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JN403, *in vitro* characterization of a novel nicotinic acetylcholine receptor α7 selective agonist

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Abstract

This report describes the *in vitro* features of a novel selective nicotinic acetylcholine receptor (nAChR) α7 agonist, JN403, (S)-(1-Aza-bicyclo[2.2.2]oct-3-yl)-carbamic acid (S)-1-(2-fluoro-phenyl)-ethyl ester. JN403 was evaluated in a number of *in vitro* systems of different species, at recombinant receptors using radioligand binding, signal transduction and electrophysiological studies. When using [^{125}I] α-bungarotoxin (α-BTX) as a radioligand, JN403 has high affinity for human recombinant nAChR α7 ($pK_D = 6.7$). Functionally, JN403 is a partial and potent agonist at human nAChR α7. The compound stimulates calcium influx in GH3 cells recombinantly expressing the human nAChR with an pEC_{50} of 7.0 and an E_{max} of 85% (compared to the full agonist epibatidine). In *Xenopus* oocytes expressing human nAChR α7 JN403 induces inward currents with an pEC_{50} of 5.7 and an E_{max} of 55%. In both recombinant systems JN403 is a partial agonist and the agonistic effects are blocked after pre-administration of methyllycaconitine (MLA, 100 nM), a nAChR α7 antagonist. In functional calcium influx assays, JN403 displays a significantly lower potency for other subtypes of human nAChRs like α4β2, α3β4, α1β1γδ as well as 5HT$_3$ receptors when tested functionally as an antagonist ($pIC_{50} < 4.8$) and is devoid of agonistic activity ($pEC_{50} < 4$). Similarly, JN403 shows low binding activity at a wide panel of neurotransmitter receptors. Thus, JN403 is a potent and selective nAChR α7 agonist and will be a useful tool for the characterization of nAChR α7 mediated effects both *in vitro* and *in vivo*.

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Nicotinic acetylcholine receptors (nAChRs) belong to a super-family of ligand gated ion channels that include the receptors for GABA, glycine and serotonin (5-HT$_3$). For centrally expressed nAChRs twelve genes have been cloned to date: nine α subunits (α2–α10) and three β subunits (β2–β4). The nAChR α7 binds α-bungarotoxin with high affinity and can form functional homopentamers, although recent findings indicate that the α7 subunit may assemble with other subunits [9,4].

Agonism at the nAChR α7 has been discussed to be of therapeutic potential in a variety of neurological disorders like age associated memory impairment (AAMI), Alzheimer's disease and schizophrenia. Peripherally, the nAChR α7 is expressed in macrophages and stimulation of the nAChR α7 inhibits the release of inflammatory cytokines (*e.g.* TNF-α, IL-1) as well as HMGB-1 [13]. Thus, the clinical use of agonists of the nAChR α7 could represent a strategy against inflammatory diseases.

Recently several nAChR α7 agonists have been described: PSAB-OFB [1] and tropisetron [11] are potent nAChR α7 agonists which additionally block the 5-HT3 receptor. A well described nAChR α7 agonist is the anabaseine derivative GTS-21 [2] which displays antagonistic effects at other nAChRs. GTS-21 has been assessed in human healthy volunteers and several measures of cognitive functions were improved upon treatment [5]. GTS-21 is a prodrug with very low efficacy at the human nAChR α7. The pharmacologically active metabolite is 4-OH-GTS-21.

The current report describes the *in vitro* characterization of (S)-(1-Aza-bicyclo[2.2.2]oct-3-yl)-carbamic acid (S)-1-(2-fluoro-phenyl)-ethyl ester (JN403) [8], a novel partial, selective and potent nAChR α7 agonist.

All cell lines were cultured at 37 °C, 5% CO$_2$ and 95% relative humidity. GH3 cells expressing the human nAChR α7 have been previously described [3]. These GH3-ha7-22 cells

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were grown in Ham's/F12 medium supplemented with 10% fetal bovine serum and 50 µg/ml G418. Rat nAChR α7 GH3 were generated as described for the GH3-ha7-22 cells and grown in the same medium. HEK293-a4b2 and HEK293-a3b4 cells have been previously described [7] and were cultured in a 1:1 mixture of Dulbecco's modified eagle medium and Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 µg/ml G418 and 100 µg/ml hygromycin B. TE671 were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. N1E115 cells were grown in Dulbecco's modified eagle medium supplemented with 20% fetal bovine serum. For passaging, the cells were detached from the cell culture flask by washing with phosphate-buffered saline (PBS) and brief incubation with trypsine (0.5 mg/ml)/EDTA (0.2 mg/ml) (Gibco BRL). The cells were passaged every 3 days.

Radioligand binding was performed as reported previously in cells expressing nAChRs [3,7].

Determination of changes in intracellular calcium was performed as described previously [3,7]. For the N1E115 the same protocol was used as described for HEK293-a4b2 and HEK293-a3b4 cells.

Oocyte preparation and injection was done as described earlier [3,6].

For electrophysiology, currents were recorded using a Labview-based software (KooL, New Vision Engineering, Winterthur, CH) and analyzed using Prism 3.0 software (GraphPad, San Diego, USA). Baseline current drifts were corrected using linear interpolations. For concentration response curves, the induced inward peak current was measured. Data from different oocytes were normalized to the response evoked by 1 mM acetylcholine and fitted by the following equation using non-linear regression:

$$I(c) = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{n(\log(EC_{50}) - \log(c))}}$$

with $I(c)$ as the current amplitude evoked by the agonist concentration c, I_{min} the asymptotic minimal, I_{max} the asymptotic maximal current, EC_{50} the half-maximal activating concentration and n as the Hill coefficient. Errors of EC_{50} values and Hill coefficients were calculated from the covariance matrix by the fitting routine (Prism 3.0). EC_{50} values are given as mean (lower to upper boundaries of the 95% confidence interval) if not stated otherwise.

For measuring concentration response curves, the amplitude of the peak currents in response to bath application of JN403 were analyzed. To investigate steady state currents, the following application protocol and analysis procedure were used: on every oocyte acetylcholine (at EC_{50} concentration) was washed in for 120 s. After a washout period, long enough to allow for recovery from desensitization, JN403 (EC_{50} concentration) was washed in for 120 s. The steady state current was measured as the average amplitude during the time period from 61 to 120 s. The steady state response of JN403 was normalized to the acetylcholine response measured on the same oocyte.

For analyzing the recovery from desensitization, a one phase exponential curve was fitted to the data points using Igor-software with the upper value fixed to 100%. The intersection of

the fitted data curve with the *y*-axis (A) was set to 0%. The time constant τ is the time that it takes the response to reach 63% of the control response.

The synthesis of JN403 [8] was performed as follows: to a solution of (S)-1-(2-fluorophenyl)ethanol 1.25 ml (10.0 mmol) in 10 ml tetrahydrofurane, *N,N'*-carbonyldiimidazole 1.70 g (10.5 mmol) is added. The white suspension is heated up to 50 °C and stirred for 40 min at this temperature. The reaction mixture is cooled and evaporated. The crude product is purified by flash chromatography (cyclohexane/ethyl acetate 90/10) to yield imidazole-1-carboxylic acid (S)-1-(2-fluoro-phenyl)-ethyl ester as colorless oil (analytical data: Rf = 0.20; TLC silicagel, cyclohexane/ethyl acetate 50/50). In the next step, to a solution of (S)-imidazole-1-carboxylic acid 1-(2-fluoro-phenyl)-ethyl ester 0.54 g (2.31 mmol) in 5 ml dimethylformamide, (S)-3-aminoquinuclidine dihydrochloride 0.46 g (2.31 mmol) and sodium carbonate 0.49 g (4.62 mmol) are added. The suspension is heated up to 80 °C and stirred for 18 h at this temperature. The reaction mixture is then cooled and extracted with water and ethyl acetate. The combined organic phases are dried and evaporated. The oily residue is dried, dissolved in ether and acidified with a 4 M hydrochloric acid dioxane solution. The precipitating crystals are filtered, washed with ether and dried to obtain 0.55 g (72% yield) of a white powder, the (S)-(1-Aza-bicyclo[2.2.2]oct-3-yl-carbamic acid (S)-1-(2-fluoro-phenyl)-ethyl ester hydrochloride, with a melting point of 218.7–219.4 °C (decomposition) and an optical rotation of −36.3° (*c* = 0.6 in dichloromethane, of the free base; analysis found (calc.) C 58.19 (58.45), H 6.52 (6.74), N 8.45 (8.52), Cl 10.90 (10.78), F 5.85 (5.78), O 9.90 (9.73)). All reagents are commercially available. The absolute (S,S)-stereochemistry was confirmed by X-ray analysis using a salt derived from camphersulfonic acid.

Fig. 1 shows the structure of JN403.

JN403 displayed high affinity for human nAChR α7 with high selectivity towards other nAChRs. JN403 had a pK_D of 6.7 ± 0.04 (*n* = 5) at recombinantly expressed human nAChR α7 as determined in radioligand binding studies using [^{125}I] α-bungarotoxin (α-BTX) as a radioligand. At other recombinantly expressed nAChR subtypes labeled with [^{125}I]epibatidine JN403 showed the following affinity values (pK_D ± S.E.M.): human α3β4: 5.2 ± 0.3 (*n* = 4); human α4β2: 3.8 ± 0.04 (*n* = 4). The pK_D of JN403 at endogenously expressed 5-HT3 receptors in the murine neuroblastoma cell line N1E-115 labeled with [^{3}H] zacopride was 4.9 ± 0.1 (*n* = 3). Further, JN403 showed selectivity over a wide range of neurotransmitter receptors, ion channels and transporters (see supplementary Table 1). Thus, radioligand
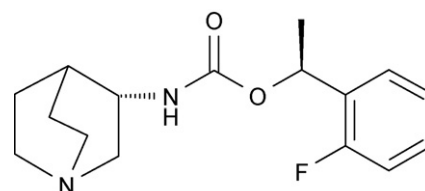

Fig. 1. Structure of JN403.