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In vitro and *ex vivo* characterization of (−)-TZ659 as a ligand for imaging the vesicular acetylcholine transporter

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ABSTRACT

The loss of cholinergic neurons and synapses relates to the severity of dementia in several neurodegenerative pathologies; and the vesicular acetylcholine transporter (VAChT) provides a reliable biomarker of cholinergic function. We recently characterized and ^{11}C-labeled a new VAChT inhibitor, (−)-TZ659. Here we report the *in vitro* and *ex vivo* characterization of (−)-TZ659. A stably transfected PC12$^{A123.7}$ cell line which expresses human VAChT (hVAChT) was used for the *in vitro* binding characterization of (−)-[^{3}H]TZ659. A saturated binding curve was obtained with $K_d = 1.97 \pm 0.30$ nM and $B_{max} = 3240 \pm 145.9$ fmol/mg protein. In comparison, a PC12$^{A123.7}$ cell line that expresses mutant hVAChT showed decreased binding affinity ($K_d = 15.94 \pm 0.28$ nM). Competitive binding assays using a panel of other CNS ligands showed no inhibition of (−)-[^{3}H]TZ659 binding. On the other hand, binding inhibitions were observed only using VAChT inhibitors ($K_i = 0.20–31.35$ nM). An *in vitro* assay using rat brain homogenates showed that (−)-[^{3}H]TZ659 had higher binding in striatum than in cerebellum, with a target: non-target ratio > 3.46. Even higher *ex vivo* striatum-to-cerebellum ratios (9.56 ± 1.11) were observed using filtered homogenates of brain tissue after rats were injected intravenously with (−)-[^{11}C]TZ659. *Ex vivo* autoradiography of (−)-[^{11}C]TZ659 confirmed high striatal uptake, with a consistently high striatum-to-cerebellum ratio (2.99 ± 0.44). In conclusion, (−)-TZ659 demonstrated high potency and good specificity for VAChT *in vitro* and *in vivo*. These data suggest that (−)-[^{11}C]TZ659 may be a promising PET tracer to image VAChT in the brain.

1. Introduction

Loss of cholinergic neurons/terminals in the brain strongly correlates with cognitive impairment in patients with dementia (Armstrong, 2013; Davies and Maloney, 1976; Schliebs and Arendt, 2011). Postmortem and neuroimaging studies in patients with Parkinson's disease dementia (PDD) reveal a similar relationship between cholinergic function and cognitive performance (Bohnen and Albin, 2011; Pavese, 2012). Moreover, cholinergic degeneration in Parkinson's disease (PD) likely begins prior to dementia (Shimada et al., 2009). These findings highlight the importance of cholinergic dysfunction which extend to acetylcholine–dopamine interactions in the striatum (Bohnen and Albin, 2011; Lester et al., 2010). Therefore, *in vivo* measurement of cholinergic dysfunction may provide a critical biomarker for understanding pathophysiology and treatment of these diseases.

Positron emission tomography (PET) imaging is a non-invasive modality that can assess the density of neuronal biomarkers in the central nervous system (CNS), and thus provide neurological information regarding molecular and cellular functions in living subjects. Tremendous efforts have been put forth towards the identification of a PET tracer suitable for evaluating cholinergic function in the human brain. PET imaging of acetylcholinesterase (AChE) is one strategy that has been successfully used to study patients with dementia (Kikuchi et al., 2013). Unfortunately, AChE enzyme activity may not functionally correlate with loss of cholinergic terminals. Another reliable marker of cholinergic neurons, choline acetyltransferase (ChAT), has not been imaged successfully *in vivo*. Vesicular acetylcholine transporter (VAChT), which loads acetylcholine into presynaptic vesicles, maps to cholinergic cells in the brain and displays a good correspondence with ChAT (Weihe et al., 1996). A PET tracer for VAChT could thus be used to assess cholinergic function in human subjects and monitor the therapeutic efficacy of VAChT inhibition strategies.

A number of radiolabeled benzovesamicol analogs have been evaluated as VAChT imaging agents (Giboureau et al., 2010). However, when tested in pre-clinical studies and human subjects, most of these radiotracers showed poor selectivity over σ receptors in brain, low extraction from the blood, slow brain kinetics, or rapid metabolism (Li et al., 2013). Despite a slow brain kinetic, [^{18}F]FEOBV is the only PET

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tracer approved for imaging VAChT in human brains (Giboureau et al., 2007; Kilbourn et al., 2009; Petrou et al., 2014). Recently, our group designed a new class of VAChT inhibitors including (−)-**TZ659**, which contain a carbonyl group attached to the 4-position of the piperidine ring (Efange et al., 2010; Li et al., 2013; Tu et al., 2009, 2012). (−)-**TZ659** has a high affinity for VAChT, and a high selectivity for VAChT over σ_1 and σ_2 receptors; (−)-[^{11}C]**TZ659** was successfully radiolabelled and showed a higher accumulation in the striatum of rats and nonhuman primates than in other non-target brain regions (Li et al., 2013). Here we further reported the *in vitro* and *ex vivo* pharmacology of (−)-[^{11}C]**TZ659** and its tritiated counterpart, (−)-[^{3}H]**TZ659**. (−)-**TZ659** binds to VAChT with a high potency and good specificity for VAChT versus other CNS targets. Both (−)-[^{11}C]**TZ659** and (−)-[^{3}H]**TZ659** showed higher uptake in the VAChT-enriched striatum than the reference brain region (cerebellum).

2. Materials and methods

2.1. Radioligand preparation

The two-step synthesis of (−)-[^{11}C]**TZ659** was carried out as previously reported (Li et al., 2013). The tritiated compound (−)-[^{3}H]**TZ659** was custom synthesized by American Radiolabeled Chemicals, Inc. (St. Louis, MO) using the Boc-protected amino precursor and the synthetic strategy employed for ^{11}C-labeling.

2.2. Drugs and preparation of stock solutions

Reagents and standard compounds for *in vitro* assays were purchased from Sigma (St. Louis, MO) and Tocris Biosciences (R&D Systems, Minneapolis, MN) unless otherwise noted. Novel compounds were synthesized in-house. Test compounds (structures are shown in Fig. 1) were dissolved in *N,N*-dimethylformamide or ethanol to create a stock solution; the desired concentration for *in vitro* assays was subsequently obtained by further dilution in the assay buffer (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4).

2.3. Radioligand binding assays in cell post-nuclear supernatant

2.3.1. Cell culture

PC12$^{A123.7}$ cells stably transfected with human VAChT (hVAChT) wild type (GenBank: AAA20497.1) or W331A mutant cDNA were grown at 37 °C in 5% CO_2 in complete Dulbecco's modified Eagle's medium mixed 1: 1 with Ham's F-12 medium. The complete medium was supplemented with 10% horse serum, 5% fetal bovine serum, 100 units penicillin/ml, 100 μg streptomycin/ml, and 10 μg blasticidin/ml. Site-directed mutagenesis of W331 previously identified the

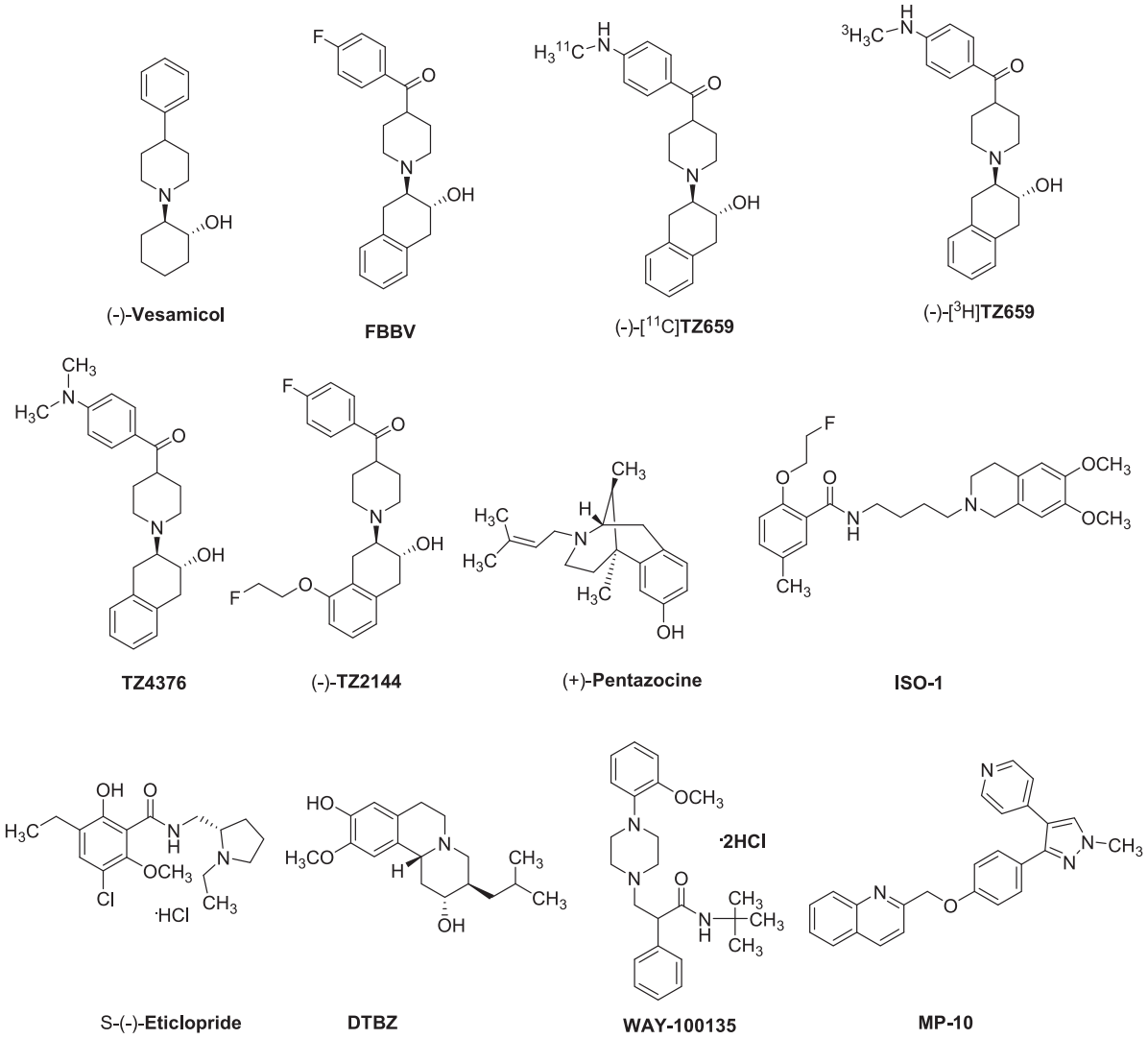

Fig. 1. Chemical structures of (−)-[^{11}C]**TZ659**, (−)-[^{3}H]**TZ659** and other compounds used in the competitive binding assay.