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Synthesis and Characterization of a Specific Iodine-125 Labeled TRPC5 Radioligand

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Abstract

The nonselective Ca²⁺ permeable transient receptor potential channel subfamily member 5 (TRPC5) belongs to the transient receptor potential canonical (TRPC) superfamily and it is widely expressed in the brain. Compelling evidence reveals that TRPC5 plays crucial roles in depression and other psychiatric disorders. To develop a TRPC5 radioligand, following up our previous effort, we synthesized the iodine compound **TZ66127** and its I-125 labeled counterpart [¹²⁵I]**TZ66127**. The synthesis of **TZ66127** is achieved by replacing the chloride using iodide in the structure of **HC608** and the [¹²⁵I]**TZ66127** was radiosynthesized using its corresponding tributylstannylated precursor. We established a stable human TRPC5 overexpressed HEK293-hTRPC5 cell line and performed the Ca²⁺ imaging and cell binding assay study of **TZ66127**, indicating that **TZ66127** had good inhibition activity for **TRPC5**, and the inhibitory efficiency of **TZ66127** toward TRPC5 presented a dose-dependent manner. Our *in vitro* autoradiography and immunohistochemistry study of the rat brain sections suggested that [¹²⁵I]**TZ66127** had binding specificity toward TRPC5. Altogether, [¹²⁵I]**TZ66127** has high potential to serve as radioligand for screening the binding activity of other new compounds toward TRPC5. The availability of [¹²⁵I]**TZ66127** may facilitate the development of therapeutic drugs and PET imaging agents targeting TRPC5.

Graphical Abstract

Baseline **Blocking**

Let's image: A potent and specific I-125 radioligand targeting TRPC5 was achieved and the preliminary evaluation in HEK293-hTRPC5 and autoradiography studies of rat brain sections were performed.

Keywords

TRPC5; iodine-125; radioligand; Ca²⁺ imaging; autoradiography; PET tracer; stable transfected cell line

Introduction

The transient receptor potential canonical 5 (TRPC5) channel belongs to one of the seven TRPC members (TRPC1 to TRPC7), which are nonselective Ca²⁺ permeable cation channels.^[1] It is generally considered that the TRPC5 is widely expressed in the brain.^[2] In addition, this channel has been recognized as a potential therapeutic target for various neurological diseases and psychiatric disorders including but not limited to depression, anxiety, epilepsy, memory, addiction and pain.^[3–8]

Up to now, extensive medicinal chemistry efforts have yielded several selective small molecule TRPC5 inhibitors, which contribute to the understanding of the biological functions and mechanisms on TRPC5.^[9] Nevertheless, this series of compounds generally suffer from low potency and selectivity, unfavorable pharmacological properties. A breakthrough was made by the discovery of xanthine analogues **HC608** (IC₅₀ = 6.2 nM) and **HC070** (IC₅₀ = 9.3 nM), identified by Hydro/Boehringer-Ingelheim.^[3,10] Encouraged by this pioneering work, we have reported our effort on synthesis and evaluation of a carbon-11 (t_{1/2} = 20.4 min) PET radiotracer for imaging TRPC5 recently (Figure 1).^[11] Although there is an exigent need for a clinical suitable PET radiotracer for imaging TRPC5, only limited ones were reported to show promise *in vitro* and *in vivo* evaluation. To achieve this goal, a reliable platform including ligand binding potency assessment and characterization is indispensable. Traditionally, patch clamp protocol is the gold standard to study the

activity and physiological roles of ion channels.^[12] Consequently, the binding potency of new compound toward TRPC5 and other TRPC family member was mainly determined using patch clamp combined with fluorescent or electrophysiological assays currently.^[9] In comparison, the radioactivity competitive assay using a suitable radioligand to determine compounds' binding potency, one of the most sensitive quantitative procedure *in vitro*, even in low receptor-expression cells, has seldom been realized for ion channels study.^[13] This may largely ascribable to lack of desirable radioligand. Radioligand with appropriate half-life isotopes such as I-125 or H-3 and having good binding potency and selectivity with TRPC5 will provide the convenience for screening new compounds toward TRPC5.

Radioiodine provides powerful tools for imaging, diagnostic and therapeutic and has extensive applications in nuclear medicine.^[14] For radioligand binding assay and autoradiography study, the Auger electron-emitting radionuclide I-125 owes favorable nuclear properties due to its long half-life (59.4 days), low-energy photons emission (maximum energy 35 keV). Compared with ³H, the high specific activity that ¹²⁵I labeled radioligands possess make them particularly useful when the density of receptors is low, or if the amount of tissue is very small. In addition, the long half-life of H-3 (12.32 years) results in the decontamination of facility and equipment to produce ³H-radiotracer pretty expensive, thus exploration of I-125 radioligands for binding assay is much convenient and practical for research.^[15–17]

To develop a I-125 labeled TRPC5 radiotracer, herein we report our efforts on the radiosynthesis of the iodine-125 labeled radiotracer [¹²⁵I]**TZ66127** (Figure 1) and *in vitro* characterization of this radioligand using the human TRPC5 stable transfected cell line HEK293-hTRPC5, as well as the intracellular live cell Ca²⁺ imaging and binding assay techniques. In addition, we also performed *in vitro* autoradiography studies of [¹²⁵I]**TZ66127** in the brain sections of rats, H&E staining and immunohistochemistry (IHC) staining of the adjunctive tissue sections.

Results and Discussion

Chemistry and radiochemistry

HC608 having low nanomolar IC₅₀ value toward TRPC5 is an attractive lead compound that can be modified for introducing different isotope to develop TRPC5 radiotracers. We hypothesized that the replacement of the chloride atom with an iodide substituent to the benzene ring of **HC608** would not cause dramatic binding potency change. Furthermore, addition of a radioiodine substituent to the aryl ring provide a versatile route for generating the I-125 labeled radiotracer.

The non-radioactive iodinated compound **TZ66127** was prepared in a four steps synthetic procedure following the pathway described before with necessary modification (Scheme 1).^[18] Briefly, the reaction of 8-bromo-3-methyl-xanthine **1** with 1-(bromomethyl)-4-iodobenzene gave the benzyl xanthine **2**, which was converted to intermediate **3** upon *N*-alkylation at 60 °C in *N,N*-dimethylformamide (DMF). Hydrolysis of the tetrahydropyranyl ether and subsequent aryl etherification afforded the reference compound **TZ66127** in 24% overall yield (4 steps). The tributylstannylated precursor **5** was synthesized by palladium

(0)-catalyzed reaction of **TZ66127** with bis(tributyltin) in 55% yield. It is worth noting that for the remove of organotin impurities in this reaction, a highly effective stationary phase containing 10% w/w anhydrous potassium carbonate-silica was employed for the column chromatography purification.^[19] Finally, [¹²⁵I]iododestannylation of **5** using [¹²⁵I]NaI in the presence of H₂O₂ (aq.), NaOAc and HOAc afforded [¹²⁵I]**TZ66127** in 73% radiochemical yield with >95% radiochemical purity. The calculated molar activity of this radioligand was ~93 GBq/μmol based on the molar activity of [¹²⁵I]NaI provided by the supplier. The authentication of [¹²⁵I]**TZ66127** was confirmed by co-injection with the non-radioactive reference compound.

Establishment of HEK293-hTRPC5 Stable cell lines

The human embryonic kidney cell line (HEK293) is a particularly interesting candidate used as a vehicle for heterologous expression to conduct functional studies of TRP channels. Not merely serving as a transient expression system, this cell line also represents a conducive system for stable transfection. Using the HEK293 cell line as a host cell has the advantage of enabling the properties of a specific ion channels to be studied in isolation from other proteins.^[20] However, the expression of these channels cannot always be supposed in HEK293 cells, it is important to identify whether a protein of interest is truly expressed in these cells.

In double restriction enzymes-digestion with Kpn I & Xba I, the recombinant plasmid construct pCMV3-TRPC5 was correctly confirmed, carrying 2.92 kb of human TRPC5 cDNA and 6.1 kb of vector pCMV3. After stable transfection screening under hygromycin selection and cell based-binding assay with [¹²⁵I]**TZ66127**, ten stable cell lines of HEK293-hTRPC5 were selected (data not shown). The expression levels of recombinant human TRPC5 protein in the stable transfected cell lines were evaluated by western blotting analysis with anti-human TRPC5 polyclonal antibodies, and a single protein band at about 110 kDa position (the right MW for human TRPC5) was detected (data not shown). Thus, we obtained stable transfected human cell lines HEK293-hTRPC5 in which human TRPC5 protein is overexpressed.

Intracellular live cell Ca²⁺ imaging study

Ca²⁺ is an extremely important intracellular messenger that drives many cell functions. Hence, quantitative measurements of cytosolic free Ca²⁺ by monitoring the fluorescence of an indicator such as Fura-2 AM is a popular measurement method to characterize the activity of TRPC channels in mammalian cells.^[21]

With the human TRPC5 stable transfected cell line HEK293-hTRPC5 in hand, the inhibitory effect of **TZ66127** is evaluated by exploration of Ca²⁺ responses in TRPC5 overexpression HEK293-hTRPC5 cells. As anticipated, this compound did not stimulate any calcium mobilization itself, this confirmed **TZ66127** represent antagonist role acting at the hTRPC5-bearing cells.

Riluzole has been reported to be a TRPC5 channels specific activator.^[22,23] In our experiments, we observed that Riluzole produced robust Ca²⁺ responses in 100 μM but

not in 50 μM , and it didn't activate TRPC4 channel (data not shown). Then we performed comparison experiments among the TRPC5 antagonist **HC070**, **HC608**, and **TZ66127** on the inhibition of Riluzole stimulating Ca^{2+} responses in TRPC5. We found that **TZ66127** at 500 nM completely suppressed Riluzole-induced Ca^{2+} responses in TRPC5-bearing cells (Figure 2C). The inhibitory effects of **HC608** was essentially similar to **TZ66127**, and **HC070** was slightly weaker than **TZ66127** in the same concentration (Figure 2A & 2B). Furthermore, we monitored the Ca^{2+} response-ligand concentration curve in a separate experiment. We analyzed the effects of **TZ66127** with a range of tested concentrations from 10 pM to 500 nM on Riluzole-evoked Ca^{2+} responses (Figure 2D). Indeed, the inhibition of **TZ66127** on Riluzole-evoked Ca^{2+} responses in TRPC5 increases as the concentration of **TZ66127** rising. That is, compound **TZ66127** inhibited the calcium responses stimulated by Riluzole in a concentration-dependent manner. Based on the results above, we conclude that, like **HC608**, compound **TZ66127** is also a high potential and selective inhibitor for TRPC5.

Based-Binding Assay of [^{125}I]TZ66127

Encouraged by the favorable results from the calcium imaging study results above, we also sought to investigate the cell based-binding assay of this radiotracer by utilizing human TRPC5 stable transfected cell line HEK293-hTRPC5. The saturation binding property of [^{125}I]TZ66127 was initially explored. Unfortunately, we found the binding potency of this radioligand toward TRPC5-bearing cells was unable to saturated when increasing the amounts of radioligand [^{125}I]TZ66127. Instead, some precipitates were observed when high concentrate radioligand were employed, suggesting that **TZ66127**, like its analogue **HC070**, has defect in solubility.^[3]

Nevertheless, competition-binding curves were utilized to examine the relative affinities of other cold ligands against [^{125}I]TZ66127. The results of [^{125}I]TZ66127 binding assay at the presence of TRPC5 inhibitors **HC070**, **HC608** and **TZ66127** are shown in Figure 3. High binding levels of [^{125}I]TZ66127 to HEK293-hTRPC5 cells cultured in 48 wells plate were observed, and the binding of HEK293-hTRPC5 was significantly reduced at the presence either **HC070** or **HC608** (2 μM). The uptake of [^{125}I]TZ66127 was also self-blocked when cold reference compound **TZ66127** (2 μM) was added. This competition study confirmed that **TZ66127** owes comparative binding affinity as **HC070** and **HC608**, thus indicated that the inhibitory efficiency have remained relatively constant when **HC608** was modified by iodine incorporation.

In vitro autoradiography (ARG), H&E and IHC staining study

Having achieved a desired cell-based assay profile on HEK293-hTRPC5 cells, we embarked on further characterizing of [^{125}I]TZ66127 on rat brain tissues. *In vitro* autoradiographic studies in tissues have played an important role in quantifying the proteins distribution in the relative regions of animals in contemporary neuroscience and pharmacology. Iodine-125 is highly favorable to *in vitro* autoradiography, particularly for tissues that have relative lower protein expression.¹⁸

The *in vitro* autoradiography study of [^{125}I]TZ66127 was performed with frozen SD rat brain sections (20 μm) to assess the distribution of [^{125}I]TZ66127 in the TRPC5-enriched

brain regions of interest (Figure 4). Coronal, sagittal, and horizontal crossing sections of rat brains were used in the [¹²⁵I]**TZ66127** autoradiography. Under optimized condition, a positive ARG signal lighted up at all major regions essentially. The strong ARG signals is observed on thalamus, colliculi, cortex, striatum, hippocampus, midbrain, brain stem and spinal cord regions, thus suggests TRPC5 channel on the frozen rat brain tissue remain biologically active and could be strongly bonded by the radiotracer. Of particular interest, the [¹²⁵I]**TZ66127** ARG signal on the rat brain sections is significantly attenuated at the presence of TRPC5 specific antagonist **HC608** at 1 μM, indicating the specific binding of this radiotracer to TRPC5 in the rat brain slides.

In addition, the TRPC5 binding signal of the [¹²⁵I]**TZ66127** ARG is confirmed by the Hematoxylin and Eosin (H&E) and anti-TRPC5 immunohistochemistry (IHC) staining of the adjacent rat brain sections. Like the [¹²⁵I]**TZ66127** ARG signal, TRPC5 H&E and IHC staining also shows up essentially on the whole brain section with higher signal on thalamus, colliculi, cortex, striatum, hippocampus, midbrain, brain stem and spinal cord regions. In other words, the localization pattern of the [¹²⁵I]**TZ66127** ARG signal matches well with the pattern of TRPC5 IHC staining signal in the rat brain sections. These results are in agreement with previous findings for TRPC5 region-specific protein expression.^[2,24–27] Together, our results clearly demonstrate a widespread distribution of TRPC5 specific binding sites in the rat brain.

Conclusions

In conclusion, the synthesis of the first I-125 labeled radioligand [¹²⁵I]**TZ66127** for TRPC5 was accomplished with good radiochemical yield, high radiochemical purity, and high molar activity. Ca²⁺ imaging study revealed **TZ66127** at 500 nM completely suppressed Riluzole-induced Ca²⁺ increase. Moreover, the inhibitory effect of **TZ66127** on Riluzole-evoked Ca²⁺ responses of TRPC5 channels is dose dependent. *In vitro* cell-binding assay, autoradiography, H&E and IHC staining study showed the accumulation of [¹²⁵I]**TZ66127** is correlated to the distribution of TRPC5.

Compared with **HC608** and **HC070**, the iodine compound **TZ66127** has similar or even slightly greater inhibitory effect on TRPC5. Collectively, [¹²⁵I]**TZ66127** is a specific TRPC5 radioligand which may be used screening other compounds binding toward TRPC5. Although additional studies will be needed to improve its physicochemical properties of low solubility, the availability of [¹²⁵I]**TZ66127** will facilitate the development of therapeutic drugs or imaging agents targeting on TRPC5 for investigating the mechanisms and functions of the TRPC5 channel in relevant diseases.

Experimental Section

General Information.

Commercially available reagents and solvents were used as purchased. All reagents and solvents (ACS or HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise stated. [¹²⁵I]NaI was purchased from Perkin-Elmer (Waltham, MA, USA). Recombinant plasmid construct pCMV3-TRPC5 for overexpression

human TRPC5 in mammalian cells was obtained from Sino Biological Inc (Beijing, China). HEK 293 human embryonic kidney cell line was from the American Type Culture Collection (ATCC, Manassas, VA, USA) Lipofectamine 2000 reagent is the product of Invitrogen. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded at room temperature on a 400 MHz Varian spectrometer. Chemical shifts are reported in δ units (ppm) downfield relative to the chemical shift of tetramethylsilane (TMS). Signals are quoted as s (singlet), d (doublet), dd (double doublet), dt (double triplet), t (triplet), q (quartet) or m (multiplet). Copy of ^1H , ^{13}C NMR spectrum characterization of compounds is available in the Supporting Information.

High-resolution mass spectra (HRMS, m/z) was acquired with Bruker MaXis 4G Q-TOF mass spectrometer with an electrospray ionization source.

All animal experiments were conducted under Washington University's Institutional Animal Care and Use Committee (IACUC)-approved protocols in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals.

Chemistry

1-(3-Hydroxypropyl)-7-(4-iodobenzyl)-3-methyl-8-(3-(trifluoromethoxy)phenoxy)-3,7-dihydro-1H-purine-2,6-dione (TZ66127).—To a solution of 8-bromo-3-methyl-1H-purine-2,6(3H,7H)-dione (1.49 g, 6.1 mmol) and K_2CO_3 (0.84 g, 6.1 mmol) in DMF (10 mL) at RT was added 1-(bromomethyl)-4-iodobenzene (1.81 g, 6.1 mmol) subsequently. The mixture was stirred for 1.5 hours and another equivalent of K_2CO_3 (0.84 g, 6.1 mmol) and 2-(3-bromopropoxy)tetrahydro-2H-pyran (1.42 g, 6.4 mmol) were added and stirred at 60 °C for additional 12 hours. After cooling, the resultant mixture was cooled to RT, diluted with ethyl acetate, and washed with LiCl solution (10% solution). The combined organic phase was dried over anhydrous Na_2SO_4 , concentrated *in vacuo* to yield a yellow solid, which was then dissolved in EtOH (40 mL), slowly added conc. HCl (5 mL) and heated at reflux. Upon completion, the reaction mixture was concentrated *in vacuo* and diluted with ethyl acetate, washed with NaCl (sat.), dried to yield a light yellow solid. Finally, the product obtained above was mixed with 3-(trifluoromethoxy)phenol (1.09 g, 6.1 mmol), K_2CO_3 (0.84 g, 6.1 mmol) in DMF (15 mL) and heated at 80 °C overnight. The mixture was cooled to RT, diluted with water, extracted with ethyl acetate. The combined organic layers were washed with LiCl (10% solution) and brine sequentially, dried (Na_2SO_4), and concentrated before purified by flash column chromatography (hexane /ethyl acetate, 2/1, v/v) to yield **TZ66127** (902 mg, 24% overall) as a white solid. Mp 100–101 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.71 – 7.68 (m, 2H), 7.46 (td, J = 8.2, 2.3 Hz, 1H), 7.27 – 7.15 (m, 5H), 5.40 (t, J = 2.4 Hz, 2H), 4.22 – 4.18 (m, 2H), 3.53 (t, J = 6.1 Hz, 2H), 3.45 (s, 3H), 1.92 – 1.89 (m, 2H). ^{13}C NMR (100 Hz) δ 155.19, 153.48, 152.78, 151.53, 146.21, 138.06, 135.27, 130.63, 130.22, 118.23, 117.90, 113.00, 102.93, 94.33, 58.55, 46.75, 37.76, 30.87, 29.93; HRMS (m/z) [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{23}\text{H}_{21}\text{F}_3\text{IN}_4\text{O}_5$, 617.0509, found 617.0499.

1-(3-hydroxypropyl)-3-methyl-7-(4-(tributylstannyl)benzyl)-8-(3-(trifluoromethoxy)phenoxy)-3,7-dihydro-1H-purine-2,6-dione (5).—In a flame-dried Schlenk tube under nitrogen atmosphere were added **TZ66127** (277 mg, 0.45 mmol), Pd(PPh₃)₄ (26 mg, 0.0225 mmol, 5 mol %), LiCl (95 mg, 2.25 mmol), followed by dioxane (8.0 mL) and bis(tributyltin) (524 mg, 0.9 mmol). The tube was screw capped and heated to 110 °C on oil bath. After stirring for 12 h, the reaction mixture was cooled to room temperature, concentrated and purified through column chromatography (10% K₂CO₃ in SiO₂ as a stationary phase,^[19] hexane /ethyl acetate = 2/1 as elute phase) to provide **5** (193 mg, 55% yield) as colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.42–7.31 (m, 5H), 7.18–7.13 (m, 2H), 7.05 (d, *J* = 8.2 Hz, 1H), 5.35 (s, 2H), 4.12 (t, *J* = 5.8 Hz, 2H), 3.53 (t, *J* = 6.9 Hz, 1H), 3.48–3.43 (m, 2H), 3.36 (s, 3H), 1.85–1.81 (m, 2H), 1.47–1.39 (m, 6H), 1.27–1.18 (m, 7H), 0.97–0.93 (m, 5H), 0.78 (t, *J* = 7.3 Hz, 9H). ¹³C NMR (100 Hz) δ 155.25, 153.64, 152.87, 151.62, 149.64 (q, *J* = 1.9 Hz), 146.13, 142.64, 136.98, 135.22, 130.49, 127.69, 120.31 (q, *J* = 258.2 Hz), 118.05, 117.91, 113.02, 103.10, 58.46, 47.38, 37.67, 30.89, 29.89, 29.00, 27.31, 13.60, 9.53. HRMS (*m/z*) [M + H]⁺ calcd for C₃₅H₄₈F₃N₄O₅Sn, 781.2593, found 781.2585.

I-125 radiochemistry

The radiosynthesis of [¹²⁵I]**TZ66127** was using the tin precursor **5** to reacting with [¹²⁵I]NaI by following the reported procedure with some modification.^[28] To an Eppendorf vial was added 50 μL of tributylstannane precursor (4 mg/1 mL acetonitrile) and 50 μL of freshly prepared 5% sodium acetate in glacial acetic acid. Then, [¹²⁵I]NaI (25 μL, 22.2 MBq) was added to the vial followed by adding 50 μL of freshly prepared hydrogen peroxide(aq)/acetic acid (1/2, v/v). The mixture was proceeded at room temperature for 15 min during which the solution was mixed occasionally using a vortex. The mixture was injected onto HPLC and purified by semi-preparative reversed HPLC system (Agilent 250 × 9.4 mm, 5 μm, UV = 254 nm, 4.0 mL/min, mobile phase: 65% CH₃CN in 0.1 M ammonium formate buffer, pH = 4.5). The desired compound's retention time is 18–20 min and the eluent was collected in a glass bottle containing 50 mL of water, then passed through a C-18 Sepak cartridge (Part No. WAT020515, Waters Corporation, Milford, MA) to trap the product. The desired product (~16.3 MBq) was eluted out with 0.88 mL of DMSO. For authentication, the tracer sample (20 μL) was injected onto HPLC and purified by an analytical reversed HPLC system (Agilent 250 × 4.6 mm, 5 μm, UV = 254 nm, 1.0 mL/min, mobile phase: 75% CH₃CN in 0.1 M ammonium formate buffer, pH = 4.5).

Cell cultures, transfection and confirmation

After confirmation of the recombinant plasmid construct pCMV3-TRPC5 by restriction enzyme digestion and DNA mapping, the plasmid DNA pCMV3-TRPC5 was used to transfect HEK 293 cells with Lipofectamine 2000 reagent. The transfected HEK 293 cells in 24-wells plate were grown for 24 to 48 hours before the addition of hygromycin (50 μg/mL). Two to three weeks late, stable cell clones were formed under hygromycin selection. The established HEK293-hTRPC5 stable cell lines were further screened by western blotting analysis and cell based-binding assay with radiotracer [¹²⁵I]**TZ66127**.

For Western blot analysis, 4–20% SDS-PAGE gel was used and 40 µg of cellular protein extracts for each HEK293-hTRPC5 stable cell line was loaded into the well. Proteins separated on the gel were transferred on the immobilon membrane. The membrane was blocked with 5% milk in TBS-T buffer over night at 4 °C. Rabbit anti-human TRPC5 polyclonal antibodies (0.25 µg/mL) and goat anti-rabbit secondary antibodies (HRP-linked IgG, 1:2000 dilution) were used to detect the recombinant human TRPC5 protein in the stable transfected cell lines.

For cell binding assay condition optimization, host cells (HEK 293) were used as control. Cells of HEK293-hTRPC5 stable clones were cultured in 24-wells plate in DMEM with 40 µg/mL of Hygromycine. For each well of cells, [¹²⁵I]TZ66127 in 200 µL of Ringer solution was added. After 30 minutes of binding incubation at room temperature, the cells were washed twice with 0.5 mL 1X PBS for 5 minutes. Then cells in the wells were lysed with 200 µL of NP40 lysis buffer, and the cell lysate in the well was collected for radioactivity counting.

Ca²⁺ imaging study

Cultured 293 cells were loaded with 4 µM Fura-2 AM (Invitrogen) in culture medium at 37°C for 60 min as previously described.^[29] Cells were then washed three times and incubated in HBSS at room temperature for 30 min before use. Fluorescence at 340 and 380 nm excitation wavelengths was recorded on an inverted Nikon Ti-E microscope equipped with 340 and 380 nm excitation filter wheels using NIS Elements imaging software (Nikon). Fura-2 ratios (F340/F380) were used to reflect changes in intracellular Ca²⁺ upon stimulation. Values were obtained from 200 to 250 cells. Drug perfusion was performed as follows: flow in extracellular fluid for 2 minutes, inhibitor compound for 4 minutes, and TRPC5 activator Riluzole for 2 minutes. The inhibitory effect on Riluzole-activated TRPC5 calcium channels was reported by the Fura-2 fluorescent signal. Ionomycin (1 µM) was applied as positive control for Ca²⁺ imaging.

Cell Based-Binding Assay

Competitive binding assay was carried out by utilizing HEK293-TRPC5 stable transfected cells grown in 48 wells-plates in DMEM + 40 µg/mL of Hygromycine. Binding buffer is Ringer solution, tracer [¹²⁵I]TZ66127 (0.74 kBq/well in 100 µL of binding buffer) and compounds (2 µM) were added to cells in 48-wells plate right after dilution, 30 minutes of binding incubation at room temperature, washing three times with 0.2 mL 1X PBS, 2 minutes each washing. Cells in the wells were lysed with NP40 lysis buffer, and the cell lysate in the well was collected for activity counting by a Beckman LS 3801 scintillation counter.

Autoradiography (ARG), H&E and IHC staining study

The *in vitro* autoradiography experiment was conducted by incubating frozen cross-sections (12 micron) of rat brain tissue and 1.85 kBq/slide of [¹²⁵I]TZ66127 in 600 µL of binding buffer (1x Ringer Buffer + 1% BSA) to check the uptake of the radioactivity. The blocking study was performed by incubating the above solution with 1 µM of HC608, a well-known specific TRPC5 inhibitor. After incubation for 60 minutes at room temperature, the brain

sections were washed for 2 minutes each using the following buffers sequentially: 1x TBST, 15% ethanol in 1x TBST, 30% ethanol in 1X TBST, 1X TBST (1X TBST buffer: 20 mM of Tris, 150 mM of NaCl, 0.1% of Tween 20). After air drying for 20 minutes, the section slides were placed into a cassette and exposed to a phosphor sensor sheet for 24 hours. Autoradiography signal was visualized by phosphor-imaging of FLA7000.

After autoradiography, the brain sections were air dried for several minutes before being stained with filtered 0.1% Mayers Hematoxylin (Sigma; MHS-16) for 10 minutes in a 50 mL conical tube. Subsequently, the sections were rinsed in cool running double-distilled water for 5 minutes in a Coplin jar. Then the sections were dipped in 0.5% Eosin (1.5 g dissolved in 300 mL 95% EtOH) for 20 seconds and ringed with distilled water until the eosin stops streaking. Next, sections were dipped in 50% and 70% EtOH 10 times separately before being equilibrated in 95% and 100% EtOH for 30 seconds and 1 minute respectively. Finally, coverslips were cleaned with kimwipes and mounted with Cytoseal XYL (Stephens Scientific; cat# 8312-4) before scanning.

Immunohistochemistry Staining study was conducted on frozen rat brain section that was adjacent to the section used for *in vitro* autoradiography studies. In brief, frozen sections were warmed at RT for 15 min and fixed with 4% paraformaldehyde in PBS (Alfa Aesar, Tewksbury, MA) for 15 min. After being washed in PBS and blocked with 2.5% normal horse serum for 1 hour, sections were incubated with rabbit anti-TRPC5 antibody diluted at 1:100 dilution (Alomone Labs, Jerusalem, Israel) in a humidified chamber overnight at 4 °C. Afterwards, sections were washed 3 times (5 minutes each) with PBS and blocked with BLOXALL Blocking Solution (Vector Laboratories, Burlingame, CA) for 15 min at RT. Next, sections were incubated with Biotinylated Anti-rabbit Antibody (R&D Systems, Minneapolis, MN) for 2 hours, washed 3 times (5 minutes each) with PBS and incubated with VECTASTAIN Elite ABC reagent for 30 min. After final wash, sections were incubated in Mix ImmPACT DAB Ekv solutions (Vector Laboratories, Burlingame, CA) and developed at desired time. All slides were then scanned automatically using a Hamamatsu NanoZoomer slide scanning system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Representative structures of TRPC5 inhibitors and radioligands

Figure 2. Compounds inhibit efficacy on Riluzole-induced Ca^{2+} responses in overexpression HEK293-hTRPC5 cells. **A.** HC608 (500 nM). **B.** HC070 (500 nM). **C.** TZ66127 (500 nM). **D.** Dose-dependent effect of TZ66127 on Riluzole-evoked Ca^{2+} response. Ionomycin (1 μM) was applied as positive control for Ca^{2+} imaging.

Figure 3.
Blocking effect of the three TRPC5 inhibitors (2 μ M) on binding of [125 I]TZ66127 to HEK293-hTRPC5 cells (mean $\pm$ SE) with 3 samples per group.

Figure 4.

In Vitro autoradiography of rat frozen brain sections (12 micron) by [^{125}I]TZ66127 (1.85 kBq/slide) and blocked by HC608 (1 μM). The ARG signal is shown in linear scale. Coronal, horizontal, and sagittal sections were used in the study. After the autoradiography, the brain sections were subjected to H&E staining to show rat brain anatomic structure and the regions of interesting are marked. TRPC5 IHC staining of the adjacent rat brain sections provides a comparison with ARG signal.

Scheme 1.

Synthesis of standard reference compound **TZ66127** and precursor **5**. *Reagents and conditons:* a) 1-(bromomethyl)-4-iodobenzene, K_2CO_3 , DMF, RT; b) 2-(3-bromopropoxy)tetrahydro-2H-pyran, K_2CO_3 , DMF, 60 °C; c) HCl (conc.), EtOH, reflux; d) 3-(trifluoromethoxy)phenol, K_2CO_3 , DMF, 80 °C; 24% for 4 steps; e) $\text{Pd(PPh}_3)_4$, bis(tributyltin), LiCl, dioxane, 110 °C, 12 h, 55%; f) Na^{125}I , H_2O_2 , NaOAc, AcOH, RT, 15 min.