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ORIGINAL ARTICLE

Preclinical evaluation and dosimetric assessment of [¹⁷⁷Lu]Lu-FAP-2286 as a targeted therapy for FAP-expressing tumors

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ABSTRACT

Introduction: Fibroblast activation protein (FAP), predominantly expressed in the tumor microenvironment, represents a promising target for targeted radionuclide therapy (TRT). This study evaluates [¹⁷⁷Lu]Lu-FAP-2286 as a FAP-targeted radiopharmaceutical for FAP-expressing tumors.

Methods: [¹⁷⁷Lu]Lu-FAP-2286 was radiolabeled, and its radiochemical purity (RCP) was assessed by RTLC and HPLC. Stability was evaluated in PBS and human serum. Binding affinity, cellular internalization, and cytotoxicity were assessed in HEK FAP+ cells. Biodistribution was performed in normal and tumor-bearing mice. Organ absorbed doses were estimated by extrapolating murine biodistribution data using the MIRD formalism and OLINDA/EXM.

Results: RCP exceeded 99% under optimal conditions. The radiotracer exhibited high affinity for FAP+ cells (K_d = 0.45 nM) with >38% internalization at 6 h. MTT assays demonstrated significant cytotoxicity, reducing FAP+ cell viability to 16–38% at the highest concentration. Biodistribution revealed rapid blood clearance, marked tumor uptake, and low background activity. Dosimetric calculations identified the kidneys as the dose-limiting organ (0.27 mGy/MBq), with substantially lower doses to other organs.

Conclusion: [¹⁷⁷Lu]Lu-FAP-2286 demonstrated high RCP, strong FAP-binding affinity, favorable biodistribution with significant tumor uptake, and a promising dosimetric profile. These preliminary findings support further investigation of this radiopharmaceutical as a candidate for TRT of FAP-positive tumors; however, comprehensive in vivo therapeutic studies and subsequent clinical trials are warranted.

Keywords: Biodistribution studies; Preclinical evaluation; Dosimetry; FAP-2286; ¹⁷⁷Lu

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INTRODUCTION

Cancer remains one of the leading causes of morbidity and mortality worldwide, contributing to 14.57% of all deaths and 8.8% of total disability-adjusted life years (DALYs) in 2021 [1]. Cancer contributes substantially to global mortality and DALYs. Early detection and timely treatment can significantly improve survival and quality of life by limiting disease progression, metastasis, and resistance to conventional therapies [2]. Despite advancements in cancer treatments, many cancers remain difficult to treat, particularly those at advanced stages. In this context, targeted therapies utilizing radiopharmaceuticals offer significant advantages over traditional therapies. By targeting specific molecular markers expressed on tumor cells, these agents can deliver high doses of radiation directly to cancerous cells, minimizing damage to surrounding healthy tissues [3, 4].

In recent years, FAP has been introduced as an ideal target for diagnostic and therapeutic applications in oncology, with its expression largely restricted to cancerous tissues [5]. FAP is a membrane-bound protease predominantly expressed in the TME, particularly in cancer-associated fibroblasts (CAFs) of several tumor types [6]. The development of small-molecule FAP inhibitors (FAPIs) has gained significant attention for their potential in both imaging and treatment of various cancers, since 2018 [7, 8]. While most FAP tracers are still under investigation, the initial results are encouraging. Previous studies have demonstrated the ability of FAPI-based radiopharmaceuticals, such as [^{68}Ga]Ga-FAPI, to identify tumor tissues more effectively compared to other radiotracers like [^{18}F]FDG PET/CT [9]. Additionally, numerous studies indicate that FAP tracers have the potential to deliver significant therapeutic efficacy while maintaining manageable side effects [9]. To date, several FAP-targeting tracers, such as FAPI-04, FAPI-46, FAP-2286, and DATAGA, have been investigated for diagnostic and therapeutic applications. (SA.FAPi)₂, have been studied for use in TRT, with distinct characteristics and potential advantages [10].

Recent studies have identified FAP-2286 as a promising FAP-targeting tracer because of its high tumor retention and strong binding affinity, highlighting its potential for theranostic applications in cancers including pancreatic, breast, colorectal, and sarcoma. Lately, FAP-2286 has shown remarkable efficacy when labeled with [^{68}Ga]Ga for imaging and [^{177}Lu]Lu for TRT [11]. Additionally, clinical trials utilizing [^{177}Lu]Lu-FAP-2286 have reported effective targeting of FAP-expressing tumors, supporting its potential as a targeted therapy for refractory cancers [12, 13].

The present study aimed to radiolabel FAP-2286 with [^{177}Lu]LuCl₃ and to conduct a comprehensive preclinical evaluation of its potential as a FAP-targeted radiopharmaceutical. Radiochemical purity and stability were evaluated, and in vitro assays were performed to assess receptor binding, cellular internalization, and cytotoxic effects. Biodistribution studies were carried out in normal and tumor-bearing mice to investigate tissue uptake, tumor accumulation, and clearance kinetics. Furthermore, human organ dosimetry was estimated by extrapolating murine biodistribution data according to the MIRD formalism. While previous reports have provided absorbed dose estimates for selected critical organs such as the kidneys and red marrow, organ-level dosimetric information for [^{177}Lu]Lu-FAP-2286 remains incomplete. In this context, the present study expands the available dosimetric dataset by providing preliminary absorbed dose estimates for a broader range of organs, offering a more comprehensive preclinical perspective on the normal-organ radiation burden of [^{177}Lu]Lu-FAP-2286.

METHODS

[^{177}Lu]LuCl₃ and FAP-2286 were obtained from Pars Isotope Co. (Tehran, Iran) and MedChemExpress LLC (USA), respectively. Other chemical reagents were obtained from Aldrich

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Chemical Company (Germany). RCP was determined using Whatman No. 2 paper (Whatman, UK) and analyzed with a Bioscan AR2000 scanner (Paris, France). Sample activity was measured with a p-type coaxial high-purity germanium (HPGe) detector (model: EGPC 80-200R) connected to a multichannel analyzer system, along with an ISOMED 1010 dose calibrator (Dresden, Germany). HPLC analysis was carried out on a ZORBAX C-18 column (250 × 4.6 mm) coupled to a JASCO 880-PU intelligent pump and monitored using a multiwavelength UV detector and a flow-through Raytest-Gabi γ detector (Ohio, USA). Fetal Bovine Serum (FBS) and RPMI 1640 Medium were purchased from GIBCO Co. (UK). HEK FAP⁺ cell lines and Syrian male rats (180-220 g) were acquired from the Pasteur Institute (Tehran, Iran). The nude mice (4–8-week-old, 20-25 g) were provided by Royan Institute (Tehran, Iran). All animal studies were conducted in compliance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, second edition. Data were analyzed using the Student's T-test, with values presented as mean $\pm$ standard deviation. A P-value of less than 0.05 was considered statistically significant.

Preparation and quality control of [¹⁷⁷Lu]Lu-FAP-2286

740 MBq of [¹⁷⁷Lu]LuCl₃ was moved to a 10-mL borosilicate reaction vial including 2 mL of sodium acetate buffer (pH=4.5) and different amounts of FAP-2286 solution (20-50 μ g). The vial was sealed and heated to 80-100 °C for 30-60 min. The final product was sterilized by filtration through a 0.22- μ m filter, and the pH was adjusted to 5.5. RCP was assessed using both RTLC and HPLC. For RTLC analysis, Whatman No. 2 paper was used as the stationary phase, and a methanol: saline (5:1) mixture was employed as the mobile phase. HPLC was carried out using the two different solvent systems (A = Ultrapure water + 1% TFA, B = Acetonitrile). The column was washed with a flow rate of 2.6 mL/min for 15 min in line with the following gradient: 100% A: 0% B for 0-3 min, 50% A: 50% B for 3-10 min, 0% A: 100% B for 10-15 min.

In-vitro stability studies of [¹⁷⁷Lu]Lu-FAP-2286

The stability of [¹⁷⁷Lu]Lu-FAP-2286 was assessed in both PBS buffer and human serum. A 100 μ L (37 MBq) sample of [¹⁷⁷Lu]Lu-FAP-2286 was mixed with 500 μ L of buffer or human serum, and incubated at 4°C and 37°C for 48 h, respectively. RCP was then evaluated at 4, 24, and 48 h intervals using the RTLC method.

Binding studies

To evaluate the binding specificity and affinity of [¹⁷⁷Lu]Lu-FAP-2286, HEK FAP⁺ cells (FAP-expressing, target-positive) and CHO FAP⁻ cells (FAP-negative control) were used. Approximately $0.8\text{--}1.0 \times 10^6$ cells were seeded into each well of 6-well plates and cultured overnight to allow adequate cell attachment. Before the assay, the culture medium was replaced with fresh medium, and the plates were pre-incubated on ice for 30 min to minimize ligand internalization and restrict radioligand binding predominantly to the cell surface. For evaluation of binding specificity, HEK FAP⁺ cells were incubated with [¹⁷⁷Lu]Lu-FAP-2286 for 120 min at 4 °C either in the absence of unlabeled ligand (total binding) or in the presence of an excess of unlabeled FAP-2286 (10 μ M) to determine non-specific binding. In parallel, CHO FAP⁻ cells were incubated with the radioligand under identical conditions as a negative control to confirm FAP-specific binding. Following incubation, the cells were washed three times with ice-cold PBS to remove unbound radioligand, lysed with 0.1 M NaOH, and the cell-associated radioactivity was measured using a gamma counter. Specific binding was calculated by subtracting non-specific

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binding from total binding. Binding affinity was determined by saturation binding analysis using HEK FAP⁺ cells incubated with increasing concentrations of [¹⁷⁷Lu]Lu-FAP-2286 (0.1–100 nM). This concentration range was selected to encompass both sub-saturating and saturating binding conditions, enabling reliable estimation of receptor binding parameters. Cells were incubated for 120 min at 4 °C to achieve equilibrium binding while minimizing receptor-mediated internalization. At each radioligand concentration, non-specific binding was determined in the presence of 10 μM unlabeled FAP-2286. Washing, cell lysis, and radioactivity measurements were performed as described above. The equilibrium dissociation constant (K_d) was determined by nonlinear regression using a one-site specific binding model in GraphPad Prism. All experiments were performed in triplicate (n = 3).

Internalization studies

To study the internalization of [¹⁷⁷Lu]Lu-FAP-2286, HEK FAP⁺ cell lines were utilized. Approximately 10⁵ cells were seeded into individual wells of 24-well plates and incubated for 24 h in a CO₂ incubator. After this incubation, the culture medium was discarded, and the cells were washed and re-incubated with fresh culture medium for 1 h at 37°C. Following this, 0.25 picomoles of radioligand were added to each well and incubated for 30, 60, 120, 240, and 360 min at 37°C and 5% CO₂ (n=3).

At each time point, the internalization process was halted by removing the medium and washing the cells twice with ice-cold PBS. To remove radioligand bound to the cell surface, a washing step with 0.1 M glycine buffer (pH = 2.8) was performed for 5 min on ice, which disrupted the interaction between [¹⁷⁷Lu]Lu-FAP-2286 and the receptor. The radioactivity of this wash solution was considered as the amount of [¹⁷⁷Lu]Lu-FAP-2286 bound to the cell surface and released from the cells. Finally, 1 N NaOH was added to the cells. The radioactivity in the culture medium, radioligand bound to the cell surface, and the internalized fraction were then measured using a gamma counter.

In vitro cytotoxicity studies

The cytotoxicity of [¹⁷⁷Lu]Lu-FAP-2286 was evaluated using the MTT assay. Briefly, the MTT reagent was prepared by dissolving 50 mg of MTT powder in 10 mL of serum-free culture medium, sterilized through a 0.22 μm membrane filter, aliquoted into 1 mL portions, and stored at -20°C until use. HEK-FAP⁺ cells were seeded in 24-well plates at a density of 1 × 10⁵ cells per well and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then exposed to [¹⁷⁷Lu]Lu-FAP-2286 at final concentrations ranging from 0.1 to 1000 nM. Based on the molar activity of the radiolabeled compound and the final treatment volume used in each well, this concentration range corresponded to approximately 0.363 to 363 kBq/well. To evaluate non-radioactive ligand-related effects, non-radiolabeled FAP-2286 (cold FAP-2286) was tested at the same molar concentrations as the radiolabeled compound, namely 0.1 to 1000 nM. Thus, the cold peptide group represented equimolar ligand exposure in the absence of radioactivity. To evaluate non-specific radiation-induced cytotoxicity, [¹⁷⁷Lu]LuCl₃ was included as a radiotoxicity control at 363 kBq/well, corresponding to the highest activity administered in the [¹⁷⁷Lu]Lu-FAP-2286 group. Specificity of the cytotoxic effect was assessed indirectly by comparing cell viability after treatment with targeted [¹⁷⁷Lu]Lu-FAP-2286 with that observed after exposure to equimolar cold FAP-2286 and activity-matched [¹⁷⁷Lu]LuCl₃. A greater reduction in cell viability in the [¹⁷⁷Lu]Lu-FAP-2286 group compared with both control groups was interpreted as evidence supporting a FAP-targeted, radiopharmaceutical-mediated cytotoxic effect rather than cytotoxicity

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caused solely by the peptide backbone or by non-specific radiation exposure. All treatment groups were incubated under identical exposure conditions, including the same culture medium, incubation temperature, exposure duration, and washing procedure. Treatments were performed in serum-containing medium for 1 h at 37°C. After incubation, cells were washed six times with serum-free medium to remove unbound material. Subsequently, 300 µL of complete culture medium was added to each well, and cells were further incubated for 24, 48, and 72 h. Cell viability was assessed by adding 10 µL of MTT solution to each well, followed by incubation for 3-5 h at 37°C. The medium was then removed, and 1 mL of DMSO/ethanol solution (50:50, v/v) was added to dissolve the formazan crystals. Plates were shaken for 20 min in a warm water bath, and absorbance was measured at 560 nm. All experiments were performed in triplicate (n = 3).

Tumor implantation

A 50 µL suspension of HEK FAP+ cells (containing 1×10^7 cells) was subcutaneously injected into the flanks of nude mice (4 to 8 weeks old, weighing 20-25 g). Biodistribution studies were performed when the tumor reached a diameter of 7-8 mm³.

Biodistribution of [¹⁷⁷Lu]Lu-FAP-2286 in normal and tumor-bearing mice

The biodistribution of the radiolabeled complex was evaluated in both normal and tumor-bearing mice following intravenous injection of (5.5 ± 0.5 MBq, 100 ± 10 µL per mouse) of [¹⁷⁷Lu]Lu-FAP-2286 (pH = 7) via the tail vein. Five mice were used per time point. The total injected activity was determined by measuring the radioactivity in the syringes before and after injection using a calibrated dose calibrator. At the designated time points, the animals were euthanized using CO₂ asphyxiation, in accordance with institutional ethical guidelines. Major organs were then harvested, weighed, and their radioactivity was measured using a calibrated gamma counter. The percentage of injected dose per gram of tissue (%ID/g) for each organ was calculated by dividing the measured radioactivity in the excised tissue by the decay-corrected injected activity and the tissue mass.

Absorbed dose estimation

The estimation of radiation absorbed dose to human organs for [¹⁷⁷Lu]Lu-FAP-2286 was performed using biodistribution data obtained from tumor-bearing mice. The non-decay-corrected %ID/g for each organ was plotted against time to generate time-activity curves. The cumulated activity for each organ was then calculated by integrating the area under the respective time-activity curve. For extrapolation of the animal data to humans, the relative organ mass scaling method was applied to estimate the accumulated activity in corresponding human organs, following previously described methodologies [14, 15].

Subsequently, the absorbed dose to each target organ was determined using the MIRD formalism, as shown in Equation (1) [16, 17]:

$$\tilde{D}(r_k) = \sum_h \tilde{A}_h S(r_{k \leftarrow h}) \quad (1)$$

where $\tilde{A}_h$ represents the cumulated activity in source organ h, and $S(r_{k \leftarrow h})$ denotes the S-value, which describes the mean absorbed dose to target organ k per unit cumulated activity in source organ h. The S-values applied in this study were obtained from the OLINDA/EXM dosimetry software [18].

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Statistical analysis

Data are presented as mean ± SD (n = 5 per time point). For each organ, differences among the five time points were analyzed using one-way ANOVA. When the ANOVA indicated a significant overall difference, Dunnett's post-hoc test was performed to compare each time point with the 4 h group. P-values less than 0.05 were reported as p < 0.05. The precision of the absorbed dose calculation was evaluated through propagation of uncertainty based on Eq. 2:

$$\sigma_D^2 = \left(\frac{\sigma_D}{\sigma_A}\right)^2 \sigma_A^2 + \left(\frac{\sigma_D}{\sigma_S}\right)^2 \sigma_S^2 \quad (2)$$

In this calculation, the uncertainty assigned to the final dose value reflects the cumulative effect of uncertainties associated with the input parameters used in the dosimetric model, particularly the residence-related activity term and the S coefficient. The combined uncertainty for each organ dose estimate is provided in the dosimetry tables as mean ± SD.

RESULTS

Preparation and quality control of [¹⁷⁷Lu]Lu-FAP-2286

The optimal conditions for radiolabeling FAP-2286 with ¹⁷⁷Lu were determined by systematically varying key experimental parameters. Specifically, 30 µg of the peptide was added to a vial containing 740 MBq of [¹⁷⁷Lu]LuCl₃, and the pH of the reaction mixture was adjusted to 4.5 using sodium acetate buffer. The reaction was conducted for 40 min at 95°C. The RTLC chromatogram of [¹⁷⁷Lu]LuCl₃ and [¹⁷⁷Lu]Lu-FAP-2286 is presented in Figure 1. As depicted, [¹⁷⁷Lu]LuCl₃ remains at the origin, whereas [¹⁷⁷Lu]Lu-FAP-2286 migrates to a higher R_f value of 0.9. The HPLC chromatogram, shown in Figure 2, reveals that [¹⁷⁷Lu]LuCl₃ elutes from the column at 3.51 min due to its more polar and hydrophilic nature, while [¹⁷⁷Lu]Lu-FAP-2286 is eluted at 17.73 min. Under optimal labeling conditions, the RCP of [¹⁷⁷Lu]Lu-FAP-2286 exceeded 99%.

In-vitro stability studies of [¹⁷⁷Lu]Lu-FAP-2286

The stability of [¹⁷⁷Lu]Lu-FAP-2286 was evaluated by measuring its RCP in PBS and human serum for up to 48 h (Table 1). The results indicated the high RCP of more than 96% in both PBS buffer and human serum, even after 48 h.

Binding studies

The binding specificity of [¹⁷⁷Lu]Lu-FAP-2286 was confirmed by a marked reduction in radioligand binding in HEK FAP⁺ cells following receptor blockade with an excess of unlabeled FAP-2286, with approximately 56% of the total binding being FAP-specific. In contrast, only negligible binding was observed in CHO FAP⁻ cells, confirming the specificity of the radioligand for FAP-expressing cells (Figure 3). Furthermore, saturation binding analysis yielded an equilibrium dissociation constant (K_d) of 0.45 ± 0.11 nM, demonstrating the high binding affinity of [¹⁷⁷Lu]Lu-FAP-2286 toward FAP-expressing HEK cells.

Internalization studies

The internalization percentage of [¹⁷⁷Lu]Lu-FAP-2286 in HEK-FAP⁺ cells is shown in Figure 4, with more than 38% internalization observed after 6 h.

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In vitro cytotoxicity studies

The effects of [¹⁷⁷Lu]LuCl₃, FAP-2286, and [¹⁷⁷Lu]Lu-FAP-2286 on the viability of HEK-FAP+ cells over a 72-h period are presented in Figures 5, 6, and 7, respectively.

As shown, treatment with [¹⁷⁷Lu]Lu-FAP-2286 at the maximum radioactivity of 363 kBq/well significantly reduced cell viability to approximately 16–38% (ranging from 38% at 24 h to 16% at 72 h). In comparison, the cold peptide FAP-2286 at its highest concentration of 1000 nM exhibited a moderate cytotoxic effect, with cell viabilities ranging from 53–68%. On the other hand, treatment with [¹⁷⁷Lu]LuCl₃ at 363 kBq/well resulted in relatively high cell viability (65–85% over 72 h). These findings demonstrate the potent and targeted cytotoxic efficacy of [¹⁷⁷Lu]Lu-FAP-2286 on HEK FAP⁺ cells.

Biodistribution of [¹⁷⁷Lu]Lu-FAP-2286 in normal and tumor-bearing mice

Each mouse received approximately 5.5 ± 0.5 MBq of [¹⁷⁷Lu]Lu-FAP-2286 in 100 ± 10 μ L via tail vein injection. The % ID/g in normal mice organs was determined up to 168 h post-injection (Table 2). The results showed very fast blood clearance; less than 0.5% of the activity was observed in blood after 4 h. A major portion of the injected activity accumulated in the kidneys, indicating that renal excretion is the primary route of elimination.

The biodistribution of [¹⁷⁷Lu]Lu-FAP-2286 in tumor-bearing mice showed the significant accumulation of [¹⁷⁷Lu]Lu-FAP-2286 in tumors at different time intervals (Table 3). Also, the considerable accumulation of activity was observed in the kidney after injection of [¹⁷⁷Lu]Lu-FAP-2286, which is related to the high hydrophilicity of the compound and its excretion via the urinary tract.

Absorbed dose estimation

The non-decay-corrected %ID/g values for each organ are shown in Figure 8. Based on these data, the absorbed dose to human organs was estimated using the MIRD formalism, and the resulting dosimetric values are presented in Table 4.

DISCUSSION

In recent years, the use of FAP as a therapeutic and imaging target has garnered significant attention in many cancers. FAP is predominantly expressed in CAFs within the tumor stroma, and its overexpression is observed in several malignant tumors such as breast, pancreatic, colorectal, and other types of cancer [19]. FAP-targeting compounds, particularly those labeled with theranostic radionuclides like ¹⁷⁷Lu, have become a focal point for both imaging and therapeutic applications.

In this study, [¹⁷⁷Lu]Lu-FAP-2286 was synthesized with the RCP greater than 99%, and a specific activity of 36.3 TBq/mmol under optimized radiolabeling conditions (30 μ g of peptide, pH = 4.5, temperature = 95°C, duration = 40 min) for targeting FAP-positive tumors. The complex exhibited high RCP (>95%) in both PBS buffer and human serum, maintaining stability for up to 48 h post-preparation.

The HEK FAP⁺ xenograft model was employed as a controlled preclinical platform for evaluating the FAP-specific targeting characteristics of [¹⁷⁷Lu]Lu-FAP-2286. This model provides stable and relatively uniform overexpression of FAP, enabling reproducible assessment of tumor uptake, cellular internalization, and biodistribution while reducing biological variability. Similar HEK293-FAP (HEK FAP⁺) xenograft models have been used in other preclinical investigations of FAP-targeted radiopharmaceuticals to assess FAP-specific tumor accumulation, pharmacokinetics, and

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biodistribution [20-22]. While this approach is useful for preliminary evaluation of FAP-targeted radiotracers, its inherent limitations and translational considerations should also be taken into account.

The binding studies exhibited exceptional binding affinity of the compound for FAP + HEK cell line ($K_d = 0.45 \pm 0.11$ nM). The MTT assay enabled comparison of the cytotoxic effects of [¹⁷⁷Lu]LuCl₃, FAP-2286 peptide, and the radiolabeled construct [¹⁷⁷Lu]Lu-FAP-2286 in HEK-FAP+ cells over 72 h. At the highest tested activity, [¹⁷⁷Lu]Lu-FAP-2286 reduced cell viability from approximately 38% at 24 h to 16% at 72 h, indicating a time-dependent decrease in viability. In contrast, the cold FAP-2286 peptide at 1000 nM resulted in higher residual viability values of approximately 53–68%, suggesting that peptide exposure alone only partially contributed to the observed cytotoxicity. [¹⁷⁷Lu]LuCl₃ at 100 MBq/well showed even higher residual cell viability, ranging from approximately 65–85% over 72 h, indicating that nonspecific exposure to ¹⁷⁷Lu was insufficient to reproduce the cytotoxic effect of [¹⁷⁷Lu]Lu-FAP-2286 construct.

Preclinical studies on [¹⁷⁷Lu]Lu-FAP-2286 have provided promising evidence of its effectiveness, particularly its ability to target tumors and deliver radiation to FAP-expressing tissues. Biodistribution studies in animal models have shown significant accumulation of the compound in FAP-positive tumor sites, with minimal background activity in non-target tissues. This high specificity for FAP-positive tumors, highlights the potential of [¹⁷⁷Lu]Lu-FAP-2286 for therapeutic use. In both healthy and tumor-bearing mice, the biodistribution study demonstrated substantial accumulation of [¹⁷⁷Lu]Lu-FAP-2286 in the kidneys. The pronounced renal uptake, followed by urinary excretion, indicates that the kidneys play a major role in the clearance of the [¹⁷⁷Lu]Lu-FAP-2286 from the body. Notably, the rapid clearance from the bloodstream and other tissues underscores a key advantage of [¹⁷⁷Lu]Lu-FAP-2286. These findings are in accordance with the previously reported data on the biodistribution of FAP-2286 labeled with different diagnostic and therapeutic radionuclides [22, 23].

These results are also in agreement with the work of Zboralski et al. (2022), who reported similar favorable biodistribution profiles and anti-tumor activity for [¹⁷⁷Lu]Lu-FAP-2286 in various cancer models, including sarcoma and pancreatic cancer. Their study also emphasized the minimal accumulation of the radiotracer in normal tissues, suggesting an advantageous tumor-to-background ratio, which aligns with the findings of the present study [24].

[¹⁷⁷Lu]Lu-FAP-2286 exhibits a favorable biodistribution pattern, with significant tumor-to-nontarget ratios (Table 5) demonstrating excellent target selectivity. Notably, the tumor-to-blood (T/B) ratio reached 55.05 at 72 h, reflecting efficient blood clearance, a critical factor for reducing systemic radiation exposure and sparing the bone marrow from myelotoxicity. Furthermore, the tumor-to-muscle (T/M) ratio peaked at 92.30 at 48 h, indicating exceptional targeting specificity with minimal retention in non-target soft tissues.

Dosimetric calculations summarized in Table 4 identify the kidneys as the organ receiving the highest absorbed dose (0.27 mGy/MBq). This observation aligns with the predominant renal clearance pathway of [¹⁷⁷Lu]Lu-FAP-2286, positioning the kidneys as the primary dose-limiting organs. Conversely, absorbed doses to other critical structures—including the liver, red marrow, and lungs—remained notably low, underscoring a favorable toxicity profile. Compared with previously published clinical dosimetry data for [¹⁷⁷Lu]Lu-FAP-2286, the absorbed doses to the kidneys and red marrow were within the reported ranges, whereas the total-body absorbed dose was lower than the published values. This discrepancy may be attributed to interspecies pharmacokinetic differences between mice and humans, the limitations of animal-to-human extrapolation methods, and differences in residence time estimation.

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A comparative analysis with other FAPI-based radioligands (Table 6) underscores the dosimetric advantages of [¹⁷⁷Lu]Lu-FAP-2286. Notably, the renal radiation burden was substantially lower than that of [¹⁷⁷Lu]Lu-FAPI-46 (0.27 vs. 0.89 mGy/MBq) and remained on par with [¹⁷⁷Lu]Lu-FAPI-04, suggesting a reduced risk of long-term nephrotoxicity. This favorable profile extends to the liver and red marrow doses, which were consistently lower than those reported for FAPI-04 and FAPI-46. Such findings indicate that FAP-2286 may offer a wider therapeutic window, potentially allowing for higher cumulative activities compared to earlier-generation FAPI derivatives. Furthermore, when evaluated against established theranostic agents, [¹⁷⁷Lu]Lu-FAP-2286 demonstrated a superior safety profile regarding critical organs. Its absorbed doses to the kidneys, liver and red marrow were markedly lower than those of [¹⁷⁷Lu]Lu-satoreotide tetraxetan. Compared to the current gold standard, [¹⁷⁷Lu]Lu-PSMA-617, our findings reveal a lower radiation exposure to the kidneys and liver, albeit with a slightly higher red marrow dose. These dosimetric divergencies are likely rooted in the distinct pharmacokinetic trajectories, receptor-binding affinities, and metabolic clearance pathways unique to each ligand. While these preliminary results highlight the safety and translational potential of [¹⁷⁷Lu]Lu-FAP-2286, rigorous clinical dosimetry remains essential to validate these findings and to optimize personalized treatment protocols [12, 25-29]. Recent advancements in FAP-targeted therapy have focused on overcoming the rapid pharmacokinetics of first-generation tracers. While conventional derivatives like [¹⁷⁷Lu]Lu-FAPI-46 show promising tumor suppression in pancreatic models, their efficacy is often limited by rapid renal clearance and short biological half-life. To address this, novel strategies such as the development of bivalent agents like [¹⁷⁷Lu]Lu-DOTA-NI-FAPI-04 have demonstrated significantly enhanced tumor retention (up to 50.75%ID/g) by leveraging hypoxia-sensitive motifs. In comparison, our findings with [¹⁷⁷Lu]Lu-FAP-2286 align with these modern trends, showing a favorable balance between selective tumor accumulation and rapid non-target organ clearance [30, 31].

This study has certain limitations that should be acknowledged. Although the HEK FAP+ xenograft model provided a well-controlled platform with stable FAP overexpression for assessing targeting specificity, it may not completely reflect the biological complexity, tumor heterogeneity, and tumor microenvironment (TME) observed in human cancers. Therefore, further studies employing clinically relevant FAP expressing tumor models are necessary to better evaluate the translational potential of [¹⁷⁷Lu]Lu-FAP-2286. In addition, organ dosimetry calculations were derived from murine biodistribution data, and species dependent pharmacokinetic variations, especially differences in renal clearance, could influence the accuracy of human dose estimations. Moreover, the present study did not investigate long term therapeutic outcomes or potential toxicities, which should be addressed in future investigations.

CONCLUSION

The present study demonstrated the successful radiolabeling of FAP-2286 with ¹⁷⁷Lu, achieving high radiochemical purity (>99%) and acceptable stability under the tested conditions. In vitro experiments confirmed the binding affinity of [¹⁷⁷Lu]Lu-FAP-2286 to FAP-expressing cells, along with measurable internalization and cytotoxic effects. Biodistribution studies in tumor-bearing mice revealed rapid systemic clearance, high tumor accumulation, and favorable tumor to background ratios. The estimated human organ dosimetry identified the kidneys as the primary dose limiting organ, consistent with the renal clearance of the compound, while radiation exposure to other organs remained relatively low. Overall, these preclinical findings are generally consistent

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with previously reported studies and support further investigation of [¹⁷⁷Lu]Lu-FAP-2286 as a potential agent for FAP-targeted radionuclide therapy.

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Table 1. Radiochemical purity (RCP) of [¹⁷⁷Lu]Lu-FAP-2286 at various time points (n=3)

	RCP (%) in PBS buffer	RCP (%) in human serum
4 h	98.6	98.7
24 h	97.4	97.0
48 h	96.3	95.1

Table 2. Biodistribution of [¹⁷⁷Lu]Lu-FAP-2286 in normal mice (%ID/g) at various time points

Organs	4h	24h	48h	72h	168h	p-value
Blood	0.31± 0.05	0.12± 0.01	0.06± 0.01	0.03± 0.00	0.01± 0.00	0.031
Skin	0.09± 0.01	0.08± 0.01	0.037± 0.00	0.02± 0.00	0.02± 0.00	0.024
Large intestine	0.73± 0.06	0.12± 0.03	0.07± 0.03	0.04± 0.00	0.02± 0.00	0.018
Small intestine	0.61± 0.05	0.11± 0.03	0.06± 0.02	0.04± 0.00	0.02± 0.00	0.026
Spleen	0.45± 0.05	0.10± 0.01	0.05± 0.01	0.04± 0.00	0.03± 0.00	0.013
Muscle	0.27± 0.04	0.10± 0.02	0.03± 0.00	0.13± 0.02	0.04± 0.00	0.017
Kidneys	5.11± 0.31	3.40± 0.23	2.19± 0.34	1.15± 0.13	0.72± 0.08	0.029
Stomach	0.35± 0.03	0.20± 0.03	0.05± 0.01	0.04± 0.00	0.01± 0.00	0.039
Heart	0.59± 0.07	0.22± 0.03	0.09± 0.04	0.06± 0.02	0.05± 0.02	0.026
Liver	0.62± 0.07	0.28± 0.04	0.17± 0.03	0.09± 0.03	0.08± 0.03	0.032
Bones	0.34± 0.04	0.16± 0.02	0.08± 0.02	0.06± 0.01	0.04± 0.00	0.014
Lungs	0.47± 0.03	0.23± 0.01	0.09± 0.03	0.06± 0.02	0.04± 0.00	0.036

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Table 3. Biodistribution of [^{177}Lu]Lu-FAP-2286 in tumor-bearing mice (%ID/g) at specified time points

Organs	4h	24h	48h	72h	p-value
Blood	0.22± 0.03	0.10± 0.02	0.05± 0.01	0.02± 0.00	0.034
Skin	0.12± 0.02	0.07± 0.01	0.03± 0.00	0.02± 0.00	0.047
Large intestine	0.73± 0.11	0.12± 0.02	0.06± 0.01	0.04± 0.00	0.024
Small intestine	0.59± 0.09	0.10± 0.02	0.06± 0.01	0.03± 0.00	0.035
Spleen	0.29± 0.05	0.09± 0.01	0.03± 0.00	0.04± 0.00	0.021
Muscle	0.19± 0.03	0.09± 0.02	0.02± 0.00	0.08± 0.01	0.012
Kidneys	3.27± 0.49	2.88± 0.43	1.85± 0.28	0.92± 0.14	0.033
Stomach	0.39± 0.06	0.19± 0.03	0.05± 0.01	0.03± 0.00	0.031
Heart	0.47± 0.07	0.20± 0.03	0.08± 0.01	0.05± 0.01	0.031
Liver	0.58± 0.09	0.25± 0.04	0.14± 0.02	0.08± 0.01	0.025
Bones	0.29± 0.04	0.13± 0.02	0.06± 0.01	0.05± 0.01	0.033
Lungs	0.39± 0.06	0.21± 0.03	0.08± 0.01	0.04± 0.00	0.025
Tumor	9.45± 1.42	3.11± 0.47	2.12± 0.32	1.12± 0.17	0.036

Table 4. Estimated human organ absorbed doses for [^{177}Lu]Lu-FAP-2286 (mGy/MBq) extrapolated from mouse biodistribution data

Organ	Absorbed dose (mGy/MBq)
Adrenals	0.002±0.000
Small intestine	0.023±0.002
Stomach	0.006±0.000
Large intestine	0.013±0.002
Heart wall	0.024±0.007
Kidneys	0.267±0.075
Liver	0.036±0.010
Lungs	0.018±0.005
Muscle	0.001±0.000
Pancreas	0.001 ±0.000
Red marrow	0.027±0.003
Bone	0.072±0.003
Spleen	0.017±0.005
Total body	0.009±0.001

Table 5. Target-to-nontarget ratios (T/B and T/M) of [¹⁷⁷Lu]Lu-FAP-2286 in tumor-bearing mice over a 72-hour period, demonstrating the uptake kinetics and target selectivity

Ratio	4h	24h	48h	72h
T/B	43.96	30.51	40.83	55.05
T/M	50.81	32.08	92.30	13.59

Table 6. Comparison of organ absorbed doses for selected [¹⁷⁷Lu]Lu-labeled radiopharmaceuticals (mGy/MBq). Values are from the present study and previously published reports

Organs	[¹⁷⁷ Lu]Lu-FAP-2286	[¹⁷⁷ Lu]Lu-FAP-2286	[¹⁷⁷ Lu]Lu-FAPI-46	[¹⁷⁷ Lu]Lu-FAPI-04	[¹⁷⁷ Lu]Lu-satoreotide-tetrexetan	[¹⁷⁷ Lu]Lu-PSMA-617
Liver	0.04	-	0.00-0.20	0.11	0.04–0.21	0.10
Kidneys	0.27	0.18–2.00	0.08-1.39	0.25	0.52–1.46	0.60
Red marrow	0.03	0.03–0.09	-	0.04	0.07–0.38	0.01
Total body	0.01	0.04-0.10	0.02-0.03	-	0.05–0.21	-
Ref	This study	[25, 26]	[27]	[28]	[29]	[30]

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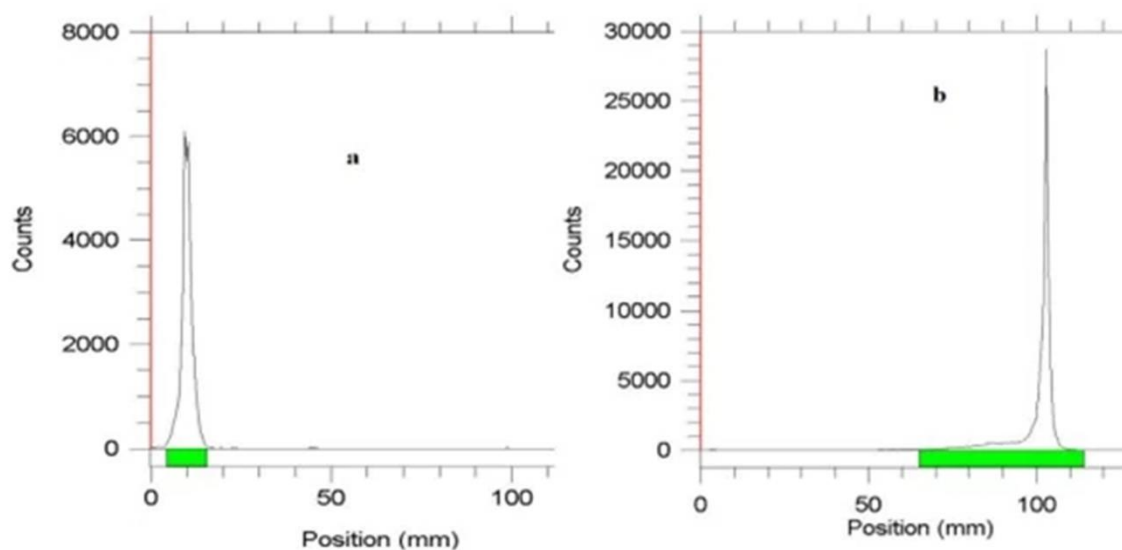


Figure 1. RTLC of (a) [^{177}Lu]LuCl₃ ($R_f \approx 0.1$) and (b) [^{177}Lu]Lu-FAP-2286 ($R_f \approx 0.9$) on Whatman No. 2 paper using methanol:saline (5:1, v/v). Radioactivity peaks (counts) are shown at 10 mm (origin) and 100 mm (front) positions, respectively

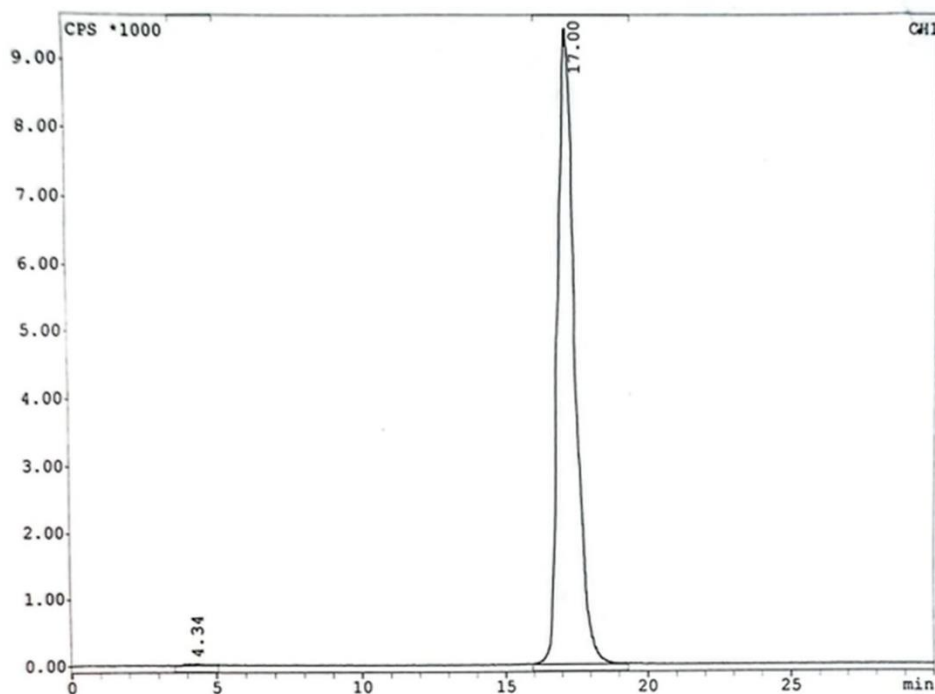


Figure 2. HPLC of [^{177}Lu]Lu-FAP-2286 ($t_R = 17.73$ min) and [^{177}Lu]LuCl₃ ($t_R = 4.34$ min). Conditions: C18 column; mobile phase A: H₂O + 1% TFA, B: MeCN; flow rate 2.6 mL/min. Gradient: 0–3 min 0% B, 3–10 min 50% B, 10–15 min 100% B

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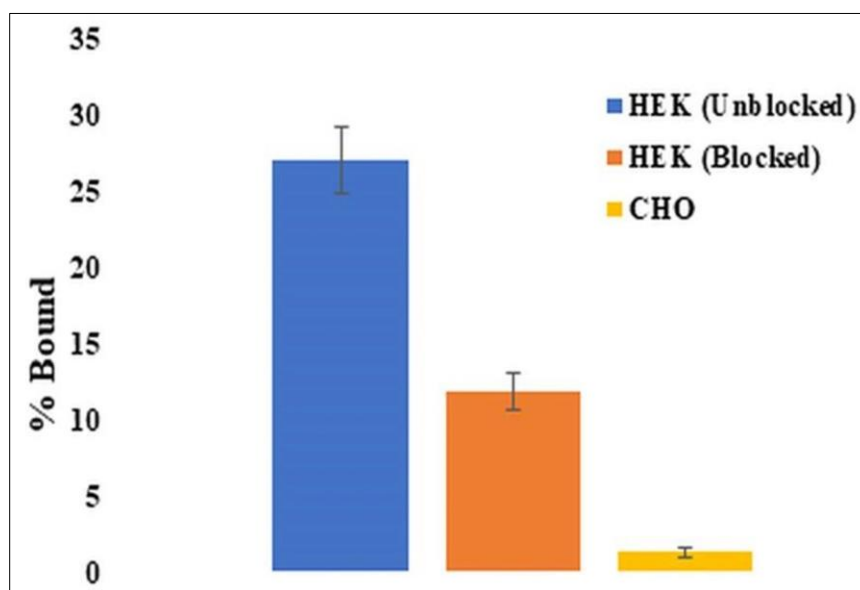


Figure 3. In vitro binding specificity of [^{177}Lu]Lu-FAP-2286 in FAP-positive (HEK-FAP+) and FAP-negative (CHO-FAP-) cells. Total binding was determined in HEK-FAP+ cells, while non-specific binding was assessed by blocking receptors with an excess of unlabeled FAP-2286 (10 μM). The minimal uptake in CHO-FAP- cells further confirms the FAP-specific binding of [^{177}Lu]Lu-FAP-2286. Data represent the mean $\pm$ SD of three independent experiments (n=3)

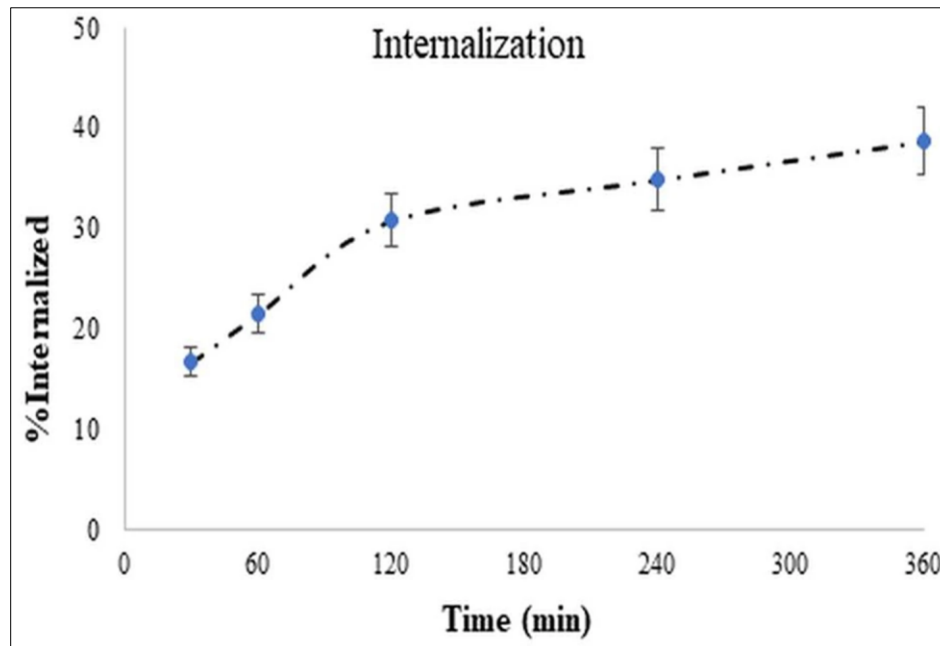


Figure 4. Time-dependent internalization of [^{177}Lu]Lu-FAP-2286 in HEK-FAP+ cells. Cells were incubated with [^{177}Lu]Lu-FAP-2286 for 30, 60, 90, 120, 240, and 360 min at 37 °C. Surface-bound activity was removed using 0.1 M glycine buffer (pH 2.8), and the remaining internalized radioactivity was quantified with a gamma counter. Results are presented as mean $\pm$ SD (n = 3)

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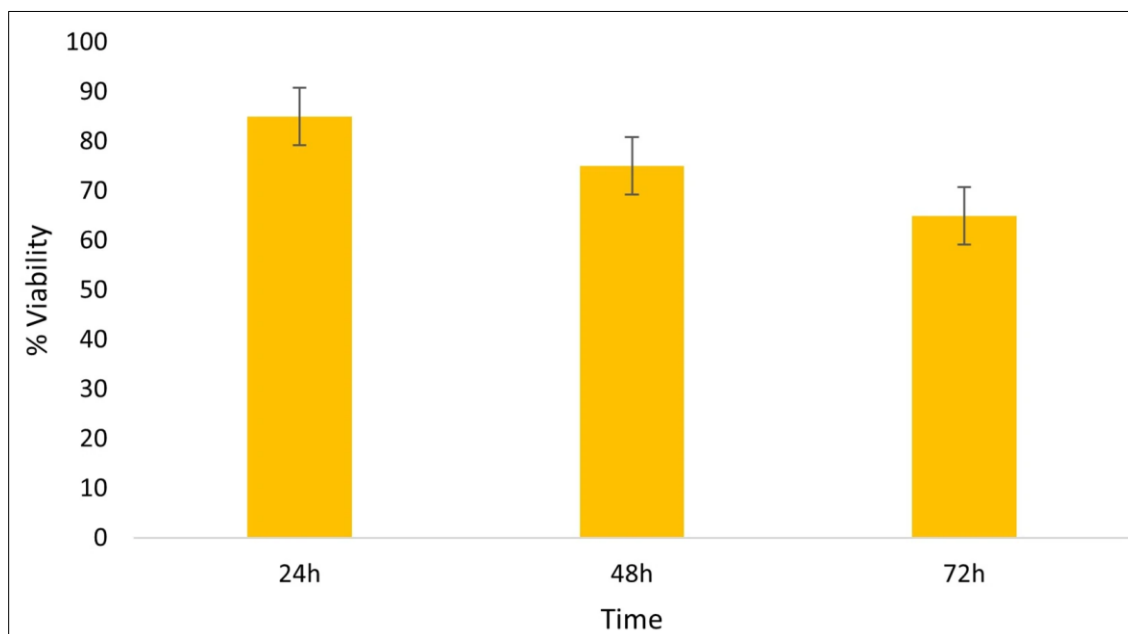


Figure 5. Effect of 100 MBq/well [^{177}Lu]LuCl₃ used as a control on the viability of HEK-FAP⁺ cells at 24, 48, and 72 h. Data represent mean $\pm$ SD (n = 3)

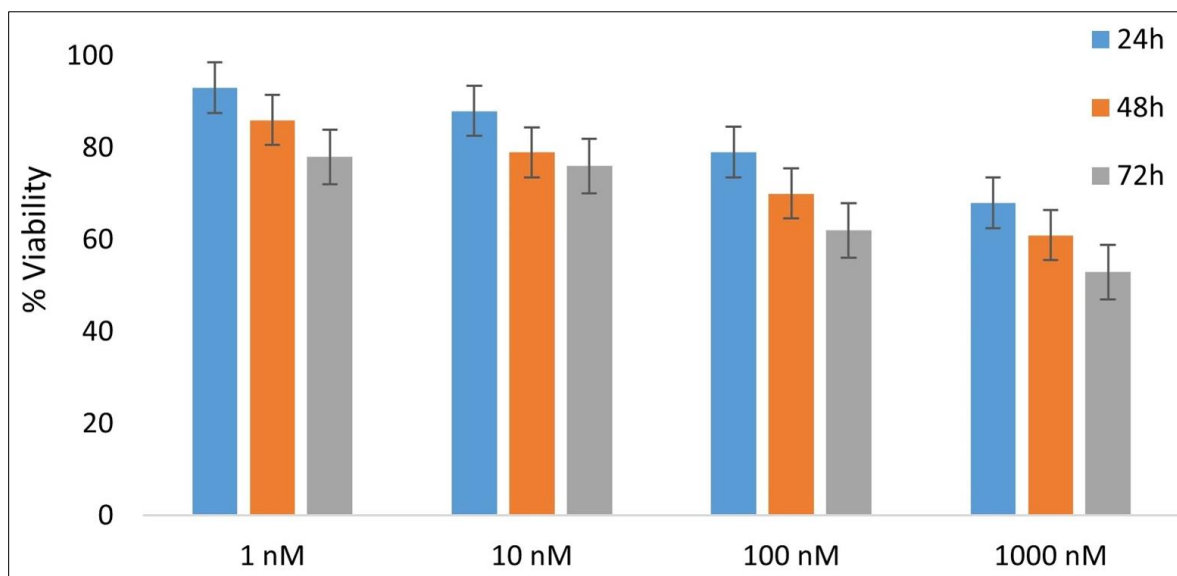


Figure 6. Effect of different concentrations of the non-radioactive (cold) peptide on the viability of HEK-FAP⁺ cells at 24, 48, and 72 h. Cell viability was assessed using the MTT assay. Data are presented as mean $\pm$ SD (n = 3)

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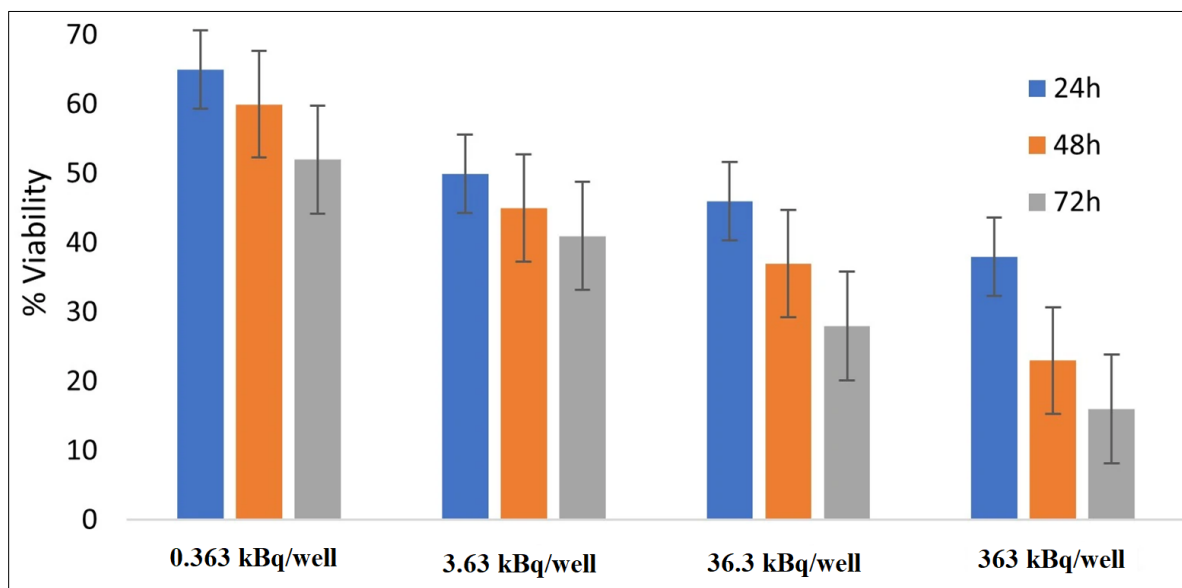


Figure 7. Effect of different concentrations of [^{177}Lu]Lu- FAP-2286 on the viability of HEK-FAP⁺ cells at 24, 48, and 72 h. Cell viability was evaluated using the MTT assay. Data are presented as mean $\pm$ SD (n = 3)

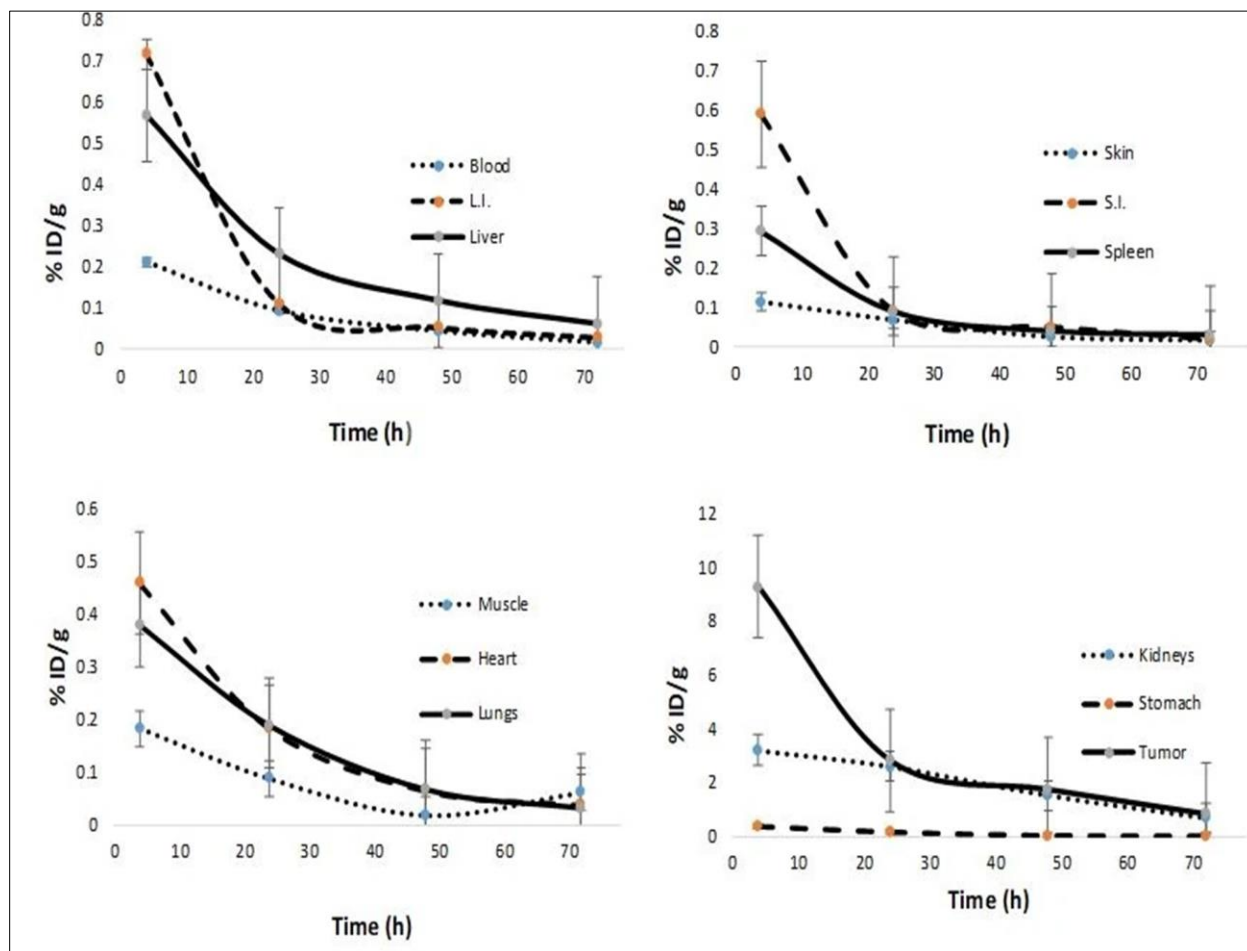


Figure 8. Non-decay-corrected percentage of injected dose per gram (%ID/g) versus time for major organs in tumor-bearing mice after administration of [^{177}Lu]Lu-FAP-2286