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Facile synthesis and ^{18}F-radiolabeling of $\alpha_4\beta_1$-specific LLP2A-aryltrifluoroborate peptidomimetic conjugates

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ABSTRACT

The peptidomimetic, LLP2A, is a specific, high-affinity ligand for $\alpha_4\beta_1$ integrin receptors. Previously, several PEGylated LLP2A conjugates were evaluated in vivo as imaging agents for the detection of lymphoma, leukemia, multiple myeloma and melanoma tumours via NIR-fluorescence, ^{111}In-SPECT, and ^{64}Cu- and ^{68}Ga-PET imaging. Despite these successes, to date there is no report of an ^{18}F-labeled LLP2A conjugate. Notably, fluorine-18 is a preferred radionuclide for PET imaging, yet its short half-life and general inactivity under aqueous conditions present challenges for peptide labeling. A simple method for labeling complex biomolecules can be achieved with arylboronic acids that readily capture aqueous [^{18}F]-fluoride ion resulting in an ^{18}F-labeled aryltrifluoroborate ([^{18}F]-ArBF$_3^-$) radioprosthetic group. Herein, we present the first radiosynthesis of an ^{18}F-labeled LLP2A conjugate by both one-step ^{18}F-labeling and one-pot two-step ^{18}F-labeling post-'click' conjugation of the ^{18}F-alkynyl-ArBF$_3^-$ prosthetic. Competition with a fluorescent conjugate of LLP2A demonstrated specific binding of the non-radioactive isotopolog ArBF$_3^-$-PEG$_2$-LLP2A to $\alpha_4\beta_1$ integrin-expressing MOLT-4 leukemia cells, as evidenced and confirmed by fluorescence microscopy. This work provides a key first step in the development of an expanding library of [^{18}F]-R-BF$_3^-$-LLP2A radiotracers for PET imaging.

Integrins are heterodimeric proteins consisting of non-covalently associated α and β subunits that link distinct extracellular matrix proteins to the cytoskeleton, of which two dozen subtypes play essential roles in cell adhesion, cell mobility, and signal transduction for normal cellular and tissue function.[1–3] Many cancers overexpress particular integrins to promote tumour cell migration, angiogenesis, tumour growth, metastasis, and drug resistance.[3] Crystallographic structural elucidations of the binding domains of integrins, namely for $\alpha_V\beta_3$,[4] $\alpha_{IIb}\beta_3$,[5] and $\alpha_x\beta_2$ have each shown that integrins contain extracellular ligand binding domains. Hence these are key targets for imaging and therapy, especially the integrins $\alpha_V\beta_3$, $\alpha_5\beta_1$, $\alpha_V\beta_6$, $\alpha_4\beta_1$, that are highly overexpressed compared to healthy adult epithelial cells.[3]

More specifically, the $\alpha_4\beta_1$ integrin, or very late antigen-4 (VLA-4), is involved in lympho-hemopoiesis,[6,7] leukocyte trafficking,[8] and cell motility[9] primarily via VCAM-1 binding and the downstream signalling resulting from such interactions.[10] Several reports have revealed that the $\alpha_4\beta_1$ integrin plays pivotal roles in promoting the progression of osteosarcomas,[11] multiple myelomas,[12–15] lymphomas,[16–18] leukemias[19,20] and melanomas[21–25] and can contribute to angiogenesis,[5,26] and tumour metastasis.[5,21,26]

The peptidomimetic, LLP2A, discovered by one-bead-one-compound (OBOC) library screening, shows extraordinarily high-affinity (IC_{50} = 2 pM) to the $\alpha_4\beta_1$ integrin receptor.[27] A subsequent study reported in vivo NIR-fluorescence imaging activity of a Cy5.5-modified PEGylated LLP2A conjugate to visualize $\alpha_4\beta_1$ integrin-overexpressing MOLT-4 tumour xenografts.[28] LLP2A derivatives containing extended PEG spacers, as well as dimers and tetramers thereof were affixed to metal chelators for one-step labeling with [^{111}In]-indium that demonstrated successful in vivo imaging.[29] Their report showed that extending the PEG length reduced $\alpha_4\beta_1$ integrin receptor binding, while multimers of the LLP2A ligand enhanced binding affinities in vitro. Nevertheless, biodistribution studies revealed that multimeric constructs showed enhanced kidney retention and high muscle retention. Subsequent reports on radiometallated LLP2A-chelator conjugates, labeled either with [^{64}Cu]-copper for PET or [^{99m}Tc]-technetium for SPECT, revealed bone metastasis of breast carcinoma,[30] non-Hodgkin lymphoma,[31] multiple myeloma,[32] primary melanoma tumours,[33] and melanoma metastases.[34] In each report, a modified PEG_4-spacer first reported by Lam et al. was used.[27]

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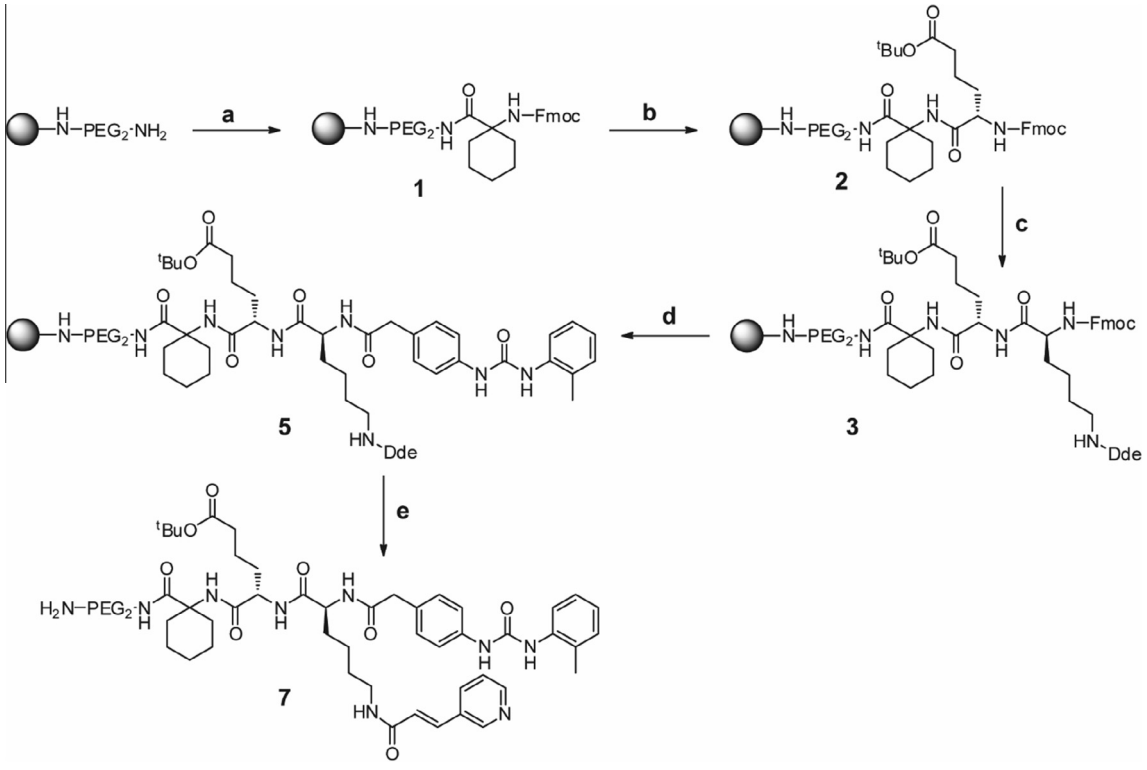
These promising studies indicate the potential for using LLP2A for imaging several cancers, made possible by the facility of radiometal chelation. Yet various potential disadvantages of radiometals including lower resolution, lower specific activity, increased radiotoxicity, higher costs, challenges to scalable production, and the possibility for off-target transchelation in vivo would suggest the need for a radiofluorinated version of LLP2A. Surprisingly, to date, there has been no report of an ^{18}F-labeled LLP2A. Previously, we demonstrated that peptide-boronic acid bioconjugates effectively capture aqueous [^{18}F]-fluoride for one-step and one-pot-two-step click labeling to give the corresponding [^{18}F]-ArBF$_3^-$-bioconjugates to several ligands including biotin,[35] marimastat,[36,37] bisRGD,[38] RGD,[39] and bombesin.[40] In these cases, radiochemical yields (RCYs) ranged from 15% to 60% and specific activities (SAs), which depend on the ratio of NCA [^{18}F]-fluoride ion to [^{19}F]-fluoride ion, ranged from 0.1–15 Ci/μmol. Most importantly, corresponding in vivo images showed specific target binding and good serum stability with minimal bone uptake. Here, we apply this labeling method to the LLP2A scaffold to afford the first-ever examples of ^{18}F-labeled LLP2A-bioconjugates. The synthesis, radiosynthesis, and in vitro binding properties form the basis of this Letter.

To begin, *O*-bis-(aminoethyl)ethylene glycol trityl resin was chosen for the solid-phase peptide synthesis (SPPS) of the PEGylated LLP2A; this strategy affords a C-terminal amide-linked PEG$_2$ spacer terminated with a primary amine for conjugation upon resin cleavage following SPPS. The focus of this Letter is to present the first ^{18}F-labeling of LLP2A conjugates, and to investigate their targeting specificity in vitro. Therefore, the PEG$_2$ linker would not influence ^{18}F-labeling, allows the facile introduction of a standard spacer at the beginning of peptide synthesis, and was not foreseen to hinder in vitro binding. Standard Fmoc-chemistry was used to synthesize LLP2A using previously described methods.[27] Briefly, HBTU and DIPEA in DMF afforded efficient amide coupling within 1 h at room temperature for each residue. Fmoc cleavage was achieved using piperidine in DMF. These methods were used to sequentially couple the residues Fmoc-Ach-OH, Fmoc-Aad(O^{t}Bu)-OH, and Fmoc-Lys(Dde)-OH to provide resin-PEG$_2$-Ach-Fmoc (**1**), resin-PEG$_2$-Ach-Aad(O^{t}Bu)-Fmoc (**2**), and resin-PEG$_2$-Ach-Aad(O^{t}Bu)-Lys(Dde)-Fmoc (**3**), respectively (Scheme 1). The urea, 2-(4-(3-*o*-tolylureido)phenyl)acetic acid (**4**) (Fig. S1), was synthesized as previously reported[27] and coupled to **3** using the aforementioned conditions to produce resin-PEG$_2$-Ach-Aad(O^{t}Bu)-Lys(Dde)-[2-(4-(3-*o*-tolylureido)phenyl)acetyl] (**5**). After Dde deprotection of **5** with hydrazine, 3-(3-pyridyl)acrylic acid was coupled to the ε–NH$_2$ of Lys to provide resin-PEG$_2$-Ach-Aad(O^{t}Bu)-Lys(3-(3-pyridyl)acrylyl)-[2-(4-(3-*o*-tolylureido)phenyl)acetyl] (**6**). Treatment with (3:7) HFIP/DCM and preparatory TLC purification then provided 20.03 μmol of LLP2A-PEG$_2$-NH$_2$ (**7**) for an overall yield of 7%. Compound **7** was identified using HRMS and the purity (>95%) was assessed by RP-HPLC analysis (Fig. S2).

The prosthetic, 2,4,6-trifluoro-3-(4,4,5,5-tetraphenyl-1,2,3-dioxaborolan-2-yl)benzoic acid (**8**), was synthesized as previously described[41] and characterized by ESI-MS, ^{1}H NMR (Fig. S3) and ^{19}F NMR (Fig. S4). The tetraphenylpinacol (TPP) boronate ester, **8**, was conjugated to the terminal PEG$_2$-NH$_2$ of the LLP2A, **7**, using EDC–HCl in the presence of HOBt-hydrate in pyridine-THF. The O^{t}Bu group was removed in the presence of TFA to provide ArB(TPP)-PEG$_2$-LLP2A (**9**) (Scheme 2). The identity and purity (>95%) of **9** was confirmed by HRMS and RP-HPLC analysis, respectively (Fig. S5). Compound **9** (100 nmol) was converted to the nonradioactive trifluoroborate derivative, ArBF$_3^-$-PEG$_2$-LLP2A (**10**), as previously reported for the conversion of arylboronyltetraphenylpinacolates into aryltrifluoroborates.[35,37] The identity of



Scheme 1. Synthesis of (**7**). Reagents and conditions: (a) Fmoc-Ach-OH, HBTU, DIPEA, DMF, 1 h, RT. (b) (i) 2 × (1:4) piperidine/DMF, 10 min, RT. (ii) Fmoc-Aad(O^{t}Bu)-OH, HBTU, DIPEA, DMF, 1 h, RT. (c) (i) 2 × (1:4) piperidine/DMF, 10 min, RT. (ii) Fmoc-Lys(Dde)-OH, HBTU, DIPEA, DMF, 1 h, RT. (d) (i) 2 × (1:4) piperidine/DMF, 10 min, RT. (ii) 2-(4-(3-(*o*-Tolyl)ureido)phenyl)acetic acid (**4**), HBTU, DIPEA, DMF, 1 h, RT. (e) (i) 2 × 2% hydrazine in DMF, 4 min, RT. (ii) 3-(3-Pyridyl)acrylic acid, HBTU, DIPEA, DMF, 1 h, RT. (iii) (3:7) HFIP/DCM, 30 min, RT.

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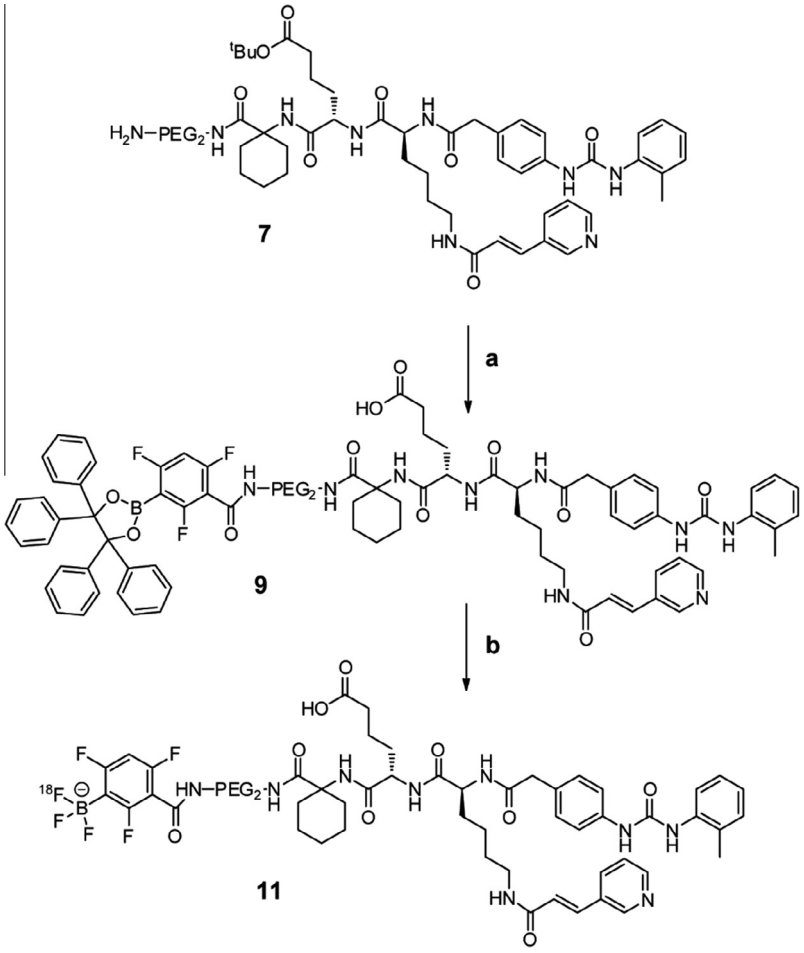


Scheme 2. Synthesis of (**11**). Reagents and conditions: (a) (i) 2,4,6-Trifluoro-3-(4,4,5,5-tetraphenyl-1,2,3-dioxaborolan-2-yl)benzoic acid (**8**), EDC–HCl, HOBt–H_2O, pyridine, THF, 16 h, RT. (ii) (1:1) TFA/DCM, 1 h, RT. (b) (i) [^{18}F]-Fluoride, THF, $HCl_{(conc.)}$, 1 h, RT. (ii) (5:45:50) $NH_4OH_{(conc.)}/H_2O$/EtOH.

10 (the nonradioactive isotopolog not shown) was confirmed by ESI-MS and was used as an HPLC standard (Fig. S6) for the subsequent analysis of its ^{18}F-labeled counterpart. One-step aqueous ^{18}F-labeling of **9** (100 nmol) was achieved using [^{18}F]-fluoride ion (995 μCi) and 750 nmol [^{19}F]-fluoride ion in (1:4) $HCl_{(conc.)}$/THF within 1 h at room temperature. After quenching with diluted $NH_4OH_{(aq)}$ in EtOH, a sample was used for RP-HPLC analysis of [^{18}F]-$ArBF_3^-$-PEG_2-LLP2A (**11**) (Fig. 1A). Radiochemical yields were reproducibly found to be in the range of 15% with effective specific activities of 3.9 mCi/μmol; activities that are low, in keeping with sub-millicurie amounts of radioactivity used (Scheme 2).

To complement a one-step labeling via the conversion of an arylboronate ester into an ^{18}F-labeled aryltrifluoroborate, we also sought a one-pot-two-step ^{18}F-labeling. For this approach, we first synthesized N_3-$(CH_2)_4$-C(O)NH-PEG_2-LLP2A (**13**) by coupling **7** with 5-azidopentanoic acid (**12**), which was synthesized in two steps from commercially available 5-bromo ethyl pentanoate (Fig. S7). This precursor was then treated with (1:1) TFA/DCM to remove the O^{t}Bu protecting group to afford **13**. The identity of **13** was confirmed by ESI-MS and the purity (>95%) was assessed by RP-HPLC (Fig. S8). A 100 nmol sample of alkynyl-ArB(dan) (B(dan) = diaminonaphthyl borimidine) (**14**) as previously reported[38,39] was ^{18}F-labeled under aqueous conditions within 22 min at room temperature using 1.28 mCi of [^{18}F]-fluoride in (1:8) $HCl_{(conc.)}$/THF. After quenching with diluted $NH_4OH_{(aq)}$ in EtOH, a sample was used for RP-HPLC analysis of [^{18}F]-alkynyl-$ArBF_3^-$ (**15**) (Fig. S9). This showed an overall radiochemical conversion of 40%. The quenched solution of **15** was then used to dissolve **13**. Copper-catalysed 'click' chemistry was used to conjugate the ^{18}F-labeled alkynyl arytrifluoroborate (**15**) to the azide-modified LLP2A derivative, **13**. This was accomplished using Cu(II) SO_4 and sodium ascorbate within 36 min at room temperature (Scheme 3). The solution was then analysed by RP-HPLC to assess the conversion to [^{18}F]-$ArBF_3^-$-triazolyl-$(CH_2)_4$-C(O)NH-PEG_2-LLP2A (**16**) (Fig. 1B). This analysis showed a ~79% conversion of **15** to **16** post ^{18}F-labeling, for an overall radiochemical conversion of ~30% by the described one-pot two-step ^{18}F-labeling conditions with an effective specific activity of ^{18}F = 1.31 mCi/μmol.

With methods in hand for ^{18}F-labeling the LLP2A derivatives **9** and **13** either by a one-step direct labeling method or a one-pot two-step reaction (respectively), a fluorescent derivative of **11** was prepared to investigate the $\alpha_4\beta_1$ integrin binding specificity by fluorescence microscopy. Briefly, fluorescein isothiocyanate (FITC) was conjugated to **7** under aqueous conditions (pH = 8.4) after 12 h at 4 °C. This precursor was subjected to (1:1) TFA/DCM to remove the O^{t}Bu protecting group and provided FITC-PEG_2-LLP2A (**17**). The identity of **17** was then confirmed by ESI-MS and was characterized by UV–vis to illustrate the presence of the FITC moiety (Fig. S10). The $\alpha_4\beta_1$ integrin binding specificity of **17** was assessed using $\alpha_4\beta_1$-expressing human MOLT-4 leukemia cells. Fluorescence microscopy found significant binding of **17** following 1 h incubations of 1 μM (Fig. 2A) and 400 μM (Fig. 2B) of **17** (in 1 mL of TBS containing 1 mM Mn^{2+} and 10 mM glucose). As expected, fluorescence was not observed when 100 μM of the

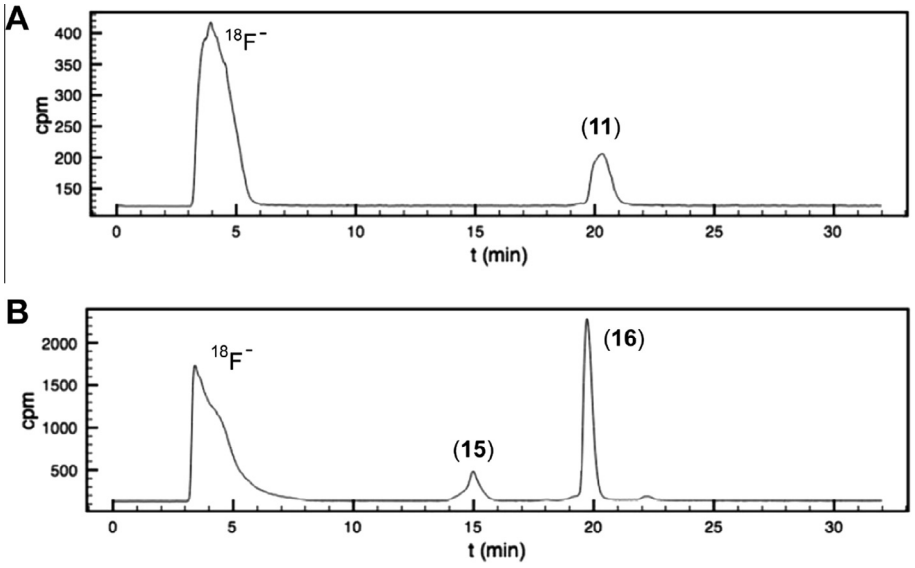
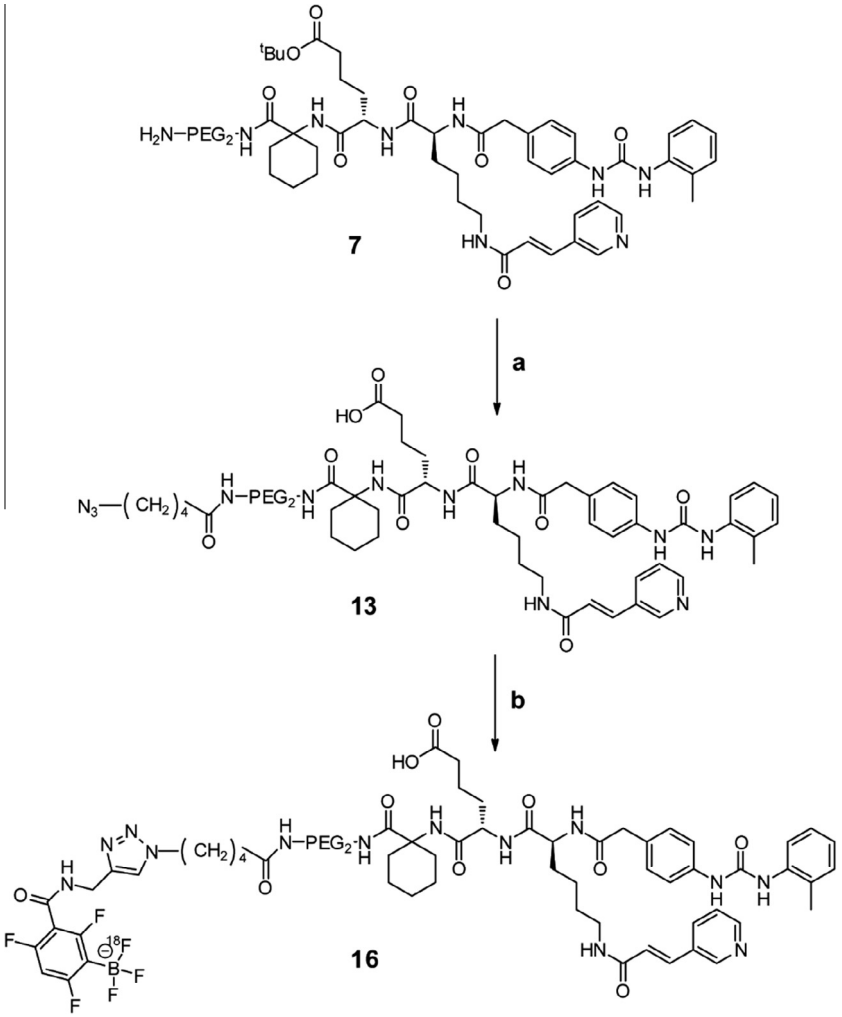


Figure 1. Representative RP-HPLC radio-chromatogram analysis of (A) [^{18}F]-ArBF$_3^-$-PEG$_2$-LLP2A (**11**, t_R = 20.3 min) following one-step ^{18}F-labeling and (B) [^{18}F]-ArBF$_3^-$-triazolyl-(CH$_2$)$_4$-C(O)NH-PEG$_2$-LLP2A (**16**, t_R = 19.7 min) following one-pot two-step ^{18}F-labeling using HPLC program 3 (with unreacted [^{18}F]-alkynyl-ArBF$_3^-$ (**15**) at t_R = 15.0 min and unreacted [^{18}F]-fluoride at t_R = 3.0–6.0 min).



Scheme 3. Synthesis of (**16**). Reagents and conditions: (a) (i) 5-Azidopentanoic acid (**12**), HBTU, DIPEA, DMF, 1 h, RT. (ii) (1:1) TFA/DCM, 1 h, RT. (b) (i) ^{18}F-Alkynyl-ArBF$_3$ (**15**), Na-ascorbate$_{(aq)}$, CuSO$_{4(aq)}$, 36 min, RT. (ii) (5:45:50) NH$_4$OH$_{(conc.)}$/H$_2$O/EtOH.

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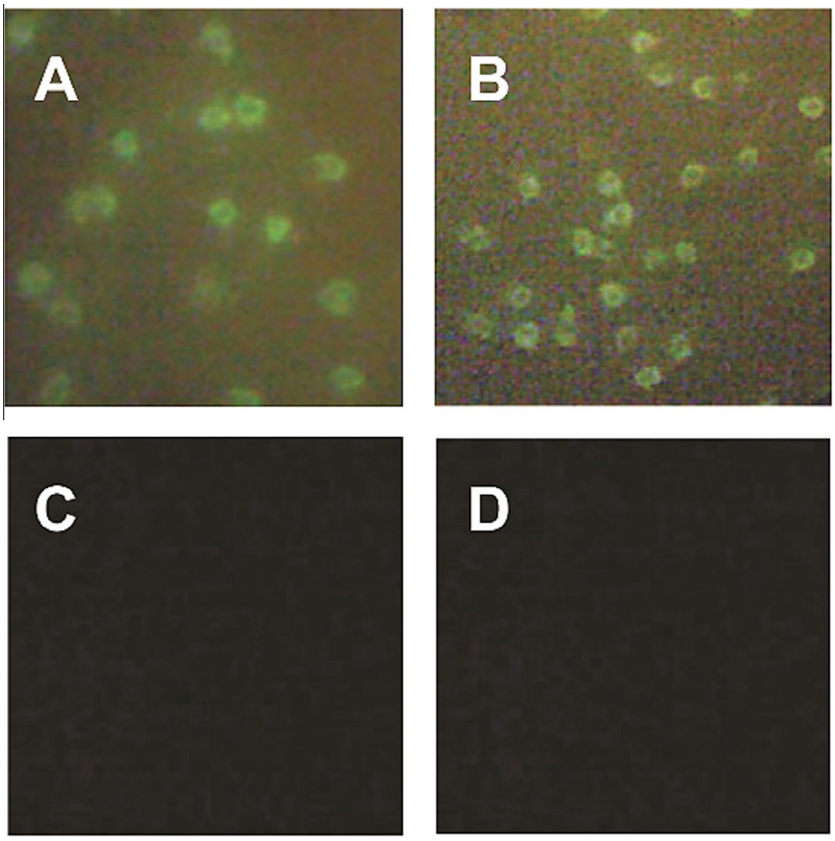

Figure 2. In vitro analysis of the binding and specificity of FITC-PEG_2-LLP2A (**17**) to $\alpha_4\beta_1$-expressing MOLT-4 leukemia cells by fluorescence microscopy. Cells (1×10^6 per well) were incubated with (A) 1 μM of **17** for 1 h at 37 °C, (B) 400 μM of **17** for 1 h at 37 °C, (C) 100 μM of $ArBF_3^-$-PEG_2-LLP2A (**10**) for 1 h at 37 °C, washed and incubated with 1 μM of **17** for 1 h at 37 °C, and (D) 100 μM of **10** for 1 h at 37 °C, washed and incubated with 400 μM of **17** for 1 h at 37 °C in TBS containing 1 mM Mn^{2+} and 10 mM glucose.

non-fluorescent trifluoroborate, $ArBF_3^-$-PEG_2-LLP2A (**10**), was pre-incubated with cells for 1 h before incubation with either 1 μM (Fig. 2C) or 400 μM (Fig. 2D) of **17**. While 1 μM incubations of **17** were required to obtain sufficient fluorescence intensities for visualizing cells, the relatively high concentration of **17** (400 μM) was chosen to illustrate that there was little fluorescence enhancement compared to 1 μM incubations—owing to the high binding affinity of the LLP2A targeting moiety. Although a priori this ligand should bind at much lower concentrations, we used higher concentrations to ensure rapid on-rates and complete saturation on cells. These in vitro fluorescence assays confirmed the binding specificity of **10** and also shows that this non-radioactive counterpart of **11** has over ~4× the binding affinity to $\alpha_4\beta_1$ integrin receptors in vitro compared to the fluorescent derivative, **17**. Therefore, this shows that the ^{18}F-labeled counterpart of **10**, **11**, will also have specificity to $\alpha_4\beta_1$ integrin receptors.

To the best of our knowledge, we report the first ^{18}F-labeling of the $\alpha_4\beta_1$ integrin-specific LLP2A peptidomimetic. Herein, we have used facile boronate-based ^{18}F-labeling in the context of one-step labeling and one-pot-two-step click labeling to prepare two ^{18}F-labeled bioconjugates of the integrin-binding peptidomimetic, LLP2A. The one-step ^{18}F-labeling procedure afforded effective specific activities ~3 times greater than the one-pot-two-step. However, the latter procedure provided ~2 times higher radiochemical yields than the former method. Although a one-step method would arguably be of greater interest and utility, because others have preferred one-pot-two-step click methods for labeling, we have featured this as well with similar success. While herein radiolabeling was performed at low activity levels (~2 mCi), which in turn resulted in low specific activities, we have previously showed for several peptides and fluorophores that increasing the amount of [^{18}F]-fluoride ion activity and/or reducing the amount of boronate conjugate, it is easy to achieve very high specific activities (7–15 Ci/μmol).[42] While we have not explored the use of isotope exchange herein, this technique also affords good-to-excellent radiochemical yields at very high specific activities (up to 16 Ci/μmol) when working with Curie-levels of no-carrier added [^{18}F]-fluoride ion.[43,44] There is nothing to suggest that this could not be applied to LLP2A for future imaging studies. Henceforth, future efforts will involve optimizing the ^{18}F-labeling conditions to improve radiochemical yields and the specific activity of **11** and **16**. In addition, we will explore the utility of other organotrifluoroborates that we have recently disclosed for imaging[45,46] with an eye to tuning the tumour uptake and pharmacokinetics of ^{18}F-labeled LLP2A-trifluoroborate bioconjugates with $\alpha_4\beta_1$ integrin-overexpressing tumour models.

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Supplementary data

Supplementary data (details for materials and equipment, synthetic methods, structural characterization spectra, ^{18}F-labeling methods and characterizations, and in vitro binding study methods) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.011.

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