Synthesis of a non-peptidic PET tracer designed for $\alpha_5\beta_1$ integrin receptor

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Arginine–glycine–aspartic acid (RGD)-containing peptides have been traditionally used as PET probes to noninvasively image angiogenesis, but recently, small selective molecules for $\alpha_5\beta_1$ integrin receptor have been developed with promising results. Sixty-one antagonists were screened, and tert-butyl (5)-3-(2-((3R,5S)-1-3-(1-(2-fluoroethyl)-1H-1,2,3-triazol-4-yl)propanoyl)-5-((pyridin-2-ylamino)methyl)pyrrolidin-3-yloxy)acetamido)-2-(2,4,6-trimethylbenzamido)propanoate (FPMt) was selected for the development of a PET tracer to image the expression of $\alpha_5\beta_1$ integrin receptors. An alkynyl precursor (PMT) was initially synthesized in six steps, and its radiolabeling was performed according to the azide–alkyne copper(II)-catalyzed Huisgen’s cycloaddition by using 1-azido-2-[18F]fluoroethane ([18F]12). Different reaction conditions between PMt and [18F]12 were investigated, but all of them afforded [18F]FPMt in 15 min with similar radiochemical yields (80–83%, decay corrected). Overall, the final radiopharmaceutical ([18F]FPMt) was obtained after a synthesis time of 60–70 min in 42–44% decay-corrected radiochemical yield.

Keywords: $\alpha_5\beta_1$ integrin receptor; PET, 1-azide-2-[18F]fluoroethane; click chemistry; peptido-mimetic

Introduction

Integrins are an extensive group of transmembrane cell adhesion receptors, composed by noncovalently linked $\alpha$ and $\beta$ subunits.1 They mediate cell migration and cell proliferation by the connection of the extracellular matrix with the intracellular cytoskeleton.2 Activation of the integrins by ligand binding promotes cell proliferation, migration, and survival, but unligated or antagonized integrins may activate ‘integrin-mediated death’.3 Integrins are of crucial support for the formation of capillaries and angiogenesis in physiological and pathological processes,4 and three of them ($\alpha_5\beta_3$, $\alpha_6\beta_1$, and $\alpha_3\beta_1$) have a prominent role for the development of a new vascular system.5,6

The involvement of these integrins in many diseases such as tumor, thrombosis, cardiovascular, and inflammatory diseases makes them an appealing target for the development of antiangiogenic therapies.7,8 Of them, $\alpha_5\beta_1$ is the most targeted integrin, and several antagonists such as monoclonal antibodies9,10 and arginine–glycine–aspartic acid (RGD)-based peptides11 are currently in clinical trials.7,12,13 Moreover, radiolabeled analogs of the RGD peptides have been evaluated in preclinical and clinical studies, as tracers, to visualize angiogenesis in growing tumors.14,15

“In the last years, integrin $\alpha_5\beta_1$ has gained great interest, not only because of its involvement in tumor angiogenesis but also for its fundamental role in brain angiogenesis.16 Recently, several small molecules have been developed to selectively antagonize this integrin receptor.17-19 The promising biological results obtained have enhanced the interest in non-$\alpha_5$ integrins as target for new therapies and for imaging to monitor tumor angiogenesis and neurovascular remodeling after ischemic stroke.16,20

In this manuscript, we advance a novel nonpeptidic PET tracer designed to image the $\alpha_5\beta_1$ integrin receptor. We report herein a reliable and reproducible procedure to radiolabel this pyrrolidine derivative that relies on the following: (i) the preparation and purification of a widely used radiolabeled synthon 1-azido-2-[18F] fluoroethane and (ii) its subsequent conjugation to our alkynyl precursor (PMT) via the Huisgen’s 1,3-dipolar cycloaddition.

Experimental

Reagents and instrumentation

All the solvents and reagents were purchased from Sigma-Aldrich and Fluka (Basel, Switzerland) or VWR (Nyow, Switzerland) and were used without further purification. Flash chromatography columns were performed on VWR silica gel (0.063–0.200 mm). Thin layer chromatography (TLC) was performed on precoated silica gel 60F254 plastic sheets from VWR. The compounds were monitored under ultraviolet (UV) light at 254 nm or by brief immersion in potassium permanganate solution. Radioactive spots were detected by autoradiography.

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were detected with a Cyclone Phosphorimager from PerkinElmer (Waltham, Massachusetts). The radiochemical yields were determined by TLC analysis of reaction mixture samples taken at specified times. High-performance liquid chromatography (HPLC) was performed on an UlTimate 3000 Rapid Separation LC system (Dionex, Basel, Switzerland) with Chromelon 6.8 software package. The UV detector was set at four different wavelengths (215 nm, 220 nm, 254 nm, and 280 nm). Radioactivity was detected with a Nal scintillation detector (Nuclear Interface, Munich, Germany) connected on the outflow of the UV detector. The HPLC column used for the purification of the product and the characterization of radioactive compounds against standards was a Phenomenex Gemini 5 μ (C18) 21.0 × 110 mm (250 × 4.60 mm) column operated at a flow rate of 1 mL/min using a gradient of 5% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) to CH3CN/TFA (99.9:0.1) over 20 min. The radiochemical purities were determined by HPLC analyses. NMR data were recorded with a Varian 300 MHz NMR Spectrometer and Oxford 300 Nuclear Magnetic Instrument (Oxfordshire, UK). The samples were dissolved either in CDCl3 or in acetone-d6 (Cambridge Isotope Laboratories Inc., Burgdorf, Switzerland), and the data were processed by MestRe-C 4.8 software. Chemical shifts (δ) are expressed in ppm relative to the signals of the solvents and coupling constants (J) in Hz. Mass spectrometry (MS) analyses were performed using an ESI-MS instrument, API 150EX from AB/MDMS (Sciex, Framingham, Massachusetts), or ES TQ-detector Aqui (Waters, Baden-Dättwil, Switzerland). High-resolution mass spectrometry (HRMS) spectra were recorded by ESI/nanoESI-IT Esquire 3000 plus (Bruker, Fällanden, Switzerland).

**Chemistry**

**Methyl (2S,4R)-4-hydroxy-1-(pent-4-ynoyl)pyrrolidine-2-carboxylic acid (2)**

Methyl-(2S,4R)-4-hydroxy-2-carboxylic acid (1) (1.00 g, 5.48 mmol), 4-pentyonic acid (0.54 g, 5.48 mmol), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) (1.26 g, 6.75 mmol), and 4-dimethylaminopyridine (DMAP) (1.00 g, 8.21 mmol) were dissolved in 20 mL of dichloromethane (DCM). Et3N (0.20 mL) was added dropwise, and the reaction mixture was stirred at room temperature overnight. Then, the mixture was extracted with EtOAc (3 × 30 mL). The organic layer was dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel hexane/EtOAc (1:1) to obtain colorless oil. Yield: 90%. 1H-NMR (300 MHz, CDCl3) δ: 4.42 (1H, d, J = 10.2 Hz), 4.39 (2H, t, J = 7.7 Hz), 4.21 (1H, bs), 3.66–3.59 (4H, m), 2.48–2.35 (4H, m), 2.16 (1H, t, J = 10.65 Hz), and 1.95–1.86 (2H, m). 13C-NMR (300 MHz, CDCl3) δ: 172.75, 170.35, 83.44, 70.53, 69.03, 57.77, 55.16, 52.59, 38.02, 33.69, and 14.17. MS (ESI): m/z 226.4 [M + H]+.

**Methyl (2S,4R)-4-(2-tert-butoxy)-2-oxoethoxy)-1-(pent-4-ynoyl)pyrrolidine-2-carboxylic acid (3)**

NaH 60% in paraffin oil (0.31 g, 7.75 mmol), tert-butylbromoacetate (2.00 mL, 13.37 mmol), and n-Bu3PO (1.49 mL, 3.90 mmol) were suspended under argon in 28 mL of dry THF. Then, 1 (1.10 g, 4.88 mmol) was dissolved in 2 mL of dry THF, slowly added to the reaction mixture, and stirred overnight. The solvent was removed under reduced pressure. The crude product was purified by chromatography on silica gel using ETOAc/hexane (2:3) to obtain light brown oil. Yield: 82%. 1H-NMR (300 MHz, CDCl3) δ: 5.53 (1H, t, J = 7.65 Hz), 4.28 (1H, q, J = 4.3 Hz), 3.96 (2H, d, d, J = 2.2 Hz, 1.7 Hz). 3.72 (2H, s), 3.65 (1H, dd, J2 = 4, J1 = 3 Hz), 2.57–2.50 (4H, m), 2.41–2.22 (1H, m), 2.16–2.03 (1H, m), 1.96 (1H, t, J = 2.4 Hz), 1.68 (1H, bs), and 1.47 (9H, s). MS (ESI): m/z 288.3 [M + H]+.

**tert-Butyl 2-((3R,5S)-5-(hydroxyethyl)-1-(pent-4-ynoyl)pyrrolidin-3-yl)oxoacetate (4)**

To a cooled solution of 3 (1.15 g, 3.39 mmol) in anhydrous THF/ EtOH (65:35, 26 mL) at 0°C, LiCl (0.80 g, 18.87 mmol) and NaHBU3 (0.30 g, 7.93 mmol) were added. After 1 h, the ice bath was removed, and the reaction mixture was stirred. Then, the reaction mixture was concentrated, and 20 mL of water was added. The aqueous layer was extracted with ethyl acetate (3 × 30 mL). The organic layer was dried over Na2SO4, filtered, and concentrated under vacuum. The crude product was purified by chromatography on silica gel using ETOAc/hexane (1:1) to obtain light yellow oil. Yield: 88%. 1H-NMR (300 MHz, CDCl3) δ: 4.96 (1H, d, J = 8.1 Hz), 4.30 (1H, q, J = 8.5 Hz), 4.20 (1H, t, J = 1.8 Hz), 3.92 (5H, d, J = 5.4 Hz), 3.76–3.5 (4H, m), 2.62–2.47 (4H, m), 2.26–2.18 (1H, m), 1.73–1.64 (2H, m), and 1.46 (9H, s). 13C-NMR (300 MHz, CDCl3) δ: 172.07, 169.25, 82.08, 77.42, 72.71, 69.00, 68.70, 66.68, 60.13, 53.61, 34.12, 33.86, 28.07 (3C), and 14.86. MS (ESI): m/z 312.1 [M + H]+.

**tert-Butyl 2-((3R,5S)-5-(tert-butidimethylsilyloxy)methyl)-1-(pent-4-ynoyl)pyrrolidin-3-yl)oxoacetate (5)**

(0.80 g, 2.57 mmol), imidazole (0.44 g, 6.42 mmol), and tert-butidimethylsilyl chloroform (0.43 g, 2.83 mmol) were dissolved in 15 mL of dimethylformamide (DMF) and stirred for 3 h at room temperature. The solvent was removed by evaporation, and 20 mL of ETOAc was added and extracted with water, saturated NaHCO3, and dried over Na2SO4, then filtered. The solvent was removed by evaporation, and the product was purified by chromatography on silica gel using ETOAc to obtain colorless oil. Yield: 82%. 1H-NMR (300 MHz, CDCl3) δ: 4.37–4.24 (2H, m), 3.37–3.63 (1H, dd, J2 = 4.2, J1 = 6 Hz), 3.58–3.51 (2H, m), 2.59–2.41 (4H, m), 2.26–2.17 (1H, m), 2.07–1.95 (2H, m), 1.47 (9H, s), 0.85 (9H, s), and 0.04–0.01 (6H, m). 13C-NMR (300 MHz, CDCl3) δ: 169.40, 156.50, 81.88, 78.43, 73.85, 68.60, 67.08 (2C), 63.11, 57.73, 52.91, 33.91, 33.27, 28.21 (3C), 25.85 (3C), 14.06, and −5.50 (2C). MS (ESI): m/z 426.5 [M + H]+.
additional hours. Saturated NaHCO₃ solution (2.70 mL) was added, and the mixture was stirred for 1 h. It was then extracted with DCM, dried with Na₂SO₄, and the solvent removed by evaporation. Light brown oil was obtained in 67% yield. H-NMR (300 MHz, CDCl₃) δ: 8.09–8.02 (2H, m), 7.40 (1H, dd, J₁ = 3.9 Hz, J₂ = 8.1 Hz), 7.35–7.30 (2H, t, J = 7.8 Hz), 6.80 (1H, s), 6.63–6.53 (2H, m), 6.64 (2H, td, J₁ = 8.4, J₂ = 16.5 Hz), 6.01 (1H, bs), 5.09–4.99 (1H, m), 4.90–4.78 (1H, m), 4.71–4.52 (2H, m), 4.28 (1H, t, J = 4.5 Hz), 4.05–3.58 (5H, m), 2.49–1.94 (13H, m), 1.47 (2H, s), 1.28–1.19 (6H, m), and 0.87 (1H, bs). ¹³C-NMR (300 MHz, CDCl₃) δ: 158.28, 157.79, 157.44, 147.79, 137.64, 133.96 (2C), 128.15 (2C), 113.79, 108.53, 83.27, 83.09, 78.12, 77.34, 68.76, 68.17, 62.95, 60.57, 52.79, 42.09, 33.64, 30.79, 27.28 (3C), 20.95, 18.95 (2C), and 13.96. HRMS (ESI) for C₃₆H₄₇FN₈O₆: calculated m/z 618.3286 and found m/z 618.3282.

tert-Butyl (S)-3-[(2-((3R,5S)-1-((2-fluoroethyl)-1H-1,2,3-triazol-4-yl)propanoyl)-5-((pyridin-2-ylamino)methyl)pyrrolidin-3-yl)oxy]acetamido)-2-(2,4,6-trimethylbenzamido)propanoate (FPMt, 8)

10 (0.16 mmol) was dissolved in 1 mL of water/THF, and 12 (62% m/m in DMF, 20.70 mmol) was added. Sodium acetate (0.5 mmol, 2.1 M) and CuSO₄ (0.5 mmol, 0.57 M) were slowly added, and the reaction was stirred at room temperature for 2 h. The crude was filtered, and the product was purified by C-18 chromatography to obtain a light brown solid. Yield: 45%. H-NMR (300 MHz, CDCl₃) δ: 9.33 (1H, d, J = 3.9 Hz), 7.75–7.63 (3H, m), 6.82 (2H, s), 4.92 (1H, t, J = 4.7 Hz), 4.76–4.68 (4H, m), 4.64–4.21 (2H, m), 4.01–3.59 (7H, m), 2.96–2.70 (7H, m), 2.24–2.18 (4H, d, J = 5.7 Hz), 1.49 (4H, s), and 0.88 (2H, t, J = 5.6 Hz). ¹³C-NMR (300 MHz, CDCl₃) δ: 171.35, 169.75, 169.41, 168.76, 161.61, 155.39, 149.30, 146.83, 138.85, 134.06 (2C), 133.42, 128.30 (2C), 126.55, 116.39, 102.89, 83.52, 81.51 (d, J = 171.3 Hz), 80.39, 78.82, 68.24, 62.86, 52.94, 52.64, 50.26, 42.55, 33.06, 31.23, 29.68, 27.95 (3C), 21.07, and 19.04 (2C). MS (APPI⁺) for C₃₆H₄₇FN₈O₆: m/z 707.00 [M + H⁺].

2-Fluoroethyl 4-methylenesulfonyl methane (11)

Fluoroethanol (10) (1.00 mL, 30.25 mmol) was stirred overnight with p-toluenesulfonyl chloride (4.80 g, 51.20 mmol) and 7 mL of Et₃N in DMF solution. The crude obtained was purified by distillation giving a DMF solution of [18O]water (Marshall Isotopes, Tel-Aviv, Israel) by a nuclear reaction ¹⁸O(p,n)¹⁸F by irradiation (10 min at 10 μA) of 2 mL of highly enriched (>97%) ¹⁸O-water (Marshall Isotopes, Tel-Aviv, Israel) by a proton beam using a Cyclone-18/9 cyclotron (IBA, Louvain-la-Neuve, Belgium). The initial radioactivity of [¹⁸F]fluoride was estimated to be around 5 GBq.

1-Azido-2-[¹⁸F]fluoroethane (12)

11 (1.52 g, 7.09 mmol) was dissolved in 3.5 mL of DMF, and Na₂N₃ (1.34 g, 20.60 mmol) was added. The mixture was stirred overnight at 80°C. The product was purified by azotropic distillation giving a DMF solution of 12 (62% m/m). H-NMR (300 MHz, CDCl₃, in DMF solution) δ: 4.56 (2H, dt, J₁ = 4.2, J₂ = 38.1 Hz) and 3.49 (2H, d, J = 27.9 Hz). ¹³C-NMR (300 MHz, CDCl₃, extrapolarized from a DMF solution) δ: 99.90 and 82.14 (d, J = 170 Hz). HRMS (ESI): m/z 204.0642 [M + H⁺].

¹⁸F-Fluoride production

No-carrier-added fluorine-18 was produced according to the nuclear reaction ¹⁸O(p,n)¹⁸F by irradiation (10 min at 10 μA) of 2 mL of highly enriched (>97%) ¹⁸O-water (Marshall Isotopes, Tel-Aviv, Israel) by a proton beam using a Cyclone-18/9 cyclotron (IBA, Louvain-la-Neuve, Belgium). The initial radioactivity of [¹⁸F]fluoride was estimated to be around 5 GBq.

Results and discussion

Chemistry

Based on docking studies, a pyrrolidine pharmacophore has been reported to yield selective ligands for integrin αβ3. Indeed, the 2-amino-5-pyridine and 2,4,6-trimethyl benzonic acid moieties
divided by an appropriate linker determine the selectivity for α5β1 integrin receptor. The 2-aminopyridine interacts with the (α5)-Phe187 on the (α5)-β-propeller domain, while the mesitylene group enables π–π interaction with (β1)-Tyr127 placed in a pocket on the β1 subunit, which is not available on the αvβ3 surface.

tert-Butyl (S)-3-(2-((3R,5S)-1-(3-(1-(2-fluoroethyl)-1H-1,2,3-triazol-4-yl)propanoyl)-5-((pyridin-2-ylamino)methyl)pyrrolidin-3-yl)oxy)acetamido)-2-(2,4,6-trimethylbenzamido)propanoate (FPMt, 8) has been identified, among 61 α5β1 antagonists, as the most appropriate molecule for the development of 18F-labeled α5β1 radioligand. FPMt has a structure closely related to the selective α5β1 antagonist JSM6427. A fluorinated substituent is incorporated on the pyrrolidine nitrogen in order to not interfere with the binding of this peptidomimetic molecule with α5β1 integrin receptor. Synthesis of 7 (Scheme 1) was adapted from the synthesis of JSM6427, as reported by Stragies et al.

An alkynyl function was introduced into compound 2 by coupling of 4-pentynoic acid to the 4-hydroxyproline methyl ester (1) to allow subsequent attachment of the radiolabeled azido synthon to the alkynyl precursor 7 by the copper(II)-catalyzed Huisgen’s cycloaddition. Conversion of the secondary alcohol into aldehyde and Williamson ether synthesis with tert-butyl bromoacetate provided intermediate 3 in 60% yield. Deprotection and concomitant reduction of the methyl ester in position 2 with sodium borohydride in the presence of lithium chloride yielded intermediate 4. Protection of the primary alcohol of 4 as tert-butylidemethylsilyl ether afforded compound 5 in 82% yield. The tert-butyl group was then selectively removed with lithium hydroxide in methanol, and the resulting carboxylic acid underwent an amidation by treatment with tert-butyl (S)-3-aminoo-2-(2,4,6-trimethylbenzamido)propanoate (9) in presence of 4-dimethylaminopyridine and the coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Deprotection in position 2 occurred simultaneously to afford the primary alcohol 6 in 40% yield. Compound 9 was synthesized in two steps starting from commercially available asparagine tert-butyl ester as previously described. Treatment of 6 with the SO3-pyridine complex to oxidize the primary alcohol into aldehyde, followed

![Scheme 1. Synthesis of FPMt (8).](http://doc.rero.ch)
by the reductive amination of the aldehyde with 2-aminopyridine in the presence of Ti(OiPr)₄ and NaBH₄(OAc)₂, gave the alkyne precursor 7 in 67% yield. Our optimized synthetic approach allowed us to obtain the alkyne pyrrolidine derivative 7 in seven steps, whereas the strategy described by Stragies provided similar analogs in nine steps. Moreover, 7 was obtained with an overall satisfactory yield of 45%.

1-azido-2-fluoroethane (12) was synthesized in two steps. Activation of the hydroxyl group of 2-fluoroethanol (10) by a tosylation, followed by a nucleophilic substitution with NaN₃, afforded 12, which was recovered by distillation and obtained as DMF solution (62% m/m of 12) in 42% yield.²²,²³ Finally, the Cu(II)-catalyzed Huisgen 1,3-dipolar cycloaddition between 7 and the azido synthon 12 gave the final peptidomimetic molecule (FPMt) in only 2 h with 45% yield.²⁵ The time of reaction and the yield obtained suggested that this strategy could be applied for the development of an ¹⁸F-labeled analog.

Radiochemistry

Nucleophilic ¹⁸F-fluorination of 2-azidoethyl-4-methylbenzenesulphonate (13) was carried out in anhydrous CH₃CN with a standard K[¹⁸F]F⁻ / Kryptofix complex. The reaction was conducted in 15 min at 80°C, and 1-azido-2[¹⁸F]fluoroethane ([¹⁸F]12) was isolated by distillation at 130°C into a vial containing anhydrous CH₃CN (Scheme 2).²²,²⁴ [¹⁸F]12 was obtained in 30 min from the EOB with a radiochemical yield of 64% (decay corrected) in accordance with the data reported in literature²²,²³ (Figure 1). The copper(II)-catalyzed Huisgen’s 1,3-dipolar cycloaddition between 7 and the azido synthon 12 gave the final peptidomimetic molecule (FPMt) in only 2 h with 45% yield.²⁵ The time of reaction and the yield obtained suggested that this strategy could be applied for the development of an ¹⁸F-labeled analog.

Conclusions

Herein, we report the synthesis of [¹⁸F]FPMt ([¹⁸F]8), a nonpeptidic radiopharmaceutical for PET imaging with a structure derived from a selective antagonist of $\alpha_5\beta_1$ integrin receptor.²¹ Our alkyne precursor 7 was successfully synthesized, and all the steps were optimized to provide good yields. 1-azido-2-fluoroethane (12) was chosen as synthon for the development of the PET tracer, and it was clicked to 7 following the standard Cu catalyzed alkyne-azole click reaction. Future studies will be aimed at determining the affinity and specificity of [¹⁸F]FPMt for integrin $\alpha_5\beta_1$, and demonstrating how useful this imaging agent may be in assessing angiogenesis in tumors and monitoring therapy.

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Conflict of Interest

The authors did not report any conflict of interest.

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