aging, National Institutes of Health, Gerontology Research Center, 5600 Nathan Shock Drive, Baltimore, MD 21224-6825, USA*
[b] *Medical University of Lublin, Staszica 6, 20-081 Lublin, Poland*
[c] *Shionogi and Co. Ltd., Hyogo 660-0813, Japan*
[d] *Department of Chemistry, Indian Institute of Technology, Kharagpur 721302, India*

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Abstract

Liquid chromatography columns containing stationary phases based upon immobilized nicotinic acetylcholine receptors (nAChRs) were used to screen a series of conformationally constrained nicotine and anabasine derivatives for agonist activity. The α3β4 nAChR and α4β2 nAChR subtypes were used to prepare the chromatographic columns and [^{3}H] epibatidine dihydrochloride ([^{3}H] EB) was used as the marker ligand. Single displacement experiments were conducted with the test ligands and with nicotine and carbachol. Nicotine was used as an internal control for compounds with agonist activity and carbachol was used as an internal control for compounds with very weak agonistic activity (K_d > 4700 nM for α3β4). The displacement of [^{3}H] EB by each of the test compounds and internal controls was calculated and expressed as Δml. Functional studies were then conducted using a stably transfected cell line that expresses the α3β4 nAChR and EC_{50} values were determined for the test compounds and the internal controls. A comparison of the Δml and EC_{50} values indicated that 9/11 compounds had been correctly identified as agonists or non-agonists of the α3β4 nAChR. A similar comparison could not be made for the α4β2 nAChR, since the intact cell line was not available for testing. The results of the study suggest that the immobilized nAChR columns can be used for the rapid on-line screening of compounds for their relative affinities for the immobilized receptor and as an initial determination of qualitative functional activities.

Keywords: On-line screening; Immobilized nicotinic receptors; Nicotinic agonists

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of ligand gated ion channels, which play a role in the regulation of synaptic activity. nAChRs consists of five transmembrane subunits oriented around a central pore [1,2]. At present, 12 different subunits have been identified, 9 α subunits (α2–α10) and 3 β subunits (β2–β4), which combine to form a wide variety of homomeric and heteromeric nAChRs. These subtypes are found in diverse locations in the central and peripheral nervous systems.

The nAChRs have been associated with a variety of clinical states including Alzheimer's and Parkinson's diseases, schizophrenia and addiction [3] and have become targets for new drug development. For example, the α3β4 nAChR and

* Corresponding author. Tel.: +1 410 558 8498; fax: +1 410 558 8409.
E-mail address: Wainerir@grc.nia.nih.gov (I.W. Wainer).

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α4β2 nAChR have been targeted for their potential roles in pain (α3β4 nAChR), gastrointestinal tract motility (α3β4 nAChR), short-term memory (α4β2 nAChR) and nicotine addiction (α4β2 nAChR) [4].

A key goal in the development of therapeutic agents aimed at the nAChR is to produce subtype-specific agonists in order to overcome the non-selective pharmacological profile of nicotine [5]. The primary binding sites for agonists are located on the extracellular portion of the receptor in a pocket at the interface between the N-terminals of the α and β subunits [3,6,7]. One approach has been to construct pharmacophores of the agonist binding site [8,9] and to synthesize drug candidates which fit the conformational requirements of this site. This has led to the syntheses of conformationally constrained nicotine and anabasine analogues [10–15].

The determination of the nAChR agonist activity of newly synthesized compounds is a key component of the drug discovery process. This has been approached using both binding and functional assays. Binding assays have been used to determine binding affinities, usually disassociation constants, K_d, which are considered a reflection of pharmacological activity. The predominate experimental approach used to determine K_d values is competitive membrane binding using a radio-ligand as the marker and filtration techniques, although surface plasmon resonance spectroscopy has also been used with a muscular nicotinic receptor [16]. However, binding affinities of a number of other proteins have also been determined by frontal affinity chromatography and this approach has been reviewed (cf. [17,18]).

We have reported an alternative method for the study of binding interactions at the agonist binding site, which utilized liquid chromatographic stationary phases containing immobilized nAChRs. In the initial studies, chromatographic columns containing either the α3β4 nAChR subtype [19,20] or the α4β2 nAChR subtype [20,21] were used in frontal displacement chromatography, with epibatidine (EB), a high affinity agonist, as the marker and multiple concentrations of the test ligands in the mobile phase. The results demonstrated that the immobilized nAChR columns could be used to accurately rank known agonists based upon their K_d values both within and between nAChR subtypes and that chromatographically determined K_d values can be correlated with those obtained using standard membranes binding techniques.

However, binding affinities, K_d values, do not necessarily reflect functional properties such as the EC_{50} values, which are defined as the concentration of a ligand required to obtain 50% of the maximal elicited response [22]. Thus, binding assays are primarily used as the initial screen, while functional assays are necessary to characterize lead drug candidates. In the case of nAChRs, EC_{50} values have been determined using rubidium ($^{86}Rb^+$) efflux assays [23–25], fluorescent assays using ion-sensitive dyes [26] and a variety of electrophysiological approaches [27]. All of these assays are time consuming and labor intensive.

The current study has explored the possibility of the on-line determination of EC_{50} values using chromatographic columns containing the α3β4 nAChR and the α4β2 nAChRs. The objective was to produce a rapid and inexpensive assay which can be automated. In this approach, a single chromatographic experiment has been used to sort a series of conformationally constrained nicotine and anabasine compounds based upon their ability to reduce the chromatographic retention of EB relative to the reductions produced by nicotine, a strong nAChR agonist, and carbachol, a weak nAChR agonist. The reductions in the retention of EB observed on the α3β4 nAChR column were compared with EC_{50} values determined using ^{86}Rubidium chloride ($^{86}Rb^+$) efflux studies carried out in a cell line expressing the α3β4 nAChR (the same cell line used to create the chromatographic column). The results of these studies are presented below.

2. Materials and methods

2.1. Materials

Minimum essential medium with Earles salts and L-glutamine (MEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S), geniticin were purchased from Gibco (Carlsbad, CA, USA). ^{86}Rubidium chloride was purchased from Perkin-Elmer (Boston, MA, USA). [^{3}H] Epibatidine dihydrochloride ([^{3}H] EB) was purchased from Amersham Life Science Products (Boston, MA, USA). 24-Well plates were purchased from Fisher Scientific (Fairlawn, NJ, USA). Carbachol hydrochloride, (*S*)-nicotine tartrate, benzamidine, NaCl, $MgCl_2$, $CaCl_2$, KCl, $MgSO_4$, cholate, leupeptin, phenyl methyl sulfonyl fluoride (PMSF), EDTA, trizma, poly D-lysine, and HEPES were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol, ammonium acetate and 0.1 M ammonium hydroxide solution were purchased from Fisher Scientific (Pittsburgh, PA, USA). Immobilized Artificial Membrane PC Stationary Phase (IAM-PC, 12 μm, 300 A) was purchased from Regis Chemical Co. (Morton Grove, IL, USA).

2.2. Test compounds

The synthesis and characterization of the test compounds used in this study have been previously reported [13]. The structures of these compounds are presented in Fig. 1.

2.3. Methods

2.3.1. Preparation of the α3β4 nAChR and α4β2 nAChR columns

The membranes containing the α3β4 nAChR and α4β2 nAChR subtypes were obtained from previously reported cell lines and were provided by K. Kellar (Department of Pharmacology, Georgetown University, Washington, DC, USA). The nAChRs were immobilized on the IAM stationary phase as previously described [19,21]. In brief, membranes prepared

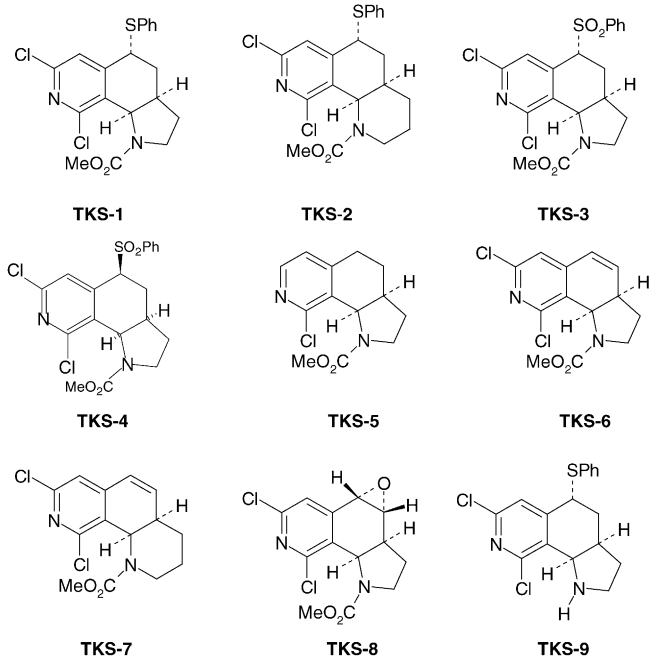

Fig. 1. Chemical structure of a series of novel compounds studied for activity on the α3β4 and α4β2 nicotinic receptor.

from 10^6 cells expressing the desired nAChR subtype were suspended in 30 ml of Tris–HCl [50 mM, pH 7.4], containing 5 mM EDTA, 3 mM benzamidine and 0.2 mM PMSF. The suspension was homogenized for 3 × 10 s at the setting of 15 on a Model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland), intermittently placing the tissue in an ice bath. The mixture was centrifuged for 10 min at 4 °C at 32,000 × *g* and the supernatant was discarded. The pellet was suspended in 10 ml of Tris–HCl [50 mM, pH 7.4] containing 100 mM NaCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 5 mM KCl, 2% sodium cholate and 10 μg/ml leupeptin. The resulting mixture was stirred for 18 h at 4 °C and centrifuged at 100,000 × *g* for 30 min, and the supernatant containing nAChR-cholate solution was collected.

The supernatant (nAChR-cholate suspension) was mixed with 200 mg of dried IAM-PC packing material and stirred gently for 1 h at 25 °C, transferred into dialysis tubing and dialyzed for 24 h at 4 °C against 1 L of Tris–HCl [50 mM, pH 7.4] containing 5 mM EDTA, 100 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM PMSF. The resulting mixture was centrifuged for 3 min at 4 °C at 700 × *g* and the supernatant was discarded. The pellet (nAChR-IAM) was washed with 5 ml of Tris–HCl [50 mM, pH 7.4] and centrifuged. This process was repeated until the supernatant was clear.

The nAChR-IAM (200 mg) was packed into a HR 5/2 glass column (Amersham Pharmacia Biotech, Uppsala, Sweden) to yield a 150 mm × 5 mm (i.d.) chromatographic bed. The resulting column contained 346 fmoles of active binding sites as determined using frontal chromatography with [^{3}H] epibatidine as the marker ligand.

2.3.2. Chromatographic studies

The nAChR-IAM column was placed in a chromatographic system consisting of a LC-10AD isocratic HPLC pump (Shimadzu, Columbia, MD, USA), a 50 ml sample Superloop (Amersham Pharmacia Biotech) and an IN/US system β-ram Model 3 on-line scintillation detector (IN/US, Tampa, FL, USA) with a dwell time of 2 s and running Laura lite 3 software. The mobile phase consisted of ammonium acetate (10 mM, pH 7.4) delivered at 0.4 ml/min at room temperature.

The marker ligand for the chromatographic studies was [^{3}H] EB applied at a concentration of 25 pM for the studies carried out using the α3β4 nAChR column and at a concentration of 20 pM for the studies carried out using the α4β2 nAChR column. In the studies, the 50 ml sample Superloop was used to apply 16 ml solutions containing the marker ligand and one of the test compounds. The test compounds were applied at concentrations of 2 nM for studies carried out using the α3β4 nAChR column and at concentration of 1 nM for the studies carried out using the α4β2 nAChR column. Each series of experiments also included the application of solutions containing the marker ligand and nicotine and the marker ligand and carbachol at the same concentrations used for the test compounds.

The applied volume corresponding to the half-height of the breakthrough curve was used to determine the retention volume of the marker ligand. The displacement of [^{3}H] EB from the nAChR by a test compound was defined as the difference in breakthrough volume of [^{3}H] EB applied to the column alone and the breakthrough volume for [^{3}H] EB observed after the addition of the test compound, and this values was designated as Δml.

2.3.3. Determination of EC_{50} values

The KXα3β4R2 cell line used in this study was established and maintained as described previously [28] and the EC_{50} values for a cohort of the test compounds were determined using a previously reported $^{86}Rb^+$ efflux assay [24]. Briefly, cells were grown in selection growth medium (500 ml MEM, 10% FBS, 1% p/s, 350 mg geniticin). Once the cells reached greater than 90% confluence, they were plated (1 ml/well) on 24-well plates coated with poly-D-lysine (>300,000 MW). These plates were then incubated for 48 h at 37 °C to reach greater than 90% confluence. The media was then removed and the cells were incubated with 0.5 ml of [$^{86}Rb^+$] (2 μCi/well) in growth medium for 4 h at 37 °C. The media was then aspirated and the cells were washed for 2 min with 1 ml of Buffer A (15 mM Hepes, 140 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, pH 7.4)/well. The process was repeated two additional times, first with a wash of 2 min and a final wash of 7 min.

The efflux studies were then initiated by the addition to the wells of 1 ml solutions of either Buffer A (control) or Buffer A with the test compounds (experimental) which were applied at concentrations ranging from 0.279 to 1000 μM. The solutions were kept in the wells for 2 min, after which they were

collected, transferred into scintillation vials and counted for [$^{86}Rb^{+}$] content using liquid scintillation counting. The EC_{50} of the test compounds was determined by relating the amount of [$^{86}Rb^{+}$] present in the solutions to the concentration of the test compound. The data was analyzed by non-linear regression with a sigmoidal response curve using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA) running on a personal computer.

3. Results and discussion

Elution profiles showing a front and plateau regions were obtained for the marker ligand, [^{3}H] EB in all of the chromatographic experiments on the α3β4 nAChR columns. The effects of the test ligands, nicotine and carbachol on the retention volume of [^{3}H] EB, expressed as Δml, are presented in Table 1.

Nicotine is a potent agonist of the α3β4 nAChR with an EC_{50} value of 19.8 μM while carbachol is essentially inactive at this nAChR. The objective was to use these compounds as internal standards in order to sort the test compounds into active and inactive categories based upon their relationship to the Δml values produced by nicotine and carbachol on the α3β4 column. In these studies, the Δml produced by the addition of nicotine to the mobile phase was 0.16 ml, and four of the test compounds produced an equivalent or greater displacement (Table 1). Carbachol had essentially no effect on the retention of [^{3}H] EB, as did three of the test compounds, while the addition of TKS-3 and TKS-5 increased the breakthrough volume of [^{3}H] EB by 0.12 and 0.32 ml, respectively (Table 1).

The EC_{50} values that were determined for the test compounds are presented in Table 1. The calculated EC_{50} values were compared to the chromatographically determined Δml values in order to determine if there was a qualitative relationship between the two values. The experimental question was, if the Δml value was greater than or equal to the displacement produced by nicotine, did the compound have agonist activity and if the Δml value was less than or equal to the value observed with carbachol, was the compound essentially inactive at the α3β4 nAChR. The method correctly identified three of the four test compounds that had agonist activity and four of the five compounds that were inactive at the α3β4 nAChR (Table 1).

Table 1
The comparison between the agonist activity of constrained nicotine and anabasine derivatives, expressed as the EC_{50} value, determined using a cell line expressing the α3β4 neuronal nicotinic acetylcholine receptor (α3β4 nAChR) and their effect on the retention of epibatidine (EB) in singlicate, expressed as Δml, calculated as breakthrough volume of EB alone minus the breakthrough volume of EB after the addition of the test ligand, on the immobilized α3β4 nAChR and α4β2 nAChR liquid chromatography stationary phases

Sample	EC_{50} (μM)	Δml α3β4	Δml α4β2
[^{3}H] EB	$(28.3 \pm 1.6) \times 10^{-3}$	–	–
TKS-9	26.4 ± 1.8	1.60	0.34
TKS-6	20.8 ± 2.9	0.28	0.28
TKS-8	> 300	0.28	0.26
TKS-7	13.8 ± 3.0	0.24	0.20
Nicotine	19.8 ± 1.6	0.16	0.26
TKS-2	18.2 ± 3.4	0	−0.05
Carbachol	> 1000	−0.04	−0.06
TKS-4	> 1000	−0.04	−0.07
TKS-1	> 1000	−0.04	0.17
TKS-3	> 1000	−0.12	0.13
TKS-5	> 1000	−0.32	0.18

TKS-8 effectively displaced [^{3}H] EB indicating that the compound binds at the agonist binding site, but it did not display any functional activity. These results suggest that TKS-8 may act as a competitive antagonist of α3β4 nAChR. The opposite results were observed with TKS-2, which did not displace [^{3}H] EB but had a functional activity similar to that of nicotine in the $^{86}Rb^{+}$ efflux assay. The reason for this result is not clear.

Previous chromatographic studies with the α3β4 nAChR column have demonstrated that quantitative data in the form of binding affinities, i.e. K_d values, can be obtained using five or more concentrations of the test compound in a series of competitive displacement experiments [19–21]. The relative order of the K_d values were the same as those obtained using membrane binding studies and the two data sets correlated with an $r^2 = 0.9972$ ($p = 0.0014$) [21].

The objective of this study was the rapid determination of qualitative, functional data in the form of relative EC_{50} values. Thus, only a single concentration of each test compound and internal standard were used. It has been previously shown that the frontal retention volumes were reproducible for the α3β4 nAChR [20] and for the PGP transporter [29]. The only realistic outcome was to sort the test compounds into active or inactive categories. With the limited experimental set utilized in this study, it appears that the objective of this study has been accomplished.

The relationship between binding affinities and functional properties was previously established for competitive inhibitors of enzymes by Cheng and Pursoff [30]. In this approach, the binding affinity of the inhibitor (K_i), the functional strength of the inhibitor (IC_{50} value) and the EC_{50} of a specific marker agonist are related in the following manner:

$$K_i = \frac{I_{50}}{1 + (S/K_m)}$$

where S is substrate concentration and K_m is the affinity of the substrate for the enzyme.

This approach can also be applied to the determination of relative IC_{50} values between different inhibitors [30]. The conditions for this comparison are that the inhibitory compounds have an identical mechanism of action and that the assays are performed under the same conditions.

In our studies, the Cheng–Pursoff equation could not be used to calculate the EC_{50} values as our studies were carried out using only agonists. However, since in displacement affinity chromatography, binding affinities (K_d values) are

directly related to Δml [31], and since with the nAChR there is a relationship between EC_{50} and binding at the agonist binding site [8,9], comparative competitive displacement studies should be capable of the relative, qualitative determination of EC_{50} values. As with the Cheng–Pursoff approach, the necessary requirements are that the agonists have an identical mechanism of action and that the experiments are performed under the same conditions.

In this study, the test compounds were designed using the known agonists, nicotine and anabasine as the template [13]. Thus, it is assumed that they will all bind at the agonist binding site, albeit with differing affinities. The relationship between the chromatographic displacement and binding to the nAChR is controlled by the use of EB, a specific marker for the nAChR. Previous studies have demonstrated that the displacement of EB by nicotine is independent of the presence of co-immobilized receptors, such as γ-amino-butyric acid ($GABA_A$) and *N*-methyl-D-aspartate (NMDA), as well as other non-specific cellular components [32].

The other condition for the comparison, the same experimental conditions, was also met during this study. The chromatographic conditions were held constant and the nAChR columns were stable throughout the study. In addition, the same concentration of the marker ligand, [^{3}H] EB and the same concentrations of nicotine, carbachol and the test compounds were used in the chromatographic experiments. Thus, the observed effects on the breakthrough volume of the marker ligand should be relative measures of the effect of the test compounds on the nAChR.

The displacement experiments were repeated using an immobilized α4β2 nAChR column. When the test compounds, nicotine and carbachol were studied on the α4β2 nAChR, elution profiles showing a front and plateau regions were obtained for the marker ligand, [^{3}H] EB in all of the chromatographic experiments (cf. Fig. 2). The effects of the test ligands, nicotine and carbachol on the retention volume of [^{3}H] EB, expressed as Δml, are presented in Table 1.

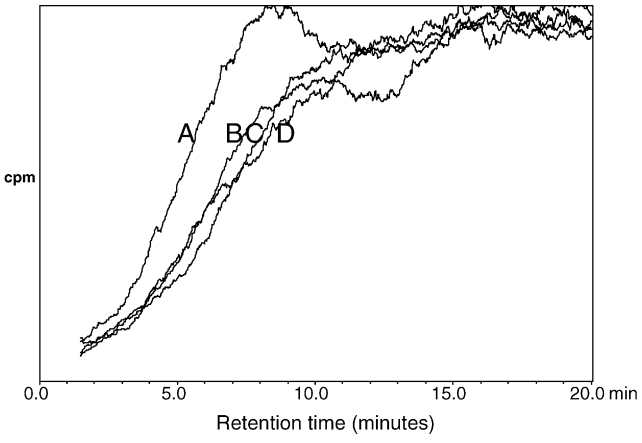

Fig. 2. Example of the elution profiles of [^{3}H] epibatidine on α4β2 nAChR-IAM stationary phase (0.5 cm × 1.6 cm). Frontal elution curves of 1 nM cytisine (A), 1 nM TKS-9 (B), 20 pM [^{3}H] epibatidine (C) and 1 nM TKS-1 (D). Mobile phase 10 mM ammonium acetate, pH 7.4 with a flow rate at 0.4 ml/min.

The results demonstrated that TKS-1, -3, -5, -6, -7, -8, and -9 should have affinity for the α4β2 nAChR while TKS-2 and -4 produced no displacement of EB. The data indicated that there were differences in the selectivity of TKS-1, -3, -5 for the α3β4 nAChR and α4β2 nAChR, and that these compounds should be active at the α4β2 nAChR but not at the α3β4 nAChR. Unfortunately, functional tests for the α4β2 nAChR are not running in this laboratory and the significance of the observed differences will, of necessity, be reported in a later communication.

4. Conclusions

The results from this study demonstrate that single displacement chromatography studies utilizing immobilized nAChR stationary phases can be used to qualitatively rank compounds according to their EC_{50} values, and that the approach may be able to identify subtype specific activities with a success rate exceeding 80%. Although, the potential for missing potential ligands is present, this method has a comparable if not better success rate than most available rapid throughput methods. In addition, each experimental run took 130 min, 40 min to apply the 16-ml solution containing the [^{3}H] EB and the displacers followed by a 90 min washout period, and the immobilized α3β4 nAChR and α4β2 nAChR columns were stable for at least 6 months. This is in contrast to the functional studies, which took 1 week for three compounds, and a total of 4 weeks to complete these studies.

References

[1] E.X. Albuquerque, M. Alkondon, E.F.R. Pereira, N.G. Castro, A. Schrattenholz, C.T.F. Barbosa, R. Bonfante-Cabarcas, Y. Aracava, H.M. Eisenberg, A. Maelicke, J. Phamacol. Exp. Ther. 280 (1999) 1117.
[2] F. Hucho, V.I. Tsetlin, J. Machold, Eur. J. Biochem. 239 (1996) 539.
[3] M.W. Holladay, M.J. Dart, J.K. Lynch, J. Med. Chem. 40 (1997) 4169.
[4] K.G. Lloyd, M. Williams, J. Pharmacol. Exp. Ther. 292 (2000) 461.
[5] H.R. Arias, Biochem. Biophys. Acta 1376 (1998) 173.
[6] H.R. Arias, Neurochem. Int. 36 (2000) 595.
[7] A. Karlin, Nat. Rev. Neurosci. 3 (2002) 102.
[8] L. Curtis, F. Chiodini, J.E. Spary, S. Bertrand, J. Palt, G. Westera, D. Bertrand, Eur. J. Pharm. 393 (2000) 155.
[9] R.A. Glennon, J.L. Herndon, M. Dukat, Med. Chem. Res. 4 (1994) 461.
[10] T.E. Catka, E. Leete, J. Org. Chem. 43 (1978) 2125.
[11] M.I. Damaj, W. Glassco, M. Dukat, E.L. May, R.A. Glennon, B.R. Martin, Drug Dev. Res. 38 (1996) 177.
[12] S. Lindström, L. Ripa, A. Hallberg, Org. Lett. 2 (2000) 2291.
[13] T.K. Sarkar, S. Basak, I.W. Wainer, R. Moaddel, R. Yamaguchi, K. Jozwiak, H.-T. Chenand, C.-C. Lin, J. Med. Chem., in press.
[14] S.W. Szczepanski, K.G. Anouna, Tetrahedron Lett. 37 (1996) 8841.
[15] T. Ullrich, S. Krich, D. Binder, K. Mereiter, D.J. Anderson, M.D. Meyer, M. Pyerin, J. Med. Chem. 45 (2002) 4047.

[16] E.K. Schmidt, T. Liebermann, M. Kreiter, A. Jonczyk, R. Naumann, A. Offenhausser, E. Neumann, A. Kukol, A. Maelicke, W. Knoll, Biosens. Bioelectron. 13 (6) (1998) 585.
[17] I. Gottschalk, C. Lagerquist, S.S. Zuo, A. Lundqvist, P. Lundahl, J. Chromatogr. B 768 (1) (2002) 31.
[18] R. Moaddel, L. Lu, M. Baynham, I.W. Wainer, J. Chromatogr. B 768 (1) (2002) 41.
[19] I.W. Wainer, Y. Zhang, Y. Xiao, K.J. Kellar, J. Chromatogr. B 724 (1999) 65.
[20] R. Moaddel, I.W. Wainer, J. Pharm. Biomed. Anal. 30 (2003) 1715.
[21] Y. Zhang, Y. Xiao, K.J. Kellar, I.W. Wainer, Anal. Biochem. 24 (1998) 22.
[22] E.J. Nestler, S.E. Hyman, R.C. Malenka, Molecular Neuropharmacology: A Foundation to Clinical Neuroscience, McGraw-Hill, 2001, 9.
[23] Y. Xiao, E.L. Meyer, J.M. Thompson, A. Surin, J. Wroblewski, K.J. Kellar, Mol. Pharmacol. 54 (1998) 322.
[24] Y.X. Xiao, R.D. Smith, F.S. Caruso, K.J. Kellar, J. Pharmacol. Exp. Ther. 299 (2001) 366.
[25] J.M. Meyer, J. Clin. Psychopharmacol. 21 (2001) 569.
[26] R.W. Fitch, Y. Xiao, K.J. Kellar, J.W. Daly, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 4909.
[27] R.L. Papke, P.R. Sanberg, R.D. Shytle, J. Pharmacol. Exp Ther. 297 (2001) 646.
[28] Y. Xiao, M. Baydyuk, H.P. Wang, H.E. Davis, K.J. Kellar, Bioorg. Med. Chem. Lett. 14 (8) (2004) 1845.
[29] R. Moaddel, P. Bullock, I.W. Wainer, J. Chromatogr. B 799 (2) (2004) 255.
[30] Y. Cheng, W. Prusoff, Biochem. Pharm. 22 (1973) 3099.
[31] B.M. Dunn, I.M. Chaiken, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 2382.
[32] R. Moaddel, J.F. Cloix, G. Ertem, I.W. Wainer, Pharm. Res. 19 (1) (2002) 104.