Bioorganic & Medicinal Chemistry Letters 23 (2013) 6068–6072

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and in vitro evaluation of a novel radioligand for αvβ3 integrin receptor imaging: [^{18}F]FPPA-c(RGDfK)

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ARTICLE INFO

Article history:
Received 27 July 2013
Revised 7 September 2013
Accepted 10 September 2013
Available online 19 September 2013

Keywords:
αvβ3 Integrin
c(RGDfK)
Click chemistry
[^{18}F]-labeling
Positron emission tomography

ABSTRACT

The development of RGD-based antagonist of αvβ3 integrin receptor has enhanced the interest in PET probes to image this receptor for the early detection of cancer, to monitor the disease progression and the response to therapy. In this work, a novel prosthetic group (*N*-(4-fluorophenyl)pent-4-ynamide or FPPA) for the ^{18}F-labeling of an αvβ3 selective RGD-peptide was successfully prepared. [^{18}F]FPPA was obtained in three steps with a radiochemical yield of 44% (decay corrected). Conjugation to c(RGDfK(N_3)) by the Cu(II) catalyzed Huisgen azido alkyne cycloaddition provided the [^{18}F]FPPA-c(RGDfK) with a radiochemical yield of 29% (decay corrected), in an overall synthesis time of 140 min.

Angiogenesis is an important process for tumor growth, invasion and metastasis.[1] Integrins, which are cell-surface heterodimers formed by the noncovalent association of two subunits α and β play an important role in the formation of new blood vessels.[2] The αvβ3 integrin is the most promiscuous of all integrins and is up-regulated in angiogenic endothelial cells during wound healing and cancer. In addition, αvβ3 overexpression is associated with increased metastatic potential and, as result, αvβ3 has been chosen as therapeutic targets.[3] The arginine–glycine–aspartate (RGD) motif is the natural ligand sequence recognized by several integrins, including αvβ3, and RGD-based soluble peptides have been developed as antagonistic compounds (such as cilengitide) to selectively inhibit αvβ3 integrin. Such peptides are currently tested in clinical trials for their antiangiogenic and anti-tumoral activities.[4–6]

Ligands for molecular imaging based on RGD peptides have already been radiolabeled with ^{99m}Tc, ^{111}In and ^{123}I for single photon emission computed tomography (SPECT) imaging, while ^{68}Ga, ^{64}Cu and ^{18}F ($t_{1/2}$ = 109.8 min, 97% β^+, E_{max} = 0.64 MeV) have been widely investigated to radiolabel RGD peptides for positron emission tomography (PET).[7–10] All those radiotracers have been evaluated in animal models and recently the FDA has approved first trial with [^{18}F]FPP(RGD)$_2$ (IND104150).[11] The specificity of this probe against αvβ3 integrin has been demonstrated in vivo and on tissue sections with competition with unlabeled peptides or irrelevant peptides. However, some concerns were raised since the fixation was not solely restricted to endothelial cells. Moreover, the preparation of this ^{18}F-labeled RGD dimer is challenging, which may considerably hamper its applications in patients.[12] Thus, there is a need for a more accessible probe to provide noninvasive quantitative imaging of αvβ3 integrin expression for the evaluation and optimization of novel antiangiogenic therapeutic compounds, as well as for the appropriate selection of cancer patients entering clinical trials with such therapeutic drugs.

Labeling of biomolecules with fluorine-18 is usually accomplished through the conjugation of a targeting entity with a radioactive prosthetic group.[13] In the present study, we aimed at labeling c(RGDfK), a cyclic peptide known to bind with high affinity and selectivity to αvβ3 integrin, with a radiolabeled prosthetic group *N*-(4-[^{18}F]fluorophenyl)pent-4-ynamide ([^{18}F]**3** or [^{18}F]FPPA).

In silico molecular docking was initially performed to check whether the presence of the prosthetic group (FPPA) into this structurally constrained cyclic pentapeptide might influence the binding with the receptor. The minor contact with the protein

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http://dx.doi.org/10.1016/j.bmcl.2013.09.031

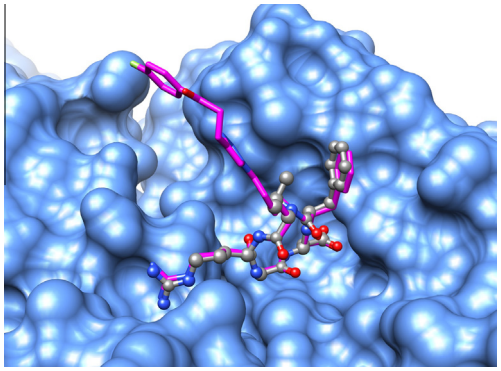
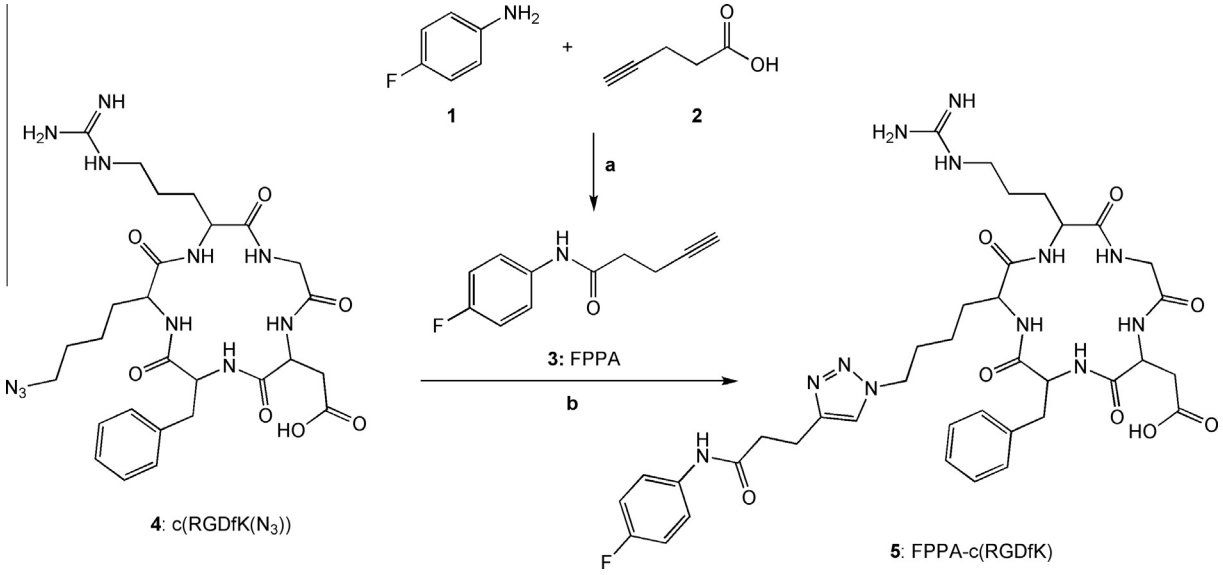


Figure 1. Binding mode of cilengitide and FPPA-c(RGDfK). The experimental binding mode of cilengitide (ball and stick model colored according to the atom types) on the αvβ3 surface (in cornflower blue), compared to the calculated binding mode of FPPA-c(RGDfK) (thick lines, with carbon atoms in magenta).

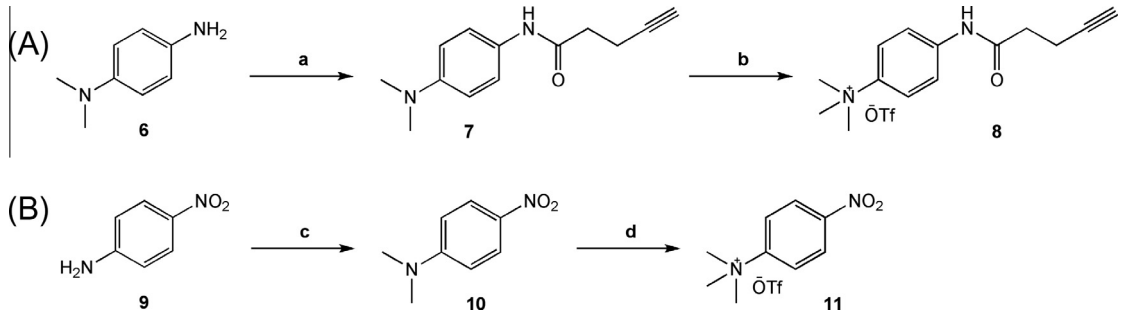
surface of the valine residue of cilengitide suggests that it could be easily replaced by a lysine to provide an anchor point to generate multivalent ligands or to conjugate drugs or radiolabeled moieties. Suggestion of a sequence modification of the cyclic pentapeptide allowing the addition of FPPA without compromising the binding affinity towards αvβ3 integrin was made by the calculation of the binding free energy contributions made by each residue of c(RGDfV) (cilengitide), in complex with αvβ3 integrin (PDB ID 1L5G).[14] We chose to convert the primary amine on the side chain of the lysine residue into an azide to facilitate the addition of the prosthetic group to the RGD peptide by using the Huisgen's click reaction. This reaction was selected due to its high selectivity and efficiency, the rapid formation of the triazole in presence of copper catalyst, and the ease of purification of the final product.[15] All those advantages are of crucial importance while we perform labeling with trace amount of a short half-life radionuclide, such as fluorine-18. Several cyclic peptides coupled to the prosthetic group were docked to the surface of αvβ3 using the EADock program.[16,17] The results demonstrated that the binding mode of the RGDf fragment of the peptide, which is responsible for the major part of the interaction between the ligand and the binding domain of αvβ3, is conserved. The prosthetic group is oriented towards solvent and remains at the surface of the integrin receptor. Thus, the modification on the lysine residue and the incorporation of our radiolabeled prosthetic group does not affect the binding of the pentapeptide (Fig. 1).

FPPA (Scheme 1) has been specially designed to bear a fluorine atom and to possess a terminal alkynyl function. The fluorine atom was implemented at the 4-position of the phenyl ring of FPPA to provide better in vivo stability. Indeed, although incorporation of fluorine-18 into prosthetic groups via substitution reactions usually proceeds better with aliphatic substrates than aromatic compounds, defluorination of aliphatic C–F bond is often observed and leads to bone uptake and artifacts on the PET images.[18–20] Then, a terminal alkynyl group was introduced to the prosthetic group to allow attachment of FPPA to the peptide according to the Huisgen's click reaction.[21] FPPA was synthesized in one step in 91% yield starting from commercially available 4-fluoroaniline (**1**) and 4-pentynoic acid (**2**) (Scheme 1).[22] The reaction was performed in dichloromethane in presence of the standard coupling agents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt). Then, FPPA was conjugated to c(RGDfK(N_3)) by Huisgen's 1,3-dipolar cycloaddition. The peptide has been synthesized according to a reported method.[23] The reaction occurred in water/THF and was catalyzed by Cu(II) in presence of sodium ascorbate. The latter was used as reducing agent in slight excess (Scheme 1).[24] Following the click reaction, the salts were removed by filtration and the final product was purified by HPLC. Identity of FPPA-c(RGDfK) (**5**) was confirmed by MALDI-TOF. Subsequently, **5** has been used as nonradioactive standard for purification and quality control of its radiolabeled analog [^{18}F]-**5** and to confirm binding and selectivity of this RGD peptide to αvβ3 integrin.

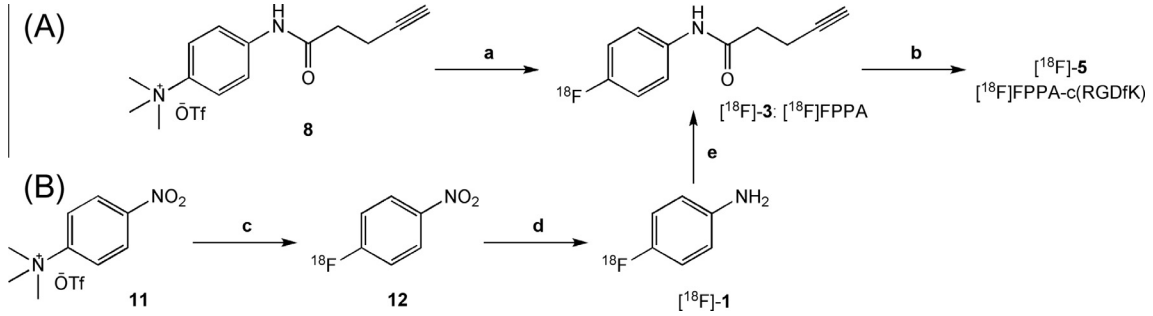
Considering that fluoride is a poor nucleophile and the low activation of the 4-position on the phenyl ring of FPPA, due to the lack of adjacent electron withdrawing groups, we decided to investigate two radiochemical routes for the preparation of [^{18}F]FPPA ([^{18}F]-**3**) (Scheme 2). Trimethylammonium salts were synthesized as precursors for the ^{18}F-fluorination reaction. *N*-(4-(Trimethylammonium)phenyl)pent-4-ynamide trifluoromethanesulfonate (**8**) and *N*-(4-trimethylammonium)nitrobenzene trifluoromethanesulfonate (**11**) were obtained in two steps from 4-(dimethylamino)aniline (**6**) (Scheme 2A) and 4-nitroaniline (**9**) (Scheme 2B), respectively.



Scheme 1. Synthesis of FPPA-c(RGDfK) (**5**). Reagents and conditions: (a) EDC, HOBt, DCM, rt, overnight, 91%; (b) water/THF, $CuSO_4$ (0.1 M), sodium ascorbate (0.3 M), rt, 1 h, 86%.



Scheme 2. Synthesis of the trimethylammonium trifluoromethanesulfonate precursors (**8**) (A) and (**11**) (B). Reagents and conditions: (a) **2**, EDC, HOBt, DCM, rt, overnight, 90%; (b) methyl trifluoromethanesulfonate, DCM, 2 h, 0 °C, 66%; (c) (i) NaH, THF, 5 min, rt; (ii) MeI, THF, 24 h, 73%; (d) methyl trifluoromethanesulfonate, DCM, 0 °C, 68%.



Scheme 3. Synthesis [^{18}F]FPPA ([^{18}F]-**3**). Reagents and conditions: (a) [^{18}F]F$^-$, K_{222}/K_2CO_3, CH_3CN or DMSO, 60–165 °C, 10–15 min, 0.4–5%; (b) **4**, water/THF, $CuSO_4$ (0.3 M), sodium ascorbate (0.7 M), rt, 15 min, 66%; (c) [^{18}F]F$^-$, K_{222}/K_2CO_3, CH_3CN, 10 min, 95 °C, 69%; (d) (i) $NaBH_4$, MeOH, Pd/C, rt, 2 min; (ii) 12 M HCl, rt, 53%; (e) **13**, Et_3N, DCM, 75 °C, rt, 10 min, 44%.

Treatment of **6** with 4-pentynoic acid gave *N*-(4-(dimethylamino)phenyl)pent-4-ynamide (**7**) in 90% yield, while dimethylation of **9** with iodomethane yielded *N*,*N*-dimethyl-4-nitroaniline (**10**). Finally, methylation of the dimethylamino intermediates **7** and **10** with methyl trifluoromethanesulfonate afforded the trimethylammonium precursors **8** and **11** in 66% and 68% yield, respectively.

The radiolabeled RGD peptide [^{18}F]-**5** or [^{18}F]FPPA-c(RGDfK) was prepared starting with the standard kryptofix–K_2CO_3-mediated nucleophilic ^{18}F-exchange reaction with the trimethylammonium triflate precursors **8** or **11** (Scheme 3). Introduction of the fluorine-18 using a no-carrier-added nucleophilic substitution with K[^{18}F]F$^-$/K_{222} was initially conducted with precursor **8** in acetonitrile at 60 °C for 10 min, but low radiochemical yield (0.4% decay noncorrected) was observed. Therefore, we investigated whether we could improve the nucleophilic incorporation of [^{18}F]fluoride into this trimethylammonium triflate salt by changing the reaction conditions (temperature, time, and solvent). We obtained our best radiochemical yield of 4.9% (decay noncorrected) with respect to initial [^{18}F]fluoride when the reaction was performed in DMSO at 165 °C for 12 min. The lack of electron withdrawing groups on the aromatic ring that would favor the fluorination at the 4-position considerably limited the efficacy of this reaction. Therefore, although this one step approach would obviate the need of sophisticated radiochemical route to prepare [^{18}F]FPPA, we considered that it would not provide quantities which are practical for use as PET radiopharmaceuticals. Consequently, a three steps radiochemical route has been established. The first radiochemical step (Scheme 3B), which consisted of the ^{18}F-fluorination of our second activated trimethylammonium precursor **11** resulted in [^{18}F]fluoro-nitrobenzene (**12**), with radiochemical yield of 69% (decay noncorrected) when the reaction mixture was heated at 95 °C for 10 min in acetonitrile. Then, reduction of the nitro group of [^{18}F]fluoro-nitrobenzene (**12**) with sodium borohydride, in presence of palladium on activated carbon, gave 4-[^{18}F]fluoroaniline ([^{18}F]-**1**) as an hydrochloric salt after quenching the reaction with either 1 M HCl or 12 M HCl, with a radiochemical yields of 45% and 53% (decay corrected), respectively.[25] Identity of [^{18}F]-**1** was confirmed by HPLC analysis by co-elution with authentic unlabeled 4-fluoroaniline (Fig. 1-SD). Subsequent coupling of 4-[^{18}F]fluoroaniline with the 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (**13**)[26] in dichloromethane in presence of triethylamine afforded the ^{18}F-labeled prosthetic group [^{18}F]-**3** in 44% radiochemical yield (decay corrected) within 40 min (Fig. 2-SD).

Finally, [^{18}F]-**3** was conjugated with c(RGDfK(N_3)) following the same procedure used for the preparation of the nonradioactive conjugate (**5**), to obtain the ^{18}F-labeled peptide [^{18}F]-**5** with 66% yield (decay corrected) (Scheme 3). [^{18}F]FPPA-c(RGDfK) was then obtained in about 140 min with a total radiochemical yield of 29% (decay corrected). Identity of the new radiopharmaceutical was confirmed by comparing its HPLC mobility with the retention time of the nonradioactive analogue (Fig. 3-SD).

The affinity of the fluorinated c-(RGDfK) was then experimentally tested with an ELISA in vitro assay using recombinant soluble integrins binding to their immobilized natural ligands in the absence or presence of gradual amounts of the test peptide. Soluble recombinant αvβ3 and α5β1 integrins were expressed in HEK293T cells transfected with cDNA encoding for the α and β subunits of the integrins of interest.[27] All cDNAs encoded for truncated integrins (i.e., all integrin subunits lacked their respective transmembrane and C-terminal domains) and were tagged with either a His-tag or fused with a human IgG-Fc-fragment. As a result expressed integrins were secreted in the culture medium ('conditioned medium'). Soluble truncated αvβ3 and α5β1 (α5β1 was used for specificity control) are assumed to adopt a rod-like shape conformation associated with high affinity

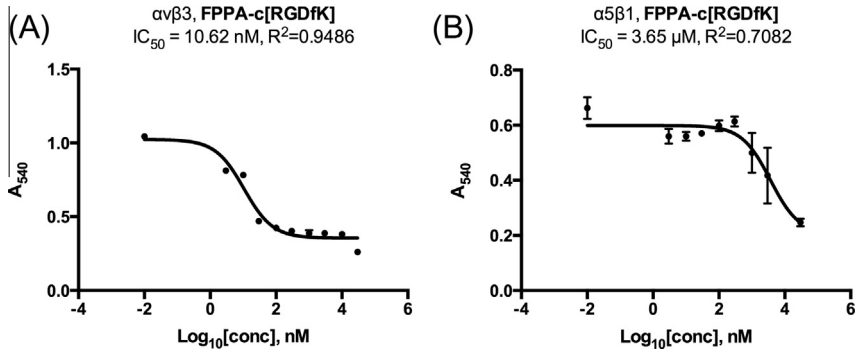


Figure 2. IC_{50} determination of the new peptide for soluble αvβ3 and α5β1 integrins. (A) Results of the IC_{50} determination test for the new peptide towards the αvβ3 integrin showing a 10.6 nM value. (B) Corresponding IC_{50} value for the α5β1 is proximally 300 times lower than the affinity for αvβ3. The data are the results of experiments done in triplicate, and the standard deviations have been reported as vertical bars.

ligand-binding for their substrate.[27] Then, IC_{50} values were obtained by challenging binding of the integrins to the surface-immobilized matrix protein in the presence of increasing amounts of peptide **5**. A cyclic peptide c(RGDfV), which is the equivalent structure of the cilengitide, was used as control peptide to validate the method. An IC_{50} of 0.51 nM was obtained for c(RGDfV) (Fig. 4-SD), which is in accordance with what has been previously reported in the literature (IC_{50} ranging from 0.13 to 1.89 nM).[28] For FPPA-c(RGDfK) (**5**), IC_{50} values of 10.6 nM and 3.6 μM were obtained for αvβ3 and α5β1, respectively (Fig. 2). The difference of binding affinity of **5** between the two integrins tested clearly demonstrates the high specificity of FPPA-c(RGDfK) for αvβ3 integrin. To further characterize the properties of FPPA-c(RGDfK), we performed flow-cytometry analysis on HUVEC (human umbilical vascular endothelial) cells, which are known to highly express αvβ3 integrin. When bound to integrins, high affinity ligands activate the receptor.[27] The presence of activated αvβ3 integrin can then be detected with a specific antibody LIBS-1 (ligand induced binding site), which solely recognizes the activated form (ligand bound) of the β3 integrin subunit. To validate that our peptide (**5**) retains the same activity of c(RGDfV) on αvβ3 integrin after binding, cells were incubated with either media (negative control), 1 mM of Mn^{2+} or 10 μM of c(RGDfV) (both are positive controls for activation), or with FPPA-c(RGDfK). The activation of the receptor was observed in cells treated with Mn^{2+}, c(RGDfV) and FPPA-c(RGDfK) (Fig. 5-SD). This confirms that the cyclic pentapeptide is active towards its receptor, and the presence of the FPPA moderately affects the interaction.

In summary, a novel prosthetic group *N*-(4-fluorophenyl)pent-4-ynamide (FPPA) for labeling of biomolecule via click reaction has been proposed. Docking studies confirmed that the presence of FPPA on c(RGDfK) does not have a negative influence on the interaction with the αvβ3 integrin receptor. Modeling predictions were correlated with the in vitro measurements of the affinity and the activation of integrin αvβ3 by FPPA-c(RGDfK). [^{18}F]FPPA ([^{18}F]-**3**) was synthetized from *N*-(4-(trimethylammonium) phenyl)pent-4-ynamidetrifluoromethanesulfonate (**8**) by direct [^{18}F]fluorination in position 4, or from *N*-(4-trimethylammonium)nitrobenzene trifluoromethanesulfonate (**11**) by a nucleophilic aromatic fluorination, a reduction of the nitro group and a conjugation with the alkynyl chain. The three steps strategy gave a better radiochemical yield (44% decay corrected). The Cu(II)-catalyzed azido-alkyne Huisgen's cycloaddition has been adopted to attach our prosthetic group with the RGD-based peptide. This strategy yielded the final radiopharmaceutical [^{18}F]FPPA-c(RGDfK) in 140 min total synthesis with a radiochemical yield of 29% (decay corrected).

Acknowledgments

This research was carried out at the cyclotron unit of the University Hospital of Geneva, using resources were provided by the Center of Biomedical Imaging (CIBM), the MS facility of the University of Geneva. This work was supported by the Leenaards Foundation. We would like to thank Dr. Sébastien Tardy for helpful discussion and Dr. Elisabeth Rivara-Minten, for her support at the NMR facility.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.09.031.

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