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Research Article

Preparation of the iodine-124 derivative of the Bolton–Hunter reagent ([^{124}I]I-SHPP) and its use for labelling a VEGF antibody as a PET tracer

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Summary

This study describes the radioiodination of an antibody specific to the vascular endothelial growth factor (VEGF), VG76e, with [^{124}I]iodine to obtain a novel PET tracer for measurement of angiogenesis. *In vitro* binding assays showed a significantly higher immunoreactive fraction with the protein labelling reagent *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate ([^{125}I]Bolton–Hunter reagent, [^{125}I]I-SHPP) (34.0 $\pm$ 4.0%) as compared with *N*-succinimidyl 3-[^{125}I]iodobenzoate (10.9 $\pm$ 6.4%) or direct radioiodination using [^{125}I]iodide and IodoGen (3.1 $\pm$ 3.0%). Consequently, the cyclotron–produced positron–emitting [^{124}I]iodine ($T_{1/2}$ = 4.2 days) was employed to prepare [^{124}I]I-SHPP.

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Using an improved radioiodination methodology, [^{124}I]I-SHPP was prepared from sodium [^{124}I]iodide with IodoGen at pH 6.5. The [^{124}I]Bolton–Hunter reagent was isolated with 25–58% ($n=3$) radiochemical yield and 88–95% ($n=3$) radiochemical purity by the conventional extraction procedure. The conjugate of VG76e with [^{124}I]I-SHPP was prepared with 17–18% (n=3) labelling efficiency and 98% radiochemical purity. The immunoreactive fraction was determined to be 33.5% ($n=2$). Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: ^{124}I; Bolton–Hunter reagent; antibody labelling; VEGF; angiogenesis

Introduction

Angiogenesis is defined as the formation of new blood vessels from pre-existing vasculature. It is involved in complex biological processes such as wound healing, inflammatory disorders and tumor growth. Anti-angiogenic therapy is therefore a promising approach for targeting cancer. The success of the angiogenesis inhibitors such as angiostatin and endostatin in animal models have triggered numerous studies in this field.[1–3] Currently, there are 18 angiogenic inhibitors under investigation in phases I–III clinical trials [4] and this number is increasing.

Vascular endothelial growth factor (VEGF) is a key regulator of the angiogenic processes. It is over-expressed in various human and mouse tumors and strongly upregulated by tumor endothelial cells under hypoxic conditions.[4,5] Thus, targeting this protein with a radiolabelled tracer could provide a means to monitor therapies that rely on the VEGF pathway. The first antibody against VEGF, reported in 1993, was shown to inhibit tumor growth by neutralizing endogenous VEGF and, thus, down–regulating angiogenesis.[6] While our own work with anti-VEGF VG76e was in progress, others published a brief note describing the use of a [^{124}I]radioiodinated anti-VEGF antibody (HuMV833) for positron emission tomography (PET). However, the authors did not provide substantial data on the biological activity of the radiolabelled conjugate.[7] The monoclonal antibody used in this study (VG76e) recognizes the 121, 165, and 189 isoforms of human VEGF, and also neutralizes endogenous VEGF.[8]

The positron-emitting iodine-124 with its relatively long half–life of 4.2 days is ideally suited to match the relatively slow pharmacokinetics of antibodies. Although the decay of iodine-124 produces only 23%

positrons, this isotope is still regarded as useful for positron emission tomography.[9] So far, investigators have used [^{124}I]iodine labelled antibodies to study malignancies such as breast cancer,[10,11] carcinoembryonic antigen–producing tumors,[12] colorectal cancer,[13] ovarian cancer,[14] and neuroblastoma.[15,16] It should be noted that all these papers are based on direct methods for labelling tyrosine residues of antibodies with iodine-124.

In 1972 Bolton and Hunter radioiodinated *N*-succinimidyl 3-(4-hydroxyphenyl) propionate[17] with iodine-125 as a labelling reagent for proteins under mild conditions (i.e. in absence of oxidant).[18,19] Limitations of that method are the ease of hydrolysis side–reaction of the *N*-succinimidyl ester group and de–iodination *in vivo* (Figure 1).

However, although the labelled Bolton–Hunter reagent is structurally similar to iodotyrosine residues – as obtained with the IodoGen method – it was found to be more inert to dehalogenation.[20] Research effort was directed at improving the indirect radiohalogenation method e.g. by the use of *N*-succinimidyl benzoates.[21–23] Other approaches are given in a comprehensive review by Wilbur.[24]

McGarry *et al.* reported in a preliminary account the first preparation of *N*-succinimidyl 3-(4-hydroxy-5-[^{124}I]iodophenyl) propionate

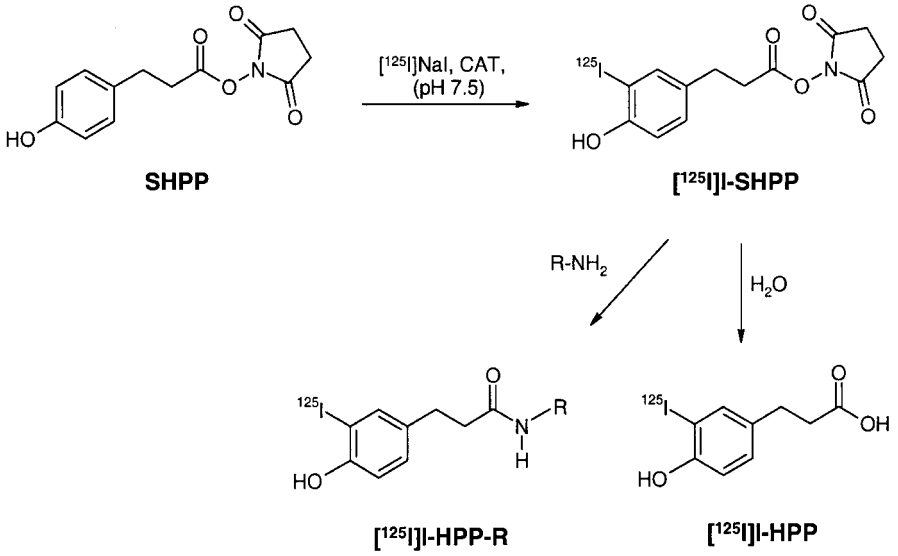

Figure 1. Original radiosynthesis of the Bolton–Hunter reagent,[19] its coupling with a protein (R-NH_2), and hydrolysis side–reaction leading to 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionic acid ([^{125}I]I-HPP).

([^{124}I]I-SHPP) and its use for indirect labelling of lysine residues of human anti-A and anti-B antibodies.[25] However, no experimental details about the preparation of [^{124}I]I-SHPP were mentioned.

In this paper, we present an improvement upon the classical Bolton–Hunter method suitable for the preparation of [^{124}I]I-SHPP and its use for labelling the monoclonal antibody VG76e as a potential angiogenesis–targeted PET probe.

Results and Discussion

Prior to the iodine-124 labelling of the VG76e, no–carrier–added iodine-125 was used in order to find an appropriate method that would provide a radiotracer which could be produced both with sufficient radiochemical yield and high biological activity.

Direct labelling of VG76e using the IodoGen method [26,27] produced [^{125}I]VEGF with a radiochemical yield of $60 \pm 7\%$ ($n = 3$) and a pseudo–specific radioactivity of 77.7 GBq/μmol (i.e. amount of radioactive iodine per mole protein). However, an immunoassay indicated a very low immunoreactive fraction of $3.1 \pm 3.0\%$. By switching to the indirect radioiodination with *N*-succinimidyl 3-[^{125}I]iodobenzoate ([^{125}I]*m*-SIB) [23,28] the VG76e conjugate was obtained in a radiochemical yield of $52 \pm 11\%$ ($n = 4$) and a pseudo–specific radioactivity of 1.3 GBq/μmol. Although the FPLC analysis of the [^{125}I]*m*-SIB conjugate with VG76e indicated less side–products of higher molecular weight (Figure 2), the improvement in immunoreactivity was modest ($10.9 \pm 6.4\%$).

Changing labelling conditions such as incubation temperature, buffer and time did not further increase the immunoreactive fraction. However, a significantly improved immunoreactivity of $34.0 \pm 4.0\%$ was observed by labelling with [^{125}I]I-SHPP. The [^{125}I]Bolton–Hunter modified antibody was prepared with a labelling efficiency of $28 \pm 8\%$ ($n = 5$) and a pseudo–specific radioactivity of 33 GBq/μmol.

The relatively low biological activity of the labelled antibody, found both with direct and indirect methods was not anticipated. However, the VG76e belongs to the subclass of IgG_1 antibodies. This group of antibodies is known to contain an above average number of tyrosine residues in their complementarity determining regions.[29] A possible reason for the different immunoreactivities of *m*-SIB and

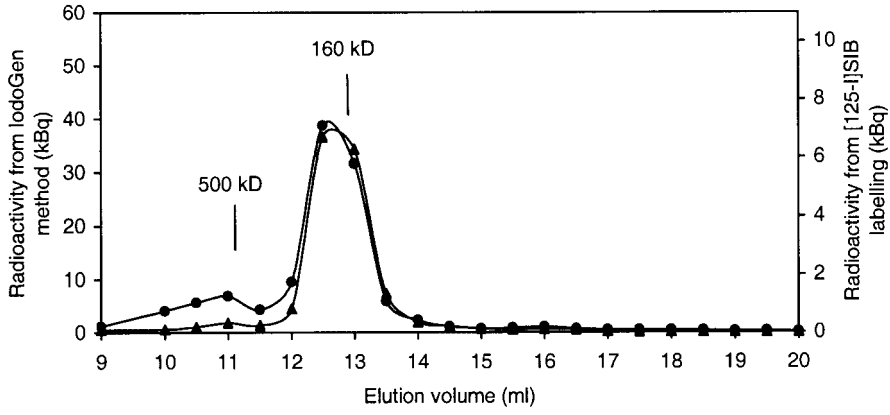

Figure 2. Size–exclusion chromatography of VG76e labelled by the IodoGen method (circles) and by [^{125}I]*m*-SIB (triangles). The peaks at elution volume 12.5 and 13.0 ml correspond to UV absorption peaks of unlabelled VG76e ($\lambda = 280$ nm).

Bolton–Hunter labelled antibody might be the higher lipophilicity of the iodobenzoyl moiety of the SIB reagent that could cause conformational changes in the variable regions of the antibody.

When working with [^{124}I]iodine, the synthesis of [^{124}I]I-SHPP should be as simple as possible. Also, the sensitivity of the *N*-succinimidyl ester group in the radioiodinated Bolton–Hunter reagent to hydrolysis demands a very short preparation time. For instance, to a vial containing [^{125}I]iodide, the components chloramine-T, SHPP, sodium metabisulfite, potassium iodide, DMF, and benzene should be 'added ... as quickly as two people working together can ... within 10 s'.[30]

Wood *et al.* suggested a 'combi method' with IodoGen as an alternative oxidant for the preparation of [^{125}I]I-SHPP – however, explicit details were not reported.[31] The iodination efficiency of IodoGen was reported to be pH independent in the pH range 6.0–8.5.[27]

Also, the hydrolysis of *N*-succinimidyl esters occurs at a relatively slow rate at lower pH as shown from literature examples in Table 1. Thus, IodoGen was used for the preparation of [^{125}I]I-SHPP at pH 6.5. The radioiodination was terminated after 60 s and the product extracted into benzene/DMF.

The isolated radiochemical yield was 51–67% ($n = 3$). TLC analysis showed radiochemical purity of 98% in a first extract that contained 86% of the total non–aqueous radioactivity. However, two additional

Table 1. Hydrolysis half–life times of some *N*-succinimidyl esters

Substrate	pH	Buffer	Temperature (°C)	$T_{1/2}$	Reference
N-Succinimidyl agarose	8.6	Carbonate (100 mM)	4	6 min	[32]
N-Sulfosuccinimidyl propionate or *N*-sulfosuccinimidyl *p*-sulfo benzoate	8.0	Sodium phosphate or HEPES (20 mM)	25	1 h	[33]
N-Succinimidyl 4-(2-carboxy-ethylthio) testosterone	8.0, 7.3	Phosphate[a] (50 mM)	26	0.5 h, 1.0 h	[34]
Dithiobis (*N*-succinimidyl propionate)	7.0	Sodium phosphate (5 mM)	0	4.5 h	[35]

[a] Phosphate buffer-dioxane 1:5 (v/v)

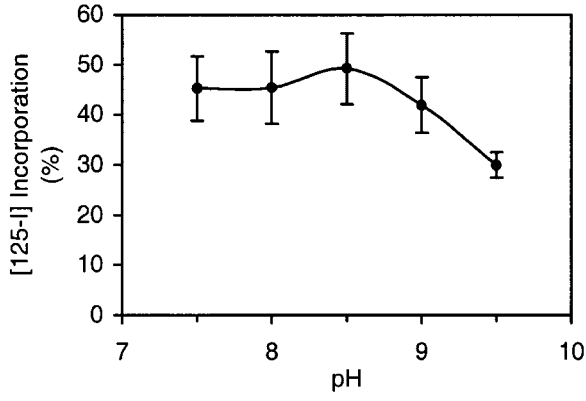

Figure 3. ITLC analysis of labelling mixtures derived from incubations of [^{125}I]I-SHPP with VG76e at different pH [error bars are S.D., $n = 3$; sodium phosphate buffer (pH 7.5 and 8.0; 100 mM) or sodium borate buffer (pH 8.5, 9.0, and 9.5; 100 mM), 1 h @ 0°C].

extracts revealed a trace of 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionic acid.

In order to optimize the labelling efficiencies of the [^{124}I]I-SHPP incubations with VG76e, several preparations using [^{125}I]I-SHPP in the pH range 7.5–9.5 were carried out. As shown in Figure 3, a maximum appears at pH 8.5 with 49.2 $\pm$ 7.1% [^{125}I]iodine incorporation. Figure 4 summarizes the labelling steps.

The higher percentage of [^{125}I]iodine incorporation compared to the lower values of isolated radiochemical yields illustrates the extent of loss

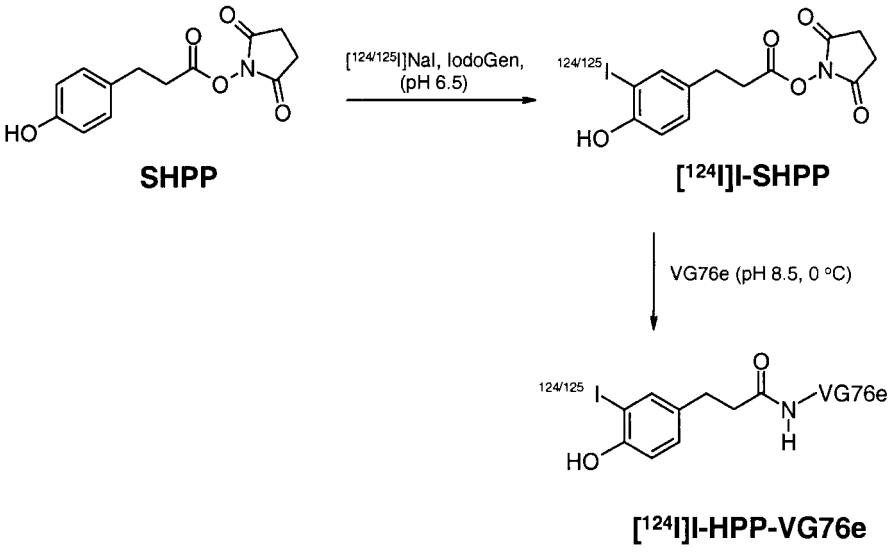

Figure 4. Preparation of [$^{124/125}$I]I-SHPP and labelling of the monoclonal VEGF antibody VG76e.

of radioactively labelled protein by gel–filtration due to non–specific binding.

In the initial preparations of [^{124}I]I-SHPP it was found that the radiochemical yield could be improved by adding a small amount of carrier sodium iodide matching the amount of [^{125}I]iodine used in previous experiments. The [^{124}I]I-SHPP obtained using this latter modification was isolated with radiochemical yields of 25–58% ($n = 3$). TLC analysis of [^{124}I]I-SHPP revealed a relatively pure product with radiochemical purities of 88–95% ($n = 3$, Figure 5).

The material was used for protein labelling without further purification. The labelling efficiency with VG76e after gel–filtration was 12–18% (n=3). An analytical FPLC chromatogram is shown in Figure 6.

The labelling yield might be improved by using a more concentrated antibody solution. However, there were indications that highly concentrated stock solutions of VG76e lead to aggregation of the protein and thus reducing the actual concentration again (data not shown). The radiochemical purity of [^{124}I]I-SHPP labelled VG76e was 98.0% and an immunoreactive fraction of 33.5% (n=2) was measured. After storing for 21 h at 4°C this value was slightly reduced to 96.1%. The pseudo–specific radioactivity of the [^{124}I]I-SHPP conjugate with VG76e was estimated to be 9.88 GBq/μmol.

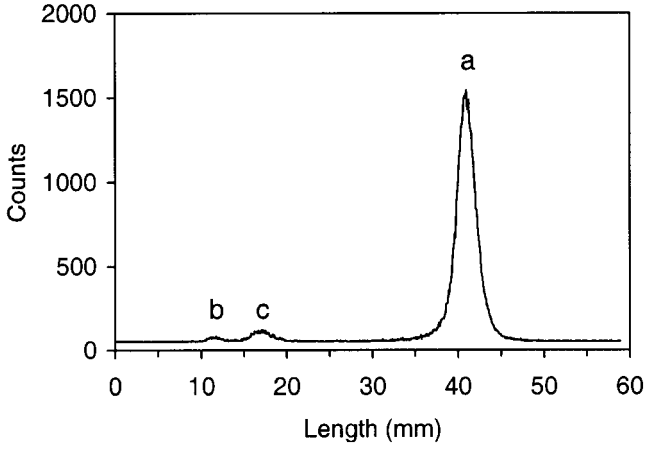

Figure 5. Typical TLC profile of a [^{124}I]I-SHPP extract: (a) [^{124}I]I-SHPP, (b) and (c) unidentified by-products.

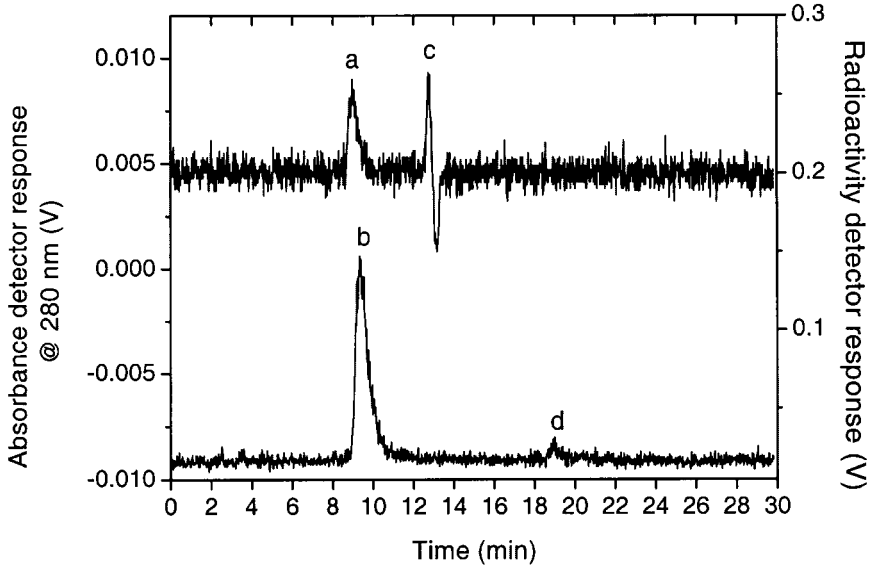

Figure 6. Typical FPLC profile of VG76e labelled with [^{124}I]I-SHPP showing the UV peak of VG76e (peak a, top chromatogram) and the radioactivity peak of labelled VG76e (peak b, bottom chromatogram). Peak (c, top chromatogram) appears to be an artefact due to buffer from the gel–filtration (molecular weight <1 kD). Peak (d, bottom chromatogram) arises from non–specific adsorption on the size–exclusion chromatography column.

A detailed study on the biological properties of the antibody conjugates with [$^{124/125}$I]I-SHPP including a PET study will be published elsewhere.[36]

Experimental

General

[^{125}I]Sodium iodide (specific radioactivity: >80 GBq/μmol) and *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate ([^{125}I]I-SHPP, specific radioactivity: ~ 74 GBq/μmol) were purchased from Amersham Pharmacia Biotech (Little Chalfont, England). *N*-succinimidyl 3-(4-hydroxyphenyl) propionate was supplied by Pierce and Warriner (Chester, England). All the other chemicals were obtained from Sigma-Aldrich (Gillingham, England), Merck (Lutterworth, England) and Fisher Scientific UK (Loughborough, England).

The preparation of the antibody VG76e has been previously described.[8] *N*-succinimidyl 3-(4-hydroxyiodophenyl)propionate (I-SHPP) was obtained according to O'Kennedy *et al.*[37] VG76e was directly labelled with [^{125}I]iodide by the IodoGen method[26,27] and indirectly with [^{125}I]*m*-SIB.[23,28] Protein concentrations for the determination of pseudo-specific radioactivity were measured by a Bio-Rad assay.[36]

The ^{1}H NMR data were aquired using a JEOL Eclipse machine (500 MHz) with dimethylsulfoxide-d_6 as solvent and tetramethylsilane as internal standard. The melting points were measured by an SMP-1 apparatus (Fisher Scientific UK).

Chromatography systems

The radiochemical purity of [^{124}I]sodium iodide was determined using a TLC system comprising methanol–water (3:1 v/v) as mobile phase and a cellulose stationary phase (Polygram Cel 300 UV_{254}; Macherey-Nagel, Middleton-Cheney, England). The incorporation of [^{125}I]I-SHPP into VG76e and the radiochemical purity of isolated, labelled antibody were measured by ITLC (methanol–water 4:1 v/v; Gelman, Ann Arbor, USA). Radioactivity was visualized by autoradiography (PhosphorImager 445 SI; Molecular Dynamics, Sunnyvale, USA).

The radiochemical purity of [$^{124/125}$I]I-SHPP was assessed using silica gel (Silica Gel 60 F_{254}; Merck) and ethyl acetate–methanol (9:1 v/v) as mobile phase.

For the FPLC analysis of the [^{125}I]iodine labelled protein, a Superdex^{TM}200 HR 10/30 column (Amersham Pharmacia Biotech UK) equipped with an HPLC pump (Beckman 110B), a Rheodyne injector (Model 0594), a UV monitor (Lambda-Max model 480; Waters), a chart recorder (Model PM 8252A; Phillips) and a fraction collector (Frac-100; Pharmacia) were used. The mobile phase (PBS) was eluted at flow rate of 0.5 ml/min. Fractions were counted for radioactivity using a well-counter (1282 CompuGamma; Wallac-Pharmacia, Turku, Finland).

The [^{124}I]iodine labelled antibody was analyzed by a TSK-Gel column (G3000SW_{xl}; TOSOHAAS, Linton, England) equipped with an SW_{xl} guard column. The FPLC system consisted of a Beckman pump (System Gold®), an injector (Model 7725; Rheodyne), a UV-detector (UV-1; Pharmacia), and a γ-counter (Flow-count; Bioscan). The data were collected using an in–house chart-recording PC program (LabView 5.0.1, National Instruments, Newbury, England). The flow rate of the mobile phase (100 mM sodium phosphate buffer, 100 mM sodium sulfate, pH 6.7) was set to 1.0 ml/min.

For gel–filtration, Sephadex-G25 columns were used (PD-10; Pharmacia) with PBS as the mobile phase. The effluent was collected in fractions of 0.5 ml.

Preparation of N-succinimidyl 3-(4-hydroxyphenyl) propionic acid (I–HPP)

To a stirred suspension of the *N*-succinimidyl ester I-SHPP (20 mg, 0.0514 mmol) in methanol (0.5 ml) a sodium hydroxide solution (77 µl, 2 M) was added. After stirring for 24 h, the methanol was evaporated by a stream of nitrogen and the mixture acidified with hydrochloric acid solution ($\sim$0.1 ml, 6 N). The product was extracted into ethyl acetate (0.25 ml), washed with water (0.2 ml) and brine (0.2 ml). Drying with sodium sulfate and evaporation of the solvent by a stream of nitrogen afforded I-HPP in analytical purity. Yield: 10.2 mg (68%), m.p. 102–105°C (literature: 109–111°C [38]), ^{1}NMR (500 MHz): 2.450 (t, 7.55 Hz, 2 H), 2.676 (t, 7.55 Hz, 2 H), 6.775 (d, 8.25 Hz, 1 H), 7.036 (dd, 8.25 and 2.1 Hz, 1 H), and 7.509 ppm (d, 2.1 Hz, 1 H).

Production of [^{124}I]Iodide

[^{124}I]Iodide was produced by the ^{124}Te(p,n)^{124}I reaction using irradiation of a [^{124}Te]tellurium(IV) oxide target with 12.5 MeV protons followed by dry distillation using a quartz apparatus.[39–41] The typical batch yield was 5.4 MBq/μA h and an isotopic distribution of 11% ^{123}I and 0.05% of ^{125}I at EOB was determined. The iodine-124 was trapped in an NaOH solution (300 μl, 8 mM) with a radiochemical purity of 98.7%. This was followed by a volume reduction in a ReactiVial (1 ml) fitted with a charcoal trap using a stream of nitrogen (flow rate: 1 l/min). The dry residue was redissolved with water (10 μl) using a sonicator (U50/D, Ultrawave, Cardiff, England) for 1 min. This procedure lead to 96% recovery of radioactivity.

Preparation of [^{124}I]I-SHPP

An aqueous solution of [^{127}I]NaI carrier (5 μl, 100 μM) was mixed with the concentrated [^{124}I]iodide (10 μl, 22 MBq). After adding HCl solution (6 μl, 100 mM) and sodium phosphate buffer (30 μl, 250 mM, pH 6.5) the solution was transferred into a ReactiVial (100 μl) plated with IodoGen (10 μg). A solution of *N*-succinimidyl 3-(4-hydroxyphenyl)-propionate (SHPP) (1,4-dioxane, 5 μl, 3.8 mM) was then added. The components were mixed by pipette. After 1 min the reaction was terminated by transferring the solution into a second ReactiVial (100 μl) containing anhydrous benzene–DMF (40:1 v/v). After mixing with a pipette, the organic phase was removed and dried by passing through a shortened Pasteur pipette containing sodium sulfate (200 mg) on a tissue plug and washing with additional benzene (100 μl). The extraction of the aqueous phase was repeated twice following the steps described above. Each extract fraction of [^{124}I]I-SHPP was analyzed by TLC (I-SHPP: $R_f = 0.92$, I-HPP: $R_f = 0.86$).

Labelling VG76e with [^{124}I]I-SHPP and [^{125}I]I-SHPP

The solvent of the [^{124}I]SHPP extract was completely removed with a stream of nitrogen. VG76e (8 μl, 30 μg, 1.97×10^{-10} mol, PBS) was mixed with sodium borate buffer (8 μl, pH 8.5, 100 mM) and incubated with [^{124}I]I-SHPP at 0°C for 1 h. The labelled antibody was isolated by gel–filtration.

The labelling of VG76e with [^{125}I]I-SHPP was carried out as described above for ^{124}I.

Immunoreactivity of VG76e labelled with[^{124}I]I-SHPP and [^{125}I]I-SHPP

ELISA plates (Corning Ltd., Buckingham, England) were coated with human $VEGF_{165}$ (10 μg/ml; R&D Systems, Oxfordshire, England) overnight in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4°C. Afterwards, the wells were treated with BSA (1%, 100 μl; Sigma) and three times washed with PBS containing Tween 80 (0.1%, Sigma). The radiolabelled antibody was diluted to 10 ng/ml and added to the wells. After the incubation (2 h, room temperature), unbound antibody was removed, the wells washed three times (PBS and 0.1% Tween 80), and the bound antibody dissolved with sodium hydroxide solution (200 mM). The radioactivity of solubilized bound antibody was measured in a well-counter (1282 CompuGamma). Experiments were done in triplicate and the results expressed as percentage of the total radioactivity added to each well.

Conclusion

In conclusion we report on the simplified synthesis of the Bolton–Hunter reagent. We employed this improved method to prepare the corresponding iodine–124 modified derivative for labelling the anti–VEGF antibody VG76e for potential use as a PET ligand in angiogenic studies.

Acknowledgements

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