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A fluorous and click approach for screening potential PET probes: Evaluation of potential hypoxia biomarkers

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ABSTRACT

Radiopharmaceuticals for nuclear imaging are essentially targeting molecules, labeled with short-lived radionuclides (e.g., F-18 for PET). A significant drawback of radiopharmaceuticals development is the difficulty to access radiolabeled molecule libraries for initial in vitro evaluation, as radiolabeling has to be optimized for each individual molecule. The present paper discloses a method for preparing libraries of ^{18}F-labeled radiopharmaceuticals using both the fluorous-based ^{18}F-radiochemistry and the Huisgen 1,3-dipolar (click) conjugation reaction. As a proof of concept, this approach allowed us to obtain a series of readily accessible ^{18}F-radiolabeled nitroaromatic molecules, for exploring their structure–activity relationship and further in vitro evaluation of their hypoxic selectivity.

1. Introduction

Radiopharmaceutical and drug development methodologies are similar: both relying on the identification of a lead compound and later optimization via the generation of analog libraries. However chemical libraries synthesized for drug screening can not be directly used for identification of targeted nuclear imaging probes, due to the numerous challenges in radiochemistry: direct ^{18}F-labeling of radiopharmaceuticals is especially tedious and limited to specific substrates,[1] furthermore, the relative short half-lives of nuclear imaging isotopes (e.g., 109.7 min for F-18) limit the synthesis and evaluation of the radiopharmaceutical. We have identified a need for identifying candidates for nuclear imaging (PET, SPECT) that can be readily radiolabeled: the present article hence describes the use of the fluorous technology and click chemistry for the development of such radiopharmaceuticals.

The 'click chemistry' and more specifically the practical and reliable Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between an alkyne and an azide is being used increasingly in drug discovery.[2–4] The approach is restricted to easy-to-make molecules but provides a means for the rapid discovery of lead compounds as well as the lead optimization (enables rapid structure–activity relationship profiling through the generation of large screening libraries). Similarly radiopharmaceutical development could benefit from the very efficient 'click chemistry' approach, readily providing freshly produced libraries of radiolabeled candidates that could be evaluated in vitro at the trace level.[2] For this purpose we propose using a simple radiolabeled synthon to tag libraries of chemicals. Furthermore, the synthesis of the radiolabeled fragment would benefit from a simple and reliable synthesis and purification method, such as the fluorous radiochemistry approach. Fluorous chemistry is best considered as solution phase synthesis with the benefit of separation tagging: rapid purification of crude reaction mixtures by using fluorous solid-phase extraction (FSPE) is allowed by the unique separation properties of highly polyfluorinated molecules.[5,6] An ^{18}F-labeled synthon can therefore be obtained by detagging a fluorous-tagged precursor upon nucleophilic ^{18}F-fluorination and subsequent purification by FSPE.[7]

As a practical demonstration of the approach, we decided to evaluate potential PET probes for hypoxia in cancer. Hypoxia is actually a common attribute of a solid tumor microenvironment and is considered a significant factor in the hallmarks of cancers.[8,9] So far, the development of hypoxia biomarker has focused on 2-nitroimidazole derivatives which are reduced and trapped within hypoxic cells.[10] Several derivatives, labeled with ^{18}F, have hence been investigated and reported for molecular imaging of hypoxia using PET: ^{18}FMISO,[11] ^{18}FAZA,[12] ^{18}FETNIM,[13] ^{18}FETA,[14] [^{18}F]HX4.[15] However those compounds exhibit a relatively low uptake in hypoxic tumors (passive transport across cell membranes), and require long examination protocols (slow clearance from other tissues), limiting the clinical potential of these PET biomarkers.

There is considerable evidence that 2-nitroimidazoles have electron affinity under hypoxic conditions and recently, we reported that the crucial irreversible binding step occurring after the initial

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reduction step may have been overlooked.[16] Hence we postulate that the development of a new hypoxia selective biomarker might arise from the optimization of the binding rate of the reduced metabolites within hypoxic cells. We evaluate how a modification of the structure of the potential biomarkers can improve the binding of their reduced metabolites within hypoxic cells.

2. Materials and methods

2.1. Chemistry

2-Azidoethyl 1*H*,1*H*,2*H*,2*H*-perfluorodecane-1-sulfonate and 2-fluoroethyl azide were prepared as described previously.[7] 2-Nitroimidazole, 2-methyl-4-nitroimidazole, 5-nitrobenzimidazole, 5-nitro-1*H*-pyrazole, 2-nitropyridine and 2-amino-5-nitrothiazole were purchased from Sigma–Aldrich, 3-amino-2-nitropyridine was purchased from Alfa Aeser.

N-Propargyl derivatives of nitroaromatics were prepared by treating the N-heteroaromatics and aromatic amines with propargyl bromide in the presence of potassium carbonate (Scheme 1). Primary aromatic amines are protected by N-formylation with acetic formic anhydride generated in situ, prior to the formation of *N*-propargyl derivatives. Purifications were performed on silica gel cartridge using ethyl acetate:hexane as the eluent.

Propargyl-functionalized nitroaromatics were conjugated with 2-fluoroethylazide in the presence of copper sulfate and sodium ascorbate (Scheme 2). Purifications were performed on silica gel cartridge using ethyl acetate:hexane as the eluent.

2.2. Partition coefficient

The log*P* values were measured using a standard shake flask method. Approximately 0.01–0.1 mg/mL of the nitroaromatic compound was shaken in a mixture of 1-octanol (0.5–1.0 mL) and 50 mM phosphate buffer (0.5–1.0 mL, pH 7.4) for 30 min at room temperature, after which equal amounts of both phases were analyzed by HPLC for UV-absorbance quantification (Phenomenex Gemini NX, C18, 150 × 4.6 mm, 1 mL/min isocratic ethanol:water 5:95 for **1**, **2**, **3**, **5**; 10:90 for **6**; 20:80 for **4**, **7**). The experiment was carried out in triplicate.

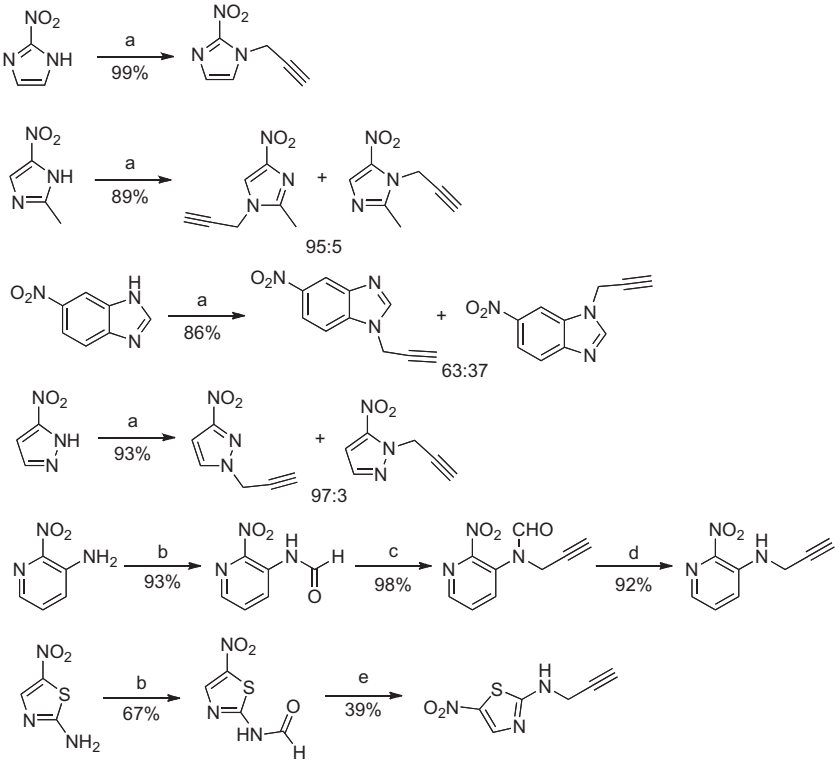
2.3. Electrochemistry

Voltammetry measurements were recorded in a glass cell located inside a Faraday cage at room temperature. Cyclic voltammograms of approximately 5 mM solutions of the nitroaromatic compound and 0.1 M *n*-tetrabutyl ammonium tetrafluoroborate in 3.0 mL of anhydrous and degassed acetonitrile were recorded on an electrochemical analyzer (CH Instruments), using a platinum working electrode, a platinum wire counter-electrode/auxiliary electrode, and a silver/silver ion reference electrode. Ferrocene was used as an internal reference for which the one electron redox process occurs at $E_{1/2}(Fc/Fc^{+}) = 0.42$ versus SCE in anhydrous acetonitrile.

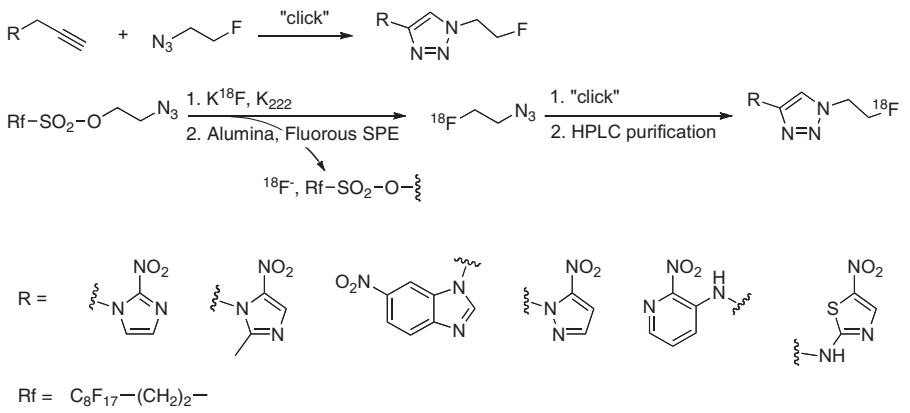
2.4. Enzymatic reduction

Enzymatic reduction of the nitroaromatic compound by xanthine oxidase was measured spectrophotometrically using the method described in the literature.[16,17] Specific activity of the enzyme was assessed by measuring the conversion of xanthine to uric acid in air. Xanthine oxidase (from bovine milk, grade III, Sigma–Aldrich, about 0.02 units) in PBS (100 μL) was added to a solution of xanthine (58 μM) in PBS (pH 7.5, 50 mM, 0.6 mL), maintained at 25 °C, and the formation of uric acid was recorded at 292 nm. One unit converts 1.0 μmol/min of xanthine to uric acid at pH 7.5 at 25 °C.

Xanthine and the nitroaromatic compounds were used as the reducing and oxidizing substrates, respectively. Anoxic conditions were produced by bubbling humidified argon (BOC Pureshield



Scheme 1. Propargyl-functionalization of nitroaromatics. Reagents and conditions: (a) Propargyl bromide, K_2CO_3, acetone, RT, overnight. (b) Acetic formic anhydride, Et_2O, RT, overnight. (c) Propargyl bromide, K_2CO_3, acetone, RT, overnight. (d) cat. K_2CO_3, MeOH, RT, 5 min. (e) (1) Propargyl bromide, K_2CO_3, acetone, RT, overnight. (2) MeOH, RT, 20 min.



Scheme 2. Click coupling to 2-fluoroethylazide.

Argon) for 20 min in the substrates and enzyme PBS solutions (pH 7.5, 50 mM). Xanthine oxidase in PBS (0.118 ± 0.002 unit in 100 μL) was added to a solution of xanthine (0.29 mM) and nitroaromatic substrate (0.12 mM) in PBS (pH 7.5, 50 mM, 0.6 mL), maintained at 37 °C in a degassed cuvette sealed with a rubber vaccine cap, and the solution was mixed by inversion. The nitroaromatic substrate reduction was determined from the initial loss of absorption at the wavelength of maximum absorbance (>325 nm) or at 249 nm, the isosbestic point for the conversion of xanthine to uric acid in air. The losses of absorbance were converted to nmol/min using the extinction coefficients.

2.5. Radiochemistry

HPLC analysis was performed with a Shimadzu Prominence HPLC system equipped with a NaI/PMT-radiodetector (Gamma-RAM, IN/US) and a diode array detector (Shimadzu).

No-carrier-added [^{18}F] fluoride was produced by the $^{18}O(p,n)^{18}F$ nuclear reaction with a 16 MeV proton beam generated by a GE PETtrace cyclotron in a silver target using [^{18}O]H_2O (Singapore Radiopharmaceuticals Pte Ltd).

A GE TracerLab FX_{FN} radiochemistry module was used for processing [^{18}F]fluoride, nucleophilic fluorination and purification of 2-[^{18}F]fluoroethylazide. Isolation of the [^{18}F]fluoride from [^{18}O]H_2O was achieved by trapping on a QMA ion exchange cartridge (Sep-Pak light, Waters) and further elution of the column with a solution containing 1.8 mg K_2CO_3 and 10 mg of Kryptofix 2.2.2 in 2.0 mL of 22:1 acetonitrile:water into a glassy carbon reaction vessel. Anhydrous [^{18}F]fluoride was obtained after azeotropic evaporation for 7 min at 60 °C under a flow of helium and 7 min at 120 °C under vacuum.

2.5.1. [^{18}F]FMISO

FMISO was prepared as described previously by nucleophilic fluorination of 1-(2′-nitro-1′-imidazolyl)-2-*O*-tetrahydropyranyl-3-*O*-toluenesulfonyl-propanediol (5 mg, ABX) at 110 °C for 10 min in anhydrous acetonitrile (0.8 mL), and further hydrolysis with HCl 1 N (1 mL) for 5 min at 100 °C, neutralization with 30% sodium acetate (0.5 mL), dilution with 3 mL water and purification by HPLC (Phenomenex Luna 5 μm C18(2), 100 Å, 150 × 10 mm, 5 mL/min, 5% ethanol).[18] The collected peak (t_R: 7 min, 3.5–4.0 mL) was diluted with 0.1 mL 35% NaCl solution and 5 mL PBS, pH 7. Analysis by radio-HPLC indicated >99% radiochemical purity and specific activity was 100–200 GBq/μmol.

2.5.2. 2-[^{18}F]Fluoroethylazide

In the sealed reaction vessel, 2-azidoethyl 1*H*,1*H*,2*H*,2*H*-perfluorodecane-1-sulfonate (10 mg) in 0.6 mL anhydrous acetonitrile was added to and heated for 10 minutes at 110 °C. The crude solution was cooled down to 50 °C, diluted with 1.5 mL water and eluted through neutral alumina (Sep-Pak light, Waters) and fluorous silica gel (SPE cartridge, 100 mg, 40 μm, Fluorous Technologies Inc.).

HPLC analysis (Phenomenex Gemini NX, C18, 150 × 4.6 mm, water:ethanol gradient 100:0 to 40:60 in 10 min, 1 mL/min) indicated a radiochemical purity of 2-[^{18}F]fluoroethylazide >95%. The non-decay-corrected radiochemical yield was >50% estimated from the cyclotron yield and the obtained radioactivity. Noteworthy, the azide is volatile and cannot be analyzed by radio-TLC.

2.5.3. Conjugation of 2-[^{18}F]fluoroethylazide with nitroaromatics

To propargyl-functionalized nitroaromatics (2.0 ± 0.5 mg) in acetonitrile (100 μL) was added aqueous solutions of 1 M copper(II) sulfate (20 μL) and 1 M sodium ascorbate (20 μL) and 2-[^{18}F]fluoroethylazide (200 μL, 100 MBq). The mixture was stirred for 30 min at room temperature and further purified by HPLC (Phenomenex Gemini NX, C18, 150 × 4.6 mm, water:ethanol gradient 100:0 to 40:60 in 10 min, 1 mL/min) using an autosampler and a fraction collector. The collected fraction was directly diluted with PBS at 0.5–1.0 MBq/mL. Analysis by radio-HPLC indicated >95% radiochemical purity. Specific activity was determined in the range of 0.1–2 GBq/μmol.

2.6. In vitro assay

MCF-7 cells, a human breast adenocarcinoma cell line, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% *penicillin–streptomycin (P/S)*. Prior to the uptake experiment, MCF-7 cells (2×10^5 cells/mL) were seeded in 24-well plates (0.5 mL, 100,000 cells) and cultured for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. Cells are cultured at normoxic or hypoxic conditions for 4 h. Hypoxic conditions were obtained as described previously[19]: cells were placed in a humidified air-tight chambers (Billubs–Rothenberg) and a hypoxic air mixture (5% CO_2/95% N_2) was infused into the air-tight chambers at a flow rate of 10 L/min for 15 min. After closing the inflow and outflow valves, the humidified air-tight chamber was kept at 37 °C for 4 h.

^{18}F-labeled nitroaromatics (0.5–1.0 MBq/mL in PBS; 0.1–2 GBq/μmol,[20] were added to the normoxic and hypoxic cells (50 μL) and incubated for 1 h at 37 °C: normoxic cells in a humidified atmosphere of 5% CO_2 and hypoxic cells in a humidified air-tight chambers after infusion of the hypoxic air mixture at a flow rate of 10 L/min for 15 min. Subsequently, cells were washed twice with PBS and lysed in 0.5 mL 1 M sodium hydroxide for 30 min. Cell lysates were counted with an automatic gamma counter (2470 Wizard[2], Perkin Elmer).

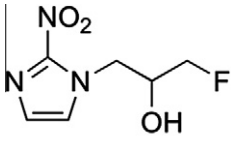
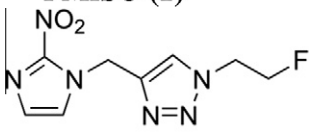
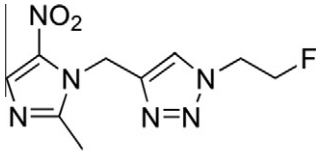
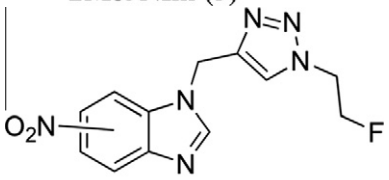
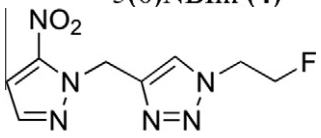
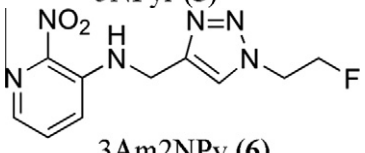
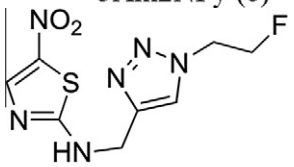
The radioactivity associated with the cell was calculated as the percentage of added radiopharmaceuticals. Each compound was tested using both normoxic and anoxic conditions in 6× replicates.

The washout assay was performed by replacing the radioactive media after 1 h incubation with fresh RPMI 1640 (10% FBS, 1% *P/S*) and incubation for 1 h under normoxic or hypoxic conditions. Subsequently, cells were washed with PBS and cell lysate counted with an automatic gamma counter as described previously.

3. Results and discussion

The fluorous-click approach is exemplified in this paper by the readily preparation of a library of ^{18}F-radiolabeled chemicals and screening for their ability to bind hypoxic cells. In detail, the radiolabeled synthon 2-[^{18}F]fluoroethylazide was synthesized by nucleophilic ^{18}F-fluorination of the polyfluorinated sulfonate precursor and purified rapidly by elution through alumina and fluorous silica gel.[7] The synthon was subsequently conjugated to propargyl-functionalized molecules in the copper(I) catalyzed Huisgen [3+2] cycloaddition ('click reaction'). Various substrates were hence successfully ^{18}F-radiolabeled without the need for protective groups nor intensive radiochemical optimization, and could be further evaluated in vitro. Six nitroaromatic substrates have

Table 1
Physico-chemical properties of various fluorinated nitroaromatics

Entry	Structure	Lipophilicity log*P*[a]	Redox potential $E_{1/2}$ (vs SCE) (V)[a]	XOD reduction rate (nmol/min/unit enzyme)[a] [λ/extinction coefficient]
1	 FMISO (**1**)	−0.33 ± 0.02	−0.98 ± 0.02	37 ± 7 [325 nm/7.8 mM^{-1} cm^{-1}]
2	 2NIm (**2**)	−0.41 ± 0.01	−1.05 ± 0.01	36 ± 2 [325 nm/7.8 mM^{-1} cm^{-1}]
3	 2Me5NIm (**3**)	−0.38 ± 0.02	−1.37 ± 0.01	5 ± 1 [325 nm/6.2 mM^{-1} cm^{-1}]
4	 5(6)NBIm (**4**)	0.94 ± 0.03	−1.21 ± 0.01	11 ± 2 [325 nm/6.6 mM^{-1} cm^{-1}]
5	 5NPyr (**5**)	0.03 ± 0.05	−1.21 ± 0.01	20 ± 10 [278 nm[b]/6.4 mM^{-1} cm^{-1}]
6	 3Am2NPy (**6**)	0.04 ± 0.01	−1.12 ± 0.01	58 ± 11 [415 nm/7.0 mM^{-1}. cm^{-1}]
7	 2Am5NTh (**7**)	0.79 ± 0.06	−1.07 ± 0.02	277 ± 38 [395 nm/9.2 mM^{-1} cm^{-1}]

[a] Carried out in triplicates (mean value ± SD).
[b] Isosbestic point.

been selected according to their reported properties and their commercial availability. Noteworthy, an ^{18}F-labeled derivative of 2-methyl-4-nitroimidazole had been used for biodistribution studies[21]; a ^{99m}Tc complex of 6-nitrobenzoimidazole had been evaluated in vitro as a hypoxia marker,[22] and a derivative had been evaluated as a radiosensitizing agent in vitro[23]; finally 2-amino-5-nitrothiazole and derivatives of 3-nitropyrazole had been evaluated as radiosensitizing agents in vitro.[24–26]

Non-radioactive reference compounds were synthesized and their physico-chemical properties determined: lipophilicity, redox potential and xanthine oxidase (XOD) reduction rate (Table 1). The lipophilicity of such compounds was actually proven to be affecting the pharmakinetics (e.g., tumor penetration) of radiosensitizers and radiopharmaceuticals.[27,28] The enzymatic anaerobic reduction is also correlated to the reduction potential of the one-electron reduction to the nitro-radical anion.[29] With redox potential $E_{1/2}$ (vs SCE) comprised between −1.37 and −0.98 V, it is likely that those compounds can accept electrons in anaerobic conditions, depending on the enzyme-substrate affinity (K_m not measured). Hence the compound with highest electron affinity amongst the evaluated ones was determined to be the 2-amino-5-nitrothiazole (**7**) with a nitroreduction rate of 277 ± 38 nmol min^{-1} U^{-1} xanthine

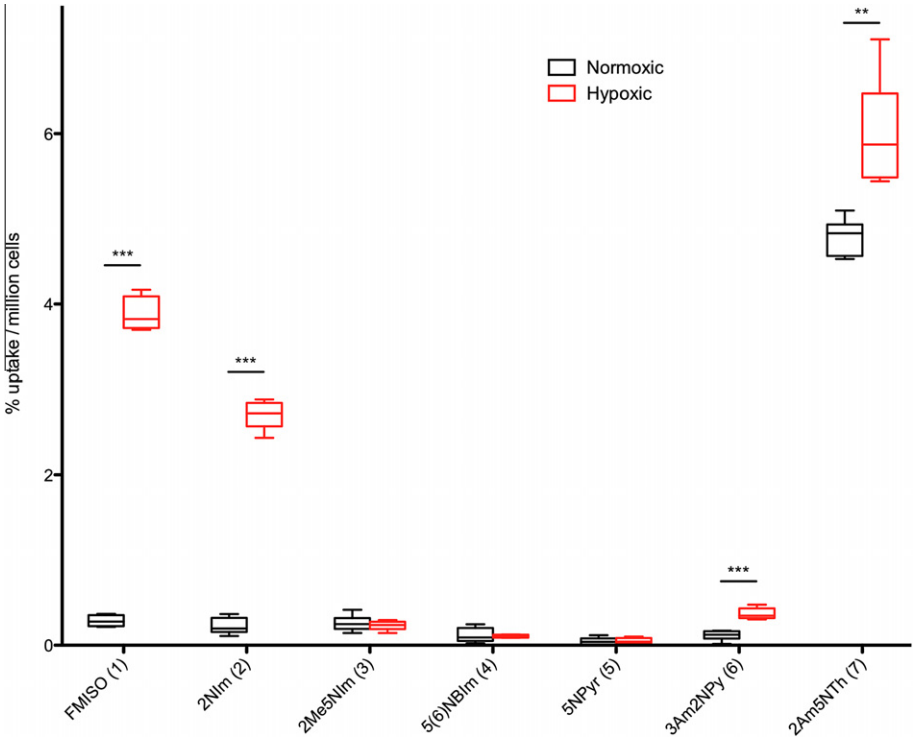


Figure 1. Cellular uptake of ^{18}F-labeled nitroaromatics in normoxic and hypoxic conditions (***P <0.0001; **P <0.001).

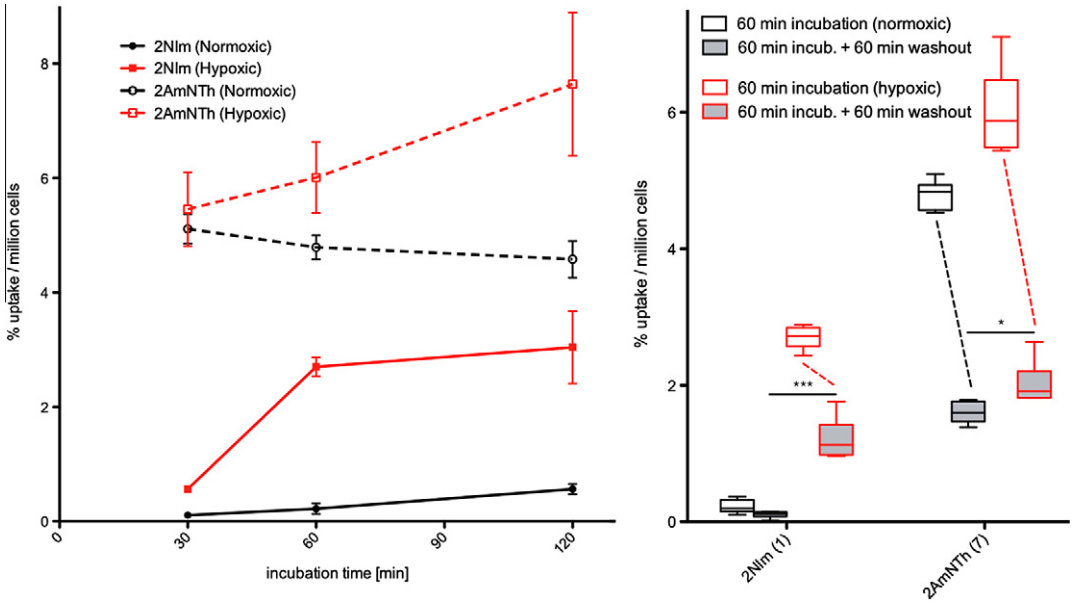


Figure 2. Time course uptake of **2** and **7** (left). Wash-out of **2** and **7** after 60 min incubation (right). (***P <0.0001; *P <0.05).

oxidase. Compounds **1**, **2** and **6** proved to have a medium electron affinity, and compounds **3**, **4** and **5** to have the lowest.

2-[^{18}F]Fluoroethylazide, whose synthesis and purification were recently reported using the fluorous technology,[7] was used to access ^{18}F-labeled compounds for screening. A single batch of 2-[^{18}F]fluoroethylazide could be readily synthesized and purified by FSPE within a shielded hot cell, using a remotely controlled module (GE Tracerlab FX_{FN}). FSPE-purified aliquots of 2-[^{18}F]fluoroethylazide could then be used for parallel conjugation with various propargyl-functionalized nitroaromatics, within a shielded fume hood. Further purification with an automated HPLC afforded the various ^{18}F-labeled nitroaromatic-based molecules for evaluation of their uptake within both hypoxic and normoxic cells (Fig. 1). ^{18}FMISO was also prepared and used to validate the cell uptake assay.

From the in vitro cell assays, it appears that three compounds are worth mentioning. The substrates with a medium-to-high electron affinity (**2**, **6** and **7**) display a significant uptake difference between hypoxic and normoxic cells, whereas those with a lower electron affinity (**3**, **4** and **5**) don't display any hypoxic selectivity (Fig. 1). As expected from the various well-validated 2-nitroimidazolyl hypoxia biomarkers (e.g., ^{18}FMISO, [^{18}F]HX4), the 2-nitroimidazole derivative (**2**) has significant hypoxia selectivity. The new substrate 3-amino-2-nitropyridine (**6**) exhibits hypoxia selectivity as well but the uptake is much lower than the reference compound ^{18}FMISO. This lead compound could eventually be a candidate for optimization, especially improving the transport through the cell membrane. 2-Amino-5-nitrothiazole (**7**) had been shown to have radiosensitizing properties due to its electron affinity.[26] In this study, the 2-amino-5-nitrothiazole derivative displays a high uptake within both hypoxic and normoxic cells. But it turns out that most of the 5-nitrothiazole-related radioactivity within cells isn't irreversibly bound as exemplified by the washout experiment (Fig. 2). Finally, the high electron affinity of **7** may explain the low selectivity between hypoxic and normoxic cells
(P <0.001): if a substrate is reduced too rapidly, it may actually accumulate and bind to cellular macromolecules irrespective of the oxygenation of the cell.

4. Conclusion

We have already demonstrated that multiple radiolabeled PET probes can be obtained from a single radiolabeled precursor by application of the same conjugation strategy: the radiolabeled 2-[^{18}F]fluoroethylazide synthon has actually proven valuable in the catalyst-free traceless Staudinger ligation process, providing ^{18}F-radiolabeled molecules through the formation of an amide bond.[30] In this paper, the same 2-[^{18}F]fluoroethylazide synthon benefits from the Cu(I)-mediated 1,3-dipolar cycloaddition ('click') for radiolabeling multiple PET probes with high reaction specificity. The strategy doesn't require lengthy radiochemistry optimization for every compound of interest nor protective groups, as usually encountered in the challenging ^{18}F-radiochemistry. Furthermore purification of the final ^{18}F-radiopharmaceutical is achieved remotely using an HPLC, equipped with an autosampler and a fraction collector. An important feature of the method is its operational simplicity and potential to be automatized: the fluorous ^{18}F-fluorination and SPE purification of the ^{18}F-labeled prosthetic group combined with the 'click' reaction represent a straightforward and very powerful approach to access novel radiolabeled compounds for in vitro screening. And as exemplified with the proof of concept hypoxic-selectivity assay, the fluorous-click method could eventually allow for identification of membrane permeable and oxygene-sensitive biomarker candidates for ^{18}F-PET.

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Supplementary data

Supplementary data (details of synthesis and characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.084.

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