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Synthesis and characterization of ^{18}F-labeled active site inhibited factor VII (ASIS)

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Activated factor VII blocked in the active site with Phe–Phe–Arg–chloromethyl ketone (active site inhibited factor VII (ASIS)) is a 50-kDa protein that binds with high affinity to its receptor, tissue factor (TF). TF is a transmembrane glycoprotein that plays an important role in, for example, thrombosis, metastasis, tumor growth, and tumor angiogenesis. The aim of this study was to develop an ^{18}F-labeled ASIS derivative to assess TF expression in tumors.

Active site inhibited factor VII was labeled using *N*-succinimidyl-4-[^{18}F]fluorobenzoate, and the [^{18}F]ASIS was purified on a PD-10 desalting column. The radiochemical yield was 25 ± 6%, the radiochemical purity was >97%, and the pseudospecific radioactivity was 35 ± 9 GBq/μmol. The binding efficacy was evaluated in pull-down experiments, which monitored the binding of unlabeled ASIS and [^{18}F]ASIS to TF and to a specific anti-factor VII antibody (F1A2-mAb). No significant difference in binding efficacy between [^{18}F]ASIS and ASIS could be detected. Furthermore, [^{18}F]ASIS was relatively stable *in vitro* and *in vivo* in mice.

In conclusion, [^{18}F]ASIS has for the first time been successfully synthesized as a possible positron emission tomography tracer to image TF expression levels. *In vivo* positron emission tomography studies to evaluate the full potential of [^{18}F]ASIS are in progress.

Keywords: PET; ^{18}F; active site inhibited factor VII; molecular imaging; protein labeling; [^{18}F]SFB

Introduction

Factor VII (FVII) is one of the several protein constituents involved in the blood coagulation cascade. Upon vessel injury, FVII binds to the transmembrane glycoprotein 'tissue factor' (TF), forming a complex in which FVII is activated to FVIIa.[1] This is the first in a series of reactions that ultimately leads to the generation of thrombin and of a fibrin network. Thus, FVIIa has previously been labeled with ^{99m}Tc and tested as a TF-targeting agent, with the ultimate aim of locating injured vessels in cases of intestinal bleeding.[2,3] Although TF is well known for its role in triggering coagulation, there is increasing evidence to suggest that it is also implicated in the etiology of several types of cancer. TF is highly expressed on the surface of many tumor cells,[4] and the TF/FVIIa complex promotes many tumor-related processes through intracellular signaling induced by protease-activated receptors (PARs).[5] When present on the tumor cell surface, the TF/FVIIa complex is capable of activating PAR2, which promotes tumor growth and angiogenesis. More indirectly, TF/FVIIa promotes metastasis via thrombin generation and PAR1 activation.[6] Diagnostic imaging of TF is therefore an attractive tool that can provide valuable information to inform decisions regarding the treatment of cancer patients.[4] Recently, a diagnostic TF-imaging reagent has been produced by labeling a chimeric antibody with ^{64}Cu.[4] The relative accumulation of the resulting ^{64}Cu-NOTA-ALT-836 antibody derivative (molecular weight: 150 kDa) in tumors was at a maximum after 48 h. In general, the major limitations of antibody-based imaging are slow tumor accumulation and a high background signal in the reticuloendothelial system.[4] These disadvantages can be circumvented using peptides, small molecules, smaller proteins, or antibody fragments. These entities accumulate more quickly and usually show superior tumor-to-liver and tumor-to-muscle ratios than antibodies. FVII (molecular weight: 50 kD), and FVII derivatives bind with a high affinity to TF[7] and are cleared from the circulation with a relatively short half-life of about 2 h.[8,9] This pharmacokinetic profile of FVII derivatives is likely to result in a favorable accumulation time frame that allows for ^{18}F labeling. Compared with labeling with ^{64}Cu, labeling with ^{18}F results in a better radiodosimetry and a lower radiation burden and also has the additional advantage of superior β^+-branching ratio (branching ratio β^+ [%]: ^{18}F : 96.7% and ^{64}Cu : 17.6%). Finally, labeled derivatives of hemostatically active FVIIa, such as [^{99m}Tc]-FVIIa, may not be optimal for TF diagnostic imaging because applying of FVIIa in patients with extensive tumoral TF expression could produce localized clot formation and interfere

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with the binding of labeled FVIIa derivatives. We therefore applied an inactive derivative of FVIIa, ASIS (active site inhibited factor VII), which was produced by blocking the active site of FVIIa with a Phe–Phe–Arg–chloromethyl ketone residue (Figure 1).[10] ASIS does not trigger the coagulation cascade and has the additional advantage of having an affinity for FVIIa that is five times that of FVII.[7,11] Thus, we developed a strategy for ^{18}F-labeling ASIS and tested the labeled compound in initial studies.

Experimental

Materials and methods

General methods

Active site inhibited factor VII in HEPES buffer (50-mM HEPES, 10-mM $CaCl_2$, 150-mM NaCl, and pH 7.8) was obtained from Novo Nordisk A/S, Malov, Denmark. The *N*-succinimidyl 4-fluorobenzoate (SFB) (standard and precursor) and QMA cartridges were purchased from ABX advanced biochemical compounds (Radeberg, Germany). PD-10 desalting column, Sephadex™ G-25 medium was purchased from GE Healthcare (Uppsala, Sweden). Reversed-phase extraction C18 Plus Sep–Pak cartridges were obtained from Waters (Sollentuna, Sweden) and were pretreated with ethanol and water before use. Gly–Gly buffer contained 10-mM Gly–Gly, 150-mM NaCl, and 10-mM $CaCl_2$, adjusted to pH 7.5.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the XCell SureLock system (Life Technologies, a Thermo Fisher Scientific Brand, Waltham, MA, USA) and the power supply Power PAC 300 (Bio-Rad, Hercules, CA, USA).

NuPAGE® 4–12% Bis-Tris Gel, NuPage MES SDS running buffer, NuPAGE LDS sample buffer, NuPAGE® sample reducing agent (10×), SimplyBlue SafeStain, and SeeBlue® Plus2 pre-staining standard were purchased from Thermo Fisher Scientific Inc, Life Technologies. All other chemicals and anhydrous solvents were bought from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. An automated module was used for the radiochemistry synthesis (Scansys Laboratorieteknik, Vaerlose, Denmark), and centrifugation was performed on a Megafuge 1.0R (Heraeus, Hanau, Germany). A Packard Cobra γ-Counter TM with auto-gamma (GMI, Ramsey, MN, USA) was used for measuring gamma counts. The computer program ImageJ[12] was used for quantification calculations. Analytical liquid chromatographic analysis was performed on an HPLC system (Gilson, Middleton, WI, USA) equipped with a Dionex UVD170U photodiode array detector (Thermo Fisher Scientific Inc, Sunnyvale, CA, USA) and β^+-flow detector (Scansys Laboratorieteknik) connected in series. Data collection and liquid chromatography control used a Chromeleon 6.8 SR 10 Build 2818 (Thermo Fisher Scientific Inc). Preparative liquid chromatographic was performed with Knauer advance scientific instrument (Berlin, Germany). [^{18}F]Fluoride was produced using an EclipsedTM HP Cyclotron (Siemens Healthcare, Munich, Germany). ^{18}*O*-Enriched water was from Taiyo Nippon Saso Corporation, Tokyo, Japan.

Radiochemistry

Synthesis of N-succinimidyl 4-[^{18}F]fluorobenzoate

N-Succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) was synthesized on an automated radiochemistry module using a three-step, one-pot procedure modified from that of Tang *et al.*[13] [^{18}F]Fluoride was transferred from the cyclotron target with helium gas and trapped on a QMA cartridge. The cartridge was purged with helium for 1 min before absorbed [^{18}F]fluoride was eluted into a reaction vial with 1-mL (1:20) methanol/water mixture containing 20-mg Kryptofix 2.2.2 (K2.2.2) and 5.4-mg potassium carbonate. This procedure took 30 min. The solution was evaporated and co-evaporated to dryness in a nitrogen stream at 110 °C for 24 min with anhydrous acetonitrile (MeCN, 2 × 1 mL). A solution of 10-mg trimethylbenzeneaminium triflate precursor in 0.5-mL dimethyl sulfoxide was added to the dried [K/K2.2.2]$^{+}$^{18}F^{-} complex. The mixture was heated at 90 °C for 10 min before tetrapropylammonium hydroxide solution (1 M) in 700 µL MeCN was added, and the reaction was heated to 120 °C for 6 min. The mixture was then co-evaporated with anhydrous MeCN (2 × 1 mL) to dryness in a helium stream. Next, a solution of 50-mg *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate in 0.6-mL MeCN was added, and the reaction further heated at 90 °C for 10 min. The crude product was mixed with 1.5% acetic acid solution (2 mL) and injected onto the semi-preparative liquid chromatography column (C18(2) Luna, 250 × 10 mm) and eluted using 3 mL/min of 50:50 MeCN/H_2O containing 0.1% trifluoroacetic acid (TFA, R_t [^{18}F]SFB = 11.6 min) The collected fraction was diluted with H_2O and transferred to a Sep–Pak C18 Plus cartridge where it eluted with 2-mL MeCN. The eluate was evaporated to dryness (110 °C for 50 min) to yield the final product, [^{18}F]SFB. The total synthesis time was 140 min. The product was analyzed using a C4 Grace Vydac column,

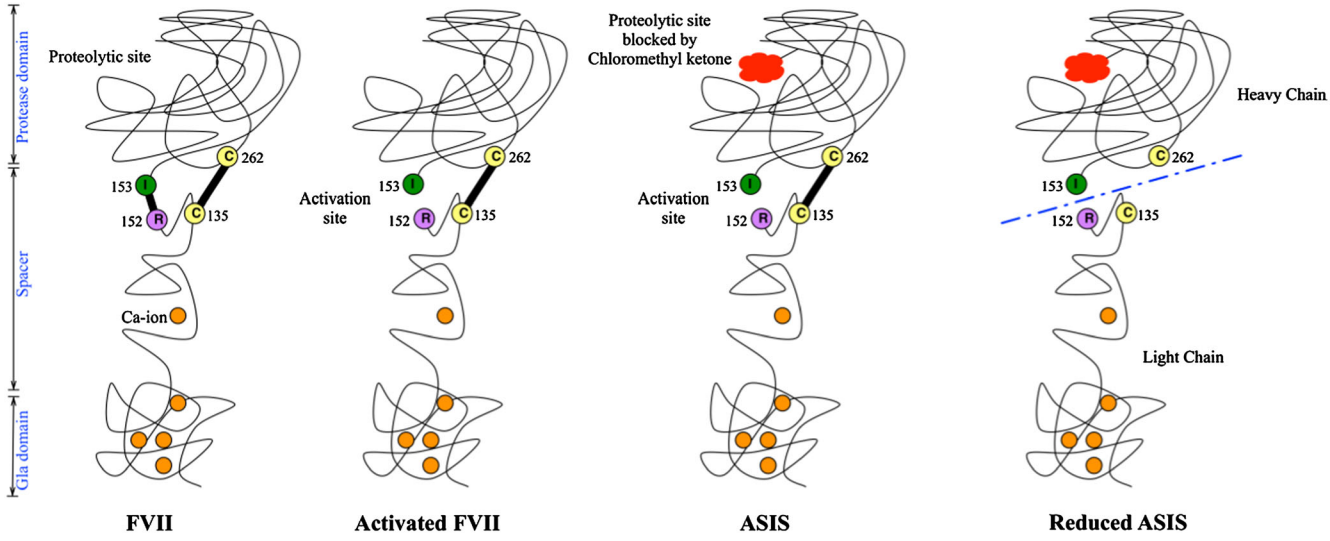


Figure 1. Schematic diagram of factor VII (FVII) showing, from left to right, the activated FVII (obtained by cleavage of amino acids Arg-152–Ile-153[15]), active site inhibited factor VII (ASIS), and reduced ASIS. See Results and discussion for further information on reduced ASIS. This figure is available in colour online at wileyonlinelibrary.com/journal/jlcr

250 × 4.6 mm in 1-mL/min solvent flow. A gradient system with two eluents, A and B, was used, with the fraction of B varying from 0% to 100% over 25 min, A = 1:9 MeCN : H_2O, 0.1% TFA; B = 9:1 MeCN/H_2O, 0.1% TFA. The R_t values of [^{18}F]SFB and the reference compound were 12.7 and 12.3 min, respectively. [^{18}F]SFB was obtained in a radiochemical purity (RCP) >99%, and a decay-corrected radiochemical yield (RCY) 30 ± 6% (n = 3). The specific radioactivity was 95 ± 1 GBq/μmol (n = 3) at the end of synthesis.

Synthesis of ^{18}F-labeled active site inhibited factor VII

Active site inhibited factor VII (1 mg in 400-μL HEPES buffer, pH 7.8) was added to purified and dried [^{18}F]SFB and stirred gently for 1 h at room temperature. The final purification of [^{18}F]ASIS was conducted on a PD-10 desalting column eluting with Gly–Gly buffer (6 min). Twenty-five fractions containing 0.5-mL eluent each were collected, and the most radioactive fractions (usually fractions 5–10) were collected as the final product (Figure 2B; peak 1 was collected). The product was analyzed using a C4 Grace Vydac column, 250 × 4.6 mm in 1-mL/min solvent flow. A gradient system with two eluents, A and B, was used, with the fraction of B varying from 0% to 100% over 25 min. A = 1:9 MeCN : H_2O, 0.1% TFA; B = 9:1 MeCN : H_2O, 0.1% TFA. The R_t of [^{18}F]ASIS was 15.6 min, and the total synthesis time was 66 min. The decay-corrected RCY of purified [^{18}F]ASIS was 25 ± 6% (n = 5) calculated from the starting amount of [^{18}F]SFB. The RCP was >97%.

Determination of specific radioactivity/pseudospecific radioactivity

The specific or pseudospecific radioactivity (pA_s) of the radiotracers was calculated from three to five consecutive HPLC analyses, determined by measuring the area of the ultraviolet (UV) absorbance peak corresponding to the radiolabeled product on the HPLC chromatogram (see the foregoing conditions) and comparing it with a standard curve that related mass to UV absorbance.

Remark: At this point, we want to emphasize that the starting material, ASIS, was not completely separated from the final ^{18}F-labeled product, [^{18}F]ASIS; thus, we report the pA_s.

Determination of the radiochemical purity of ^{18}F-labeled active site inhibited factor VII

The RCP of [^{18}F]ASIS was assessed by analytical HPLC (see the aforementioned applied conditions) and protein precipitation.

Protein precipitation: [^{18}F]ASIS (1 μL of the final product) was mixed with human serum albumin (500 μL of a 1% solution) and $NaHSO_4$ (50 μL of a 0.1 M solution). Afterwards, thrichloroacetic acid (450 μL of a 0.4 M solution) was carefully added. The resulting mixture was vortexed (10 s) and centrifuged (4000 r.p.m., 4 min). The supernatant (500 μL) was removed, and the activity of this fraction and of the remaining sample (500-μL supernatant + the precipitate) was determined. The percentage of protein-bound radioactivity were calculated as follows:

X 500-μL supernatant + the precipitate (counts per minute)
Y 500 μL of the supernatant (counts per minute)

$$\text{Protein bound radiactivity } (\%) = \frac{X - Y(\text{c.p.m.})}{(X+Y) - (2 \times \text{background})(\text{c.p.m.})} \cdot 100\%$$

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

NuPAGE® 4–12% Bis-Tris Gel and NuPage MES SDS running buffer were used in all experiments. The SDS-PAGE system was set up as described.[14]

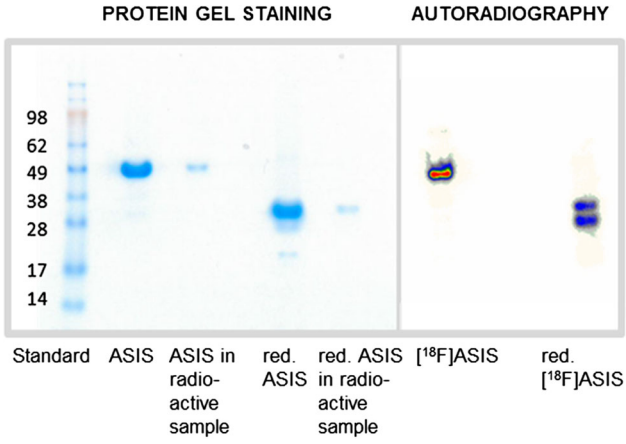


Figure 3. ^{18}F-labeled active site inhibited factor VII (ASIS) and its reduced (red.) forms. The same gel was used for radioactive and non-radioactive samples. This figure is available in colour online at wileyonlinelibrary.com/journal/jlcr

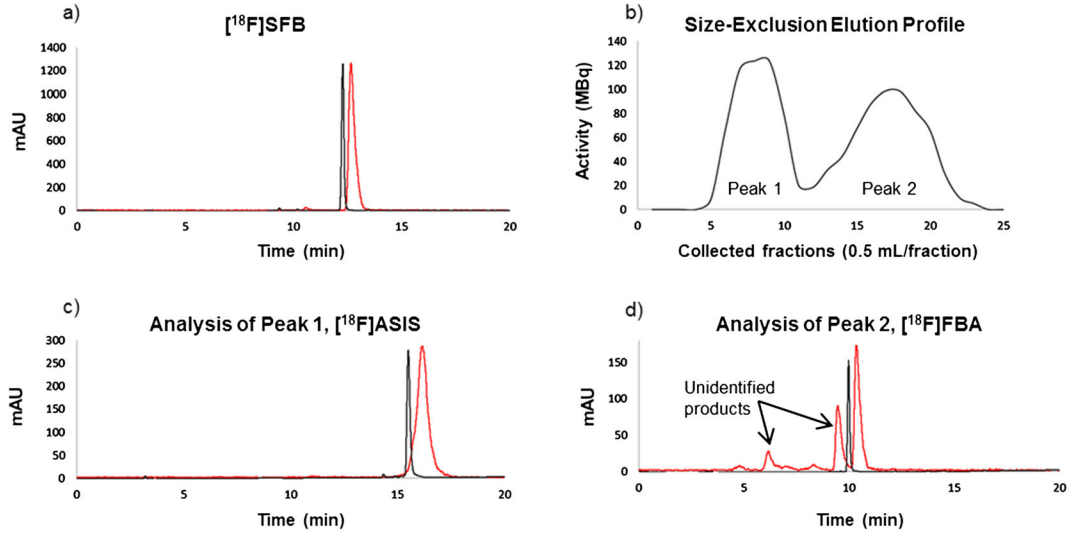


Figure 2. HPLC analysis of ^{18}F-labeled active site inhibited factor VII (ASIS) reaction sequence. (a) Chromatogram of purified *N*-Succinimidyl 4-[^{18}F]fluorobenzoate (SFB) (——) and SFB reference (– –); (b) size exclusion purification of [^{18}F]ASIS; (c) analysis of peak 1, [^{18}F]ASIS (——), and ASIS(– –); (d) analysis of the second peak, [^{18}F] fluorobenzoic acid (FBA) and two other unidentified products (——), and FBA reference (– –). This figure is available in colour online at wileyonlinelibrary.com/journal/jlcr

Gel sample preparation

(Gel plate 1) [^{18}F]ASIS and its reduced forms (Figure 3):

Sample 1: 13 μL of SeeBlue® Plus2 was used as a pre-staining standard.
Sample 2: 13 μL of ASIS solution, 6-μL NuPAGE LDS sample buffer (4×), and 5-μL H_2O were mixed (non-reducing conditions).
Sample 3: [^{18}F]ASIS, 5 μL of [^{18}F]ASIS solution, 6-μL NuPAGE LDS sample buffer (4×), and 13-μL H_2O were mixed (non-reducing conditions).
Sample 4: 13 μL of ASIS solution, 6-μL NuPAGE LDS sample buffer (4×), 2.6-μL H_2O, and 2.4-μL NuPAGE® sample reducing agent (10×) were mixed (reducing conditions).
Sample 5: 5 μL of [^{18}F]ASIS solution, 6-μL NuPAGE LDS sample buffer (4×), 10.6-μL H_2O, and 2.4-μL NuPAGE® sample reducing agent (10×) were mixed (reducing conditions).

(Gel plate 2) *In vitro* stability of [^{18}F]ASIS in plasma (Table 1):

Sample 1–3: Same as for gel plate 1.
Sample 4–7: [^{18}F]ASIS was added to the mouse plasma and incubated (see details as follows). Afterwards, 10-μL NuPAGE LDS sample buffer and 10-μL H_2O were added.

(Gel plate 3) *In vivo* metabolite study of [^{18}F]ASIS (Table 1):

Sample 1–3: Same as for gel plate 1.
Sample 4–7: The blood samples were centrifuged, and the mouse plasma separated. Afterwards, 18 μL of the plasma samples were mixed with 6-μL NuPAGE LDS sample buffer.

After this initial preparation, each sample was heated for 10 min at 70 °C. Subsequently, 13 μL of the appropriate solution was applied to the gel well, and electrophoresis was performed at constant voltage (200 V for 30 min). The gels were then exposed to a phosphor imaging plate, and radioactivity was analyzed using a Fuji Bass system (Bass 1800, Fuji Film, Tokyo, Japan) or a Cyclone Plus Phosphor Imager (Perkin Elmer, Waltham, MA, USA). Afterwards, SimplyBlue™ SafeStain staining agent was added to the gels, which were then slowly shaken on a horizontal rotator overnight. Finally, any excess developing agent was washed away with water.

Stability of ^{18}F-labeled active site inhibited factor VII

Purified [^{18}F]ASIS was dissolved in Gly–Gly buffer, pH 7.5, and incubated at room temperature for 0.5, 1, 2, or 4 h. After incubation, three samples were withdrawn from each mixture and analyzed for RCP by analytical HPLC : C4 Grace Vydac column, 250 × 4.6 mm and flow rate at 1 mL/min. Gradient system is mentioned previously; see 'Synthesis of [^{18}F]ASIS'.

Animals

Female NMRI mice (Taconic, Lille Skensved, Denmark) were used for *in vivo* stability studies. All animal procedures were performed under a protocol approved by the National Animal Experiments Inspectorate (Denmark).

Table 1. The stability of ^{18}F-labeled active site inhibited factor VII (ASIS) in various media

Time (h)	Buffer stability of [^{18}F]ASIS (%) ($n = 3$)	*In vitro* stability of [^{18}F]ASIS (%) ($n = 3$)	*In vivo* stability of [^{18}F]ASIS (%) ($n = 2$)
0.5	98 ± 1	89 ± 2	92 ± 4
1	97 ± 1	85 ± 4	79 ± 3
2	96 ± 1	76 ± 3	73 ± 1
4	96 ± 1	73 ± 4	65 ± 4

Stability of ^{18}F-labeled active site inhibited factor VII in mice plasma (gel plate 2)

Purified [^{18}F]ASIS (10 μL) was dissolved in murine plasma (10 μL) and incubated at 37 °C for 0.5, 1, 2, or 4 h. After incubation, three samples were withdrawn from each mixture and analyzed and prepared as mentioned previously; see 'SDS-PAGE'.

Metabolite study in mice (gel plate 3)

Mice were injected with 10-MBq [^{18}F]ASIS, which corresponded to 14 ± 4-μg ASIS (calculated from the pseudospecific activity). At each of the time points 0.5, 1, 2, and 4 h, two samples were withdrawn and analyzed and prepared as mentioned previously; see 'SDS-PAGE'.

Pull down experiments with sepharose 4B-coupled TF(1–209) and sepharose 4B-coupled-anti-factor VII monoclonal antibody F1A2

The sepharose 4B-coupled TF(1–209) suspension (sTF) and sepharose 4B-coupled-anti-FVII monoclonal antibody F1A2 suspension (FVII mcab Seph. 4B) were obtained from Novo Nordisk A/S.[2]

Matrix preparation

Sepharose 4B-coupled TF(1–209) suspension (250 μL) and FVII mcab Seph. 4B (250 μL) suspensions were taken out in separate vials and equilibrated with 250-μL assay buffer (10-mM Gly–Gly, 150-mM NaCl, 10-mM $CaCl_2$, 0.5% bovine serum albumin, and pH 7.5). Equilibration was performed by spinning down the matrices using a centrifuge for 30 s. After spinning down the matrix, the supernatant was removed, and 500-μL assay buffer was added to the matrix. This washing procedure was performed three times. Finally, 125 μL of assay buffer was added. Each of the matrices was divided in 4 × 30 μL in 500-μL polymerase chain reaction (PCR) vials.

^{18}F-labeled active site inhibited factor VII solution preparation

The concentration of ASIS was determined in the [^{18}F]ASIS solution by analytical HPLC using the same conditions as described previously. The concentration of ASIS was determined by measuring the area of the corresponding UV absorbance peak and comparing it with a standard curve that related mass to UV absorbance. Depending on the ASIS concentration, the final product (the [^{18}F]ASIS solution) was adjusted to approximately 5 μg/mL with assay buffer.

Sample preparation

Assay buffer (30 μL) was added to the aforementioned PCR vials, and to an additional vial, 60 μL of assay buffer was added. Afterwards, 170 μL of an approximately 5-μg/mL [^{18}F]ASIS solution was added to all PCR vials. All vials were swirled to mix and placed on the plate shaker for 60 min at room temperature and then centrifuged for 30 s. Finally, 50 μL of each supernatant was removed to a counting vial and counted for 1 min in the γ-counter.

The binding capacity was calculated as follows:

Counts in 50-μL supernatant (sTF or FVII mcab Seph. 4B) : C (counts per minute)

Counts in 50-μL assay buffer supernatant: D (counts per minute)

$$\%\,\text{Binding} = \frac{(D - C)}{D} \cdot 100\%$$

Results and discussion

^{18}F labeling of active site inhibited factor VII

N-Succinimidyl 4-[^{18}F]fluorobenzoate[13] was used as a prosthetic group to label ASIS with ^{18}F, as it conjugates to amino groups at physiological pH and is therefore suitable for labeling

theavailable lysine residues or the amino terminals present in ASIS.[15]

N-Succinimidyl 4-[^{18}F]fluorobenzoate was synthesized on a fully automated radiochemistry module, in a three-step, one-pot reaction sequence similar to the procedure recently published by Tang *et al.*[13] In brief, the synthesis started with an aromatic nucleophilic ^{18}F substitution of a trimethylammonium salt, which was followed by hydrolysis of the resulting ethyl ester and activation with *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (Scheme 1). When a solid-phase extraction cartridge purification was used for this compound, a small amount of impurities remained, and these interfered with the conjugation to ASIS. This procedure was therefore replaced with a preparative HPLC purification.[16] That yielded [^{18}F]SFB in an RCP of >99% (Figure 2A) and a decay-corrected RCY of 30 ± 6% (n = 3), consistent with the reported procedure.[13] The specific radioactivity was 95 ± 1 GBq/μmol (n = 3) at the end of synthesis. The total duration of our procedure is about 140 min, due primarily to the long trapping/eluting/drying procedure (54 min) and the evaporation process to produce dry [^{18}F]SFB (50 min). No optimization for reduction of the duration of our procedure was conducted. Although this procedure is somewhat longer than the 40-min (including the Sep–Pak cartridge step) and 90-min procedures reported by Tang *et al.*[13] and by Jahan *et al.*,[16] respectively, the resulting radioactivity levels were satisfactory for all kinds of evaluation studies.

Dried [^{18}F]SFB was combined with ASIS in HEPES buffer, pH 7.8, at room temperature for 1 h, and the resulting [^{18}F]ASIS was purified on a PD-10 desalting column. These coupling and purification steps required ~65 min. Two peaks eluted during size exclusion chromatography (Figure 2B) and were analyzed by HPLC. The first contained pure [^{18}F]ASIS (Figure 2C), whereas the second contained [^{18}F]fluorobenzoic acid and two unidentified side products (Figure 2D). [^{18}F]SFB was completely consumed or degraded during the coupling procedure. The decay-corrected RCY of purified [^{18}F]ASIS was 25 ± 6% (n = 5) calculated from the starting amount of [^{18}F]SFB.

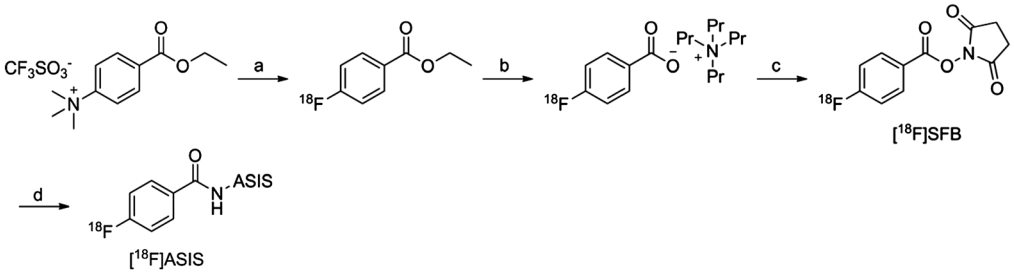
The precursor (ASIS) and the final product ([^{18}F]ASIS) were not completely separated by the present procedure, which is not unusual for larger molecules like antibodies, proteins, or nanoparticles. However, the procedure did separate ~50% of the ASIS initially present. The pA_s, which is based on the determination of the precursor concentration, was 35 ± 9 GBq/μmol (n = 5) at the end of synthesis. The pseudospecific activity was calculated as follows: pA_s = activity of [^{18}F]ASIS/ASIS amount (precursor). For *in vivo* stability experiments, 10 MBq of [^{18}F]ASIS was used; this corresponded to 14 ± 4 μg of the starting material (ASIS) as calculated from the pseudospecific activity. With a clinical dose of 220 MBq per patient (comparable with a fluorodeoxyglucose scan for a 75-kg patient), this corresponds to less than 0.01-mg/kg ASIS. A single dose of up to 0.40-mg/kg ASIS has been reported safe in clinical studies.[8,17,18] The RCP of [^{18}F]ASIS was >97% as determined by protein precipitation[2] and analytical HPLC.

Determination of labeling ratio between the heavy and light chains of ^{18}F-labeled active site inhibited factor VII

Active site inhibited factor VII contains 17 lysines and two *N*-termini, all of which could, in principle, be conjugated to [^{18}F]SFB in a nonspecific way. We were interested in whether [^{18}F]SFB coupling resulted in equipotent labeling of the light and heavy chains of ASIS and investigated this via experiments in which [^{18}F]ASIS was subjected to reducing conditions followed by SDS-PAGE. Activation of FVII to FVIIa results in cleavage of the R_{152}–I_{153} band that separates the protein into two chains held together by the C_{135}–C_{262} disulfide bond (Figure 1). Cleavage of disulfide bond under reducing conditions prior to SDS-PAGE makes it possible to separate the protein on the gel, producing heavy and light chains having apparent molecular weight values of approximately 30 and 28 kDa, respectively.[19] Analysis of the radioactivity of the two bands on the SDS-PAGE gel allowed us to evaluate whether preferred sites were labeled on either the heavy or the light chains. The theoretical ratio between [^{18}F]ASIS's heavy (eight lysines and one *N*-terminus) and light (nine lysines and one *N*-terminus) chains is 0.9 when all 19 positions are labeled with equal probability. We measured a heavy-chain-to-light-chain ratio of 0.74, indicating a slight preference for labeling of the light chain amine groups.

Stability studies of ^{18}F-labeled active site inhibited factor VII

The stability of [^{18}F]ASIS was investigated in Gly–Gly buffer solution, mouse plasma, and in living mice (Table 1). In the buffer solution, more than 96 ± 1% (n = 3) of [^{18}F]ASIS was intact after 4 h. The molecule was metabolized relatively slowly in mouse plasma, with 89 ± 2%, 85 ± 4%, 76 ± 3%, and 73 ± 4% (n = 3) intact [^{18}F]ASIS detected after 0.5, 1, 2, and 4 h, respectively. *In vivo* metabolism in mice was also relatively slow, with 92 ± 4%, 79 ± 3%, 73 ± 1%, and 65 ± 4% (n = 2) intact [^{18}F]ASIS detected after 0.5, 1, 2, and 4 h, respectively (Figure 4 and Table 1).



Scheme 1. Radioactive multi-step synthesis of ^{18}F-labeled active site inhibited factor VII (ASIS). (a) dimethyl sulfoxide, 90 °C, 10 min; (b) tetrapropylammonium hydroxide, anhydrous acetonitrile (MeCN), 120 °C, 6 min; (c) *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate, MeCN, 90 °C, 10 min; (d) ASIS (1 mg in 400-μL HEPES buffer), pH 7.8, room temperature, 1 h.

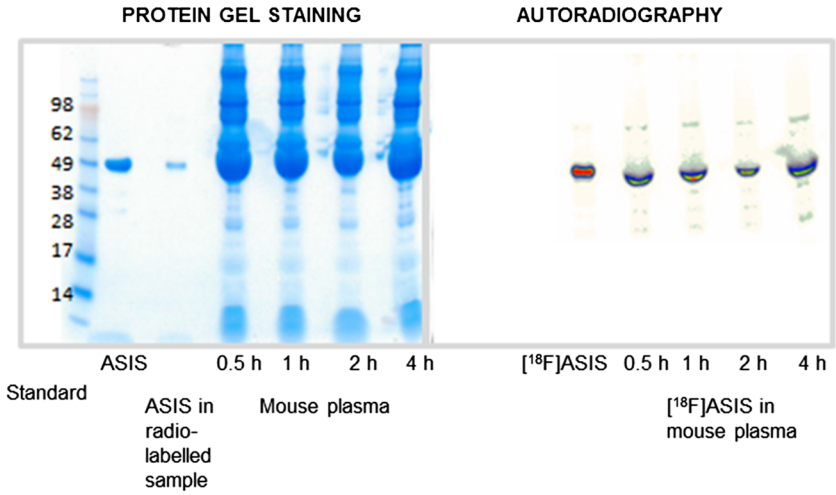


Figure 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis gel for ^{18}F-labeled active site inhibited factor VII (ASIS) *in vivo* metabolism stability in mice. The same gel was used for radioactive and non-radioactive samples. This figure is available in colour online at wileyonlinelibrary.com/journal/jlcr

Binding efficacy of ^{18}F-labeled active site inhibited factor VII

The binding efficacy of [^{18}F]ASIS (sample 1) to TF and to a specific anti-FVII monoclonal antibody (F1A2) was investigated in pull-down experiments and compared with the binding of unlabeled ASIS (sample 2). The concentration of ASIS in each sample was 5 μg/mL. The pull-down experiments showed that the binding efficacies of [^{18}F]ASIS (sample 1) and ASIS (sample 2) to F1A2-mAb-sepharose and TF (1–209)-sepharose were essentially the same (83 ± 4% vs 87 ± 5% and 90 ± 2% vs 91 ± 2%, respectively), after 1-h incubation (n = 4). Thus, the additional introduction of the ^{18}F tag did not alter the binding efficacy of ASIS.

Conclusion

Active site inhibited factor VII has been successfully radiolabeled with [^{18}F]SFB, resulting in [^{18}F]ASIS with satisfactory RCY, RCP, and pseudospecific activity in a total synthesis time of approximately 200 min. The ^{18}F-labeling procedure was shown to produce a [^{18}F]ASIS molecule in which available amino groups were randomly labeled. The *in vitro* stability studies in buffer and in murine plasma as well as the *in vivo* stability studies in mice showed relatively slow degradation. In addition, the binding capability of [^{18}F]ASIS to TF and a specific monoclonal FVII antibody was essentially unchanged after ^{18}F labeling. Thus, our results indicate that [^{18}F]ASIS may be a promising candidate positron emission tomography tracer to image TF expression levels *in vivo*.

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Conflict of Interest

The authors did not report any conflict of interest.

References

[1] P-O. Fresgard, O. H. Olsen, E. Persson, *Protein Sci.* **1996**, *5*, 1531.
[2] J. Madsen, J. B. Kristensen, O. H. Olsen, C. L. Christoffersen, L. C. Petersen, M. Tranholm, A. Kjaer, B. Hessen, *J. Label. Compd. Radiopharm.* **2011**, *54*, 214.
[3] A. Nalla, I. Buch, M. Sigvardt, R. P. Bodholdt, A. Kjaer, B. Hesse, *Inter. J. Mol. Imag.*, **2012**, 2012.
[4] H. Hong, Y. Zang, T. R. Nayak, J. W. Engle, H. C. Wong, B. Liu, T. E. Barnhart, W. Cai, *J. Nucl. Med.* **2012**, *53*, 1748.
[5] J. E. Bluff, N. J. Brown, M. W. R. Reed, C. A. Staton, *Breast Cancer Res.*, **2008**, *10*, 204.
[6] W. Ruf, N. Yokota, F. Schaffner, *Thromb. Res.* **2010**, *125*, S36–S38.
[7] B. B. Sørensen, E. Persson, P.-O. Freskgård, M. Kjalke, M. Ezban, T. Williams, L. V. M. Rao, *J. Biol. Chem.* **1997**, *272*, 11863.
[8] T. Klitgaard,T. G. Nielsen, *Br. J. Clin. Pharmacol.* **2008**, *65*, 3.
[9] E. Erhardtsen, P. Nilsson, M. Johannessen, M. S. Thomsen, *J. Clin. Pharmacol.* **2001**, *41*, 880.
[10] A. C. W. Pike, A. M. Brzozowski, S. M. Roberts, O. H. Olsen, E. Persson, *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 8925.
[11] B. J. N. Leonard, B. J. Clarke, S. Sridhara, R. Kelley, F. A. Ofosu, M. A. Blajchman, *J. Biol. Chem.* **2000**, *275*, 34894.
[12] http://imagej.nih.gov/ij/ [3 March 2014].
[13] G. Tang, X. Tang, X. Wang, *J. Label. Compd. Radiopharm.* **2010**, *53*, 543.
[14] https://www.lifetechnologies.com/se/en/home/references/protein-analysis-guide/protein-separation.html [10 January 2014].
[15] D. W. Banner, A. D'Arcy, C. Chène, F. K. Winkler, A. Guha, W. H. Komigsberg, Y. Nemerson, D. Kirchhofer, *Nature* **1996**, *380*, 41.
[16] M. Jahan, S. Nag, R. Krasikova, U. Weber, A. Muhs, A. Pfeifer, C. Spenger, D. Willbold, B. Gulyás, C. Halldin, *Nucl. Med. Biol.* **2012**, *39*, 315.
[17] B. Jilma, C. Marsik, F. Mayr, M. T. Graninger, F. B. Taylor, M. C. Ribel, E. Erhardtsen, S. Handler, H. G. Eichler, *Clin. Pharmacol. Therapeut.* **2002**, *72*, 403.
[18] J. L. Vincent, A. Artigas, L. C. Petersen, C. Meyer, *Crit. Care Med.* **2009**, *37*, 1874.
[19] C. D. Dickinson, W. Ruf, *J. Biol. Chem.* **1997**, *272*, 19875.