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Development and in vivo characterization of the first peptide-based ^{99m}Tc-Nitrido-radiopharmaceuticals for FAP-targeted SPECT imaging

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Abstract:

Background: Fibroblast activation protein (FAP) is a well-established target for molecular imaging and targeted radionuclide therapy. Although several technetium-99m-labelled FAP imaging agents have been reported, these predominantly employ small-molecule FAP inhibitors. This study aimed to develop and characterize technetium-99m-labelled radiopharmaceuticals based on the cysteine-modified FAP-targeting peptide scaffold (cysFAP) derived from the DOTA-FAP-2286 sequence, and the highly chemically modulable [^{99m}Tc][Tc(N)PNP]-synthon (PNP= bisphosphinoamine). Two PNP ligands with distinct physicochemical properties were assessed: **PNP3**= {[(CH₃OCH₂CH₂)₂P(CH₂)₂]₂N(CH₂)₂OCH₃}; **PNP3OH**= {[(HOCH₂)₂P(CH₂)₂]₂N(CH₂)₂OCH₃}. The resulting [^{99m}Tc][Tc(N)PNP]cysFAP compounds were evaluated for their potential for single-photon emission computed tomography (SPECT) imaging.

Results: [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP were obtained with radiochemical yields of 95.4 ± 1.9% and 86.2 ± 3.0%, respectively. Both compounds demonstrated favourable serum stability and specific binding to FAP-expressing cells. The more hydrophilic PNP3OH derivative showed higher cellular uptake and a lower equilibrium dissociation constant (3.0 ± 1.5 nM) than the PNP3 derivative (9.8 ± 3.1 nM). In mice bearing paired FAP-positive and FAP-negative xenografts, both radiopharmaceuticals demonstrated selective tumour accumulation and low uptake in most non-target tissues. At approximately 4.5 h post-injection, FAP-positive tumour uptake was 6.60 ± 1.15 %ID/g for [^{99m}Tc][Tc(N)PNP3]-cysFAP and 4.55 ± 1.27 %ID/g for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP, while uptake in FAP-negative tumours remained below 0.2 % %ID/g. SPECT/CT clearly visualized FAP-positive tumours at 1 and 4 h. The two compounds exhibited distinct tumour kinetics, with PNP3 showing higher early tumour accumulation and PNP3OH demonstrating increasing tumour uptake between 1 and 4 h.

Conclusions: The FAP-targeting peptide scaffold of DOTA-FAP-2286 was successfully adapted for technetium-99m labelling while retaining high-affinity, FAP-specific tumour targeting. The choice of the bisphosphinoamine ligand substantially influenced the biological profile, establishing the feasibility of fine-tuning radiopharmaceutical pharmacokinetics through rational modification of the radiochemical framework. [^{99m}Tc][Tc(N)PNP3]-cysFAP showed the most favourable overall in vivo profile and warrants further optimization as a ^{99m}Tc-based FAP-targeted radiopharmaceutical for imaging or as intra-operative probe.

Keywords: Single Photon Emission Tomography (SPECT), fibroblast activation protein, oncology, inflammation, technetium-99m

Introduction

Fibroblast activation protein (FAP) is a type II transmembrane serine protease that is overexpressed in cancer-associated fibroblasts within the tumour microenvironment of many epithelial malignancies. FAP expression is reported in up to 90% of epithelial cancers. Since its expression in healthy tissue is negligible, FAP is highly attractive for theranostic nuclear medicine applications. With considerable

advances in FAP-targeted radiopharmaceuticals, it can now be considered an established molecular marker for both imaging and targeted radionuclide therapy. [1-4].

Several FAP-targeting vectors such as DOTA-FAPI-46 and DOTA-FAP-2286, have demonstrated high affinity and specificity for FAP, and this is ideal for diagnostic applications with excellent tumour-to-background uptake [5,6]. Numerous studies have demonstrated that FAP Positron Emission Tomography (PET) can outperform [^{18}F]FDG (^{18}F -Fluorodeoxyglucose) in cancers associated by abundant stromal activation [7] such as breast [8], gastric [9, 10], hepatic [11] and pancreatic malignancies [10, 12]. Consequently, FAP PET has emerged as a prominent radiopharmaceutical for tumour detection, staging and treatment response assessment [1, 2, 9].

Despite these advances, most clinically investigated FAP-targeting radiopharmaceuticals rely on PET radionuclides such as gallium-68 and fluorine-18. Although PET imaging provides excellent sensitivity and spatial resolution, the infrastructure required for radionuclide production, radiopharmaceutical preparation and imaging remains unavailable in many parts of the world, especially in low- and middle-income countries [13]. Technetium-99m remains the most widely used diagnostic radionuclide due to its favourable physical characteristics (140 keV gamma-emission, 6 hours physical half-life) for single photon emission tomography (SPECT) imaging, and widespread availability from relatively inexpensive $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ -generators. Last year, it was reported that a total of 23 488 SPECT or SPECT/Computed Tomography (CT) systems are currently in operation worldwide, compared to only 5381 PET/CT scanners [13]. The development of $^{99\text{m}}\text{Tc}$ -FAP radiopharmaceuticals therefore remains crucial for expanding equitable global access to nuclear medicine [14].

In parallel with diagnostic imaging, FAP-targeting radiopharmaceuticals open the possibility for theranostic applications. Unlike [^{18}F]FDG which is restricted to diagnostic use, FAP targeting has the flexibility to allow for the incorporation of therapeutic radionuclides to enable targeted radionuclide therapy (TRT). Several FAP-targeting compounds labelled with beta-minus-emitting radionuclides, particularly lutetium-177, are currently under investigation for therapeutic applications, with encouraging preliminary results. Among these, the DOTA-FAP-2286 compound, a cyclic peptide conjugated to tetraazacyclododecane tetraacetic acid (DOTA) for the coordination of radiometals for

77 imaging and therapeutic applications, has emerged as a promising candidate due to its serum stability
78 and prolonged tumour retention compared to small-molecule FAP inhibitor (FAPI) derivatives. In
79 contrast, several small-molecule derivatives exhibit rapid tumour wash-out, potentially resulting in
80 insufficient tumour residence time for effective deposition of the therapeutic radiation dose [5, 14].

81 Although several technetium-99m FAP targeting radiopharmaceuticals have been reported, these agents
82 are all based on small-molecule FAPI scaffolds [15]. Technetium-99m based imaging agents are not so
83 advanced as their PET counterparts, with the limitation remaining in the detection of metastatic lesions,
84 particularly in lymph nodes and distant organs [15]. Since the peptide-based ligand FAP-2286
85 demonstrates prolonged tumour retention and higher lesion uptake through enhanced receptor-mediated
86 internalisation [14] these may partially compensate for the lower sensitivity of SPECT by increasing
87 tumour-to-background contrast and extending the imaging window. This makes DOTA-FAP-2286 a
88 worthwhile lead compound in the future development of FAP based technetium imaging agents.

89 To the best of our knowledge, this is the first report of a diagnostic technetium-99m-labelled FAP-
90 targeted radiopharmaceutical based on the DOTA-FAP-2286 sequence, in which the DOTA chelator was
91 replaced with a cysteine residue and the [^{99m}Tc][Tc(N)PNP]- synthon (Figure 1) [16, 17]. Based on
92 previous work [18], two bisphosphinoamine ligands with distinct physicochemical properties were
93 investigated to assess their influence on the biological profile of the resulting radiolabelled peptides.
94 N,N-bis[(dimethoxypropylphosphino)ethyl]methoxyethylamine (PNP3) provides a bulkier and more
95 lipophilic ligand environment, whereas N,N-bis[(di-hydroxymethylphosphinoethyl)]methoxyethylamine
96 (PNP3OH) is more hydrophilic and water-soluble [18]. The resulting radioligands were subjected to
97 comprehensive in vitro stability and binding studies using HEK293T.hFAP cells, followed by ex vivo
98 biodistribution and SPECT/CT imaging in HEK293T.hFAP xenograft-bearing mice. These studies
99 enabled assessment of their in vivo pharmacokinetic profiles, metabolic stability, and tumour
100 accumulation, as well as the influence of the bisphosphinoamine ligand on their overall biological
101 behaviour.

102

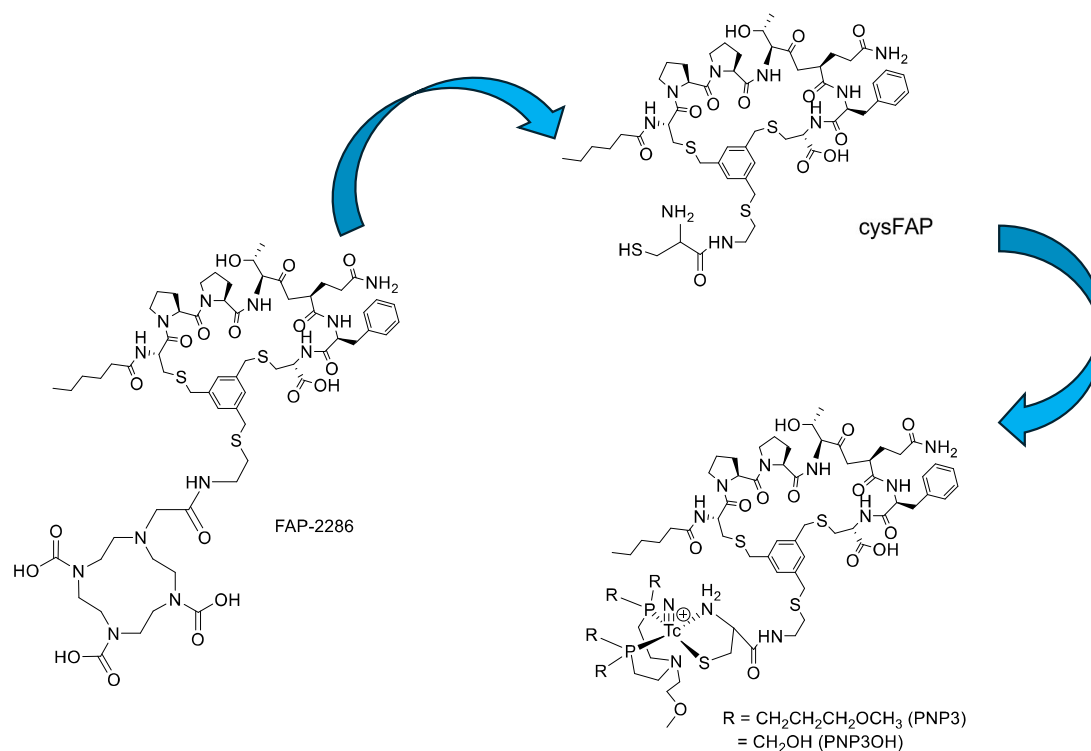


Figure 1: Chemical drawing of FAP-2286 and cys-FAP ligands and of $[^{99m}\text{Tc}][\text{Tc}(\text{N})\text{PNP3}]\text{-cysFAP}$ and $[^{99m}\text{Tc}][\text{Tc}(\text{N})\text{PNP3OH}]\text{-cysFAP}$ compounds evaluated in this study.

Materials and Methods

1) Materials

The bisphosphinoamine ligands (PNP3= N,N-bis[(dimethoxypropylphosphino)ethyl]methoxyethylamine; PNP3OH= N,Nbis[(di-hydroxymethylphosphinoethyl)]methoxyethylamine) were synthesized in-house as previously described [18]. Sodium pertechnetate ($[^{99m}\text{Tc}]\text{NaTcO}_4$) was obtained by elution of either a $^{99}\text{Mo}/^{99m}\text{Tc}$ -DryTec generator (GE Healthcare, Milan, Italy) or an Ultra-Technekow FM generator (Curium, Mechelen, Belgium).

Succinic dihydrazide (SDH), oxalic acid, mouse serum and human serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stannous chloride dihydrate ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) and sodium hydroxide were obtained from Thermo Fisher Scientific (Waltham, MA, USA), (Geel, Belgium). All solvents were purchased from Chemlab (Zedelgem, Belgium). $\text{H}_3\text{NSCysFAP}$ (cysFAP) was obtained from Pepmic

(Suzhou, China), and UAMC-1110 was purchased from Ambeed (Arlington Heights, IL, USA). Solid-phase extraction cartridges (Oasis HLB) were obtained from Waters Corporation (Milford, MA, USA). The Sep-Pak C18 Light cartridge was procured from Waters Corporation (Milford, MA, USA). Cell culture reagents, including Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), TrypLE™ Express Enzyme, puromycin, Poly-D-lysine, Reagent A100 and Reagent B, were purchased from Thermo Fisher Scientific (Waltham, MA, USA). A 50 mM glycine-HCl buffer (pH 2.8) was prepared in-house using Sigma-Aldrich reagents. General laboratory consumables, including cell culture flasks, six-well plates, Falcon tubes and pipette tips, were obtained from Thermo Fisher Scientific. Trypan Blue solution and EVE™ disposable counting slides were purchased from NanoEntek (Seoul, Republic of Korea), while NucleoCounter® cassettes were obtained from ChemoMetec (Allerød, Denmark). The stable human fibroblast activation protein (hFAP)-expressing HEK293T cell line (HEK293T.hFAP and HEK293T control) was obtained from Creative Biogene Biotechnology (Shirley, NY, USA). SCID Beige mice (C B17.Cg-Prkdc scid Lystbg-J/Crl) were procured from Charles River Laboratories (Sulzfeld, Germany). Cultrex® Basement Membrane Extract was obtained from R&D Systems (Minneapolis, MN, USA).

2) Analytical procedures

High-performance liquid chromatography (HPLC) was used to determine radiochemical yield, purity and stability of the radiolabelled compounds. HPLC was performed on a Dionex Ultimate 3000 equipped with a Gabi Raystest Radiometric detector. A symmetry 300 RP-18 column (5 µm 4.6 x 250 mm) with a Symmetry 300 RP-C18 precolumn ((=5 µm 4.6 x 45 mm) from Waters Corporation (Milford, MA, USA) were used. LC/MS was used to confirm product formation. Chromatography was performed using a HPLC Finnigan Surveyor Plus system from Thermo Fisher (San Jose, CA, USA) with a photodiode PDA detector, interfaced with an LCQ Fleet ion trap mass spectrometer from Thermo Fisher (San Jose, CA,

USA). A symmetry 300 RP-18 column (5 μ m 4.6 x 250 mm) with a Symmetry 300 RP-C18 precolumn (5 μ m 4.6 x 45 mm) from Waters Corporation (Milford, MA, USA) was used.

Analytical method for $[^{99m}\text{Tc}]/[\text{Tc}(\text{N})\text{PNP3}]/\text{-cysFAP}$: $\lambda = 230$ nm. Flow rate = 1 ml/min. Solvents: A) 0.1% TFA in H_2O ; B) 0.1% TFA in CH_3CN . Gradient: 0–1 min, %B = 40; 1–17 min, %B = 42; 17–23 min, %B = 42; 23–24 min, %B = 90; 24–28 min, %B = 90; 28–29 min, %B = 40; 29–30 min, %B = 40. $[^{99m}\text{Tc}]/[\text{Tc}(\text{N})\text{PNP3OH}]/\text{-cysFAP}$: $\lambda = 230$ nm. Flow rate = 1 ml/min. Solvents: A) 0.1% TFA in H_2O ; B) 0.1% TFA in CH_3CN . Gradient: 0–1 min, %B = 25; 1–17 min, %B = 32; 17–23 min, %B = 32; 23–24 min, %B = 90; 24–28 min, %B = 90; 28–29 min, %B = 25; 29–30 min, %B = 25.

3) Radiosynthesis

Radiolabelling was performed according to the procedure previously described [19, 20] with minor modifications. All syntheses were carried out under an oxygen-free nitrogen atmosphere using freshly degassed reagents. Briefly, succinic dihydrazide (SDH, 2.5 mg) was added to a reaction vial, which was sealed and purged with nitrogen. A freshly prepared solution of stannous chloride (50 μ l, 1 mg/ml in 0.9% saline) was added, followed by 200 μ l of degassed ethanol. Sodium pertechnetate ($[^{99m}\text{Tc}]\text{NaTcO}_4$; 100 MBq–1 GBq) was subsequently added in a total volume of 800 μ l using 0.9% saline as required and the reaction vial was vortexed for 10–20 seconds. The subsequent radiolabelling procedure depended on the phosphinoamine ligand employed.

$[^{99m}\text{Tc}]/[\text{Tc}(\text{N})\text{PNP3}]/\text{-cysFAP}$: PNP3 (50 μ l, 1 mg dissolved in 100 μ l degassed ethanol) was added to the reaction mixture, immediately followed by cysFAP (50 μ g, 42.1 nmol; 1 mg/ml in degassed ethanol) and phosphate-buffer (PB, 200 μ l, 0.2 M, pH 7.4). The reaction mixture was heated at 80 $^\circ\text{C}$ for 30 min. The final reaction pH was 7. $[^{99m}\text{Tc}]/[\text{Tc}(\text{N})\text{PNP3OH}]/\text{-cysFAP}$: PNP3OH $\cdot$ HCl was dissolved in water (1 mg/100 μ l). An aliquot (50 μ l) of this solution was diluted with 400 μ l of PB (0.2 M, pH 7.4), and 225 μ l of the buffered ligand solution was subsequently added to the reaction rapidly followed by cysFAP (50 μ g, 42.1 nmol; 1 mg/ml in degassed ethanol). The reaction mixture was incubated at room temperature for 30 min. The final reaction pH was 7.

Purification of crude mixture: During both the radiolabelling of [^{99m}Tc][$\text{Tc}(\text{N})\text{PNP3}$]-cysFAP and [^{99m}Tc][$\text{Tc}(\text{N})\text{PNP3OH}$]-cysFAP, the final product was purified for all batches. The crude reaction mixture was purified using a Sep-Pak C18 Light cartridge conditioned prior to use. The reaction mixture was diluted to a final volume of 10 ml with 0.1 M NaOH and loaded onto the cartridge. The cartridge was subsequently washed with 15 ml of 0.1 M NaOH, followed by 20 ml phosphate-buffered saline (PBS), 10 ml of 20% (v/v) ethanol in 0.1 M NaOH, 5 ml of 30% (v/v) ethanol in 0.1 M NaOH, and finally 10 ml ultrapure water. The purified radiopharmaceutical was eluted with an 80:20 (v/v) ethanol/0.9% saline solution. The fraction containing the highest radioactivity concentration was collected, the ethanol was removed under a gentle stream of nitrogen, and the product was reconstituted in sterile 0.9% saline for subsequent in vitro and in vivo studies. Quality control was performed on the final formulated product immediately prior to biological evaluation. Molar activity was determined through UV-HPLC, with a predetermined calibration curve.

Carrier-added synthesis of [^{99g}Tc][$\text{Tc}(\text{N})\text{PNP3}$]-cysFAP and [^{99g}Tc][$\text{Tc}(\text{N})\text{PNP3OH}$]-cysFAP and characterization through UV-radioHPLC and LC-MS: Carrier-added [$\text{Tc}(\text{N})\text{PNP3}$]-cysFAP and [$\text{Tc}(\text{N})\text{PNP3OH}$]-cysFAP was synthesized to enable LC-MS characterization. All procedures were performed under an oxygen-free nitrogen atmosphere using degassed reagents. Succinic dihydrazide (15 mg) was combined with stannous chloride (2 mg) dissolved in 50 μl of 0.9% saline, followed by the addition of 200 μl degassed ethanol. Sodium pertechnetate (approximately 500 MBq in 400 μl of 0.9% saline) was added, followed by carrier technetium-99g (100 μg in 100 μl saline). The reaction mixture was mixed thoroughly and incubated for 30 min at room temperature to generate the nitrido intermediate. Subsequently, PNP3 or PNP3OH (1 mg in 100 μl degassed ethanol) was added, followed by cysFAP (300 μg dissolved in 300 μl degassed ethanol). Finally, 200 μl PB 0.2 M pH 7.4 was added, and the reaction mixture was heated at 80 $^{\circ}\text{C}$ for 60 min. The reaction progress and the RCY of the final compounds was monitored by UV-radio HPLC. After SEP-Pak purification the product was analysed by LC/MS to confirm chemical identity/formulation. ESI-MS: calc. for [^{99m}Tc][$\text{Tc}(\text{N})\text{PNP3}$]-cysFAP: $\text{C}_{78}\text{H}_{129}\text{N}_{11}\text{O}_{17}\text{P}_2\text{S}_4\text{Tc} = 1782.04$, found $m/z = 892.42$ [$\text{M}^+ \text{H}$] $^{2+}$ and 1782.34 [M^+]. ESI-MS: calc. for

205 $[^{99m}\text{Tc}][\text{Tc}(\text{N})\text{PNP3OH}]\text{-cysFAP}$: $\text{C}_{66}\text{H}_{105}\text{N}_{11}\text{O}_{17}\text{P}_2\text{S}_4\text{Tc}$ = 1613.72, found m/z = 808.26 $[\text{M} + \text{H}]^{2+}$ and
1614.08 $[\text{M}^+]$.

207 4) Distribution coefficient

208 To determine the n-Octanol/PBS or n-Octanol/water partition coefficients, 3 ml of n-octanol was mixed
209 with either 3 ml of PBS or 3 ml of ultrapure water. To this, 100 μl of the purified radiolabelled compound
210 was added and vortexed for 30 minutes. Then, the sample was centrifuged (3000 g for 10 min) and 25
211 μl of both the organic and the aqueous phases were collected and counted with a gamma counter.

212 5) Stability assays

213 *Serum stability*: The purified radiopharmaceuticals were incubated in a ratio of 1:9 in either human
214 serum or mouse serum. The samples were incubated over time (0 min, 30 min, 60 min, 180 min and 24
215 h) at 37°C in a shaking bath. At each time point, 100 μl of sample was treated with 200 μl of ice cold
216 MeCN 0.1% TFA and centrifuged (3500 g for 10 min, 4°C). The supernatant was diluted with saline
217 (1:3) and assessed by HPLC using the previously described analytical methods.

218 To determine the protein binding, the radioactivity of the pellet and 25 μl of supernatant was measured
219 in the gamma counter. Protein binding was determined by using the following equation: pellet activity/
220 activity (pellet + supernatant) x 100.

221 *In vitro enzymatic stability*: Mouse kidney/liver homogenates were prepared and stored at -20 °C.
222 Preparation: the organs freshly excised from mice were rapidly rinsed with PBS and homogenized in 20
223 mM HEPES buffer (pH 7.3) with an Ultra-Turrax T25 homogenator (IKAWorks, Inc.) for 1 min at 4 °C.
224 The purified radiopharmaceuticals were incubated in a ratio of 1:5 in mouse kidney and mouse liver
225 homogenates. The samples were incubated over time (0 min, 30 min, 60 min, 180 min and 24 h) at 37°C
226 in a shaking bath. A sample was taken at the corresponding time. An OASIS HLB extraction cartridge
227 was preconditioned (1 ml MeOH followed by 1 ml water and finally 3 ml 5% MeOH:H₂O). The sample
228 was loaded on the preconditioned OASIS HLB cartridge, and for $[^{99m}\text{Tc}][\text{Tc}(\text{N})\text{PNP3}]\text{-cysFAP}$, elution
229 from the cartridge was performed with 80% EtOH/saline (1 ml) and for $[^{99m}\text{Tc}][\text{Tc}(\text{N})\text{PNP3OH}]\text{-cysFAP}$
230 the elution from the cartridge was performed with a 60% EtOH-Saline (1 ml) mixture. The recovered

sample was indicated as in-tact. Additionally, a 100 µl of sample was extracted with 200 µl of ice cold MeCN 0.1% TFA, diluted with saline and analysed on HPLC using the previously described analytical methods.

In vivo stability: For each radiopharmaceutical, three healthy female SCID Beige mice were intravenously injected with 15–20 MBq of the respective tracer and sacrificed 10 min post-injection for radiometabolite analysis. Blood and urine were collected immediately after sacrifice, and the radiopharmaceutical was extracted and processed as previously described [20]. Samples were analysed using the same HPLC method employed for radiochemical purity determination during synthesis. For samples with low radioactivity (below the limit of detection of the radiodetector on the HPLC), the HPLC eluant was collected in 1-min fractions, which were subsequently quantified using a Wallac Wizard 1480 gamma counter (PerkinElmer, Waltham, MA, USA).

6) In vitro studies

Cell culture: Human embryonic kidney-HEK293T cells and the corresponding cell line engineered to express a stable human fibroblast activation protein HEK293T.hFAP were obtained from Creative Biogene (Shirley, NY, USA) and cultured as previously described [21]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were routinely passaged upon reaching approximately 80% confluency using TrypLE™ Express Enzyme according to the manufacturer's instructions. Cell passage numbers were maintained below 20 throughout the study, while only cells below passage 10 were used for animal inoculations. Cultures were routinely screened for mycoplasma contamination using the MycoStrip® detection kit (InvivoGen, San Diego, CA, USA).

Cellular binding assay: Cellular binding assays were performed according to our previously reported methodology [21, 22]. Internalization data are not reported here, as our findings concur with FAP-ligands literature, showing a significant variability upon current acid-wash methods [24]. For the evaluation of novel diagnostic compound in an overexpression cell-line such as HEK293T.hFAP,

accurate total cell-associated activity combined with in-depth kinetic binding parameters as provided by the Ligand Tracer experiments, should provide sufficient validation.

Briefly, six-well plates were coated with 2 ml of 0.1 mg/ml Poly-D-lysine for 60 min at 37 °C, washed three times with PBS and allowed to dry under sterile conditions. HEK293T.hFAP and wild-type HEK293T (control) cells were subsequently seeded at the desired cell density (typically 1.5×10^5 cells per well in 500 µl medium) and allowed to adhere overnight under standard culture conditions. The next day, following radiolabelling and quality control, fresh culture medium containing the radiopharmaceutical (360 kBq/ml, 500 µl per well) was added to the cells. The plates were incubated at 37 °C in 5% CO₂ and harvested at predetermined time points (1 hours, 3 hours). As an additional blocking control, 100 µM vector molecule CysFAP was added 30 minutes, before the radiopharmaceutical was added (360 kBq/ml, 500 µl per well), followed by a 1-hour incubation. Following incubation, the culture medium was collected, and the wells were washed three times with 500 µl ice-cold PBS. The remaining cell-associated radioactivity was recovered by lysis with 1 ml Reagent A100 followed by 1 ml of reagent B according to the manufacturer's protocol. Radioactivity in each collected fraction was measured using a Wallac Wizard 1480 gamma counter (PerkinElmer, Waltham, MA, USA). Correction for cell density was performed with cell numbers determined counting the lysate with a NucleoCounter[®] NC-100 (ChemoMetec, Allerød, Denmark), and radioactivity uptake was normalized to 1×10^6 cells.

Binding kinetic assay: The binding kinetics of [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP to hFAP were evaluated using a LigandTracer Yellow instrument (Ridgeview Instruments AB, Uppsala, Sweden). The manufacturer's standard protocol was modified to optimise cell attachment and measurement conditions. Briefly, Petri dishes were coated in two circular areas opposite each other with 0.5 ml of 0.1 mg/ml poly-D-lysine each, for 60 min at 37 °C, washed three times with PBS, and allowed to dry under sterile conditions. Subsequently, 0.5×10^6 HEK293T.hFAP cells and 0.5×10^6 wildtype HEK293T cells (negative control) were seeded on opposite sides of each dish. Wild-type HEK293T cells served as the background control for signal correction. Cells were allowed to adhere and stabilise overnight before measurements. Binding kinetics were

determined using three consecutive association phases at radioligand concentrations of 1, 3, and 9 nM, followed by a dissociation phase in ligand-free medium (0 nM). The resulting association and dissociation curves were analysed using TraceDrawer software (Ridgeview Instruments AB) to determine the equilibrium dissociation constant (K_D). Experiments were performed in triplicate for each radiopharmaceutical ($n = 3$).

7) In vivo evaluation

All animal procedures were approved by the KU Leuven ethical review board (P079-2023) and were carried out following the Directive 2010/63/EU.

SCID Beige mice were grafted with FAP-positive tumours (HEK293T.hFAP, 1×10^6 cells, right shoulder in the prone position) and FAP-negative tumours (HEK293T, 1×10^6 cells, left shoulder in the prone position). Cells were prepared for inoculation in Cultrex (1:1; Cultrex Basement Membrane Extract, R&D systems, Minneapolis, MN) as described previously [21]. After an average of three weeks, the animals were enrolled in the study with a success rate of 75 % for development of usable positive and negative tumours.

For the *in vivo* evaluations a total of 4 animals per group were injected with an activity of 21.9 ± 0.1 MBq for [^{99m}Tc][Tc(N)PNP3]-cysFAP and 19.2 ± 0.6 MBq for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP, formulated in 100 μl of saline and a molar activity adapted to 30 nmol/kg per injection. The molar activity was determined by a calibration curve based on the UV chromatogram and 30 nmol/kg was chosen based on previously determined optimal molar activity to reduce off-target binding [21]. The animals were allowed to wake up during the first hour of biodistribution, whereafter a $\mu\text{SPECT/CT}$ scan was taken 1-hour post-injection ($N=3$), and 4 hours post-injection ($N=3$). All the animals were sacrificed ($N=4$) after the last scanning procedure was completed by decapitation after being anesthetized with (5% isoflurane) and the organs were harvested, weighed and measured a Wallac Wizard gamma counter (Perkin-Elmer, Waltham, MA, USA).

For the scanning procedures, anaesthesia was induced at 5% isoflurane in a constant flow of oxygen at 1L/minute, reduced to 2.5% during the imaging procedures. The animals were positioned head-first

prone during the imaging procedure. An initial scout scan was obtained using the X-CUBE CT system (Molecubes, Ghent, Belgium), followed by a CT acquisition lasting 5 minutes. After completion of the CT scan, the bed with the animals was transferred to the γ -CUBE SPECT system for a 30 -minute acquisition, with energy windows centred at 140 keV ($\pm 10\%$). The acquired SPECT images were reconstructed through a maximum-likelihood expectation-maximization (MLEM) algorithm using 50 iterations (Molecules). A predetermined q-factor (13.45) was applied during postprocessing to enable the generation of SUV-scaled images by converting counts/cc to activity/cc. SPECT/CT fusion and analysis was performed with PFUS version 4.0 (PMOD Technologies, Switzerland). For image analysis, volumes of interest (VOIs) were manually drawn. Tumours were delineated on CT images, kidneys were primarily defined on SPECT with CT used as guidance, and the liver was sampled using a standard 1.5 mm spherical VOI placed over the left lobe.

8) Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD) unless stated otherwise. Statistical analysis was performed using GraphPad Prism 10.6 (GraphPad Software, LLC, Boston MA, USA). Means were compared using the paired and unpaired t-test, when samples were correlated and uncorrelated respectively. Statistically significance was defined as $P < 0.05$.

Results

Radiolabelling

The compounds [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP were successfully prepared and purified according to prescribed methods. The radiochemical yields and HPLC data are provided in Table 1, with representative radio-HPLC chromatograms reported in the Supplementary Information (Figure Supplement A). Both radiocompounds exhibited two characteristic isomers, with their identities and molecular mass confirmed by LC/MS Carrier-added syntheses using mixed ^{99}Tc and ^{99m}Tc were performed to verify the structural identity of [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP. Dual UV/radiometric HPLC profiling showed co-eluting peaks (Figure Supplement B), establishing that the ^{99}Tc - and ^{99m}Tc species are isostructural. Mass spectrometry

[ESI(+)-MS] of the isolated fractions confirmed the expected mass patterns (Experimental Section), consistent with mononuclear monocationic complexes with the [^{99m}Tc][Tc(N)PNP]-synthon (PNP=PNP3, PNP3OH) bound to a cysteine ligand carrying the FAP targeting peptide as a mixture of diastereoisomers.

Table 1: Chromatographic properties and yields of compounds

Complex	HPLC Rt min		a/b isomeric ratio	%RCY* (N=3)	Log-P _{O/W}	Log-D _{O/PBS}
	^{99m} Tc	^{99m} Tc				
[^{99m} Tc][Tc(N)PNP3]-cysFAP	a) 13.4; b) 16.5	a) 13.7; b) 16.8	68: 32	95.4 ± 1.9 %	1.18	1.19
[^{99m} Tc][Tc(N)PNP3OH]-cysFAP	a) 20.0; b) 23.8	a) 20.3; b) 24.1	73:27	86.2 ± 3.0%	-0.074	-0.177

* as sum of a+b.

Stability

An extensive in vitro stability analysis was performed to predict if there would be any enzymatic biotransformation of the compounds in vivo. When incubated in human and mouse serum, [^{99m}Tc][Tc(N)PNP3]-cysFAP (Table 2) demonstrated low protein binding (<10%) at all timepoints up to 24 hours. Low protein binding was demonstrated for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP (Table 3) up to 24 hours of incubation, with an increase up to 24 hours of 28.87 ± 3.82% in human serum and 16.2 ± 0.65% in mouse serum. Both radiopharmaceuticals are adequately stable following incubation in human and mouse serum for 3 h, exhibiting only minor degradation (RCP >70%) for both radiopharmaceuticals after prolonged incubation in human serum (Table3 and Figure Supplement C).

The stability of the radiopharmaceuticals was further evaluated in murine tissue homogenates to have a better prediction on their in vivo degradation profiles. Before HPLC analyses the % radioactivity recovered from the tissues with respect to the total amount of incubated radioactivity was determined. [^{99m}Tc][Tc(N)PNP3]-cysFAP showed high recovery from both kidney and liver homogenates during the first 3 h, ranging from 89.0–94.4% for kidney homogenates and 88.7–98.3% for liver homogenates.

After 24 h, recovery remained around 70% in both tissue homogenates. [^{99m}Tc][Tc(N)PNP3OH]-cysFAP displayed lower recovery in kidney homogenates throughout the incubation period (49.2–72.0%), while liver recovery remained comparatively stable (71.1–87.6%) over 24 h.

Radiochromatograms of the extracted solutions revealed the presence of species, characterized by shorter retention times (Figure Supplement C) starting from 30 min of incubation, whose peak shapes and relative ratio are consistent with a truncated complex containing the [^{99m}Tc(N)PNP]-synthon bound to a cysteine residue as *syn* and *anti*-isomers. Due to the rich pool of active peptidase present in renal tissues, both radiopeptides degraded more rapidly in murine kidney homogenate (MKH) than in Murine Liver Homogenate (MLH).

[^{99m}Tc][Tc(N)PNP]-radiolabelled peptides are in general characterized by a very rapid blood clearance and distribution profiles that occurred within the first 15 min after injection. Thus, for in vivo metabolism evaluation blood and urine samples were collected at 10 minutes after in vivo administration. Analysis of biological samples demonstrated serum stability of $76.9 \pm 4.6\%$ for [^{99m}Tc][Tc(N)PNP3]-cysFAP and $86.6 \pm 5.9\%$ for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP. Corresponding urinary samples contained $52.5 \pm 3.8\%$ and $24.7 \pm 5.0\%$ intact radiopharmaceutical, respectively. Radio-HPLC analysis of the urinary samples revealed earlier-eluting radiometabolites in addition to the intact radiopharmaceutical (Figure Supplement C3–C6). The urinary metabolite profiles showed qualitative correspondence with the more hydrophilic degradation products observed following in vitro incubation in murine kidney homogenates, supporting renal peptide degradation as a likely contributor to the radiometabolites observed in vivo (Figure Supplement C3-6).

Cell binding & Ligand tracer

Cellular uptake of both radiopharmaceuticals was evaluated in HEK293T.hFAP cells after 1 and 3 h of incubation (Figure 2). [^{99m}Tc][Tc(N)PNP3OH]-cysFAP demonstrated higher uptake than [^{99m}Tc][Tc(N)PNP3]-cysFAP at both time points. After 3 h, uptake remained high for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP ($17.8 \pm 0.6\%$), whereas uptake of [^{99m}Tc][Tc(N)PNP3]-cysFAP decreased to $8.2 \pm 0.2\%$. Only low levels of uptake were observed in wild-type HEK293T cells $0.67 \pm$

0.20% for [^{99m}Tc][Tc(N)PNP3]-cysFAP and $1.47 \pm 0.79\%$ for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP. Pre-incubation with an excess of unlabelled FAP ligand reduced cellular uptake in HEK293T.hFAP cells to $3.14 \pm 0.65\%$ for [^{99m}Tc][Tc(N)PNP3]-cysFAP and $0.83 \pm 0.22\%$ for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP. Binding kinetics were evaluated using LigandTracer analysis (Figure 2). Both radiopharmaceuticals exhibited concentration-dependent association and reversible dissociation following sequential exposure to 1, 3 and 9 nM radioligand concentrations. Analysis of the association and dissociation curves yielded an equilibrium dissociation constant (K_D) of 9.8 ± 3.1 nM for [^{99m}Tc][Tc(N)PNP3]-cysFAP and 3.0 ± 1.5 nM for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP respectively.

Table 2: Stability* parameters of [^{99m}Tc][Tc(N)PNP3]-cysFAP

	%Protein Binding Human serum	%Protein Binding Mouse Serum	PBS	Human serum stability	Mouse Serum stability	Mouse Kidney	Mouse Liver	In Vivo Stability Serum	In Vivo stability Urine
0	4.05 ± 1.44	1.65 ± 0.05	96.97 ± 1.31	98.2 ± 0.8	98.2 ± 0.8	98.2 ± 0.8	98.2 ± 0.8		
10 min								76.9 ± 4.6%	52.5 ± 3.8%
30 min	2.68 ± 0.68	2.59 ± 0.06	95.23 ± 0.36	97.3 ± 0.5	97.8 ± 0.5	69.9 ± 4.3	96.23 ± 0.5		
60 min	5.82 ± 2.50	2.46 ± 0.02	94.23 ± 2.63	94.7 ± 0.9	96.0 ± 0.7	48.33 ± 3.5	93.2 ± 2.8		
180 min	3.87 ± 0.4	2.89 ± 0.94	95.12 ± 0.96	84.3 ± 1.5	87.4 ± 2.1	25.36 ± 4.1	85.23 ± 3.6		
24 hours	7.33 ± 0.80	9.74 ± 2.62	Not Available	76.34 ± 2.22	87.75 ± 1.89	17.93 ± 1.02	20.95 ± 3.01		

*Stabilities are expressed as sum of a+b %RCP; experiments were performed in triplicate

Table 3: Stability* parameters of [^{99m}Tc][Tc(N)PNP3OH]-cysFAP

	%Protein Binding Human serum	%Protein Binding Mouse Serum	PBS	Human serum stability	Mouse Serum stability	Mouse Kidney	Mouse Liver	In Vivo Stability Serum	In Vivo stability Urine
0	4.40 ± 3.82	2.8 ± 1.02	92.52 ± 3.97	93.10 ± 0.25	90.73 ± 0.65	76.36 ± 2.81	87.57 ± 2.54		
10 min								86.6 ± 5.9	24.7 ± 5.0
30 min	5.27 ± 3.82	6.4 ± 1.82	95.26 ± 0.96	90.94 ± 0.87	87.99 ± 0.05	62.88 ± 4.22	86.79 ± 0.57		
60 min	5.09 ± 3.82	8.9 ± 2.02	95.68 ± 0.96	90.01 ± 0.63	87.65 ± 0.87	59.52 ± 3.01	66.22 ± 2.87		
180 min	12.20 ± 3.82	17.3 ± 2.76	93.47 ± 0.96	89.88 ± 1.64	86.08 ± 1.18	47.36 ± 10.41	41.94 ± 0.87		
24 hours	28.87 ± 3.82	16.2 ± 0.65	72.92 ± 1.97	70.88 ± 3.13	84.75 ± 2.59	26.29 ± 5.84	Not detectable		

*Stabilities are expressed as sum of a+b %RCP; experiments were performed in triplicate

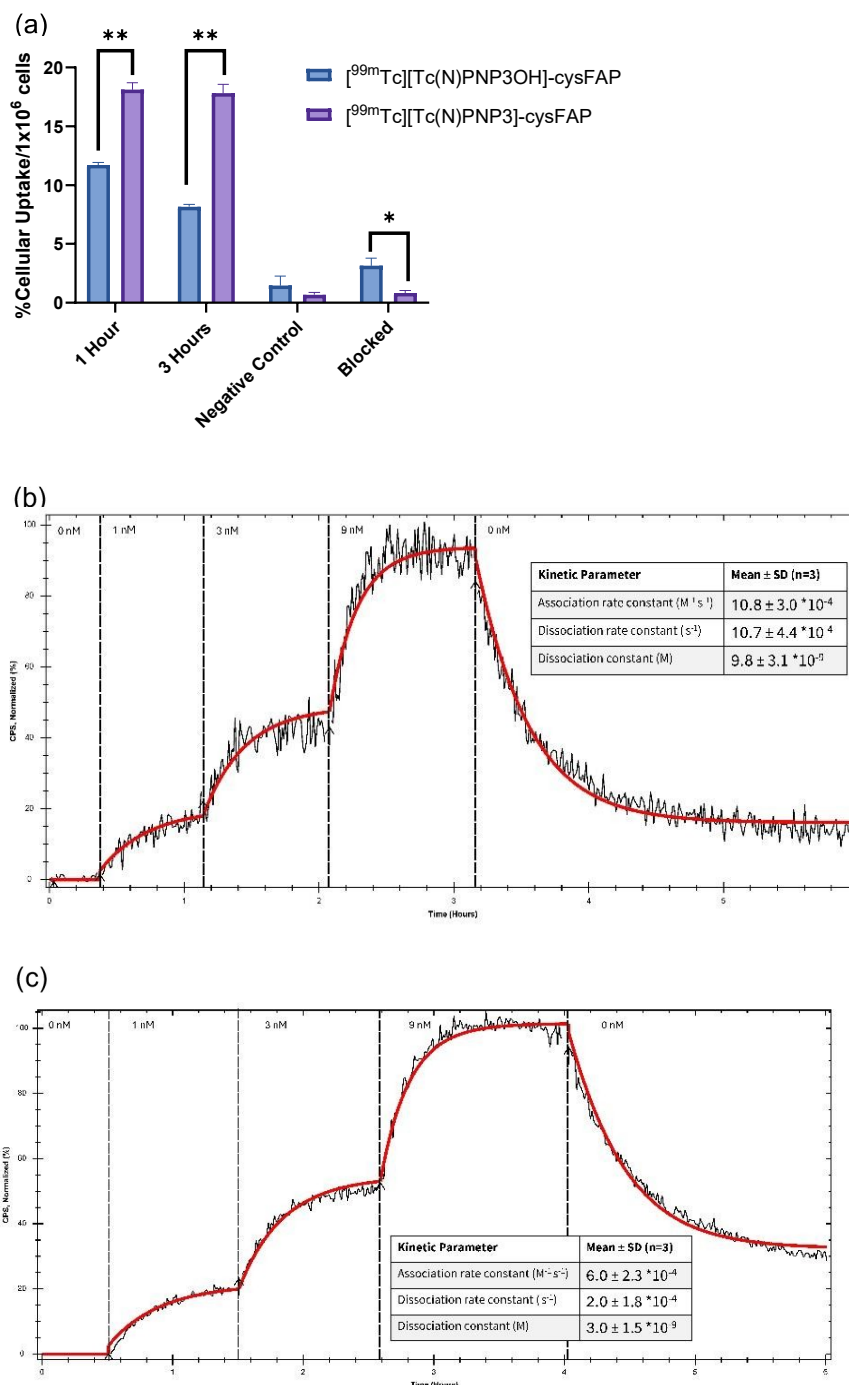


Figure 2: In vitro characterization of technetium-99m based cysFAP compounds. (a) results of the 1- (p= 0.002) and 3-hour (p=0.003) binding assay on HEK293T.hFAP, including negative control (HEK293T) and blocking assay (cysFAP self-block). B) representative binding curve of [^{99m}Tc][Tc(N)PNP3OH]-cysFAP with three consecutive association phases and one dissociation phase and C) [^{99m}Tc][Tc(N)PNP3]-cysFAP.

19 Biological evaluation

20 The biodistribution of [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP was
 21 evaluated in HEK293T.hFAP and HEK293T animal model (Figure 3). Uptake and clearance were
 22 monitored using SPECT/CT imaging at multiple time points. Tumor and organ uptake were quantified
 23 as percent injected activity per gram (%IA/g). Both radiopharmaceuticals demonstrated the highest
 24 uptake in the HEK293T.hFAP tumour and kidneys, while uptake in all other tissues remained low.
 25 [^{99m}Tc][Tc(N)PNP3]-cysFAP exhibited tumour uptake of 6.60 ± 1.15 %ID/g, compared with 4.55 ± 1.27
 26 %ID/g for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP. Uptake in the contralateral FAP-negative tumour was low
 27 for both radiopharmaceuticals, measuring 0.18 ± 0.05 %ID/g for [^{99m}Tc][Tc(N)PNP3]-cysFAP and 0.13
 28 ± 0.05 %ID/g for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP.

29 Renal uptake was 4.53 ± 0.71 %ID/g for [^{99m}Tc][Tc(N)PNP3]-cysFAP and 2.68 ± 0.30 %ID/g for
 30 [^{99m}Tc][Tc(N)PNP3OH]-cysFAP. On the contrary, liver uptake was higher for [^{99m}Tc][Tc(N)PNP3OH]-
 31 cysFAP (1.53 ± 0.46 %ID/g) than for [^{99m}Tc][Tc(N)PNP3]-cysFAP (0.53 ± 0.50 %ID/g). Both
 32 radiopharmaceuticals showed minimal accumulation in the spleen, pancreas, lungs, heart, brain, bone
 33 and muscle, with uptake values below 0.5 %ID/g in all tissues. The compounds are monocationic, thus
 34 brain uptake was undetectable.

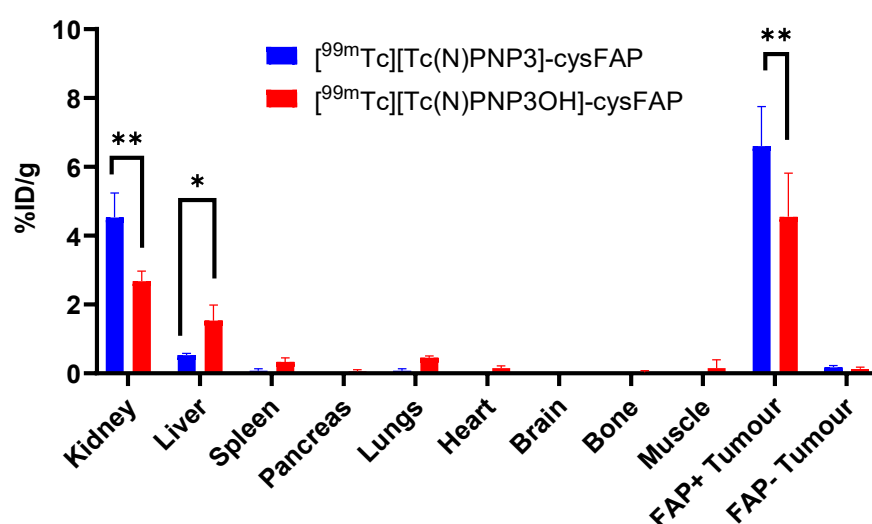
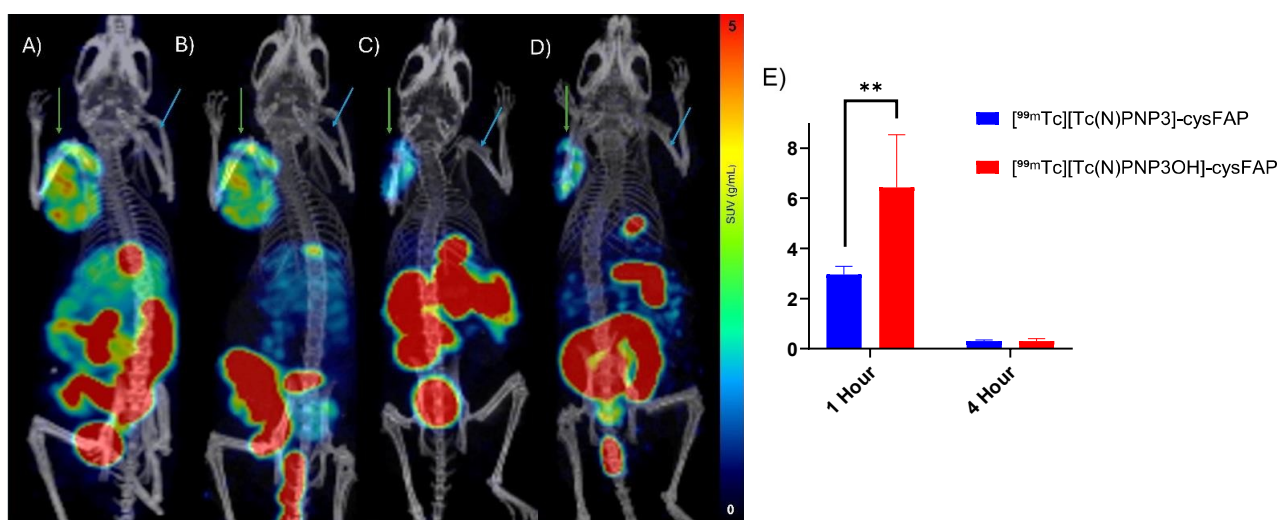


Figure 3: Ex-vivo biodistribution for [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP (N=4), with ex-vivo collection 4.5 hours post-injection, expressed as %IA/g. Statistically significant differences exist between the kidney (p=0.0074), liver (p=0.025) and the HEK293T.hFAP tumour (p=0.0083).

Small-animal SPECT/CT imaging was performed at 1 hour and 4 hours following administration of [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP (N=3) in mice bearing paired HEK293T.hFAP-positive and HEK293T wild-type tumours (Figure 4). In both imaging groups, clear visualization of the FAP-positive tumour was observed at both imaging time points, whereas uptake in the contralateral FAP-negative tumour remained low.



51

Figure 4: Maximum-intensity-projection SPECT/CT images of [^{99m}Tc][Tc(N)PNP3]-cysFAP at a) 1 hour b) 4 h p.i. and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP at c) 1 hour d) 4 h p.i.. e) Kidney-to-tumour ratios for both radioligands at 1 h and 4 h p.i. negligible residual accumulation in the kidney. The location of the positive tumour is indicated with a green arrow, and the negative tumour with a blue arrow.

All quantitative image data are reported as SUV_{max} . Quantitative image analysis demonstrated renal uptake of an SUV of 14.87 ± 1.60 and 1.28 ± 0.20 for [^{99m}Tc][Tc(N)PNP3]-cysFAP at 1 hour and 4

hours, respectively. For [^{99m}Tc][Tc(N)PNP3OH]-cysFAP, kidney uptake measured 16.32 ± 1.47 at 1 hour and decreased to 1.06 ± 0.14 at 4 hours post-injection. Liver uptake of [^{99m}Tc][Tc(N)PNP3]-cysFAP decreased from 2.89 ± 1.12 at 1 hour to 0.503 ± 0.39 at 4 hours post-injection. Corresponding liver uptake values for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP were 0.68 ± 0.21 and 0.47 ± 0.31 at 1 hour and 4 hours, respectively. No notable thyroid and stomach uptake is present in any of the timepoints for both tracers, with no visual signs of tracer instability and free pertechnetate visible.

FAP-positive tumour uptake remained detectable for both radiopharmaceuticals throughout the imaging period. [^{99m}Tc][Tc(N)PNP3]-cysFAP demonstrated tumour uptake of $5.03 \pm 0.14 \text{ SUV}_{\text{max}}$ at 1 hour and $4.21 \pm 0.21 \text{ SUV}_{\text{max}}$ at 4 hours post-injection. [^{99m}Tc][Tc(N)PNP3OH]-cysFAP exhibited tumour uptake values of $2.54 \pm 0.79 \text{ SUV}_{\text{max}}$ at 1 hour and $3.52 \pm 1.08 \text{ SUV}_{\text{max}}$ at 4 hours post-injection. Uptake in the contralateral FAP-negative tumour remained low for both tracers at both time points. SUV_{max} values for [^{99m}Tc][Tc(N)PNP3]-cysFAP were 0.54 ± 0.18 and 0.33 ± 0.12 at 1 hour and 4 hours, respectively, while [^{99m}Tc][Tc(N)PNP3OH]-cysFAP demonstrated values of 0.46 ± 0.36 and 0.44 ± 0.27 at the corresponding time points.

Discussion

In this study, we investigated the use of the [^{99m}Tc (N)PNP]-technology where PNP represent the choice of two bisphosphinoamines to generate ^{99m}Tc -nitride based radiopharmaceuticals targeting FAP. The peptide targeting vector was based on DOTA-FAP-2286 [14], a well-known lead peptide for development of FAP-targeting theranostic compounds, in which the DOTA chelator was replaced with a cysteine residue to allow coordination with the [^{99m}Tc][Tc(N)PNP]-synthon. Both [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP were efficiently prepared in physiological conditions suitable for a kit formulation using a ligand amount of 42.1 nmol in a final volume of 1.5 ml. [^{99m}Tc][Tc(N)PNP3]-cysFAP was obtained with a higher radiochemical yield of $95.4 \pm 1.9\%$ after heating at 80°C for 30 min, whereas [^{99m}Tc][Tc(N)PNP3OH]-cysFAP was prepared at room temperature within 30 min, albeit with a somewhat lower radiochemical yield of $86.2 \pm 3.0\%$. The ability of the PNP3OH framework to achieve efficient labelling under mild conditions is consistent with

84 previous investigations of the water-soluble [^{99m}Tc][Tc(N)PNP3OH]-synthon [18, 19] and may be
85 advantageous for extending this labelling strategy to temperature-sensitive targeting vectors. Following
86 solid phase extraction, radiochemical purities exceeding 95% were obtained, while the excess of the
87 unlabelled cysFAP was removed to obtain radiolabelled peptides in very high specific activity (<500
88 GBq/ μl in all cases). Radiochromatograms of both complexes displayed two characteristic product
89 peaks, which were identified by LC/MS as two distinct structural isomers. This was consistent with the
90 previously described syn/anti isomers resulting from the orientation of the cysteine substituent with
91 respect to the Tc $\equiv$ N core [18, 19].

92 The choice of the PNP ligand produced a marked difference in the physicochemical properties of the
93 two radiopharmaceuticals. [^{99m}Tc][Tc(N)PNP3]-cysFAP demonstrated a logP_{O/W} of 1.18, compared with
94 -0.074 for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP, confirming the substantially greater hydrophilicity
95 imparted by the hydroxymethyl substituents of PNP3OH. A similar relationship between PNP
96 substitution and hydrophilicity was previously reported for [^{99m}Tc][Tc(N)(PNP)]-labelled cRGDfK
97 peptides [18], where the replacement of the bulky PNP3 ligand with PNP3OH increased the
98 hydrophilicity and altered the subsequent biological behaviour of the radioconjugate. However, despite
99 their different physicochemical properties, both FAP-targeted radioconjugates exhibited high stability
100 in injectable solutions (saline and PBS), low serum protein binding and good metabolic stability upon
101 in vitro incubation in sera. Meanwhile, metabolic differences became apparent after incubation in
102 murine tissue homogenates and *in vivo*. Thus, the formation of new hydrophilic species was detected
103 after 30 minutes of incubation in mouse kidney homogenates, consistent with the peptidase-mediated
104 degradation/cleavage of the peptide moiety. The amount of these degradation products increased over
105 time, revealing a more rapid degradation kinetics for the less lipophilic compound
106 [^{99m}Tc][Tc(N)PNP3OH]-cysFAP compared to [^{99m}Tc][Tc(N)PNP3]-cysFAP. This behaviour was partially
107 confirmed in vivo. Although FAP-2286 is expected to be highly stable in blood after in vivo
108 administration, data attained from this study revealed the presence of metabolites in
109 radiochromatograms of blood and urine samples collected at 10 min post injections (*vide infra*).
110 Nevertheless, the serum level of intact radiopeptides measured 10 min post-injection (RCP 75-80%)

demonstrates adequate *in vivo* stability for both compounds during the window time of initial target localization. Higher amounts of degradation products were detected in HPLC profiles of urine.

Both radiopeptides maintained high FAP-binding specificity in cell-based assays and provided clear visualization of FAP-positive xenografts by SPECT/CT, confirming that conjugation of either [^{99m}Tc][Tc(N)PNP] synthon to the cys-FAP peptide does not compromise its FAP-targeting capability.

In vitro cellular studies showed substantially higher uptake for both radiopharmaceuticals in HEK293T.hFAP cells than in wild-type HEK293T cells, while pre-incubation with an excess unlabelled FAP ligand markedly reduced cellular uptake, confirming target specificity. Ligand Tracer analysis further demonstrated concentration-dependent and reversible binding, with equilibrium dissociation constants (K_D) in the low-nanomolar range. Specifically, [^{99m}Tc][Tc(N)PNP3OH]-cysFAP exhibited a lower K_D (3.0 ± 1.5 nM) than [^{99m}Tc][Tc(N)PNP3]-cysFAP (9.8 ± 3.1 nM). This difference was confirmed by a significantly greater and stable cellular accumulation of the PNP3OH derivative, which remained at $17.8 \pm 0.6\%$ after 3 h compared to $8.2 \pm 0.2\%$ for the PNP3 analogue. These findings suggest that the radiochemical synthon can modulate target interaction and cellular retention for two radiopharmaceuticals with the same cysFAP vector, but different [^{99m}Tc][Tc(N)PNP] synthons. Similar synthon-dependent effects have previously been observed for [^{99m}Tc][Tc(N)(PNP)]-labelled RGD peptides [19], where PNP modification altered the cellular uptake and internalized radioactivity while preserving receptor-specificity. Importantly, since internalization was not directly quantified in the present study, the differences observed represent total cell-associated radioactivity rather than demonstrable differences in intracellular retention.

Despite the superior *in vitro* binding characteristics of the PNP3OH analogue, [^{99m}Tc][Tc(N)PNP3]-cysFAP displayed the higher absolute tumour accumulation *in vivo*. At approximately 4.5 h post-injection, *ex-vivo* uptake in the FAP-positive xenograft reached 6.60 ± 1.15 % ID/g for [^{99m}Tc][Tc(N)PNP3]-cysFAP compared to 4.55 ± 1.27 %ID/g for [^{99m}Tc][Tc(N)PNP3OH]-cysFAP (which is a statistical significant difference). The marked difference between FAP-positive and FAP-negative tumour uptake (35- to 37-fold) provides strong *in vivo* evidence that tumour localization was FAP-mediated. Importantly, the low uptake in most non-target tissues (< 0.5 %ID/g), resulted in

138 favourable tumour-to-background ratio yielding high contrast target visualization, at the later imaging
139 time point. to be mentioned that these compounds had substantially increased bowel presence compared
140 to other FAP targeting radiopharmaceuticals. This could be problematic when peritoneal metastases or
141 colorectal tumours are visualised, or if technetium-99m FAP radiopharmaceuticals are applied for
142 radioguided abdominal surgery. However, since the PNP system has demonstrated the possibility to
143 adjust pharmacokinetics, alternative PNP systems might be incorporated to adapt towards lower
144 hepatobiliary clearance.

145 Overall, these findings establish that the incorporation of spatially demanding [^{99m}Tc][Tc(N)(PNP)]
146 moieties does not impair the FAP-targeting characteristics of the DOTA-FAP-2286-derived peptide.
147 Dynamic differences between the two compounds became particularly apparent from quantitative
148 SPECT/CT. [^{99m}Tc][Tc(N)PNP3]-cysFAP demonstrated relatively rapid tumour accumulation, reaching
149 an SUV_{max} of 5.03 ± 0.14 at 1 h, followed by only a modest washout to 4.21 ± 0.21 at 4 h. Conversely,
150 [^{99m}Tc][Tc(N)PNP3OH]-cysFAP showed lower initial tumour uptake of $2.54 \pm 0.79 \text{ SUV}_{\text{max}}$ but
151 increased to 3.52 ± 1.08 at 4 h. The latter pattern is consistent with the higher and more sustained cellular
152 uptake observed for PNP3OH in vitro and suggests slower tumour accumulation or retention kinetics.
153 The two [^{99m}Tc][Tc(N)PNP] frameworks therefore appear to produce different kinetic profiles:
154 [^{99m}Tc][Tc(N)PNP3], favours higher and earlier absolute tumour accumulation, whereas
155 [^{99m}Tc][Tc(N)PNP3OH] displays slower accumulation accompanied by continued tumour uptake over
156 the investigated imaging interval. Whether the latter translates into superior tumour retention at time
157 points beyond 4 h cannot be determined from the present study and would require extended kinetic
158 evaluation.

159 The biodistribution profile also indicates that both hepatobiliary and renal elimination represents
160 important clearance pathways for both radiopharmaceuticals. Some differences were observed between
161 imaging-derived, and ex vivo estimates of organ uptake, particularly for the liver. These measurements
162 are not directly equivalent: ex vivo biodistribution measures radioactivity within the complete excised
163 organ, whereas quantitative SPECT analysis in the present study sampled hepatic activity using a defined

spherical VOI positioned within the left liver lobe. Therefore, activity associated with adjacent biliary structures or differences in the regional tracer distribution could contribute to the apparent discrepancy. The present compounds differ conceptually from the [^{99m}Tc]Tc-FAP-targeting agents reported to date, as they apply technetium chemistry to the peptide-based DOTA-FAP-2286 targeting vector rather than to the more extensively investigated small-molecule FAPI scaffolds [24]. Current development in this field has focused primarily on small molecule FAPI derivatives [24-27], and nanobody-based compounds [29]. To the best of our knowledge, this is the first report of a peptide-based technetium-99m FAP-targeted imaging probe. A non-exhaustive summary of reported ^{99m}Tc-compounds and their corresponding tumour uptake values is provided in Table 4 as well as those of the corresponding [¹¹¹In][In-FAP-2286 and [⁶⁸Ga][Ga-FAP-2286 radiopharmaceuticals. Direct comparison of the biological behaviour of the compounds investigated in the present study with previously reported technetium-99m FAP-targeting radiopharmaceuticals is challenging because of differences in the animal models employed. Nevertheless, the most promising compound in the present study, [^{99m}Tc][Tc(N)PNP3]-cysFAP demonstrated a tumour uptake of 6.6 ± 1.15 %ID/g and an SUV_{Max} of 5.03 ± 0.14 in HEK293T.hFAP-positive xenograft, which compares favourably with values reported for other technetium-99m FAP-targeting radiopharmaceuticals.

Table 4: Reported %ID/g values and SUV values reported for technetium-99m radiopharmaceuticals in literature.

Compound	Animal Model	%ID/g and time p.i	Comment	Ref
[^{99m} Tc][Tc(N)PNP3]-cysFAP-2286	HEK293T.hFAP -positive xenograft	6.6 ± 1.15 %ID/g [4.5 h]	Rapid, sustained tumor uptake. predominant renal excretion; low off target accumulation (< 0.5 %ID/g); tumor-to-kidney 1.45 at 4.5 h <i>p.i.</i>	Current study

[^{99m} Tc][Tc(N)PNP3O H]-cysFAP-2286	HEK293T.hFAP -positive xenograft	4.55 ± 1.27 %ID/g [4.5 h]	Rapid, sustained tumor uptake. predominant renal excretion; low off target accumulation (< 0.5 %ID/g); tumor-to-kidney 1.70 at 4.5 h <i>p.i.</i>	Current study
[^{99m} Tc]Tc-FAPI-L3	MDA-MB231 solid tumour	2.23% ID/g [2 h]	First reported ^{99m} Tc-FAP, localized in tumour.	29
[^{99m} Tc]Tc-(CN- PEG4-FAPI)6+	U87MG bearing tumours	8.05 ± 1.48% ID/g [1 h]	Unsatisfactory renal and off-target accumulation	30
[^{99m} Tc]Tc-FAPI-34	HT-1080-FAP	5.4 ± 2.05 [1 h] and 4.3 ± 1.95 %ID/g [4 h]	This compound was furthered to human evaluation	27
[^{99m} Tc]Tc-iFAP	Hep-G2 tumour- bearing nude mice	7.05 ± 1.13 % ID/g [30 min] and 5.18 ± 0.82 % ID/g [2 hours]	Demonstrated stability in human serum, tumour uptake and fast kidney elimination.	25
[^{99m} Tc]Tc-HYNIC- FAPI-04	U87MG-derived FAP-positive tumors	2.67 ± 0.35 % ID/g [1.5 hours]	The authors concluded that this radiopharmaceutical could be used for imaging of fibrosis over longer time periods.	31
[^{99m} Tc]Tc-AFN13	U87MG bearing tumours	4.41 ± 0.13 % ID/cc [30 min]	Preliminary clinical imaging confirmed safety and specificity.	28
[¹¹¹ In][In-FAP-2286	HEK293T.hFAP -positive xenograft	11.1% ID/g at 1 h <i>p.i.</i> and 9.1% ID/g at 48 h	hFAP $K_D = 0.2 \pm 0.1$ nM; rapid, sustained tumor uptake with predominant renal excretion. Off- target retention in liver, salivary glands, and shoulder was minimal. The tumor-to-kidney ratio steadily increased from 3 at 1h <i>pi</i> , to 4.5 at 3 h <i>p.i</i> reaching a maximum of 7.5 at 48 h <i>p.i.</i>	32

[⁶⁸ Ga][Ga-FAP-2286	HEK293T.hFAP	9.8 %ID/g (0.5 h p.i);	hFAP K _D =1.4±0.5 nM; rapid, high-	32
	-positive	10.8 %ID/g (3 h p.i)	specificity tumor uptake in HEK-	
	xenograft		FAP xenografts with	
			predominantly renal elimination;	
			retention in liver, decreases from	
			2.36 ± 0.46 to 0.26 ± 0.12 within 3	
			h p.i; the tumor-to-kidney ratio	
			steadily increased from 1.5 at 0.5 h	
			pi, to 4.5 at 3 h p.i.	

A comparison of the biodistribution profile [^{99m}Tc][Tc(N)PNP]-cysFAP in the same animal model was provided by evaluating the biological performance of [¹¹¹In][In-FAP-2286 and [⁶⁸Ga][Ga-FAP-2286 radiopharmaceuticals. Although ^{99m}Tc-radiopeptides exhibited a good in vitro target affinity and uptake with K_D values in the low nanomolar range comparable to those observed for the [¹¹¹In]In-FAP-2286 and [⁶⁸Ga]Ga-FAP-2286 [32], they showed a lower tumour accumulation in vivo and target-to-background ratio, likely due to the rapid blood clearance and reduced serum stability. To overcome these drawbacks, future design strategies such as linker modification, structural peptide stabilisation against degradation, incorporation of albumin binder moieties or dimeric constructs, will be considered to extend blood circulation times, increase the target accumulation, and maximize tumour retention.

The current results demonstrate that this peptide scaffold can be successfully adapted to accommodate the bulky [^{99m}Tc][Tc(N)(PNP)] and [^{99m}Tc][Tc(N)(PNP3OH)] synthons without loss of FAP specificity and can generate high-contrast SPECT images. Such a probe could also be useful for intra-operative guidance. Although the two PNP ligands produced clear differences in affinity, cellular uptake and pharmacokinetics, the present experiments cannot establish the individual contributions of lipophilicity, metabolism and binding kinetics to these observed differences.

Conclusion

This study demonstrates the successful development of the first peptide-based technetium-99m FAP-targeted radiopharmaceuticals for SPECT imaging. Both [^{99m}Tc][Tc(N)PNP3]-cysFAP and [^{99m}Tc][Tc(N)PNP3OH]-cysFAP retained high FAP affinity and specificity and enabled clear visualization of FAP-positive tumours. Furthermore, modification of the bisphosphinoamine ligand significantly influenced affinity, cellular uptake and in vivo pharmacokinetics, proving that the [^{99m}Tc(N)(PNP)]-framework can be strategically tailored to fine-tune biological behaviour. Although, the moderate in vivo stability may be responsible of a reduced absolute tumour uptake compared to ¹¹¹In- or ⁶⁸Ga-labelled FAP-2286 counterpart, these finding establish a strong proof-of-concept. Further structural optimizations of the [^{99m}Tc][Tc(N)PNP]-cysFAP platform, including peptide backbone stabilization and linker modification will be pursued to enhance the stability, tumour accumulation and overall pharmacokinetics.

Declarations

Ethics approval: All animal procedures were approved by the KU Leuven ethical review board (P079-2023) and were carried out following the Directive 2010/63/EU.

Consent for publication: All the authors consent for publication of this manuscript

Availability of data and material: additional data and material available upon reasonable request.

Competing interests: The authors declare no competing interests.

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Authors' contributions

JK: funding acquisition, project administration, investigations, methodology, formal analysis, data curations, writing-original draft, writing-review and editing. **NV:** methodology, data curation, writing-review and editing. **CG:** investigations, methodology, Formal analysis **CMD:** funding acquisition, project administration, supervision, writing-review and editing. **FC:** methodology, writing-original draft, funding acquisition, project administration, supervision, writing & editing. **CB:** funding, conceptualization, project administration, investigations, methodology, formal analysis, writing-review & editing.

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