Electronic Supplementary Material (ESI) for Medical Chemistry Communications

Supporting Information

Online parallel fragment screening and rapid hit exploration for nicotinic acetylcholine receptors

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Table S-1. Data of 51 validated hits.

Code	Ls		Ac		Ls/Ac selectivity
	pK_i	s.e.m.	pK_i	s.e.m.	
1	4,84	0,09	4,36	0,03	3,05
2	6,01	0,00	4,24	0,05	58,60
3	4,34	0,01	4,44	0,01	0,80
4	4,98	0,10	3,18	0,00	62,14
5	4,66	0,08	<3		>46,22
6	4,63	0,03	3,43	0,00	16,18
7	4,65	0,02	<3		>44,70
8	4,36	0,07	4,39	0,11	0,92
9	4,12	0,03	4,06	0,06	1,13
10	5,91	0,17	4,18		53,89
11	4,31	0,09	<3		>20,30
12	4,56	0,10	3,62	0,10	8,73
13	5,05	0,11	4,94	0,37	1,27
14	4,01	0,07	3,84	0,05	1,49
15	4,03	0,14	<3		>10,64
16	4,20	0,13	3,37	0,05	6,77
17	6,47	0,01	5,78	0,02	4,89
18	4,56	0,07	4,21	0,22	2,24
19	5,50		4,82		4,75
20	3,89	0,11	<3		>7,74
21	4,24	0,25	<3		>17,24
22	4,83	0,14	4,80	0,04	1,07
23	4,69	0,08	3,94	0,04	5,67
24	4,22	0,08	3,32	0,00	8,07
25	3,87	0,09	3,37	0,06	3,11
26	4,71	0,10	3,70	0,03	10,29
27	4,03	0,09	3,32	0,00	5,19
28	4,49	0,08	4,34	0,01	1,42
29	4,45	0,07	3,46	0,14	9,78
30	4,69	0,06	3,78	0,11	8,21
31	4,66	0,08	3,42	0,24	17,43
32	4,58	0,08	3,98	0,09	3,93

33	4,55	0,08	4,14	0,29	2,57
34	4,04	0,05	<3		>10,87
35	4,08	0,09	3,44	0,01	4,34
36	4,24	0,06	3,56	0,04	4,86
37	4,87	0,21	3,73	0,00	13,94
38	4,41	0,13	3,63	0,03	6,09
39	5,78	0,00	4,03	0,05	55,86
40	4,03	0,09	<3		>10,79
41	4,68	0,10	3,96	0,02	5,19
42	3,97	0,08	3,56	0,04	2,58
43	4,53	0,10	3,92	0,02	4,15
44	4,53	0,10	3,70	0,03	6,88
45	4,58	0,11	3,70	0,03	7,61
46	4,37	0,16	3,50	0,08	7,42
47	5,97	0,03	4,42	0,04	35,95
48	4,50		3,31	0,12	15,45
49	4,61	0,11	3,70	0,03	8,08
50	4,75	0,09	3,65	0,02	12,64
51	4,28	0,06	3,95	0,00	2,13

Table S-2. Similarity matrix for the ten hits presented in Table 1[i]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1	0.12	0.08	0.13	0.11	0.13	0.12	0.10	0.18	0.15
2	0.12	1	0.08	0.07	0.06	0.05	0.06	0.33	0.10	0.07
3	0.08	0.08	1	0.12	0.21	0.04	0.19	0.07	0.20	0.22
4	0.13	0.07	0.12	1	0.15	0.08	0.08	0.08	0.23	0.23
5	0.11	0.06	0.21	0.15	1	0.02	0.13	0.05	0.16	0.10
6	0.13	0.05	0.04	0.08	0.02	1	0.04	0.06	0.13	0.10
7	0.12	0.06	0.19	0.08	0.13	0.04	1	0.08	0.21	0.28
8	0.10	0.33	0.07	0.08	0.05	0.06	0.08	1	0.10	0.10
9	0.18	0.10	0.20	0.23	0.16	0.13	0.21	0.10	1	0.37
10	0.15	0.07	0.22	0.23	0.10	0.10	0.28	0.10	0.37	1

[i]Tanimoto scores based on ECFP4 fingerprints in Pipeline Pilot.

Experimental Procedures

General Remarks. The ELISA blocking reagent (BR) was obtained from Hoffmann-La Roche (Mannheim, D) and the Bovine Serum Albumin (BSA) came from Gibco BRL (Breda, NL). For the AChBP assays (in microplate reader and in online format), a TRIS/PBS buffer was used with the following composition: 1 mM KH_2PO_4, 3 mM Na_2HPO_4, 0.16 mM NaCl and 20 mM Trizma-base at pH 7.5 with 400 mg/L ELISA BR.

Protein expression and purification. Recombinant Ac-AChBP and Ls-AChBP with a C-terminal His-tag in pFastbac vector (Invitrogen, San Diego, CA, USA) was expressed in baculovirus in SF9 insect cells following the manufacturer's recommendations. Secreted protein was purified on an ÄKTA purifier, using a HisTrap HP Cartridge (GE Healthcare, Uppsala, Sweden). The purity of the protein was checked on a SDS gel and the protein concentration was determined by Bradford analysis. Protein aliquots were stored at -80˚C until use.

Human neuroblastoma cells (SH-SY5Y) expressing human α7 nAChRs were kindly provided by Dr. Christian Führer (University of Zurich, Switzerland). They were grown in DMEM/F12 with glutamax, 10% heat-incativated FCS and 100 µg/ml Geneticin. Cells were washed with PBS, harvested and pelleted by centrifugation, washed 2x with PBS and aliquots stored at -80°C until use.

Human α4β2 receptors were obtained using a transient transfection of HEK293t cells. To this end, HEK293t cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 50 IU/mL penicillin, and 50 µg/mL streptomycin in 5% CO_2 humidified atmosphere at 37°C. Approximately 2 million cells were seeded in a 10 cm dish and cultured overnight before transfection. For transfection of each dish of cells, the transfection mixture was prepared in 0.6 mL PBS and contained 0.3 µg of human a4 subunit plasmid, 2.7 µg of human b2 subunit plasmid, 3.0 µg of RIC3 and 24 µL of 1 mg/mL 25 kDa linear polyethyleneimine (Polyscience, Inc., USA). The mixture was incubated for 10–15 min at room temperature before it was added into the monolayer cell

culture loaded with 6 mL of fresh and pre-warmed cell culture medium. Two days after transfection, the cells were washed with PBS, collected as pellet by centrifuging, and stored at –80°C until use.

Radioligand binding assay. Competition binding assays were performed with His-tagged Ls-AChBP or Ac-AChBP in buffer (PBS, 20 mM Tris, pH 7.4/ 0.05% Tween) in a final assay volume of 120 µL in Optiplates (white, Perkin-Elmer Life Science, Inc., USA,). Ligands were added at 10^{-3} to 10^{-11} M (stock concentrations: 10mM in DMSO and further diluted in a 20% DMSO H2O solution, final concentration in assay 4%). The concentration of radioligand, [^{3}H]epibatidine (Perkin-Elmer, specific activity ~ 56 Ci/mmol) was around the K_d value for the target protein, i.e., 1 nM for Ls-AChBP and 8 nM for Ac-AChBP. The amount of protein was chosen such that we obtained a clear window in the displacement curve, sufficient amount of counts in our scintillation counting and a radioligand depletion of less than 10%. PVT Copper His-Tag SPA beads (Perkin Elmer) were added at 2 mg/ml final concentration. Plates were incubated at room temperature under continuous shaking, protected from light, for 1.5 h. SPA beads were allowed to settle for 3 hours in the absence of light before counting. The label-bead complex was counted in a Wallac 1450 MicroBeta (PerkinElmer, USA). All radioligand binding data were evaluated by a non-linear, least squares curve fitting procedure using Graphpad Prism® (version 5, GraphPad Software, Inc., San Diego, CA). All data are represented as mean ± SEM from at least three independent experiments.

Binding assays for human α4β2 and α7 receptors were performed in a similar way as described for AChBP, but with a filtration assay and in a final volume of 100 µl. The cells were homogenized and sonicated immediately before use. In the α4β2 assay, [^{3}H]epibatidine was used at a final concentration of 2 nM and [^{3}H]MLA (K_D = 1.81 nM, (American Radiolabeled Chemicals, Inc, specific activity ~100 Ci/mmol) was used at a final concentration of 2 nM for the α7 assay. Bound radioligand was collected on 0.3% polyethyleneimine-pretreated Unifilter-96 GF/C filters (Perkin Elmer) using ice-cold 50 mM Tris buffer at pH 7.4. After drying the filters, scintillation fluid (MicroScint, Perkin Elmer) was added and the radioactivity was measured in a Wallac 1450 MicroBeta liquid scintillation counter. Radioligand

saturation experiments were performed with nicotine to determine non-specific binding. The concentration of nicotine was 100 μM for the $\alpha4\beta2$ receptor and 1 mM for the $\alpha7$ receptor.

Online bioaffinity analysis. A similar setup described by de Vlieger *et al*[1] was used but now in flow injection analysis (FIA) mode (utilizing no HPLC separation prior analysis). Figure 1 shows a schematic drawing of the system. A Shimadzu ('s Hertogenbosch, the Netherlands) SIL20 auto-injector introduced the samples into the system (40 µl injections for the whole library analysis; 2 µl for all subsequent analyses). The mobile phase for direct sample introduction consisted of MeOH-H_2O-Formic Acid (98.9 %-1 %-0.1 %). The bioaffinity analysis part employed five Agilent 1100 HPLC pumps, two Agilent 1100 fluorescence detectors (λ_{ex} = 485 nm, λ_{em} = 526 nm) and one Kratos UV-vis detector (λ = 254 nm). Four Agilent pumps were used to operate the superloops at flow-rates of 70 µl/min (with H_2O-MeOH-Formic Acid (98.9 %-1 %-0.1 %) as eluent), while one pump was used (at 100 µl/min) to provide an eluent flow of the mobile phase for transport of injected compounds to the biochemical and UV detection. The two parallel online assays either housed Ls- or Ac-AChBP in the respective $P1_b$ superloops. For the actual online assays, the Ls- and Ac-AChBP concentrations were 25 nM and 100 nM respectively in superloop 1a and 1b (representing 11 nM and 46 nM respectively in the assays). The concentration of tracer ligand DAHBA was 100 nM in superloop 2a (representing 46 nM in Ls-AChBP assay) and 2000 nM in superloop 2b (representing 911 nM in Ac-AChBP assay). The superloops were kept on ice. The assays were performed in a thermostated oven at 37°C. The volumes of the first and second reaction coils (of striped blue PEEK material; ID of 250 µm) were 5 µl and 50 µl, respectively.

Autofluorescence of fragments. An intrinsic weakness of fluorescence-based assays is the interference of fluorescence of the ligands under evaluation, at the same wavelength as the displacement ligand. Since the fragment library is screened at a high concentration, autofluorescence was expected for a certain part of the library. To investigate this, the fluorescence (at ex=485; em=535) of all fragments was measured in the platereader, at the estimated assay concentration ($1*10^{-5}$ M) in the same buffer;

3.8% of the fragments gave a significant amount of fluorescence. This means that 3.8% of the library could not be evaluated for binding to AChBP.

Screening of fragment library. The fragment library consisted of $5*10^{-4}$ M solutions in water (5% DMSO); the injection volume was 40 µL. Controls were taken along on every 96-well plate. Nicotine was used as a control for Ls-AChBP, in a $5*10^{-4}$ M concentration, lobeline for Ac-AChBP in a $5*10^{-5}$ M concentration. The thirteen plates of the fragment library were measured with 1.5 minute injection intervals in 3.5 days (n=1). The results were only used to determine the hit compounds; potential hits (signals ≥ three times the noise) were freshly weighted and measured again to rank the hits according to their relative peak heights. For hit validation, 10^{-2} M solutions in DMSO were used. Here, the injection volume was 2 µL and injection intervals of 3.5 minutes were used; fragments were evaluated at least in duplicate. For fragments with displacements near 100%, 10^{-2} M solutions were diluted 2, 10 and 20 times in DMSO and re-injected. The signal of the then resulting signals which was closest to 50% displacement was used for the pK_i estimation.

Synthesis of ketone building blocks. Secondary amine 1,4-dioxa-8-azaspiro-[4,5]decane (0.26 mL, 2.0 mmol) and the aldehyde or ketone were dissolved in 1,2-dichloroethane (7 ml). Sodiumtriacetoxyborohydride (0.59 g, 2.8 mmol, 1.4 eq) and acetic acid (120 µl, 2.0 mmol, 1 eq) were added. The reaction mixture was stirred overnight at room temperature. The reaction was acidified by adding 5 mL of a 1M HCl solution, and washed twice with dichloromethane (5 mL). The water layer was basified with Na_2CO_3, the product was extracted with DCM (3x 5 mL). The DCM layer was dried with Na_2SO_4 and concentrated. The ketal-protected ketones were deprotected by refluxing in TFA/DCM (1:1) for 4 hrs. The reaction mixture was concentrated, basified with 2.5M NaOH solution. The ketones were extracted with DCM, dried with Na_2SO_4, filtered and concentrated.

1-phenethylpiperidin-4-one (V4). Synthesized as described above, using phenylacetaldehyde (0.27 mL, 2.4 mmol, 1.2 eq.). A colorless oil was obtained (62 mg, 0.3 mmol, 15%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.14 (m, 5H), 2.83 (dd, J = 10.1, 6.3 Hz, 2H), 2.70 – 2.56 (m, 6H), 1.85 – 1.72 (m, 4H).

1-isopropylpiperidin-4-one (V5). Synthesized as described above, using acetone (0.22 mL, 3.0 mmol, 1.5 eq.). A colorless oil was obtained (93 mg, 0.7 mmol, 33%). ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.92 – 2.70 (m, 1H), 2.70 – 2.56 (m, 4H), 1.79 (dd, J = 7.2, 4.2 Hz, 4H), 1.09 (d, J = 6.6 Hz, 6H).

1-cinnamylpiperidin-4-one (V6). Synthesized as described above, using *trans*-cinnemaldehyde (0.30 mL, 2.4 mmol, 1.2 eq.). A colorless oil was obtained (112 mg, 0.5 mmol, 26%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.37 (m, 2H), 7.36 – 7.17 (m, 3H), 6.54 (d, J = 15.8 Hz, 1H), 6.31 (dt, J = 15.8, 6.8 Hz, 1H), 3.23 (dd, J = 6.8, 1.2 Hz, 2H), 2.72 – 2.55 (m, 4H), 1.81 (t, J = 5.7 Hz, 4H).

Construction of combinatorial libraries in 96-well format. The hydroxylamines and hydrazines, and five of the ketones, were purchased at Sigma Aldrich. For the ketone and hydroxylamine/hydrazine building blocks, $6*10^{-2}$ M stock solutions in DMSO were prepared. A stock solution of $240*10^{-2}$ M acetic acid in DMSO was prepared as well. Of each solution, 20 µL was used to obtain final concentrations of $2*10^{-2}$ M for the building blocks and $80*10^{-2}$ M for the catalyst. All building blocks were taken along separately as well, for control purposes. The reaction mixtures were shaken overnight at room temperature. The solutions were then diluted two times to reach the injection concentration of $1*10^{-2}$ M.

Control experiments were performed to validate the experimental setup. Five of the best compounds were synthesized at larger scale. The DMSO was removed by freeze-drying. LC-MS and ^{1}H-NMR spectra were recorded to confirm product formation. These five compounds were all formed with > 95% purities (as determined by UV detection at 230 nm, calculated as the percentage peak area of the analyzed compound). Dilution series were made (eight dilutions between $1.36*10^{-4}$ M and $1.65*10^{-10}$ M), 7 nM Ls-AChBP and 33 nM DAHBA were added and full curve affinities in triplicate were determined by measuring fluorescence in plate reader format. The NMR data for the two best compounds, **V4H19** and **V2H12**, is reported.

V4H19: ^{1}H NMR (400 MHz, DMSO) δ 7.34 – 7.12 (m, 5H), 7.11 – 6.99 (m, 2H), 6.49 – 6.38 (m, 1H), 3.08 (t, J = 6.0 Hz, 2H), 3.05 – 2.96 (m, 4H), 2.95 – 2.84 (m, 2H), 2.69 (t, J = 6.1 Hz, 2H), 2.54 (t, J = 6.0 Hz, 2H).

V2H12: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (s, 1H), 7.45 (d, J = 8.2 Hz, 1H), 7.26 (t, J = 3.9 Hz, 1H), 7.20 (t, J = 7.8 Hz, 1H), 6.81 (t, J = 7.5 Hz, 1H), 3.95 (s, 2H), 3.68 (d, J = 15.7 Hz, 1H), 3.42 (d, J = 15.1 Hz, 1H), 2.82 (d+s, J = 20.9 Hz, 4H), 2.62 (d, J = 13.6 Hz, 1H), 2.31 (s, 2H), 2.07 (d, J = 11.0 Hz, 1H), 1.87 (t, J = 9.7 Hz, 1H).

Dilution factor. The dilution factor after injection into the online bioaffinity analysis system was determined by a calibration process. For this, the fluorescent compound fluorescein was pumped into the online bioaffinity analysis system in different concentrations (1 µM – 60 nM concentrations in buffer) via one superloop, while the other superloop only contained buffer. The resulting increased fluorescent signals were recorded. Then, the fluorescein solution was replaced by buffer and a concentration range of fluorescein was subsequently injected (2 µl) into the online bioaffinity analysis system. The peak heights of eluting fluorescein peaks were compared with the elevated fluorescent signals from which the dilution factor of fluorescein in the online bioaffinity analysis system was determined. The dilution factor found for a 2 µl injection volume was 66.35.

Calculations estimated pK_i values. The formula describing sigmoidal-dose response curves, (%displacement = 100% / (1+10$^{\wedge\log IC50 - \log [ligand]}$)), was used to calculate IC_{50} values using displacement values relative to full displacement by a high concentration of nicotine (2 µl of 10^{-2}M solution). Concentrations in the bioassay were injected concentrations (2 µl) divided by the dilution factor. pK_i values were calculated using the Cheng-Prusoff equation, pK_i = pIC_{50} / log (1 + [DAHBA]/K_i DAHBA).

References

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