Synthesis and evaluation of an ^{18}F-labeled pyrimidine-pyridine amine for targeting CXCR4 receptors in gliomas

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ABSTRACT

Introduction: Chemokine receptor-4 (CXCR4, fusin, CD184) is expressed on several tissues involved in immune regulation and is upregulated in many diseases including malignant gliomas. A radiolabeled small molecule that readily crosses the blood–brain barrier can aid in identifying CXCR4-expressing gliomas and monitoring CXCR4-targeted therapy. In the current work, we have synthesized and evaluated an [^{18}F]-labeled small molecule based on a pyrimidine–pyridine amine for its ability to target CXCR4.

Experimental: The nonradioactive standards and the nitro precursor used in this study were prepared using established methods. An HPLC method was developed to separate the nitro-precursor from the nonradioactive standard and radioactive product. The nitro-precursor was radiolabeled with ^{18}F under inert, anhydrous conditions using the [^{18}F]-kryptofix 2.2.2 complex to form the desired N-(4-(((6-[^{18}F]fluoropyridin-2-yl)amino)methyl)benzyl)pyrimidin-2-amine ([^{18}F]-3). The purified radiolabeled compound was used in serum stability, partition coefficient, cellular uptake, and in vivo cancer targeting studies.

Results: [^{18}F]-3 was synthesized in 4–10% decay-corrected yield (to start of synthesis). [^{18}F]-3 ($t_R \approx 27$ min) was separated from the precursor ($t_R \approx 30$ min) using a pentafluorophenyl column with an isocratic solvent system. [^{18}F]-3 displayed acceptable serum stability over 2 h. The amount of [^{18}F]-3 bound to the plasma proteins was determined to be > 97%. The partition coefficient ($LogD_{7.4}$) is 1.4 ± 0.5. Competitive in vitro inhibition indicated 3 does not inhibit uptake of ^{67}Ga-pentixafor. Cell culture media incubation and ex vivo urine analysis indicate rapid metabolism of [^{18}F]-3 into hydrophilic metabolites. Thus, *in vitro* uptake of [^{18}F]-3 in CXCR4 overexpressing U87 cells (U87 CXCR4) and U87 WT indicated no specific binding. *In vivo* studies in mice bearing U87 CXCR4 and U87 WT tumors on the left and right shoulders were carried out using [^{18}F]-3 and ^{68}Ga-pentixafor on consecutive days. The CXCR4 positive tumor was clearly visualized in the PET study using ^{68}Ga-pentixafor, but not with [^{18}F]-3.

Conclusions: We have successfully synthesized both a radiolabeled analog to previously reported CXCR4-targeting molecules and a nitro precursor. Our *in vitro* and *in vivo* studies indicate that [^{18}F]-3 is rapidly metabolized and, therefore, does not target CXCR4-expressing tumors. Optimization of the structure to improve the *in vivo* (and *in vitro*) stability, binding, and solubility could lead to an appropriate CXCR4-targeted radiodiagnositic molecule.

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1. Introduction

Chemokine receptor-4 (CXCR4, fusin, CD184) is a G-protein coupled receptor (GPCR) that functions as an immunomodulatory receptor. It is expressed on several tissues involved in immune regulation, including bone marrow, spleen, CD4 + T cells and is specific for its ligand C-X-C motif chemokine 12 (CXCL12, SDF-1) [1–5]. Binding of CXCL12 to CXCR4 leads to downstream activation of PI3K/AKT and MAPK pathways leading to survival, proliferation, and other chemotactic functions [6,7]. CXCR4 overexpression has been observed in several diseases including different cancers, rheumatoid arthritis, and post-traumatic stress disorder [1–5].

CXCR4 expression has been shown to be upregulated in gliomas with expression correlating with tumor grade [8]. Targeting CXCR4 with positron-emitting radiopharmaceuticals that penetrate an intact blood–brain barrier (BBB) could provide clinicians with a diagnostic tool necessary for identifying CXCR4 + gliomas and monitoring anti-

CXCR4 based therapies. Currently, the only FDA-approved drug that targets CXCR4 is plerixafor. Unfortunately, attempts to radiolabel plerixafor with ^{64}Cu for PET imaging have resulted in compounds with reduced affinity for CXCR4 [9–12]. Another radiopharmaceutical undergoing clinical investigation is ^{68}Ga-pentixafor, a ^{68}Ga labeled pentapeptide that binds to CXCR4 [13–15]. Studies have shown the utility of this tracer in imaging CXCR4 positive tumors, but the peptide-based agent would be unable to penetrate an intact BBB. Thus, it would be useful to identify alternative molecules, with better BBB penetrating properties, that can be labeled with radiohalogens and that can target CXCR4.

Dipyrimidine-based agents – where the two pyrimidines were linked through a para-xylyl-endiamine – and pyrimidine-pyridine amine-based agents – where the two heterocycles were linked through a para-xylyl-endiamine – have been suggested by Zhu et al. to specifically target CXCR4 [16] and *in silico* calculations of the partition coefficients indicated that these compounds have potential to cross the BBB. Zhu et al. reported that many of the pyrimidine-pyridine amine-based agents, including fluorine containing variants – **2** and **3** (Fig. 1), had low nM affinities for CXCR4 [16] based on a fluorescence inhibition binding affinity assay utilizing MDA-MB-231 cells, a biotinylated peptide (TN14003-biotin), and secondary staining with rhodamine. Though both **2** and **3** showed limited efficacy in matrigel invasion inhibition assays, compound **3** displayed significant paw inflammation suppression *in vivo* [16]. Our goal was to develop an ^{18}F-labeled compound that can penetrate the BBB and therefore we investigated ^{18}F-labeled **3** as a potential PET imaging agent of CXCR4-positive tumors. Work from the same group to develop an ^{18}F-labeled CXCR4 targeting agent utilized a radiolabeled compound to determine the IC$_{50}$ value for uptake in MDA-MB-231 cells, but no imaging data were reported [17].

The current study describes the synthesis of a nonradioactive analog (**3**) and the corresponding nitro precursor (**4**), an HPLC method development for separation of the precursor from the final product, and radiofluorination to produce [^{18}F]-**3**. Preliminary *in vitro* and *in vivo* evaluations of [^{18}F]-**3** with comparison to [$^{67/68}$Ga]-pentixafor were performed.

2. Experimental

All chemicals were commercially available, unless otherwise stated. All nonradioactive synthesized materials were analyzed using a Bruker Ultrashield Plus 600 MHz NMR and Acquity-SQD mass spectrometer (Waters, Milford, MA) or Shimadzu LC–MS (Kyoto, Japan) with Gemini-NX column (Phenomenex, C-18, 5 μm, 110 Å, 250 × 4.6 mm) 10–95% AcN in water with 0.05% formic acid over 15 min at 1 mL/min. The human glioblastoma cell line U87 (U87 WT) and mouse embryonic fibroblast cell line NIH/3 T3 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's

Fig. 1. Compounds used in this study.

modified Eagle's medium (DMEM) with 10% fetal bovine serum (FCS), 2 mM L-glutamine, 1500 mg/L NaHCO$_3$, 100 units/mL penicillin G and 100 μg/mL streptomycin. A CXCR4 over-expressing U87 cell line (U87 CXCR4) was provided by the NIH AIDS Reagent Program (cat. no. 4036) [18] and cultured in Dulbecco's modified Eagle's high glucose medium (DMEM-HG) with 10% FCS, 1 μg/mL puromycin, 300 μg/mL G418, 100 units/mL penicillin G and 100 μg/mL streptomycin. The human mammary gland/breast adenocarcinoma cell line MDA-MB-231 was purchased from ATCC (Manassas, VA) and cultured in DMEM-HG with 10% FCS, 100 units/mL penicillin G and 100 μg/mL streptomycin. All culture media were prepared by the MSKCC Media Preparation Core.

1 was prepared in two steps as reported [16] in 7.28% overall yield and confirmed by NMR. **2** and **3** were previously reported by Zhu et al. [16], but were purified in the present study by semi-prep HPLC (Phenomenex, Jupiter C-18, 5 μm, 300 Å, 250 × 10 mm, 3 mL/min). **2** eluted from 15 to 17.5 min (10 to 67% AcN in water over 13 min) and **3** eluted from 26.4 to 29 min (10 to 67% AcN in water over 30 min). The fractions were lyophilized to dryness and confirmed by ^{1}H-NMR and MS.

2.1. Synthesis of N-(4-(((6-nitropyridin-2-yl)amino)methyl)benzyl) pyrimidin-2-amine (4)

To a dry mixture of **1** (49.9 mg, 0.234 mmol) and 2-amino-6-nitropyridine, 5 mL of 1,2-dichloroethane were added. The resulting mixture was stirred at room temperature to dissolve the solids. After dissolution, acetic acid (14.76 μL, 0.2579 mmol), molecular sieves (191 mg, flame dried), and sodium triacetoxyborohydride (106.5 mg, 0.5025 mmol) were added sequentially. The mixture was stirred at room temperature for 3 d. Ethyl acetate (5 mL) was added and the soluble material transferred to a new vial. To the supernatant, 1 M NaOH (3 mL) was added and the mixture extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried with anhydrous Na$_2$SO$_4$, gravity filtered, and rotary evaporated. The solids were dissolved in 5 mL of 50:50 AcN:water and filtered through a 0.2 μm nylon filter (Pall Life Sciences, Port Washington, NY). The vial was washed with 2 mL of EtOAc, then 2 mL CH$_2$Cl$_2$, and then 1 mL MeOH, which were sequentially filtered through the nylon filter and collected. The combined filtrate was purified by HPLC in 1 mL injections using a semi-prep HPLC method (Phenomenex, Jupiter C-18, 5 μm, 300 Å, 250 × 10 mm, 3 mL/min, 10 to 67% AcN in water over 30 min) to elute the product from 26.5 to 27.7 min. The fraction was lyophilized to yield 13.4 mg of **4** as a yellow solid (0.0398 mmol, 17.0% yield) and HPLC analyzed using an analytical method (Kinetex F5, Phenomenex, 100 × 4.6 mm, 2.6 μm, 100 Å, 0.5 mL/min, 35% EtOH in 50 mM NH$_4$OAc) to elute **4** at 30.0 min. ^{1}H-NMR (600 MHz, CDCl$_3$) δ 8.30 (d, *J* = 4.8 Hz, 2H, CHC**H**N-pyrimidine), 7.64 (t, *J* = 7.9 Hz, 1H, NC(NO$_2$)CHC**H**CH-nitropyridine), 7.49 (d, *J* = 7.6 Hz, 1H, NC(NO$_2$)CHCHC**H**-nitropyridine), 7.34 (s, 4H, benzyl), 6.65 (d, *J* = 8.3 Hz, 1H, NC(NO$_2$)-**CH**CHCH-nitropyridine), 6.57 (t, *J* = 4.8 Hz, 1H, C**H**CHN-pyrimidine), 5.42 (s, 1H, CH$_2$N**H**-nitropyridine), 5.34 (s, 1H, CH$_2$N**H**-pyrimidine), 4.64 (d, *J* = 6.0 Hz, 2H, C**H$_2$**NH-nitropyridine), 4.55 (d, *J* = 5.8 Hz, 2H, C**H$_2$**NH-pyrimidine). ^{13}C-NMR (600 MHz, CDCl$_3$) δ 162.21 (quaternary **C** pyrimidine), 158.13 (CHC**H**N-pyrimidine), 157.50 (quaternary **C** nitropyridine), 156.04 (N**C**(NO$_2$)CHCHCH-nitropyridine), 140.11 (NC(NO$_2$)CH**C**HCH-nitropyridine), 138.67 (quaternary **C** benzyl), 136.71 (quaternary **C** benzyl), 127.82 (**C**H-benzyl), 112.32 (NC(NO$_2$) **C**HCHCH-nitropyridine), 111.04 (**C**HCHN-pyrimidine), 106.27 (NC (NO$_2$)CHCH**C**H-nitropyridine), 46.03 (**C**H$_2$NH-pyrimidine), 45.00 (**C**H$_2$NH-nitropyridine). LC–MS (C$_{17}$H$_{16}$N$_6$O$_2$, 12.73 min) [M + H]$^+$ 337.05; [M-H]$^-$ 334.95.

2.2. Radiosynthesis of [^{18}F]-**3** (N-(4-(((6-[^{18}F]fluoropyridin-2-yl) amino)methyl)benzyl)pyrimidin-2-amine)

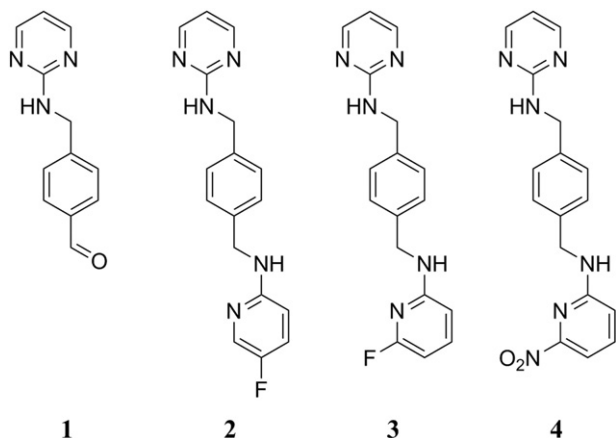Prior to beginning the reaction, reaction vials were dried overnight in an oven to remove residual water. Fluorine-18 irradiated target