Development and preclinical evaluation of [^{68}Ga]Ga-Alb-FAPtp-01, a novel tumour-associated fibroblast activation protein tracer for PET imaging of xenograft glioma

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Research Article

Keywords: FAP, Gallium-68, Glioblastoma, PET/CT imaging, Tracer development

Posted Date: May 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-500662/v1>

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Abstract

Purpose

Dynamic changes in tumour-associated fibroblast activation protein (FAP) expression in tumours of different stages may be helpful for prognostic evaluation and treatment response monitoring, making this protein a promising surveillance biomarker for timely diagnosis of malignant tumours and effective planning of patient care. Thus, novel FAP-PET imaging tracers were developed and evaluated for the diagnosis of xenograft glioma tumours.

Methods

To prospectively verify the prognostic value of the developed FAP tracers, [^{68}Ga]Ga-FAPtp and [^{68}Ga]Ga-Alb-FAPtp-01, measurements of FAP expression and cell uptake and specific binding assays were conducted in U87MG glioma cells. Dynamic/static PET/CT scans were acquired for tumour targeting studies *in vivo* and in comparison with the reference tracer [^{68}Ga]Ga-FAPI-04 to evaluate diagnostic efficacy. Tumour autoradiography and immunohistochemistry images were acquired to confirm the tracer distribution within the tumour to determine whether it was in accordance with the pathologic data.

Results

Both [^{68}Ga]Ga-FAPI-04 and [^{68}Ga]Ga-FAPtp demonstrated marked tumour uptake and comparable pharmaceutical profiles in 1 h dynamic PET scans, and [^{68}Ga]Ga-FAPtp had marginally favourable tumour uptake and less kidney and liver uptake. However, both tracers demonstrated rapid clearance from tumours. Thus, the optimized rationally designed FAP-targeting PET tracer [^{68}Ga]Ga-Alb-FAPtp-01, with albumin binding capability, demonstrated prominent longitudinal tumour uptake in tumour xenografts and displayed significant tumour-to-background contrast over time. The tracer uptake values and T/M ratio were 1.775 ± 0.179 SUV and T/M = 5.9, 1.533 ± 0.222 SUV and T/M = 6.7, and 1.425 ± 0.204 SUV and T/M = 9.5, respectively, at 1 h, 2 h and 3 h. Major organs, such as the heart ($0.504 \pm 0.125\%$ ID/g), muscle ($0.156 \pm 0.043\%$ ID/g) and brain ($0.119 \pm 0.039\%$ ID/g), all displayed comparatively low levels of tracer uptake.

Conclusion

Its improved tumour uptake and pharmacokinetics suggest that the [^{68}Ga]Ga-Alb-FAPtp-01 tracer can noninvasively detect FAP activation *in vivo*, permitting a precise definition of its roles in tumours of different stages and yielding insights regarding novel FAP-targeted radiotherapeutic strategies at the molecular level.

Introduction

Malignant tumours can usually be distinguished from nonmalignant cells, such as immune and endothelial cells, and extracellular matrix components by their ability to modulate the peritumoural environment [1–4]. Thus, the dynamic, constantly changing stromal tumour microenvironment (TME) provides cues for assessing tumour proliferation and invasion and the ability to metastasize to distant sites. In contrast, most localized tumours exhibit limited diffuse growth. Thus, the interactions between the tumour and the TME and even microvascular proliferation status, which indicates tumour growth and progression, are essential for tumour characterization.

Distinct from normal fibroblasts, cancer-associated fibroblasts (CAFs) in the TME express high levels of a unique protein called fibroblast activation protein (FAP), which has been recognized as a potential prognostic marker [5–6]. FAP, also known as FAP α or seprase, is a membrane-bound type II serine protease and a member of the S9 family of proline-specific proteases, which includes dipeptidyl peptidase IV (DPP IV), DPP8, DPP9, and prolyl endopeptidase (PREP) [7]. FAP possesses both dipeptidyl peptidase and endopeptidase activities. Importantly, the expression of FAP in adult mammals is usually low in most normal tissues but elevated in activated fibroblasts during tissue remodelling at sites of inflammation, such as sites of rheumatoid arthritis, osteoarthritis, fibrosis of the heart and liver, and in a variety of tumour-promoting environments [8–11].

Although the molecular mechanism underlying the contribution of FAP to tumour severity remains elusive, intense studies of potential FAP-targeted inhibitors, diagnostic agents and therapeutic interventions have been expanding in recent decades [12–14]. The rapidly growing field of FAP-targeting agent development, including but not limited to antibodies, chimaeric antigen receptor T cells, immunoconjugates, peptide-drug complexes and small molecules for the inhibition of FAP enzyme activity, has received increasing attention. Among these agents, small molecules such as FAP inhibitors (FAP Is) have had the most promising results from the perspective of FAP-expressing tumour cell binding, internalization, efflux, tumour xenograft accumulation and theranostic application in patients [15–19].

Noninvasive positron emission tomography (PET) imaging with suitable radioligands may quantify the distribution of biomarkers of interest through the measurement of radioactivity profiles in living subjects. Such radiopharmaceutical approaches may reveal the network of molecular mechanisms underlying diseases and assist in the development and evaluation of effective therapies. The development of optimal suitable PET tracers is especially challenging because they must satisfy a wide array of chemical, biochemical, and pharmacological requirements. To reduce unnecessary radioexposure, an important aspect of PET radiotracer development is to consider variations in specific binding affinities and concentrations across species in relevant native tissues.

Therefore, in the current investigation, a prototype peptide-based FAP-specific PET tracer, [^{68}Ga] Ga -FAPtp, based on the structure of a potent FAP inhibitor (known as UAMC-1110) [20], were designed, characterized and optimized for use as novel FAP-targeted diagnostic agents and potential theranostic

agents. In contrast to the original quinoline-based moiety, peptide amide bond core structures with an additional short PEGylating linker were contemporaneously adopted. Furthermore, to enhance the plasma retention time, the optimized [^{68}Ga]Ga-Alb-FAPtp-01 tracer with an endogenous albumin-binding moiety, 4-p-chlorophenyl butyric acid [21], was designed to improve pharmacokinetics and was tested in glioma xenograft models. Characteristics including FAP binding specificity and affinity, were validated by *in vitro* cell uptake assays and biolayer interferometry (BLI), and preclinical pharmacokinetics were determined in a U87MG xenograft model by small-animal PET/CT imaging, *ex vivo* histochemical staining and biodistribution studies. These first proof-of-concept tracers are unique in that they can be effectively synthesized through the standard solid-phase peptide synthesis (SPPS) procedure without any sophisticated chemical methods. Thus, the peptide probes can be easily accessed via custom order from any certificated peptide vendor in any amount requested. Importantly, the cyclic chelator DOTA can be utilized as the functional component for ^{68}Ga isotope radiolabelling for PET imaging and, possibly, for endoradiotherapy interventions involving labelling with therapeutic isotopes, such as ^{90}Y and ^{177}Lu .

Materials And Methods

General

All commercially available chemical reagents were analytical grade, purchased from Aldrich (St. Louis, MO, USA) and used without further purification. The FAPI-04 precursor was purchased from KriSan Biotech Co. (Tainan, Taiwan), a PICS/GMP-certified pharmaceutical company. The FAP-targeting peptides DOT-PEG₈-Gly-Pro-Trp-Gly-Pro (4,4-F₂) (DOTA-FAPtp) and DOTA-Lys (4-p-chlorophenyl) butyric acid)-PEG₈-Gly-Pro-Trp-Gly-Pro (4,4-F₂) (Alb-FAPtp-01) (Fig. 1) were designed by us and purchased from Mission Biotech (Taipei, Taiwan). The $^{68}\text{GaCl}_3$ solution was produced by elution with 0.05 N HCl through a $^{68}\text{Ge}/^{68}\text{Ga}$ generator (ITG, Germany). The radiochemical purity of the radiolabelling product was determined on an analytical reverse-phase HPLC system equipped with a dual ultraviolet absorbance detector (Waters 2487, Waters, Milford, MA, USA) using a Phenomenex (Torrance, CA, USA) synergi 4 m Hydro-RP 80A° (150×3×4.6 mm, 4 μm). The flow rate was 1 mL/min, with the mobile phase starting with 98% solvent A (0.1% trifluoroacetic acid (TFA) in water) and 2% solvent B (0.1% TFA in acetonitrile) (0–2 min) and transitioning to 35% solvent A and 65% solvent B over 30 min.

Radiochemistry

The details of the ^{68}Ga labelling procedures have been reported previously, with a radiochemical purity over 95% [22–23]. Briefly, [^{68}Ga]Ga-acetate was prepared by adding 555–370 MBq/mL $^{68}\text{GaCl}_3^{3+}$ to 250 μL of 0.1 M ammonium acetate buffer (pH 5.5), which was then added to the DOTA-conjugated peptide solution (approximately 25–35 μg in 20 μL of 0.1 N ammonium acetate buffer). The vial was subsequently sealed and heated for 5–10 minutes at 95 °C to form the radioactive ^{68}Ga -DOTA-peptide complex. The overall synthesis time was approximately 20–25 minutes. The radiochemical yield of the

tracers, which was determined by radio-TLC, was > 95%, and the molar activity of the solution was 185.09 ± 13.5 GBq/ μ mol. The radiochemical purity (RCP) was >95%, as determined by HPLC. Solution stability experiments were performed to demonstrate that ^{68}Ga -DOTA-peptide was intact before the tracer was injected into tumour-bearing mice. DOTA-conjugated peptides remained stable for at least 2 hours (only 5-6% of the ^{68}Ga was dissociated from the DOTA complex, supplementary Fig. S3).

Biolayer interferometry (BLI)

An eight-channel Octet RED96 BLI system (ForteBio, Menlo Park, California) was used to conduct the titration binding experiments at room temperature with shaking at 1000 rpm. Due to the small size of the peptide tracers, Super Streptavidin tips ("SSA," ForteBio, Pall Life Sciences) were used for biotinylated FAP protein loading in these binding assays; the tips were hydrated for at least 30 minutes in PBS buffer, loaded with biotinylated FAP in PBS buffer for 180 s, and moved to baseline buffer to wash unbound biotinylated FAPs as a baseline step (60 s). Then, the biotinylated FAP was associated with sequentially diluted tracers (from 5 μ M to 150 μ M) in a 200 μ L volume in black 96-well plates (association step, 720 s). Finally, the tips were submerged in baseline buffer for dissociation analysis (300 s); the association and dissociation profiles were obtained for data fitting analysis with Octet Data Analysis software (9.0.0.6.). The control study with tips with no peptides was also conducted in parallel, and the results showed low nonspecific binding. The average values were subtracted for later kinetic binding analysis. The mathematical model used assumes a simple 1:1 stoichiometry, with only one analyte in solution binding to one binding site on the surface. A Savitzky-Golay filter was applied to smooth the data.

Cell line

U87MG cells, a human glioblastoma cell line, were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and maintained in minimum essential Eagle's medium (MEM) with 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin-streptomycin and 10% FBS. The cells were cultured as an adherent monolayer in an incubator with a 5% CO_2 /95% air atmosphere at 37 °C. All media and reagents were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, USA).

Cell-based uptake and competition studies

Briefly, U87MG glioma tumour cells were seeded in 24-well plates. When the cells reached 80 to 90% confluence, 18.5 kBq of radiolabelled tracers were added to each well in 0.5 ml of Hank's buffered salt solution (HBSS) for different times (15 min, 30 min, 60 min, 90 min and 120 min). Furthermore, competition assays were performed by incubating 2×10^5 U87MG cells in 24-well plates with ^{68}Ga -labelled

tracers (~18.5 kBq) and various concentrations of cold FAPI-04 tracers in the medium at 37 °C for 1 h. In all experiments, the cells were washed twice with 1 mL of ice-cold PBS and dissolved in lysis buffer containing 0.2 M NaOH and 1% SDS. The remaining cell-bound radioactivity after washing was determined by gamma counting (Cobra II, Packard Instruments, Downers Grove, IL) as counts per minute (CPM) and calculated as the percentage of the applied dose. All experiments were performed in triplicate and repeated three times. The binding affinity value was calculated by fitting the data with nonlinear regression using Prism6 (GraphPad software).

Subcutaneous tumour xenografts

All experiments were conducted according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Laboratory Animal Center, Chang Gung Memorial Hospital, Taiwan. Female BALB/c nude mice, 6 to 8 weeks old, were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and housed under a regular 12 h light/dark schedule. Food and water were available *ad libitum*. A subcutaneous tumour model was established by inoculation of 5×10^6 U87-MG tumour cells in exponential phase into the front flank or shoulder region. When the tumours reached a volume of approximately 300-900 mm³ ($0.5 \times \text{length} \times \text{width}^2$), the mice were ready for *in vivo* imaging experiments.

Small-animal PET/CT imaging of tumours

PET imaging of mice was performed by using nano PET/CT (Mediso Medical Imaging Systems). For PET-based monitoring of tumour-targeting capability, radiotracers ([⁶⁸Ga]Ga-FAPI-04, [⁶⁸Ga]Ga-DOTA-FAPtp, and [⁶⁸Ga]Ga-Alb-FAPtp-01) were administered as a bolus in phosphate-buffered saline at an activity dose of approximately 14 MBq by rapid manual tail vein injection *in situ* to assess the xenograft status. For tracer imaging evaluation, dynamic (1 h for [⁶⁸Ga]Ga-FAPI-04 and [⁶⁸Ga]Ga-DOTA-FAPtp) and serial static (for [⁶⁸Ga]Ga-Alb-FAPtp-01) PET images were acquired at 10 min, 15 min and 20 min at 1 hour, 2 hours and 3 hours post-injection. Animals were anaesthetized using 2.0% isoflurane (Benson Medical Industries, Markham, ON, Canada) prior to and during imaging. All images were generated with scatter correction, random correction and attenuation correction (AC). CT images were acquired on a small-animal Nano CT (Mediso Medical Imaging Systems) scanner with an 8.5-cm axial-by-5.0-cm transaxial field of view. Coregistered PET/CT images were recorded and mapped to a matrix with PMOD image analysis software, version 4.0 (PMOD Technologies Ltd., Zurich, Switzerland). We determined the regions of interest (ROIs) by determining 50% of the maximum minus minimum activity level in the coronal plane of fused CT images, and we determined the ROIs of muscles by analysing the shoulder muscle using PMOD image analysis software, version 4.0 (PMOD Technologies Ltd., Zurich, Switzerland). The average radioactivity concentration within the tumour or organ of interest was calculated semiquantitatively as a percentage of the administered activity per gram of tissue (% ID/g) or as the standard uptake value (SUV)

(SUV_{mean}). Maximum intensity projection (MIP) images were created by collapsing across one dimension with the maximum value in each column of voxels. The imaging ROI-derived tumour-to-normal organ radioactivity ratios, which were calculated for organs such as the muscles, heart, lung, liver, kidney and brain, were also calculated for comparisons.

***Ex vivo* histological analysis and autoradiography**

To investigate the expression level of FAP histologically, immunohistochemistry and autoradiography were conducted to confirm the colocalization of activated FAP and the accumulation of the imaging agents. The tumour tissues were embedded in OCT compound (Tissue-Tek), snap-frozen in a dry ice-isopentane slurry and stored at -80 °C. The tissue samples were cut into 10 µm thick sections, which were then stained with primary antibodies against FAP (rabbit anti-human; AB28244). Staining for FAP was performed by immunohistochemistry on histologic sections that were fixed in 4% paraformaldehyde and incubated with anti-FAP monoclonal antibodies (dilution, 1:200 in Tris buffer) at 4 °C overnight. After washing with PBS, the slides were incubated with a biotinylated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. Streptavidin-conjugated peroxidase (ABC Elite, Vector Laboratories) was applied, and the reaction was developed by staining with 3,3'-diaminobenzidine (Sigma). The autoradiogram images were collected by a Cyclone Pulse Storage Phosphor system (Perkin-Elmer, USA) with an exposure time of 24 h. The white-light pictures of these tumour sections were taken by a Leica MZ75 (Leica Inc., Germany) high-performance stereomicroscope equipped with a 2.5 x Plano objective.

Statistical analysis

Quantitative data are expressed as the mean with standard deviation ($\pm$ SD). All data were analysed with GraphPad Prism 6 statistical software. Student's *t* test was used to analyse the differences between the control group and the experimental group. A *p*-value less than 0.05 was considered statistically significant.

Results

Chemistry and radiochemistry

$^{68}\text{Ga}^{3+}$ was used under previously reported radiolabelling conditions to radiolabel FAPI-04 and generate FAP-targeting peptides with the chelator DOTA, with high radiochemical purity (> 95%), as validated by analytic radio-HPLC. The molar radioactivity of all tracers was estimated to be $\sim 185.09 \pm 13.5$ GBq/µmol. The octanol/water partition coefficient (logP) was calculated as -2.92 (FAPI-04), -2.64 (FAPtp) and -2.88 (Alb-FAPtp-01), and the negative log *p* values indicate the high hydrophilicity of all tracers. Higher hydrophilicity is desirable for PET tracer design to reduce radioexposure, since the radioligands will

quickly wash out through the urine. The proposed FAP tracers all demonstrated low liver uptake, which might be due to the PEGylating linker and highly hydrophilic peptide residues. The purity of all radiolabelled tracers was sufficient; thus, the tracers were passed through a 0.22 μm syringe filter for the following *in vitro* cell binding affinity assay or *in vivo* animal imaging studies without further purification.

Binding specificity/affinity

To elucidate and quantify the kinetic binding ability of FAP with the proposed peptide tracers, a fast, real-time, high-throughput and label-free analysis method based on BLI was performed (Fig. 2). In these experiments, the association profile of FAP-04 was determined as an inhibitor, and the binding signals gradually increased over time and had a minimal dissociation drop pattern. The K_d value was determined as $17.4 \pm 3 \times 10^{-6}$ M with BLI. However, the Alb-FAPtp-01 peptide tracers demonstrated the typical quick up-and-down profile pattern of small molecules with fast association and dissociation rates, and the K_d value was calculated as $18.5 \pm 2.4 \times 10^{-6}$ M. Due to the potential cleavage site (-Gly-Pro-) of FAP in the FAPtp peptides, the association rate of FAP is quite different than those the other two tracers. The association rate quickly increased, similar to Alb-FAPtp-01, at the early time points, but instead of reaching a plateau, the signals gradually increased over time but were not as high as those of FAP-04. This complex association pattern might be due to the peptide being cleaved by the FAP enzyme and cannot be simply determined by a 1:1 or 1:2 stoichiometry. These results were also confirmed with proteomic analysis experiments, in which the FAPtp peptide was found to be cleaved into two fragments when incubated with FAP enzymes, but the other two tracers were found to be intact under the same processing conditions (Supplementary Fig. S4). When DPP4 was used in this binding assay, only FAPI-04 demonstrated low binding and a quick dissociation profile, with a K_d value of 2.55×10^{-4} M ($R^2=0.96$) (Fig. 2b). FAPtp and Alb-FAPtp-01 both demonstrated nonspecific binding throughout the experimental period. All these data indicated the specificity of the developed peptide tracers.

In vitro cell uptake and competition assays

The upregulated FAP expression in U87MG cells was confirmed by western blotting (Fig. 3 (a)) Then, the cellular uptake of [^{68}Ga]Ga-FAPI-04, [^{68}Ga]Ga-FAPtp and [^{68}Ga]Ga-Alb-FAPtp-01 into U87MG cells was examined. In the cellular uptake experiment, U87MG cells were seeded into 24-well plates at a density of 0.5 million cells per well and incubated overnight. The cellular uptake was normalized to total added radioactivity. All tracers demonstrated comparable and specific cell uptake within 30 min of incubation and plateaued afterward, and [^{68}Ga]Ga-Alb-FAPtp-01 showed slightly higher cellular uptake (> 2%) at 120 min but did not reach statistical significance (Fig. 3b). Note that the cellular uptake protocol used in this study does not distinguish between cell surface-bound and internalized radioactivity. In the competition study, the half maximal inhibitory concentrations [IC_{50}] of [^{68}Ga]Ga-FAPI-04, [^{68}Ga]Ga-FAPtp and

[⁶⁸Ga]Ga-Alb-FAPtp-01 were 2.489 nM, 1.197 nM and 25.76 nM, respectively (Fig. 3c). The inhibition value of [⁶⁸Ga]Ga-FAPI-04 was similar to that reported in previous papers [28]; however, a 10-fold higher concentration of FAPI-04 was needed to block the cellular uptake of [⁶⁸Ga]Ga-Alb-FAPtp-01, indicating that Alb-FAPtp-01 may have a stronger ability to bind to U87MG cells than FAPI-04.

Dynamic PET imaging analysis of [⁶⁸Ga]Ga-FAPI-04 and [⁶⁸Ga]Ga-DOTA-FAPtp

Armed with promising data from *in vitro* assays, we were able to proceed with radiolabelling and evaluation of the FAP tracers in animal models with PET. [⁶⁸Ga]Ga-FAPI-04 is believed to be a highly selective and potent FAP ligand (IC₅₀ = 6.5 nM) and has emerged as a lead PET imaging agent [15-16] that might compensate for the functional role that has been dominated by ¹⁸F-FDG in recent decades. [⁶⁸Ga]Ga-FAPI-04 has been proven to be highly correlated with FAP expression status clinically and was used as a reference tracer in this study. To validate the efficacy of the proposed [⁶⁸Ga]Ga-DOTA-FAPtp tracer, 1-hour dynamic PET imaging of [⁶⁸Ga]Ga-DOTA-FAPtp and potent FAP-targeting [⁶⁸Ga]Ga-FAPI-04 tracers was acquired and compared side by side (see Supplementary Movies S1 & S2). After intravenous injection of both tracers, very good overall tumour radioactivity uptake and rapid whole-body washout patterns were observed (Fig. 4a). The time-activity curve of U87MG tumours displayed rapid peak uptake of both tracers, with approximately 1 standardized uptake value (SUV) by 10 min, but [⁶⁸Ga]Ga-DOTA-FAPtp showed a delayed signal decline during the 20-40 min period, with a tumour uptake value of 0.5 SUV compared with 0.42 SUV for [⁶⁸Ga]Ga-FAPI-04 (Fig. 4b). In addition to the higher tumour uptake observed for [⁶⁸Ga]Ga-FAPtp, there was a much faster washout rate in the two major excretion organs, kidneys and liver, especially in the liver, and the clearance rate of [⁶⁸Ga]Ga-FAPtp was 3-fold faster than that of [⁶⁸Ga]Ga-FAPI-04, which may be due to the hydrophilic PEGylation linker of [⁶⁸Ga]Ga-FAPtp that accelerated the tracer excretion time (Fig. 5). In addition, the higher heart accumulation signals indicated the longer plasma half-life of [⁶⁸Ga]Ga-FAPtp. For brain and muscle tissues, the potential adverse side effects were minimal, as evidenced by indistinguishably low tracer uptake (SUV= 0.03 and 0.042, respectively) at late time points. These preclinical imaging results proved that the proposed FAP-targeting peptide tracer may be an effective positron emission tomography radioligand for quantifying FAP expression levels in U87MG tumours. The continuous washout of radioactivity occurred mostly through the renal pathway. Collectively, [⁶⁸Ga]Ga-FAPtp peptide tracers bound reversibly to FAP and demonstrated comparable or even higher tumour targeting capability and more favourable biodistribution profiles than [⁶⁸Ga]Ga-FAPI-04 and thus warrant further improvements in follow-up tracer development studies.

In vitro albumin binding affinity and *in vivo* pharmacokinetics of [⁶⁸Ga]Ga-Alb-FAPtp-01

The major focus of this work was to modify and optimize current FAPI derivative-based tracers, such as FAPI-04, FAPI-21, and FAPI-46, to prolong tumour retention and improve the tumour-to-normal ratio based on the structural framework of the peptides, eventually making them potential theranostic agents. Therefore, we designed and synthesized prototypic [^{68}Ga]Ga-FAPtp-targeted PET imaging agents with critical features of the peptide substrate and experimentally substantiated these agents. However, the prototype [^{68}Ga]Ga-FAPtp tracer exhibited a rapid washout rate; thus, an intriguing in situ albumin binding strategy was incorporated into the tracer design with 4-p-chlorophenyl butyric acid. The concept of optimizing the tracer pharmacokinetic profile and prolonging tracer stability in the plasma has been proven to be effective for several published PSMA-relevant tracers. With adoption of the albumin-binding agent 4-p-chlorophenyl butyric acid, [^{68}Ga]Ga-Alb-FAPtp-01 was first validated via *in vitro* FAP binding (Fig. 2), tumour cellular uptake (Fig. 3-b), albumin binding assays and *in vivo* half-life experiments (Fig. 6). The HSA binding affinity can reach a K_d value of 1.9 μM , and the half-life of the [^{68}Ga]Ga-Alb-FAPtp-01 tracer in living animals was calculated to be approximately 120 mins, which reflected a significantly prolonged circulation time for such small peptide constructs that usually have circulation times of less than 10 min. Overall, these results demonstrated the high selectivity and albumin binding ability of the tracer, which may substantially enhance the opportunity for higher tumour uptake due to the long retention time in the plasma.

***In vivo* PET imaging evaluation of [^{68}Ga]Ga-Alb-FAPtp-01**

Therefore, intravenous administration of the optimized albumin binding [^{68}Ga]Ga-Alb-FAPtp-01 tracer for PET/CT imaging was conducted to validate the FAP-targeting specificity. The imaging results showed profound rapid tumour uptake followed by moderately slow washout compared with the non-albumin-binding [^{68}Ga]Ga-FAPI-04 and [^{68}Ga]Ga-FAPtp peptide tracers (Fig. 7). The peak tumour uptake reached an SUV of 1.775 and subsequently slightly declined to 1.425 SUV at 180 min after injection. The percentage of radioactivity in the heart represented by the circulating tracers decreased to approximately $63.154 \pm 1.779\%$ within the initial 30 min scan interval and thereafter slowly decreased to $\sim 12.334 \pm 3.052\%$ at the end of the scan (180 min), indicating that 4-p-chlorophenyl butyric acid played a role in optimizing the pharmacokinetics of the tracers. The tumour margins were visualized at early time points but may be confused with nonspecific perfusion imaging; thus, the persistent tracer signals detected at 2 or 3 hours post-injection in [^{68}Ga]Ga-Alb-FAPtp-01 PET imaging reflect the specificity of tumour lesion detection. Furthermore, due to the low normal uptake background, the tumour contrast ratio of tumour/muscle increased significantly from 5.904 ± 0.824 (1 hour) to 6.704 ± 0.903 (2 hour) and 9.459 ± 1.330 (3 hour), which indicates precise whole-tumour localization for preoperative planning and delineates the tumour margins from coregistered PET/CT imaging data with high sensitivity and high spatial resolution.

In addition, both the [^{68}Ga]Ga-FAPI-04 and [^{68}Ga]Ga-FAPtp tracers were quickly washed out through the kidney within 60 min. The higher blood circulation half-life of [^{68}Ga]Ga-Alb-FAPtp-01 was attributable to the albumin-binding ability, which maintained a greater availability of radioligands in the plasma. Although there was somewhat higher liver uptake, the tumour uptake of the [^{68}Ga]Ga-Alb-FAPtp-01 peptide tracer was significantly improved due to the prolonged and increased availability of stable radioligands in the plasma. These results validated the FAP-targeting specificity in animals injected with [^{68}Ga]Ga-Alb-FAPtp-01 tracers.

Longitudinal monitoring of tumour growth with FAPI-targeted PET imaging

An elevated FAP expression level in tumour neoplasia was correlated with high tumourigenic and metastatic potential and a high rate of mortality in clinical patients, justifying its use as a potential prognostic marker. To further confirm the application of imaging with tracers for staging, longitudinal PET scans were conducted. The stable [^{68}Ga]Ga-Alb-FAPtp-01 PET tracer demonstrated prominent longitudinal tumour uptake in subcutaneous U87MG xenografts with sequential imaging acquisition time points at different tumour sizes/stages (tumour size from 200-870 mm³). The tracer uptake in malignancies was 0.540 $\pm$ 0.096, 1.198 $\pm$ 0.155 and 1.829 $\pm$ 0.100 (SUV_{mean}) at the small, medium and large stages (late stage), respectively. The increased tumour uptake observed in PET/CT imaging was consistent with the immunostaining profile of FAP, and tumour growth was accompanied by upregulation of FAP expression levels. Overall, the developed albumin-binding FAP-targeting [^{68}Ga]Ga-Alb-FAPtp-01 tracer was able to differentiate the malignant neoplasia of U87MG tumours, and this feature may serve as a prognostic marker to identify the specific tumour phenotype for anti-FAP therapies. These pilot studies demonstrated that [^{68}Ga]Ga-Alb-FAPtp-01 tracers may be superior to FAPI-04 PET for phenotypic tumour imaging for patient diagnosis. Furthermore, it is worth noting that albumin-binding FAPtp-01 tracers are more suitable for treatment response monitoring, with potential applications for anti-FAP interventions or combination therapy with conventional treatments, such as radiotherapy and chemotherapy drugs. These follow-up studies are currently being performed.

Histopathologic analysis

The *in vivo* three-dimensional (3D) PET imaging is presented as a movie (see supplementary S3), and the PET imaging was strongly correlated with *ex vivo* autoradiography images, suggesting that FAP can be accurately validated by the proposed [^{68}Ga]Ga-Alb-FAPtp-01 PET tracer (Fig. 9). We next explored the relationship between radioactivity signal images and the *ex vivo* histopathologic analysis of FAP and vascular maturity (CD31-positive blood vessels) on a per-tumour basis in tumour xenograft models (Fig. 9b). U87MG xenograft tumours were collected and stained with antibodies against FAP and CD31 to evaluate FAP and vascular density by histochemistry. The PET imaging and *ex vivo* autoradiography

images were compared spatially with the immunostaining data. The results demonstrated that the vascular density and resulting tumour perfusion were not directly correlated with [^{68}Ga]Ga-Alb-FAPtp-01 tracer uptake, as shown in Fig. 9. After investigating the histopathologic data in greater depth, it was obvious that the tumour regions with higher FAP expression levels but relatively low vascular density (weaker CD31 immunostaining) have significantly high tracer uptake in both *in vivo* 3D volume-rendered PET imaging and *ex vivo* autoradiography. The spatial colocalization and cooccurrence of the histopathologic staining with tracer accumulation confirmed the accurate registration of FAP expression status. However, vascular function and perfusion status were not significantly related to tracer accumulation but to FAP-binding specificity. Overall, in addition to FDG, the albumin tracer [^{68}Ga]Ga-Alb-FAPtp-01 might provide complementary information for the noninvasive assessment of tumour status and thus support better patient stratification and subsequent monitoring of therapeutic response.

Discussion

FAP, a recognized hallmark of tumours, is a cell-surface serine protease that acts on various hormones and extracellular matrix components. FAP expression is highly upregulated in a wide variety of cancers, especially epithelial tumours, of which 90% overexpress FAP [24]. To date, massive efforts have been put into targeted diagnostic imaging and peptide receptor radionuclide therapy (PRRT) based on the identified upregulated FAP [13–18, 25–26]. However, despite this growing field of research and the finding that tumour-derived activation of FAP signalling augments aggressive behaviours in certain types of tumours, the molecular mechanisms underlying the functional roles of FAP in various tumours remain elusive. Cancer biology is far more complex than originally expected, and a deeper understanding of the causes of FAP expression and relevant complications during different stages of tumour progression must be obtained to meet the unmet needs of early tumour diagnosis, therapeutic prognosis prediction and metastasis prevention.

However, the development of FAP-targeting small molecules requires extensive organic synthesis knowledge and skills. The whole panel of small modifications of the original parent structures has to be designed and fine-tuned, and then extensive follow-up experimental tests have to be carried out both *in vitro* and *in vivo* [27–28]. For most investigators, accordingly, the more effective and easy-to-use peptide-based FAP-targeted tracer proposed here would offer an amiable choice or a key noninvasive FAP imaging strategy for tumour phenotyping, staging and/or intervention monitoring studies.

In vitro cell uptake and competition assays and *in vivo* translational imaging evaluation studies of FAP-targeting ligands were the major focus of the current tumour diagnosis studies. In this study, the structure and radioligand properties of FAP-targeted peptide tracers were fully elucidated. The rationale of the proposed FAP-targeted PET agents is based on the natural FAP dipeptide substrate ligand sequence (-Gly-Pro-), which can be synthesized by a facile SPPS procedure, as a surrogate biomarker for early detection of the functional status of FAP, with the aim of determining whether such a biomarker can be used for early detection and to phenotypic differentiation of tumours.

Unlike most published papers using transgenic FAP-expressing HT-1080 cells, however, the permanent glioma tumour cell line U87MG was used here for *in vitro* and *in vivo* tracer evaluation. Because U87MG cells evolved from a rather heterogeneous cell population, the expression profile of FAP, tumour perfusion and interactions with surrounding tissues may be closer to those of real human tumours than those of the homogeneous HT-1080 cell population, which possesses consistent FAP protein expression. In an *in vivo* xenograft model, U87MG cells recruited murine fibroblast cells, which were FAP positive and resulted in specific FAP-targeted PET imaging. FAP expression is also observed in tumour vessels, and this may facilitate and enhance FAP tracer binding to the tumour vasculature, contributing to higher tracer uptake in tumours. Overall, the heterogeneous nature of U87MG cells may assist the interpretation of the tumour-binding and pharmacokinetic profiles of different tumour entities in general. This valuable information may be extrapolated to support clinical translation of FAP-based imaging evaluation or even endoradiotherapy applications.

To confirm the advantage of the newly developed tracers over widely used FAP inhibitor tracers, the proposed FAP-targeting tracers were subjected to direct head-to-head comparison with [^{68}Ga]Ga-FAPI-04, the reference FAP tracer, and both *in vitro* binding affinity and *in vivo* PET/CT image results were comparable between [^{68}Ga]Ga-FAPI-04 and the first prototype peptide tracer, [^{68}Ga]Ga-FAPtp, in the U87MG model. The rapid overall washout rate in normal tissues and significant tumour uptake were similar for both tracers, but [^{68}Ga]Ga-FAPtp demonstrated marginally higher tumour uptake. As reported by the Weir-Torn Jiaang and Pieter Van der Veken groups [28–29], the difluorinated proline residue in the [^{68}Ga]Ga-FAPtp structure can decrease the K_m value of FAP binding due to the higher lipophilicity and better fitting. The double fluorine substitution in position 3 of the pyrrolidine ring was also reported to dramatically improve the binding potency up to 6-fold for the nitrile-based inhibitor and to improve FAP/PREP selectivity [29–30]. Taken together, these features show that difluorinated proline might be the crucial component in the current FAP-targeting tracer design. On the basis of these favourable initial assessments, we designed the albumin-binding FAP tracer [^{68}Ga]Ga-Alb-FAPtp-01 to prolong the circulation time and increase the retention time in tumour lesions. Additionally, the potential cleavage site of FAP, the Gly-Pro-Trp-Gly-difluorinated Pro-OH sequence in [^{68}Ga]Ga-FAPtp, was reduced to Trp-Gly-difluorinated Pro-OH to prevent confusing imaging analysis results. Unlike the non-albumin-binding FAPI tracers ([^{68}Ga]Ga-FAP-04, [^{68}Ga]Ga-FAPtp) for which tumour uptake was much lower and quickly washed out through the renal pathway within 1 hour, [^{68}Ga]Ga-Alb-FAPtp-01 demonstrated quick and prolonged tracer accumulation in U87MG tumours, indicating that the [^{68}Ga]Ga-Alb-FAPtp-01 tracers were not only targeted to FAP but also steadily diffused and transported into the tumour by the intrinsic blood vessels or internal fluid pressure due to its albumin-binding capability. Thus, the [^{68}Ga]Ga-Alb-FAPtp-01 targeted tracer not only provides a facile strategy for tracer synthesis but also improves the pharmacokinetic profile of the [^{68}Ga]Ga-FAPtp tracer with a 4-p-chlorophenyl butyric acid component for endogenous albumin-binding capability *in vivo*. This feature enables prolonged stability in the systemic circulation and precise marker targeting in the blood, highlighting its potential for clinical application in theranostic agent development. However, it is a concern that [^{68}Ga]Ga-Alb-FAPtp-01 uptake does not necessarily

reflect only the FAP expression level but also the neovasculature of U87MG tumours due to the intrinsic blood-targeting agent in [^{68}Ga]Ga-Alb-FAPtp-01. In fact, the kinetic profile of tracer distribution clearly demonstrates two regions: “rapid washout” and retaining regions. The higher tracer uptake in the latter region was confirmed by the immunostaining results, showing that FAP expression is upregulated but not associated with the immature and leaky vasculature; thus, the increase in tracer accumulation over time may be due to specific FAP binding (Fig. 9). Consequently, with high overall scores for pharmacokinetic performance, suitability for tumour diagnosis, and accessibility to the wider clinical and research communities, the [^{68}Ga]Ga-Alb-FAPtp-01 tracer has emerged as a tracer of choice for further studies of potential clinical application. The tracer may play a role in ascertaining the links between FAP expression, disease state, and disease progression in the near future. The use of a DOTA chelator as a dual functional component that may be labelled with therapeutic radioisotopes, such as Y-90 and Lu-177, shows promise for theranostic applications, and related therapeutic effect experiments are currently underway.

Conclusion

Dynamic changes in tumour FAP expression are a characteristic marker of cancer; however, precise the protein fluctuation changes that occur and their correlations with tumour progression are not known. Thus, we try to provide an effective and noninvasive imaging strategy to advance the understanding of the functions of FAP in cancer, particularly its prognostic value for various tumour types, and as a complement to diagnostic uses, its potential theranostic application is under investigation in preclinical investigations. We highlight this unique and accessible solution to the existing limitations in the field, which could shape preclinical and clinical studies of FAP tracer development.

Declarations

Acknowledgements

The authors wish to thank for technical assistance of Instrumentation Center, Chang Gung University, Tao-Yuan, Taiwan. The authors are grateful to the staffs of Technology Commons, College of Life Science, and the Instrumentation Center sponsored by Ministry of Science and Technology, National Taiwan University.

Funding information

This work was supported by Ministry of Science and Technology of Taiwan (107-2314-B-182A-149-MY3 to C.W.H.), Chang Gung Memorial Hospital (CMRPG3K1451) and National Taiwan University (108L7866 and 109L7866 to F.T.H.).

Compliance with ethical standards

Conflict of interest

The authors declare no conflicts of interest.

Research involving human participants and/ or animals

All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (No. NTU108-EL-00021) and Chang Gung Memorial Hospital, Linkou, Taiwan (No.201612060).

Disclaimer The current work has not been submitted for review or is not under acceptance for publication in any journal.

Author Contributions

Jia-Jia Lin contributed to *in vitro* cell experiments, generated figures, and contributed to *in vivo* animal experiments. Chia-Pao Chuang and Jia-Yu Lin contributed to biological study design, experiments, and figure generation. F.T. Huang designed and coordinated the study, wrote the manuscript, and edited the manuscript. C.W. Huang designed and coordinated the study, performed chemical experiments, generated figures, and wrote and edited the manuscript.

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Figures

Fig. 1 Representative chemical structures of (a) UAMC-1110, (b) FAPI-04 and the developed peptide tracers (c) FAPtp and (d) Alb-FAPtp-01

Figure 1

[See figure]

Fig. 2 Validation of FAPI-04, FAPtp and Alb-FAPtp-01 tracer binding to (a) FAP and (b) DPP4, assayed with BLI. The FAP and DPP4 proteins were loaded into the sensor tip from a 10 nM solution and then incubated with various concentrations of labelled free FAP tracers in binding buffer. The association and dissociation binding curves were monitored with an eight-channel Octet RED96 BLI system and Kd values were calculated with Octet Data Analysis software (9.0.0.6.) (green: FAPI-04, blue: FAPtp, red: Alb-FAPtp-01.)

Figure 2

[See figure]

Fig. 3 FAP expression and cell binding and inhibition assay. (a) The western blot results confirmed FAP expression and GAPDH was used as internal control. (b) Cell uptake assay of FAPI-04, FAPtp and Alb-FAPtp-01 tracers. (c) Inhibition of FAPI-04, FAPtp and Alb-FAPtp-01 tracers binding to FAP in U87MG cells by nonradiolabelled inhibitor, FAPI-04 (n=3, mean $\pm$ SD).

Figure 3

[See figure]

Fig. 4 (a) Dynamic PET imaging of $[^{68}\text{Ga}]\text{Ga-FAPI-04}$ and $[^{68}\text{Ga}]\text{Ga-FAPtp}$ and (b) time-activity curves of radiotracers in major organs and U87MG tumours.

Figure 4

[See figure]

Fig. 5 Comparison of the time-activity curves of $[^{68}\text{Ga}]\text{Ga-FAPI-04}$ and $[^{68}\text{Ga}]\text{Ga-FAPtp}$ in major organs and U87MG tumour based on ROIs drawn from dynamic imaging data.

Figure 5

[See figure]

Fig. 6 (a) The albumin binding affinity assay of [⁶⁸Ga]Ga-Alb-FAPtp-01. (b)The proportion of plasma-bound activity of [⁶⁸Ga]Ga-Alb-FAPtp-01 in healthy nu/nu mice after injection.

Figure 6

[See figure]

Figure 7

[See figure]

[See figure]

[See figure]

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