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Synthesis and *in vitro* characterization of a P2X7 radioligand [^{123}I]TZ6019 and its response to neuroinflammation in a mouse model of Alzheimer disease

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ABSTRACT

The purinergic receptor P2X ligand-gated ion channel 7 (P2X7 receptor) is a promising imaging target to detect neuroinflammation. Herein, we report development of a potent iodinated radiotracer for P2X7 receptor, [^{123}I]TZ6019. The radiosynthesis of [^{123}I]TZ6019 was accomplished by allylic-tin precursor iodination using [^{123}I]NaI with good radiochemical yield of 85% and high radiochemical purity of > 99%. Human embryonic kidney 293 (HEK-293) cell line stably transfected with the human P2X7 receptor was used to characterize the binding affinity of TZ6019 by fluorescence, radioactive competitive, and saturation binding assays. A radioligand competitive binding assay with [^{123}I]TZ6019 demonstrated that the nonradioactive compound TZ6019 has an IC_{50} value of 9.49 ± 1.4 nM, and the known P2X7 receptor compound GSK1482160 has an IC_{50} value of 4.30 ± 0.86 nM, consistent with previous reports. The radioligand saturation binding assay and competitive assay revealed that [^{123}I]TZ6019 specifically bound to the human P2X7 receptor with high affinity (K_i = 6.3 ± 0.9 nM). *In vitro* autoradiography quantification with brain slices collected from 9-month old P301S tau transgenic mice along with wild type controls, revealed higher binding of [^{123}I]TZ6019 (35% increase) in the brain of P301S transgenic mice (n = 3, p = 0.04) compared to wild type controls. The immunofluorescence microscopy confirmed that expression of P2X7 receptor was colocalized with astrocytes in the tauopathy P301S transgenic mice. [^{123}I]TZ6019 has specific binding for P2X7 receptor and has great potential to be a radiotracer for screening new compounds and quantifying expression of P2X7 receptor in neuroinflammation related diseases.

1. Introduction

Neuroinflammation plays an important role in progression of many neurodegenerative diseases including Alzheimer disease (AD) and Parkinson disease (PD) (Bhattacharya and Biber, 2016; Monif et al., 2010). Elevated purinergic receptors expression has shown close association with neuroinflammation due to the large release of adenosine/uridine derivatives from the damage site (Bhattacharya et al., 2013; Boumechache et al., 2009). Purinergic receptors are adenosine-triphosphate (ATP) gated non-selective ion channels which are expressed on

Abbreviations: **ACN**, Acetonitrile; **AD**, Alzheimer's disease; **AMF**, ammonium formate; **ATP**, adenosine-triphosphate; **BzATP**, 3′-O-(4-Benzoyl)benzoyl ATP, strong agonist for P2X7 receptor; **CNS**, central nervous system; **DMEM**, Dulbecco's Modified Eagle's Medium; **DMF**, dimethylformamide; **EDAC**, 1-ethyl-3-(3-dimethy-laminopropyl) carbodiimide hydrochloride; **EtOAc**, ethyl acetate; **ESI**, electrospray ionization; **GFAP**, Glial fibrillary acidic protein; **FACS**, fluorescence activation cell sorting; **HEK-293**, human embryonic kidney cells 293; **HEPES**, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; **HOBt**, 1-hydrobenzotrizole; **HPLC**, high-performance liquid chromatography; **HRMS**, high resolution mass spectrum; hr, hour; **IC_{50}**, the concentration of an inhibitor where the response (or binding) is reduced by half; **IF**, Immunofluorescence; **K_d**, the equilibrium dissociation constant, inverse of association constant (Ka); **K_i**, inhibition dissociation constant; **LC-MS**, liquid chromatography–mass spectrometry; **LiHMDS**, Lithium bis(trimethylsilyl)amide; **MAPT**, human microtubule-associated protein tau; **NHS**, normal horse serum; **NMR**, nuclear magnetic resonance; **PBS**, phosphate buffered saline; **PD**, Parkinson's disease; **PET**, positron emission tomography; **Prnp**, prion protein promoter; **PSL/mm^2**, photo-stimulated luminescence signals per square millimeter; **P2X7 receptor**, P2X ligand-gated ion channel 7; **ROI**, region of interest; **SDS**, sodium dodecyl sulfate; **TMS**, tetramethylsilane; **THF**, tetrahydrofuran; **TLC**, Thin layer chromatography

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the surface of many different cell types (Able et al., 2011; Burnstock, 2009a, 2009b). Among these purinergic receptors, P2X7 receptor has been implicated in microglial activation, astrogliosis, or phagocytosis at sites of injury (Bartlett et al., 2014; Bhattacharya and Biber, 2016; Franke and Illes, 2014; Grygorowicz et al., 2016), and release of pro-inflammatory cytokines (Bianco et al., 2006; Collo et al., 1997; Ferrari et al., 2006). Upregulation of extracellular ATP in neuroinflammatory conditions significantly increases expression of P2X7 receptor. The P2X7 receptor knockout mice display a significantly reduced neuroinflammatory response (Basso et al., 2009; Monif et al., 2009). Therefore, P2X7 receptor represents a novel molecular target for imaging and treating neuroinflammation in neurological diseases. A potent P2X7 receptor antagonist, GSK1482160, has recently been labeled with carbon-11 (half-life 20.4 min) for positron emission tomography (PET) imaging of neuroinflammation in an animal model (Gao et al., 2015; Han et al., 2017; Ory et al., 2016). However, this radioligand showed tight binding to human P2X7 receptor, but weak binding to rodents P2X7 receptor (Gao et al., 2015; Ory et al., 2016). And the human applications of this tracer remain to be explored. New potent ligands specifically targeting both human and rodent P2X7 receptor, and capable to be translated for clinic are still in demand. Screening new analogues for P2X7 ligand development requires a reliable assay platform for P2X7 receptor. For the *in vitro* assays, labeled probes with longer half-life radioisotopes facilitate implementation of these assays. Previously two tritium labeled ligands ([^{3}H]A804598 and [^{3}H]JNJ54232334) have been reported for P2X7 receptor (Donnelly-Roberts et al., 2009; Lord et al., 2015), but they have limited availability. Herein we report the radiosynthesis of the Iodine-123 (half-life 13.3 h) labeled radiotracer [^{123}I]TZ6019 and initial characterization of [^{123}I]TZ6019 for P2X7 receptor. The radiosynthesis of [^{123}I]TZ6019 was accomplished by allylic-tin precursor iodination. The binding properties of [^{123}I]TZ6019 were determined using P2X7 receptor expressed HEK-293 living cells, and *in vitro* autoradiography studies with [^{123}I]TZ6019 were performed with postmortem brain tissue from P301S transgenic mice, a tauopathy mouse model. Our results showed that [^{123}I]TZ6019 is a potent and specific radiotracer for P2X7 receptor, and the binding of P2X7 receptor was associated with the neuroinflammation in the tauopathy mouse model.

2. Materials and methods

2.1. Chemistry

The structures of several known P2X7 receptor radioligands including [^{123}I]TZ6019 are summarized in Fig. 1. Compound GSK1482160 was synthesized by following the reported procedure (Gao et al., 2015; Han et al., 2017). The standard compound **5** (TZ6019) was also synthesized from demethyl-GSK1482160, depicted in Scheme 1A. All reagents and chemicals were obtained from standard commercial sources and used without further purification, unless otherwise stated. The organic reaction was carried out under inert nitrogen and moisture-free conditions with a dry solvent. Thin layer chromatography (TLC) was used to monitor the reaction with precoated glass plates of silica gel 60 F_{254} from EMD Chemicals Inc. (Gibbstown, NJ). The product of each step was purified by silica gel 40–63 μm from SiliCycle Inc. (Quebec City, Quebec, Canada). ^{1}H and ^{13}C spectra were recorded at 400 MHz on a Varian Mercury-VX spectrometer with $CDCl_3$ as solvents and tetramethylsilane (TMS) as the internal standard. The chemical shifts were reported in δ (ppm) values relative to $CHCl_3$ (δ 7.26 ppm for ^{1}H NMR and δ 77.2 ppm for ^{13}C NMR), multiplicities were indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). High resolution mass spectrum (HRMS) was performed on a Waters ZQ 4000 single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) LC–MS interface.

2.1.1. (E)-N-(2-chloro-3-(trifluoromethyl)benzyl)-5-oxo-1-(3-(tributylstannyl)allyl)pyrrolidine-2-carbox-amide (4)

At 0 °C, 3 ml Lithium bis(trimethylsilyl)amide (LiHMDS, 1 M in tetrahydrofuran, THF) was added into an oven-dried 100 ml round bottle flask with a solution of compound **3** (600 mg, 1.8 mmol, 1.0 equiv.) in 10 ml dimethylformamide (DMF) (Gao et al., 2015; Han et al., 2017). Then 2.0 equiv. of tributyl-(3-chloro-propenyl)-stannane was added into the flask at 0 °C. The mixture was stirred overnight at room temperature, then extracted with ethyl acetate (EtOAc). The organic solution was dried using Mg_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column using hexane/EtOAc (1/1, v/v) to yield product **4** as a yellow solid (320 mg, 27% yield). ^{1}H NMR (400 MHz,$CDCl_3$): δ 7.66 (d, J = 7.9 Hz, 1H), 7.57 (d, J = 7.5 Hz, 1H), 7.35 (t, J = 7.8 Hz, 1H), 6.41 (t, J = 5.5 Hz, 1H), 6.02 (d, J = 19.0 Hz, 1H), 5.67–5.79 (m, 1H), 4.58 (d, J = 6.0 Hz, 2H), 4.48–4.56 (m, 1H), 4.03 (dd, J = 8.8, 3.4 Hz, 1H), 3.26 (dd, J = 15.3, 7.3 Hz, 1H), 2.44–2.56 (m, 1H), 2.24–2.42 (m, 2H), 1.99–2.11 (m, 1H), 1.36–1.54 (m, 6H), 1.20–1.32 (m, 6H), 0.77–0.94 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.5, 171.5, 140.5, 137.5, 134.1, 133.8, 131.6, 129.2 (q, J_{C-F} = 28.0 Hz), 127.2 (q, J_{C-F} = 5.4 Hz), 126.9, 122.7 (q, J_{C-F} = 272 Hz), 60.5, 47.6, 41.7, 29.65, 29.0 ($J_{C\text{-}Sn}$ = 20.7 Hz), 29.6, 27.2 ($J_{C\text{-}Sn}$ = 54.7 Hz), 23.5, 13.7, 9.4 ($J_{C\text{-}Sn}$ = 345 Hz). HRMS (ESI) calculated for $C_{28}H_{42}ClF_3N_2O_2Sn$ $[M + H]^+$ 651.1982 revealed $[M + H]^+$ 651.1978.

2.1.2. (S,E)-N-(2-chloro-3-(trifluoromethyl)benzyl)-1-(3-iodoallyl)-5-oxopyrrolidine-2-carboxamide (5)

A solution of iodine in chloroform (0.1 M, 2 ml) was added dropwise to a solution of **4** (130 mg, 0.2 mmol) in dry chloroform (10 ml). The reaction was stirred at room temperature for 20 h. The solution then was diluted with chloroform (15 ml), washed with saturated $Na_2S_2O_3$ (6 × 25 ml) and then saturated aqueous NaCl solution. The combination of organic layers were dried over anhydrous sodium sulfate, concentrated under reduced pressure. The residue was purified by flash silica gel chromatography using hexane/EtOAc (1/4, v/v) as the eluent to yield the final product **5** (TZ6019) in a brown solid (86 mg, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.62 (d, J = 7.7 Hz, 1H), 7.53 (d, J = 7.5 Hz, 1H), 7.32 (t, J = 7.8 Hz, 1H), 6.67 (t, J = 5.5 Hz, 1H), 6.42–6.24 (m, 1H), 6.18 (d, J = 14.7 Hz, 1H), 4.64–4.44 (m, 2H), 4.14 (dd, J = 14.9, 5.2 Hz, 1H), 3.99 (dd, J = 8.5, 3.6 Hz, 1H), 3.31 (dd, J = 15.3, 7.9 Hz, 1H), 2.55–2.35 (m, 1H), 2.33–2.18 (m, 2H), 1.92–2.06 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.4, 171.1, 139.2, 137.5, 133.9 (q, J_{C-F} = 1.6Hz), 129.2 (q, J_{C-F} = 32 Hz), 127.3 (q, J_{C-F} = 5.4 Hz), 127.0, 122.7 (q, J_{C-F} = 272 Hz), 80.6, 60.7, 46.0, 41.8, 29.5, 23.5. HRMS (ESI) calcd for $C_{16}H_{15}ClF_3IN_2O_2$ $[M + H]^+$ 487.6651 revealed $[M + H]^+$ 487.6656.

2.1.3. (S)-N-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-fluoroethyl)-5-oxopyrrolidine-2-carboxamide (6)

Compound **6** (TZ5038) was prepared similarly to compound **4**. Compound **6** white solid (360 mg, 54% yield) was derived from compound **3** (600 mg, 1.8 mmol, 1.0 equiv.) and 1-bromo-2-fluoroethane (2.0 equiv.) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (d, J = 8 Hz, 1H), 7.57 (d, J = 7.2 Hz, 1H), 7.34 (t, J = 7.6 Hz, 1H), 4.59 (d, J = 6 Hz, 2H), 4.39–4.53 (m, 1H), 4.19–4.24 (m, 1H), 3.74–3.90 (m, 1H), 3.14–3.29 (m, 1H), 2.43–2.56 (m, 1H), 2.26–2.37 (m, 2H), 1.97–2.06 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.0, 171.4, 137.6, 133.6, 131.6, 129.1 (q, J_{C-F} = 30.8 Hz), 127.1 (q, J_{C-F} = 5.4 Hz), 126.9, 122.7 (q, J_{C-F} = 272 Hz), 82.8 (d, J_{C-F} = 166.7 Hz), 62.3 (d, J_{C-F} = 2.1 Hz), 42.5 (d, J_{C-F} = 19.4 Hz), 41.7, 29.3, 28.8. HRMS (ESI) calculated for $C_{15}H_{15}ClF_4N_2O_2$ $[M + H]^+$ 367.0831 revealed $[M + H]^+$ 367.0836.

2.2. Radiochemistry

The radiosynthesis of [^{123}I]TZ6019 was depicted in Scheme 1B. Briefly, 111 MBq [^{123}I]NaI (Nordion International Inc., Ottawa,