Targeting fibroblast activation protein (FAP): Next generation PET radiotracers using squaramide coupled bifunctional DOTA and DATA5m chelators

Euy Sung Moon

Johannes Gutenberg-Universitat Mainz Fachbereich Chemie Pharmazie und Geowissenschaften
<https://orcid.org/0000-0003-0447-1355>

Filipe Elvas

Universiteit Antwerpen Faculteit geneeskunde en gezondheidswetenschappen

Gwendolyn Vliegen

Universiteit Antwerpen Faculteit Farmaceutische Biomedische en Diergeneeskundige Wetenschappen

Stef De Lombaerde

Universiteit Antwerpen Faculteit geneeskunde en gezondheidswetenschappen

Christel Vangestel

Universiteit Antwerpen Faculteit geneeskunde en gezondheidswetenschappen

Sven De Bruycker

Universiteit Antwerpen Faculteit geneeskunde en gezondheidswetenschappen

An Bracke

Universiteit Antwerpen Faculteit Farmaceutische Biomedische en Diergeneeskundige Wetenschappen

Elisabeth Eppard

PositronPharma

Lukas Greifenstein

Johannes Gutenberg-Universitat Mainz Fachbereich Chemie Pharmazie und Geowissenschaften

Benedikt Klasen

Johannes Gutenberg-Universitat Mainz Fachbereich Chemie Pharmazie und Geowissenschaften

Vasko Kramer

PositronPharma

Steven Staelens

Universiteit Antwerpen Faculteit geneeskunde en gezondheidswetenschappen

Ingrid De Meester

Universiteit Antwerpen Faculteit Farmaceutische Biomedische en Diergeneeskundige Wetenschappen

Pieter Van der Veken

Universiteit Antwerpen Faculteit Farmaceutische Biomedische en Diergeneeskundige Wetenschappen

Frank Roesch (✉ frank.roesch@uni-mainz.de)

Original research

Keywords: DOTA, DATA5m, 68Ga, FAP, PREP, squaric acid, squaramide, HT-29

Posted Date: April 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-24915/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at EJNMMI Radiopharmacy and Chemistry on July 29th, 2020. See the published version at <https://doi.org/10.1186/s41181-020-00102-z>.

Abstract

Background

Fibroblast activation protein (FAP) is a proline selective serine protease that is overexpressed in tumor stroma and in lesions of many other diseases that are characterized by tissue remodeling. In 2014, a most potent FAP-inhibitor (referred to as UAMC1110) with low nanomolar FAP-affinity and high selectivity toward related enzymes such as prolyl oligopeptidase (PREP) and the dipeptidyl-peptidases (DPPs): DPP4, DPP8/9 and DPP2 were developed. This inhibitor has been adopted recently by other groups to create radiopharmaceuticals by coupling bifunctional chelator-linker systems. Here, we report squaric acid containing bifunctional DATA^{5m} and DOTA chelators relied on UAMC1110.

Results

The radiopharmaceuticals DOTA.SA.FAPi and DATA^{5m}.SA.FAPi were synthesized, labeled with gallium-68 and further characterized for *in vitro* stability, inhibitory efficiency, *in vivo* targeting properties and *ex vivo* biodistribution. [⁶⁸Ga]Ga-DOTA.SA.FAPi and [⁶⁸Ga]Ga-DATA^{5m}.SA.FAPi showed high complexation after already 10 minutes and high stability over a period of 2 h. Affinity to FAP of DOTA.SA.FAPi and its ^{nat}Ga and ^{nat}Lu-labeled derivatives were in low nanomolar range. Comparable results were obtained for DATA^{5m}.SA.FAPi and its ^{nat}Ga analogue. Additionally, all five compounds showed low affinity for the related protease PREP (high μM range). First proof-of-principle *in vivo* PET-imaging animal studies of the [⁶⁸Ga]Ga-DOTA.SA.FAPi precursor in a HT-29 human colorectal cancer xenograft mouse model indicated promising results with high accumulation in tumor and low background signal. *Ex vivo* biodistribution showed high tumor uptake at 60 min post injection with overall low uptake in healthy tissues.

Conclusion

In this work, novel PET radiotracers targeting fibroblast activation protein (FAP) were synthesized and biochemically investigated. Critical substructures of the novel compounds are a squaramide linker unit derived from the basic motif of squaric acid, DOTA and DATA^{5m} bifunctional chelators and a FAP-targeting moiety. In conclusion, these new FAP-ligands appear promising, both for further research and development as well as for first human application.

Background

The proline-selective serine protease fibroblast activation protein (FAP) is a type II transmembrane glycoprotein with 760 amino acids. It is related to the dipeptidyl peptidases (DPPs) DPP2, DPP4, DPP8 and DPP9 and furthermore related to the endopeptidase prolyl oligopeptidase (PREP).[1, 2] FAP combines DPP and endopeptidase activities.[3–6] With respect to FAP's endopeptidase activity, a remarkable

preference is present for cleavage after Gly-Pro motifs in peptides.[7] FAP is not detectable in most healthy adult tissues and therefore considered non-essential under normal circumstances. However, it is clearly expressed in pathophysiological lesions, characterized by tissue remodeling. Such lesions can be found in, e.g., cancer, atherosclerosis, arthritis and several fibrosis types.[8, 9] Over the past two decades, significant attention has gone to FAP in solid tumors, where it is mainly expressed on so-called cancer associated fibroblasts (CAFs).[10, 11] These are activated fibroblasts with a myofibroblast phenotype.[12] There is growing evidence that CAFs have a regulatory role in tumor biology and extracellular matrix composition.[13–15] FAP⁺-CAFs are present in the stromal tissue of more than 90% of epithelial carcinomas, including pancreatic, colon, ovarian, lung and breast cancer.[16, 17] Generally speaking, the tumor stroma contains a large part of the tumor mass (> 90% of tumor mass in carcinomas) and is therefore an attractive target for diagnostic and therapeutic radiopharmaceuticals. Conferring stroma affinity to these radiopharmaceuticals by incorporating a FAP-inhibitor moiety is, based on the presence of FAP⁺-CAFs, a potentially rewarding strategy.

Several highly potent FAP-inhibitors have been reported earlier.[18, 19] First-generation compounds with a boronic acid warhead, however, are plagued by a lack of selectivity with respect to the related enzymes and are also characterized by lower chemical stability. More recently, compounds with a more stable carbonitrile warhead have been reported.[20, 21] One of the most promising molecules to date is UAMC1110 (Fig. 1). This molecule combines low nanomolar FAP affinity and high selectivity with respect to both the DPPs and PREP. The high FAP-selectivity of UAMC1110 is particularly attractive for tumor-targeting, when taking into account the near-ubiquitous expression of the DPPs and PREP in humans. In addition, this molecule possesses a satisfactory pharmacokinetic profile.

UAMC1110 is currently still under evaluation as a potential therapeutic in diseases characterized by FAP expression. At the same time, the molecule is being used as a FAP-targeting moiety in so-called activity-based probes that can be used to visualize and quantify FAP activity in tissues and organisms.[22] Highly relevant examples have also been published that rely on radionuclide-based reporter systems, such as XY-FAP-02 developed by Yang et al.[23] They used a DOTAGA chelator combined with an alkyl chain as linker system bound to the FAP-inhibitor.

Further developments by Lindner and Loktev et al. using radiolabeled [UAMC1110-chelator]-conjugates have shown promising results in preclinical and first clinical patient studies. Applications of these molecules cover both diagnosis and therapy.[24–29] First, a DOTA-FAPI conjugate using piperazine as linker (referred to as FAPI-02 in the original reference) was synthesized and characterized with respect to binding, internalization, and efflux in cells expressing human and murine FAP as well as CD26. PET-imaging studies of HT-1080 tumor xenografts showed low [⁶⁸Ga]Ga-FAPI-02 accumulation in normal tissues and a rapid clearance from the blood via kidneys and bladder. In addition, a high tumor uptake resulting in high tumor-to-normal organ-ratio was determined. By structural variation, especially in the linker region, more analogous ⁶⁸Ga-labeled compounds were obtained. Several of these had improved imaging parameters, with FAPI-04, FAPI-21 and FAPI-46 being relevant examples.[25, 26] These

compounds also had low nanomolar FAP-affinity ($IC_{50} = 6.5$ nM for FAPI-04, $IC_{50} = 6.7$ nM for FAPI-21 and $IC_{50} = 13.5$ nM for FAPI-46), higher tumor uptakes *in vivo* and longer tumor retention times. First PET/CT imaging studies of patients diagnosed with different tumor entities were performed with the ^{68}Ga -compounds indicating high tumor uptake and low background in healthy organs. As an example of a first therapeutic application, patients diagnosed with metastatic breast cancer were treated with [^{90}Y]Y-FAPI-04. The $^{68}\text{Ga}/^{90}\text{Y}$ -DOTA-derivatives represent promising tracers for both diagnostic imaging and, possibly, targeted therapy of malignant tumors with high accumulated activated fibroblasts.

In this work, novel FAP-targeting radiotracers were evaluated using bifunctional DOTA and DATA^{5m} chelators coupled by squaramide as linker moiety. The basic motif squaric acid (SA) is a cyclic aromatic diacid.[30, 31] One advantage of SA is the simple chemistry regarding coupling to chelator and target vector including that no protecting groups are necessary due its selectivity for primary amines. Especially reactions with biomolecules are attractive and no side reactions are observed. The coupling with SA-diester is a highly selective, pH controlled asymmetric amidation under mild conditions.[32] In a neutral pH, only one ester of the SA-diester reacts with an amine and by increasing the pH to basic conditions, amidation of the second ester takes place. The use of SA as a linker unit between a chelator-biomolecule conjugate as a radiopharmaceutical was demonstrated using DFO and conjugation on a peptide to complex iron and using DFO-squaric acid coupled to antibodies for complexing ^{89}Zr .[33, 34] Recently, our group published the usage of SA as a linker forming a radiotracer with the bifunctional hybrid chelator AAZTA⁵ coupled to a PSMA inhibitor unit (KuE) and evaluated those AAZTA⁵.SA.PSMA conjugate with various radionuclides such as ^{44}Sc , ^{64}Cu , ^{68}Ga and ^{177}Lu .[35] Additionally, we indicate a second feature of SA beyond coupling chemistry. In several cases we could observe a positive impact on pharmacology of the final products. [^{68}Ga]Ga-NODAGA.SA.PSMA, [^{68}Ga]Ga-TRAM.SA.PSMA and [^{68}Ga]Ga-DOTAGA.SA.PSMA showed high tumor uptake and overall high tumor-to-organ ratio. [^{68}Ga]Ga-DOTAGA.SA.PSMA provided *in vivo* in LNCaP-tumor bearing mice comparable results to [^{68}Ga]Ga-PSMA-617 and [^{68}Ga]Ga-PSMA-11 with significant tumor accumulation.[36]

Here, the preparative synthesis of DOTA.SA.FAPi and DATA^{5m}.SA.FAPi and the metal-analogs [$^{\text{nat}}\text{Ga}$]Ga-DOTA.SA.FAPi, [$^{\text{nat}}\text{Ga}$]Ga-DATA^{5m}.SA.FAPi and [$^{\text{nat}}\text{Lu}$]Lu-DOTA.SA.FAPi are described. The macrocyclic chelator DOTA was used to allow labeling with both ^{68}Ga and ^{177}Lu . However, one disadvantage of these chelator types are the requirement of high temperatures for complexation.[37] DATA^{5m}, a bifunctional version of the hybrid chelator DATA, was used to allow instant ^{68}Ga -labeling at room temperature.[38–40] Radiochemical evaluation with regard to labeling and *in vitro* stability studies were performed with ^{68}Ga for DOTA.SA.FAPi and DATA^{5m}.SA.FAPi. For all the five cold compounds, inhibition assays were carried out and IC_{50} values obtained for FAP and PREP. In a first proof-of-principle PET-study, [^{68}Ga]Ga-DOTA.SA.FAPi was tested *in vivo* using a FAP-expressing HT-29 human colorectal adenocarcinoma xenograft model.

Results

Synthesis of DOTA.SA.FAPi

The commercially available DO3A-*t*Bu-*N*-(2-aminoethyl)ethanamide **1** was treated with trifluoroacetic acid (TFA) to deprotect the *tert*-butyl groups. Since the coupling of squaric acid diethyl ester (SADE) with primary amines is selective, no protective groups were necessary for the next synthesis steps. The deprotected DO3A-*N*-(2-aminoethyl)ethanamide was coupled to SADE in phosphate buffer (pH 7) at ambient temperature and purified via HPLC to receive DOTA.SA **2**. The free coupling side of **2** was afterwards coupled to (*S*)-6-(4-aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-quinoline-4-carboxamide **3** (termed NH₂-FAPi) in phosphate buffer (pH 9) at room temperature. After successful HPLC purification, DOTA.SA.FAPi **4** was obtained (Fig. 3).

Synthesis of DATA^{5m}.SA.FAPi

DATA^{5m}-3^tBu **5** was synthesized as described by Seemann et al. (Figure 4).[38] DATA^{5m}-3^tBu provides a bifunctional carbonyl group for further coupling with spacer molecules or target vectors. Terminal primary amines are required for binding to SA-diethylester. Therefore *N*-*boc*-ethylenediamine was attached to the carboxylic acid group of DATA^{5m} via common coupling reagents HATU in DIPEA and MeCN receiving **6**. Amidation of SA-monoester **7** with the terminal amine of NH₂-FAPi was executed analogously to DOTA.SA.FAPi to receive DATA^{5m}.SA.FAPi **8**.

Synthesis of cold complexes and enzyme inhibition assays

Cold complexes of [^{nat}Ga]Ga-DOTA.SA.FAPi, [^{nat}Ga]Ga-DATA^{5m}.SA.FAPi and [^{nat}Lu]Lu-DOTA.SA.FAPi were synthesized. The corresponding precursors were reacted with a 10 mM solution of the metal chlorides or nitrates in 0.2 M NaAc buffer pH 4.5. The solutions of ^{nat}Ga and ^{nat}Lu complexed DOTA.SA.FAPi were shaken for 3 h at 95 °C and the solution of ^{nat}Ga-metalled DATA^{5m}.SA.FAPi was shaken for 2 h at RT. Complexations were monitored by ESI LC-MS and the metal complexes were purified via HPLC.

In the inhibition assays, DOTA.SA.FAPi and DATA^{5m}.SA.FAPi, along with their non-radioactive, metal complexed analogues were characterized for inhibitory potency towards FAP and PREP. Earlier work had shown that the lack of a basic amine function in UAMC1110-based molecules, precludes DPP-affinity in this series.[21,22] Nonetheless, the FAP/PREP selectivity was shown to be a particularly important parameter to check. Obtained results are summarized in Table 1. Parent compound UAMC1110 was used as a reference in this assay. All the evaluated molecules displayed highly satisfactory, low nanomolar FAP potencies, in the same range as the parent inhibitor UAMC1110. This implies that introduction of a linker, a chelator and a metal ion at the selected position of the quinoline ring are tolerated by FAP and have no negative influence on target affinity. Likewise, equally satisfactory compound selectivities with respect to PREP were measured, again comparable with UAMC1110.

Table 1 IC₅₀-values of DOTA.SA.FAPi, the ^{nat}Ga and ^{nat}Lu-complexes and DATA^{5m}.SA.FAPi and the ^{nat}Ga-complex with regard to FAP and PREP. Selectivity index gives the ratio FAP to PREP.

	IC ₅₀ FAP (nM)	IC ₅₀ PREP (µM)	Selectivity index (IC ₅₀ (FAP/PREP))
DOTA.SA.FAPi – uncomplexed	0.9 ± 0.1	5.4 ± 0.3	6000
DOTA.SA.FAPi – ^{nat} Ga	1.4 ± 0.2	8.7 ± 0.9	6214
DOTA.SA.FAPi – ^{nat} Lu	0.8 ± 0.2	2.5 ± 0.4	3125
DATA ^{5m} .SA.FAPi – uncomplexed	0.8 ± 0.2	1.7 ± 0.1	2113
DATA ^{5m} .SA.FAPi – ^{nat} Ga	0.7 ± 0.1	4.7 ± 0.3	6714
FAP-inhibitor UAMC1110	0.43 ± 0.07 ^a	1.8 ± 0.2 ^b	4186

^a Determined under the conditions of this study. ^b data from Jansen *et al.*[21]

Radiochemical evaluations with gallium-68

Radiolabeling of DOTA.SA.FAPi with ⁶⁸Ga was performed with varying amounts of the precursor (10-40 µg) and at 95 °C (Figure 5). Labeling was performed in 1 M AmAc buffer (pH 5.5) at 95 °C in triplicate n=3 with around 200 MBq of ⁶⁸Ga. For precursor amounts of more than 15 µg (16 nmol), a quantitative radiochemical yield of >97 % could be achieved in less than 5 minutes. At 10 µg (11 nmol) a decreased RCY of 44 % after 15 minutes could be observed. For further *in vivo* studies, 20 µg was used as standard and C18 purification of [⁶⁸Ga]Ga-DOTA.SA.FAPi was executed which results in a radiochemical purity of >98 %. HPLC retention time of free gallium-68: *t_R*(⁶⁸Ga) was 4 min and the retention time of the complex *t_R*([⁶⁸Ga]Ga-DOTA.SA.FAPi) was 9 min. The *R_f* values of the radio-TLC were *R_f*(⁶⁸Ga) = 0.9 and *R_f*([⁶⁸Ga]Ga-DOTA.SA.FAPi) = 0.1 using citrate buffer pH 4 as mobile phase.

Carrying out labeling at different temperatures (70, 80 and 95 °C) with a defined tracer amount of 30 µg (31 nmol) resulted in quantitative RCYs >97 % at temperatures of 80 °C and 95 °C after 15 minutes. At 70 °C, complexation of ⁶⁸Ga via DOTA.SA.FAPi showed decreased radiolabeling efficiency, nevertheless resulting in >83 % after 15 min. (SI, Figure S3).

Stability studies were performed in EtOH, HS and saline 0.9 % over a period of 2 h at 37 °C. In all three media, [^{68}Ga]Ga-DOTA.SA.FAPi showed high stabilities over 98 % intact conjugate (SI, Figure S4). In addition, stability against transmetallation and transchelation were carried out (SI, Figure S5, S6). Against DTPA and EDTA the stability values were >98 % and against Cu, Mg and Ca the stabilities were >95 % after 2 h. Stabilities against Fe showed >95 % after 90 minutes and a slightly lower value however still over 92 % after 2 h.

For radiolabeling of DATA^{5m}.SA.FAPi, various precursor amounts (1-20 µg) in 1 M AmAc buffer (pH 5.5) were labeled with ^{68}Ga . The reaction mixture was shaken for 10 min at room temperature to afford [^{68}Ga]Ga-DATA^{5m}.SA.FAPi.

First kinetic studies were performed with 200-230 MBq of ^{68}Ga in 1 M AmAc buffer (pH 5.5) at RT (n=1). Quantitative RCYs >98 % could be achieved for precursor amounts of 15 µg (16 nmol) and 20 µg (21 nmol) in less than 1 minute. Analogous to [^{68}Ga]Ga-DOTA.SA.FAPi, [^{68}Ga]Ga-DATA^{5m}.SA.FAPi also showed significantly lower RCY below 15 µg. At 10 µg (10 nmol) only a RCY of 38 % and for 5 µg a RCY of just 12 % after 10 minutes could be observed. HPLC retention times of free gallium-68 $t_R(^{68}\text{Ga})$ and of the complex $t_R([^{68}\text{Ga}]Ga-DATA^{5m}.SA.FAPi)$ were 4 min and 8.5 min, respectively. The R_f values of the radio-TLC were $R_f(^{68}\text{Ga}) = 0.9$ and $R_f([^{68}\text{Ga}]Ga-DATA^{5m}.SA.FAPi) = 0.1$ using citrate buffer pH 4 as mobile phase. In addition, it was investigated whether a lower amount of activity has an influence on better complexation. Therefore 10 µg of the precursor were labeled with around 60 MBq of ^{68}Ga in triplicate. The lower activity improved complexation of [^{68}Ga]Ga-DATA^{5m}.SA.FAPi. Using 10 nmol of precursor, a quantitative yield could be achieved instead of 38 % as before. Stability of [^{68}Ga]Ga-DATA^{5m}.SA.FAPi was determined in PBS, HS and saline 0.9 % over a period of 120 min. In all three media, the stability of [^{68}Ga]Ga-DATA^{5m}.SA.FAPi remained >95 % (SI, Figure S7).

PET/CT-imaging and *ex vivo* biodistribution data of [^{68}Ga]Ga-DOTA.SA.FAPi

For investigation of the tumor uptake by [^{68}Ga]Ga-DOTA.SA.FAPi HT-29 tumor-bearing mice (n=3) were sacrificed after PET/CT scans and an *ex vivo* biodistribution study was executed. In the PET images, the tumor accumulation is clearly visible and the ratio to nonspecific organs and tissues is very high. Figure 7 shows the maximum intensity projection (MIP) images of three mice. *Ex vivo* biodistribution is shown in Figure 8a. The highest accumulation was observed in the tumor with an uptake of 5.2 ± 0.2 %ID/g 1 h post injection (p.i.). In general, the tumor-to-organ ratios are high after 1 h p.i. which is shown by, e.g., tumor-to-blood (9.2 ± 1.1), tumor-to-large intestine (24.9 ± 1.7) and tumor-to-muscle (11.5 ± 2.2) ratios (Figure 8b). Uptake in other organs are also low such as in heart, lungs, liver, spleen, pancreas, stomach, fat and skin. In addition to the tumor accumulation, a slightly higher accumulation at the bones and

small intestine was found, which cannot yet be fully explained. One suggestion could be that FAP is also expressed in these tissues. However, it is important that the main accumulation is located in the tumor.

Discussion

Two novel bifunctional chelator-linker conjugates based on the FAP inhibitor UAMC1110 were developed. As bifunctional chelators, macrocyclic DOTA as well as the hybrid chelator DATA^{5m} were used. Squaric acid is the main component of the linker system forming a squaramide unit and accordingly substitutes the heterocyclic nitrogen moieties in the structures reported by the Heidelberg group.[24–26] The asymmetrically substituted squaramide unit in the target compounds was efficiently installed with SADE, relying on the latter's elegant pH-dependent chemistry and selectivity for primary amines. Due to this selectivity, no protecting groups are required. In addition, the pH dependent reactivity of SADE is explained by changes of the aromatic stabilization energy in the ring system over the course of the sequential amidation steps.[32] After the first amidation, which is carried out at neutral pH, the obtained uncharged monoamide is characterized by a higher aromatic stabilization than the starting material, squaric acid diester. This stabilization prevents addition of a second amine molecule. By increasing the pH, the monoamide is deprotonated, loses aromatic stabilization, and this allows for addition of a second amine molecule to provide the diamide.[30, 41] Correspondingly, C₂-symmetric diamide derivatives of squaric acid can be obtained, if the reaction is performed at higher pH: in that case, two equivalents of amine will directly substitute the ethoxy groups in SADE.

Both DOTA.SA.FAPi and DATA^{5m}.SA.FAPi could be well used for radiolabeling with ⁶⁸Ga. DATA has already demonstrated good complexation with ⁶⁸Ga and has the advantage of labeling even under mild conditions such as room temperature. Quantitative gallium-68 labeling results were observed for both FAPi-conjugates with ⁶⁸Ga. The DOTA complex showed quantitative radiochemical yields at temperatures of 95 °C with precursor amounts of > 15 nmol. For the DATA^{5m} conjugate quantitative yields could be achieved at room temperature for amounts > 15 nmol. In addition, it could be seen that with a lower activity of ⁶⁸Ga, less precursor amount (≥ 10 nmol) is required to achieve quantitative complexation, whereas with higher gallium activity (> 200 MBq) more substance > 15 nmol is needed for quantitative yields.

The stability for both derivatives against different media was high with > 95% intact conjugates. [⁶⁸Ga]Ga-DOTA.SA.FAPi proved stable in EtOH, HS and saline and was stable against transmetallation (Cu, Mg, Ca and Fe) and transchelation (DTPA and EDTA). Stability of [⁶⁸Ga]Ga-DATA^{5m}.SA.FAPi in HS, PBS and in saline is very high with > 95% intact conjugates over a period of 2 h.

All five measured compounds, DOTA.SA.FAPi, [^{nat}Ga]Ga-DOTA.SA.FAPi, [^{nat}Lu]Lu-DOTA.SA.FAPi, DATA^{5m}.SA.FAPi and [^{nat}Ga]Ga-DATA^{5m}.SA.FAPi demonstrated very high affinity to FAP with low nanomolar IC₅₀ values and high IC₅₀ values with respect to PREP. Therefore, all measured FAP inhibitors

have a potency in the same order of magnitude as the original FAP inhibitor. In addition, they all presented excellent selectivity for FAP with regard to PREP.

Preclinical *in vivo* animal studies were performed with HT-29 xenograft mice. The HT-29 cancer cell line is a human colorectal adenocarcinoma cell line with epithelial morphology.[42, 43] When inoculated into nude mice, they produce undifferentiated tumors with modest stroma. Significant FAP expression is present in this stroma, that typically trabeculates between nests of HT-29 cells. Notably, and similar to the situation in most tumor types, FAP staining is distinctly absent from the actual HT-29 cancer cells within the tumors.[43]

The highest accumulation in the tumor was found in both the PET images and biodistribution with an overall uptake of 5.2%ID/g 60 min post injection. In addition, the tumor-to-organ ratios were quite high (tumor-to-blood (9.2 ± 1.1), tumor-to-large intestine (24.9 ± 1.7) and tumor-to-muscle (11.5 ± 2.2)), which is also reflected by the high contrast in the images. Besides the high tumor uptake, accumulation in the bladder could also be observed, suggesting renal clearance to be the predominant excretion.

Conclusion

In this work, two potential theranostic radiopharmaceuticals were successfully synthesized, based on the selective FAP-inhibitor UAMC1110. Key elements of these compounds are a squaramide motif (introduced via amidation of SADE) and a DOTA or DATA^{5m}-type chelator. Due to the unique chemistry of SADE, it was possible to avoid complex synthesis routes and protective group strategies. In recent works from our group, SA has shown good results, both in chemistry and physiologically, as a linker unit coupled with PSMA inhibitors.[35, 36] DOTA.SA.FAPi and DATA^{5m}.SA.FAPi showed very good *in vitro* complexations of ⁶⁸Ga and a very high stability in different media of more than 95% intact conjugate. In general, the hybrid chelator DATA^{5m} shows a quantitative complexation under mild conditions and is therefore very well suited to label temperature sensitive target molecules with radiometals. Additionally, DATA is well known for fast and stable complexation of ⁶⁸Ga and to show high stabilities over a long period of time. DOTA is interesting because of its ability to complex other radiometals preferring higher coordination numbers, e.g., the long-lived therapy radionuclide ¹⁷⁷Lu but also shorter-lived therapeutic radionuclides such as ²¹³Bi, ²¹⁰Pb and ⁹⁰Y. DOTA.SA.FAPi allows to use the same precursor for both diagnosis with ⁶⁸Ga and therapy with ¹⁷⁷Lu in nuclear medicine.

Both FAPi-precursors as well as their gallium and lutetium versions showed excellent affinity and selectivity to FAP, in the low nanomolar range, with IC₅₀-values between 0.7 and 1.4 nM. Conversely, PREP IC₅₀-values were found to be in the μM-range, implying excellent FAP/PREP selectivity indices.

In the HT-29 colon cancer xenograft model, first proof-of-concept animal studies with [⁶⁸Ga]Ga-DOTA.SA.FAPi showed good tumoral accumulation with high uptake of $5.2 \pm 0.2\%$ ID/g on average at 60 min p.i. and low background activity, *i.e.* an overall good tumor-to-organ ratio.

The potential of the novel compound family to target FAP could be clearly demonstrated. In further preclinical characterizations we will also investigate the *in vivo* properties of [⁶⁸Ga]Ga-DATA^{5m}.SA.FAPi and publish these results at a later stage. We expect to apply these new radiotracers for various tumor types in nuclear medicine both in diagnosis and therapy.

Materials And Methods

Reagents and instrumentations for synthesis

All basic chemicals were acquired from Sigma-Aldrich (St. Louis, USA), Merck KGaA (Darmstadt, Germany), TCI Deutschland GmbH (Eschborn, Germany) and VWR International GmbH (Darmstadt, Germany). DOT3AtBu-*N*-(2-aminoethyl)ethanamide **1** was purchased from CheMatech (Dijon, France), (*S*)-6-(4-aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-quinoline-4-carboxamide **3** was purchased from KE Biochem Co. (Shanghai, China). Thin-layer chromatography plates from Merck, Kieselgel 60 F254 coated aluminum plates, were used for the analysis. Detection was carried out by fluorescence extinction at $\lambda = 254$ nm and by staining with potassium permanganate. Silica gel 60 (core size 0.063 0.200 mm) from Acros Organics (Schwerte, Germany) was used for purification by column chromatography. The LC/MS spectra were measured on an Agilent Technologies 1220 Infinity LC system coupled to an Agilent Technologies 6130B Single Quadrupole LC/MS system. The ¹H and ¹³C NMR measurements were performed at 400 MHz (400 MHz FT NMR spectrometer AC 400, Bruker Analytik GmbH). For analytical and semi-preparative HPLC a 7000 series Hitachi LaChrom with a Phenomenex (Aschaffenburg, Germany) Luna C18 (250 × 4.6 mm, 5 μ) column, a Phenomenex Luna C18 (250 × 10 mm, 10 μ) column and a Phenomenex Synergi C18 (250 × 10 mm, 4 μ) column were used.

Organic Synthesis

Synthesis of DOTA.SA.FAPi

DOTA.SA (2) [2,2',2''-(10-(2-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl]triacetic acid]

1 (48.0 mg; 78.1 μ mol) was reacted with 1 mL 80% TFA in DCM for 6 h at room temperature for deprotection of *tert*-butyl protecting groups. After evaporating TFA/DCM, the residue was reacted with 3,4-diethoxycyclobut-3-ene-1,2-dione (13.3 mg; 78.1 μ mol) in 500 μ L 0.5 M Na₂HPO₄/NaH₂PO₄ phosphate buffer pH 7 and shaken at room temperature overnight. The chelator-linker conjugate DOTA.SA **2** could be isolated via HPLC purification. After HPLC purification (Phenomenex® Luna® 10 μ m C18 (2) 100 Å, gradient 6–8% MeCN (+ 0.1% TFA)/ 94–92% Water (+ 0.1% TFA) in 20 min with a 5 mL/min flow) and lyophilization the product was obtained as white powder (28.2 mg; 49.4 μ mol; 63%). ¹H-NMR (D₂O, 600 MHz, δ [ppm]): 4.64–4.53 (dq, 2 H); 3.93–2.89 (m, 28 H); 1.41–1.33 (m, 3 H). MS (ESI⁺): *m/z* (%): 571.3 (M + H⁺), 593.3 (M + Na); calculated for C₂₄H₃₈N₆O₁₀: 570.26

DOTA.SA.FAPi (4) [(S)-2,2',2''-(10-(2-((2-((2-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid]

Coupling of DOTA.SA **2** (10.3 mg; 17.5 μ mol) and NH₂-FAPi **3** (11.4 mg; 26.3 μ mol) to form DOTA.SA.FAPi **4** was performed by amidation at pH 9 in 500 μ L 0.5 M Na₂HPO₄ phosphate buffer at room temperature. The reaction was shaken for 12 h. DOTA.SA.FAPi **4** was isolated via HPLC purification (Phenomenex® Luna® 10 μ m C18(2) 100 Å) with a linear gradient condition of 15–20% MeCN (+ 0,1% TFA)/85 – 80% Water (+ 0,1% TFA) in 20 min with a 5 mL/min flow. After lyophilization the product was obtained as yellow powder (12.2 mg; 12.7 μ mol, 73%). MS (ESI⁺): m/z (%): 956.4 (M + H⁺), 978.4 (M + Na); calculated for C₄₃H₅₅F₂N₁₁O₁₂: 955.40

Inhibitory Potency Determination

Enzymes: A gateway-entry clone for human FAP was purchased from Dharmacon (Accession number DQ891423) and the human secretion signal was replaced with the HoneyBee mellitin secretion signal. For transfection and expression of FAP in Sf9 insect cells, the C-terminal BaculoDirect kit from LifeTechnologies was used. The enzyme was purified from the supernatant of the insect cells using immobilized Ni-chelating chromatography (GE healthcare, Diegem, Belgium), followed by anion-exchange chromatography using a 1 mL HiTrap Q (GE healthcare, Diegem, Belgium). Human recombinant PREP was expressed in BL21(DE3) cells and purified using immobilized Co-chelating chromatography (GE healthcare) followed by anion-exchange chromatography on a 1 ml Mono Q column (GE healthcare).

FAP: IC₅₀ measurements of the inhibitors were carried out using Z-Gly-Pro-7-amino-4-methylcoumarine (AMC) (Bachem, Switzerland) as the substrate at a concentration of 50 μ M at pH 8 (0.05 M Tris-HCl buffer with 0.1% glycerol, 1 mg/mL BSA and 140 mM NaCl). Eight concentrations of inhibitors were tested. The final DMSO concentration was kept constant during the experiment to exclude any solvent effects. Inhibitors were pre-incubated with the enzyme for 15 minutes at 37 °C, afterwards the substrate was added and the velocities of AMC release were measured kinetically at $\lambda_{\text{ex}} = 380$ nm, $\lambda_{\text{em}} = 465$ nm for at least 10 minutes at 37 °C. The Infinite 200 (Tecan Group Ltd.) micro-titer plate reader and the Magellan software were used for measurement and data processing respectively.

Note

a slightly different protocol, involving a different FAP substrate (Ala-Pro-pNA), was used to determine the originally published FAP IC₅₀-value for reference UAMC1110 (3.2 +/- 0.4 nM). This accounts for the non-identical value published here.

PREP: IC₅₀ measurements of the inhibitors were carried out using *N*-succinyl-Gly-Pro-AMC (Bachem, Switzerland) as the substrate at a concentration of 250 μ M at pH 7.4 (0.1 M K-phosphate, 1 mM EDTA, 1 mM DTT). Eight concentrations of inhibitors were tested. The final DMSO concentration is kept

constant during the experiment to exclude any effects. Inhibitors were pre-incubated with the enzyme for 15 minutes at 37 °C, afterwards the substrate was added and the velocities of AMC release were measured kinetically at $\lambda_{\text{ex}} = 380 \text{ nm}$, $\lambda_{\text{em}} = 465 \text{ nm}$ for at least 10 minutes at 37 °C. The Infinite 200 (Tecan Group Ltd.) micro-titer plate reader and the Magellan software were used for measurement and data processing, respectively.

The data were fitted using a non-linear fit model in GraFit 7 software, according to the following equation:

$$y = \frac{\text{range}}{1 + \left(\frac{x}{IC_{50}}\right)^s}$$

where y is the value of the residual enzymatic activity compared to a non-inhibited sample, x is the final inhibitor concentration in the assay, s is the slope factor and the IC_{50} is the half maximal inhibitory concentration.

Synthesis Of DATA^{5m}.SA.FAPi

DATA^{5m}-en [1,4-Di(tert-butylacetate)-6-((5-(2-((tert-butoxy-carbonyl)aminoethyl)amino)-5-oxopentyl)-6-(amino(methyl)-tert-butylacetate)-perhydro-1,4-diazepane] (6)

5 (100 mg; 0.18 mmol) was added to 1 mL dry MeCN, HATU (66.3 mg; 0.18 mmol), HOBt (70.9 mg; 0.53 mmol) and DIPEA (89.3 μL ; 0.53 mmol) were added and stirred for 1 h at room temperature. *N*-boc-ethylenediamine (56.1 mg; 0.35 mmol) was added to the solution and stirred overnight. After completion of the reaction, the solution was concentrated under vacuum and the residue was purified by column chromatography ($\text{CHCl}_3/\text{MeOH}$, 20:1, $R_f = 0.23$). The product was obtained as yellow oil (114 mg; 0.16 mmol; 91%). ¹H-NMR (DMSO, 400 MHz, δ [ppm]): 3.36 (s, 2 H); 3.23 (s, 4 H); 3.07–3.01 (m, 2 H); 2.97–2.91 (m, 2 H); 2.79 (d, $J = 13.7 \text{ Hz}$, 2 H); 2.72–2.67 (m, 2 H); 2.59–2.54 (m, 2 H); 2.51 (d, $J = 13.7 \text{ Hz}$, 2 H); 2.17 (s, 3 H); 2.03 (t, 2 H); 1.45–1.41 (m, 4 H); 1.40 (s, 18 H); 1.39 (s, 9 H); 1.37 (s, 9 H); 1.22–1.18 (m, 2 H). ¹³C-NMR (CDCl_3 , 100 MHz, δ [ppm]): 172.25 (s); 171.72 (s); 170.28 (s); 169.58 (s); 155.62 (s); 80.19 (s); 80.08 (s); 77.63 (s); 62.37 (s); 61.87 (s); 61.73 (s); 58.72 (s); 56.06 (s); 51.50 (s); 37.10 (s); 35.55 (s); 28.24 (s); 27.87 (s); 27.77 (s); 26.11 (s); 25.50 (s); 21.55 (s). MS (ESI⁺): m/z (%): 714.4 (M + H⁺); 736.5 (M + Na⁺); calculated for $\text{C}_{36}\text{H}_{67}\text{N}_5\text{O}_9$: 713.49

DATA^{5m}.SA [1,4-Di(acetate)-6-((5-(2-((2-ethoxy-3,4-dioxo-cyclobut-1-en-1-yl)aminoethyl)amino)-5-oxopentyl)-6-(amino(methyl)-acetate)-perhydro-1,4-diazepane] (7)

6 (100 mg; 0.14 mmol) was dissolved in DCM/TFA (1:1; vol %) and stirred for 3 hours. After complete deprotection of the *tert*-butyl groups, the solution was concentrated under vacuum and 3 mL 0.5 M phosphate buffer pH 7 was added to the residue. After adding 3,4-diethoxycyclobut-3-ene-1,2-dione (61.7 μL ; 0.42 mmol) to the solution, the pH was adjusted again to pH 7 with 1 M NaOH and stirred

overnight at room temperature. After completion, the reaction solution was purified by HPLC (Phenomenex® Luna® 10 µm C18(2) 100 Å) with a linear gradient condition of 8–12% MeCN (+ 0,1% TFA)/92 – 88% Water (+ 0,1% TFA) in 20 min with a 5 mL/min flow. After lyophilization the product was obtained as white powder (24.8 mg; 43.6 µmol, 31%). ¹H-NMR (D₂O, 600 MHz, δ [ppm]): 4.73–4.66 (m, 2 H); 3.79 (s, 2 H); 3.70 (s, 4 H), 3.67–3.47 (m, 6 H); 3.39–3.22 (m, 6 H); 2.98 (d, J = 8.7 Hz, 3 H); 2.22 (t, 2 H); 1.71–1.68 (m, 2 H); 1.53–1.48 (m, 2 H); 1.43–1.38 (m, 2 H); 1.35–1.29 (m, 2 H). ¹³C-NMR (D₂O, 150 MHz, δ [ppm]): 188.70 (s); 183.25 (s); 177.21 (s); 176.42 (s); 173.82 (s); 170.00 (s); 117.19 (s); 115.26 (s); 70.66 (s); 68.77 (s); 54.14 (s); 43.89 (s); 39.22 (s); 37.76 (s); 35.09 (s); 29.53 (s); 25.69 (s); 25.54 (s); 22.09 (s); 15.03 (s); 14.94 (s). MS (ESI⁺): m/z (%): 570.3 (M + H⁺); 593.3 (M + Na⁺); calculated for C₂₅H₃₉N₅O₁₀: 569.27

DATA^{5m}.SA.FAPi (8) [(S)-2,2'-(6-((carboxymethyl)(methyl)amino)-6-(5-((2-((4-((4-((2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)ethyl)amino)-5-oxopentyl)-1,4-diazepane-1,4-diyl)diacetic acid]

DATA^{5m}.SA **7** (8.7 mg, 15.3 µmol) and NH₂-FAPi **3** (19.8 mg, 45.9 µmol) were reacted to form DATA^{5m}.SA.FAPi **8** via amidation at pH 9 in 500 µL 0.5 M Na₂HPO₄ phosphate buffer at room temperature stirred overnight. DATA^{5m}.SA.FAPi was isolated via HPLC purification (Phenomenex® Luna® 10 µm C18(2) 100 Å) with a linear gradient condition of 18–20% MeCN (+ 0,1% TFA)/82 – 80% Water (+ 0,1% TFA) in 20 min. The product was obtained as yellowish powder (6.2 mg, 6.5 µmol; 42%). MS (ESI⁺): m/z (%): 955.4 (M + H⁺); calculated for C₄₄H₅₆F₂N₁₀O₁₂: 954.40

^{nat}Ga/ ^{nat}Lu-complexes of DOTA.SA.FAPi

The ^{nat}Ga-metallated species [^{nat}Ga]Ga-DOTA.SA.FAPi was obtained after treatment of DOTA.SA.FAPi (5.2 mg; 5.4 µmol) with stoichiometric amount (1 eq) of 10 mM ^{nat}Ga(NO₃)₂ in 1 mL 0.2 M AmAc buffer pH 4.5 shaken for 3 h at 80 °C. Complexation was confirmed by ESI-MS and HPLC-purification was performed (Phenomenex® Synergi® 10 µm (C18) 100 Å (250 mm ⊆ 10 mm, 10 µm), linear gradient of 5–95% MeCN (+ 0,1% TFA)/95 – 5% Water (+ 0,1% TFA) in 10 min. The product was obtained as yellowish powder (4.6 mg, 4.5 µmol; 83%). MS (ESI⁺): m/z (%): 1022.2 (M + H⁺), 1044.2 (M + Na); calculated for C₄₃H₅₃F₂GaN₁₁O₁₂: 1021.30

The ^{nat}Lu-metallated species [^{nat}Lu]Lu-DOTA.SA.FAPi was obtained after treatment of DOTA.SA.FAPi (6.0 mg; 6.3 µmol) with stoichiometric amount (1 eq) of 1 mM ^{nat}LuCl₃ in 1 mL 0.2 M AmAc buffer pH 4.5 shaken for 3 h at 80 °C. Complexation was confirmed by ESI-MS and HPLC purification was done analogously to the gallium species. The product was obtained as yellowish powder (5.5 mg, 4.9 µmol; 77%). MS (ESI⁺): m/z (%): 1028.3 (M + H⁺), 1051.2 (M + Na); calculated for C₄₃H₅₂F₂LuN₁₁O₁₂: 1027.32

^{nat}Ga-complexes of DATA^{5m}.SA.FAPi

The ^{nat}Ga -metallated species $[^{nat}\text{Ga}]\text{Ga-DATA}^{5m}\text{.SA.FAPi}$ was obtained after treatment of $\text{DATA}^{5m}\text{.SA.FAPi}$ (7.2 mg; 7.5 μmol) with stoichiometric amount of $^{nat}\text{Ga}(\text{NO}_3)_2$ in 1 mL 0.2 M AmAc buffer pH 4.5 shaken for 2 h at 25 °C. Complexation was confirmed by ESI-MS and HPLC-purification was performed (Phenomenex® Luna® 10 μm (C18) 100 Å (250 mm $\times$ 10 mm, 10 μm), linear gradient of 5–95% MeCN (+ 0,1% TFA)/95 – 5% Water (+ 0,1% TFA) in 10 min. The product was obtained as yellowish powder (4.4 mg, 4.3 μmol ; 57%). MS (ESI⁺): m/z (%): 1021.3 (M + H⁺), 1043.2 (M + Na); calculated for $\text{C}_{44}\text{H}_{53}\text{F}_2\text{GaN}_{10}\text{O}_{12}$: 1020.31

Inhibitory potency determination

Enzymes: A gateway-entry clone for human FAP was purchased from Dharmacon (Accession number DQ891423) and the human secretion signal was replaced with the HoneyBee mellitin secretion signal. For transfection and expression of FAP in Sf9 insect cells, the C-terminal BaculoDirect kit from LifeTechnologies was used. The enzyme was purified from the supernatant of the insect cells using immobilized Ni-chelating chromatography (GE healthcare, Diegem, Belgium), followed by anion-exchange chromatography using a 1 mL HiTrap Q (GE healthcare, Diegem, Belgium). Human recombinant PREP was expressed in BL21(DE3) cells and purified using immobilized Co-chelating chromatography (GE healthcare) followed by anion-exchange chromatography on a 1 ml Mono Q column (GE healthcare).

FAP: IC₅₀ measurements of the inhibitors were carried out using Z-Gly-Pro-7-amino-4-methylcoumarine (AMC) (Bachem, Switzerland) as the substrate at a concentration of 50 μM at pH 8 (0.05 M Tris-HCl buffer with 0.1 % glycerol, 1 mg/mL BSA and 140 mM NaCl). Eight concentrations of inhibitors were tested. The final DMSO concentration was kept constant during the experiment to exclude any solvent effects. Inhibitors were pre-incubated with the enzyme for 15 minutes at 37 °C, afterwards the substrate was added and the velocities of AMC release were measured kinetically at $\lambda_{\text{ex}} = 380 \text{ nm}$, $\lambda_{\text{em}} = 465 \text{ nm}$ for at least 10 minutes at 37 °C. The Infinite 200 (Tecan Group Ltd.) micro-titer plate reader and the Magellan software were used for measurement and data processing respectively.

Note: a slightly different protocol, involving a different FAP substrate (Ala-Pro-pNA), was used to determine the originally published FAP IC₅₀-value for reference UAMC1110 (3.2 +/- 0.4 nM). This accounts for the non-identical value published here.

PREP: IC₅₀ measurements of the inhibitors were carried out using *N*-succinyl-Gly-Pro-AMC (Bachem, Switzerland) as the substrate at a concentration of 250 μM at pH 7.4 (0.1 M K-phosphate, 1 mM EDTA, 1 mM DTT). Eight concentrations of inhibitors were tested. The final DMSO concentration is kept constant during the experiment to exclude any effects. Inhibitors were pre-incubated with the enzyme for 15 minutes at 37 °C, afterwards the substrate was added and the velocities of AMC release were measured kinetically at $\lambda_{\text{ex}} = 380 \text{ nm}$, $\lambda_{\text{em}} = 465 \text{ nm}$ for at least 10 minutes at 37 °C. The Infinite 200 (Tecan Group Ltd.) micro-titer plate reader and the Magellan software were used for measurement and data processing, respectively.

The data were fitted using a non-linear fit model in GraFit 7 software, according to the following equation:

$$y = \frac{\text{range}}{1 + \left(\frac{x}{IC_{50}}\right)^s}$$

where y is the value of the residual enzymatic activity compared to a non-inhibited sample, x is the final inhibitor concentration in the assay, s is the slope factor and the IC_{50} is the half maximal inhibitory concentration.

Radiolabeling And Stability Studies With ^{68}Ga

^{68}Ga was eluted manually utilizing ethanol post-processed ^{68}Ga -eluate from a $^{68}\text{Ge}/^{68}\text{Ga}$ -generator (ITG Garching, Germany). ^{68}Ga was eluted with 5 mL 0.05 M HCl, purification was carried out with 1 mL 80% EtOH/ 0.15 M HCl and the Ga(III) elution from the column with 400 μL 90% EtOH/ 0.9 M HCl.[44] The pH of the ^{68}Ga -eluate ^{68}Ga was adjusted to pH 4.0 – 4.5 using 400 μL 1 M AmAc buffer.

Reaction controls for radiochemical purity were executed using radio-TLC (TLC Silica gel 60 F₂₅₄ Merck) with citrate buffer pH 4 and radio-HPLC using an analytical HPLC 7000 series Hitachi LaChrom with a Phenomenex (Aschaffenburg, Germany) Luna C18 column (250 $\times$ 4.6 mm, 5 μ), linear gradient of 5–95% MeCN (+ 0,1% TFA)/ 95 – 5% Water (+ 0,1% TFA) in 10 min). TLC's were measured in TLC imager CR-35 Bio Test-Imager from Duerr-ndt (Bietigheim-Bissingen, Germany) with the analysis software AIDA Elysia-Raytest (Straubenhardt, Germany). The citrate TLCs show free radio metal with a R_f value of 0.8–0.9. The labeled complexes are observed at a R_f value of 0.1–0.2.

^{68}Ga stability studies against transmetallation (Fe, Cu, Ca, Mg), transchelation (EDTA, DTPA) as well as in HS, ethanol and saline (0.9% isotone NaCl-solution) were performed. 50 μL of the [^{68}Ga]Ga-DOTA.SA.FAPI labeling solution with > 95% radiochemical purity were added to 1 mL of the respective media. The measured time points for ^{68}Ga were 15, 30, 45, 60, 90, 120 min. Human serum (human male AB plasma, USA origin) was bought from Sigma Aldrich, PBS was purchased from Sigma Aldrich and 0.9% saline from B. Braun Melsungen AG (Melsungen, Germany).

In vivo **animal studies** and ex vivo **biodistributions**

After quantitative radiolabeling of [^{68}Ga]Ga-DOTA.SA.FAPI (20 nmol), the solution was purified via C-18 column (Sep-Pak Light C18, Waters Corporation, Massachusetts, USA). Conditioning of the SPE was performed using 5 ml abs. ethanol and 5 mL water. Precursor solution was pressed over the SPE and then washed with 5 ml water. Afterwards, the ^{68}Ga -labeled precursor was eluted with 1 mL of 50 vol% ethanol.

Finally, the ethanol was evaporated and the tracer was reformulated in 5% ethanol in saline solution (500 µl total volume). The completed solution was used as injection solution.

In vivo tumor model: HT-29 (human colon adenocarcinoma, ATCC, Rockville, Maryland) cells were routinely cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat inactivated foetal bovine serum (FBS), 2 mM glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin (Gibco, Life technologies). After detaching the cells, the number of viable cells was counted with the automated Muse™ Cell Analyzer (Merck Millipore). For the HT-29 subcutaneous model, 10×10^6 viable cells, suspended in 100 µl PBS, were inoculated in the right hind leg of female 6-week-old CD1^{-/-} Foxn1nu mice (n = 3), obtained from Charles River Laboratories (L'Arbresle, France). The animals were kept under environmentally controlled conditions (12 h light/dark cycle, 20–24 °C and 40–70% relative humidity) with food and water ad libitum. When tumors reached an approximate volume of 400 mm³, 3 mice underwent µPET imaging. All experimental procedures and protocols were performed in accordance with European Directive 86/609/EEC Welfare and Treatment of Animals and were approved by the local ethical commission (2017-070, University of Antwerp, Belgium).

Micro-PET imaging: Micro-PET scans were carried out using an Inveon small-animal PET/CT scanner (Siemens), after i.v. injection of 4 nmol of [⁶⁸Ga]-DOTA.SA.FAPi (8.6 MBq) into tumor bearing mice (n = 3), under isoflurane anesthesia (5% for induction, 2% for maintenance). Static whole-body PET images were acquired 60 min after injection of the radiotracer. Following each PET acquisition, a whole-body CT scan was acquired to obtain the animal's anatomical information individually.

For quantitative analysis, PET data were reconstructed using 3-dimensional ordered subset expectation maximization (OSEM3D, 16 subsets and 2 iterations) and 18 maximum a posteriori (MAP) iterations including scatter and attenuation correction (matrix size, 128 × 128 × 159; voxel size, 0.776 × 0.776 × 0.776 mm;). Volumes of interest (VOIs) were manually drawn on the PET/CT images using PMOD (version 3.6; PMOD Technologies) to delineate the tumor, heart and muscle.

Ex vivo biodistribution: Immediately after the CT scans, the animals were sacrificed, the blood, tissues and organs were collected, weighed and the radioactivity was measured using an automatic γ-counter (Wizard² 2480, PerkinElmer). Values were expressed as percentage of the injected dose per gram (%ID/g).

List Of Abbreviations

DATA: 2,2'-(6-((carboxymethyl)amino)-1,4-diazepane-1,4-diyl)diacetic acid); DATA^{5m}-3^tBu: 5-[1,4-bis tertbutoxycarbonylmethyl-6-(tert-butoxycarbonylmethyl-methyl-amino)-[1, 4]diazepan-6-yl]-pentanoic acid; DOTA: 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA: cyclohexyldiethylene-triaminepentaacetic acid; EDTA: ethylenediaminetetraacetic acid; FAP: fibroblast activation protein; PREP: prolyl endopeptidase; DPP: dipeptidyl peptidase; CAF: cancer associated fibroblast; IC₅₀: Half-maximal inhibitory concentration; MIP: maximum intensity projection; PET: positron emission tomography; CT: computed tomography; Boc: *tert*-Butyloxycarbonyl; ^tBu: *tert*-Butyl; ESI: electrospray ionization; HS: human

serum; HPLC: high-performance liquid chromatography; LC: liquid chromatography; MS: mass spectrometry; AmAc: ammonium acetate; NaAc: sodium acetate; NMR: nuclear magnetic resonance; PBS: phosphate buffered saline; RCY: radiochemical yield; RT: room temperature; SA: squaric acid; SADE: squaric acid diethyl ester; TLC: thin layer chromatography; AMC: Z-Gly-Pro-7-amino-4-methylcoumarine; FBS: foetal bovine serum; p.i.: post injection.

Declarations

Ethics approval and consent to participate: All experimental procedures and protocols were performed in accordance with European Directive 86/609/EEC Welfare and Treatment of Animals and were approved by the local ethical commission (2017-070, University of Antwerp, Belgium).

Consent for publication: Not applicable.

Availability of data and materials: Data sharing is not applicable to this article as no datasets were generated. Please contact authors for data request.

Competing interests: The authors declare that they have no competing interests.

Funding: No specific funding was received.

Authors contribution: EM carried out the preparative organic and radiochemical synthesis and evaluations, chemical separations, and analytics. Additionally, EM wrote the manuscript. GV carried out the *in vitro* inhibitory studies and AB is responsible for the expression of FAP and PREP. FE, SDL and EM carried out the labeling for *in vivo* studies and FE was responsible for analysis of the *in vivo* and *ex vivo* data. CV was responsible for the tumor model and has carried out the animal handling and SDB was also involved in the organ/tumor manipulation during the biodistribution determination. EE helped with radiochemical evaluation and LG, BK with organic synthesis. SS, IDM and PVV directed the *in vitro* inhibitory and *in vivo/ex vivo* studies in Antwerp. FR supervised the whole project. All authors read and approved the manuscript.

Acknowledgements: The authors thank the employees and technicians of the nuclear medicine (UZA) and the animal PET-team of the Molecular Imaging Center Antwerp (MICA). An Bracke is supported by the GOA BOF 2015 grant (No. 30729) of the University of Antwerp. Especially thanks to Philippe Joye (MICA) for the *in vivo* measurements, Jonathan Dewulf and Lucas Beroske (UZA) for helping with ^{68}Ga -elution for the *in vivo* studies.

References

1. Brennen WN, Isaacs JT, Denmeade SR. Rationale behind targeting fibroblast activation protein-expressing carcinoma-associated fibroblasts as a novel chemotherapeutic strategy. *Mol Cancer Ther.* 2012;11:257–66.

2. Dvořáková P, Bušek P, Knedlík T, Schimer J, Etrych T, Kostka L, et al. Inhibitor-Decorated Polymer Conjugates Targeting Fibroblast Activation Protein. *J Med Chem*. 2017;60:8385–93.
3. Aertgeerts K, Levin I, Shi L, Snell GP, Jennings A, Prasad GS, et al. Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein α . *J Biol Chem*. 2005;280:19441–4.
4. Levy MT, McCaughan GW, Abbott CA, Park JE, Cunningham AM, Muller E, et al. Fibroblast activation protein: A cell surface dipeptidyl peptidase and gelatinase expressed by stellate cells at the tissue remodelling interface in human cirrhosis. *Hepatology*. 1999;29:1768–78.
5. Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J Biol Chem*. 1999;274:36505–12.
6. Edosada CY, Quan C, Wiesmann C, Tran T, Sutherlin D, Reynolds M, et al. Selective inhibition of fibroblast activation protein protease based on dipeptide substrate specificity. *J Biol Chem*. 2006;281:7437–44.
7. 10.1016/j.cca.2019.04.063
Bracke A, Van Elzen R, Van Der Veken P, Augustyns K, De Meester I, Lambeir AM. The development and validation of a combined kinetic fluorometric activity assay for fibroblast activation protein alpha and prolyl oligopeptidase in plasma. *Clin Chim Acta* [Internet]. Elsevier; 2019;495:154–60. Available from: <https://doi.org/10.1016/j.cca.2019.04.063>.
8. Hamson EJ, Keane FM, Tholen S, Schilling O, Gorrell MD. Understanding fibroblast activation protein (FAP): Substrates, activities, expression and targeting for cancer therapy. *Proteomics - Clin Appl*. 2014;8:454–63.
9. Liu R, Li H, Liu L, Yu J, Ren X. Fibroblast activation protein: A potential therapeutic target in cancer. *Cancer Biol Ther*. 2012;13:123–9.
10. 10.1038/s41573-018-0004-1
Chen X, Song E. Turning foes to friends: targeting cancer-associated fibroblasts. *Nat Rev Drug Discov* [Internet]. Springer US; 2019;18:99–115. Available from: <http://dx.doi.org/10.1038/s41573-018-0004-1>.
11. Jiang G-M, Xu W, Du J, Zhang K-S, Zhang Q-G, Wang X-W, et al. The application of the fibroblast activation protein alpha-targeted immunotherapy strategy. *Oncotarget* [Internet]. 2016;7. Available from: <http://www.oncotarget.com/fulltext/8098>.
12. Liu T, Zhou L, Li D, Andl T, Zhang Y. Cancer-associated fibroblasts build and secure the tumor microenvironment. *Front Cell Dev Biol*. 2019;7:1–14.
13. Zi F, He J, He D, Li Y, Yang L, Cai Z. Fibroblast activation protein α in tumor microenvironment: Recent progression and implications (Review). *Mol Med Rep*. 2015;11:3203–11.
14. De Vlieghere E, Verset L, Demetter P, Bracke M, De Wever O. Cancer-associated fibroblasts as target and tool in cancer therapeutics and diagnostics. *Virchows Arch*. 2015;467:367–82.
15. Tao L, Huang G, Song H, Chen Y, Chen L. Cancer associated fibroblasts: An essential role in the tumor microenvironment (review). *Oncol Lett*. 2017;14:2611–20.

16. Busek P, Mateu R, Zubal M, Kotackova L, Sedo A. Targeting Fibroblast activation protein in cancer - Prospects and caveats. *Front Biosci - Landmark*. 2018;23:1933–68.
17. Scanlan MJ, Raj BKM, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, Healey JH, et al. Molecular cloning of fibroblast activation protein α , a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. *Proc Natl Acad Sci U S A*. 1994;91:5657–61.
18. Poplawski SE, Lai JH, Li Y, Jin Z, Liu Y, Wu W, et al. Identification of selective and potent inhibitors of fibroblast activation protein and prolyl oligopeptidase. *J Med Chem*. 2013;56:3467–77.
19. Connolly BA, Sanford DG, Chiluwal AK, Healey SE, Peters DE, Dimare MT, et al. Dipeptide boronic acid inhibitors of dipeptidyl peptidase IV: Determinants of potency and in vivo efficacy and safety. *J Med Chem*. 2008;51:6005–13.
20. Jansen K, De Winter H, Heirbaut L, Cheng JD, Joossens J, Lambeir AM, et al. Selective inhibitors of fibroblast activation protein (FAP) with a xanthine scaffold. *Medchemcomm*. 2014;5:1700–7.
21. Jansen K, Heirbaut L, Verkerk R, Cheng JD, Joossens J, Cos P, et al. Extended structure-activity relationship and pharmacokinetic investigation of (4-quinolinoyl)glycyl-2-cyanopyrrolidine inhibitors of fibroblast activation protein (FAP). *J Med Chem*. 2014;57:3053–74.
22. De Decker A, Vliegen G, Van Rompaey D, Peeraer A, Bracke A, Verckist L, et al. Novel Small Molecule-Derived, Highly Selective Substrates for Fibroblast Activation Protein (FAP). *ACS Med Chem Lett*. 2019;10:1173–9.
23. Yang X, Nimmagadda S, Rowe S, Slania S, Pomper MG. Imaging and Radiotherapeutics Agents Targeting Fibroblast-Activation Protein-Alpha (FAP-Alpha). Patent. 2019.
24. Loktev A, Lindner T, Mier W, Debus J, Altmann A, Jaeger D, et al. A Tumor-Imaging Method Targeting Cancer-Associated Fibroblasts. *J Nucl Med*. 2018;59:1423–9.
25. Lindner T, Loktev A, Altmann A, Giesel F, Kratochwil C, Debus J, et al. Development of Quinoline-Based Theranostic Ligands for the Targeting of Fibroblast Activation Protein. *J Nucl Med*. 2018;59:1415–22.
26. Loktev A, Lindner T, Burger EM, Altmann A, Giesel F, Kratochwil C, et al. Development of fibroblast activation protein-targeted radiotracers with improved tumor retention. *J Nucl Med*. 2019;60:1421–9.
27. Giesel FL, Kratochwil C, Lindner T, Marschalek MM, Loktev A, Lehnert W, et al. ^{68}Ga -FAPI PET/CT: Biodistribution and preliminary dosimetry estimate of 2 DOTA-containing FAP-targeting agents in patients with various cancers. *J Nucl Med*. 2019;60:386–92.
28. Giesel FL, Heussel CP, Lindner T, Röhrich M, Rathke H, Kauczor HU, et al. FAPI-PET/CT improves staging in a lung cancer patient with cerebral metastasis. *Eur J Nucl Med Mol Imaging European Journal of Nuclear Medicine Molecular Imaging*. 2019;46:1754–5.
29. Kratochwil C, Flechsig P, Lindner T, Abderrahim L, Altmann A, Mier W, et al. ^{68}Ga -FAPI PET/CT: Tracer uptake in 28 different kinds of cancer. *J Nucl Med*. 2019;60:801–5.
30. Wurm FR, Klok HA. Be squared: Expanding the horizon of squaric acid-mediated conjugations. *Chem Soc Rev*. 2013;42:8220–36.

31. Ian Storer R, Aciro C, Jones LH. Squaramides. Physical properties, synthesis and applications. *Chem Soc Rev.* 2011;40:2330–46.
32. Tietze LF, Arlt M, Beller M, Glüsenkamp K -H, Jähde E, Rajewsky MF. Squaric Acid Diethyl Ester: A New Coupling Reagent for the Formation of Drug Biopolymer Conjugates. Synthesis of Squaric Acid Ester Amides and Diamides. *Chem Ber.* 1991;124:1215–21.
33. Yoganathan S, Sit CS, Vederas JC. Chemical synthesis and biological evaluation of gallidermin-siderophore conjugates. *Org Biomol Chem.* 2011;9:2133–41.
34. Rudd SE, Roselt P, Cullinane C, Hicks RJ, Donnelly PS. A desferrioxamine B squaramide ester for the incorporation of zirconium-89 into antibodies. *Chem Commun.* 2016;52:11889–92.
35. Greifenstein L, Grus T, Nagel J, Sinnes JP, Rösch F. Synthesis and labeling of a squaric acid containing PSMA-inhibitor coupled to AAZTA⁵ for versatile labeling with ⁴⁴Sc, ⁶⁴Cu, ⁶⁸Ga and ¹⁷⁷Lu. *Appl Radiat Isot.* 2019;108867.
36. Greifenstein L, Engelbogen N, Lahnif H, Sinnes J-P, Bergmann R, Bachmann M, et al. Synthesis, labeling and preclinical evaluation of a squaric acid containing PSMA-inhibitor labeled with ⁶⁸Ga – a comparison with PSMA-11 and PSMA-617. *ChemMedChem.* 2020;1–11.
37. Price EW, Orvig C. Matching chelators to radiometals for radiopharmaceuticals. *Chem Soc Rev.* 2014;43:260–90.
38. Seemann J, Waldron B, Parker D, Roesch F. DATATOC: a novel conjugate for kit-type ⁶⁸Ga labelling of TOC at ambient temperature. *EJNMMI Radiopharm Chem.* 2017;1:4.
39. Seemann J, Waldron BP, Roesch F, Parker D. Approaching “kit-type” labelling with ⁶⁸Ga: The DATA chelators. *ChemMedChem.* 2015;10:1019–26.
40. Sinnes JP, Nagel J, Waldron BP, Maina T, Nock BA, Bergmann RK, et al. Instant kit preparation of ⁶⁸Ga-radiopharmaceuticals via the hybrid chelator DATA: clinical translation of [⁶⁸Ga]Ga-DATA-TOC. *EJNMMI Res.* 2019;9.
41. Quiñonero D, Frontera A, Ballester P, Deyà PM. A theoretical study of aromaticity in squaramide and oxocarbons. *Tetrahedron Lett.* 2000;41:2001–5.
42. Henry LR, Lee HO, Lee JS, Klein-Szanto A, Watts P, Ross EA, et al. Clinical implications of fibroblast activation protein in patients with colon cancer. *Clin Cancer Res.* 2007;13:1736–41.
43. Cheng JD, Dunbrack RL, Valianou M, Rogatko A, Alpaugh RK, Weiner LM. Promotion of tumor growth by murine fibroblast activation protein, a serine protease, in an animal model. *Cancer Res.* 2002;62:4767–72.
44. Eppard E, Wuttke M, Nicodemus PL, Rošch F. Ethanol-based post-processing of generator-derived ⁶⁸Ga Toward kit-type preparation of ⁶⁸Ga-radiopharmaceuticals. *J Nucl Med.* 2014;55:1023–8.

Figures

UAMC1110

Figure 1

FAP-inhibitor lead structure UAMC 1110

Figure 2

Chelator-linker conjugates coupled with UAMC1110. A) XY-FAP-02; B) FAPI-02; C) FAPI-04; D) FAPI-21; E) FAPI-46; F) DOTA.SA.FAPi; G) DATA5m.SA.FAPi.

Figure 3

Synthesis scheme of DOTA.SA.FAPi 4: (a) 80 % TFA in DCM; (b) 3,4-diethoxycyclobut-3-ene-1,2-dione, Phosphate-buffer pH 7, 1 M NaOH; (c) Phosphate-buffer pH 9, 1 M NaOH.

Figure 4

Synthesis scheme of DATA5m.SA.FAPi 8: (a) N-Boc-ethylenediamine, HATU, HOBt, DIPEA, MeCN; (b) (i) 80 % TFA in DCM; (ii) 3,4-diethoxycyclobut-3-ene-1,2-dione, phosphate buffer pH 7, 1 M NaOH; (c) 3, phosphate-buffer pH 9, 1 M NaOH.

Figure 5

Radiolabeling kinetics for various amounts of [68Ga]Ga-DOTA.SA.FAPi complex at 95 °C, precursor amounts >15 nmol result in RCY >97 % after 15 min.

Figure 6

Radiolabeling kinetics of various amounts of [68Ga]Ga-DATA5m.SA.FAPi complex at RT, precursor amounts >15 nmol result in RCY >98 % after 15 min

Mouse 1

Mouse 2

Mouse 3

Min Max

Figure 7

In vivo evaluation of $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$ uptake in a HT-29 xenograft mouse model. Representative coronal small-animal PET/CT image (MIP) 60 min after injection of $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$.

Figure 8

a) Ex vivo biodistribution of [68Ga]Ga-DOTA.SA.FAPi in HT-29 xenograft mice (n=3) 1 h p.i. b) Tumor-to-organ ratios of [68Ga]Ga-DOTA.SA.FAPi in HT-29 xenograft mice (n=3) 1 h p.i.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supportinginformationFAPimanuskript.docx](#)