Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 17 April 2013, Revised 29 May 2013, Accepted 18 June 2013 Published online 16 July 2013 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.3095

Scavenging strategy for specific activity improvement: application to a new CXCR4-specific cyclopentapeptide positron emission tomography tracer†

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Huisgen cycloaddition is attractive to label peptide because of its rapidity and bioorthogonality. However, for larger tracers, the physico-chemical differences between the precursor and the tracer are usually insufficient to allow their separation by HPLC, reducing the specific activity. This is of importance for peptidic tracers because the combination of their high-affinity receptor with low specific activity results in the precursor saturating the receptors, causing non-specific tracer binding. Here, we report a fast, one-pot, general strategy to circumvent this issue, yielding a tracer of improved specific activity. It consists in adding a lipophilic azide after the labeling step to scavenge unreacted precursor into a more lipophilic species that does not co-elute with the tracer. We applied this strategy to a new fluorinated cyclopentapeptidic CXCR4 antagonist for the PET imaging of cancer, CCIC15, for which we managed to reduce the apparent peptide concentration by a factor of 34 in 10 min. This tracer was radiolabeled by click chemistry with 2-[^{18}F]fluoroethylazide, yielding the tracer in 18 ± 6% (n = 5) end-of-synthesis radiochemical yields (EOS-RCY) in 1.5 h from [^{18}F]fluoride with a specific activity of 19.4 GBq μmol^{-1}. Preliminary biological evaluation of the probe confirmed potency and specificity for CXCR4; further biological evaluation is underway.

Keywords: scavenging; specific activity; CXCR4; cancer; FC131

Introduction

The chemokine receptor CXCR4 is a heptahelical transmembrane G protein-coupled receptor. With its native ligand SDF-1 (stromal cell-derived factor-1), it is a key part of a signal transduction pathway that is exploited for instance during embryo development and inflammatory responses.[1] Over-expression of this receptor is associated with various diseases, including particularly aggressive types of cancer and/or metastatic tumors. As a consequence, CXCR4 appears to be a particularly attractive marker to specifically image invasive and metastatic phenotypes.

Most of the CXCR4 inhibitors reported in the literature are based on peptide (T140, FC131) or cyclam (AMD3100) scaffolds,[2] although the number and classes of inhibitors are expected to increase following the recent disclosure of the receptor's crystal structure.[3] A selection of these inhibitors has been derivatized to allow CXCR4-specific labeling for different imaging modalities, mostly fluorescence, PET, and single-photon emission computed tomography.[4]

Positron emission tomography is a non-invasive and highly sensitive imaging technique that requires the incorporation of a positron-emitting radioisotope into administered probe molecules.[5] Fluorine-18 is an attractive radionuclide for PET as its 110-min half-life allows multistep radiolabeling strategies and enables the transfer of radiotracers to hospitals without an on-site cyclotron.[6]

In general, peptides tend to show favorable properties for cancer imaging, such as their rapid pharmacokinetics, their specific tumor-targeting properties, and their efficient tumor penetration.[7] Furthermore, the precursors for the labeling step are also easily available via solid-phase peptide synthesis, which can even be automated and therefore allow a molecule of high binding affinities to be engineered without much effort. However, a low IC_{50} value increases the tracer's vulnerability to its specific activity: unlabeled species, for example, the unreacted precursor, are likely to saturate the receptors causing non-specific binding of the tracer and thus reducing the signal-to-noise ratio.[8]

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In labeling experiments of low-molecular-weight compounds utilizing a fluorine-18-labeled prosthetic group, a large excess of precursor is often used to increase the reaction rate in order to be compatible with the radionuclide's short half-life. The starting material and the product have distinctive physico-chemical properties (*e.g.*, charge and lipophilicity) permitting the isolation by reverse-phase HPLC (RP-HPLC) of the radioactive product with high specific activity. In the case of high-molecular-weight precursors, such as peptides, differences in physico-chemical properties can be insufficient to prevail over the unmodified parts of the molecule, leading to the precursor and the product to co-elute and resulting in low apparent specific activities.[9]

For instance, the use of the 2-[^{18}F]fluoroethylazide ([^{18}F]FEA) prosthetic group to label an alkyne-containing peptide by click chemistry is attractive because of the bioorthogonality of this reaction. Nevertheless, the relatively high amounts of precursor needed for the reaction to occur reduce the apparent specific activity of the tracer thus produced, limiting the use of such methods for the production of radiotracers.

In this study, we report the design and synthesis of an FC131 analogue as a potential CXCR4-specific tracer. Modifications to FC131 were designed following the structure-activity relationship analysis of the structures reported in the literature as described in the succeeding texts. We also present the development of a method, unprecedented to the best of our knowledge, that allows scavenging of the unreacted precursor, thus affording the tracer in much higher apparent specific activity.

FC131 is a *cyclo*(-Gly^1-D-Tyr^2-Arg^3-Arg^4-Nal^5-) pentapeptide, which was first synthesized by Fujii *et al.*[10] in 2003 and displayed good inhibition of [^{125}I]SDF-1 binding to CXCR4-transfected Chinese hamster ovary cells. Over 100 analogues of FC131 have been published in the literature along with their inhibitory potencies,[11] providing valuable insight into its structure-activity relationship. Notably, substitution of Arg^3 seems to have a relatively moderate effect on the binding provided the replaced residue is not a negatively charged residue such as glutamate. This position is therefore attractive for the introduction of the required terminal alkyne and subsequent triazole following Huisgen alkyne-azide cycloaddition.

Materials and methods

General

All reagents and solvents were purchased from Sigma-Aldrich (Gillingham, U.K.), Fluka, or Fluorochem (Hadfield, U.K.) and used without further purification. Flash column chromatography was carried out on silica gel (Fluka 230–400 mesh, for flash chromatography). Thin layer chromatography was performed on aluminum plates pre-coated with silica (200 μm, 60 F_{254}), which were visualized either by quenching of ultraviolet fluorescence (λ_{max} = 254 nm) or by charring with a $KMnO_4$ dip. Peptides were shaken on an IKA (Staufen, Germany) KS 130-basic device. The m.p.'s were determined on a Khofler hot stage and are uncorrected. The $[\alpha]_D^{20}$ were recorded on a Perkin-Elmer (Waltham, MA, U.S.A.) 241 polarimeter at 589 nm (Na D-line) with a path length of 1 dm. Concentrations (*c.*) are quoted in g 100 mL^{-1}, and specific rotations are quoted in units of 10^{-1} deg cm^2 g^{-1} at 20 °C. Infrared spectra were recorded neat on Perkin-Elmer Paragon 1000 Fourier transform spectrometer. Only selected absorbances are reported. ^{1}H, ^{13}C, and ^{19}F nuclear magnetic resonance (NMR) spectra were obtained at 300 K on Bruker (Billerica, MA, U.S.A.) AV-400, DRX-400, or AV-500 instruments. Chemical shifts (δ) are given in parts per million (ppm) as referenced to the appropriate residual solvent peak (for Me_2SO-d_6 ^{1}H 2.50 ppm and ^{13}C 39.5 ppm, for MeOH-d_4 ^{1}H 3.31 ppm and ^{13}C 49.0 ppm) or to fluorobenzene (^{19}F −113.5 ppm). The ^{13}C chemical shifts are assigned as s, d, t, and q for C, CH, CH_2, and CH_3, respectively. Coupling constant (*J*) are given in Hertz (Hz). Mass spectra were obtained in positive ESI mode on a Micromass LCT Premier, Atlantis, 600 pump, Empower Pro software etc.: Waters (Elstree, U.K.) equipped with a Waters Atlantis C18 3 μ column 2.1 × 30 mm. Mobile phase (A) water (0.1% formic acid) and (B) acetonitrile. High resolution mass spectrometry values are valid up to ±5 ppm. A Mettler Toledo (Leicester, U.K.) pH meter was used to prepare the acetate buffer. The [^{18}F]fluoride was acquired from PETNet Solutions (Manchester, UK). Conventional heating was carried out with a heating block, and the temperature was measured in the heating block by using a digital thermometer. Preparative ultraviolet HPLC (UV-HPLC) was carried out using a Waters 600 pump and EMPOWER PRO software (Waters) equipped with a linear Water 2487 UV detector (λ = 254 and 270 nm) and a Phenomenex (Macclesfield, U.K.) Luna C18(2) 5 μ 75 × 30.00 mm HPLC column. Injection loop 5000 μL. Mobile phase (A) water [0.1% trifluoroacetic acid (TFA)] and (B) MeCN (0.1% TFA). Gradient 5–95% B over 10 min. Flow rate 20 mL min^{-1}. Semi-preparative UV-radio-HPLC was carried out using a Beckman Pump 127 (Beckman Coulter, High Wycombe, U.K.) and Laura 3 software (Lablogic) equipped with a linear Hewlett Packard (Wokingham, U.K.) Series 1100 G1314A UV detector (λ = 254 nm), a Bioscan (Washington, D.C., U.S.A.) Flowcount FC-3400 PIN diode detector (Lablogic) and a Phenomenex Luna C18(2) 5 μ 100 × 10.00 mm HPLC column. Injection loop 2000 μL. Mobile phase (A) water (0.1% TFA) and (B) MeCN (0.1% TFA). Gradient 10% B for 1 min, 10–40% over 9 min, 40–80% over 5 min, and 80% for 1 min. Flow rate 3.15 mL min^{-1}. Analytical UV-radio-HPLC was carried out as aforementioned but by using a Hewlett Packard Series 1100 G1312A pump and a Phenomenex Luna C18(2) 5 μ 150 × 4.6 mm HPLC column. Injection loop 20 μL. Flow rate 1 mL min^{-1}.

Chemistry

Preparation of 2-([(9H-fluoren-9-ylmethoxy)carbonyl]amino)-3-[1-(2-fluoroethyl)-1H-1,2,3-triazol-4-yl]propanoic acid (Fmoc-Fta-OH)

The CuI (5.78 g, 30.4 mmol, 2 equiv.) and *N*,*N*-diisopropylethylamine (DIPEA) (10.4 mL, 60.8 mmol, 4 equiv.) were added to a solution of Fmoc-propargyl-Gly-OH (5.08 g, 15.2 mmol, 1 equiv.) and 2-[^{18}F]FEA (91.2 mL at 0.5 M in DMF, 45.6 mmol, 3 equiv.) and stirred at room temperature (r.t.) under N_2 for 2 h. The reaction mixture was diluted with 0.5 N HCl aqueous solution (500 mL) and extracted with EtOAc (100 mL). The organic layer was washed with 0.5 N HCl aqueous solution (4 × 500 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (AcOH – MeOH – CH_2Cl_2 1:10:90, R_f 0.33) to afford 6.01 g (14.2 mmol, 93%) of amorphous dark yellow powder. The m.p. is 78–79 °C; $[\alpha]_D^{20}$ −5.0 (*c.* 0.08, MeOH); infrared (IR): 1713 (s), 1220 (s), 739 (vs) cm^{-1}; ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.79 (3H, d, *J* = 7.4 Hz, H-16, H-20, and H-25), 7.63 (2H, m, H-19, and H-23), 7.39 (2H, t, *J* = 7.4 Hz, H-17, and H-21), 7.31 (2H, t, *J* = 7.4 Hz, H-18, and H-22), 4.72 (4H, tt,

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J = 4.5, 27.1 Hz, H-29, and H-30), 4.54 (1H, dd, J = 4.8, 9.1 Hz, H-2), 4.30 (2H, d, J = 7.1 Hz, H-9), 4.20 (1H, t, J = 7.0 Hz, H-11), 3.23 (3H, m, H-4); ^{13}C NMR (101 MHz, MeOH-d_4): δ 173.1 (s, C-3), 157.0 (s, C-7), 143.9 (s, C-12, and C-15), 143.6 (s, C-24), 141.2 (s, C-13, and C-14), 127.4 (d, C-17, and C-21), 126.8 (d, C-18, and C-22), 124.9 (d, C-19, and C-23), 123.6 (d, C-25), 119.5 (d, C-16 and C-20), 81.4 [t (d, J = 170.6 Hz), C-30], 66.7 (t, C-9), 53.7 (d, C-2), 50.4 [t (d, J = 20.3 Hz), C-29], 46.9 (d, C-11), 27.4 (t, C-4); ^{19}F NMR (471 MHz, MeOH-d_4): δ -223.7 (tt, J = 47.1 and 27.2 Hz, -CH_2F); Time of Flight (TOF) Mass spectroscopy (MS) Electrospray (ES) + m/z [elative intensity(rel. int.)] 426 (25), 425 $[M+H]^+$ (100); High resolution mass spectrometry (HRMS) ES + $[M+H]^+$ m/z calcd for $C_{22}H_{21}FN_4O_4$ 425.1625, found 425.1616, Δ = –2.1 ppm.

Preparation of cyclopentapeptides

In a syringe with a plastic filter and a stopper at its bottom, H-Gly-2ClTrt resin (0.46 mmol g^{-1} and 100 μmol) was swollen in dimethylformamide (DMF) for 30 min in a shaker and the solvent removed by filtration. Hydroxybenzotriazole (HOBt) (40 mg), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (113 mg), Fmoc-protected amino acid (300 μmol), and DIPEA (52 μL) were mixed in 3.2 mL DMF; this coupling mixture was added to the syringe and the reaction mixture was shaken for 2 h at r.t. The resin was then washed with DMF (3 × 30 s); the Fmoc protecting group was removed by the treatment of the resin with 20% piperidine in DMF (4 mL) for 30 min and the resin was washed again with DMF (3 × 30 s). The sequence coupling–washing–deprotection–washing was repeated with the appropriate Fmoc-protected amino acids until the desired resin-bound pentapeptide was synthesized. The pentapeptide was cleaved from the resin by treatment with a 1:1:3 mixture of AcOH – TFE – CH_2Cl_2 (1 × 2 h and 3 × 2 min, 10 mL each), and the combined filtrates were concentrated under reduced pressure. To a solution of the resulting unprotected linear pentapeptide and $NaHCO_3$ (60 mg) in DMF (40 mL) at –40 °C was added diphenylphosphorylazide (100 μL) and the reaction mixture was allowed to stir at r.t. for 4 days. DMF was removed under reduced pressure; the residue was suspended in MeOH–$CHCl_3$ (9:1) and filtered through a small column of basic alumina. The filtrate was concentrated *in vacuo*; the residue was taken up in a minimum of CH_2Cl_2 and precipitated with Et_2O. The solid was filtered and washed with Et_2O, affording the protected cyclopentapeptide in good to high yields (73% to >99%) as white powders. It was dissolved in TFA – triisopropylsilane – H_2O (190:5:5) (10 mL) and the reaction mixture was stirred at r.t. for 6 h. It was concentrated *in vacuo* and the residue was suspended in Et_2O and filtered affording a white powder. It was purified by HPLC on a preparative column: Luna C_{18}(2) 5 μ 75 × 30.00 mm, solvent A: water + 0.1% TFA, solvent B: MeCN + 0.1% TFA, flow: 20 mL min^{-1}, gradient 5% B to 95% over 10 min. The collected product fraction was freeze-dried to afford the TFA salt of the desired product as a white powder. Alkyne-containing deprotected cyclopentapeptide **1** (5 mg) underwent Huisgen alkyne-azide cyclization with $CuSO_4.5H_2O$ (0.32 mg, 1.3 μmol, 0.2 equiv.), sodium ascorbate (5.6 mg, 32 μmol, 5 equiv.), 2-fluoroethylazide (250 μL, 1.84 M in DMF) and acetate buffer (250 μL, pH 5, 250 mM) at r.t. over a week. The reaction mixture was subjected to the same HPLC purification conditions as described in the previous texts and freeze-dried to afford CCIC15 (**2**) a white powder in quantitative yield.

cyclo(– Gly – D-Tyr – Prg – Arg – Nal –). TFA, (1)

TOF MS ES + m/z (rel. int.) 669 $[M+H]^+$ (100), 670 (40), 671 (10); HRMS ES + $[M+H]^+$ m/z calcd for $C_{35}H_{40}N_8O_6$ 669.3149, found 669.3127, Δ = –3.3 ppm.

cyclo(– Gly – D-Tyr – Fta – Arg – Nal –). TFA, (2)

The ^{19}F NMR (376 MHz, MeOH-d_4-$CDCl_3$ 1:1): δ −76.2 (s, TFA), −222.6 (tt, J = 45.8 and 25.6 Hz, -CH_2F); TOF MS ES + m/z (rel. int.) 820 $[M+Cu]^+$ (60), 758 $[M+H]^+$ (100); HRMS ES + $[M+H]^+$ m/z calcd for $C_{37}H_{44}FN_{11}O_6$ 758.3538, found 758.3530, Δ = –1.1 ppm.

Radiochemistry

Preparation of 2-[^{18}F]Fluoroethylazide[12]

2-[^{18}F]Fluoroethylazide[12] was synthesized from 740 MBq (20 mCi) of [^{18}F]fluoride as reported in the literature in 47 ± 7% decay-corrected radiochemical yield (n = 6) affording a solution of the product in 300 μL in 40 min with >99% radiochemical purity.

Preparation of cyclo(– Gly – D-Tyr – [^{18}F]Fta – Arg – Nal –), ([^{18}F] CCIC15)

To a 1 mL Wheaton vial sealed with a PTFA/Silicon septum under N_2 were successively added $CuSO_4$ (12.5 μL, 238 mM in water), sodium ascorbate [12.5 μL, 262 mM in acetate buffer (pH 5, 250 mM)], bathophenanthrolinedisulfonic acid disodium salt (12.5 μL, 298 mM in water), 2-[^{18}F]fluoroethylazide (50 μL, in MeCN, 178 MBq) and *cyclo*(– Gly – D-Tyr – Prg – Arg – Nal –) (**1**) (50 μL, 15.0 mM in DMF) and the mixture was allowed to react at r.t. for 10 min. A solution of BnN_3 in MeOH (10 μL, 1.50 M) was added to the Wheaton vial and the reaction carried out 10 min under the same conditions. The reaction mixture was then diluted to 1.9 mL with water and purified by reverse phase semi-preparative HPLC, column: Luna C_{18}(2) 5 μ 100 × 10.0 mm, solvent A: water + 0.1% TFA, solvent B: MeCN + 0.1% TFA, flow: 3.15 mL.min^{-1}, gradient: 10% B for 1 min, 10–40% over 9 min, 40–80% over 5 min, and 80% for 1 min. The decay-corrected yield was 63 ± 16% (n = 5) in approximately 30 min, starting from 178 MBq [^{18}F]FEA. The non-decay-corrected EOS-RCY were of 18 ± 6% (n = 5) in approximately 1.5 h from aqueous [^{18}F]fluoride. The purified fraction (t_R = 11:00–12:20) was diluted with water (3 mL) and >99% of the radioactivity was immobilized on Waters (Elstree, U.K.) SepPak tC18 Light cartridge (preconditioned with 5 mL MeOH and 10 mL water). The cartridge was washed with water (5 mL) and dried with air (10 mL), and the title compound was eluted with 25 mM HCl in EtOH in 8 × 100 μL fractions. Over 90% of the radioactivity was collected in two fractions. The labeled peptide was stable in 10% EtOH in phosphate buffered saline (PBS) for at least 4 h at r.t. Analytical HPLC showed a radiochemical purity of >98%.

Labeling optimization

Optimization of the radiolabeling was carried out following the aforementioned detailed procedure except with one variable. In particular, we followed the evolution through time of the reaction advancement for various reaction temperatures and various peptide concentrations. The advancement was calculated as the ratio between the integration of the product's radio-peak to the total integration of the chromatogram (*i.e.*, the integration of the product's radio-peak + the integration of [^{18}F]FEA).

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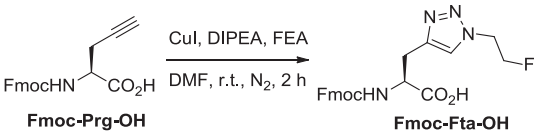
Biological evaluation

Binding assay

U87.CD4. CXCR4 cells were aspirated with EDTA and incubated at 37 °C in complete media for 2 h to recover, then resuspended in binding buffer (PBS containing 2 mg mL^{-1} bovine serum albumin) at a concentration of 1.5×10^6 cells mL^{-1}. To each tube, 100 µl of this cell mixture was added [1.5×10^5 cells], along with 10 µl of antagonist prepared in dimethyl sulfoxide to the final concentration indicated, and [^{125}I]SDF-1 radioligand (PerkinElmer) is added to give a final concentration of 0.1 nM in a 200 µl total assay volume. The tubes were then incubated on ice for 60 min, with frequent agitation. Bound and unbound radioactivities were separated by filtration using a vacuum manifold, followed by three washes in assay buffer. The filters were then punched out and counted in a gamma counter for 240 s. The bound radioactivity was expressed as a percentage of the maximum bound radioactivity using GRAPHPAD (La Jolla, CA, U.S.A.) Prism 5.

Cell-uptake assay

U87.CD4 and U87.CD4.CXCR4 cells were seeded at 48 h prior to the experiment in complete Dulbecco's Modified Eagle Medium in triplicate wells of 6-well plates at a density of 3×10^5 cells/well. At the commencement of the experiment, the wells were incubated with 0.74 MBq (20 µCi) of [^{18}F]CCIC15 for 60 min at 37 °C. After incubation, the unbound radioactivity was washed from the cells twice with ice-cold PBS, scraped in PBS, and collected by centrifugation at 5000 rpm for 3 min at 4 °C and then counted by gamma counter. The decay-corrected counts per minute was normalized to the protein content of the wells using GraphPad Prism 5.

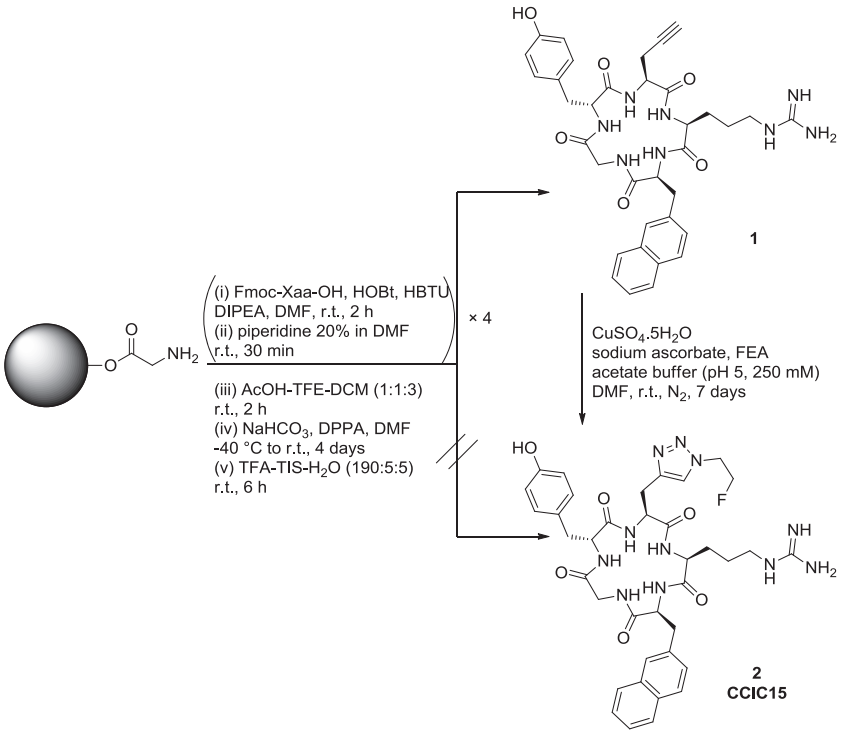


Scheme 1. Synthesis of Fmoc-Fta-OH.

Results and discussion

To test our hypothesis, we decided to synthesize an FC131 analogue by substituting its Arg[3] residues for either 1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-ylalanine (Fta) or propargylglycine (Prg) in order to afford the reference compound and the precursor for radiolabeling.

We built both the precursor and the cold isotopologue by solid phase peptide synthesis deploying a 2-chlorotrityl/Fmoc strategy. Fmoc-Fta-OH was synthesized from commercially available Fmoc-protected propargyl glycine (Fmoc-Prg-OH) via copper(I)-mediated alkyne-azide cyclisation with 2-FEA in good yields (Scheme 1). Solid-phase peptide synthesis was carried out with both of these non-proteogenic amino acids to introduce the desired acetylenic and fluoroethyltriazolyl side-chains (Scheme 2). A mixture of HOBt/HBTU/DIPEA was used to perform the amide bond formation and treatment of the resin with a mixture of AcOH – trifluoroethanol – CH_2Cl_2 (1:1:3) was used to afford the protected linear pentapeptides. After successful cyclisation with diphenylphosphorylazide, the resulting protected cyclic pentapeptides underwent side-chain deprotection in TFA – triisopropylsilane – H_2O (190:5:5). This last step was followed by RP-HPLC purification to afford the desired alkyne-containing analogue **1** in 29% overall yield. Although the deprotection conditions used are common for peptide synthesis, they were too harsh for the triazole-containing analogue, resulting in isolation of insufficient quantities of the



Scheme 2. Synthesis of an FC131 analogue, precursor, and reference compound.

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desired triazole-containing peptide. Instead, reaction of its alkyne counterpart **1** by Huisgen alkyne-azide cycloaddition with FEA followed by RP-HPLC purification efficiently afforded the desired product in 89% isolated yield.

Binding activity of the TFA salt of compound **2** was assessed by competitive displacement of [^{125}I]SDF-1 on U87.CD4.CXCR4 cells. The concentration of compound **2** that reduced the amount of radioactivity bound to the cells by 50% compared with controls (IC_{50} value) was determined after 60 min of incubation over ice. CCIC15 (**2**) showed an IC_{50} of 1.23 μM which is, as expected, relatively close to what we found for FC131 (0.55 μM). It was therefore decided to evaluate the biological properties of compound CCIC15 (**2**).

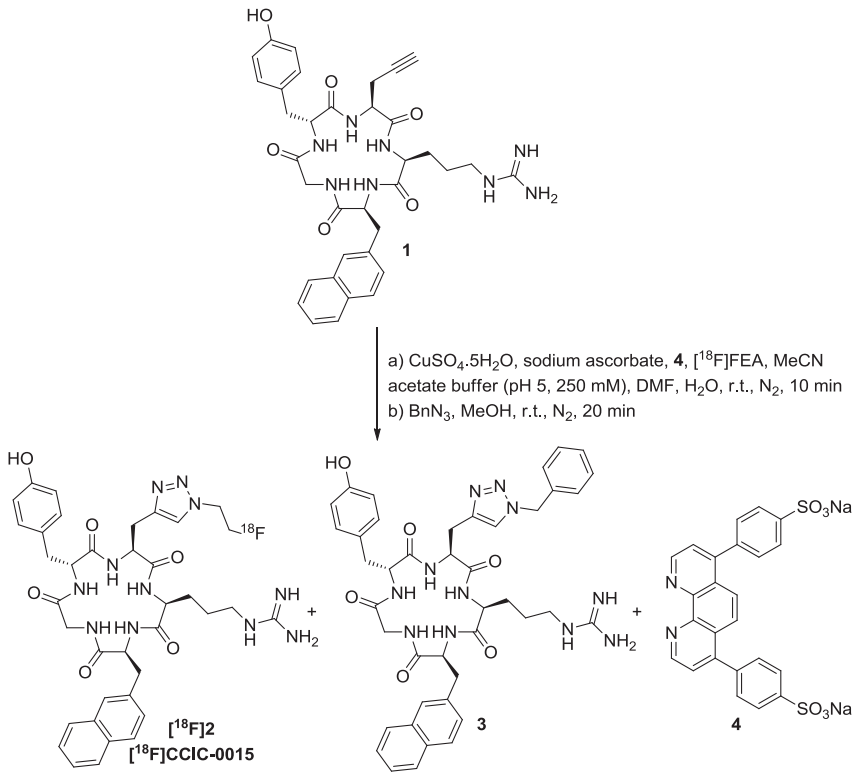
The [^{18}F]FEA was synthesized according to literature procedure[13] affording a solution of the product in approximately 300 μL of MeCN in 47 ± 7% decay-corrected radiochemical yield ($n = 6$) in about 40 min.

The radiochemical yields of the subsequent Huisgen alkyne-azide cycloadditions were strongly dependent on reaction conditions: temperature, duration, concentration, pH, and the order of addition of reagents. Previous work in our group[12] also showed that both the addition of a copper(I)-stabilizing bidentate ligand such as bathophenanthrolinedisulfonic acid disodium (**4**) and the use of acetate buffer substantially increased the reaction rate of this type of cycloaddition and that the optimal order of addition of reagents is $CuSO_4$, sodium ascorbate, the ligand, [^{18}F]FEA and the precursor, sequentially. Taking these data into account, focussed screening was performed to determine the influence of peptide concentration, temperature, and the duration of the reaction (Scheme 3, Table 1).

As expected, the cycloaddition reaction rate was highly dependent on the peptide precursor concentration. However, a low precursor concentration is desirable as it impacts the apparent specific activity. Although the optimal peptide concentration was found to be 5.4 mM, reaching full conversion, the reaction rate associated to a peptide concentration of 2.2 mM was satisfactory. A peptide concentration of 1.1 mM caused reaction progress to plateau at about 20% conversion. The identity of the radiolabeled product was confirmed by co-elution with the cold reference compound. On the other hand, temperature had a lesser effect on the reaction rate. Four temperatures were tested (0 °C, 20 °C, 50 °C, and 80 °C) at 5.4 mM. With the exception of the coldest experiment, the reaction rates were relatively similar; however, higher reaction temperatures tended to deform the HPLC-radio peak. This behaviour, which likely stems from epimerization of the peptide's stereogenic centers, encouraged by heat and the presence of the Lewis acidic copper, was not observed at 20 °C. These observations made 20 °C the temperature of choice for further experiments. The

Table 1. Radiolabeling of FC131 analogue by Huisgen alkyne-azide cycloaddition

Precursor concentration (mM)				
Reaction temperature (°C)	1.1	2.2	5.4	10.9
0	25		35	
20	25	91	96	95
40	35			
50			100	
60	41			
80	48		100	



Scheme 3. Radiolabeling and scavenging of FC131 analogue by Huisgen alkyne-azide cycloaddition.

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optimal labeling conditions were found to be a 2.2 mM peptide concentration at room temperature for ~5 min.

Unsurprisingly, the unreacted precursor and the radiotracer co-eluted on the HPLC, although it was not possible to reduce the precursor concentration of peptide **1** any further to increase the apparent specific activity. To overcome this challenge, we developed a strategy wherein the unreacted peptide alkyne **1** was scavenged by a lipophilic azide. The retention time of the corresponding triazole was anticipated to differ significantly from the radioactive product, allowing an easy HPLC purification. Additionally, we envisaged that such a scavenging reaction could be performed under the same conditions as the labeling, generating a practical one-pot labeling-scavenging procedure.

Benzyl azide[14] was chosen as a potential scavenging agent. A test reaction demonstrated that this azide was fit for purpose, leading to a satisfactory 2-min difference in retention times between the radioactive product [^{18}F]CCIC15 and the benzyl triazole derivative **3**; thus, unreacted benzyl azide was easily removed by preparatory HPLC. More detailed monitoring of the scavenging reaction showed that the apparent peptide concentration, obtained from the integration of the UV-HPLC traces and a calibration curve, had decreased from 3.48 mM to 103 μM in 10 min and to 71.2 μM in 20 min ($n = 2$), that is, a factor of 34 in 10 min and of 49 in 20 min ($n = 2$).

The click labeling – scavenging step afforded the tracer in 63 ± 16% ($n = 5$) (decay corrected) yields in approximately 30 min starting from 178 MBq of [^{18}F]FEA but overall, the desired radiolabeled peptide was obtained in EOS-RCY of 18 ± 6% ($n = 5$) from aqueous [^{18}F]fluoride in approximately 1.5 h, with an apparent specific activity of 19.4 GBq.μmol^{-1}. The tracer was stable in 10% EtOH/PBS for 4 h as assessed by analytical HPLC.

The distribution coefficient of the tracer [^{18}F]CCIC15 was then evaluated. At the physiological pH of blood serum, the product is expected to be exclusively present in its monoprotonated form as the pK_a of guanidine is ~12. The distribution ratio at physiological pH was assessed by measuring the distribution of radioactivity between *n*-octanol and PBS (pH 7.4) using the 'shake flask' method.[15] The logarithm of the ratio was calculated as log$D_{ph\ 7.4} = -0.11 \pm 0.02$ ($n = 5$). This balanced lipophilicity was assumed to be appropriate for a short-lived radiotracer as it should account for a optimal renal elimination,[16] faster pharmacokinetics, and increased contrast.[17]

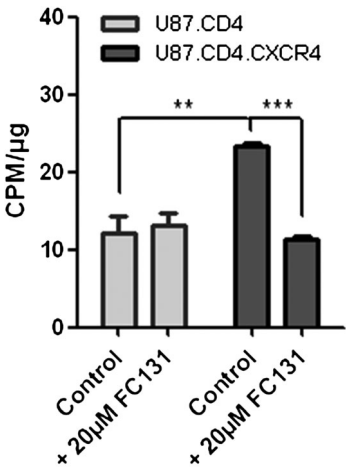

Figure 1. Cell uptake profile at 1 h of [^{18}F]CCIC15 in U87.CD4.CXCR4 cells compared with U87.CD4 cells.

Cellular uptake of [^{18}F]CCIC15 was assessed in U87.CD4 cells transfected with CXCR4 (U87.CD4.CXCR4) (Figure 1). Sixty-minute uptake of [^{18}F]CCIC15 showed a two-fold higher uptake in the CXCR4 over-expressing cells compared with isogenic CXCR4 negative cells, confirming binding of the tracer to CXCR4. Furthermore, addition of 20 μM of FC131 showed a decrease of the cellular uptake to the level of the CXCR4 negative cells control, demonstrating specificity of the tracer for CXCR4. These biological data, along with the IC_{50} value measured for our tracer, validated our structure-activity relationship analysis.

Conclusions

We devised an easy, rapid, one-pot method to circumvent the common issue met when labeling large molecules, namely the poor apparent specific activity afforded. This was achieved by treating the unreacted precursor with an excess of scavenging reagent at the end of the labeling process. We successfully applied it to a fluoroethyl triazole analogue of FC131 of our own design, of which the ^{18}F-isotopologue, [^{18}F]CCIC15, was radiosynthesized by Huisgen alkyne-azide cycloaddition with [^{18}F]FEA in a copper(II) – ascorbic acid system. Although, as anticipated, the radioactive product and its unlabeled precursor co-eluted on the HPLC, resulting in a low apparent specific activity, scavenging of the unreacted precursor with benzyl azide allowed easy purification, reducing the peptide concentration from 3.48 mM to 103 μM, that is, by a factor of 34, in 10 min. Overall, the labeling – scavenging step was achieved in 63 ± 16% ($n = 5$) yield (decay corrected) in approximately 30 min, whereas our new tracer was obtained in an overall 18 ± 6% ($n = 5$) EOS-RCY in 1.5 h from aqueous [^{18}F]fluoride with a specific activity of 19.4 GBq μmol^{-1}. *In vitro* biological assay showed specificity for CXCR4 and further biological evaluation of CCIC15 is underway.

Acknowledgements

This work was funded by Cancer Research UK–Engineering and Physical Sciences Research Council grant [in association with the Medical Research Council and Department of Health (England)] grant C2536/A10337. Professor Eric O. Aboagye (E.O.A.'s) laboratory receives core funding from the UK Medical Research Council (MC_A652_5PY80).

Conflict of Interest

The authors did not report any conflict of interest.

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