^{99m}Tc SPECT imaging agent based on cFLFLFK for the detection of FPR1 in inflammation.

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Non-invasive imaging of the inflammatory process can provide a great deal of insight into a wide variety of diseases states, aiding diagnosis, evaluation and effective targeted treatment. During inflammation, blood borne leukocytes are recruited, through a series of activation and adhesion steps, to the site of injury or infection where they migrate across the blood vessel wall into the tissue. Thus, tracking leukocyte recruitment and accumulation provides a dynamic and localised read out of inflammatory events. Current leukocyte imaging techniques require *ex vivo* labelling of patient blood, involving laborious processing and potential risks to both patient and laboratory staff. Utilising high affinity ligands for leukocyte specific receptors may allow for injectable tracers that label leukocytes *in situ*, omitting potentially hazardous *ex vivo* handling. Formyl peptide receptors (FPRs) are a group of G-protein coupled receptors involved in the chemotaxis and inflammatory functioning of leukocytes. Highly expressed on leukocytes, and up regulated during inflammation, these receptors provide a potential target for imaging inflammatory events.

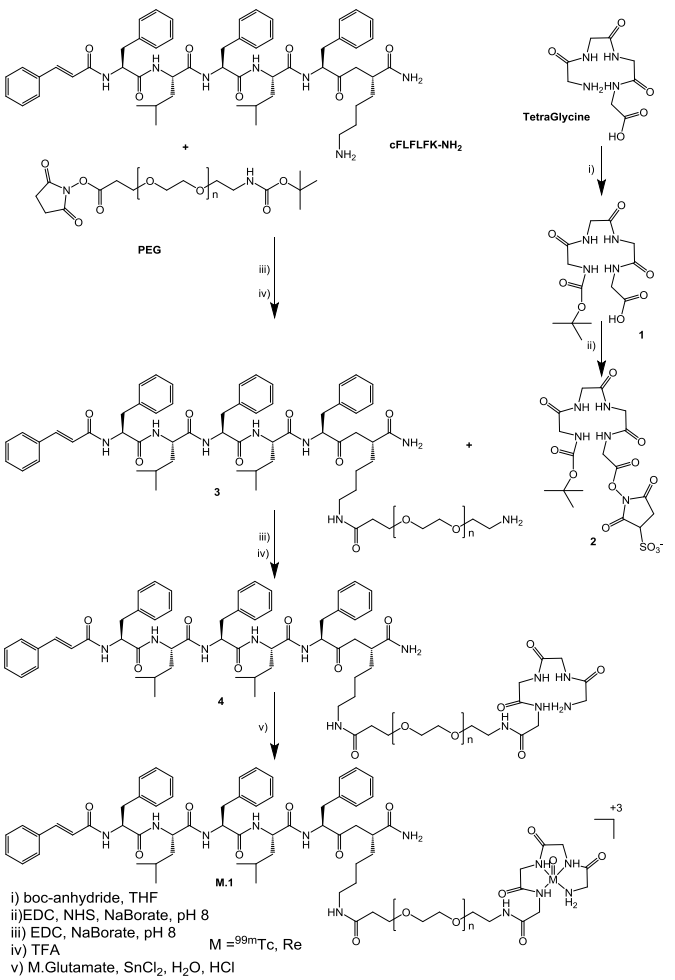
Herein we present the synthesis and initial *in vitro* testing of a potential Single Photon Emission Computed Tomography (SPECT) leukocyte tracer. The FPR1 antagonist cFLFLFK-NH_2, which displays high affinity with little physiological effect, has been linked via a PEG motif to a ^{99m}Tc chelate. This tracer shows *in vitro* binding to human embryonic kidney cells expressing the FPR1 receptor, and functional *in vitro* tests reveal cFLFLFK-NH_2 compounds to have no effect on inflammatory cell functioning. Overall, these data show that ^{99m}Tc.cFLFLFK-NH_2 may be a useful tool for non-invasive imaging of leukocyte accumulation in inflammatory disease states.

Introduction

Single Photon Emission Computed Tomography (SPECT) is a highly sensitive and versatile nuclear medical imaging technique. Specifically designed gamma-emitting radiotracers can provide accurate 3D localisation of a desired target or process, providing useful diagnostic and prognostic information. SPECT tracers have been widely used e.g. for tumour imaging (indium-111 octreotide) [1], bone scintigraphy (technetium-99m-MDP) [2] myocardial perfusion imaging (Myoview) [3] and brain functional imaging (Ceretec) [4].

The inflammatory response is increasingly being recognised as playing a significant role in a wide variety of disease states including; autoimmune diseases such as rheumatoid arthritis [5] and lupus [6], vascular diseases [7], infection, neurodegenerative disorders [8] and cancers [9]. Effective and non-invasive imaging tools are vital not only in the diagnosis of disease, but also as a screening tool to facilitate drug discovery. Imaging of the increased metabolic rate and elevated glucose transporters associated with inflammation using fluorine-18-fluorodeoxyglucose-positron emission tomography (FDG-PET) has proved of some use in monitoring inflammation. However, FDG is not specific for inflammatory conditions and has a high rate of false-positives [10, 11]. Similarly, ^{67}Ga-labelled citrate which binds a variety of plasma proteins (including transferrin) and accumulates at sites of inflammation, can been used to image inflammatory foci, however, it too suffers from a lack of specificity[11].

Neutrophils are the most abundant phagocytic leukocytes in the body, and are the earliest leukocyte subtype recruited to the site of tissue damage [9]. These cells harbour secretory vesicles containing a variety of cytotoxic substances allowing for a rapid and indiscriminate response to pathogens, but in the case of pathogenic inflammation may also damage the body's own cells. Due to their abundance and often pathological role in the inflammatory disorders, tracking this leukocyte subset with a non-invasive technique, such as SPECT, may provide a powerful clinical and pre-clinical tool.



Scheme 1. Synthesis of **^{99m}Tc.1/Re.1**

Formyl peptide receptors (FPRs) are a family of seven transmembrane domain G-protein-coupled receptors (GPCRs), with three FPRs (FPR1, FPR2, and FPR3) having been identified in humans [12]. Originally identified as a receptor for chemoattractants e.g. N-formyl-Met-Leu-Phe (fMLP), FPR1 is a highly abundant cell surface receptor on neutrophils, and is up-regulated under inflammatory conditions [13]. Consequently FPR1 ligands such as fMLF and i-Boc-MLF have previously been investigated as nuclear imaging tracers for neutrophilic inflammation [14]. However, these peptides have been found to enhance key inflammatory processes such as activation and chemotaxis [15, 16]. cFLFLFK-NH_2 is a hexapeptide (with a cinnamoyl group at the c-terminus), and an antagonist of FPR1, with little reported inflammatory activity [14].

To date, the 'gold standard' for radioimaging of inflammation is through *ex-vivo* cell labelling, where indium-111 or technetium-99m are bound intracellularly to isolated leukocytes [11, 17]. As discussed above, the recruitment of leukocytes to sites of tissue damage is a hallmark of the inflammatory response and as such, tracking leukocytes provides a high level of specificity. *Ex vivo* labelling requires processing of blood samples, posing risks of infection for both patient and laboratory staff. Furthermore, neutrophils which are highly sensitive to inflammatory activation, may be activated during processing, causing brief but intense signal in pulmonary vessels following administration.[11] A tracer that could be safely administered to a patient and label leukocytes *in situ,* would have significant benefits over current methodologies. While antibody based strategies have so far proved of limited success [18] the use of ligands with high affinity for leukocyte cell surface receptors may prove fruitful. The cFLFLFK-NH_2 peptide, modified with a polyethylene glycol (PEG) moiety to increase solubility and bioavailability, has previously been investigated as a near infrared (NIR) fluorescence imaging probe using a Cyanine 7 fluorophore [19] and also as a PET imaging agent, being further modified with a DOTA motif to chelate the a ^{64}Cu radio-isotope [14, 20]. However, the limited tissue-penetration depth of NIR imaging and the relatively high cost of PET (along with the requirement for cyclotron facilities for ^{64}Cu production) may limit the use of these imaging agents. Zhang *et* al. have labelled cFLFLFK-NH_2 peptide with ^{99m}Tc as a SPECT agent, however their studies use coordination through TKPPR. TKPPR is an antagonist of the yet to be fully characterised tuftsin receptor (proposed to be neurophilin-1) which has been shown to be expressed on neutrophils, macrophages and also to a high degree on endothelial cells and in cancers [21]. This lack of specificity and that the target receptor is relatively poorly characterised may limit the use of this imaging compound. Furthermore the method of synthesis used only produced a radiochemical yield of 75% [22]. More recently Stasiuk *et al.* have reported a MRI contrast agent based on cFLFLFK-NH_2 and DOTA chelate showing detection of neutrophils in an inflammatory response [23].
In the present study we utilise ^{99m}Tc, the most widely used radio-isotope in medicinal imaging, as a gamma emitting tracer. With a half-life of 6.5 hours, ^{99m}Tc allows for an excellent window of time for imaging whilst also limiting patient exposure[24]. Furthermore the ready availability for production from molybdenum-99 (66 hour half-life and the parent isotope used in technetium generators) allows ^{99m}Tc to be used in hospitals with limited radiochemistry facilities [24]. Here, ^{99m}Tc has been encapsulated within an organic framework (chelate) and conjugated to the FPR1 ligand, cFLFLFK-NH_2. This novel tetraglycine chelate for ^{99m}Tc, produces a SPECT tracer which has been assessed *in vitro* and *in vivo.*

Results and discussion

1 was synthesised in 82% yield from tetra glycine and ^{t}BOC anhydride, described by Shendage *et al*. as a general synthesis for protection of amino acids (Scheme 1).[25] The purification of which was less facile than first anticipated, due to the high water solubility of **1** and starting materials. **1** was purified by precipitation of the tetraglycine from the starting material and inorganic salts by the addition of MeOH giving a pure product. This is highlighted in the ^{1}H NMR spectrum by the appearance of a 9H singlet at 1.48 ppm corresponding to the ^{t}BOC group and the shift of CH_2 groups on the glycine from 3.9 ppm to 3.8 ppm. Further indication is observed in the ESMS at 347 [M+H]. **3** has been synthesised with 29% yield, in a two-step reaction from an average weight 3000 ^{t}BOC-NH-PEG-NHS with cFLFLFK-NH_2. **3** was produced *in situ* by the cleavage of the ^{t}BOC protecting group with TFA. The crude mixture was purified using HPLC, using a gradient of H_2O 0.1% TFA: MeCN starting at 95:5 to 5:95 over 30 minutes on a c18 reverse phase column. **3** was eluted at 19 minutes, which was confirmed by mass spectrometry MALDI 3609, 3685, 3762, range of mass is due to the average weight PEG linker. **4** was synthesised in 71% yield, from the reaction of **3** with **2**, under the same conditions as formation of **3**. **2** was synthesised *in situ* from **1** and sulfo-NHS in the presence of EDC. **4** was purified *via* reverse phase HPLC on a c18 column, using a gradient of H_2O 0.1% TFA: MeCN starting at 50:50 to 5:95 over 20 minutes, with a retention time of 4.5 minutes. Formation has been confirmed by MALDI-TOF mass spectrometry (MS) with peaks at 4254, 4298 and 4342.
In order to test the tetraglycine chelates affinity for ^{99m}Tc, a cold chemistry alternative was devised. Coordination of Re(V)=O with **4** was undertaken to create a Re=O core in the same coordination environment required for ^{99m}Tc. Ammonia perrhenate and sodium glutamate in the presence of $SnCl_2$ and HCl gives the desired Re=O core with two glutamate units coordinating. This precursor is soluble in water, and formation is indicated by the change of solution colour to blue, to which **4** is added. The transmetalation reaction was indicated by a change in colour of solution to yellow, after heating to reflux for 2 h. Purification *via* HPLC, was undertaken and **Re.1** has a retention time of 4 minutes, gradient of 50:50 to 5:95 (H_2O:MeCN),with a yield of 72%.

This was confirmed via MALDI-TOF MS with peaks at 1474, 1499, 1536 [M/3], which is expected for **Re.1** with a 3+ charge. The mono-oxo core and 3+ charge were verified by the synthesis of the model compound, **Re.Tetraglycine** which showed a 3+ species in the ESMS at 149 [M/3], and a peak in the FT-IR at 943 cm^{-1}, which is indicative of the Re=O bond.[26] The complex was examined for luminescence, a peak was observed at 440 nm in Figure 1 and shows the emission spectrum (red) for **Re.1**, a Re(V)=O complex, and the excitation spectrum (blue) showing a Ligand to Metal Charge Transfer (LMCT) band at 388 nm. This is a comparable emission maxima and excitation wavelength through the LMCT to other Re(V)=O compounds, recently shown by Hann and co-workers.[27]

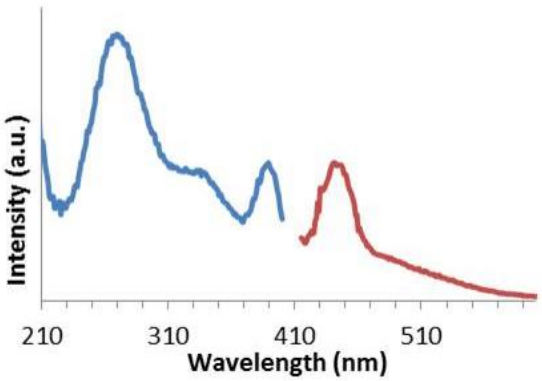


Figure 1. Luminescence Spectra for **Re.1** emission spectrum (red), excitation spectrum (blue), λ_{ex} = 388 nm λ_{em} = 440 nm, (298K, H_2O, pH 7.4).

Synthesis of **^{99m}Tc.1** was undertaken with **4** dissolved in a solution of sodium glutamate to which $SnCl_2$, in HCl was added as a reductant. $^{99m}Tc.O_4$- (4 mCi) was added to the solution and heated at 80°C for 45 minutes. The solution was passed down a G-10 Sephedex column to purify and fractions of 0.5 ml were taken and tested for radioactivity in a gamma counter. The radiolabelled peptide eluted after 1 column volume. Un-reacted ^{99m}Tc was eluted after 2 column volumes. The radiochemical yield was determined to be 93% (supplementary S4), suggesting the transmetallation reaction from the ^{99m}Tc-bis-glutamate, pre-chelate is highly efficient, and that tetraglycine is an excellent novel chelate for ^{99m}Tc. Studies at different heating times were undertaken at 15, 30 and 45 minutes, the largest concentration of **^{99m}Tc.1** eluted from the column was observed when heated at 45 minutes. This is nearly as efficient as the more standard tetraamine chelates with regards to reaction kinetics for this coordination geometry of the ^{99m}Tc.

In vitro experiments

Binding and chemotaxis assays were undertaken using **Re.1** to determine the specificity, efficacy and effect on neutrophil functional responses of using **^{99m}Tc.1** as a SPECT imaging agent, and if the modification to the native cFLFLFK-NH_2 peptide changed the antagonistic properties.

In order to validate the specificity of our compounds for FPR1, receptor-binding studies were performed using human embryonic kidney (HEK) cells stably transfected with FPR1. A competition binding studies using ^{125}I labelled fnLLFnLYK ([125I]-Form-NleLeuPheNleTyrLys) revealed **Re.1** to have an IC-50 of 18 nM giving the K_i as 9.6 nM as determined using the Cheng-Prusoff equation, demonstrating that **Re.1** binds FPR1 with an affinity not dissimilar to the native peptide (K_d = 2 nM). This illustrates that the choice of conjugation upon the lysine residue does not significantly disrupt the binding to FPR1 and therefore that **^{99m}Tc.1** will bind with a similar affinity.

In order to confirm specificity of **Re.1** for FPR1 over other neutrophil FPR family members (i.e. FPR2), radioligand binding assays were also performed using HEK cells stably transfected with FPR2. **Re.1** failed to show any specific binding to FPR2 receptor, indicating the specificity of **Re.1** for FPR1.

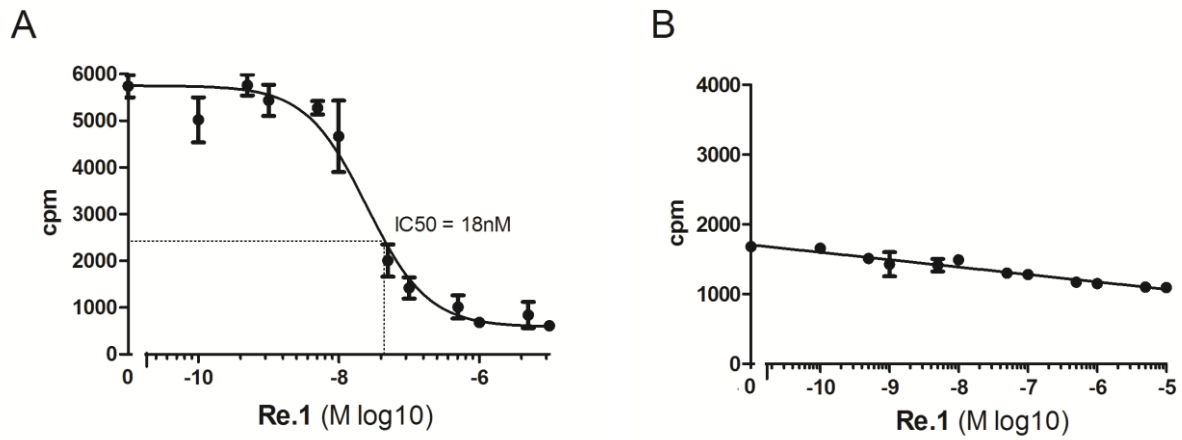


Figure 2. Competition binding curves of ^{125}I-fnLLFnLYK analogue vs **Re.1**, A) cFLFLFK-Re demonstrates competitive binding to FPR1 expressing HEK cells, against 125I fnLLFnLYK, with an IC50 of 18nM and a mathematically derived K_d of 9.6 nM B) 125I fnLLFnLYK displays a lower level of binding at HEK-FPR2 cells while **Re.1** shows no specific binding to this receptor. Data points represent mean +/- SEM from 4 independently performed experiments.

FPR1 is a known neutrophil chemoattractant receptor and administration of biologically active ligands could enhance the chemotactic response promoting tissue infiltration and degranulation of leukocytes, enhancing inflammation and tissue damage. Thus, in order to ascertain whether any of the cFLFLFK-NH_2 compounds elicited a chemotactic response, the level of isolated human neutrophil chemotaxis toward these peptides was investigated in comparison to the chemotactic peptide fMLP. Figure 3a demonstrates the number of migrating neutrophils in response to the native cFLFLFK-NH_2 peptide and **Re.1** in comparison to fMLP. In agreement with previous publications cFLFLFK-NH_2 [14] was found not to produce a chemotactic response, with concentrations of 0.1-300 nM producing no significant increase in chemotaxis compared to media alone. **Re.1** closely mirrored the effects of the native peptide producing little to no chemotactic response demonstrating that conjugation of the native peptide to a chelate upon the lysine residue does not confer chemotactic properties to the peptide.

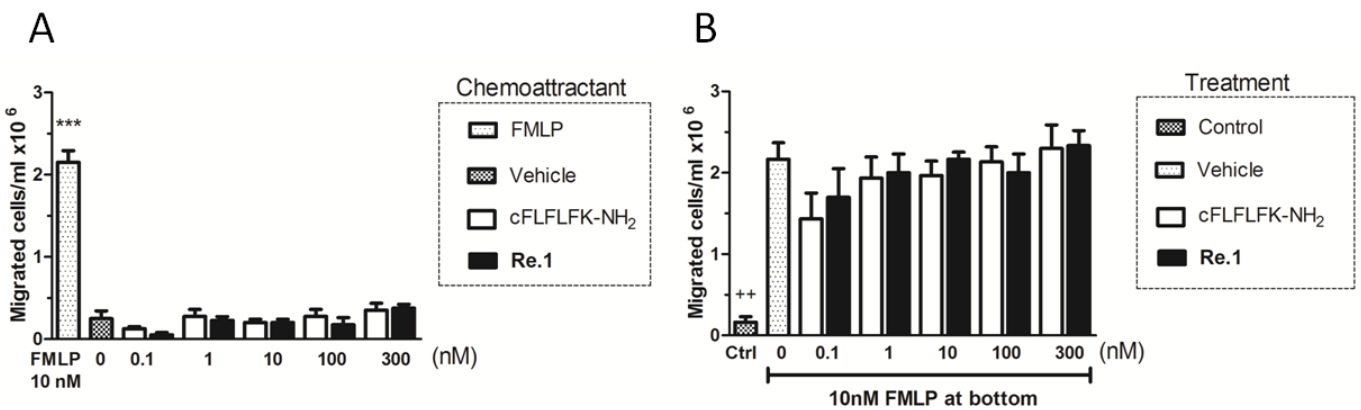


Figure 3: A) The effect of cFLFLFK-NH_2 or **Re.1** to induce neutrophil chemotaxis**.** Freshly isolated human neutrophils were added to the top wells of a modified boyden chemotaxis chamber, whilst different concentrations of the peptides were added to the bottom wells to assess their potential chemotactic effect. fMLP (10 nM. Bottom well) demonstrates a significant migration of neutrophils into the bottom well while cFLFLFK-NH_2 and **Re.1** induce similar levels of chemotaxis as found with the vehicle.. Data are overall average ± SEM of 4 separate experiments, prepared with different neutrophil donors. Each experiment was run in triplicate. *P<0.01 vs. fMLP alone (ANOVA and Bonferroni). **B)** Effect of cFLFLFK-NH_2 or **Re.1** on human neutrophil chemotaxis**.** neutrophils were treated with varying concentrations of the compounds for 10 minutes prior to addition to the chemotaxis plate where fMLP (10 nM) was added to the bottom wells.. Pre treatment had no significant effect on the chemotactic action of fMLP. Data are overall average ± SEM of 4 separate experiments, prepared with different neutrophil donors. Each experiment was run in triplicate. *P<0.01 vs. fMLP alone. ^ P<0.05 vs. cFLFLFK-NH_2 (ANOVA and Bonferroni).

To determine whether cFLFLFK-NH_2 or **Re.1** had an effect on neutrophil chemotaxis toward fMLP, neutrophils were pre-incubated

with these compounds before being exposed to an fMLP gradient (Figure 3b). Treatment with either the native peptide of **Re.1** was found to have no significant effect on the ability of neutrophils to respond to chemotactic signals. These results are promising, indicating that the **Re.1** tracer will not influence the inflammatory functioning of neutrophils during the process of imaging.

In Vivo SPECT Imaging

To show that **^{99m}Tc.1** is a viable probe for imaging FPR1 on neutrophils, an acute murine inflammatory response was induced via the injection of lipopolysaccharide (LPS), LPS is a bacterial fragment that disrupts the vascular endothelium, causing sites of inflammation and the recruitment of activated neutrophils. C57BL/6 mice were injected with LPS in the left thigh and saline in the right thigh 4 hours prior to scanning. This acute inflammation model produces neutrophil accumulation in the left thigh and not the right. **^{99m}Tc.1** (350-500 uCi) was injected intravenously *via* the tail vein, and allowed to circulate for 30 minutes prior to imaging. Figure 4 (right side of image) shows uptake in the left thigh and not the right, showing specific targeting to neutrophils, these initial studies showing the ability of imaging the localisation of neutrophils in the thigh are promising. LPS injection is a widely used model of bacterial induced inflammation and is thus relevant to infection. In demonstrating that **^{99m}Tc.1** can be used to target and image neutrophil accumulation in the thigh, we have provided proof of concept that **^{99m}Tc.1** may provide as a useful tool to image neutrophilic inflammatory events. Due to the involvement of neutrophil accumulation in a wide variety of disease states we aim to test this SPECT tracer in different models of both acute and chronic clinically relevant models of inflammatory disease.

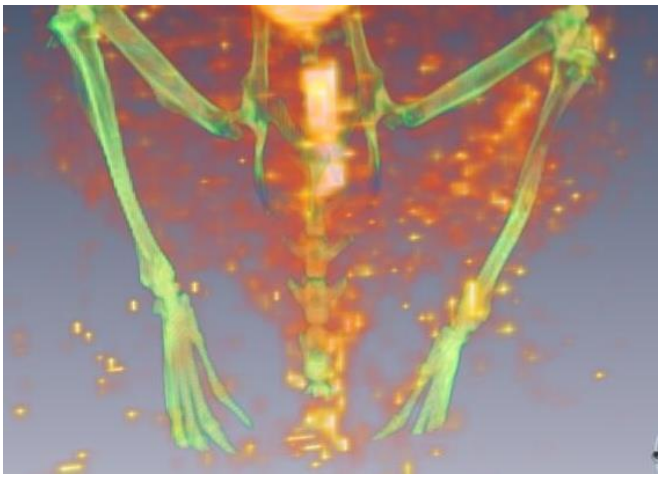


Figure 4 SPECT image of murine LPS thigh model at 30 minutes after injection with **^{99m}Tc.1**.

Conclusions

These studies were undertaken to validate **^{99m}Tc.1** as a potential SPECT imaging agent to track neutrophil recruitment and accumulation, thus giving a read out of inflammatory status. Here we have successfully shown that tetraglycine can be conjugated to cFLFLFK-NH_2 *via* a PEG linker, and to this chelate both Re and ^{99m}Tc can be coordinated with an oxo-core, forming **^{99m}Tc.1** and **Re.1**. **Re.1** shows luminescent properties and could feasibly be used to observe cells expressing FPR1 in inflammatory disease states as a fluorescent probe. These peptides have been demonstrated to bind to FPR1 expressing cell lines and isolated human neutrophils with similar affinity as the native peptide. This shows that **^{99m}Tc.1** can be used as a SPECT probe for detecting inflammatory accumulation of neutrophils without the need for *ex vivo* labeling of cells.

An ideal imaging agent should not only display selectivity for the target receptor but should also have no effect on the process being imaged, in this case the role of the neutrophil in inflammation. Following successful synthesis of cFLFLFK-NH_2 conjugates we investigated the effects of all compounds on the functional responses of human neutrophils (*via* neutrophil chemotaxis towards fMLP). In line with previous reports, the native cFLFLFK-NH_2 peptide was found to have no chemotactic actions upon neutrophils nor was it found to enhance chemotaxis toward fMLP. Furthermore modification of the antagonist at the terminal lysine residue does not change the chemotactic response of neutrophils with the compounds tested (**Re.1**, **^{99m}Tc.1** and cFLFLFK-NH_2). These *in vitro* tests of neutrophil functioning suggest that the compounds produced have no influence on the functional response of neutrophils and are thus suitable candidates for non-invasive imaging of neutrophil accumulations in inflammatory diseases. *In vivo* studies show that **^{99m}Tc.1** goes to the site of inflammation, allowing for the non-invasive imaging of neutrophil accumulation.

In conclusion, these studies reveal that, successful modification of cFLFLFK-NH_2 peptide to incorporate the tetraglycine motif as a chelate for Re(V), and ^{99m}Tc does not abolish ligand affinity for FPR1, nor does it alter specificity for this receptor. Furthermore these compounds do not interfere with neutrophil functions, thus demonstrating the potential of these FPR1 ligand analogues as possible replacements for current techniques for the non-invasive imaging of inflammation.

Experimental

Synthesis

General procedures

NMR spectra were recorded on an Advance DMX 400 Bruker spectrometer. Chemical shifts are reported in ppm with solvent as internal reference. HPLC was carried out on a HP 3000 system with a reverse phase c18 semi prep column. Electronic absorption spectra were recorded on a Varian CARY 50 probe UV/vis spectrometer. SPECT/CT scans were performed using a TriFoil Imaging Triumph Tri-Modality scanner

Materials

Solvents and starting materials were obtained from Aldrich, Fluka, Acros, and Alfa. The peptide cFLFLFK-NH_2 Cambridge biochemical. They were used without further purification unless otherwise stated. Water and H_2O refer to high purity water with resistivity value of 18 MΩ·cm, obtained from the "Millipore/MilliQ" purification system.

*^{t}BOC-tetraglycine **1***

Tetraglycine (0.1 g, 0.4 mmol) was dissolved in H_2O and tetrahydrafuran 1:1 (2 ml), cooled to 0^{o}C, to this solution ^{t}BOC-anhydride (0.11 ml, 0.48 mmol) and $NaHCO_3$ (101 mg, 1.2 mmol) were added sequentially. The solution was stirred for 30 minutes at 0^{o}C, then the solution was allowed to warm to room temperature and stirred for 12 h. The mixture was washed with diethyl ether (3 x 5 ml), the aqueous layer was acidified to pH 5 with dilute HCl and washed with DCM (3 x 5 ml) to remove excess ^{t}BOC-anhydride. The aqueous layer was then concentrated giving a white solid, which was taken up in MeOH (10 ml) and filtered. The filtrate was concentrated yielding a white solid 128 mg (82%) δ_H (400 MHz, MeOD), 1.61 (9H, s), 3.75 (2H, s), 3.95 (2H, s), 4.15 (2H, s), 4.25 (2H, s), *m/z* (ESMS+) 346 $[M + H]^+$.

*NHS-tetragylcine-^{t}BOC **2***

1 (80 mg, 0.23 mmol) was dissolved in H_2O (5 ml) at pH 8 to which *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (41 mg, 0.23 mmol) and sulfonyl *N*-Hydroxysuccinimide (50 mg, 0.23 mmol) were added sequentially at 0^{o}C. This solution was stirred for 1 h and allowed to warm up to room temperature. The solution was washed with diethyl ether (3 x 5 ml) and the aqueous phase was concentrated yielding a white hydroscopic solid.

*cFLFLFK-PEG **3***

cFLFLFK-NH_2 (10 mg, 100 µmol) dissolved in MeCN (1 ml) and NHS-PEG-NH-^{t}BOC (31 mg, 100 µmol) dissolved in sodium borate buffer (1 ml, pH 8.5) were combined and stirred for 12 hours at 4^{o}C. The solvent was concentrated, giving a white solid to which TFA (2 ml) was added. This solution was stirred at room temperature for 2 hours followed by the concentration of the solvent to give a white solid, which was purified *via* HPLC using a c18 reverse phase column with a gradient of H_2O 0.1% TFA: MeCN starting at 95:5 to 5:95 over 30 minutes. **3** has a retention time of 19 minutes, giving a white solid 12 mg, 29% yield. *m/z* (MALDI) $[M+H]^+$ 3609, 3685, 3762.

*cFLFLFK-PEG-tetraglycine **4***

To a solution of **2** (0.42 mg, 0.0013 mmol) in H_2O (0.5 ml) **4** (5 mg, 0.0013 mmol) was added in Na borate buffer (0.5 ml) and this solution was stirred at 4^{o}C for 12 h. The solvent was removed and the crude solid was purified *via* HPLC using a c18 reverse phase column with a gradient of H_2O 0.1% TFA: MeCN starting at 50:50 to 5:95 over 20 minutes. **4** has a retention time of 4.5 minutes, giving a white solid 2.6 mg, 41% yield. *m/z* (MALDI) $[M+H]^+$ 4254, 4298 ,4342.

*Re cFLFLFK **Re.1***

Ammonium perrhenate (0.16 mg, 0.069 mmol) was dissolved in a solution of sodium glutamate (0.5 M, 0.0545 g, 0.5 ml). This solution was degassed for 10 minutes, to which $SnCl_2$ (0.139 mg, 0.069 mmol), in HCl (0.1 M, 0.04 ml) was added. The solution turned blue, and was stirred for 90 minutes. To the solution **4** (2.6 mg, 0.069 mmol) was added and this solution was heated to reflux for 2 h, in which time the solution turned yellow. The solvent was removed giving a yellow solid which was purified via HPLC

using a c18 reverse phase column with a gradient of H_2O: MeCN starting at 50:50 to 5:95 over 20 minutes. The retention time was 4 minutes giving a yellow oil, 2 mg, 73% yield. *m/z* (MALDI) $[M/3]^+$ 1474, 1499, 1536.

Re tetraglycine

Ammonium perrhenate (100 mg, 0.37 mmol) was dissolved in a solution of sodium glutamate (0.5 M, 1.09 g, 10 ml). This solution was degassed for 10 minutes, to which $SnCl_2$ (71 mg, 0.37 mmol), in HCl (0.1 M, 0.8 ml) was added. The solution turned blue, and was stirred for 90 minutes. To the solution tetraglycine (92 mg, 0.37 mmol) was added and this solution was heated to reflux for 2 h, in which time the solution turned yellow. The solvent was removed giving a yellow solid which was purified via HPLC using a c18 reverse phase column with an isocratic mixture of of H_2O: MeCN 95:5 over 20 minutes. The retention time was 3.2 minutes giving a yellow oil, 120 mg, 73% yield. δ_H (400 MHz, D2O), 3.61 (2H, s), 3.67 (2H, s), 3.82 (2H, s), 3.88 (2H, s), FT-IR, (cm-1): 943 (Re=O) *m/z* (ESMS) $[M/3]^+$ 149.

*^{99m}Tc cFLFLFK **^{99m}Tc.1***

4 (100 μg, 0.02 mmol) was dissolved in a solution of sodium glutamate (0.5 M, 200 μl), to which $SnCl_2$ (4.5 mg, 0.02 mmol), in HCl (0.1 M, 20 μl) was added. $^{99m}TcO_4$- (4 mCi, 500 μl) was added to the solution and heated at 80°C for 45 minutes. The solution was passed down a G-10 Sephadex column to purify (2.1 ml), fractions of 0.5 ml were taken and tested for radioactivity using a gamma counter. The radiolabelled peptide eluted after 1 column volume. Un-reacted ^{99m}Tc was eluted after 2 column volumes.

In vitro experiments

Human blood leukocyte isolation

The local research committee approved experiments with healthy volunteers, and informed consent was provided according to the Declaration of Helsinki. Blood was collected from healthy volunteers with a 21-gauge needle and transferred to a 50 ml centrifuge tube containing 1/10 volume of 3.2% sodium citrate. Blood was diluted 1:1 with warm Roswell Park Memorial Institute (RPMI) 1640 medium before addition to a double density gradient of equal volumes (3 ml) of Histopaque 1077 over Histopaque 1119. Samples were then centrifuged at 400 g for 30 minutes at room temperature. Two distinct layers of leukocytes can be seen after centrifugation, with red blood cells (RBCs) pelleted to the bottom of the centrifuge tube. The resulting neutrophil cell layer was removed and placed in 6 ml aliquots in 15 ml centrifuge tubes to which an equal volume of RPMI 1640 was added. Samples were centrifuged at 300 x g for 15 minutes. The supernatant was then discarded and cells re-suspended with 7.5 ml of ice-cold H_2O, to lyse any contaminating RBCs, before immediately adding 2.5 ml of 3.6% sodium chloride solution, to restore isotonicity. Cells were then centrifuged at 300 x g for 10 minutes and re-suspended in 3 ml of RPMI medium, supplemented with 10% heat inactivated foetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U) and streptomycin (100mg/ml) (complete media). Cell counts were performed in Turk's solution (1:10) using a haemocytometer and cells diluted to the appropriate concentration for each assay using complete media.

Compound Preparation

cFLFLFK-NH_2 and **Re.1** were made in-house and on the day of the experiment were dissolved in pre-warmed PBS to make a stock concentration of 1 μM and diluted in RPMI1640 0.1% BSA to the desired concentrations. formyl-Met-Leu-Phe (fMLP), a pro-inflammatory ligand of FPRs, and peptide Ac2-26, LXA_4, and ionomycin were all used as controls.

Receptor binding studies

Human Embryonic Kidney (HEK293) cells transfected to express FPR1 under the CMV promoter were grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) plus L-glutamate containing 10% FCS, 0.05ug/ml gentamicin and 0.2 mg/ml geneticin. Cells were detached using 0.5mM EDTA in Dulbecco's Phosphate-Buffered Saline before being centrifuged at 1200rpm for 5 minutes and re-suspending in RBA buffer (1 M HEPS buffer + 6 μM $CaCO_3$ + 2 μM $MgCO_3$ + 0.5% BSA) and used at $1x10^5$ cells per assay. Saturation curves were initially performed using the radio labeled ligand [125I]-fNleLeuPheNleTyrLys (Cambridge Research Biochemicals, UK custom labeled by Perkin Elmer). K_d was determined using a one site saturation curve fit in Graphpad prism 5 giving a K_d of 68,350 cpm. This confirmed receptor binding to the FPRs and to inform the concentration to be use in competition assays. For competition binding studies, 400 μl of cell suspension was incubated with 50μl of a fixed concentration of [125I]-Form-NleLeuPheNleTyrLys (60,000 cpm) and 50μl of RBA buffer containing various concentrations of **Re.1**, (from $1x10^{-5}$ M – $1x10^{-10}$ M). Following 90 minutes incubation with radio ligand (in 50μl) at room temperature, cells were centrifuged for 2 minutes at 2000 RPM, the supernatant discarded and pellet re-suspended in 0.5 ml RBA buffer before being centrifuged for a further 2 minutes at 2000 RPM. The supernatant was then discarded and radiation in the cell pellet measured on a Packard Cobra II Gamma Counter for 240 seconds. Binding was expressed as counts per minute (cpm) with IC_{50} determined using a binding competitive one site fit log IC50 curve. K_i was determined using the Cheng-Prusoff equation, where the fixed concentration of labelled ligand was 60,000 and K_d was 68,350 cpm.

Chemotaxis

PMN ($4x10^6$ /ml in RPMI containing 0.1% BSA) were pre-incubated for 10 minutes with compounds (cFLFLFK-NH_2, **Re.1** and or controls) at 37°C. The chemotaxis assay was performed using commercially available Neuroprobe ChemoTx plate 96-well plates (3 µm) (Receptor Technologies Ltd, Warwick, U.K.) with fMLP (10 nM, Sigma Aldrich) as the chemotactic stimulus (concentration chosen from preliminary studies, in order to obtain ~85% of the maximal response). Thus, fMLP or medium were added to the bottom wells (27 µl), the filter was placed on top and 25 µl of the neutrophil cell suspension placed above the membrane. In some cases, cFLFLFK-NH_2, and cFLFLFK-Re were added to the bottom well to determine whether these compounds may act as chemo-attractants themselves. Chemotaxis plates were incubated for 90 minutes at 37°C and 5% CO_2 in a humidified incubator. After incubation any remaining cells were removed using cotton wool buds and the plate was washed with RPMI + 0.1 % BSA. The plate was then centrifuged at 312 g for 5 minutes to pellet the migrated cells into the plate wells. The polycarbonate filter was carefully removed and the pellets were re-suspended. A 20 µl aliquot was taken and diluted 1:10 in Turks solution for counting on a haemocytometer, viewed under a light microscope.

***In vivo* SPECT imaging**

^{99m}Tc.1 (350 - 500 µCi) in 300 µl of saline, was injected intravenously via the tail vein of a C51BL6 mouse, which had been injected with LPS (10 µg/mouse) in the left thigh and saline solution in the right thigh, 4 hours prior to scanning. The contrast agent was allowed to circulate for 30 minutes prior to scanning SPECT and CT for 30 minutes using a TriFoil Imaging Triumph Tri-Modality scanner. Image acquisition was performed using Triumph X-O® CT and SPECT imaging system software and images rendered using Volumetric Image Visualization Identification and Display VIVID™ software (TriFiol, USA).

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