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Synthesis of a new fluorine-18 glycosylated 'click' cyanoquinoline for the imaging of epidermal growth factor receptor

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This study reports the radiosynthesis of a new fluorine-18 glycosylated 'click' cyanoquinoline [^{18}F]5 for positron emission tomography imaging of epidermal growth factor receptor (EGFR). The tracer was obtained in 47.7 ± 7.5% (n = 3) decay-corrected radiochemical yield from 2-[^{18}F]fluoro-2-deoxy-β-D-glucopyranosyl azide, and the overall nondecay-corrected radiochemical yield from aqueous fluoride was 8.6 ± 2.3% (n = 3). An *in vitro* preliminary cellular uptake study showed selectivity of the tracer for EGFR-positive A431 cell lines versus EGFR-negative MCF-7 cell lines. [^{18}F]5 tracer uptake in A431 cells was significantly reduced by addition of the cold isotope analogue compound 5.

Keywords: EGFR; PET imaging; TKI; click chemistry; radiolabelling

Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane protein belonging to the ErbB receptor kinase family that is involved in cell proliferation, apoptosis, migration and angiogenesis. Over-expression of EGFR is frequently detected in a wide range of human cancers.[1] EGFR-targeted therapies including monoclonal antibodies, for example, cetuximab, and small molecule tyrosine kinase inhibitors (TKIs), for example gefitinib and erlotinib, are used clinically.[2] TKIs have proven to be beneficial in some patients with tumours expressing wild type EGFR. Moreover, patients with nonsmall cell lung cancer expressing L858R- or 746_750del-activated mutant EGFR showed significantly higher response to TKI treatment relative to wild type.[3] In terms of diagnostics, however, a noninvasive technique to image EGFR expression *in vivo* in a clinical setting has yet to emerge. As positron emission tomography (PET) imaging has the potential to give valuable insights into EGFR biology, a number of PET tracers have been published in recent years with mixed results.[4] Reversible tracers, which do not bind to the enzyme active site *via* covalent bonds, are mainly based on the quinazoline structure of gefitinib and erlotinib. These tracers, although displaying promising properties *in vitro*, were found to be generally unsuitable for *in vivo* imaging with only a recent clinical study by Bahce *et al.*[5] showing promising results for the detection of mutant EGFR with [^{11}C]erlotinib. The underlying reason for such inadequacy for *in vivo* imaging with reversible TKIs has been attributed to competition between high levels of intracellular ATP and reversible inhibitors for tyrosine kinase domain occupancy. Therefore, inhibitors bearing a Michael acceptor, which binds covalently and therefore irreversibly to cysteine-773 of the EGFR tyrosine kinase, are expected to attenuate washout by intracellular ATP and to be more promising for *in vivo* imaging.

In this context, we recently reported a fluorine-18-labelled cyanoquinoline irreversible imaging agent, [^{18}F]**1** (Figure 1), which was radiolabelled using a 'click' reaction with the prosthetic group 2-[^{18}F]fluoroethylazide ([^{18}F]FEA).[6] Compound [^{18}F]**1** displayed fourfold higher *in vivo* tumour uptake, relative to muscle, in EGFR over-expressing A431 tumour bearing mice and low tracer retention in other organs including bone, brain and heart. The tracer appeared to be metabolically stable as the parent compound was found to be the major radioactive species in liver and plasma at 60 min post-injection. The gallbladder and the urine presented the highest tissue radioactivity levels, suggesting tracer elimination via both the hepatobiliary and the renal routes. Finally, the detection of high levels of radioactivity in the intestine was a concern and led us to reevaluate the pharmacokinetic properties of the tracer. Compound [^{18}F]**1** has a calculated logarithm of distribution coefficient ($\log D_{pH\ 7.4}$) of 3.97,[7] which is deemed too high for a PET oncology imaging agent directed at somatic lesions; an optimal $\log D_{pH\ 7.4}$ would probably be in the 0–3 range.[8] The high lipophilicity of [^{18}F]**1** could account at least in part for the suboptimal excretion profile of the tracer.

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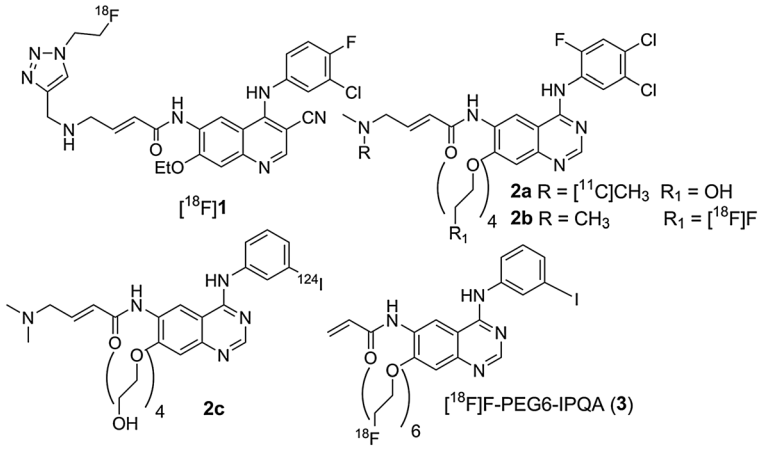


Figure 1. Epidermal growth factor receptor irreversible positron emission tomography imaging agents.

In order to circumvent this suboptimal pharmacokinetic profile, introduction of a polar group, such as a short polyethylene glycol (PEG) linker or a carbohydrate unit, was considered an attractive strategy by which to increase the hydrophilicity of our imaging agent. In the field of EGFR PET tracers, radiolabelled PEG-anilinoquinazoline derivatives have been reported by Mishani, Biasco *et al.*[9] (**2a**–**c**) and Gelovani *et al.*[10] (**3**) (Figure 1). Compounds **2a**–**c** were studied *in vitro* and *in vivo* in mice bearing xenografts derived from human glioblastoma cell lines U-138 MG (EGFR negative) and U-87 MG. wtEGFR (EGFR positive). The *in vivo* imaging protocol showed only a small increase in uptake in some tumours and no significant difference between the two model cell lines. The authors concluded that the lack of specific tumour uptake could be due to nonselective binding and fast metabolism. Compound **3**, [^{18}F]F-PEG6-IPQA, was reported to show high uptake in activated L858R mutant tumour, whereas low-to-moderate uptake was observed in wild type EGFR and resistant T790M mutant tumours. However, despite these promising properties, the tracer also displayed undesirable rapid metabolism. The observed rapid metabolism could be partially due to the presence of the PEG linker. This assumption prompted us to test the carbohydrate moiety strategy and to couple it to our lead cyanoquinoline compound by using a triazole linker, which is known to be highly metabolically stable.

Experimental

General

All reagents and solvents were purchased from Sigma-Aldrich or Fluka 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 aluminium plates precoated with silica (200 μm, 60 F_{254}), which were visualised either by quenching of ultraviolet fluorescence (λ_{max} = 254 nm) or by charring with a $KMnO_4$ dip. ^{1}H and ^{13}C spectra were obtained at 300 K on Bruker 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 peaks. ^{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 electrospray ionisation mode on a Micromass LCT Premier equipped with a Waters Atlantis C18 3 μ column 2.1 × 30 mm. Mobile phase (A) water (0.1% formic acid), (B) acetonitrile. HR-MS values are valid up to ± 5 ppm. Synthesis of [^{18}F]**5** was carried out with the help of an Advion Nanotek platform followed by an in-house developed automated system consisting of an eight two-way valve tower system, a mass flow controller, an oven and a six-port/two-way HPLC valve. ^{18}O-enriched water, >98% ^{18}O atom, was purchased from Rotem. [^{18}F]Fluoride was produced using 11 MeV Siemens Eclipse HP cyclotrons. Semi-preparative HPLC used to isolate [^{18}F]**5** was carried out on a Gilson 121 model and quality control (QC) HPLC on a QC Agilent 1100 series (quaternary pump with diode array UV detector).

Chemistry

Compounds **6**, **7** and **10** were synthesised following literature procedures.[6,11]

Synthesis of compound 5

Sugar **6** (80 mg, 0.38 mmol) and alkyne **7** (44 mg, 0.08 mmol) were dissolved in CH_3CN (1 mL) and water (1.5 mL), and $CuSO_4.5H_2O$ (16 mg, 0.06 mmol) and Na ascorbate (16 mg, 0.08 mmol) were added. The mixture was stirred at room temperature overnight. The mixture was concentrated, and the resulting Boc-protected compound **Boc-5** was purified by flash chromatography on silica gel (AcOEt then AcOEt/MeOH = 10,1). Boc-protected compound **Boc-5** (47 mg, 75%) was deprotected by adding dioxane (1 mL) and concentrated HCl (0.1 mL) at room temperature for 10 min. Compound **5**.HCl (yellow salt, 40 mg, 69%) was precipitated with MeOH and dried under vacuum.

tert-Butyl N-[(2E)-3-({4-[(3-chloro-4-fluorophenyl)amino]-3-cyano-7-ethoxyquinolin-6-yl}carbamoyl)prop-2-en-1-yl]-N-({1-[3-fluoro-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]-1H-1,2,3-triazol-4-yl}methyl) carbamate (**Boc-5**)

R_f: 0.34 (AcOEt/MeOH = 10,1); ^{1}H NMR (400 MHz, MeOD) δ 8.94 (s, 1H), 8.54 (br s, 1H), 8.20 (s, 1H), 7.40 (dd, *J* = 6.5, 1.9, 1H), 7.37–7.30 (m, 1H), 7.28–7.19 (m, 2H), 6.93–6.78 (m, 1H), 6.48–6.33 (m, 1H), 5.92 (d, *J* = 9.3, 1H), 4.81 (dt, J_{HF} = 48.9, J_{HH} = 9.1, 1H), 4.65–4.53 (m, 2H), 4.33 (q, *J* = 7.0, 2H), 4.21–4.06 (m, 2H), 3.89–3.77 (m, 2H), 3.71–3.59 (m, 2H), 3.58–3.51 (m, 1H), 1.56 (t, *J* = 6.8, 3H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.0 (s), 166.0 (s), 158.8 (s), 156.4 (s), 154.8 (s), 154.7 [s, (d, J_{CF} = 432.2)], 153.5 (s), 142.6 (d), 138.1 (s), 129.5 (s), 128.0 (d), 126.2 [d, (d, J_{CF} = 7.6)], 125.9, 125.5 (d), 124.3, 123.8 (d), 122.23 [s, (d, J_{CF} = 19.3)], 118.1 [d, (d, J_{CF} = 22.6)], 117.8 (s), 114.6 (d), 108.8 (s), 92.1 [d, (d, J_{CF} = 188.0)], 86.5 [d, (d, J_{CF} = 24.5)], 82.2 (s), 81.2 (d), 76.4 [d, (d, J_{CF} = 16.7)], 70.1 [d, (d, J_{CF} = 7.7)], 66.3 (t), 62.2 (t), 49.8 (t), 43.4, 42.8 (t), 28.6 (q, 3C), 14.7 (q); HR-MS (ESI) calcd for $C_{36}H_{40}ClF_2N_8O_8$: 785.2626, found 785.2641 (Δ 1.9 ppm); MS (ESI): *m/z* (%) 785 [MH^+] (100).

*(2*E*)-N-{4-[(3-Chloro-4-fluorophenyl)amino]-3-cyano-7-ethoxyquinolin-6-yl}-4-[({1-[3-fluoro-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]-1*H*-1,2,3-triazol-4-yl}methyl)amino]but-2-enamide* (**5**)

^{1}H NMR (500 MHz, D_2O) δ 8.88 (s, 1H), 8.64 (s, 1H), 8.41 (s, 1H), 7.56 (dd, *J* = 2.2, 6.4, 1H), 7.38–7.31 (m, 2H), 7.25 (s, 1H), 6.85 (dt, *J* = 6.7, 15.3, 1H), 6.58 (d, *J* = 15.5, 1H), 6.06 (dd, *J* = 2.6, 9.0, 1H), 4.87 (dt, *J* = 50.6, 9.0, 1H), 4.47 (s, 2H), 4.33 (q, *J* = 7.0, 2H), 4.03–3.94 (m, 3H), 3.85 (d, *J* = 10.5, 1H), 3.77–3.64 (m, 2H), 3.60 (t, *J* = 9.4, 1H), 1.47 (t, *J* = 7.0, 3H); ^{13}C NMR (126 MHz, D_2O) δ 164.7 (s), 160.5 [s, (d, J_{CF} = 352.5),] 155.6 (s), 154.5 (s), 151.3 (s), 148.2 (d), 138.3 (s), 137.6 (s), 134.2 (d), 133.1 (s), 129.7 (d), 129.5 (d), 128.6 (s), 127.7 [d, (d, J_{CF} = 8.1)], 126.0 (d), 117.5 [d, (d, J_{CF} = 22.8)], 114.2 (d), 111.4 (s), 101.5 (d), 90.4 [d, (d, J_{CF} = 187.5)], 85.6 (s), 84.7 [d, (d, J_{CF} = 24.1)], 79.0 (s), 74.1 [d, (d, J_{CF} = 16.5)], 68.5 [d, (d, J_{CF} = 8.0)], 66.6 (t), 66.5 (d), 60.1 (t), 46.9 (t), 40.9 (t), 13.4 (q); HR-MS (ESI) calcd for $C_{31}H_{32}ClF_2N_8O_6$: 685.2101, found 685.2109 (Δ 1.2 ppm); MS (ESI): *m/z* (%) 685 [MH^+] (20).

Radiochemistry

Synthesis of [^{18}F]6

Aqueous [^{18}F]fluoride (2 mL) was trapped on a QMA cartridge and eluted with a mixture of Kryptofix-2.2.2 (400 μL of a 12 mg/mL stock solution in water), K_2CO_3 (100 μL of an 18 mg/mL stock solution in water) and KH_2PO_4 (100 μL of a 18 mg/mL stock solution in water). [^{18}F]Fluoride was dried at 105 °C under a stream of nitrogen. MeCN (0.5 mL) was added and distillation continued. After cooling at room temperature, triflate precursor **8** (3 mg) in acetonitrile (300 μL) was added. This procedure was performed automatically on the Advion Nanotek platform. The mixture was delivered into an external heating block and heated at 80 °C for 5 min. The mixture was then diluted in water (8 mL), and the activity was trapped on a Sep-Pak light *t*C18 cartridge (Waters), which was then washed with water (5 mL). Activity as eluted in a vial with MeCN (2 mL) and concentrated at 80 °C under a stream of He (flow rate 20 mL/min) in ~10 min. NaOH (60 mM, 250 μL) was added and the mixture heated at 80 °C for 10 min then neutralised with HCl (60 mM, 250 μL).

Synthesis of [^{18}F]5

A mixture of $CuSO_4.5H_2O$ (50 μL of a 34.8 mg/mL stock solution in water), sodium ascorbate (50 μL of a 174 mg/mL stock solution in phosphate-buffered saline (PBS)), bathophenanthroline disulfonic acid disodium salt (BPDS) (2.5 mg in 20 μL of PBS) and alkyne **10** (1 mg in 25 μL of water and 25 μL of MeCN) was added to [^{18}F]**6**, and the mixture was left at room temperature for 5 min. [^{18}F]**5** was isolated by semi-preparative HPLC [Phenomenex Luna C18 150 × 10 mm HPLC column, isocratic mobile phase of 20% MeCN/water (5 mM H_3PO_4), flow rate 3 mL/min]. The HPLC solution was diluted with water (5 mL) and the activity trapped on a Sep-Pak light C18 cartridge (Waters, preconditioned with 5 mL of EtOH and 10 mL of PBS). The cartridge was washed with water (5 mL) and the activity eluted in EtOH/PBS: 1,1.

Epidermal growth factor receptor tyrosine kinase enzyme inhibition assay

Epidermal growth factor receptor tyrosine kinase enzyme inhibition assay was performed as reported in the literature.[6]

LogD determination

To 50 μL of [^{18}F]**5** formulated in 10% EtOH in PBS, 450 μL of preequilibrated PBS and 500 μL of preequilibrated *n*-octanol were added. The mixture was vortexed for 20 s before centrifugation at 15 krpm for 3 min. To each of five Eppendorf tubes, 75 μL from the upper *n*-octanol layer, 175 μL of preequilibrated *n*-octanol and 250 μL of preequilibrated PBS were added. The Eppendorf tubes were vortexed and centrifuged as earlier. From the upper *n*-octanol layer, 100 μL was transferred to a gamma-counting tube with a pipette. The lower PBS layer was first transferred with a syringe fitted on a thin needle to a second Eppendorf tube from which 100 μL was transferred to a gamma-counting tube with a pipette. The gamma-counting tubes were placed in a gamma counter and analysed for 1 min each.

In vitro cell uptake

A431 and MCF7 cells were seeded 48 h prior to the experiment in complete Dulbecco's modified Eagle's medium in triplicate wells of six-well plates at densities of 4×10^5 and 3×10^5 cells per well, respectively. On the day of the experiment, cells were incubated with 0.55 MBq (15 μCi) of [^{18}F]**5** for 60 min at 37 °C. After incubation, the excess radioactivity was washed from the cells twice with PBS. Cells were trypsinised and transferred into a counting tube. Following centrifugation at 5000 rpm for 2 min at 4 °C, the obtained cell pellet was resuspended in lysis buffer, and counts were measured on a gamma counter. The decay-corrected counts per minute acquired were normalised to the protein levels by carrying out a bicinchoninic acid assay.

Results and discussion

In order to improve the pharmacokinetic properties of our cyanoquinoline tracer while retaining high metabolic stability, we synthesised a glycosylated triazole cyanoquinoline [^{18}F]**5**, which was designed to be radiolabelled by 'click' chemistry with the prosthetic group 2-[^{18}F]fluoro-2-deoxy-β-D-glucopyranosyl azide ([^{18}F]**6**) (Figure 2).[11a] Not only have 2-[^{18}F]fluoro-2-deoxy-β-D-glucopyranosyl triazoles been found to be metabolically stable, but also, incorporation of these moieties have been reported previously to improve the *in vivo* properties of PET radiotracers, such as [^{18}F]fluorodeoxyglucose folate.[12] Furthermore, the calculated distribution coefficient of cyanoquinoline **5** ($\log D_{pH\ 7.4}$ = 2.40)[7] appeared to be more favourable for PET imaging of somatic lesions.

Unlabelled reference compound **5** was obtained by Cu(I)-catalysed Huisgen cycloaddition (the 'click' reaction) between 2-fluoro-2-deoxy-β-D-glucopyranosyl azide **6** and the *N*-Boc-protected alkynylcyanoquinoline **7** (Scheme 1) followed by deprotection with concentrated HCl–dioxane in 69% overall yield. Building blocks **6** and **7** were synthesised following literature procedures.[6,11]

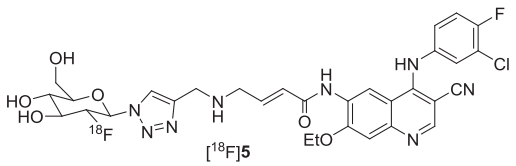
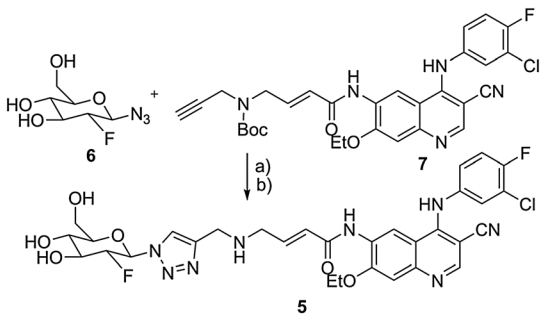


Figure 2. Epidermal growth factor receptor glycosylated 'click' cyanoquinoline [^{18}F]**5**.



Scheme 1. Synthesis of reference compound **5**. *Reagents and conditions*: a) $CuSO_4.5H_2O$, Na ascorbate, water, CH_3CN, r.t., 12 h. b) 10% concentrated HCl in dioxane, r.t., 10 min (69% overall yield).

We have previously shown[6] that modification of the terminus of the Michael acceptor does not have a significant detrimental effect on the ability of this class of inhibitors to irreversibly bind to EGFR. Receptor binding efficiency of cyanoquinoline **5** was assessed using a cell-free kinase activity inhibition assay (Perkin Elmer, UK). The inhibitor displayed an IC_{50} value—half maximal inhibitory concentration—of 4.5 ± 0.7 nM, comparable with that of cyanoquinoline **1** (1.8 ± 0.2 nM), which demonstrates that it retains high EGFR binding property and prompted the subsequent *in vitro* evaluation (Figure 3).

The radiolabelled congener of compound **5**, [^{18}F]**5**, was synthesised semi-manually with the help of an Advion Nanotek automated system. Alkyne-containing cyanoquinoline **10** was obtained from the corresponding *N*-Boc-protected compound **7** by treatment with concentrated HCl in 1,4-dioxane in quantitative yield.[6] Prosthetic group [^{18}F]**6** was synthesised as reported by Maschauer *et al.*[11a] from the β-mannosyl azide precursor **8**[11a] using [^{18}F]KF/Kryptofix-2.2.2 at 80 °C for 5 min. In the process of forming the [^{18}F]KF/cryptand complex, KH_2PO_4 was added in order to buffer the basicity of K_2CO_3 and reduce degradation of the precursor. The Advion Nanotek was used to dry the fluoride and to add precursor **8**, whereas the radiolabelling reaction to form the intermediate [^{18}F]**9** was performed in an external heating block. Intermediate [^{18}F]**9** was purified from any unreacted [^{18}F]fluoride by solid phase extraction on a Waters Sep-Pak light *t*C18 cartridge and released with acetonitrile. Acetonitrile was removed at 80 °C under a stream of nitrogen and 60-mM aqueous NaOH was added to remove the acetate protecting groups. The reaction was complete after 10 min at 80 °C, as assessed by radio-HPLC. The reaction mixture was then neutralised by addition of 60-mM aqueous HCl and used in the 'click' reaction without further purification (Scheme 2).

The 'click' reaction was performed in a $CuSO_4$/Na ascorbate solution containing BPDS (Scheme 3). BPDS is a Cu(I)-stabilising bidentate ligand, which has been shown previously to enhance greatly the rate of the 'click' reaction.[13] Precursor **10** was added to this solution, and the resulting mixture was then added to the solution of azide [^{18}F]**6**. The cycloaddition was complete in 5 min at room temperature, at which point [^{18}F]**5** was purified by semi-preparative HPLC and reformulated by solid phase extraction by using a Waters Sep-Pak light C18 cartridge. The tracer was obtained in 47.7 ± 7.5% (n = 3) decay-corrected radiochemical yield from [^{18}F]**6** and >99% radiochemical purity. The identity of [^{18}F]**5** was confirmed by co-elution with the nonradioactive compound **5**. Overall nondecay-corrected radiochemical yield from aqueous fluoride was 8.6 ± 2.3% (n = 3). The radiosynthesis took ~90 min and yielded the tracer ready for injection with specific activity of 7.3 GBq/μmol. The radioimaging agent [^{18}F]**5** was stable for at least 4 h after formulation in PBS. The distribution coefficient of the tracer [^{18}F]**5** was then evaluated by measuring the distribution of radioactivity between preequilibrated *n*-octanol and PBS (pH 7.4) using the 'shake flask' method.[14] The logarithm of the ratio was found to be $\log D_{pH\ 7.4} = 2.51 \pm 0.02$ (n = 5). This value is similar to the calculated value ($\log D_{pH\ 7.4} = 2.40$)[7] and should allow reasonable cell penetration and subsequent localisation to the tyrosine kinase domain of EGFR. Moreover, as this log*D* falls within the desired 0–3 range, this derivative is expected to display a more favourable pharmacokinetic profile.

Compound [^{18}F]**5** was tested *in vitro* in a cellular uptake experiment. A431 cells, harbouring high EGFR expression, were compared with low EGFR expressing MCF7 cells, as shown in Figure 4. The uptake of [^{18}F]**5** was twofold higher in the A431 cells than in the MCF7 cells. Moreover, the uptake of [^{18}F]**5** in the A431 cells was significantly reduced when cells were pretreated with cold **5** prior to incubation with [^{18}F]**5**. On the other hand, pretreatment with cold **5** had no effect on tracer uptake in MCF7 cells. This set of experiments strongly suggest that tracer [^{18}F]**5** exhibits selectivity for EGFR.

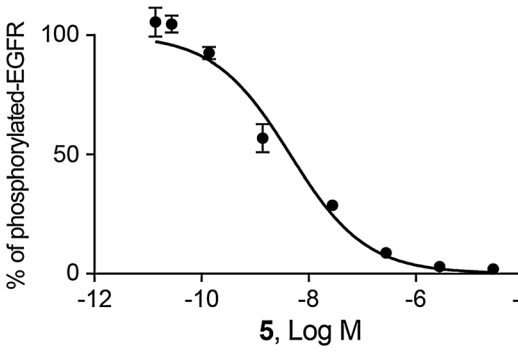
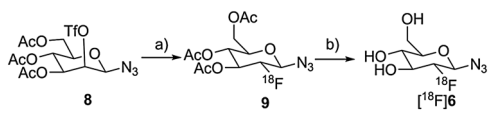
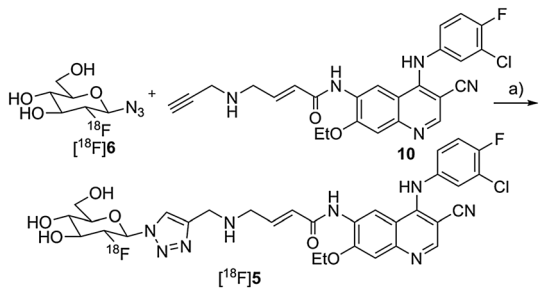


Figure 3. *In vitro* cell-free inhibition of epidermal growth factor receptor kinase activity by compound **5**.



Scheme 2. *Reagents and conditions*: a) [^{18}F]KF/K_{222}, 80 °C, 5 min. b) $NaOH_{(aq)}$ (60 mM), 80 °C, 10 min then $HCl_{(aq)}$ (60 mM).



Scheme 3. *Reagents and conditions*: a) $CuSO_4$, Na ascorbate, bathophenanthroline disulfonic acid disodium salt, water, MeCN, PBS, rt, 5 min.

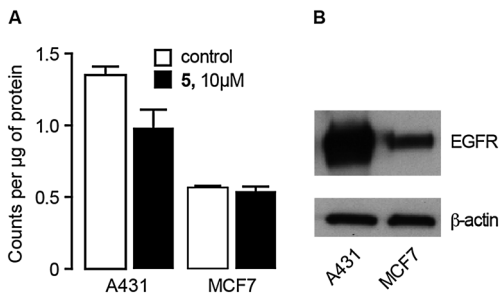


Figure 4. A. Cellular uptake of [^{18}F]**5** in A431 and MCF7 cells. Data show mean ± standard error of the mean from triplicate of three independent experiments. B. Western blot analysis of epidermal growth factor receptor (EGFR) expression in A431 and MCF7 cells indicates significantly higher EGFR levels in A431 cells as compared with MCF7 cells.

Conclusion

In conclusion, a new EGFR-specific tracer [^{18}F]**5** has been synthesised by 'click' chemistry with 2-[^{18}F]fluoro-2-deoxy-β-D-glucopyranosyl azide ([^{18}F]**6**). The tracer was obtained in 47.7 ± 7.5% ($n = 3$) decay-corrected radiochemical yield from [^{18}F]**6**, and the overall nondecay-corrected radiochemical yield from aqueous [^{18}F]fluoride was 8.6 ± 2.3% ($n = 3$). An *in vitro* preliminary study showed selectivity of the tracer for EGFR and encourages further evaluation.

Acknowledgements

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Conflict of Interest

The authors did not report any conflict of interest.

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