Iodine-125 labeled Australian frog tree host-defense peptides caerin 1.1 and 1.9 better inhibit human breast cancer cells growth than the unlabeled peptides. ^{125}I-caerin 1.9 may better be used for the treatment of breast cancer

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Abstract

Objectives: We recently showed that host defense caerin peptides isolated from Australian frog tree were able to inhibit cervical cancer tumour cell growth in vitro. We wished to determine if radioactive isotope iodine-125 (1^{25}I) can be labeled to caerin 1.9 peptide and if this peptide is bioactive for breast cancer cells treatment. **Materials and Methods:** The biological function of caerin (1.1 and 1.9) peptides were investigated by in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The anti-cancer effect of ^{125}I labeled caerin 1.9 was compared with unlabeled caerin 1.9 peptide. The tissue distribution of ^{125}I labeled caerin 1.9 peptide was further studied in mice. **Results:** In the current paper, we demonstrated that caerin peptides (1.1 and 1.9) were separately able to inhibit the viability of two breast cancer cell lines in vitro and this inhibition was more profound when these peptides were simultaneously applied. Moreover, ^{125}I can be stably attached to caerin 1.9 peptide with high efficiency. Iodine-125 labeled caerin 1.9 inhibited breast cancer cells line MCF-7 viability more efficiently than free ^{125}I and also than unlabeled caerin 1.9. Additionally, iodine-125 labeled caerin 1.9 in vivo imaging demonstrated that although slightly, it could be accumulated in tumor tissue. **Conclusion:** Our results from this totally original study indicated that radioactive isotope ^{125}I labeled to caerin peptide 1.9 may be used to treat breast cancer while at the same time the response to treatment may be monitored by simultaneous imaging.

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Introduction

Cancer continues to be a major public health problem worldwide. The incidence of cancer is still rising and it has been estimated that 14 million individuals a year would be diagnosed with cancer in 2012, while a 50% projected increase to 21.6 million a year by 2030 [1]. Thanks to tremendous effort made to better understand tumorigenesis and disease progression, the mortality rates from cancer is decreasing over recent years. However, development and testing of novel therapeutic strategies for malignances remains the most urgent priority, especially for those with advanced, treatment-refractory diseases. Recently, with the successful use of immunotherapy for multiple types of cancer, like immune check point inhibitors, development of immunomodulatory agents to change the immune suppressive tumor microenvironment (TME) become a popular research field [2].

Innate immunity polypeptides have been shown to overcome the immune suppressive TME via a unique cancer cells killing mechanism, possibly involving cell membrane lysis [3-7]. These peptides were initially discovered due to their function in clearing bacteria, while some were also highly active against cancer cells but not normal mammalian cells [8-14].

During the last three decades, more than 200 host-defense peptides have been isolated and identified from skin secretions of Australian tree frogs and toads. Many of these peptides show antimicrobial and/or neuropeptide-type activities [15]. The caerin 1 peptides have previously been shown to be potent membrane-active peptides and to stop the formation of nitric oxide by neuronal nitric oxide synthetase [16, 17]. It has been previously reported that caerin 1.1 (^{1}GLLSVLGSV^{10}ALPHVLP^{20}HVVPVIAEHL-NH_2) has an anti-cancer effect against a number of human cancer cell lines (including leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancers) [16, 17]. The caerin 1.9 peptide (^{1}GLFGVLGSI^{10}ALPHVVP^{20}VIAEKL-NH_2) showed antimicrobial activity against

a wide spectrum of Gram-positive and Gram-negative microbial strains [18]. Recently, it has been demonstrated that caerin 1.1 and 1.9 had a syngenic effect against tumour cells growth in vitro (Ni et al., Comparative Proteomic Study of the Antiproliferative Activity of Frog Host-Defence Peptide Caerin 1.9 and Its Additive Effect with Caerin 1.1 on TC-1 Cells Transformed with HPV16 E6 and E7. BioMed Research International 2018 in press). It has also been found that caerin 1.1 and 1.9 inhibit HIV-infected T cells within minutes post-exposure at concentrations non-toxic to target cells and also inhibit the transfer of HIV from dendritic cells (DCs) to T cells [19].

Radioisotope labeled peptides have been extensively studied for receptor-targeted theragnostics with the successful example of somatostatin receptor targeting peptides for the diagnosis and treatment of neuroendocrine tumors with gallium-68-dotatate and lutetium-177-dotatate, approved recently by Federal Drug Administration. These peptides, showing high specificity and minimal toxicity, can be easily synthesized, modified and easily labeled with imaging and/or therapy-based radionuclides.

In this study, we tested the anti-cancer ability of two Australian tree frog host-defense peptides, caerin 1.1 and caerin 1.9 using two breast cancer cell lines. Caerin 1.9 was further labeled with ^{125}I and its anti-cancer ability was compared with the cold peptide. The tumor cells internalization ability in vitro and the tumor targeting potential in vivo of ^{125}I-caerin 1.9 for breast cancer cells were determined by using cells uptake assay and single photon emission tomography/computed tomography (SPET/CT) imaging, respectively. The authors confirm that this is a totally original study.

Materials and Methods

Cell line and cell culture

Human breast cancer cells lines MCF-7 and Skbr-3 were a gift from Prof. Yaoqi Zhou of Griffith University Australia, purchased from ATCC USA. The two cells lines were cultured following the protocols as suggested in the product sheets. Briefly, the cells were cultured in complete RPMI 1640 media (GIBCO) supplemented with 10% heat inactivated fetal calf serum (FCS, GIBCO), 100U of penicillin/mL, 100μg of streptomycin/ml (GIBCO), 0.2mM non-essential amino acid solution, 1.0mM sodium pyruvate, 2mM L-glutamine, 0.4 mg/mL G418 and were cultured at 37°C with 5% CO_2.

Mouse breast cancer 4T1 cells were cultured in complete RPMI 1640 media (GIBCO) supplemented with 10% heat inactivated fetal calf serum (FCS, GIBCO), 100U of penicillin/mL and 100μg of streptomycin/ml (GIBCO) and were cultured at 37°C with 5% CO_2.

Mouse tumour model

4T1 cells, approximately 70% confluent were harvested with 0.25% trypsin and washed repeatedly with PBS. 4T1 tumour cells (5×10^4/mouse) were injected subcutaneously in the left flank of 4-6 week Nude mice of Balb/c background in 0.05mL of PBS. All mice were bought and were kept in SPF animal facilities at Soochow University. The experiments were approved and performed in compliance with the Animal Ethics committee of Soochow University.

Peptides

Caerin 1.1 (GLLSVLGSVALPHVLPHVVPVIAEHL-NH_2), caerin 1.9 (GLFGVLGSIALPHVVPVIAEKL-NH_2), two control peptides, one isolated from Australia tree frog F4 GLFDVIKKVASVIGGL-NH_2 one randomly designed P3 GTELPSPPSVWFEAEFK-NH_2, were synthesized by Mimotopes Proprietary Limited, Wuxi, China. The purity of the peptides was >95% as determined by reverse-phase HPLC, done at Mimotopes.

Labeling caerin 1.9 (F3) with iodine-125

Iodine-125 was obtained from the daily rinsing of a molybdenum-99/technetium-99m-generator (the China Institute of Atomic Energy) with sterile saline. Radioactivity of ^{125}I was measured after collection, and then ^{125}I was diluted to 370 MBq/mL using sterilization phosphate buffered saline (PBS, 0.01M, pH=0.4). Forty μL (0.4μg/μL) of F3 were added into the mixture solution of freshly prepared iodogen (1mg/mL) and Cl_3CH_4. The volume of the whole reaction was 100μL and adjusted to pH 7.0 with PBS (pH 7.8). Afterward, 20μL (500 μCi/1.85x104kBq) ^{125}I-NaCl solution were added into the solution which was supplemented with proper amount of normal saline to a total reaction volume of 1mL. The reac-tion system was carried out at room temperature for 5min.

Radiochemical purity of ^{125}I-F3

The Sephadex G-25 column (Amersham Phamacia Biotech) was used to separate ^{125}I-F3 from unbound reactants, and then the ^{125}I-F3 was eluted with PBS (0.05M, pH 7.0). The radiochemical purity was evaluated by paper chromatographic system. Briefly, 5μL of ^{125}I-F3 samples were spotted on a 15cm strip of qualitative filter paper as the stationary phase and developed with acetone or normal saline as the mobile phase for 30min. Free ^{125}I will be located at an Rf (retention factors) of 0.9~1.0, and ^{125}I-F3 will be located at an Rf of 0.0-0.1 while the mobile phase is acetone, nevertheless Rf of ^{125}I and ^{125}I-F3 were both equal to 0.9~1.0.

Stability of ^{125}I-F3

To determine labeled product stability, ^{125}I-F3 was incubated with fresh human serum or normal saline at 37°C for 4 hours in a water bath. Eight mL of blood were drawn into eva-cuated blood collection tubes without anticoagulant, and then stored at room temperature for 30min. The evacuated blood collection tube was centrifuged at 1500r/min for 10 minutes. The serum was carefully aspirated from the cells pellet. Fifty μL of ^{125}I-F3 were mixed with 100μL of fresh human blood serum and normal saline, respectively, and then incubated at 37°C. The radiochemical stability of ^{125}I-F3 after 4 hours incubation was measured by instant thin-layer chromatography.

MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (ATCC, USA)

following the manufactured instructions. For unlabeled peptides, 5×103 of MCF-7 were cultured in flat bottomed 96 well plates. Approximately 0-15μg of caerin 1.1 or/and caerin 1.9 peptides were added to 5×10^3 of MCF-7 cells and cultured overnight at 37°C with 5% CO_2. Ten microliters of MTT stock solution were added and cultured for another 4h, before 100 μL of DMSO were added to stop the experiment. Results were analyzed with an ELISA plate reader (BioTek, USA) at 450 nm.

To determine cells viability change after incubation with ^{125}I-F3, logarithmically growing MCF7 cells were inoculated in a 96-well plate at a concentration of 1×10^4 cells per well. Cells were cultured (5% CO_2, 37℃, 24 hours) up to 60%-70% confluence and then cultured in 100μL serum-free medium containing differently concentrated ^{125}I-F3 (radioactivity set at 12, 24, 37, 49 and 62kBq/mL, respectively). Cells cultured in the Na^{125}I containing medium with the same radioactivity were used as control. After incubation for 30 minutes, cells were washed thrice with a serum-free medium, and cultured in a serum containing medium. At 24 and 48 hours post culturing, the MTT solution (5mg/mL, 10μL) was added, followed by 4 hours of incubation. At the end of the incubation period, the supernatant was aspirated carefully and cells were washed thrice with PBS. Then, cells were treated with 100μL of DMSO. Cell survival was determined by detecting the absorbance at 570nm using an enzyme-linked immunosorbent plate reader. Three independent experiments were performed, and the results were used for plotting the relative growth rate with SD.

Iodine-125 intake rate

Logarithmically growing MCF7 cells were inoculated in a 96-well plate at a concentration of 1×10^5 cells per well. Cells were cultured (5% CO_2, 37℃, 24 hours) up to 60%-70% confluence and then cultured in 100μL serum-free medium containing differently concentrated ^{125}I-F3 (radioactivity set at 12, 24, 37, 49, 62kBq/mL, respectively). Positive control cells were cultured in the Na^{125}I containing medium with the same radioactivity, while equal volumes of cell-free medium were used as blank controls. After incubation for 16 hours, cells were washed three times with PBS and collected to a special tube for the radioactivity or to the surfactant for Liquid scintillation counter and gamma counter in order to measure, cells absorb of Na^{125}I and the radioactive ^{125}I-F3, respectively. Intake rate of the MCF7 cells was calculated as: intake rate=cells radioactive (cpm)/the total radioactive (cpm)x100%.

^{125}I-F3 micro-SPET imaging and biodistribution

When the tumors reached an average volume of ~50-60mm^3, the nude mice were divided into three groups, each group have four parallel samples. The mice in group A were injected with 100μCi of Na^{125}I and group B with 100μCi of ^{125}I-F3. All mice were imaged at 2h after injection by SPET/CT. Tumors were isolated and scanned by SPET again. Regions of interest (ROI) were drawn in the tumor (T) and in normal tissues (NT) and then the T/NT radioactive ratio was calculated.

For group C, potassium iodide pills are given to saturate the thyroid gland and prevent the uptake of radioactive iodine. Two hundred μCi/200μL ^{125}I-F3 was injected through tail vein and mice were sacrificed respectively at 1, 2, 4, 8, 24, 48h hours post injection. Accordingly, main visceral organs and tumor tissues were weighed and the radioactivity counts rate was measured. These data were required to calculate the %ID/g.

Results

Caerin 1.1 and caerin 1.9 inhibit breast cancer cells growth in vitro

Previously, it was demonstrated that caerin 1.1 and caerin 1.9 were able to inhibit cervical cancer cells growth (TC-1 cells and Hela cells), but not normal cell growth in vitro, and the inhibition was more pronounced when the two peptides were applied in conjunction with each other (Ni et al., Comparative Proteomic Study of the Antiproliferative Activity of Frog Host-Defence Peptide Caerin 1.9 and Its Additive Effect with Caerin 1.1 on TC-1 Cells Transformed with HPV16 E6 and E7. BioMed Research International 2018 in press). Now we demonstrated that caerin 1.1 and caerin 1.9 were able to inhibit cell growth of two human breast cancer cells lines MCF-7 and Skbr-3 in vitro. Both caerin 1.1 and caerin 1.9 were able to inhibit the breast cancer cells growth at 10μg/mL, although caerin 1.9 was more potent at inhibiting the tumour cell growth (Figure 1). The two control peptides, F4 and P3 were unable to inhibit the tumour cells growth even at 20μg/mL. Moreover, when the caerin 1.1 and caerin 1.9 were used together, the breast cancer cell growth was inhibited at 5μg/mL (Figure 2).

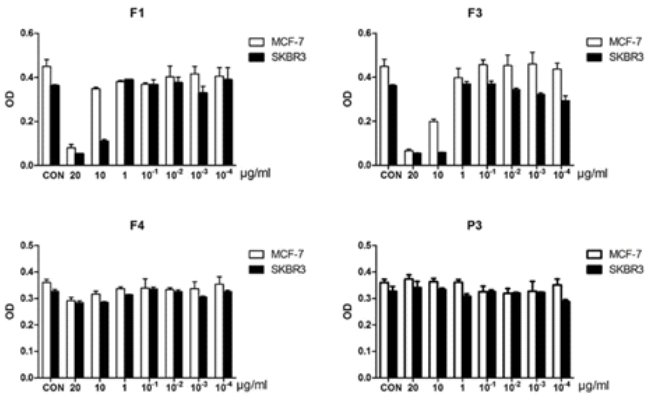

Figure 1. Cell viability evaluated by MTT assay after incubation with different peptides under different concentrations.

^{125}I-F3 radiochemical purity valuation

Next, we studied whether ^{125}I can be labeled to the caerin peptides. F3 was successfully labeled with ^{125}I (Figure 3a) and paper chromatography was used to separate ^{125}I-F3 from the labeled reaction mixture to estimate the labeling rate and radiochemical purity (RCP). Acetone was used during the mobile phase, radioactivity of qualitative filter paper (Rf from

0.1 to 1.0) was measured by a radioactivity meter. The results showed that the labeling rate was 83.33% and the radiochemical purity was 99.63%. Therefore, the specific activity of the labeled compound was 1.38MBq/mg by calculation (118.4 MBq*87.6%/74.8mg).

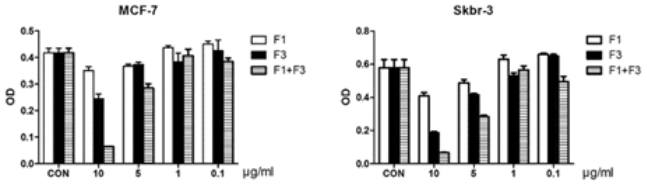

Figure 2. Cell viability evaluated by MTT assay after incubation with F1, F3 and F1+F3 under different concentrations.

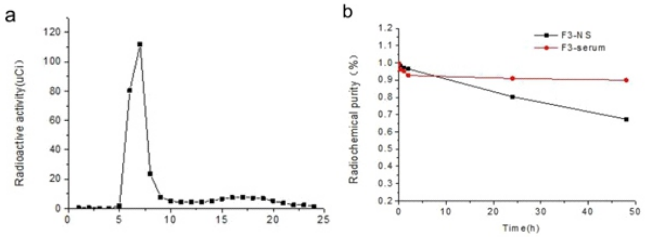

Figure 3. Radiochemical characteristics of ^{125}I-F3. a) Elution curve of ^{125}I-F3; b) Radioactive stability of ^{125}I-F3.

^{125}I-F3 stability assessment

The stability of ^{125}I-F3 was assessed by incubating ^{125}I-F3 in fresh human serum and saline at 37°C. We measured RCP by paper chromatography. After incubation for 4h, the remained ^{125}I-F3 were still greater than 97% either in saline or/ and fresh human serum (Figure 3b).

^{125}I-F3 inhibits breast cancer cells viability

First, we compared the ^{125}I uptake by MCF-7 cells treated with free ^{125} I or ^{125}I-F3, respectively. As shown in Table 1, the uptake of ^{125}I is always higher in MCF-7 cells treated with ^{125}I-F3 than ^{125}I.

The cell proliferative ability of the MCF-7 cells was decreased in a time-dependent manner. When the radioactivity of ^{125}I-F3 was 500kBq/mL, the inhibitory effect of incubating time of 48 hours was more significant than 24 hours (Figure 4A). Cell viability of MCF-7 cells treated with ^{125}I-F3 was decreased compared with Na^{125}I treated and untreated cells in a time-dependent manner (Figures 4B, C). When the concentration of F3 peptide was 3x10^{-3}μg/mL, if the F3 peptide concentration would be the same situation, ^{125}I-F3 had more significant inhibitory effect of the breast cancer cells in vitro. In another experiment, the MCF-7 cell viability was accessed by MTT assay after the cells were treated with ^{125}I-F3 and unlabeled F3. As shown in Table 2, ^{125}I-F3 was able to inhibit the viability of MCF-7 even at 3×10^{-3}μg/mL, significantly higher than unlabeled F3, which usually inhibit the viability of MCF-7 at 5-10μg/mL.

Table 1. *MCF7 cellular uptake of Na^{125}I and ^{125}I-F3 (cpm/10^{5}cells X±SD).*

Drug	Drug radioactivity concentration (kBq/mL)				
	12	24	37	49	62
Na^{125}I	2120± 137	4128± 429	6169± 375	7369± 163	7812± 174
^{125}I-F3	4623± 267	10396± 842	16197± 994	20133± 815	25254± 254

^{125}I or ^{125}I labeled caerin 1.9 at 12, 24, 37, 49, 62kBq/mL, respectively. Na ^{125}I represents sodium iodine-125, ^{125}I-F3 represents sodium iodine-125 labeled caerin 1.9.

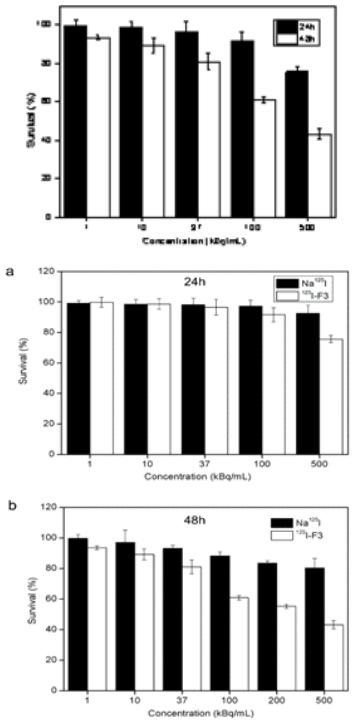

Figure 4. Cell viability evaluated by MTT assay after incubation with ^{125}I-F3 under different concentrations for 24h and 48h.

^{125}I-F3 in vivo imaging and biodistribution

^{125}I-F3 in vivo SPET imaging demonstrated that although slightly, ^{125}I-F3 could be accumulated in tumor tissue. Biodistribution data showed that the maximum tumor uptake appeared at 2 hours post i.v injection of the tracer. The peak of tumor to blood ^{125}I-F3 uptake rate appeared at 48 hours post injection, which is different from all other organ and tissues, which peak at 24 hours (Figure 5A, B).

Discussion

In this study, we found that two Australian tree frog host de-

fense peptides, caerin 1.1 and caerin 1.9 could inhibit human breast cancer cells viability in vitro and these two peptides showed synergic anti-cancer effect. Caerin 1.9 was successfully labeled with ^{125}I and its anti-cancer ability was increased when compared with the cold peptide. In vitro cell uptake assay showed ^{125}I-caerin 1.9 could be internalized by breast cancer cells and SPET/CT imaging further proved that ^{125}I-caerin 1.9 could bind to tumor tissue in vivo. These properties of ^{125}I-caerin 1.9 made it a promising novel probe for cancer imaging, diagnosis and treatment.

Table 2. *^{125}I-F3 inhibits MCF7 cells growth(survival fraction % X±SD)*

Incubation time (h)	Drug concentration (μg/mL)	MCF7 cells	
		^{125}I-F3	F3
24h	$1x10^{-4}$	99.97±0.23	99.98±0.26
	$3x10^{-4}$	99.73±0.08	99.98±1.27
	$1x10^{-3}$	99.60±0.29	99.88±1.03
	$3x10^{-3}$	90.54±2.62	96.83±2.41
	$1x10^{-2}$	51.42±1.73	95.61±3.69
48h	$1x10^{-4}$	99.73±0.23	99.99±0.08
	$3x10^{-4}$	98.82±1.17	99.95±0.96
	$1x10^{-3}$	96.15±1.63	99.16±0..13
	$3x10^{-3}$	66.27±1.64	92.54±3.06
	$1x10^{-2}$	40.23±7.26	91.39±3.51

h: hour; ^{125}I-F3: Iodine-125 labeled caerin 1.9; F3: caerin 1.9

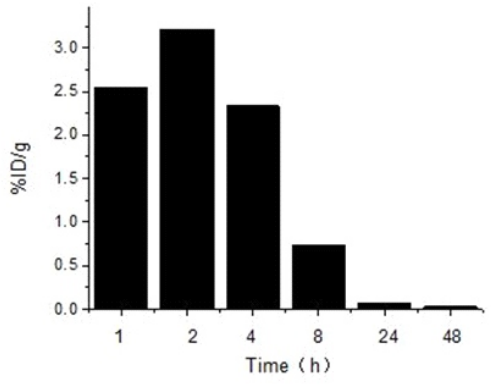

Figure 5a. The tumor tissue uptake of ^{125}I-F3 was the maximum radiation dose in 2 hours after the i.v. injection.

Caerin peptides is the largest group of antibacterial amphibian peptides isolated to date. It has been reported that more than 30 peptides were identified from over six Australian tree frog species of the Litoria genus [18]. Most of these host defence peptides like the ones we studied here, caerin 1.1 and caerin 1.9, show multifaceted activity including antimicrobial, fungicide activity and neuronal nitric oxide synthetase inhibition. Additionally, accumulating evidence has indicated that some host-defence peptides could be applied in cancer therapy. Recently, Li et al. (2016) reported that they isolated Xenopus laevis antibacterial peptide-P1 (XLAsp-P1) from the skin of Xenopus laevis. This small molecular peptide showed potent inhibitory activity against breast cancer cells with a concentration dependent manner starting from the lowest concentration of 5μg/mL [20]. Here, we confirmed the anti-cancer ability of caerin 1.1 and caerin 1.9 and found that these two peptides showed synergic effect against human breast cancer cells in vitro.

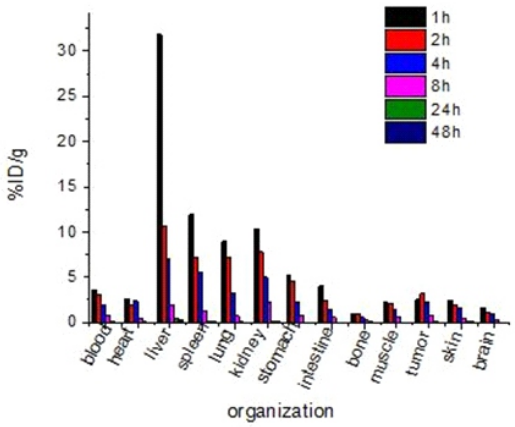

Figure 5b. In vivo tissue distribution results showed that ^{125}I-F3 could be accumulated in tumor tissue slightly.

As mentioned previously, lots of peptides have been studied and labeled with imaging/therapy-based radionuclides to test their theragnostic potential for different kinds of malignancies. Mostly, these peptides are belonging to tumor-targeting peptides and treatment strategies based on are referred to peptide receptor radionuclide therapy (PRRT). Here, we successfully labeled the host-defence peptide, caerin 1.9, with radioiodine and demonstrated that ^{125}I-caerin 1.9 had better anti-cancer effect comparing to unlabeled caerin 1.9. This result firstly provided the insight that host-defence peptide labeling with imaging and/or therapy based radionuclides could be a promising cancer theragnostic strategy by combining cancer cells toxicity from itself and radiation damage from the labeled isotope.

The anti-cancer effect of these antimicrobial peptides (A-PM) has been frequently reported, however, the underlining mechanism is still not fully understood. Immunomodulatory effect of AMP was proposed as they were increasingly recognized to interact with host cells by influencing diverse signaling cascades during infection process [2]. For example, β-defensins, one kind of small, cationic, host-derived AMP, also act as a ligand for the CCR6 and CCR2 chemokine receptors to induce chemotactic activity of lymphocytes [21]. These eviden-

ces indicate that APM may act as a bridge between the innate and adaptive host immunity to enhance host-defence effect to pathogens. It is widely accepted that immune suppression exists in the tumor microenvironment. This fact makes immunotherapy like immune check point inhibitors a great success in cancer treatment. Based on above, whether host-defence peptides like caerin 1.1 and caerin 1.9 can act as a immunomodulator to change the immune suppression status of TME and therefore increase the therapeutic effect of immunotherapy need intensive studies to prove.

In conclusion, our study confirmed the anti-cancer effect of caerin 1.1 and caerin 1.9 and these two peptides showed synergic effect against human breast cancer cells in vitro. We also found that ^{125}I-caerin 1.9 could bind to human breast cancer cells in vitro and in vivo. Iodine-125-caerin 1.9 showed better tumor cells viability inhibition effect than unlabeled caerin 1.9. Our results indicated that ^{125}I-caerin 1.9 could be a promising novel probe for cancer theragnostic.

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The authors declare that they have no conflicts of interest.

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