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Radioactivity- and time-dependent cellular cytotoxicity of astatine-211-labeled FAP-targeting radiopharmaceuticals

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Abstract

Fibroblast activation protein (FAP), which is overexpressed in cancer-associated fibroblasts (CAFs) is an attractive stromal target for a-therapy. We previously reported moderate anti-tumor effects of ^{211}At-labeled FAP inhibitors (FAPI) in a PANC-1 xenograft mouse model, where insufficient tumor accumulation and the absence of FAP expression in tumor cells limited therapeutic efficacy. In this study, we performed in vitro mechanistic follow-up experiments using FAP-negative PANC-1 cells to clarify the radioactivity–time-dependent cytotoxicity of ^{211}At-labeled FAPI compared with free ^{211}At (non-conjugated ^{211}At). We found that, whereas free ^{211}At showed only limited cytotoxicity, ^{211}At-labeled FAPI exerted significant cytotoxic effects. Systematic variation of activity concentrations and exposure durations revealed that cytotoxicity depended primarily on sustained pericellular exposure rather than initial radioactivity levels. These findings provide mechanistic, hypothesis-generating insight into how CAF-targeted ^{211}At radiopharmaceuticals can exert effective cytotoxicity without direct tumor cell targeting and may inform future optimization of stromal-targeted a-therapy, while further in vivo and translational studies will be required.

Keywords Radiopharmaceutical · Astatine-211 · α-emitter · Cytotoxic effect · Fibroblast activation protein (FAP)

Abbreviations

FAP	Fibroblast activation protein
FAPI	Fibroblast activation protein inhibitor
PEG	Polyethylene glycol
CAF	Cancer-associated fibroblast
PET	Positron emission tomography
CT	Computed tomography
RCC	Radiochemical conversion
RCP	Radiochemical purity
%ID/g	Percent injected dose per gram
logP	Logarithm of the partition coefficient
tPSA	Topological polar surface area
PSMA	Prostate-specific membrane antigen
PA	Phenylalanine
AAMT	^{211}At-labeled alphamethyltyrosine
AuNP	Gold nanoparticles
PANC-1	Human pancreatic cancer cell line

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Introduction

Fibroblast activation protein (FAP) is highly expressed in cancer-associated fibroblasts across many epithelial tumors, while its expression in normal tissues is limited [1]. Consequently, FAP-targeted radiotheranostics using FAP inhibitors (FAPIs) are attracting increasing attention [2]. Various FAPI compounds bind to the active site of FAP through key structural elements, while the quinoline moiety further stabilizes the interaction with the protein, resulting in efficient inhibition of FAP [3, 4]. Among these, gallium-68 (^{68} Ga) and fluorine-18 (18F) labeled FAPI tracers have rapidly advanced in clinical imaging, consistently demonstrating high tumor-to-background contrast, rapid tumor uptake, and favorable pharmacokinetics. Early clinical positron emission tomography (PET)/computed tomography (CT) studies using FAPI derivatives such as FAPI-02, FAPI-04, FAPI-46 and FAPI-74 have confirmed clear visualization of a wide range of tumors, and further optimization continues with the development of dimeric and polyethylene glycol(PEG)-modified variants as well as automated radiosynthesis protocols [2]. Collectively, these findings establish FAPI-based PET imaging as a clinically feasible platform for FAP-targeted radiotheranostics.

While diagnostics using FAPI-based PET agents are advancing rapidly, both a and b radiation therapy are under development, each with its own unique requirements. For β-emitters such as lutetium-177 (^{177}Lu), achieving a high tumor-to-normal tissue ratio and sufficient radiation dose delivery through cross-fire effects can result in meaningful tumor control. Indeed, [^{177}Lu]Lu-FAPI-46 demonstrated marked antitumor activity in xenograft mice at 30 MBq [5]. In contrast, a-particle therapy relies on very short path lengths and high linear energy transfer (LET); therefore, therapeutic efficacy depends more critically on sufficient tumor uptake and residence time at the target site. Consistent with this concept, actinium-225 (^{225}Ac)-labeled FAPI-04 has shown moderate antitumor activity even at low administered radioactivity [2, 3].

From the perspective of clinical feasibility, we focused on astatine-211 (^{211}At) as an a-emitter with 7.2 h half-life and a simple decay scheme producing only ^{207}Bi and ^{207}Pb. These characteristics make ^{211}At-labeled agents attractive candidates for clinical translation by potentially reducing hospitalization and improving cancer patients' quality of life [6]. Our group developed various ^{211}At-labeled candidates: Examples include sodium astatide ([^{211}At]NaAt), [^{211}At]At-phenyalanine (PA), [^{211}At]At-labeled compounds targeting prostate-specific membrane antigen (PSMA), [^{211}At]At-labeled alpha-methyltyrosine (AAMT), and [^{211}At]At-labeled gold nanoparticles (AuNPs) [7–13]. Based on these considerations, we previously synthesized a series of ^{211}At-labeled FAPI derivatives and evaluated their antitumor efficacy and biodistribution in mouse models (Fig. 1) [14–16]. In PANC-1 xenograft mice, a mouse model implanted with the human pancreatic cancer cell line, [^{211}At]At-FAPI1 and [^{211}At]At-FAPI5 significantly inhibited tumor growth without notable toxicity, as reflected by stable body weights and normal behavior [15]. Biodistribution studies revealed modest tumor uptake, with [^{211}At]At-FAPI5 showing 1.24%ID/g (percentage of injected dose per gram of tissue) at 1 h and

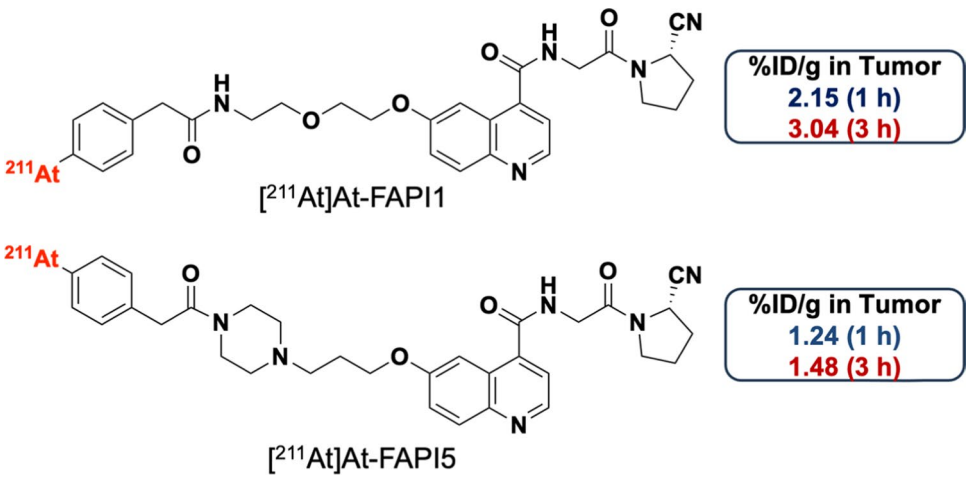

Fig. 1 Our previous animal experiment utilizing ^{211}At-labeled FAPIs ([^{211}At]At-FAPI1 and [^{211}At]At-FAPI5). Anti-tumor effect in PANC-1 xenograft mice after administration of ^{211}At-FAPIs (approximately 1 MBq). Biodistribution of [^{211}At]At-FAPI1 and [^{211}At]At-FAPI5 at 1 h (blue) and 3 h (red) after injection ([^{211}At]At-FAPI1 0.81 MBq and [.^{211}At]At-FAPI5 0.54 MBq). After the mice were dissected, the intensities were measured and the % ID/g(percentage of injection dose per gram of tissue) were calculated. All data are shown as means ± SE. All groups consisted of three mice. CC BY from. Aso A et al., *Int.J. Mol. Sci.* 2023, *24*(10), 8701 [15]

1.48%ID/g at 3 h, whereas [^{211}At]At-FAPI1 achieved higher uptake values of 2.15%ID/g and 3.04%ID/g at the corresponding time points. Although [^{211}At]At-FAPI1 exhibited slightly superior tumor retention and antitumor activity, tumor regression was incomplete and regrowth occurred after treatment. Uptake in the thyroid and stomach, organs susceptible to free halogen accumulation, suggested partial in vivo cleavage of the carbon–astatine bond [17]. In a subsequent study, we investigated the effect of linker length by comparing [^{211}At]At-FAPI1 with [^{211}At]At-FAPI2 in BxPC-3 xenograft-bearing mice, in which tumors were generated from the human pancreatic cancer cell line BxPC-3. Although the longer linker improved tumor retention, no appreciable enhancement in therapeutic efficacy was observed, and tumor regrowth occurred in all mice approximately one month after treatment. These findings indicate that neither linker modification nor the administered activity (1 MBq/mouse) was sufficient to achieve durable tumor control, underscoring the need to clarify the intrinsic pharmacological efficacy of [^{211}At]At-FAPI. In particular, the contribution of stromal targeting to therapeutic effect remains poorly understood. Because many pancreatic cancers lack FAP expression in tumor cells themselves, an important question is whether cancer-associated fibroblast (CAF)-targeted a-therapy can induce sufficient cytotoxicity in FAP-negative cancer cells, and whether instantaneous radioactivity or sustained pericellular exposure is the dominant determinant of efficacy. Building on our previous in vivo studies with [^{211}At]At-FAPI derivatives, the present work was therefore designed as an in vitro mechanistic follow-up study rather than as a comprehensive pharmacological or translational evaluation.

Herein, we addressed these questions through detailed in vitro studies using FAP-negative PANC-1 cells. We compared the cytotoxicity of ^{211}At-labeled FAPI with that of free ^{211}At (non-conjugated ^{211}At species) and systematically varied both activity concentration and exposure duration.

Materials and methods

Radioactivity of ^{211}At was determined by γ-ray spectrometry with a Ge semiconductor detector (BE-2020, Mirion Technologies (Canberra) Inc.), and Curie meter IGC-7 and IGC-8B (Hitachi Ltd.). The ^{211}At-labeled products were purified by Oasis HLB Plus Light Cartridge (weight: 30 mg, particle size: 30 µm). TLC silica gel 60 F_{254} (Merck) was used for TLC analysis of radiolabeled products. The TLC plate was exposed to an imaging plate (GE Healthcare), and the imaging plate was scanned by Typhoon FLA 7000 (GE Healthcare).

Synthesis of ^{211}At-labeled FAPI compounds

The experimental protocol for our [^{211}At]At-FAPI studies has been described in our previous reports [15, 16]. A synthetic methodology is briefly summarized here. Astatine-211 was obtained from Research Center for Nuclear Physics (RCNP, The University of Osaka, Japan), National Institutes for Quantum Science and Technology (QST, Takasaki, Japan) and RIKEN (Wako, Japan) via a short-lived radioisotope supply platform. It was produced using the ^{209}Bi (α, 2n) ^{211}At reaction and separated from the Bi target by dry distillation, then dissolved in pure water. FAPI precursors bearing dihydroxyboryl groups ("B-FAPI" precursors) were labeled with ^{211}At via substitution reaction. B-FAPIs were synthesized as precursors for ^{211}At labeling according to published procedures [14, 15]. The radiolabeled compound [^{211}At]At-FAPI1 was synthesized by reacting 10 µL of aqueous B-FAPI1 (1 mg/mL), 10 µL $NaHCO_3$ (7% w/v), 90 µL H_2O, 2–19 µL ^{211}At (0.28–1.01 MBq), and 30 µL KI (0.1 mol/L) in a polypropylene tube at room temperature, then heated at 80 °C for 45 min. The radiochemical conversion (RCC) of [^{211}At]At-FAPI1 and the radiochemical purity (RCP) after purification were analyzed by radio-TLC. Physicochemical parameters logarithm of the partition coefficient (logP) and topological polar surface area (tPSA) for the iodinated analog of [^{211}At]At-FAPI1 were calculated using ChemDraw Professional (Ver. 25.0.2.7, Revvity Signals) with default settings.

Materials and reagents

Dulbecco's modified Eagle's medium (D-MEM; high Glucose) with L-Glutamine, Phenol Red and Sodium Pyruvate and RPMI-1640 with L-Glutamine and Phenol Red were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Ninety-six-well microplates and the WST-8 reagent kit were purchased from Dojindo Laboratories (Kumamoto, Japan), and CFSE was purchased from Biotium (CA, USA). Six-well and 96-well microplates were purchased from AGC TECHNO GLASS Co., Ltd. (Shizuoka, Japan). Lewis lung carcinoma (LLC) cells, a murine lung carcinoma cell line, were kindly provided by Prof. Fei Yu (Tongji University, School of Medicine, China). The LLC cell line was grown in RPMI-1640 supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin–streptomycin solution (FUJIFILM Wako). Cells were incubated at 37 °C under 5% CO_2 atmosphere. PANC-1 cells, a human pancreatic cancer cell line, were purchased from Japanese Collection of Research Bioresources. PANC-1 cells were cultured in D-MEM (high-glucose) supplemented by 10% FBS and 1% penicillin–streptomycin (× 100), then incubated at 37 °C in a 5% carbon dioxide atmosphere.

Cell viability assay by WST-8 assay

LLC and PANC-1 cells were respectively plated in a 96 well microplate (100 µL /well) at a density of 2.5×10^4 cells / well. Background control wells (n = 3) containing the same volume of complete culture medium were included in each assay. The microplate was incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Free ^{211}At at 0, 10, 100 and 1000 kBq/mL in medium were added to it and the plate incubated at 37 °C under 5% CO_2 atmosphere. After incubating for 3 days following the addition of free ^{211}At, 10 µL of WST-8 reagent was added to each well, followed by incubation at 37 °C under 5% CO_2 atmosphere for 1 h. The absorbance was measured using an infinite F50 (Tecan) at 450 nm. For each well, the average background absorbance was subtracted, and the values were normalized to the untreated control group (no ^{211}At). Data processing was performed with GraphPad Prism (GraphPad Software, Boston, MA, USA). The results are expressed as the mean ± standard error of the mean (SEM) (no mark: not significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ vs. control group; one-way ANOVA followed by Dunnett's test, GraphPad Prism 9).

Cell viability assay by colony-forming assay

The ability of [^{211}At]At-FAPI1 to inhibit cancer cell proliferation was evaluated using a colony-forming assay. PANC-1 cells were seeded in 24-well plates at a density of 2,000 cells/well in 1 mL supplemented D-MEM high glucose and incubated overnight. The cells were treated in triplicate with various activity concentrations of [^{211}At]At-FAPI1 for 1, 2 or 24 h at 37 °C in 5% CO_2, washed with pre-warmed PBS, and the medium was changed to a fresh medium. The cells were incubated for 7 days, fixed, and stained with 0.5% crystal violet. The cells were observed by microscope, and the stained crystal violet was extracted using 0.05 M NaH_2PO_4 in 50% ethanol for optical density (OD) measurement at 570 nm, normalized to the control group. For each well, the average background absorbance was subtracted, and the values were normalized to the untreated control group. The results are expressed as the means ± standard deviation (SD). The number of PANC-1 colonies in the 1% EtOH-treated control group was set to 100%. Values are expressed as mean ± SD, n = 3, and asterisks indicate statistical significance compared to controls (no mark: no significance, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ vs. control group. One-way ANOVA followed by Dunnett's test was performed using GraphPad Prism 9 software).

Results

Cytotoxicity of free ^{211}At toward LLC and PANC-1 cells

Cell viability assays were performed using Lewis lung carcinoma (LLC) and pancreatic cancer (PANC-1) cells (Fig. 2). In experiments with LLC cells, free ^{211}At exhibited activity concentration-dependent cytotoxicity, consistent with our previous reports [18]. In contrast, when PANC-1 cells were treated with free ^{211}At at an initial concentration of 10 kBq/mL, no decrease in cell viability was observed. Even when the concentration was increased to 100 and 1000 kBq/mL, only a slight decrease in cell viability was detected, and strong cytotoxicity was not observed.

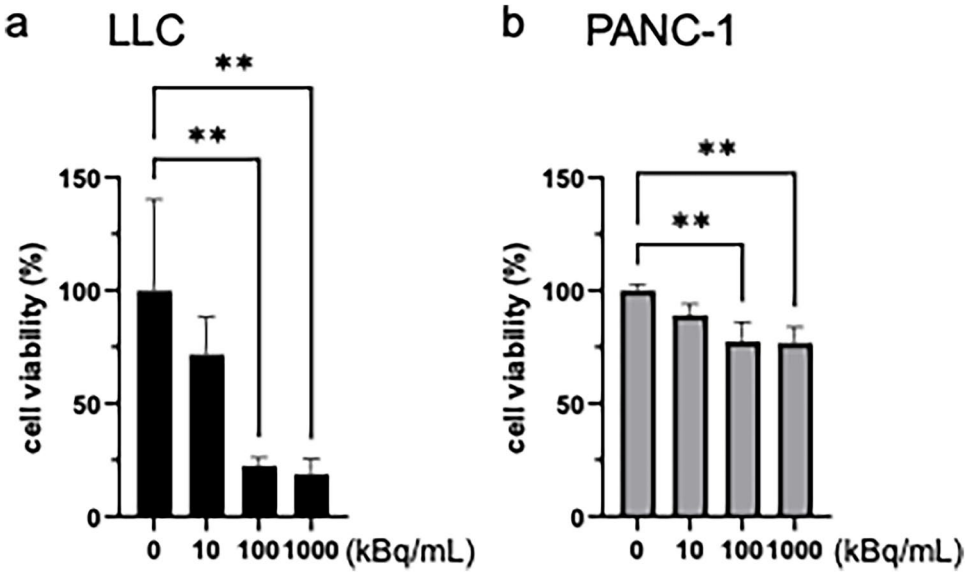

Fig. 2 Quantitative analysis of cell viability by WST-8 assay following.^{211}At solution treatment in a) LLC and b) PANC-1 cells. The number of LLC and PANC-1 by non-treated controls was set to 100%. Values are expressed as mean ± SD, n = 3, and asterisks indicate statistical significance compared to controls (no mark: no significance, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ vs. control group. One-way ANOVA followed by Dunnett's test was performed using GraphPad Prism 9 software)

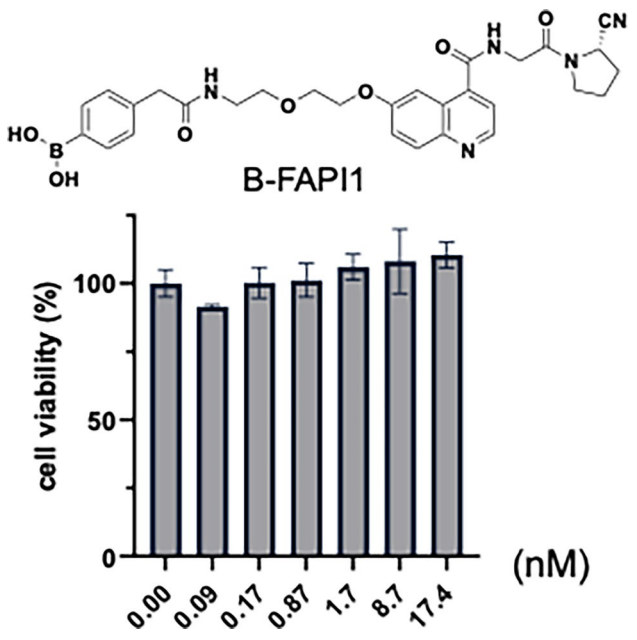

Fig. 3 Quantitative analysis of cell viability by colony forming assay with B-FAPI1(labeling precursor of [.^{211}At]At-FAPI1). The number of colonies in PANC-1 treated with 1% EtOH was set to 100% as the control. Values are expressed as mean ± SD, n = 3, and asterisks indicate statistical significance compared to controls (no mark: no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs. control group. One-way ANOVA followed by Dunnett's test was performed using GraphPad Prism 9 software)

Cellular cytotoxicity of B-FAPI1 and [.^{211}At]At-FAPI1

Based on our previous in vivo study, we focused on FAPI1, which exhibited the highest antitumor efficacy among the tested [^{211}At]At-FAPI derivatives, and evaluated its cytotoxicity against PANC-1 cells by a colony-forming assay. In the present study, a boronic acid–containing precursor (B-FAPI1) was employed for astatine radiolabeling. No cytotoxicity of B-FAPI1 was observed across a wide concentration range from 0.09 nM to 17 nM (Fig. 3). In subsequent biochemical experiments, the concentration of unreacted boronic acid precursor was at most approximately 10 nM, confirming that toxicity from the precursor would not affect the outcomes of in vitro experiments described below. Next, we prepared [^{211}At]At-FAPI1 and carried out the experiments by our reported method. The RCC of [^{211}At]At-FAPI1 ranged from 80 to 90%, and the RCP after purification was 92%, as determined by radio-TLC. The cytotoxicity of [^{211}At]At-FAPI1 toward PANC-1 cells was evaluated (Fig. 4). The incubation times with the radiolabeled compound were set to 1, 2 and 24 h. After 1 h of treatment, no clear decrease in cell survival was observed. In contrast, 2 h of treatment at a concentration of 150 kBq/mL resulted in a significant reduction in cell survival. When the treatment time was further prolonged to 24 h, decreased cell survival was observed at activity concentrations as low as 6 kBq/mL, and an activity concentration-dependent reduction in cell survival was demonstrated. Consequently, [^{211}At]At-FAPI1 induced markedly stronger and exposure-time-dependent

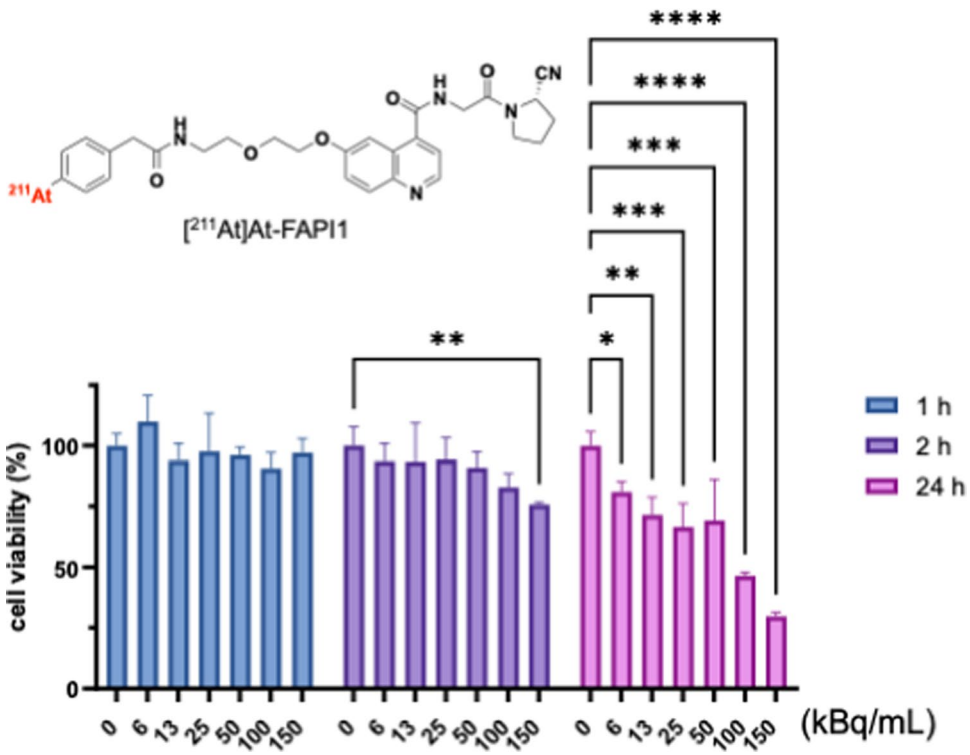

Fig. 4 Quantitative analysis of cell viability by colony forming assay with [^{211}At]At -FAPI1. Time in treatment of [.^{211}At]At-FAPI1 is 1 h (blue), 2 h (purple), and 24 h (pink). The number of PANC-1 by treated 1% EtOH colony controls was set to 100%. Values are expressed as mean ± SD, n = 3, and asterisks indicate statistical significance compared to controls (no mark: no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs. control group. One-way ANOVA followed by Dunnett's test was performed using GraphPad Prism 9 software)

cytotoxicity in PANC-1 cells. To obtain additional supportive information, we calculated the physicochemical parameters logP and tPSA for the iodinated analog of [^{211}At]At-FAPI1, which yielded values of 2.51 and 133 Å^2, respectively.

Discussion

In the present study, free ^{211}At alone exerted only limited cytotoxicity toward PANC-1 cells, whereas [^{211}At]At-FAPI1 produced markedly stronger cytotoxic effects under comparable activity concentrations and exposure conditions. This contrast suggests that the observed biological effect is primarily associated with the radiopharmaceutical itself rather than with rapidly dissociated free ^{211}At. Although we did not perform dedicated in vitro stability assays under the present experimental conditions, our previous in vivo studies provide supportive information on the radiochemical stability of [^{211}At]At-FAPI derivatives. In general, the chemical stability of ^{211}At-labeled compounds is known to depend strongly on the susceptibility of the At–C bond to oxidative cleavage. Radiochemical instability of ^{211}At-labeled aryl compounds is generally caused by oxidative deastatination, in which oxidation of astatine facilitates cleavage of the At–C bond. In our previous studies, oxidation-labile compounds such as [^{211}At]At-AAMT required the addition of ascorbic acid to maintain sufficient in vivo stability [10, 19]. In contrast, [^{211}At]At-PSMA5 retained adequate chemical integrity and tumor-targeting activity over several hours in vivo without ascorbic acid, although some physiological uptake in the thyroid and stomach was observed [9]. A similar extent of thyroid uptake (approximately 15%ID/g at 3 h) was also observed for [^{211}At]At-FAPI1 [17], suggesting that some degree of in vivo deastatination occurs but that the overall radiochemical stability of [^{211}At]At -FAPI1 is comparable to that of [^{211}At]At-PSMA5 rather than that of highly oxidation-labile compounds. Importantly, [^{211}At]At-FAPI1 and related [^{211}At]At-FAPI derivatives also exhibited similarly stable behavior during routine TLC quality control and were obtained with high radiochemical purity (RCP ≥ 90%) at the time of biological use, indicating that these compounds belong to the chemically stable class of ^{211}At radiopharmaceuticals rather than the oxidation-labile class.

Under the present in vitro conditions, free ^{211}At exerted only limited cytotoxicity toward PANC-1 cells, with no reduction in viability at 10 kBq/mL and only slight decreases even at 100–1000 kBq/mL. These results suggest that the cytotoxicity observed with [^{211}At]At-FAPI1 cannot be explained solely by dissociated free ^{211}At. The stronger cytotoxicity of [^{211}At]At-FAPI1 compared with free ^{211}At further suggests that the radiopharmaceutical is more effectively retained in association with the cell surface and/or internalized than free radionuclide alone. FAP is generally known to be expressed in cancer-associated fibroblasts (CAFs) within the tumor stroma, whereas PANC-1 pancreatic cancer cells do not express FAP [18, 20]. Therefore, the FAP-mediated ligand recognition pathway is unlikely to explain the observed cytotoxicity in this in vitro model. Given the similar chemical properties of iodine and astatine, the calculated logP (2.51) and tPSA (133 Å^2) for the corresponding iodinated analog are compatible with moderate membrane permeability and suggest that passive diffusion may contribute to cellular association of [^{211}At]At-FAPI1. Such physicochemical characteristics may allow the radiopharmaceutical to remain in close proximity to the plasma membrane and, at least partially, access intracellular compartments. However, these mechanistic considerations remain indirect, and direct uptake, internalization, and subcellular localization studies will be necessary in future work to clarify the precise mode of cellular association.

In TAT, efficient induction of lethal DNA damage generally requires that radioactive decays occur in close proximity to the cell nucleus, because the short path length and isotropic emission of α-particles markedly increase the probability of nuclear traversal when decay occurs near the nucleus. Accordingly, receptor-mediated retention on the tumor cell surface, and particularly intracellular internalization of the radiopharmaceutical, markedly enhance biological effectiveness by increasing the likelihood of perinuclear α-particle emission.

However, this efficient delivery mechanism is not applicable to FAP-targeted radiotherapy. FAP is predominantly expressed in cancer-associated fibroblasts within the tumor stroma and is not expressed on PANC-1 tumor cells themselves [18, 20]. Therefore, [^{211}At]At-FAPI1 is unlikely to benefit from receptor-mediated internalization into target cancer cells. To enhance its cytotoxic effect, it may instead be necessary to increase the cumulative number of α-particle traversals delivered to tumor-cell nuclei through prolonged stromal localization, sustained pericellular retention, and indirect cross-fire irradiation. Our recent data indicate that the α-particle exposure rate correlates with cell viability [18].

Consistent with this, the present study demonstrated that cell viability varied markedly with exposure duration. Notably, short-term exposure to a high activity concentration (150 kBq/mL, 2 h) produced a comparable level of cytotoxicity to long-term exposure to a lower concentration (6 kBq/mL, 24 h). These findings suggest that cytotoxicity is influenced not only by the administered activity but also by the effective α-particle exposure over time and the duration of pericellular retention. In the present study, activity concentration and exposure time were used as practical experimental variables, and no formal dosimetric analysis was performed. For α-particle emitters such as ^{211}At, however, the

absorbed dose and dose rate at the cellular and subcellular level are determined not only by activity and exposure time but also by geometrical factors and microdosimetric considerations, including the probability and number of nuclear traversals. A more rigorous analysis of absorbed dose and dose rate under the present in vitro conditions will therefore be required in future work to quantitatively link the observed cytotoxicity to energy deposition.

A comparison with our previous [^{211}At]At-PSMA5 colony formation assay further highlights that the direct cell-killing potency of [^{211}At]At-FAPI1 is substantially lower than that of a receptor-internalizing tumor-targeted radiopharmaceutical. Whereas [^{211}At]At-PSMA5 reduced colony formation in PSMA-high LNCaP cells with an estimated IC_{50} of approximately 5 Bq/well after only 1 h exposure [21, 22], [^{211}At]At-FAPI1 required activity levels on the order of several kBq/well to induce a comparable cytotoxic response in PANC-1 cells. This difference of roughly two to three orders of magnitude strongly suggests that [^{211}At]At-FAPI1 does not achieve the highly efficient cell-associated α-particle delivery enabled by receptor-mediated internalization.

A rough comparison of in vivo therapeutic efficacy likewise indicates that [^{211}At]At-FAPI1 is less potent than [^{211}At]At-PSMA5 on an administered-activity basis. In a PANC-1 xenograft model, a single 1 MBq administration of [^{211}At]At-FAPI1 produced approximately 60–70% tumor growth suppression [15], whereas [^{211}At]At-PSMA5 induced marked tumor regression in the LNCaP model even at 0.4 MBq, corresponding to an apparent 4- to fivefold higher therapeutic efficiency after normalization by injected activity [22]. Notably, the difference observed in vivo is considerably smaller than the two- to three-order gap in direct in vitro cytotoxic potency, implying that mechanisms other than direct intracellular delivery contribute substantially to the antitumor efficacy of [^{211}At]At-FAPI1 under tumor-bearing conditions.

Consistent with this interpretation, the present study demonstrated a marked dependence of cytotoxicity on exposure duration, indicating that even relatively modest but sustained stromal localization, pericellular retention, or limited cellular association of [^{211}At]At-FAPI1 can increase the cumulative number of α-particle traversals delivered to tumor cells over time. Thus, although the intrinsic direct cell-killing efficiency of [^{211}At]At-FAPI1 is limited, prolonged local retention of the radiopharmaceutical may still provide biologically meaningful cumulative α-particle exposure.

Although the conclusions of the present study are based solely on in vitro observations, they provide a mechanistic framework for interpreting our previously observed in vivo therapeutic effects of [^{211}At]At-FAPI derivatives [15]. The present findings suggest that prolonged tumor-associated retention and sustained α-particle exposure are key contributors to the antitumor efficacy of this class of agents. These insights may aid future molecular optimization of stromal-targeted α-therapy, although additional in vivo pharmacological and translational studies will be required.

Conclusions

In this in vitro mechanistic follow-up study, we investigated the cytotoxicity of free ^{211}At and [^{211}At]At-FAPI1. The radiolabeled compound [^{211}At]At-FAPI1 exhibited markedly stronger cytotoxicity than free ^{211}At under identical conditions, indicating that the biological effect is more consistent with sustained local association of the [^{211}At]At-FAPI1 with target cells than with the behavior of dissociated free radionuclide alone. These results are consistent with our previously reported antitumor effects in mouse models. Importantly, systematic variation of activity concentration and exposure duration suggested that cell viability was not determined by administered activity alone but rather by the rate of α-particle exposure. Consistently, short-term exposure to a high activity concentration resulted in a similar level of cytotoxicity as long-term exposure to a lower concentration, indicating that the α-particle exposure rate correlates closely with cell viability. Overall, this work provides mechanistic, hypothesis-generating insight into the cellular effects of [^{211}At]At-FAPI1 and free ^{211}At and may inform future optimization of FAP-targeted α-particle therapies, while additional in vivo and translational studies will be required to fully establish their therapeutic potential.

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Author's contribution Conceptualization, M.T. and K.F.; precursor synthesis, M.T., A.S., T.T., Y.K. and T.Y.; cellular and animal experiments, M.T., J.U., T.T., K.K.-N.; nuclide labeling, T.T. and Y.S.; chemical analysis, M.T., J.U. and T.T.; Cellular cytotoxicity M.T., J.U., T.T., K.M. and K.K.; writing, M.T., astatine production and purification, K.O., M.M., A.T. and H.H.; supervision, M.T., A.S. and K.F.; and project administration, T.W., F.L.G. and J.C., All authors have read and agreed to the published version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflicts of interest The authors declare no competing interests.

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