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Synthesis and evaluation of an ^{18}F-labelled norbornene derivative for copper-free click chemistry reactions†

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The copper-free click chemistry reaction between norbornene and tetrazine species is known to proceed in a rapid, reliable and selective manner under mild conditions. Due to these attractive properties, this reaction has recently been explored as a generally applicable method of bioconjugation. Here, we report a convenient synthetic procedure towards a novel ^{18}F-labelled norbornene derivative (**[^{18}F]NFB**) and have evaluated its ability to undergo strain-promoted copper-free click chemistry reactions with two model tetrazine species: an asymmetric dipyridyl tetrazine derivative (Tz) and a tetrazine thiourea-coupled stabilised bombesin peptide (TT-BBN). In both cases, **[^{18}F]NFB** was found to undergo rapid and high-yielding click chemistry reactions. Furthermore, as reactions of this type could also potentially be used *in vivo* to facilitate the development of a novel pretargeting approach for tumour imaging and therapy, we have also assessed the radiopharmacological profile (bioavailability, biodistribution, blood clearance and metabolic stability) of **[^{18}F]NFB** in normal BALB/c mice. This radiolabelled compound exhibits both high bioavailability and metabolic stability with approximately 90% remaining intact up to 30 min following administration.

Introduction

The development of molecular probes for positron emission tomography (PET) is an area of intense multi-disciplinary investigation.[1–4] PET offers several advantages compared to other imaging modalities, including its ability to track biochemical, physiological, and pharmacological events with exquisite sensitivity. Consequently, this technique has been widely used in biomedical and clinical settings to monitor the progression of various malignancies, especially aggressive cancers, with great success in some cases. Fluorine-18 exhibits many desirable characteristics which have made it the most widely used radionuclide for PET imaging.[5–8] For example, (*i*) it can easily be produced on a cyclotron with high specific activity, (*ii*) it has a convenient radioactive half-life ($t_{1/2}$ = 109.8 min), and (*iii*) it has a low β^+ energy (0.64 MeV) which results in a short range in tissue (max = 2.4 mm) and high spatial resolution.

The use of effective molecular probes is vital in order to maximise the imaging potential of this powerful technique and a wide variety of targeting vectors (ranging from small molecules to much larger vectors, *e.g.* antibodies) are currently used in PET imaging applications. A key stage in the development of each radiotracer is the rapid and site-selective incorporation of the radionuclide. However due to the poor nucleophilicity of the fluoride anion and its limited reactivity in protic environments, this step can be problematic. These obstacles in combination with the harsh labelling conditions for the ^{18}F-incorporation represent a challenge particularly for peptides and proteins which require mild reaction conditions. Therefore indirect radiolabelling *via* prosthetic groups[9] or novel direct radiolabelling strategies (*e.g.* aluminium–, boron–, and silicon–^{18}F bond formations[10–14]) are often required to facilitate the process. Furthermore, conventional conjugation chemistry presents some limitations, such as the requirement of large excesses of (often expensive) protein in order to speed up reaction rates and obtain reasonable radiochemical yields.

Click chemistry is now a widely applied tool in synthetic chemistry and the reactions which fall under this category are characterised by their speed, reliability, high yields and selectivity.[15–21] The inverse electron-demand Diels–Alder cycloadditions between (*i*) ring-strained norbornene or (*ii*) *trans*-cyclooctene (TCO) species with tetrazines are excellent examples of click chemistry reactions and are demonstrating much recent promise as a chemoselective method of bioconjugation.[22–25] Furthermore, unlike most other click chemistry reactions (*e.g.* the azide–alkyne Huisgen cycloaddition[26,27]), the aforementioned cycloadditions do not require the presence of a copper catalyst, potentially

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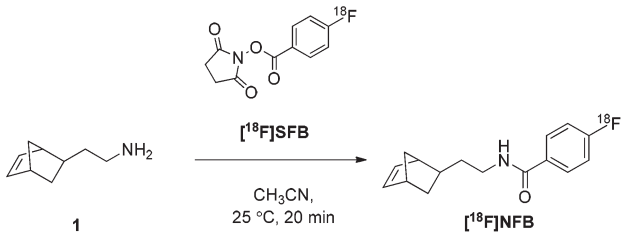

Fig. 1 Radiosynthesis of **[^{18}F]NFB**.

facilitating the development of a bioorthogonal pretargeting approach for both imaging and therapeutic applications.[28–32]

Recently, these reactions have been used in *in vitro* experiments to fluorescently label a variety of cancer cell lines (both at the cell surface[33–38] and intracellular regions[39–42]) using a two-step pretargeting strategy.

The norbornene/TCO-tetrazine ligations have also recently been applied as effective radiolabelling strategies in the development of radiotracers for both PET and SPECT imaging.[43] Their application has been successfully demonstrated by Zeglis *et al.* in their construction of a radiometalated derivative of the HER2 antibody, trastuzumab (Herceptin).[44] Similar strategies have been employed by Weissleder and co-workers[40,45] in the preparation of ^{18}F-labelled PARP1 inhibitors based on AZD2281, and also by Selvaraj *et al.* who developed an ^{18}F-labelled cyclic RGD peptide for PET imaging.[46]

In addition to the clear utility of such reactions as effective methods of bioconjugation, a report by Rossin *et al.* in 2010 provided the first example of a copper-free click chemistry reaction (in this case, between TCO and tetrazine species) being used successfully for pretargeted tumour imaging in live mice.[29]

Here, we report the reliable and high-yielding radiosynthesis of an ^{18}F-labelled norbornene derivative (**[^{18}F]NFB**; Fig. 1) which could be a useful compound for the mild, rapid and selective radiolabelling of a wide variety of targeting vectors for PET imaging. We have examined its ability to function as a prosthetic group *via* a copper-free click chemistry reaction using two model tetrazine species: (*i*) an asymmetric dipyridyl tetrazine (Tz),[47,48] and (*ii*) a tetrazine-modified bombesin peptide derivative (TT-BBN). Furthermore, we have also assessed the suitability of **[^{18}F]NFB** as a molecular probe that could potentially facilitate the development of a novel pretargeting strategy by evaluating its radiopharmacological profile (bioavailability, biodistribution, blood clearance and metabolic stability) in normal BALB/c mice.

Results and discussion

Radiosynthesis of *N*-[2-(bicyclo[2.2.1]hept-5-en-2-yl)ethyl]-4-[^{18}F]fluorobenzamide ([^{18}F]NFB)

The amine-functionalised norbornene precursor, 2-[(1*S*,2*S*,4*S*)-bicyclo[2.2.1]hept-5-en-2-yl]ethanamine (**1**), readily undergoes an acylation reaction with the prosthetic group 4-succinimidyl-[^{18}F]fluorobenzoate ([^{18}F]SFB),[49] leading to the formation of **[^{18}F]NFB**.

The radiosynthesis of **[^{18}F]NFB** was optimised based on (*i*) the concentration of **1**, (*ii*) reaction time, (*iii*) temperature, and (*iv*) solvent (ESI; Fig. S1 and S2†). It can be concluded from these experiments that the optimal conditions for this reaction involve stirring at 25 °C for 20 minutes and using 1 mg mL^{-1} of **1**. Under these conditions, the reaction proceeds with equal efficiency (quantitative yield) in acetonitrile, ethanol, hexane, and dimethyl sulphoxide (DMSO). When performed in methanol, the reaction yield was reduced to approximately 40% and more polar, unidentified by-products were observed *via* radio-TLC which most likely arise as a result of the known instability of [^{18}F]SFB in methanol. For practical reasons, we chose to use acetonitrile in our subsequent radiosyntheses of this compound. Following HPLC purification, **[^{18}F]NFB** was obtained in good radiochemical yields of 60 ± 17% (decay-corrected based on [^{18}F]SFB) within 52 minutes. The overall non-decay radiochemical yield based on starting [^{18}F]F$^-$ was approximately 18% within 106 minutes. The radiochemical purity of **[^{18}F]NFB** routinely exceeded 99% (Fig. S3†).

Copper-free click chemistry reaction between [^{18}F]NFB and a model tetrazine species (Tz) in various media

To assess the ability of **[^{18}F]NFB** to undergo a copper-free click chemistry reaction, an asymmetric dipyridyl tetrazine species (Tz) was selected to provide a convenient model. This reaction (outlined in Fig. 2) was performed in (*i*) acetonitrile, (*ii*) mouse plasma, and (*iii*) mouse blood.

In order to dissolve the tetrazine species, a small amount of the solubilising agent propylene glycol (10% v/v) was added to each reaction mixture. The reactions which were performed in plasma gave excellent yields (>97%) after only 10 minutes (Fig. S4†), and those performed in mouse blood (which were analysed at a single time point of 20 minutes) also resulted in very high yields (95%). The reactions conducted in acetonitrile were less rapid and lower yielding, reaching a maximum yield of 56% after 30 minutes (Fig. S4 and S5†). These results clearly demonstrate the potential of **[^{18}F]NFB** to function as an effective prosthetic group for radiolabelling tetrazine-modified species, particularly in aqueous solutions. Furthermore, the ability of **[^{18}F]NFB** to undergo high yielding copper-free click

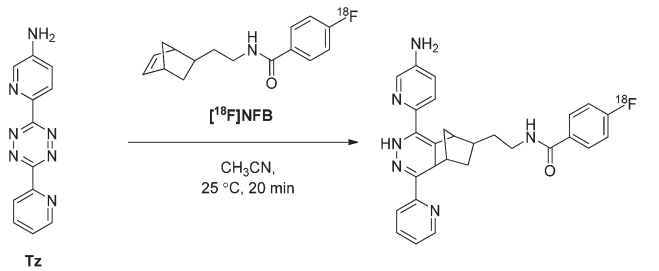

Fig. 2 Reaction scheme depicting the click chemistry reaction between **[^{18}F]-NFB** and a tetrazine (Tz) which proceeds efficiently without the requirement of a copper catalyst.

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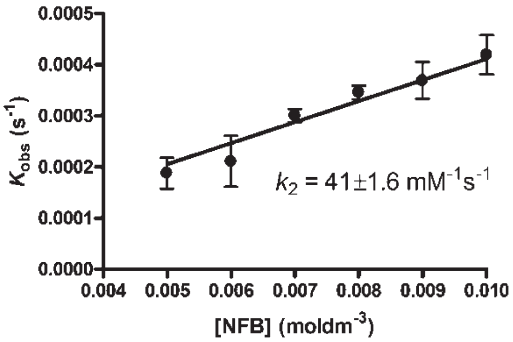

Fig. 3 Reaction kinetics between **[^{18}F]NFB** and Tz in DMSO.

chemistry reactions in complex biological media highlights the robust and highly selective nature of this ligation and suggests that this approach could potentially be applied within an *in vivo* setting to enable the pretargeted imaging of tumours using PET.

Reaction kinetics between NFB and Tz

The kinetics of the reaction between NFB and Tz were established by following an experimental protocol reported by Karver *et al.* with some minor differences (see Experimental for details).[35] Using this technique, we were able to determine a second order rate constant of 0.04 M^{-1} s^{-1} in DMSO (Fig. 3). This represents a rapid reaction which compares very favourably with many other copper-free click chemistry reactions such as the well-known Staudinger ligation.[50–52] However, this value is lower compared with other reported norbornene–tetrazine click chemistry reactions. For example, Devaraj *et al.* studied the reaction between (1*S*,2*S*,4*S*)-bicyclo[2.2.1]hept-5-en-2-yl acetic acid and 3-(*p*-benzylamino)-1,2,4,5-tetrazine which was found to proceed with a second order rate constant (k_2) of 1.9 M^{-1} s^{-1} when conducted in PBS (or 1.6 M^{-1} s^{-1} when performed in FBS).[33] Therefore, it is likely that the rate of this reaction may not be sufficiently high to enable an *in vivo* pretargeting approach in the traditional manner. However, we postulate that the reaction kinetics could be markedly improved by the adoption of a multi-valent approach involving, for example, nanoparticles substituted with multiple tetrazine species. In this case, the favourable *in vivo* radiopharmacological profile of **[^{18}F]NFB** would make it an ideally suited 'chaser' molecule for this purpose and we are currently investigating the validity of this proposed approach.

The rapid and high-yielding reaction between **[^{18}F]NFB** and the model tetrazine species, Tz, under mild conditions provides a clear indication of its utility in the preparation of targeting vectors containing short-lived positron emitters like fluorine-18.

Copper-free click chemistry reaction between [^{18}F]NFB and a tetrazine-functionalised bombesin peptide (TT-BBN)

These promising results encouraged us to examine the copper-free click chemistry reaction between **[^{18}F]NFB** and a tetrazine-functionalised model peptide in a proof-of-concept study. Therefore, we employed a stabilised bombesin derivative introduced recently by our group[53] which is comprised of the sequence Ava-Gln-Trp-Ala-Val-Sar-His-FA01010-Tle-NH_2 and selectively targets the gastrin-releasing peptide receptor (GRPR) with high affinity.

In a representative radiolabelling experiment, 100 μg (300 μL) of the tetrazine-functionalised bombesin (TT-BBN) was incubated with 0.5 MBq **[^{18}F]NFB** at room temperature over 30 min to react in an inverse electron-demand Diels–Alder cycloaddition. The reaction scheme is outlined in Fig. 4.

Monitoring of the reaction mixture *via* radio-TLC showed *ca.* 30% of the desired ^{18}F-labelled Diels–Alder 'click' bombesin [^{18}F]FDA-BBN after 5 min and increased to nearly 50% after 20 min. Furthermore, aside from the product, only non-reacted **[^{18}F]NFB** was observed in the reaction mixture. The [^{18}F]FDA-BBN yield in the reaction mixture did not change significantly after 20 min. The results obtained by radio-TLC nicely reflect the radio-HPLC yields (46% [^{18}F]FDA-BBN) recorded after 30 min (Fig. 5).

According to radio-HPLC data in Fig. 5, [^{18}F]FDA-BBN showed the expected mixture of isomers corresponding to the *endo*- and *exo*-position of the norbornene moiety as well as the isomeric dihydropyradazines as a result of the nitrogen release.[54] [^{18}F]FDA-BBN was isolated using HPLC purification in radiochemical purities exceeding 95%. Whilst a few other reports describe copper-free, strain-promoted click chemistry involving peptides,[46,55] to the best of our knowledge this study represents the first example of an ^{18}F-labelled peptide prepared *via* the inverse electron-demand Diels–Alder cycloaddition between norbornene and tetrazine species.

Small animal PET imaging of [^{18}F]NFB in BALB/c mice

Analysis of the dynamic small animal PET imaging data revealed that **[^{18}F]NFB** distributes rapidly and undergoes a relatively fast clearance from the blood based on the low molecular weight of this radiolabelled compound (Fig. 6). From the time-activity curve of the blood (heart representing the blood pool), a blood clearance half-life of 69 seconds was determined (Fig. S6†). Radioactivity seen within the liver, intestines and bladder indicates this compound undergoes both hepatobiliary and renal excretion.

Distribution of [^{18}F]NFB in the blood and metabolic stability

The majority of **[^{18}F]NFB** was found to distribute evenly between the blood cells and supernatant plasma (Fig. 7). We observed only minimal binding to plasma proteins for the duration of the experiment which indicates that the radiolabelled compound has excellent bioavailability. The results were largely uniform across all recorded time points.

Furthermore, metabolic profiling experiments revealed that **[^{18}F]NFB** has good *in vivo* stability with approximately 90% of the original compound remaining intact up to 30 minutes post-injection (Fig. 7). After 30 minutes, the radiolabelled compound was found to undergo rapid degradation and was almost entirely metabolised at 60 minutes post-injection.

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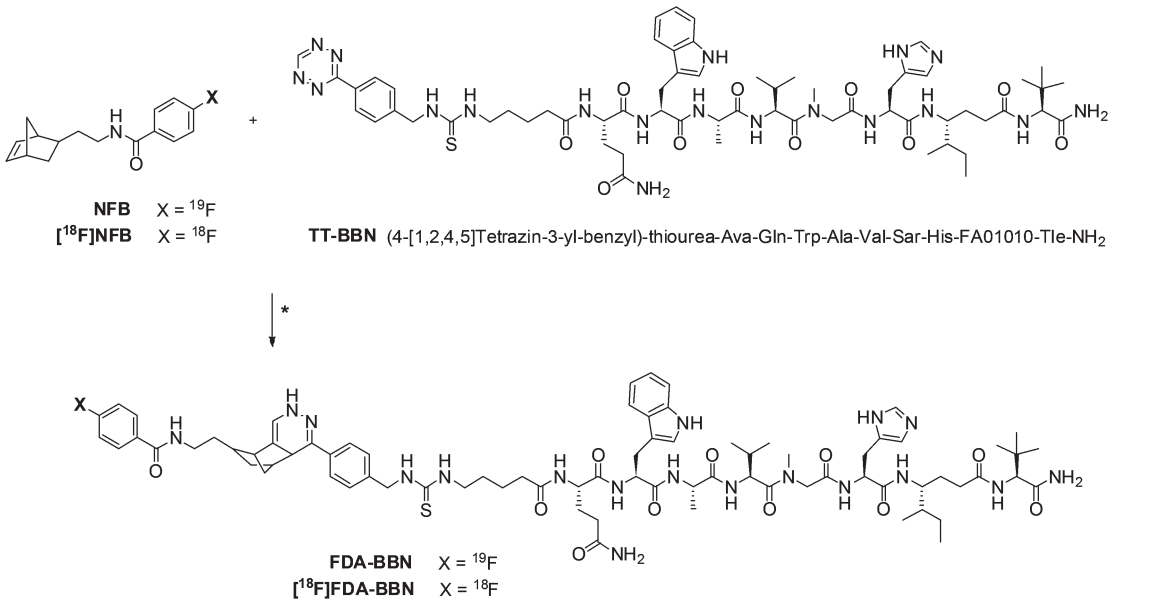

Fig. 4 Reaction scheme representing the click chemistry reaction of ^{19}F/**[^{18}F]NFB** with tetrazine-functionalised bombesin derivative TT-BBN.

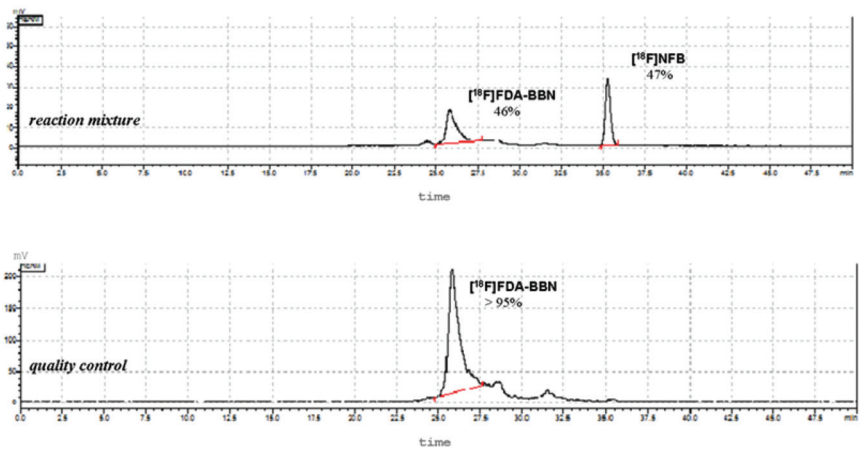

Fig. 5 Radio-HPLC traces displaying the reaction mixture (top) and the quality control (bottom) of **[^{18}F]NFB** click chemistry labelling of tetrazine-functionalised bombesin derivative TT-BBN yielding in Diels–Alder peptide [^{18}F]FDA-BBN.

Bioavailability and resistance to metabolic degradation are important requirements for an *in vivo* pretargeting strategy using PET radiopharmaceuticals. These results indicate that **[^{18}F]NFB** has favourable radiopharmacological properties which would be suitable for such an approach.

Conclusions

We have developed a reliable synthetic procedure towards a novel ^{18}F-labelled compound, **[^{18}F]NFB**, which is capable of undergoing click chemistry reactions with tetrazine species. The commercial availability of the amine-functionalised norbornene starting material (2-(bicyclo[2.2.1]hept-5-en-2-yl)-ethanamine) and the use of the common prosthetic group [^{18}F]SFB make the ^{18}F-labelled building block **[^{18}F]NFB** an attractive species for copper-free, strain-promoted click chemistry reactions involving tetrazine-modified targeting vectors.

The presented experiments clearly indicate the feasibility of using **[^{18}F]NFB** for the efficient radiolabelling of tetrazine-modified peptides which was successfully exemplified by radiolabelling a tetrazine-functionalised bombesin-like peptide with good efficacy. Furthermore, this approach is also attractive for the radiolabelling of proteins and antibodies with the short-lived positron emitter fluorine-18 due to the ability to perform these reactions in the absence of copper and under mild conditions.

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The ability of **[^{18}F]NFB** to undergo click chemistry reactions with a model tetrazine species in both plasma and blood highlights the robust and highly selective nature of this reaction and indicates the potential of this approach to facilitate an *in vivo* pretargeting strategy. However, while **[^{18}F]NFB** revealed a promising radiopharmacological profile (*e.g.* high metabolic stability, rapid blood clearance and high bioavailability) in normal BALB/c mice, the kinetics of the reaction between **[^{18}F]-NFB** and Tz indicate this approach is likely to be too slow for this purpose. We are currently optimising this particular ligation in order to obtain higher rates of reaction which will hopefully move the project in this direction.

In summary, we have demonstrated that **[^{18}F]NFB** has great potential to function as a generally applicable tool for the efficient radiolabelling of tetrazine-modified peptides (and potentially other targeting vectors) *via* copper-free click chemistry.

Experimental

Materials

N^{α}-Fmoc-protected amino acids, the 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink Amide MBHA) resin, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and ethyl cyanoglyoxylate-2-oxime (Oxyma) were purchased from Novabiochem (Darmstadt, Germany). The reactive amino acid side chains were protected as trityl (Trt) for Gln and His and *tert*-butyloxycarbonyl (Boc) for Trp. *N*,*N*-Diisopropylethylamine (DIPEA), dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), methanol, dichloromethane, acetonitrile, ethanol, diethyl ether, thioanisole, 1,2-ethanedithiol (EDT), trifluoroacetic acid (TFA) and triethylamine (TEA) were ordered from Sigma-Aldrich (Oakville, Canada). Piperidine was purchased from Caledon (Georgetown, Canada) and 3-(4-benzylamino)-1,2,4,5-tetrazine from Jena Bioscience (Jena, Germany). 3-(5-Aminopyridin-2-yl)-6-(pyridine-2-yl)-s-tetrazine (Tz) was prepared according to literature procedure.[47,48]

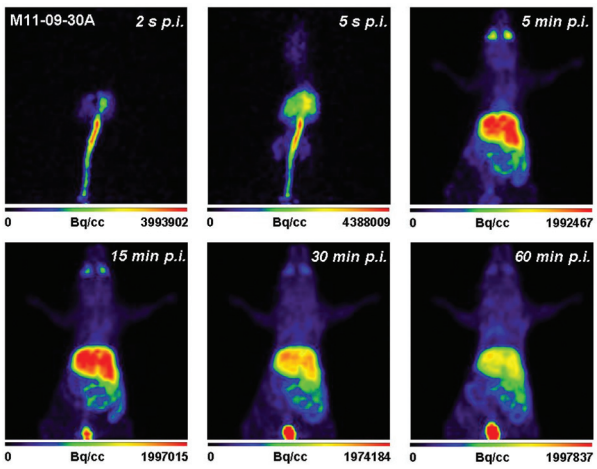

Fig. 6 Representative small animal PET images (maximum intensity projection) of a normal BALB/c mouse after single intravenous administration of 5 MBq of **[^{18}F]NFB**.

General methods

^{1}H NMR and ^{13}C NMR spectra were recorded on a 500 MHz (Varian Unity) spectrometer. Chemical shifts are given in ppm referenced to internal standards. Mass spectra were recorded using a Micromass ZabSpec Hybrid Sector-TOF by positive mode electrospray ionisation. Thin layer chromatography (TLC) was monitored using HF_{254} silica gel. Crude reaction mixtures were analysed by TLC and HPLC. HPLC purification was performed on a semi-preparative Luna C18 column (100 Å, 10 μm, 250 × 10 mm). The eluting solvent started with an acetonitrile–water gradient from (15/85 to 50/50, v/v) for 8 min at a flow rate of 3 mL min^{-1}, followed by a 8 min gradient (from 50/50 to 70/30, v/v) and finally 14 min at 70/30. UV detection was performed at 210 nm and 254 nm. Radioactivity detection was performed using a well-scintillation NaI (Tl) detector.

Chemical syntheses

***N*-[2-(Bicyclo[2.2.1]hept-5-en-2-yl)ethyl]-4-fluorobenzamide (NFB).** 2-(Bicyclo[2.2.1]hept-5-en-2-yl)ethanamine (30 μL, 0.21 mmol) was dissolved in dichloromethane (1 mL) then added dropwise to a solution of *N*-succinimidyl-4-fluorobenzoate (64 mg, 0.27 mmol, 1.3 eq.) in dichloromethane (4 mL). The reaction was stirred for 60 min at 25 °C. The reaction mixture was concentrated under reduced pressure at room

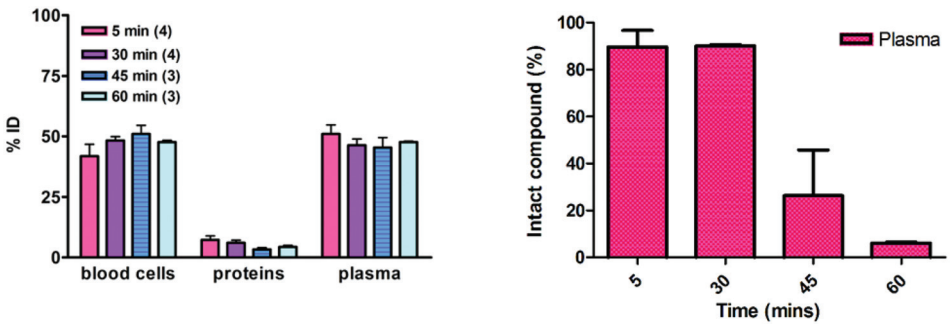

Fig. 7 Distribution of **[^{18}F]NFB** in the blood (left) revealing only minimal binding to plasma proteins and therefore good bioavailability, and evaluation of the metabolic stability of **[^{18}F]NFB** (right) following injection in BALB/c mice (n = 3). The radiotracer remains largely intact at 30 minutes p.i.

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temperature and the resulting yellow oil was then purified by column chromatography (ethyl acetate–hexane, gradient 5–50%). The collected fractions were analysed by TLC (ethyl acetate–hexane, 1 : 1; R_f (NFB) = 0.73). The pure fractions were combined and the solvent was evaporated under reduced pressure yielding a pale yellow oil. This was left overnight at 4 °C and formed a white crystalline residue (18.7 mg, 34%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.56 (m, 1H), 1.23 (m, 1H), 1.32–1.49 (m, 3H), 1.89 (m, 1H), 2.06 (m, 1H), 2.79 (bs, 1H), 2.81 (bs, 1H), 3.42 (m, 2H), 5.94 (dd, 1H), 6.07 (bs, 1H), 6.14 (dd, 1H), 7.09 (m, 2H), 7.76 (m, 2H). ^{13}C (150.8 MHz, $CDCl_3$): δ 32.2, 34.7, 36.4, 39.5, 42.5, 45.5, 49.6, 115.4, 115.6, 129.0, 129.1, 131.1, 132.1, 137.4, 164.6 (d, ^{13}C–^{19}F, ^1J = 251 Hz), 166.3. *m/z* (ESI) $C_{16}H_{18}FNO$ ([M + H$^+$]) calcd 260.1445, found 260.1445.

***N*-{2-[3-(5-Amino-pyridin-2-yl)-6-pyridin-2-yl-4,5-diaza-tricyclo-[6.2.1.0^{2,7}]undeca-3,6-dien-9-yl]-ethyl}-4-fluoro-benzamide (NFB-Tz).** NFB (14 mg, 54 μmol) in acetonitrile (2 mL) was added dropwise to a solution of 3-(5-aminopyridin-2-yl)-6-(pyridine-2-yl)-s-tetrazine (Tz, 19 mg, 76 μmol) in acetonitrile (8 mL). The reaction mixture was stirred at 25 °C for 60 min and then concentrated under reduced pressure. The crude product was purified using semi-preparative HPLC (t_R = 14.0 min) (5 mg, 19%). *m/z* (ESI) $C_{28}H_{28}FN_6O$ ([M + H$^+$]) calcd 483.2303, found 483.2300.

3-(4-Isothiocyanatomethyl-phenyl)-[1,2,4,5]tetrazine (TT-NCS). A solution of 3-(4-benzylamino)-1,2,4,5-tetrazine (51 mg, 267 μmol, 1 eq.) in chloroform (2 mL) was cooled to 0 °C and treated with thiophosgene (81.9 μL, 1068 μmol, 4 eq.) forming a red-orange coloured suspension. Addition of TEA (125 μL) led to the release of HCl gas. The reaction mixture was allowed to warm to room temperature and was stirred for 16 h at 25 °C. Deionised water (100 mL) was added to the reaction mixture and the crude product was extracted with chloroform (4 × 30 mL). The organic layer was dried with sodium sulphate and concentrated under reduced pressure. The residue was purified by flash column chromatography in 100% dichloromethane (R_f = 0.5) forming a pink-coloured solid (22.4 mg, 38%). ^{1}H NMR (500 MHz, CD_3CN): δ 4.93 (s, 2H), 7.63–7.64 (d, 2 aromatic-H), 8.59–8.61 (m, 2 aromatic-H). ^{13}C NMR (150.9 MHz, CD_3CN): δ 48.8, 128.7, 129.2, 132.9, 140.6, 158.8, 166.8.

Peptide syntheses

The stabilised bombesin derivative was synthesised on the basis of Fmoc-orthogonal solid-phase peptide synthesis (SPPS) *via* a combination of manual coupling procedures and automated synthesis performed with a peptide synthesiser (Syro I, MultiSynTech, Germany). The Rink Amide MBHA resin (loading 0.6 mmol g^{-1}) provided the solid support for synthesis of stabilised bombesin with the sequence Ava-Gln-Trp-Ala-Val-Sar-His-FA01010-Tle-NH_2. Fmoc deprotection was achieved by treatment with 40% piperidine/DMF for 5 min, followed by further incubation with 20% piperidine/DMF for 15 min. 5 eq. of each Fmoc-protected amino acid were activated and coupled using the reagents HBTU (5 eq.), Oxyma (5 eq.) and DIPEA (10 eq.) for 60 min throughout the peptide synthesis followed by washing steps with DMF, MeOH, dichloromethane and diethyl ether. The completeness of each coupling step was monitored *via* Kaiser and TNBS (2,4,6-trinitrobenzene-sulfonic acid) tests. Cleavage and deprotection of the amino acid side chains was achieved by treatment of the peptidyl resin with an acidic cocktail of 92.5% TFA, 5% water, 5% thioanisole, and 2.5% 1,2-ethanedithiol (EDT) for 4 h at room temperature. The peptide was separated from the resin by passing it through a syringe filter and precipitated by the addition of ice-cold diethyl ether. The crude peptide was obtained by removing the residual ether using a syringe filter. Purification was performed by semi-preparative HPLC using a Phenomenex Jupiter 10 μm, Proteo 90 Å , 250 × 10 mm followed by subsequent lyophilisation, giving the sufficiently pure peptide as white powder.

Ava-Gln-Trp-Ala-Val-Sar-His-FA01010-Tle-NH_2 (BBN). BBN was obtained as a white powder (17 mg, 15.9 μmol, 27%) starting from 100 mg Rink amide MBHA resin and after purification using semi-preparative HPLC at a flow rate of 2 mL min^{-1} and a gradient of 0–5 min 30% eluent B, at 15 min 35% eluent B, at 35 min 55% eluent B, at 40 min 70% eluent B. t_R = 11.8 min. MW $C_{52}H_{82}N_{14}O_{10}$ calculated 1062.6, found HR-MS (ESI) 1063.6 [M + H]$^+$, 1085.6 [M + Na]$^+$.

(4-[1,2,4,5]Tetrazin-3-yl-benzyl)-thiourea-Ava-Gln-Trp-Ala-Val-Sar-His-FA01010-Tle-NH_2 (TT-BBN). Peptide BBN (2.0 mg, 1.9 μmol, 1 eq.) dissolved in DMF (100 μL) was reacted with 3-(4-benzyl-isothiocyano)-1,2,4,5-tetrazine TT-NCS (0.9 mg, 3.8 μmol, 2 eq.) in DMF (110 μL) by adding DIPEA (0.7 μL, 3.8 μmol, 2 eq.). The reaction solution was incubated at 50 °C for 3 h followed by semi-preparative HPLC purification at a flow rate of 2 mL min^{-1} using the following gradient: 0–10 min 20% eluent B, at 25 min 50% eluent B, at 30–40 min 80% eluent B, at 40–50 min 90% eluent B (t_R = 32.8 min). TT-BBN was isolated as a pink solid (0.65 mg, 0.503 μmol, 24%). MW $C_{62}H_{89}N_{19}O_{10}S$ calculated 1291.7, found LR-MS (ESI) 1292.7 [M + H]$^+$, 646.8 [M + 2H]$^{2+}$.

4-Fluoro-*N*-{2-[3-(4-thioureidomethyl-phenyl)-4,5-diaza-tricyclo-[6.2.1.02,7]undeca-3,6-dien-9-yl]-ethyl}-benzamide-Ava-Gln-Trp-Ala-Val-Sar-His-FA01010-Tle-NH_2 (FDA-BBN). NFB (0.1 mg, 0.503 μmol, 1 eq.) dissolved in acetonitrile (100 μL) was added to a solution of TT-BBN (0.65 mg, 0.503 μmol, 1 eq.) in DMF (200 μL). The combined solutions were allowed to sit overnight at room temperature resulting in the disappearance of the pink colour. The reaction mixture was purified *via* semi-preparative HPLC at a flow rate of 2 mL min^{-1} using a gradient of 0–10 min 20% eluent B, at 25 min 50% eluent B, at 30–40 min 80% eluent B, at 40–50 min 90% eluent B (t_R = 35.0 min). FDA-BBN was isolated as a white solid (0.3 mg, 0.200 μmol, 39%). MW $C_{78}H_{107}FN_{18}O_{11}S$ calculated 1522.8, found HR-MS (ESI) 1523.6 [M + H]$^+$, 1524.8 [M + 2H]$^+$, 1525.8 [M + 3H]$^+$, 1526.8 [M + 4H]$^+$, 1545.8 [M + Na]$^+$, 1546.8 [M + Na + H]$^+$, 1547.8 [M + Na + 2H]$^+$, 1548.8 [M + Na + 3H]$^+$.

Radiosyntheses

No-carrier added aqueous [^{18}F]fluoride was produced using the TR19/9 (Advanced Cyclotron systems Inc.) cyclotron at the

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Edmonton PET Center (Ep = 17.8 MeV) by irradiation of enriched [^{18}O]water (3.0 mL, Rotem Germany, >98% enrichment) *via* the ^{18}O(p,n)^{18}F nuclear reaction. *N*-Succinimidyl-[^{18}F]-4-fluorobenzoate ([^{18}F]SFB) was synthesised following the procedure reported by Mäding *et al.*[49] The overall synthesis was usually carried out in less than 56 min, providing [^{18}F]SFB in decay-corrected yield of 60% and with a radiochemical purity greater than 95%.

***N*-[2-(Bicyclo[2.2.1]hept-5-en-2-yl)ethyl]-4-[^{18}F]fluorobenzamide ([^{18}F]NFB).** 2-(Bicyclo[2.2.1]hept-5-en-2-yl)ethanamine (2 μL, 15 μmol) was dissolved in acetonitrile (1 mL) containing [^{18}F]-SFB (typically in the range of 272–862 MBq). The reaction was stirred for 20 min at 25 °C. Progress of the reaction was monitored by radio-TLC (hexane–ethyl acetate 1/1; product R_f = 0.73). Upon completion, the reaction mixture was purified using semi-preparative HPLC (t_R = 20.0 min). **[^{18}F]NFB** was isolated in radiochemical yields of 60 ± 17% (decay-corrected based on [^{18}F]SFB, n = 14). The radiochemical purity routinely exceeded 99%.

***N*-{2-[3-(5-Amino-pyridin-2-yl)-6-pyridin-2-yl-4,5-diaza-tricyclo-[6.2.1.0^{2,7}]undeca-3,6-dien-9-yl]-ethyl}-4-[^{18}F]fluoro-benzamide ([^{18}F]NFB-Tz).** To a solution of 3-(5-aminopyridin-2-yl)-6-(pyridine-2-yl)-s-tetrazine (1 mg, 4 μmol) in acetonitrile (900 μL) was added **[^{18}F]NFB** in acetonitrile (typically 25 MBq, 100 μL). The reaction mixture was then stirred at 25 °C for 40 min. Progress of the reaction was monitored by radio-TLC (hexane–ethyl acetate 1/1; R_f ([^{18}F]NFB-Tz) = 0). Upon completion, the reaction mixture was purified using semi-preparative HPLC (t_R ([^{18}F]NFB-Tz) = 14.0 min).

General procedure for copper-free click chemistry of [^{18}F]NFB with Tz in a variety of solvents

Reactions performed in acetonitrile and mouse plasma. The HPLC-collected peak containing **[^{18}F]NFB** was diluted with water (20 mL per 1.5 mL of HPLC eluent) and passed through a Sep-Pak cartridge (Sep-Pak Plus tC18, Waters). The trapped **[^{18}F]NFB** was eluted with diethyl ether (1 mL) which was then blown dry under a stream of $N_{2(g)}$. **[^{18}F]NFB** (*ca.* 5 MBq) was then dissolved in propylene glycol (25 μL). In a separate eppendorf tube, Tz (1 mg, 4 μmol) was dissolved in propylene glycol (25 μL) and then made up to 475 μL with either acetonitrile or plasma. The tetrazine solution was then added to the **[^{18}F]NFB** solution and stirred at 37 °C for 30 min. Each of these reactions was monitored at 0.5, 1, 2.5, 5, 10, 15, 20, 25, and 30 minutes by radio-TLC (hexane–ethyl acetate, 1/1; R_f ([^{18}F]-NFB-Tz) = 0). In each case, a control reaction was performed in the absence of Tz which confirmed the presence of the unreacted starting material (R_f([^{18}F]NFB) = 0.73).

Reactions performed in mouse blood. In this case, the click chemistry reaction was performed in a smaller overall volume to allow for subsequent cell lysis within the same eppendorf tube. Dried **[^{18}F]NFB** (*ca.* 5 MBq) was dissolved in propylene glycol (5 μL). In a separate eppendorf tube, Tz (0.2 mg, 0.8 μmol) was dissolved in propylene glycol (5 μL) and then made up to 95 μL with mouse blood. The tetrazine solution was then added to the **[^{18}F]NFB** solution and stirred at 37 °C for 20 min. The red blood cells were lysed by adding deionised water (900 μL) and leaving in a thermoshaker (500 rpm) at room temperature for 5 min. The sample was centrifuged for 5 min at 10 000 rpm and the clear red solution was analysed by radio-TLC as described above. In this case, a single time point of 20 minutes was selected due to the additional processing time of the sample prior to radio-TLC analysis which rendered earlier time points meaningless. All click chemistry reactions were performed in triplicate.

Rate constant determination between NFB and Tz

Kinetic experiments were performed in a 96-well plate format using a SpectraMax 340PC^{384} microplate reader and analysed using SpectraMax Pro software version 5.4.1 (Molecular Devices). A dilution series of NFB and a solution 3-(5-aminopyridin-2-yl)-6-(pyridine-2-yl)-s-tetrazine (Tz) were prepared in DMSO and allowed to equilibrate to 37 °C for 10 minutes. The reactions between NFB and Tz were then initiated by combining these solutions in the 96-well plate, leading to final concentrations of 5, 6, 7, 8, 9, 10 mM for NFB and 1 mM of Tz, representing a 5- to 10-fold excess of the norbornene reactant. The total reaction volume in each well was 100 μL. The 96-well plate was then loaded immediately into the plate reader and the reactions were monitored by recording the intensity of the characteristic tetrazine absorption (λ_{max} = 540 nm) which diminished over the course of each reaction. Measurements were acquired at 30 second intervals over 1 h. The temperature was maintained at 37 °C over the course of the experiment. k_{obs} (s^{-1}) Values (baseline corrected) were determined using Prism 5 (GraphPad) and these values were then plotted against [NFB]. From this graph, the second order rate constant (k_2, M^{-1} s^{-1}) was calculated from the gradient. Each experiment was performed in triplicate.

Radiolabelling of (4-[1,2,4,5]tetrazin-3-yl-benzyl)-thiourea-Ava-Gln-Trp-Ala-Val-Sar-His-FA01010-Tle-NH$_2$ (TT-BBN) with [^{18}F]NFB. TT-BBN (100 μg, 77 nmol) dissolved in a 20% MeCN–PBS (pH 7.6) solution (150 μL) was treated with purified **[^{18}F]NFB** (14 μL, as obtained from semi-preparative HPLC in 70% MeCN–water) corresponding to 0.3–1.2 MBq. The overall reaction volume equaled 100 μL and consisted of 20% MeCN. The reaction mixture was incubated at room temperature for 30 min. Determination of the formed Diels–Alder peptide [^{18}F]-FDA-BBN was monitored at 5 minute intervals *via* radio-TLC (hexane–ethyl acetate 1/1; R_f ([^{18}F]FDA-BBN) = 0.0, R_f (**[^{18}F]NFB**) = 0.73). Additionally, the reaction mixture was analysed after 30 min *via* analytical radio-HPLC performed on a Luna C18 column (100 Å, 10 μm, 250 × 4.5 mm) and using a gradient of acetonitrile (solvent B) and water/0.2% TFA (solvent A) with a flow rate of 1 mL min^{-1} (0–10 min 80% A/20% B, 25 min 50% A/50% B, 30–40 min 20% A/80% B, 40–50 min 10% A/90% B). t_R ([^{18}F]FDA-BBN) = 25.8 min, t_R (**[^{18}F]NFB**) = 35.3 min.

Radiopharmacology

Small animal PET imaging in normal BALB/c mice. All animal experiments were carried out in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and

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were approved by the local animal care committee of the Cross Cancer Institute. Positron emission tomography (PET) experiments were performed using normal BALB/c mice. The mice were not fasted prior to imaging experiments. The animals were anaesthetised through inhalation of isoflurane in 40% oxygen/60% nitrogen (gas flow, 1 L min^{-1}) and body temperature was kept constant at 35 °C for the entire experiment. Mice were positioned and immobilised in the prone position with their medial axis parallel to the axial axis of the scanner in the centre of the field of view of the microPET® R4 scanner (Siemens Preclinical Solutions, Knoxville, TN, USA). A transmission scan for attenuation correction was not acquired. 4–6 MBq of **[^{18}F]NFB** in 100–150 μL saline (0.9%) containing 10% of ethanol was injected through a needle catheter into the tail vein. Data acquisition continued for 60 min in 3D list mode. The frames were reconstructed using *maximum a posteriori* (MAP) reconstruction. The pixel size was 0.085 by 0.085 by 0.12 cm and the resolution in the centre field of view was 1.8 mm. No correction for partial volume effects was performed. The image files were further processed using the ROVER v 2.0.21 software (ABX GmbH, Radeberg, Germany). Masks for defining 3D regions of interest (ROI) were set and the ROIs were defined by thresholding. ROI time-activity curves (TAC) were generated for subsequent data analysis. Standardised uptake values (SUV = (activity/mL tissue)/(injected activity/body weight), mL g^{-1}) were calculated for each ROI.

Distribution of [^{18}F]NFB in the blood and metabolic profiling. Typically, 3 to 5 MBq of **[^{18}F]NFB** in 100–150 μL of saline (0.9%) containing 10% of ethanol was injected as a bolus through a catheter into the tail vein of isoflurane anaesthetised BALB/c mice. Before radiotracer injection, mice were heparinised by subcutaneous injection of 50 μL heparin (1000 I.U.) and kept under anaesthesia during the course of the experiment. At selected time points of 5, 30, 45 and 60 min, the animal was sacrificed and a whole blood sample (approximately 500 μL) was collected. Blood cells were separated by immediate centrifugation (5 min at 13 000 rpm). Proteins within the sample were precipitated by adding methanol (*ca.* 800 μL) to the supernatant following a second centrifugation step (5 min at 13 000 rpm). The activity present in each fraction was measured using a gamma counter. For metabolic profiling, the supernatant plasma fractions from each time point were analysed by radio-TLC as described above.

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