Synthesis and Radiosynthesis of Prospective 2-Nitroimidazole Hypoxia PET Tracers via Thiazolidine Ligation with 5-Fluorodeoxy-ribose (FDR)

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Abstract The first prospective fluorinated PET tracers for imaging hypoxia obtained via thiazolidine-ligation are reported. Three 1,2-thiol-amine linkers were combined with four different 2-nitroimidazole spacers via amide or urea bond formation. The resulting compounds were submitted to thiazolidine-ring-forming ligation reaction with the fluorinated carbohydrate L-5-fluoro-5-deoxy-ribose (FDR), affording the desired candidate PET tracers in variable yields. The same ligation reactions performed on L-ribose – a by-product of [18F]FDR radiosynthesis – under conditions mimicking a radiochemical production showed that the fluorinated adducts can be efficiently purified and isolated by HPLC. Finally, one of the prospective hypoxia tracers was successfully produced in radiolabelled form in 29.2% radiochemical yield from [18F]FDR.

Key words hypoxia, radiofluorination, thiazolidines, nitroimidazole, bio-orthogonal ligation

Hypoxia occurs in cells and tissues when oxygen demand exceeds supply.1,2 The irregular vasculature typical of solid tumours does not sufficiently support cellular oxygen demand, leading to the development and progression of heterogeneous hypoxic cancer areas, which are generally poorly responsive to chemo- and radio-therapies.3,4 Accurate imaging of hypoxic regions could allow clinicians to stratify patients and develop more efficient treatment strategies to improve therapeutic outcomes.5,6 Identification and quantification of hypoxic areas in tumours – and in other pathologies – is therefore important for planning the most appropriate and personalised therapeutic approach.7,8 Owing to its high sensitivity and non-invasive nature, PET imaging is emerging as the method of choice for in vivo identification, characterization and discrimination of hypoxic areas.8,9 In the last two decades, several PET tracers for hypoxia have been described, but all of them are affected by significant drawbacks, such as low signal-to-noise ratios, slow accumulation in hypoxic regions and poor brain uptake, therefore the development of new hypoxia-targeted PET tracers remains a very active area of research.10,11 L-5-Fluoro-5-deoxy-ribose ([18F]FDR) 2 (Figure 1) has recently emerged as a promising prosthetic group for rapid, indirect radiolabelling of bioactive molecules via oxime bond formation.12–15

As an alternative to the oxime bond, thiazolidine ring formation could be used as a site-specific ligation method via reaction of a 1,2-thiol-amine function with a carbonyl group – including masked carbonyls of carbohydrates and hemiacetals – in mildly acidic or basic conditions (pH 4 to 8).16–18 Importantly, the thiazolidine ring is generally stable in a wide pH range (from 4 to 10), thus representing an attractive linkage option. In order to further investigate the efficiency of [18F]FDR as a radiolabelling agent and expand the library of prospective PET tracers for hypoxia imaging, we designed a novel class of candidate tracers [18F]FDR 1 (Figure 1) taking advantage of the last-step formation of a thiazolidine ring linkage between [18F]FDR 2 and terminal 2-amino-thiols 3 carrying a hypoxia-reactive 2-nitroimidazole group.
Three different 2-aminoethanethiol linkers 4a–c (Scheme 1) were selected to modulate the steric constraints and lipophilicity of the final candidate tracers. The synthesis was based on the conditions described by Duthaler et al.\textsuperscript{19} A mixture of racemic cysteine 5 and conc. HCl in acetone was heated at reflux for 6 h, affording the thiazolidine intermediate 6. Samples of 6 were invariably found (by \textsuperscript{1}H NMR spectroscopy) to contain 5–10\% of cysteine hydrochloride 7. The mixture of 6 and 7 was allowed to react with (Boc)\textsubscript{2}O in pyridine for 3 days to give the N-Boc-derivative 4a.\textsuperscript{19} NMR spectroscopy showed that this compound exists as a mixture of rotamers, the signals of which did not show coalescence at 60 °C either in CDCl\textsubscript{3} or in CD\textsubscript{3}OD. Compound 4a was converted into the Weinreb amide 8 by reaction with HATU and DIPEA, followed by addition of N,O-di-methylhydroxylamine hydrochloride. Reduction of 8 using LiAlH\textsubscript{4} at 0 °C provided in good yield the aldehyde 9, which was submitted to Wittig reaction with the phosphonium ylide Ph\textsubscript{3}P=CHCO\textsubscript{2}Me to give exclusively the \textit{trans} isomer of the \textit{\alpha,\beta}-unsaturated ester 10.\textsuperscript{20} Hydrogenation reaction of 10 using H\textsubscript{2} over Pd/C catalyst gave in quantitative yield the saturated intermediate 11, which afforded the free carboxylic acid 4b\textsuperscript{21} by basic hydrolysis of the ester function. The carbinol 12 was obtained upon treatment of 11 with LiAlH\textsubscript{4} at 0 °C, whereas the amine derivative 4c\textsuperscript{22} was obtained via Mitsunobu reaction of phthalimide with 12 to give compound 13, followed by phthalimide-ring cleavage with hydrazine monohydrate.

The 2-nitro-imidazole spacers 14a–d (Scheme 2 and Scheme 3) were selected with the aim of introducing structural diversity within the series. The structure of the spacer was expected to have an important effect on lipophilicity, metabolic stability and ultimately on the imaging potential of the candidate tracers 1.

Amines 14a,b were synthesised via Gabriel reaction (Scheme 2) starting respectively from commercial 1,3-dibromopropane (15a) and 1,5-dibromopentane (15b). The resulting phthalimides 16a,b\textsuperscript{23} were reacted with 2-nitroimidazole and K\textsubscript{2}CO\textsubscript{3} in DMF upon heating to 115 °C to give compounds 17a,b\textsuperscript{24} in good yields. The desired amines 14a,b were obtained by quantitative cleavage of the phthalimido group with hydrazine. The 1,2,3-triazole-amine 14c was prepared via Huisgen cycloaddition reaction between the azide 18, which was obtained by bromine displacement reaction of 16a with sodium azide\textsuperscript{25} and 1-propargyl-2-nitroimidazole 19, which was prepared according to the literature,\textsuperscript{26} to afford phthalimide derivative 20. Removal of the phthalimido group with hydrazine gave compound 14c in good overall yield.
2-Nitro-imidazolyl-acetic acid 14d\(^27\) (Scheme 3) was prepared in four steps starting from 21, which provided compound 22 after protection of the hydroxy group as tetrahydropyranyl acetal (THP) followed by introduction of the 2-nitroimidazole function in K\(_2\)CO\(_3\) and DMF upon heating to 115 °C. The resulting intermediate 23 was then dissolved in a 6 M aq. HCl solution in MeOH to cleave the THP group, followed by treatment of the resulting carbinol 24 with Jones reagent (CrO\(_3\)/H\(_2\)SO\(_4\)/acetone) in acetone to give the desired compound 14d in 41% yield over the three steps.

Assembling of linkers 4a–c and spacers 14a–d to give the tracers’ precursors 3a–f is shown in Scheme 4. Treatment of carboxylic acid derivatives 4a–c with HATU and DIPEA gave the corresponding activated esters, which were reacted in situ with the amines 14a–d to afford the amides 25a–f.\(^28\) Different conditions were used to prepare the urea derivative 25f.\(^29\) In this case, the amine 4c was added drop-wise to a solution of carbonyldiimidazole (CDI) in CH\(_2\)Cl\(_2\) at 0 °C to give the intermediate imidazocarboxyamide, which gave the desired urea 25f upon in situ treatment with the amine 14a.

The final unprotected 2-aminoethanethiol derivatives 3a–f\(^6\) (Scheme 4) were obtained by treatment of 25a–f with a TFA/H\(_2\)O/MeOH 3:2:1 mixture upon heating to 65 °C for 2–4 h, followed by solvents removal under reduced pressure at 60 °C. Then the crude compounds were dissolved in ethanol (except compound 3c, which is only soluble in aqueous solutions) and eluted through a SiliaBond\(^6\) carbonate pad (silica bound equivalent of tetramethylammonium carbonate), which trapped residual TFA, acid by-products and free-based 2-nitroimidazolium trifluoroacetate salts formed during the thiazolidine hydrolysis. In all cases, variable amounts of disulphide dimers were obtained in mixture with the desired thiol monomers 3a–f, as evidenced by both HPLC/MS analysis and NMR spectroscopy. However, we did not attempt to purify further the samples, as the disulphide dimers could be readily reduced back to the monomeric thiols by treatment with 1,4-dithiothreitol (DTT) before the following ligation reaction with FDR 2 (Scheme 5).

The thiazolidine ring formation was performed by reaction of 3a–f with cold \[^{19F}\]FDR 2 using 1 M acetate buffer as reaction medium in the presence of DTT. Acetate buffers with different molarity (from 0.1 to 4.0) and pH (from 3 to 6) were tested at different temperatures (from r.t. to 50 °C) in order to optimise the thiazolidine ring formation rate. The optimised conditions were 2.5 equiv of 3a–f reacted with 1 equiv of \[^{19F}\]FDR(2) in the presence of 2.5 equiv of DTT, using 1 M acetate buffer at pH 4.5 as reaction medium.
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for 20 min at 30 °C. The purification step was performed by gradient RP-HPLC using a mixture of H2O/ACN + 0.05% (v/v) of TFA as eluent. 1,2-Aminothiol derivatives 3a–f showed markedly different reactivity towards [19F]FDR 2, showing that the spacers’ structure plays an important role in the cyclisation reaction (see Table 1 for yields). Only the 1,2-thiol-amine derivative 3c, incorporating a triazole ring, failed to react under all the conditions explored, affording in very low yields (<5%) the corresponding thiazolidine 1c, which could not be isolated in pure form by RP-HPLC purification.

The radiosynthesis of [18F]FDR 2 is known to produce an excess of L-ribose 26 as by-product, which, although less reactive than 2, will compete with it in the thiazolidine ring formation, affording the corresponding non-fluorinated thiazolidines 27a–f (Scheme 5) and decreasing the chemical purity of the tracer.

**Scheme 4** Synthesis of the 2-amino-thiol tracer precursors 3a–f. Reagents and conditions: (a) HATU, DIPEA, CH2Cl2, r.t., 18 h; (b) CDI, CH2Cl2, from 0 °C to r.t., 18 h; (c) TFA/H2O/MeOH 3:2:1, from r.t. to 65 °C, 2–4 h.
To simulate the radiosynthesis conditions, the thiazolidine ring formation reaction was carried out in the presence of 10 equiv of 26 along with 1 equiv of \([^{19}\text{F}]\text{FDR}\) and 1,2-aminothiol derivatives 3a–f. This experiment was performed with the aim of assessing the formation of the desired FDR thiazolidines in the presence of L-ribose 26 and the possibility of performing an HPLC purification for separating the \([^{18}\text{F}]\text{FDR}\)-derived tracers 1a–f from the non-radioactive l-ribose-derived thiazolidines 27a–f. As shown in Table 1, as well as in the HPLC profiles (see the Supporting Information), the retention times of the target FDR-thiazolidines 1a–f are indeed significantly different to those of the ribose-derived thiazolidines 27a–f.

Therefore, the final cold tracers \([^{19}\text{F}]\text{1a–f}\) could be isolated and characterised by LC-MS. Their Log P values were determined by RP-HPLC (isocratic phase H2O/EtOH 90:10). Considering that the gold standard hypoxia tracer \([^{18}\text{F}]\text{FMI-SO}\) has a Log P = 0.42, candidate tracers 1 appear to have suitable lipophilicity for use in vivo. Thiazolidines 1a–f presented very complex NMR spectra owing to the presence of four diastereomers, originated by the two (R/S) thiazolidine stereogenic centres, plus different rotamers and trifluoroacetate salts. Only compound 1a\(^{31}\) was isolated in sufficient quantity for being satisfactorily characterised by NMR spectroscopy, after treatment with SiliaBond® carbonate in order to freebase the trifluoroacetate salts.

Radiolabelling tests for producing \([^{18}\text{F}]\text{1a}\)\(^{32}\) were conducted on the 1,2-aminothiol derivative 3a, which was treated with \([^{18}\text{F}]\text{FDR}\) (2)\(^\text{12,13}\) using a sodium acetate buffer solution (Scheme 6). Also in this case, different reaction conditions were tested with the aim of achieving the maximum radiochemical conversion within 40 minutes (see Table 1S, Supporting Information).

Eventually, we found that the use of a 6 M acetate buffer solution (70% v/v concentration) in the reaction mixture containing \([^{18}\text{F}]\text{FDR}\) (2), 3a and DTT (1:1) (in the range 2–4 M) at pH 4.5, provided the highest RCY (29.2%, decay corrected). No further improvements could be achieved by changing buffer concentration, pH or extending further the reaction time.

The tracer identity was confirmed by superimposition of the UV-HPLC profile of the cold reference \([^{19}\text{F}]\text{1a}\) with the semi-preparative RP-HPLC radio-chromatogram of \([^{18}\text{F}]\text{1a}\), acquired before purification of the radiotracer (Figure 2).

In conclusion, we have designed and synthesised the first candidate PET tracers (1) for hypoxia imaging based on the use of \([^{18}\text{F}]\text{FDR}\) 2 as radiolabelling agent. The synthesis is based on the formation of a thiazolidine-ring-linkage between \([^{18}\text{F}]\text{FDR}\) 2 and 1,2-thiol-amines 3, which occurs with moderate to good efficiency depending on the structure of spacer and linker featured in 3. The method was successfully tested for the radiosynthesis of \([^{18}\text{F}]\text{1a}\), which was produced in 29.2% radiochemical yield and successfully purified by RP-HPLC.
Figure 2 In black: semi-prep RP-HPLC Radio-analysis of radiotracer $[^{18}F]$FDR (2) formation using 70% v/v of a 6 M acetate buffer solution in the aqueous solution of $[^{18}F]$FDR. In red: superimposed UV chromatogram of the cold reference $[^{19}F]$FDR obtained using the same RP-HPLC conditions.

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### Supporting Information
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### References and Notes

21. Synthesis of 4b: An aqueous 1 M LiOH solution (1.64 mL, 1.64 mmol) was added at r.t. to a solution of 11 (200 mg, 0.66 mmol) in THF (1.7 mL). The reaction mixture was stirred for 18 h at r.t. and then neutralised with a 1 M aq. HCl solution, then extracted in THF (1.7 mL). The reaction mixture was stirred for 18 h at r.t.
22. Synthesis of 4c: Hydrazine monohydrate (166 mg, 9.74 mmol) was added at r.t. to a solution of 1.64 mL, 1.64 mmol of 4b in THF (1.7 mL). The reaction mixture was stirred for 18 h at r.t. and then neutralised with a 1 M aq. HCl solution, then extracted in THF (1.7 mL), dried and concentrated under reduced pressure to afford 4c (186 mg, 97.4%).
23. NMR (CDCl 3, 400 MHz): δ = 9.07 (br, 1 H), 4.35 (br, 1 H), 3.12 (dd, J = 11.9, 5.9 Hz, 1 H), 2.58 (d, J = 11.9 Hz, 1 H), 2.39–2.19 (m, 2 H), 2.12–1.93 (m, 2 H), 1.72 (s, 6 H), 1.45 (s, 9 H). 13C NMR (CDCl 3, 100 MHz): δ = 178.9, 152.7, 69.4, 63.7, 61.2, 31.3, 30.9, 30.2, 29.6 (2C), 28.4 (3C) MS (ESI): m/z calcd for C 13H 23NO 4S: 290.2 [M+H] +, 312.1 [M+Na] +, found: 290.2 [M+H] +, 312.1 [M+Na] +
25. NMR (CDCl 3, 400 MHz): δ = 4.16 (br, 1 H), 3.00 (dd, J = 11.6, 6.1 Hz, 1 H), 2.65–2.54 (m, 2 H), 2.47 (d, J = 11.6 Hz, 1 H), 1.73–1.49 (m, 4 H), 1.60 (s, 6 H), 1.44–1.41 (m, 2 H), 1.33 (s, 9 H), 13C NMR (CDCl 3, 100 MHz): δ = 152.3, 79.8, 69.4, 64.1, 41.8, 31.3, 30.9, 30.2, 29.6 (2C), 28.4 (3C). MS (ESI): m/z calcd for C 13H 26N 2O 4S: 275.1 [M+H] +, 297.2 [M+Na] +, 303.2 [M+K] +; found: 275.2 [M+H] +, 297.2 [M+Na] +, 303.1 [M+K] +
30. A shorter synthesis of 14d has been reported, see: Joyard Y., Azzouz R., Bischoff L., Papamicael C., Labar D., Bol A., Bol V., Vera P., Grégoire V., Levacher V., Bohn P.; Bioorg. Med. Chem. 2013,
Synthesis of 25a: A solution of sodium carbonate (10% w/w) in EtOH, under gentle stirring for 1 h in order to freebase trifluoroacetate salt. 1H NMR (CD3OD, 400 MHz, – four diastereoisomers – two major isomers in –3:2 ratio were identified): δ = 7.55 (d, J = 1.2 Hz, 1 H), 7.16 (d, j = 1.2 Hz, 1 H), 4.89–4.83 (m, 1 H), 4.62–4.42 (m, 4 H), 4.25 (dd, J = 7.0, 6.8 Hz, 1 H), 4.09–4.04 (m, 1 H), 3.91 (dd, J = 7.4, 4.6 Hz, 1 H), 3.66 (dd, J = 7.4, 5.8 Hz, 1 H), 3.39–3.23 (m, 3 H), 3.02–2.91 (m, 1 H), 2.16–2.05 (m, 2 H); δ (second isomer) = 7.57 (d, J = 1.2 Hz, 1 H), 7.17 (d, j = 1.2 Hz, 1 H), 4.92 (d, J = 2.6 Hz, 1 H), 4.67 (dd, J = 9.8, 3.0 Hz, 1 H), 4.63–4.42 (m, 4 H), 4.25 (dd, J = 7.0, 6.8 Hz, 1 H), 4.04–3.95 (m, 1 H), 3.85–3.75 (m, 2 H), 3.39–3.14 (m, 3 H), 3.02–2.91 (m, 1 H), 2.16–2.05 (m, 2 H). 13C NMR (CD3OD, 100 MHz, – four diastereoisomers – two major isomers in –3:2 ratio were identified): δ (first isomer) = 172.5, 144.7, 127.2, 127.1, 84.3 (dd, J = 12.5 min) gave 25a as a yellow oil. 1H NMR (CDCl3, 400 MHz, mixture of rotamers): δ = 7.35 (br, 1 H), 7.02 (br, 1 H), 5.55 (br, 2 H), 4.40 (t, J = 6.9 Hz, 2 H), 4.19 (br, 1 H), 3.24–2.94 (m, 5 H), 2.49 (d, J = 11.8 Hz, 1 H), 2.05–1.86 (m, 2 H), 1.81–1.66 (m, 2 H), 1.63 (s, 3 H), 1.61 (s, 3 H), 1.48–1.26 (m, 11 H). 13C NMR (CDCl3, 100 MHz, 2 rotamers): δ = 159.0, 152.7, 144.6, 128.2, 127.1, 80.3, 69.4, 64.0, 47.8, 39.8, 39.6, 36.3, 31.6, 31.4, 30.6, 30.1, 29.6, 28.4 (3C), 27.3. MS (ESI): m/z calcd for C14H22FN5O6S: 436.2 [M+Na]⁺, 452.0 [M+K]⁺; found: 436.1 [M+Na]⁺, 452.0 [M+K]⁺.

Synthesis of 25f: A solution of 4e (152 mg, 0.56 mmol) in CH2Cl2 (2 mL) was added dropwise to a solution of CDI (90 mg, 0.56 mmol) in anhydrous CH2Cl2 (3 mL) at 0 °C under N2 atmosphere, then the mixture was allowed to react at rt. for 1 h. After 16 h under stirring, the mixture was added via syringe to a solution of 14a (226 mg, 1.33 mmol) in CH2Cl2 (3 mL) under N2 atmosphere. After 16 h under stirring the mixture was concentrated under reduced pressure. Purification by flash chromatography (Hex/EtOAc, from 3:2 to 7:3) to afford 25f (229 mg, 72.3%) as a yellow oil. 1H NMR (CDCl3, 400 MHz, mixture of rotamers): δ = 7.32 (s, 1 H), 7.08 (s, 1 H), 6.55 (br, 1 H), 4.72 (br, 1 H), 3.42–3.11 (m, 4 H), 1.82 (s, 3 H), 1.73 (s, 3 H), 1.42 (s, 9 H). 13C NMR (CDCl3, 100 MHz): δ = 171.7, 153.3, 144.7, 128.4, 127.0, 81.8, 71.4, 67.5, 47.4, 36.0, 31.0, 29.3, 28.9, 28.4 (3C). MS (ESI): m/z calcd for C12H21N2O4S: 343.2 [M+Na]⁺, 359.2 [M+K]⁺; found: 343.1 [M+Na]⁺, 359.2 [M+K]⁺.

Optimised radiosynthesis of [18F]1a: A solution of sodium acetate buffer (pH 4.5), was added to a solution of 3a (25 mg, 64 μmol), DTT (1 mg, 64 μmol) and [18F]1 (3.5–7.0 MBq) in 0.1–0.5 mL of H2O to form a 70% v/v sodium acetate buffer solution (final concentration 4.2 M). After ~20 min the mixture was purified by RP-HPLC (Column: Phenomenex Luna C18 250 × 10.00 mm, 5 μm; mobile phase: A (H2O + 0.05% TFA), B (ACN + 0.05% TFA); gradient: from 5% B to 60% B in 15 min; flow: 5 mL min⁻¹; tR: 12.5 min) gave 1a as a white solid (75.6 mg, 84.4%). NMR analyses were performed after treatment of 1a with SilaBond® carbonate (10% w/w) in EtOH, under gentle stirring for 1 h in order to freebase trifluoroacetate salt. 1H NMR (CD3OD, 400 MHz, – four diastereoisomers – two major isomers in ~3:2 ratio were identified): δ = 7.55 (d, J = 1.2 Hz, 1 H), 7.16 (d, J = 1.2 Hz, 1 H), 4.89–4.83 (m, 1 H), 4.62–4.42 (m, 4 H), 4.25 (dd, J = 7.0, 6.8 Hz, 1 H), 4.09–4.04 (m, 1 H), 3.91 (dd, J = 7.4, 4.6 Hz, 1 H), 3.66 (dd, J = 7.4, 5.8 Hz, 1 H), 3.39–3.23 (m, 3 H), 3.02–2.91 (m, 1 H), 2.16–2.05 (m, 2 H); δ (second isomer) = 7.57 (d, J = 1.2 Hz, 1 H), 7.17 (d, j = 1.2 Hz, 1 H), 4.92 (d, J = 2.6 Hz, 1 H), 4.67 (dd, J = 9.8, 3.0 Hz, 1 H), 4.63–4.42 (m, 4 H), 4.25 (dd, J = 7.0, 6.8 Hz, 1 H), 4.04–3.95 (m, 1 H), 3.85–3.75 (m, 2 H), 3.29–3.14 (m, 3 H), 3.02–2.91 (m, 1 H), 2.16–2.05 (m, 2 H). 13C NMR (CD3OD, 100 MHz, – four diastereoisomers – two major isomers in ~3:2 ratio were identified): δ (first isomer) = 172.5, 144.7, 127.2, 127.1, 84.1 (d, JCF = 167 Hz), 73.6 (d, JCF = 7 Hz), 72.3, 72.1, 71.8 (d, JCF = 18 Hz), 71.4, 65.8, 35.8, 34.9, 30.0; δ (second isomer) = 172.4, 144.7, 127.2, 127.1, 84.3 (d, JCF = 167 Hz), 73.6 (d, JCF = 7 Hz), 72.3, 72.0, 71.9, 71.9 (d, JCF = 18 Hz), 70.2, 66.2, 36.4, 34.9, 29.9. 18F NMR (376 MHz, CD3OD): δ (first isomer) = -233.0 (dt, J = 48.0, 22.7 Hz); δ (second isomer) = -233.6 (dt, J = 48.0, 22.7 Hz); MS (ESI): m/z calcd for: C14H20FN5O5S18F: 408.1 [M+Na]⁺, 430.1 [M+Na]⁺; found: 408.0 [M+Na]⁺, 430.0 [M+Na]⁺.