Investigating the theragnostic potential of ^{131}I-caerin peptide in thyroid cancer

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Abstract

Objective: Caerin is a new peptide with tumour toxicity and its uptake by tumour cells is independent of the sodium iodide symporter (NIS). Thyroid cancer is the most common cancers of endocrine malignancy. Radioiodine (^{131}I)-refractory thyroid cancer is the most lethal subtype of the thyroid cancers and remains a clinical challenge. In the current study, we investigated the ^{131}I radiolabeling efficiency of Caerin and the effects of Caerin, ^{131}I-Caerin and free ^{131}I on differentiated and undifferentiated human thyroid cancer cell lines (B-CPAP and CAL-62) in vitro. **Materials and Methods:** Cell Counting Kit-8 was used to assess the cytotoxic effect of Caerin, ^{131}I-Caerin and free ^{131}I on B-CPAP and CAL-62 cells. Laser scanning confocal microscope was exploited to evaluate the uptake and internalization of Caerin by thyroid cancer cells. The Chloramine-T method was used to label the peptide with ^{131}I. And the stability and water partition coefficient (Log P) of ^{131}I-Caerin were studied. **Results:** Our results demonstrated that Caerin and ^{131}I-Caerin could be accumulated by B-CPAP and CAL-62 cells, resulting in killing of the thyroid cancer cells in vitro. The efficacy of ^{131}I-Caerin is much higher than ^{131}I, especially to undifferentiated CAL-62 cells. The results prove the feasibility of radioiodination of the ^{131}I-Caerin via the Chloramine-T method. Moreover, the result indicate the hydrophobic ^{131}I-Caerin was stable in 72 hours. **Conclusion:** Iodine-131-Caerin can inhibit the cell viability of thyroid cancer and hold certain promise as a theragnostic tool for human thyroid cancers.

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Introduction

Caerin is a new peptide extracted from Australian tree frogs and toads. Its uptake is independent of the sodium iodide symporter (NIS) and is probably mediated by receptors expressed by tumor cells. More importantly, Caerin could inhibit the growth of tumor cells [1-2]. Caerin could suppress the growth of bacteria and tumor cells [3], exerting no inhibitive effect on mammalian cells with similar doses [4-7]. Caerin peptide is able to modulate several immune-related proteins of signaling pathways, such as Tec kinase and ILK signaling pathway. Moreover, Caerin could activate the inflammatory signaling pathways of tumor cells and further lead to secretion of several inflammatory factors, such as TNFα, IL-1β, IL-6, which in turn promote inflammation and accelerate host anti-tumor effects [8-9]. Our previous study showed the feasibility of radiolabeling a peptide among Caerins with (iodine-125) ^{125}I, and ^{125}I-Caerin could inhibit the growth of breast cancer cells more efficiently when compared to free ^{125}I [10].

Thyroid cancer is the most frequent malignancy of the endocrine system, of which differentiated thyroid carcinoma (DTC) accounts for more than 90% of all the thyroid cancer cases [11]. Although the 5-year survival rate of DTC is above 90%, the outcome of anaplastic thyroid cancer (ATC) is dismal owing to the aggressiveness [12-13]. In addition, about two thirds of metastasis DTC patients have degenerative changes in the shape and function of tumor cells during their natural course of disease or treatment, and lose the ability to concentrate iodine, which eventually develop into radioiodine-refractory DTC (RAIR-DTC) and thus become ineffective for radioactive iodine treatment [14-15]. Radioiodine-refractory-DTC progresses rapidly and has a high mortality rate, and has been a research hotspot to explore the effective treatment of RAIR-DTC [16]. As recommended by the 2015 American Thyroid Association (ATA) guideline, patients with RAIR-DTC are no longer suitable for radioiodine treatment, in other words, RAIR-DTC patients will not benefit from radioiodine treatment since the diseases lost the ability to concentrate radioactive iodine [17]. In recent years, there is plenty of progress regarding the molecular pathogenesis of thyroid cancers, and molecular targeted therapies using small molecules are changing the therapeutic landscape of RAIR-DTC. Despite this prog-

ress, molecular targeted therapies have varying degrees of adverse reactions [16]. Therefore, there is an urgent need to explore new therapeutic strategies for RAIR-DTC.

Tumor immunotherapy is an emerging option for treating tumors by selectively killing tumor cells. It has been reported that the microenvironment of thyroid cancers is enriched in immune cells, potentially subjecting thyroid cancer as another tumor type suitable for immunotherapy [18]. In this study, we used Caerin (GLLSVLGSVAKHVLPHVLPHVVPVIAEHL-NH) as a vector for developing theranostic approaches for thyroid cancer. Caerin contains histidines and is suitable for ^{131}I radiolabeling via the Chloramine-T method. After successful conjugation of ^{131}I-Caerin, we further assess the theragnostic potential of ^{131}I-Caerin in thyroid cancers.

Materials and Methods

Cell lines and cell culture

The papillary thyroid cancer (PTC) cell line B-CPAP and the anaplastic thyroid cancer (ATC) cell line CAL-62 were kindly provided by Stem Cell Bank, Chinese Academy of Sciences, and the cells were cultured as suggested. Briefly, the culturing medium of B-CPAP contains the following components: 87% RPMI Medium 1640 (GIBCO), 10% heat inactivated fetal bovine serum New Zealand Origin (FBS, CORNING), 0.1% penicillin-streptomycin, Liquid (GIBCO), 1% MEM non-essential amino acids (NEAA, GIBCO), 1% Glutamax (GIBCO), 1% sodium pyruvate (GIBCO). The culturing medium of CAL-62 contains the following components: 90% DMEM medium (GIBCO), 10% heat inactivated fetal bovine serum New Zealand Origin (FBS, CORNING), 0.1% penicillin-streptomycin, Liquid (GIBCO). Both the two cell lines were cultured at 37°C in an incubator supplemented with 5% CO_2.

Peptides

Caerin (GLLSVLGSVAKHVLPHVLPHVVPVIAEHL-NH2) was originally extracted from the Australian tree frogs. A P3 peptide (GTELPSPPSVWFEAEF) was designed as the control and non-specific peptide. Both the two peptides were synthesized by China Peptides Co. Ltd. and the purity of the peptides was >95% as determined by Reverse-Phase HPLC. Caerin and P3 were dissolved in phosphate buffer solution (PBS) with varying concentrations (10mg/mL, 1mg/mL, and 0.1mg/mL) and stored at -20°C.

Cell proliferation assessment

B-CPAP cells and CAL-62 cells viability were determined by Cell Counting Kit-8 (CCK-8, DOJINDO) test following the manufactured instructions. Briefly, logarithmically growing B-CPAP cells or CAL-62 cells were plated in 96-well cell culture plate (EPPENDORF) at a concentration of $5x10^3$/100μL. Four duplicates for each sample was set. The plated cells were cultured for 24 hours to reach a confluence of 60%-70%, followed by addition of Caerin or P3 peptides with increasing concentrations (0, 1.25, 2.5, 5, 7.5, 10, 12.5, 15, 17.5μg/mL and cultured for another 24 hours). Ten μL CCK-8 was added to the manipulated cells and the cells were cultured for another 4-6 hours. Cell survival was determined by absorbance (OD) at 450nm using an enzyme linked immunoassay.

Determination of IC_{50} of Caerin

The same CCK-8 was used to determine the half maximal inhibitory concentration (IC_{50}) of the chosen peptide Caerin. B-CPAP or CAL-62 cells were plated ($5x10^3$/100μL) into 96-well cell culture plate (EPPENDORF) and were cultured as indicted (37°C, 5%CO_2, 24h). Four duplicates for each sample was set. Caerin of varying concentrations (0, 1.25, 2.5, 5, 10, 20μg/mL) were added and the cells were cultured for 24 hours. Cell survival was determined by absorbance (OD) at 450nm using an enzyme linked immunoassay, and the IC_{50} was calculated with GraphPad Prism 6 software.

Laser scanning confocal microscope imaging

In order to observe the uptake of Caerin peptide by thyroid cancer cells, Caerin-FITC and P3-FITC were synthesized (Wuxi Mimotopes Peptides Company). B-CPAP cells and CAL-62 cells were respectively inoculated on eight-well chamber culture slides (EPPENDORF) and the cells were cultured (37°C, 5% CO_2, 24h) with FBS free medium for adherent treatment. Caerin-FITC and P3-FITC in the control group were added to each cell sample according to the determined IC_{50} concentrations (5ug/mL for B-CPAP cells and 10ug/mL for CAL-62 cells) as the final concentration. The cells were incubated in dark for 2 hours. The supernatant was then absorbed and discarded, and the cells were gently washed with appropriate wash buffer (90% PBS + 10% FBS free medium) for about 4-5 times. The cells were then mounted with anti-fading Mounting Medium with DAPI (Solarbio) and were covered by cover glasses overnight at 4°C. In confocal imaging, fluorescence signals of FITC and DAPI were detected with excitation light at wavelengths of 405nm and 488nm, respectively. Confocal laser scanning (LSM 880 Basic Operation) was performed on cells to detect the uptake of Caerin polypeptide by thyroid cancer cells.

Radiolabeling of Caerin peptides with ^{131}I

In this study, we used the Chloramine-T method to directly radiolabel Caerin peptides with Na^{131}I. The freshly-prepared Chloramine-T (MACKLIN) was diluted to 1mg/mL and was kept in dark place. Forty μL (1mg/mL) of Caerin was added into 0.5mL EP tube, followed by addition of 100μL of Na^{131}I solution (1mCi/$3.7x10^4$KBq) and the Chloramine-T solution. The total volume of the reaction was 240μL and the mixture was left oscillating at room temperature (25°C) for 30min.

Labeling rate of ^{131}I-Caerin

The labeling rate was evaluated by paper chromatographic system. Briefly, 1μL of ^{131}I-Caerin sample was spotted on a 1×12cm strip of chromatography paper as the stationary phase and developed with normal saline as the mobile phase. After the mobile phase is completed, the paper was cut off with every 1cm and the radioactive counts were then measured step by step. Three independent experiments were performed, and the GraphPad Prism 6 software was

used to draw γ counting curve to the labeling rate.

Stability of ^{131}I-Caerin

The stability of ^{131}I-Caerin was determined at different temperatures (25°C and 37°C) and different media (FBS and Normal Saline). Samples were taken at different time points (0h, 12h, 24h, 48h, and 72h) and the stability of the radiopharmaceutical was determined by measuring the radiochemical purity (RCP). The RCP were calculated by using GraphPad Prism 6 software.

Determination of Log P

To investigate the hydrophilicity and lipophilicity of the developed ^{131}I-Caerin, 500μL of n-octanol, 500μL of Normal Saline and 40μL of ^{131}I-Caerin were added into 1.5mL centrifugal tube and the sealed tube was vibrated for 2min and then centrifuged for 5min (4000rpm/min), resulting in equilibrium state between n-octanol and Normal Saline. Samples were taken from the organic phase and aqueous phase and the radioactive counts were counted.

Cell proliferation and cytotoxicity assays

B-CPAP cells or CAL-62 cells were plated into a 96-well cell culture plate (EPPENDORF) at a concentration of 7x10^3/100 μL, and cells were cultured (5%CO_2, 37°C, 24 h). Three duplicates for each sample were set. Iodine-131-Caerin and free ^{131}I- of increasing radioactivity (0, 2000, 4000, 8000, 16000 KBq/mL) were added and the cells were cultured for 24 hours. Ten μL of CCK-8 was then added to each sample and the manipulated cells were cultured for another 4-6 hours. Cell survival was determined by absorbance (OD) at 450nm using an enzyme linked immunoassay.

Statistical analysis

The Graphpad Prism 6 software was used to carry out statistical analyses and the differences between groups were determined by ANOVA. P<0.05 was considered as statistically significant of the analyzed data.

Results

Caerin suppressed the growth of human thyroid cancer cells in vitro

We previously reported that Caerin peptide could inhibit the proliferation of Hela cells, TC-1 cells, MCF-7 cells and SKBR-3 cells [10], and therapeutic dose of Caerin had no obvious killing effect on normal mammalian cells [4-7] . In this study, we demonstrated that when compared to the control group (0μg/mL of Caerin), 2.5μg/mL of Caerin and 5μg/mL of Caerin efficiently prohibited the growth of B-CPAP cells and CAL-62 cells (P<0.01), with the survival rates of the two cell lines were 84.98%±2.95% and 86.84%±4.99%, respectively. The anti-cancer ability of Caerin increased as the concentration increased (Figure 1). Within the tested concentrations (2.5μg/mL-15μg/mL), Caerin had different inhibitory effects on B-CPAP cells and CAL-62 cells. That is to say, Caerin inhibited the growth of B-CPAP cells more thoroughly than its inhibitory effect on CAL-62 cells (P<0.05, Figure 1C). However, when the concentration of Caerin reached 17.5μg/mL, all these two cell lines lost their viability without any statistical difference (P>0.05, Figure 1C). The survival rates for the two cell lines were 0.55%±0.23% and 4.81%±1.09%, respectively. In comparison, P3 peptide did not show any suppressive effects on the growth of B-CPAP cells and CAL-62 cells even at higher concentrations (15μg/mL and 17.5μg/mL) (P>0.05, Figure 1A and B) and the survival rates for the two cell lines were 98.18%±3.39% and 95.88%±2.68%%, respectively.

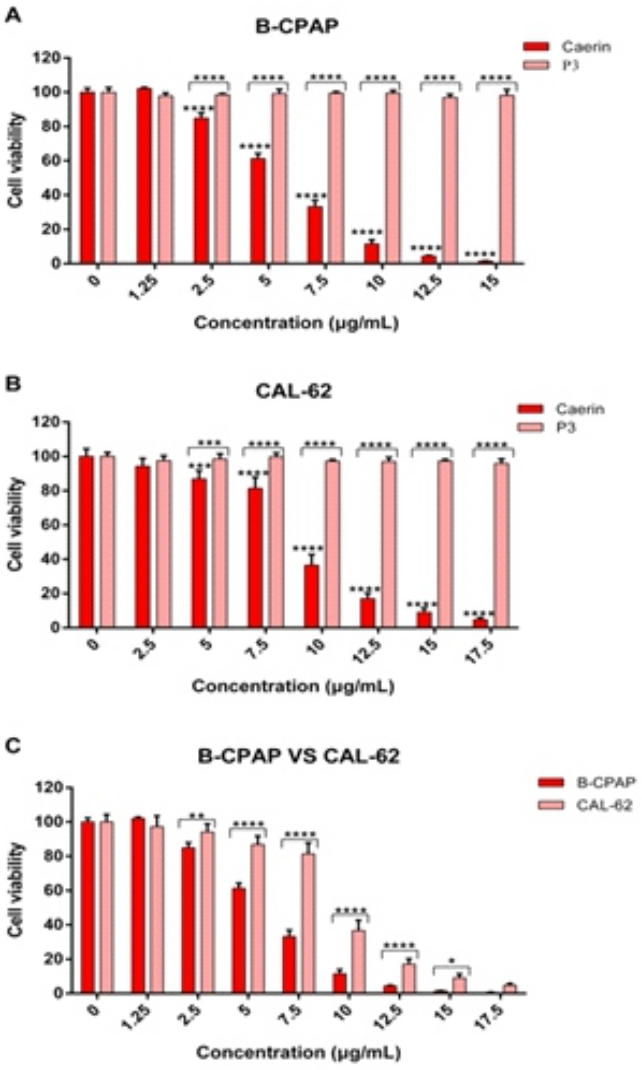

Figure 1. (A, B, C) Survival rate of B-CPAP and CAL-62 cells after treating with Caerin or P3 peptide for 24 h (n=4, mean±SD). *means statically significant, (P<0.05);** (P<0.01);***(P<0.001);****(P<0.0001).

5×10^3/100 μL of B-CPAP or CAL-62 cells were cultured either untreated, or added with different concentrations (0-17.5μg/mL) of Caerin or control peptides overnight before CCK-8 test was performed as described in Materials and Methods. Each bar represents the statistical mean from four replicates and the error bars represent the standard deviation. A: B-CPAP cells, B: CAL-62 cells, C: B-CPAP cells and CAL-62 cells.

IC_{50} of Caerin

Next, we investigated the IC_{50} of Caerin in inhibiting B-CPAP and CAL-62 cells, and the determined IC_{50} values for B-CPAP and CAL-62 cells were 4.038μg/mL and 9.856μg/mL (Figure 2), respectively. The relatively lower IC_{50} for B-CPAP cells further consolidated our above CCK-8 findings, Caerin could more efficiently suppress B-CPAP cells than CAL-62 cells.

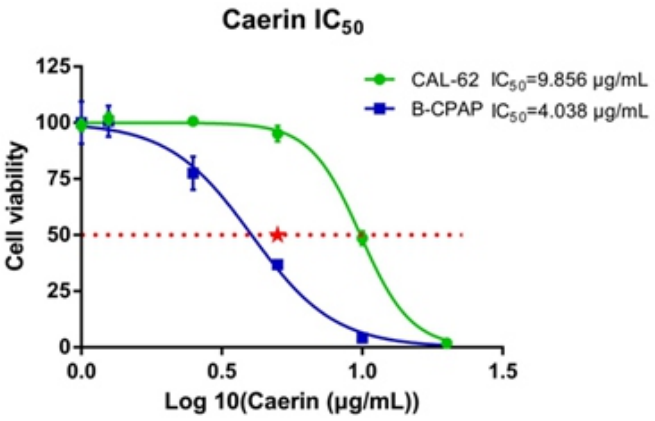

Figure 2. IC_{50} of Caerin peptide for B-CPAP and CAL-62 cells for 24 h (n= 4, mean ± SD). IC_{50}, half maximal inhibitory concentration.

5×10^3/100 μL of B-CPAP or CAL-62 cells were cultured either untreated, or added with different concentrations (0, 1.25, 2.5, 5, 10, 20μg/mL) of Caerin or control peptides overnight before CCK-8 test was performed as described in Materials and Methods. Each point represents the statistical mean from four replicates and the error bars represent the standard deviation. IC_{50} was calculated using a prism software as described in Materials and Methods.

Laser scanning confocal microscope imaging

Two hours after incubation of Caerin-FITC with B-CPAP cells and CAL-62 cells, substantial signal was observed in the cytoplasm of B-CPAP and CAL-62 cells (Figure 3), and DAPI indicated the signal from nucleus. In comparison, no obvious fluorescent signal was seen in the cytoplasm of B-CPAP and CAL-62 cells in which P3-FITC was added.

1×10^5/500μL of B-CPAP or CAL-62 cells were respectively inoculated on eight-well chamber slide overnight, and then either untreated, or added with Caerin-FITC (5ug/mL for B-CPAP cells and 10ug/mL for CAL-62 cells) or control peptides-FITC (P3-FITC) for 2 hours, after intensive washing with wash buffer, the cells were stained with DAPI and were covered by cover glasses overnight at 4°C, seal with tinfoil before examining whether Caerin enter the tumour cells using a confocal microscope.

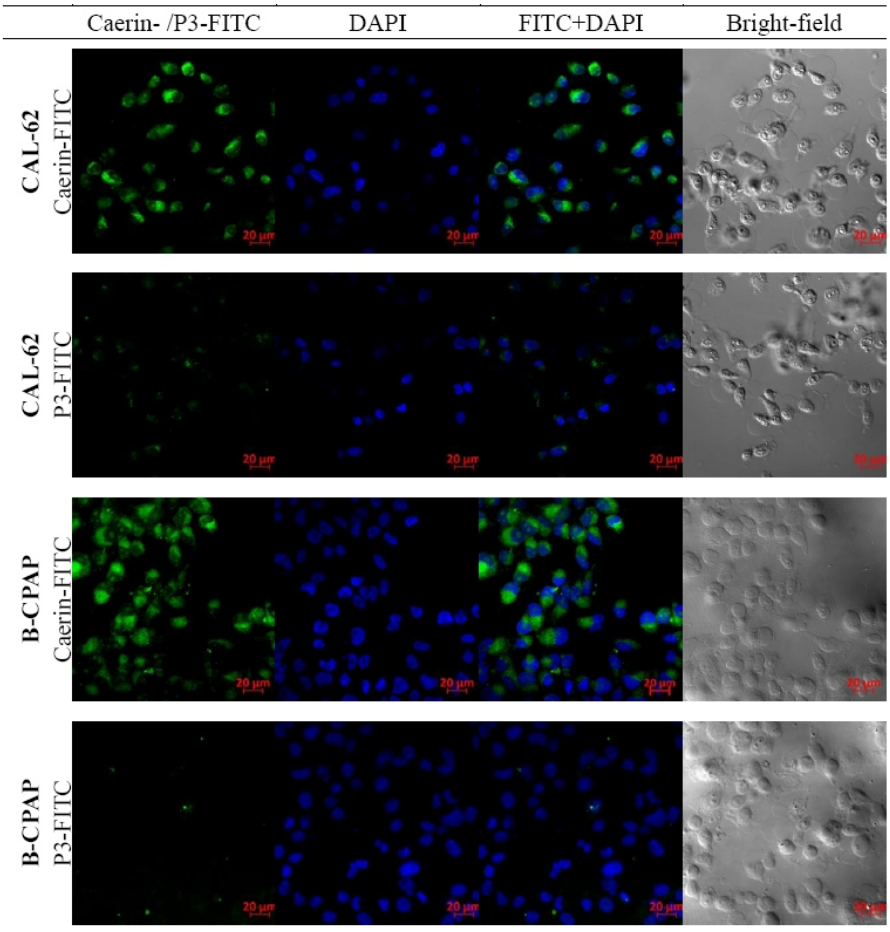

Figure 3. Confocal microscope imaging of CAL-62 and B-CPAP cells incubating with FITC conjugated Caerin and P3 respectively.

Labeling rate of ^{131}I-Caerin

Next, we investigated the IC_{50} of Caerin in inhibiting B-CPAP and CAL-62 cells, and the determined IC_{50} values for B-CPAP and CAL-62 cells were 4.038μg/mL and 9.856μg/mL (Figure 2), respectively. The relatively lower IC_{50} for B-CPAP cells further consolidated our above CCK-8 findings, Caerin co-uld more efficiently suppress B-CPAP cells than CAL-62 cells.

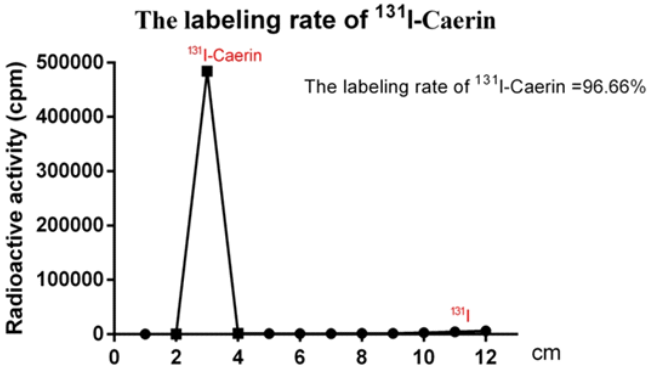

Figure 4. Radiochemical characteristics of ^{131}I-Caerin.

One μL of ^{131}I-Caerin samples were spotted on a 1×12cm strip of chromatography paper as the stationary phase and developed with normal saline as the mobile phase. After the mobile phase, the paper was cut off with every 1cm and the radioactive counts were then measured step by step.

Stability of ^{131}I-Caerin

When ^{131}I-Caerin was left for 72 hours at room temperature, the determined RCP was 85.76%±14.80%, and the corresponding value was 77.35%±13.24% when the radiopharmaceutical was left at 37°C for 72 hours. When ^{131}I-Caerin was incubated in FBS for 72 hours at room temperature (25°C), the determined RCP was 92.01%±8.77%, and the corresponding value was 90.24%±8.59% when the radiopharmaceutical was incubated in FBS at 37°C for 72 hours. When ^{131}I-Caerin was incubated in normal saline for 72 hours at room temperature (25°C), the determined RCP was 91.41%±8.76%, and the corresponding value was 91.33%±5.40% when the radiopharmaceutical was incubated in normal saline at 37°C for 72 hours. These results together demonstrate the excellent stability of ^{131}I-Caerin (Figure 5).

The stability of ^{131}I-Caerin were measured by the RCP at FBS and NS and different temperatures (room temperature 25°C (A) or 37°C (B)) for 0, 12, 24, 48 and 72h, respectively. Each bar represents the statistical mean from three replicates and the error bars represent the standard deviation.

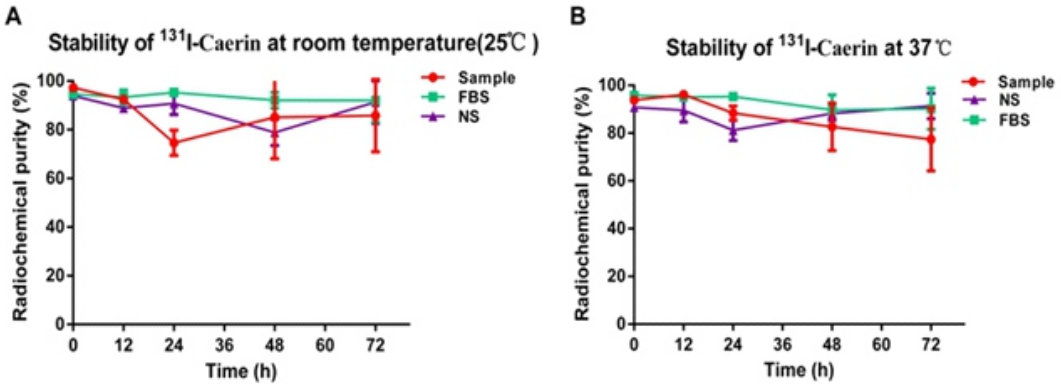

Figure 5. (A) In vitro stability of ^{131}I-Caerin in normal saline (NS) and fetal bovine serum (FBS) at room temperature(25°C) for 12, 24, 48 and 72h (n=3, mean±SD). (B) In vitro stability of ^{131}I-Caerin in NS and FBS at 37°C for 12, 24, 48 and 72h (n=3, mean±SD).

Lipo-hydro partition coefficient

Log P is a factor indicating solubility of ^{131}I-Caerin either in organic phase or in aqueous phase. The higher the Log P, the easier solubility of ^{131}I-Caerin in organic phase (Table 1). The calculated Log P=0.827±0.036 (n=3), indicating the developed ^{131}I-Caerin is lipophilic.

^{131}I-Caerin inhibited growth of thyroid cancers in vitro

We then moved to investigate the anti-cancer ability of ^{131}I-Caerin in two thyroid cancer cell lines, B-CPAP and CAL-62. We found ^{131}I-Caerin suppressed the proliferation of B-CPAP and CAL-62 cells in vitro. To be specific, when compared to the control group (0KBq/mL), 2000KBq/mL and 4000KBq/mL of ^{131}I-Caerin inhibited the growth of B-CPAP cells and CAL-62 cells (P<0.001), with the survival rates of 84.17%±4.48% and 64.24%±3.23%, respectively. Moreover, as the dose of ^{131}I-Caerin increased, the inhibitory effect increased as well (Figure 6A and B). When 2000KBq/mL-4000KBq/mL of ^{131}I-Caerin was incubated with the cells, the inhibitory effects were different for B-CPAP cells and CAL-62 cells (P< 0.01, Figure 6C), and ^{131}I-Caerin suppressed B-CPAP cells more efficiently than its effect on CAL-62 cells. However, when the radioactive dose of ^{131}I-Caerin reached 8000KBq/mL, all the B-CPAP and CAL-62 cells died with the survival rates of 8.96%±0.21% for B-CPAP cells and 9.16%±2.23% for CAL-62 cells, and there was no statistical difference between these two survival rates (P>0.05, Figure 6C). Moreover, only higher dose of free ^{131}I- (8000-16000KBq/mL) showed inhibitory effect on B-CPAP and CAL-62 cells (P<0.01, Figure 6A and B), with the survival rates of 84.62%±3.47% and 87.84%±3.08%, respectively.

Table 1. *The lipo-hydro partition coefficient of ^{131}I-Caerin.*

NO.	The organic phase-base (CPM)	The aqueous phase-base (CPM)	Log P (Mean±SD)
1	3561067	569715	0.827±0.036
2	3354493	455777	
3	3564733	541657	
Mean	3493431	522383	

500 μL of n-octanol, 500μL of Normal Saline and 40 μL of ^{131}I-Caerin were added into 1.5 mL tube and was vibrated for 2 min and then centrifuged for 5 min (4000 rpm/min). Samples were taken from the organic phase and aqueous phase and the radioactive counts were counted

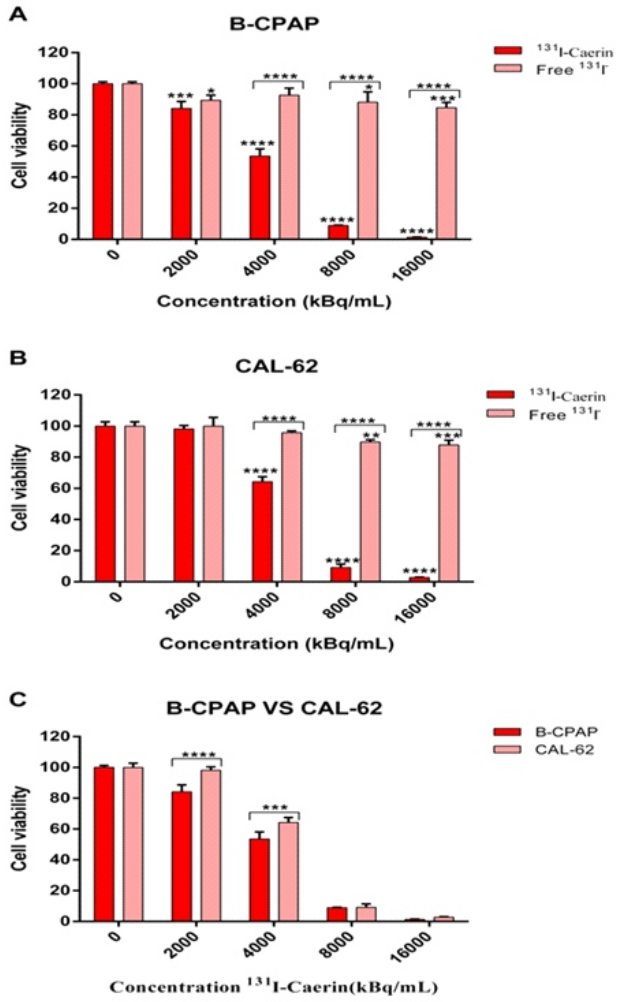

Figure 6. (A, B, C) Survival rate of B-CPAP and CAL-62 cells after treating with ^{131}I-Caerin or free ^{131}I- for 24 h (n=3, mean±SD). * means statically significant, (P<0.05);** (P<0.01);***(P<0.001);****(P<0.0001).

Discussion

In this study, we found that Caerin, an Australian frog tree host-defense peptide, can inhibit the proliferation of B-CPAP cells and CAL-62 cells in vitro. Caerin had a dose-dependent cytotoxic effect on thyroid cancer cells. The minimal inhibition dose of Carein on B-CPAP and CAL-62 cells, was 2.5μg/mL and 5μg/mL, respectively. B-CPAP cells were more sensitive to Caerin than CAL-62 cells when the concentration of Caerin peptide was in the range of 2.5μg/ml to 15μg/ml. The IC_{50} of Caerin peptide for B-CPAP cells was lower than that for CAL-62 cells, indicating that higher dose was required to kill undifferentiated thyroid cancer cells, which may result from the different amount of membrane channels or receptors associated with the Caerin peptide on these two cell lines. By utilizing proteomic analysis of Caerin treated human Hela cells, Caerin mediated Hela cell growth inhibition was shown by deregulating EGFR signaling pathway, resulting decreased expression of PI3K and pAKT and increased expression of caspase 3 and 9, and finally leading to Hela cell apoptosis (paper submitted). And our next work is to investigate the expression of EGFR in B-CPAP and CAL-62 cell lines.

B-CPAP cells express sodium iodine transporter (NIS), while CAL-62 cells do not express NIS. By confocal imaging, we demonstrated that Caerin peptide could be ingested by B-CPAP cells and CAL-62 cells, indicating that the uptake of Caerin peptide is independent of sodium iodine transporter, which may be helpful in the treatment of radioiodine-refractory thyroid cancers and anaplastic thyroid cancers.

Iodine-131 (half-time, 8.04 days) is the most commonly used radioisotope for radionuclide therapy. Iodine-131 can emit both beta rays and gamma rays, which can be used for radiotherapy and in vivo imaging, respectively. Based on its characteristics, ^{131}I has been used in the development of new drugs, such as the labeled monoclonal antibodies [19-20]. The labeling method is quite mature, among which Iodogen method and Chloramine-T method are mainly used [21-22]. In our study, the Chloramine-T method was used to obtain ^{131}I-Caerin with a high labeling rate and good stability. We further showed that ^{131}I-Caerin is a lipophilic and tumor-targeting polypeptide.

Moreover, our study showed ^{131}I-Caerin has more inhibition effect on B-CPAP cells and CAL-62 cells in vitro compared to ^{131}I. The growth of B-CPAP cells was inhibited when incubated with 2000KBq/mL of ^{131}I-Caerin, while CAL-62 cells stopped growing when incubated with >4000KBq/mL of ^{131}I-Caerin. When the dose of ^{131}I-Caerin was in the range of 2000-4000KBq/mL, B-CPAP cells were more sensitive than CAL-62 cells. Iodine-131-Caerin could kill the radioiodine-refractory thyroid cancer cell line CAL-62, indicating the potential theragnostic value of ^{131}I-Caerin for both differentiated and radioiodine-refractory thyroid cancers. Therefore, radioactive Iodine conjugated Caerin reduce the amount of radioactive Iodine, and at the same time inhibit thyroid cancer cell growth by promoting the apoptosis of the cancer cells.

In conclusion, in this work, we developed and assessed the therapeutic potential of ^{131}I-Caerin in thyroid cancers. Our results showed that ^{131}I-Caerin can inhibit radioiodine refractory thyroid cancer cells growth in vitro which provide an alternative therapeutic potential for this disease. And our ongoing work is investigating the possible diagnostic and therapeutic

effects of ^{131}I-Caerin in vivo.

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The authors declare that they have no conflicts of interest

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