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Synthesis and preliminary biological evaluation of [^{11}C]methyl (2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucinate for the fractalkine receptor (CX_3CR1)

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Competitive binding assay
Positron emission tomography (PET)

ABSTRACT

The reference standard methyl (2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucinate (**5**) and its precursor 2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucine (**6**) were synthesized from 6-amino-2-mercaptopyrimidin-4-ol and BnBr with overall chemical yield 7% in five steps and 4% in six steps, respectively. The target tracer [^{11}C]methyl (2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucinate ([^{11}C]**5**) was prepared from the acid precursor with [^{11}C]CH_3OTf through *O*-[^{11}C] methylation and isolated by HPLC combined with SPE in 40–50% radiochemical yield, based on [^{11}C] CO_2 and decay corrected to end of bombardment (EOB). The radiochemical purity was >99%, and the specific activity (SA) at EOB was 370–1110 GBq/μmol with a total synthesis time of ~40-min from EOB. The radioligand depletion experiment of [^{11}C]**5** did not display specific binding to CX_3CR1, and the competitive binding assay of ligand **5** found much lower CX_3CR1 binding affinity.

CX_3C chemokine receptor 1 (CX_3CR1), also known as fractalkine receptor or G-protein coupled receptor 13 (GPR13), is a protein in humans.[1] CX_3CR1 binds the chemokine CX_3CL1, also called fractalkine ligand or neurotactin.[2] CX_3CR1 is expressed in the brain, spleen, and in subpopulations of leukocytes, cells of monocytic lineage, and neutrophils but also in lymphocytes, and associated with various cancer, cardiovascular and neurological diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).[3] CX_3CR1 is an interesting therapeutic target, and many selective CX_3CR1 antagonists have been developed.[4,5] Methyl (2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucinate (**5**) recently developed by AstraZeneca is a potent and selective CX_3CR1 antagonist with K_i 8.3 and 1940 nM for CX_3CR1 and CXCR2, respectively, and selectivity index (SI) 230.[6] CX_3CR1 has also become a promising target for molecular imaging of CX_3CR1-mediated diseases and image-guided therapy using positron emission tomography (PET) modality. However, radionuclides including carbon-11 and fluorine-18 labeled CX_3CR1 antagonists are still not reported. In our previous work, we have developed carbon-11-labeled naphthalene-sulfonamides as potential radioligands for PET imaging of chemokine receptor 8 (CCR8), as indicated in Fig. 1.[7] In this ongoing study, we first target CX_3CR1 and develop radiolabeled CX_3CR1 antagonists. Here we report the synthesis and preliminary biological evaluation of [^{11}C]methyl (2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucinate ([^{11}C]**5**) as a new candidate PET agent for imaging of CX3CR1.

The reference standard **5** and its desmethylated acid precursor 2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucine (**6**) were synthesized as depicted in Scheme 1, according to the literature method with modifications.[6,8] The alkylation of commercially available starting material 2-amino-6-hydroxy-2-mercaptopyrimidine with benzyl bromide in 1 M NaOH gave compound **1** in 92% yield, which was collected by filtration and was sufficiently pure to be used in next step without further purification. Compound **1** was reacted with potassium thiocyanate, pyridine, and bromine in *N*,*N*-dimethylformamide (DMF) to provide intermediate **2** in 83% yield, which was followed by condensation at elevated temperature to afford the thiazole **3** in 95% yield. The 7-hydroxy group of compound **3** was then converted to the corresponding chloride **4** to introduce a better leaving group chloro via a Vilsmeier reaction in 91% yield. In this reaction, the organic base *N*, *N*-dimethylanline was removed, consequently the reaction process and workup procedure were simplified, and the yield was increased from 60% to 91%.[6] The chloro group of compound **4**

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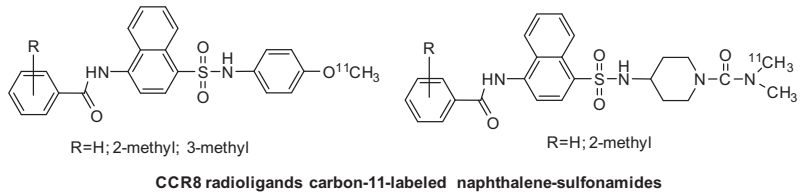
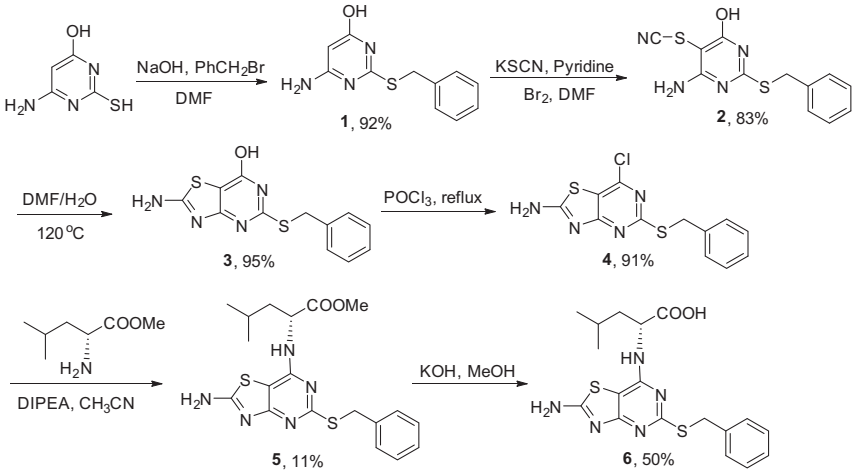


Fig. 1. PET radioligands for imaging of CCR8.

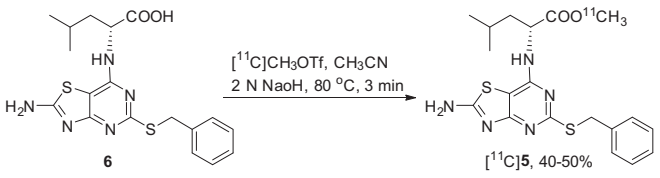


Scheme 1. Synthesis of methyl (2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucinate (**5**) and 2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucine (**6**).

was subsequently displaced in a nucleophilic aromatic substitution reaction with excess methyl D-leucinate in the solvent anhydrous CH_3CN or *N*-methylpyrrolidone and *N*,*N*-diisopropylethylamine (DIPEA) as a catalyst to give the standard compound **5** in only 11% yield. The poor yield is due to that it is difficult to displace aromatic chloro with methyl D-leucinate. The hydrolysis of compound **5** in KOH/methanol at room temperature (RT) for 21 h provided the acid precursor **6** in 50% yield.

Synthesis of the target tracer ([^{11}C]**5**) is shown in Scheme 2. The acid precursor **6** underwent *O*-[^{11}C]methylation[9–11] using the reactive [^{11}C]methylating agent [^{11}C]methyl triflate ([^{11}C]CH_3OTf)[12,13] in acetonitrile at 80 °C under basic condition (2 N NaOH). The product was isolated by semi-preparative reverse-phase (RP) high performance liquid chromatography (HPLC) with a C-18 column, and then concentrated by solid-phase extraction (SPE)[14,15] with a disposable C-18 Light Sep-Pak cartridge to produce the corresponding pure radiolabeled compound [^{11}C]**5** in 40–50% radiochemical yield, decay corrected to end of bombardment (EOB), based on [^{11}C]CO_2.

The radiosynthesis process included three stages: 1) labeling reaction; 2) purification; and 3) formulation. The radiolabeled precursor we used is more reactive [^{11}C]CH_3OTf, instead of commonly used [^{11}C]methyl iodide ([^{11}C]CH_3I),[16] in *O*-[^{11}C]methylation to improve radiochemical yield of [^{11}C]**5**. An Eckert & Ziegler Modular Lab C-11 Methyl Iodide/Triflate module is employed to produce



Scheme 2. Synthesis of [^{11}C]methyl (2-amino-5-(benzylthio)thiazolo[4,5-*d*]pyrimidin-7-yl)-D-leucinate ([^{11}C]**5**).

[^{11}C]methylating agent either [^{11}C]CH_3OTf or [^{11}C]CH_3I ([^{11}C]CH_3Br passed through a NaI column). The direct comparison between [^{11}C]CH_3OTf and [^{11}C]CH_3I confirmed the aforementioned result. The labeling reaction was conducted using a V-vial method. Addition of aqueous $NaHCO_3$ to quench the radiolabeling reaction and to dilute the radiolabeling mixture prior to the injection onto the semi-preparative HPLC column for purification gave better separation of [^{11}C]**5** from its acid precursor **6**. We used Sep-Pak trap/release method instead of rotatory evaporation for formulation to improve the chemical purity of radiolabeled product [^{11}C]**5**. In addition, a C18 Light Sep-Pak to replace a C18 Plus Sep-Pak allowed final product formulation with ≤5% ethanol.[17] Overall, it took ~40 min for synthesis, purification and dose formulation.

The radiosynthesis was performed in a self-designed automated multi-purpose [^{11}C]-radiosynthesis module.[18–20] This radiosynthesis module facilitated the overall design of the reaction, purification and reformulation capabilities in a fashion suitable for adaptation to preparation of human doses. In addition, the module is designed to allow in-process measurement of [^{11}C]-tracer specific activity (SA, GBq/μmol at EOB) using a radiation detector at the outlet of the HPLC-portion of the system. For the reported syntheses, the product SA was in a range of 370–1110 GBq/μmol at EOB. The major factors including [^{11}C]-target and [^{11}C]-radiosynthesis unit that affect the EOB SA significantly to lead to such a wide range from 370 to 1110 GBq/μmol have been discussed in our previous works.[21] The general methods to increase SA have been described as well, and the SA of our [^{11}C]-tracers is significantly improved.[21] The 'wide range' of SA we reported is for the same [^{11}C]-tracer produced in different days, because very different [^{11}C]-target and [^{11}C]-radiosynthesis unit situations would make SA in a wide range. Likewise, the methods to minimize such wide range of SA from practice perspective have been provided in our previous works.[21] At the end of synthesis (EOS), the SA of [^{11}C]-tracer was determined again by analytical HPLC,[22] calculated, decay

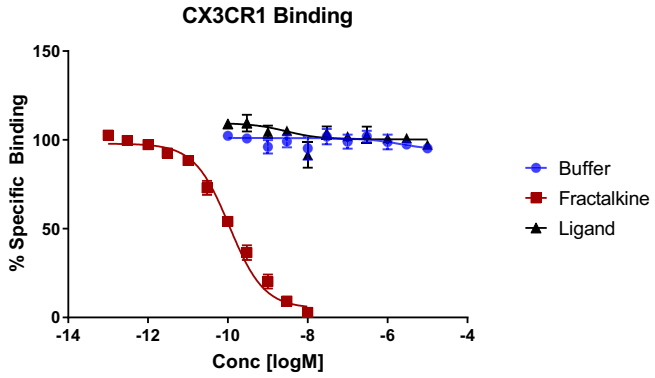


Fig. 2. The result of the competitive binding assay of ligand **5**.

corrected to EOB, and based on [^{11}C]CO_2, which was in agreement with the 'on line' determined value. In each our [^{11}C]-tracer production, if semi-preparative HPLC was used for purification, then the SA of [^{11}C]-tracer was assessed by both semi-preparative HPLC (during synthesis) and analytical HPLC (EOS); if SPE was used for purification, then the SA of [^{11}C]-tracer was only measured by analytical HPLC at EOS.[11]

Chemical purity and radiochemical purity were determined by analytical HPLC.[22] The chemical purity of the precursor and reference standard was >90%. The radiochemical purity of the target tracer was >99% determined by radio-HPLC through γ-ray (PIN diode) flow detector, and the chemical purity of the target tracer was >90% determined by reversed-phase HPLC through UV flow detector.

The preliminary biological evaluation of the radioligand [^{11}C]**5** was performed by a radioligand depletion experiment.[23] In this experiment, a wide range of protein was used for the optimization of the assay conditions. For the law of mass action to be valid, the protein and radioligand concentration need to be at levels where no more than 10% of the total radioligand added is bound to the protein. Otherwise the radioligand is considered depleted and specialized formulas for radioligand depletion must be used for analysis of the data in saturation or competitive binding assays. An optimized binding assay uses protein levels where the radioligand is depleted no more than 10%, and where sufficient signal to background binding levels, at least 5 fold total binding/non-specific binding ratio, are obtained. To achieve both of these ends, a wide range of membrane protein concentrations was tested to get the optimal level for the assay. The result indicated no specific binding of [^{11}C]**5** to CX_3CR1. The method was further optimized by improving the solubility of the tracer [^{11}C]**5**, and the result remained same. We are puzzled by this disappointing result. Then the competitive binding assay of ligand **5** was conducted following the literature method.[6] The assays were incubated at 25 °C for 2 h, and the results are shown in Fig. 2. [^{125}I]Fractalkine was used as the radioligand, and fractalkine and buffer were used as a positive control and a negative control, respectively. Likewise, there was not specific binding of **5** to CX_3CR1, since its binding curve is similar to that of the negative control buffer. We were unable to reproduce the reported result.[6] However, the result of the competitive binding assay of ligand **5** was consistent with the result of radioligand [^{11}C]**5** depletion experiment. Thus, we can conclude compound **5** is not a potent and selective antagonist of CX_3CR1.

The experimental details and characterization data for compounds **1**–**6** and for the tracer [^{11}C]**5,** as well as radioligand [^{11}C]**5** depletion experiments and ligand **5** competitive binding assays are given.[24]

In summary, synthetic routes with reasonable to high yields have been developed to produce the reference standard **5**, acid precursor **6** and target radioligand [^{11}C]**5**. The radiosynthesis employed [^{11}C]CH_3OTf for *O*-[^{11}C]methylation at the carboxylic acid position of the desmethyled precursor, followed by product purification and isolation using a semi-preparative RP HPLC combined with SPE. [^{11}C]**5** was obtained in high radiochemical yield, radiochemical purity and chemical purity, with a reasonably short overall synthesis time, and high specific activity. The preliminary biological evaluation via radioligand [^{11}C]**5** depletion experiment and ligand **5** competitive binding assay suggested the compound **5** is not a potent and selective CX_3CR1 ligand, consequently, the tracer [^{11}C]**5** is not able to be a CX_3CR1 radioligand.

Acknowledgments

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24. (a). *General:* All commercial reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific, and used without further purification. [^{11}C]CH_3OTf was prepared according to a literature procedure.[13] Melting points were determined on a MEL-TEMP II capillary tube apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 500 MHz NMR Fourier transform spectrometer at 500 and 125 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to an internal standard tetramethylsilane (TMS, δ 0.0) (^{1}H NMR) and to the solvent signal (^{13}C NMR), and coupling constants (J) are reported in hertz (Hz). Liquid chromatography–mass spectra (LC–MS) analysis was performed on an Agilent system, consisting of an 1100 series HPLC connected to a diode array detector and a 1946D mass spectrometer configured for positive-ion/negative-ion electrospray ionization. The high resolution mass spectra (HRMS) were obtained using a Waters/Micromass LCT Classic spectrometer. Chromatographic solvent proportions are indicated as volume: volume ratio. Thin-layer chromatography (TLC) was run using Analtech silica gel GF uniplates (5×10 cm^2). Plates were visualized under UV light. Normal phase flash column chromatography was carried out on EM Science silica gel 60 (230–400 mesh) with a forced flow of the indicated solvent system in the proportions described below. All moisture- and air-sensitive reactions were performed under a positive pressure of nitrogen maintained by a direct line from a nitrogen source. Analytical RP HPLC was performed using a Prodigy (Phenomenex) 5 μm C-18 column, 4.6 × 250 mm; mobile phase 70%CH_3CN/30% H_2O;

flow rate 1.0 mL/min; UV (254 nm) and γ-ray (PIN diode) flow detectors. Semi-preparative RP HPLC was performed using a Prodigy (Phenomenex) 5 μm C-18 column, 10 × 250 mm; mobile phase 70%CH_3CN/30%H_2O; flow rate 4 mL/min; UV (254 nm) and γ-ray (PIN diode) flow detectors. C18 Light Sep-Pak cartridges were obtained from Waters Corporation (Milford, MA). Sterile Millex-FG 0.2 μm filter units were obtained from Millipore Corporation (Bedford, MA). (b). *6-Amino-2-(benzylthio)pyrimidin-4-ol (**1**)*: Benzyl bromide (32.23 g, 189 mmol) was added dropwise into a solution of 4-amino-6-hydroxy-2-mercaptopyrimidine (25.77 g, 180 mmol) in 1 M NaOH (36 mL) and water (6 mL). The reaction was kept stirring at 40–50 °C for 4 h and was left at RT for 12 h. Then the mixture was neutralized with acetic acid to obtain a white solid, which was collected by filtration, washed with water and hexanes, and dried in air to give **1** as a white solid (38.70 g, 92%). R_f = 0.70 (1:9 MeOH/CH_2Cl_2), mp 230–232 °C. ^{1}H NMR (DMSO-d_6): δ 4.32 (s, 2H, CH_2), 6.52 (s, 2H, NH_2), 7.24 (t, *J* = 7.5 Hz, 1H, Ph-H), 7.30 (t, *J* = 7.5 Hz, 2H, Ph-H), 7.41 (d, *J* = 7.5 Hz, 2H, Ph-H), 11.46 (s, 1H, OH). MS (ESI): 234 ([M+H]$^+$, 100%); MS (ESI): 232 ([M−H]$^-$, 14%). (c). *6-Amino-2-(benzylthio)-5-thiocyanatopyrimidin-4-ol (**2**)*: Compound **1** (37.36 g, 160.3 mmol) and KSCN (65.44 g, 673 mmol) were suspended in DMF (760 mL) and heated to 60 °C. Pidine (22.8 g, 288.5 mmol) was added, and the solution was cooled to 5 °C. Bromine (25.62 g, 160.3 mmol) was added dropwise, and the solution was stirred at 5–10 °C for 2 h and then at RT overnight. The mixture was poured into ice water and stirred for 1 h. The resulting solid was filtered, washed with cold water, and dried in air to afford **2** as a white solid (38.45 g, 83%). R_f = 0.82 (1:9 MeOH/CH_2Cl_2), mp 235–237 °C. ^{1}H NMR (DMSO-d_6): δ 4.38 (s, 2H, CH_2), 7.25–7.27 (m, 1H, Ph-H), 7.31 (dd, *J* = 7.0, 12.5 Hz, 2H, Ph-H), 7.46 (t, *J* = 6.0 Hz, 2H, Ph-H), 7.60 and 7.80 (2 s, 2H, NH_2), 12.34 (s, 1H, OH). MS (ESI): 291 ([M+H]$^+$, 100%); MS (ESI): 289 ([M−H]$^-$, 90%). (d). *2-Amino-5-(benzylthio)thiazolo[4,5-d]pyrimidin-7-ol (**3**)*: Compound **2** (20.3 g, 70 mmol) was suspended in water (45 mL) and DMF (140 mL) and heated at 120 °C for 27 h. The reaction mixture was poured onto ice, and resulting pale-yellow precipitate was collected by filtration and washed with cold water. The solid material was suspended in water (250 mL) and heated to 75 °C, and NaOH (10 M, 17 mL) was added. The resulting suspension was filtered, and the product was precipitated by addition of concentrated HCl until pH 4. The solid was filtered, and dried in vacuo to give **3** as a white solid (19.28 g, 95%). R_f = 0.63 (1:9 MeOH/CH_2Cl_2), mp > 330 °C. ^{1}H NMR (DMSO-d_6): δ 4.41 (s, 2H, CH_2), 7.23–7.26 (m, 1H, Ph-H), 7.31 (t, *J* = 7.5 Hz, 2H, Ph-H), 7.43 (d, *J* = 7.5 Hz, 2H, Ph-H), 8.17 (s, 2H, NH_2), 12.54 (s, 1H, OH). MS (ESI): 291 ([M+H]$^+$, 100%); MS (ESI): 289 ([M−H]$^-$, 20%). (e). *5-(Benzylthio)-7-chlorothiazolo[4,5-d]pyrimidin-2-amine (**4**)*: Compound **3** (10.0 g, 34.5 mmol) was suspended in $POCl_3$ (100 mL), and the mixture was heated at reflux for 5 h. After the reaction mixture was concentrated in reduced pressure, the ice water was slowly added into above residue. The resulting precipitate was filtered, washed with cold water, and dried in air to give **4** as a yellow solid (9.67 g, 91%). R_f = 0.70 (1:1 EtOAc/hexanes), mp 217–219 °C. ^{1}H NMR (acetone-d_6): δ 4.38 (s, 2H, CH_2), 7.24 (t, *J* = 7.5 Hz, 1H, Ph-H), 7.31 (d, *J* = 7.5 Hz, 2H, Ph-H), 7.49 (d, *J* = 7.5 Hz, 2H, Ph-H), 8.13 (s, 2H, NH_2). MS (ESI): 309 ([M+H]$^+$, 100%); MS (ESI): 307 ([M−H]$^-$, 60%). (f). *Methyl (2-amino-5-(benzylthio)thiazolo[4,5-d]pyrimidin-7-yl)-D-leucinate (**5**)*: To a solution of compound **4** (0.93 g, 3.0 mmol) in anhydrous CH_3CN (120 mL) were added methyl D-leucinate (1.45 g, 10.0 mmol) and DIPEA (0.78 g, 6.0 mmol). The reaction mixture was stirred and heated at 100 °C for 4 days. Then the reaction mixture was concentrated in vacuo, and the residue was purified by column chromatographyon silica gel with eluent (1:99 to 5:95 MeOH/CH_2Cl_2) to afford **5** as a yellowish solid (138 mg, 11%). R_f = 0.38 (1:14 MeOH/CH_2Cl_2), mp 143–145 °C. ^{1}H NMR (MeOD-d_4): δ 0.92 (d, *J* = 6.5 Hz, 3H, CH_3), 0.95 (d, *J* = 6.5 Hz, 3H, CH_3), 1.62–1.68 (m, 1H, CH), 1.72–1.84 (m, 2H, CH_2), 3.65 (d, *J* = 5.5 Hz, 3H, CH_3), 4.33 (dd, *J* = 5.5, 13.5 Hz, 1H, SC*H*H), 4.41 (dd, *J* = 5.5, 13.5 Hz, 1H, SCH*H*), 4.83 (dd, *J* = 5.0, 10.0 Hz, 1H, NCH), 6.68 (s, 1H, NH), 7.20–7.24 (dd, *J* = 2.0, 8.0 Hz, 1H, Ph-H), 7.29 (dd, *J* = 2.0, 8.5 Hz, 2H, Ph-H), 7.44 (s, 2H, NH_2), 7.47 (t, *J* = 8.5 Hz, 2H, Ph-H); ^{1}H NMR ($CDCl_3$): δ 0.88 (d, *J* = 6.5 Hz, 3H, CH_3), 0.92 (d, *J* = 6.5 Hz, 3H, CH_3), 1.62 (t, *J* = 7.0 Hz, 2H, CH_2), 1.75–1.80 (m, 1H, CH), 3.71 (s, 3H, OCH_3), 4.35 (s, 2H, SCH_2), 4.77–4.78 (m, 1H, NCH), 5.96 (s, 1H, NH), 7.18 (t, *J* = 7.0 Hz, 1H, Ph-H), 7.23 (d, *J* = 7.5 Hz, 2H, Ph-H), 7.38 (d, *J* = 7.5 Hz, 2H, Ph-H). MS (ESI): 417 ([M+H]$^+$, 100%); MS (ESI): 416 ([M−H]$^-$, 15%). (g). *2-Amino-5-(benzylthio)thiazolo[4,5-d]pyrimidin-7-yl)-D-leucine (**6**)*: To a solution of compound **5** (83 mg, 0.2 mmol) in methanol (40 mL) was added KOH (0.5 g, 8.9 mmol). The reaction mixture was stirred at RT for 21 h. Then the reaction mixture was concentrated in vacuo, and the mixture was neutralized with 1 N HCl. The mixture was extracted with EtOAc (3 × 60 mL), and the combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The resulting residue was purified by column chromatography on silica gel with eluent (5:95 to 30:70 MeOH/CH_2Cl_2) to give **6** as a white off solid (40 mg, 50%). R_f = 0.24 (1:5 MeOH/CH_2Cl_2), mp 245 °C (decomposed). ^{1}H NMR (MeOD-d_4): δ 0.92 (d, *J* = 6.5 Hz, 3H, CH_3), 0.94 (d, *J* = 6.5 Hz, 3H, CH_3), 1.71–1.76 (m, 2H, CH_2), 1.77–1.79 (m, 1H, CH), 4.32 (d, *J* = 13.5 Hz, 1H, SC*H*H), 4.47 (d, *J* = 13.5 Hz, 1H, SCH*H*), 4.75–4.77 (m, 1H, NCH), 7.18 (t, *J* = 7.5 Hz, 1H, Ph-H), 7.26 (t, *J* = 7.0 Hz, 2H, Ph-H), 7.40 (d, *J* = 7.5 Hz, 2H, Ph-H). ^{13}C NMR (MeOD-d_4): δ 22.33, 23.70, 26.28, 36.10, 43.03, 55.86, 127.87, 129.38, 130.00, 139.85, 155.98, 157.13, 168.53, 169.67, 173.64, 180.60. MS (ESI): 404 ([M+H]$^+$, 100%); MS (ESI): 402 ([M−H]$^-$, 10%). HRMS (ESI) calcd for $C_{18}H_{21}N_5O_2S_2H$, 404.1215 ([M+H]$^+$); found 404.1223. (h). *([^{11}C]methyl (2-amino-5-(benzylthio) thiazolo[4,5-d]pyrimidin-7-yl)-D-leucinate ([^{11}C]**5**)*: [^{11}C]CO_2 was produced by the ^{14}N(p,α)^{11}C nuclear reaction in the small volume (9.5 cm^3) aluminum gas target provided with the Siemens RDS-111 Eclipse cyclotron. The target gas consisted of 1% oxygen in nitrogen purchased as a specialty gas from Praxair, Indianapolis, IN. Typical irradiations used for the development were 58 μA beam current and 15 min on target. The production run produced approximately 25.9 GBq of [^{11}C]CO_2 at EOB. The acid precursor (**6**, 0.1–0.3 mg) was dissolved in CH_3CN (300 μL). To this solution was added aqueous NaOH (2 N, 2 μL). The mixture was transferred to a small reaction vial. No-carrier-added (high specific activity) [^{11}C]CH_3OTf that was produced by the gas-phase production method[13] within 12 min from [^{11}C]CO_2 through [^{11}C]CH_4 and [^{11}C]CH_3Br with silver triflate (AgOTf) column was passed into the reaction vial at RT until radioactivity reached a maximum (2 min), and then the reaction vial was isolated and heated at 80 °C for 3 min. The contents of the reaction vial were diluted with aqueous $NaHCO_3$ (0.1 M, 1 mL). The reaction vial was connected to a 3-mL HPLC injection loop. The labeled product mixture solution was injected onto the semi-preparative HPLC column for purification. The product fraction was collected in a recovery vial containing 30 mL water. The diluted tracer solution was then passed through a C-18 Sep-Pak Light cartridge, and washed with water (3 × 10 mL). The cartridge was eluted with EtOH (3 × 0.4 mL) to release the labeled product, followed by saline (10–11 mL). The eluted product was then sterile-filtered through a Millex-FG 0.2 μm membrane into a sterile vial. Total radioactivity was assayed and total volume (10–11 mL) was noted for tracer dose dispensing. The overall synthesis time including HPLC-SPE purification and reformulation was ~40 min from EOB. The decay corrected radiochemical yield was 40–50%. Retention times in the analytical HPLC system were: t_R **6** = 3.08 min, t_R **5** = 5.64 min, t_R [^{11}C]**5** = 5.73 min. Retention times in the preparative HPLC system were: t_R **6** = 3.75 min, t_R **5** = 8.36 min, t_R [^{11}C]**5** = 8.48 min. (i). *Radioligand depletion experiments:* Radioligand depletion experiments were performed using CX_3CR1 cell membrane preps (Millipore) to measure the bound radioligand [^{11}C]**5** concentration. Four replicates of membranes (0.004–4.5 mg protein/mL assay medium) were incubated with 1 nM or 10 nM [^{11}C]**5** in a final volume of 0.2 mL in assay buffer (25 mM HEPES, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, pH 7.4) for30 min at 25 °C. Unlabeled compound **5** was added at 100 fold excess of [^{11}C]**5** to determine non-specific binding. In some experiments 10% Solutol (Sigma) was added to increase the solubility of the radioligand. To measure the total radioactivity added to the experiment, 20 μL of stock radioligand [^{11}C]**5** (amount added to each experimental well) was spotted onto a unifilter GF/B plate then allowed to air dry. An additional aliquot was added to a scintillation vial and counted on a Beckman LSC to determine CPMs (counts per minute) added. For termination of the binding reaction, the samples were filtered onto GF/B unifilter plates (Perkin Elmer) that had been pre-soaked in 20 mM tetrasodium pyrophosphate for 30 min using a unifilter-96 cell harvester (Perkin-Elmer). Plates were washed 6 times with ice cold saline, dried under a vacuum, and exposed to a TR2025 phosphorscreen (GE Healthcare) for 20–60 min. Exposed phophorscreens were then read on a Typhoon FLA-7000IP (GE Healthcare) along with [^{11}C]**5** calibration standards. CPMs were determined by calibrating the image to the CPMs in the calibration standards via MCID analysis Software. *(j). Ligand competitive binding assays:* To compare the affinity of the unlabeled compound **5** (ligand) with the native ligand, fracktalkine, competitive binding assays were performed using the above cell membrane. Approximately 0.5 μg per well of membrane protein was incubated with 11 different concentrations of the compound over a six log unit range. Assays were run with 0.025 nM [^{125}I]fractalkine (Perkin Elmer) in assay buffer as above with the addition of 0.5% BSA (bovine serum albumin) to reduce non-specific binding. Triplicate determinations were done at each concentration of the test compound. Unlabeled fractalkine (Peprotech) was used to determine non-specific binding. Each assay plate included a positive control (fractalkine) and a negative control (buffer). Assays were incubated at 25 °C for 2 h. After equilibrium was reached, bound radioligand was separated from free radioligand using a Perkin Elmer 96-well filtration apparatus. Plates were washed at least 3 times with ice cold saline. Filters were quickly dried under a vacuum and radioactive counts collected on a Perkin Elmer TopCount microplate scintillation counter using a counting protocol for ^{125}I. Data was analyzed with Prism 7 (GraphPad Software Inc.) to calculate *Ki* values.