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Preparation of ^{125}I Labeled PKCδ-V5 Heptapeptide and Evaluation of Its Targeting Heat Shock Protein 27 in NCI-H1299 and H460 Cells

Byoung-Soo Kim,[†,§] Eun-Jung Kim,[†] Hae-June Lee,[‡] Sang-Jin Han,[†] Tae-Hyun Choi,[†] Yun-Sil Lee,[‡] and Gi Jeong Cheon[*]

Department of Nuclear Medicine, Korea Institute of Radiological and Medical Sciences, 215-4 Gongneung-dong, Nowon-gu, Seoul 139-706, Korea. [*]E-mail: larry@kirams.re.kr
[†]Radiopharmaceuticals Research Team and [‡]Laboratory of Radiation Effects, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea
[§]College of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi-do 440-746, Korea
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PKCδ-catalytic V5 Heptapeptide (FEQFLDI, FP7) interacts with heat shock protein 27 (HSP27) and inhibits HSP27-mediated resistance to cell death against various stimuli including radiation therapy. Here, we prepared radio-iodinated heptapeptide and further investigated its uptake properties in HSP27 expression cells. Peptide sequence of FP7 and a negative control peptide (WSLLEKR, QP7) was modified by substituting their C-terminus residue to tyrosine (FP6Y and QP6Y) to label radio-iodine. Iodinated peptides were confirmed by LC mass analysis with cold iodine reaction mixture. Accumulation of [^{125}I]iodo-FP6Y and [^{125}I]iodo-QP6Y in NCI-H1299 cell line, with higher level of HSP27, and NCI-H460 cell line, with lower level of HSP27, was measured by NaI(Tl) scintillation counter. The modification of substituting C-terminus residue of FP7 to tyrosine (FP6Y) did not affect its interaction with HSP27. Accumulation of [^{125}I]iodo-FP6Y in NCI-H1299 cells was 3 fold higher than in NCI-H460 cells. The novel radio-iodinated FP6Y would be used as a tracer for targeting HSP27 protein.

Key Words: PKCδ-V5 Heptapeptide, HSP27, Radio-iodination

Introduction

Heat shock protein 27 (HSP27), an important small HSP found in human cells, has strong anti-apoptotic properties and is induced by different stresses such as heat, irradiation, oxidative stress, or anticancer chemotherapy.[1,2] An increased level of HSP27 has been detected in a number of cancers such as breast cancer, ovarian cancer, osteosarcoma, endometrial cancer and leukemia.[3] Over-expressed HSP27 has been shown to being resistant to apoptotic cell death triggered by ionizing radiation.[4,5] Cancers, where the expression of HSP27 is high, may predict a poor prognosis in terms of survival and response to radiation therapy.[6-8]

HSP27 has been shown to interact and inhibit different key apoptotic proteins.[2,9-11] Protein kinase Cδ (PKCδ) has been reported to play a critical role in the control of cell growth, proliferation and apoptosis.[12,13] Regulating the expression and function of apoptotic related proteins and being itself a target for caspases, PKCδ is actively involved in cell apoptosis.[14,15] The activation of PKCδ was found to be associated with cell cycle progression inhibition, and PKCδ down-regulation was found to be associated with tumor promotion.[12,16] HSP25, a murine form of small heat shock protein with more than 90% homology to HSP27, has been shown to inhibit PKCδ-mediated cell death through direct interaction.[17] HSP27 also was found to interact directly with the COOH terminus of the PKCδ-V5 region with ensuing inhibition of PKCδ activity and PKCδ-mediated cell death. Several experiments with various deletion mutants of the region revealed that amino acid residues 668 to 674 (F-E-Q-F-L-D-I) of the V5 region mediate its interaction with HSP27. Having efficient interaction with HSP27, the heptapeptide of the PKCδ-V5 region was found to increase radiation-induced cell death in tumor xenograft model as well as in cell based assay.[18]

Radioiodination is a technique commonly used on peptides for in-vitro radioligand investigations as well as for medical imaging and therapy. Several direct and indirect iodination methods currently exist. The following direct labeling techniques are considered to be the most widely used: Chloramine-T[19], Iodo-Gen® (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril)[20] and lactoperoxidase.[21] These procedures result in radioiodine substituted tyrosine and histidine amino acid residues.[22,23] PKCδ-catalytic V5 Heptapeptide (FEQFLDI, FP7) has no tyrosine or histidine amino acid residue. So we displace an amino acid residue of the heptapeptide to tyrosine for the further radioiodination by chloramine-T technique.

In this study, we prepared a novel radio-iodinated heptapeptide through the structure modification of the heptapeptide of the PKCδ-V5 region. To assess the applicability as a potential tracer for HSP27 protein, we evaluated its uptake in human lung cancer cells with HSP27 over-expression.

Experimentals

Chemicals and reagents. Peptides were purchased from Peptron (Daejeon, Korea). Iodine-125 was purchased from Perkin-Elmer (Waltham, MA). Chloramine-T, ascorbic acid, sodium iodide and sodium metabisulfite were from Sigma Chemical Co. Trifluoroacetic acid (Acros Chemical Co.) and acetonitrile (HPLC grade, J. T. Baker) were used for HPLC analysis. Anti-

HSP27, anti-PKCδ and anti-β-actin were purchased from Santa Cruz Biotechnology. Dulbecco's phosphate-buffered saline (DPBS) was from WelGENE (Deagu, Korea).

Cell line and culture. Human non-small cell lung cancer cell lines, NCI-H1299 (H1299; higher HSP27 expression cell line) and NCI-H460 (H460; lower HSP27 expression cell line) cells were grown in RPMI 1640 (Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS; JHR Biosciences, Lenexa, KS), glutamine, HEPES, and 1% penicillin-streptomycin (Gibco, Carlsbad, CA). Medium was changed twice or three times a week. The cells were cultured at 37 °C in a 5% CO_2 atmosphere.

Immunoblotting. Immunoblotting was done as previously described.[24] For PAGE and Western blotting, cells were solubilized with lysis buffer (120 mmol/L NaCl, 40 mmol/L Tris (pH 8.0), 0.1% Nonidet P-40) and boiled for 5 min, and equal amounts of protein were analyzed on 10% SDS-PAGE. After electrophoresis, proteins were to a nitrocellulose membrane and processed for immunoblotting.

Immunoprecipitation. Cells (1×10^7) were lysed in immunoprecipitation buffer [50 mmol/L HEPES (pH 7.6), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.1% NP40]. After centrifugation (10 min at 15,000 × *g*) to remove particulate material, the supernatant was incubated with antibodies (1:100) with constant agitation at 4 °C. The immunocomplexes were precipitated with protein A-Sepharose (Sigma) and analyzed by immunoblotting.

Cold iodination reaction of heptapeptides. Iodination reaction of heptapeptides were performed using chloramine-T as a oxidizing agent. Sodium iodide (NaI) solution was prepared resolving in 0.01 N NaOH. NaI solution (10 μL, 5 mM) was added to 20 μL of DPBS. Chloramine-T (10 μL, 2.5 mg/mL) was added the mixture and stand for 1 minute at room temperature. The iodination was initiated by adding each peptides (50 μL, 1 mM) and the reaction mixture was allowed to stand for 30 seconds at room temperature. The reaction was terminated by adding 40 μL of sodium metabisulfite (5 mg/mL). Formation of iodinated products were assessed by LC-UV-MS analysis. LC-UV-MS analysis was conducted using following system: a Agilent 1200 series HPLC system (binary pump SL, vacuum degasser, autosampler SL+, thermostatted column compartment SL and diode array detector SL), a Agilent 6530 accurate Mass Q-TOF. Reaction mixture was loaded on a Nomura Develosil ODS HG-5 column (3.0 × 150 mm) and the column temperature was kept constant at 45 °C. The analytes were eluted using gradient solvent system with flow rate of 0.7 mL/min. The initial mobile phase consisted of 15:85:0.1% acetonitrile/water/TFA. The ratio of acetonitrile was increased in up to 40% for 20 minutes. MS Q-TOF analysis was performed with ESI negative ion mode and used following paremeters. Sheath gas temperature and flow rate were 350 °C and 11 L/min, respectively. Voltage of ESI source capillary was 4,000 V. The voltage of fragmentor, skimmer and OCT 1 RF Vpp was 120, 70 and 750 V, respectively.

Varying the molar ratio of NaI/heptapeptide in the iodination reaction, the difference of iodinated products pattern was assessed by analytical HPLC. Iodination reaction procedure was the same to that described upper. The molar ratios of NaI/heptapeptide used in the reaction were 1, 0.5 and 0.1, respectively. HPLC analysis was performed following system: Waters 2690 separation module and Waters 996 PDA detector. Reaction mixture was loaded on a Nomura Develosil ODS HG-5 column (3.0 × 150 mm) and the column temperature was kept constant at 40 °C. The analytes were eluted using gradient solvent system with flow rate of 0.43 mL/min. The initial mobile phase consisted of 20:80:0.1% acetonitrile/water/TFA. The initial composition was kept for 5 minutes and the ratio of acetonitrile was increased in up to 80% for next 10 minutes.

Radiolabeling. [^{125}I]iodo-FP6Y and [^{125}I]iodo-QP6Y were prepared using chloramine-T as a oxidizing agent. Na[^{125}I]I solution (10 μL, 1 ~ 2 mCi) was added to 20 μL of DPBS. Chloramine-T (10 μL, 2.5 mg/mL) was added the mixture and stand for 1 minute at room temperature. The iodination was initiated by adding each peptide (50 μL, 1 mg/mL in DPBS) and the reaction mixture was allowed to stand for 30 seconds at room temperature. The reaction was terminated by adding 40 μL of sodium metabisulfite (5 mg/mL) and 10 μL of ascorbic acid (10 mg/mL) was added as a stabilizer. The reaction solutions were added 900 μL of distilled water (DW) and purified by high-performance liquid chromatography (HPLC). HPLC was conducted using the following system: a Gilson 321 pump combined with Gastorr BG-14 in-line degasser, a Gilson UV-vis-151 detector (λ; 295 nm) and a Bioscan Flow-count PMT radioactivity detector. The resulting products were loaded on a Waters μ-Bondapack^{TM} C18 column (5 μm, 3.9 × 300 mm) using Rhenodyne^{TM} injector with 1mL of sample loop and eluted using gradient solvent system with flow rate of 1 mL/min. The initial mobile phase consisted of 20:80:0.1% acetonitrile/water/TFA. The initial composition was kept for 5 minutes and the ratio of acetonitrile was increased in up to 80% for next 10 minutes. The radio-iodinated product fraction was collected and dried by nitrogen blowing on the heating block (40 °C). For cell uptake experiment, the product were reconstituted in 1mL of DPBS, added with ascorbic acid (10 μL, 10 mg/mL) as a stabilizer against probable radiolysis, and filtered through a 0.22 μm syringe filter into a sterile glass vial.

Cellular uptakes of radio-iodinated heptapeptides. NCI-H1299 and NCI-H460 cells were plated in 6-well plates at a density of 1×10^6 cells/well and incubated at 37 °C for 24 h. When reached 80-100% confluence, each well was incubated with [^{125}I]iodo-FP6Y or [^{125}I]iodo-QP6Y (1 μCi/2 mL) for 0.25, 0.5, 1, 2, 4, 8, 24 h at 37 °C under 5% CO_2 atmosphere. The cells rinsed three times with DPBS without Mg^{2+} and Ca^{2+}, and adherent cells were harvested. The radioactivity of harvested cells was determined by gamma scintillation counter (Perkin-Elmer, Waltham, MA). The radioactivities of triplicated samples were measured at designated time points. The accumulation of radiotracers was calculated as the percentage of the radiotracer dose added to the medium.

Results and Discussion

Structural design of the heptapeptides for radio-iodination. HSPs are overexpressed in a wide range of malignant cells and tissues. Among these proteins, HSP27 has been of special clinical interest because of its involving in radio- or chemo-resi-

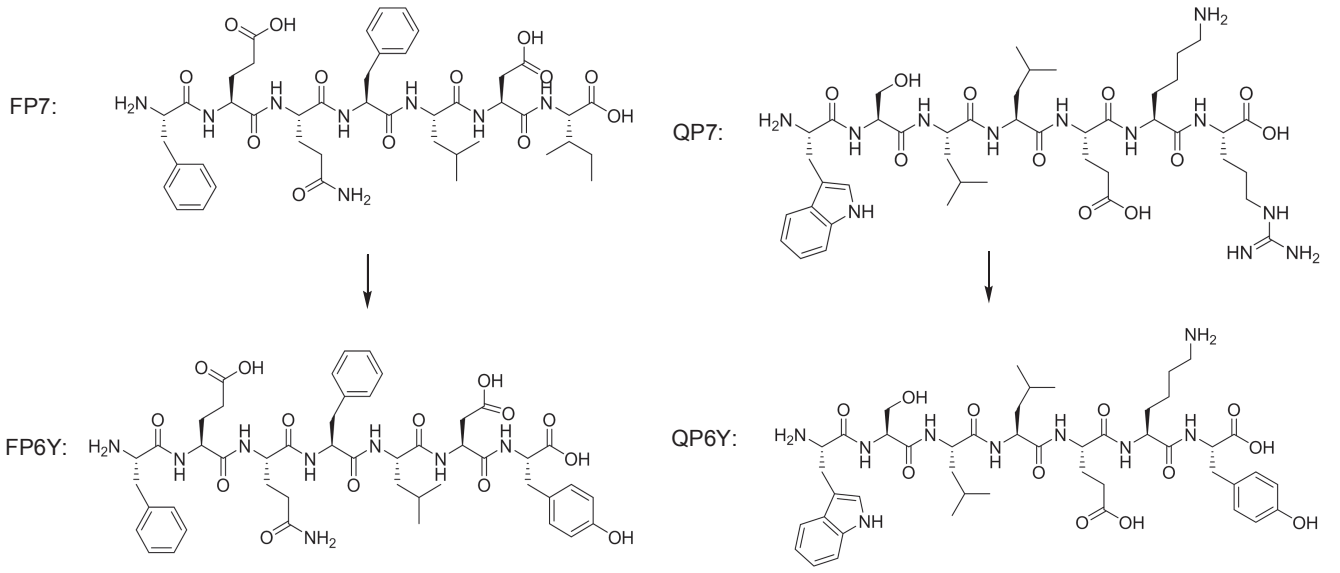


Figure 1. Modification of the heptapeptides for radioiodination. To label the heptapeptides with radio-iodine directly, their C-terminal amino acid residue was replaced with tyrosine. FP7, PKCδ-catalytic V5 heptapeptide binds to HSP27, QP7, negative control peptide, FP6Y, modified heptapeptide for radioiodination, QP6Y, modified negative control peptide.

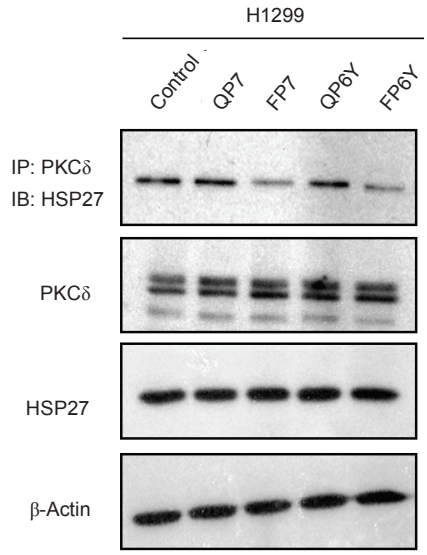


Figure 2. The ability of heptapeptides to inhibit binding between PKCδ and HSP27. NCI-H1299 cell lysates were immunoblotted (IB) with the indicated antibodies and immunoprecipitated (IP) with anti-PKCδFP7, PKCδ-catalytic V5 heptapeptide binds to HSP27, QP7, negative control peptide, FP6Y, modified heptapeptide for radioiodination, QP6Y, modified negative control peptide. FP7, PKCδ-catalytic V5 heptapeptide binds to HSP27, QP7, negative control peptide, FP6Y, modified heptapeptide for radioiodination, QP6Y, modified negative control peptide.

stance. Recently, a novel heptapeptide (FP7) targeting HSP27 has been suggested[18] and FP7 was consisted of seven amino acid of PKCδ V5 region (amino acid from 668 to 674) which is necessary for interaction with HSP27. Treatment of FP7 to NCI-H1299 cells which showed HSP27 overexpression synergistically increased the cell death by cisplatin or radiation. HSP27 overexpressed tumor xenograft mice data suggested that NCI-1299 cells were sensitized by FP7 by efficient interaction with HSP27, thereby HSP27-mediated inhibition of endogenous apoptotic pathway was restored.

Radio-iodination technique using chloramine-T has been widely used for labeling peptides, proteins and a number of small organic compounds.[25-27] Chloramine-T is used to release radioactive elemental iodine by oxidation of its salts.[28] Tyrosine or histidine residue is necessary to halogenate peptide using chloramine-T. Because of FP7 has no tyrosine or histidine residue, we had to introduce a tyrosine residue to FP7 for radioiodination. Recently, we have been found that N-terminal five amino acid residues of FP7 are more critical to interact with HSP27 (data not shown). So we substituted an amino acid of C-terminus to tyrosine.

In order to label FP7 with radioactive iodine, FP7, the PKCδ-V5 heptapeptide which interacts with HSP27, and QP7, a negative control peptide which did not interact with HSP27, were modified. The sequence of FP7 peptide was modified by substituting its isoleucine residue of C-terminus to tyrosine (FP6Y). QP7 peptide sequence was also modified by substituting its arginine residue of C-terminus to tyrosine (QP6Y). Figure 1 shows the scheme of their amino acid sequence. The modified peptides, FP6Y and QP6Y, were assessed their ability to interact with HSP27 using immunoprecipitation (IP) and immunoblotting (IB). FP6Y inhibited binding between endogenous PKCδ and HSP27, similar with FP7. However control peptide QP6Y or QP7 did not inhibit (Figure 2).

Cold iodination reaction of heptapeptides. Two different products were formed after cold iodination reaction of heptapeptide. These products were elucidated to monoiodo- and diiodo-heptapeptide by HPLC-UV-MS analysis. Figure 3A shows the HPLC elution profile of a reaction mixture. Peak (2) and peak

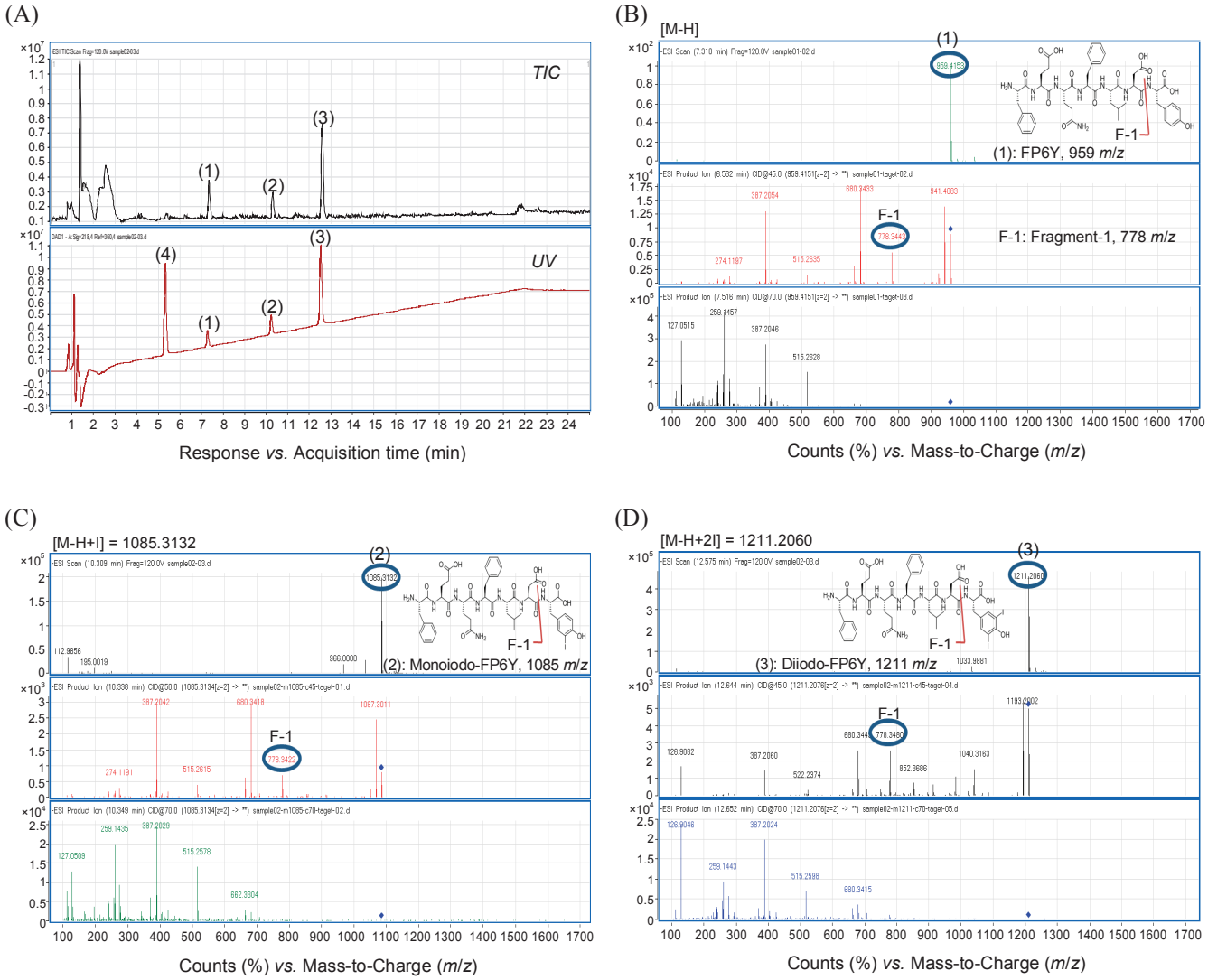
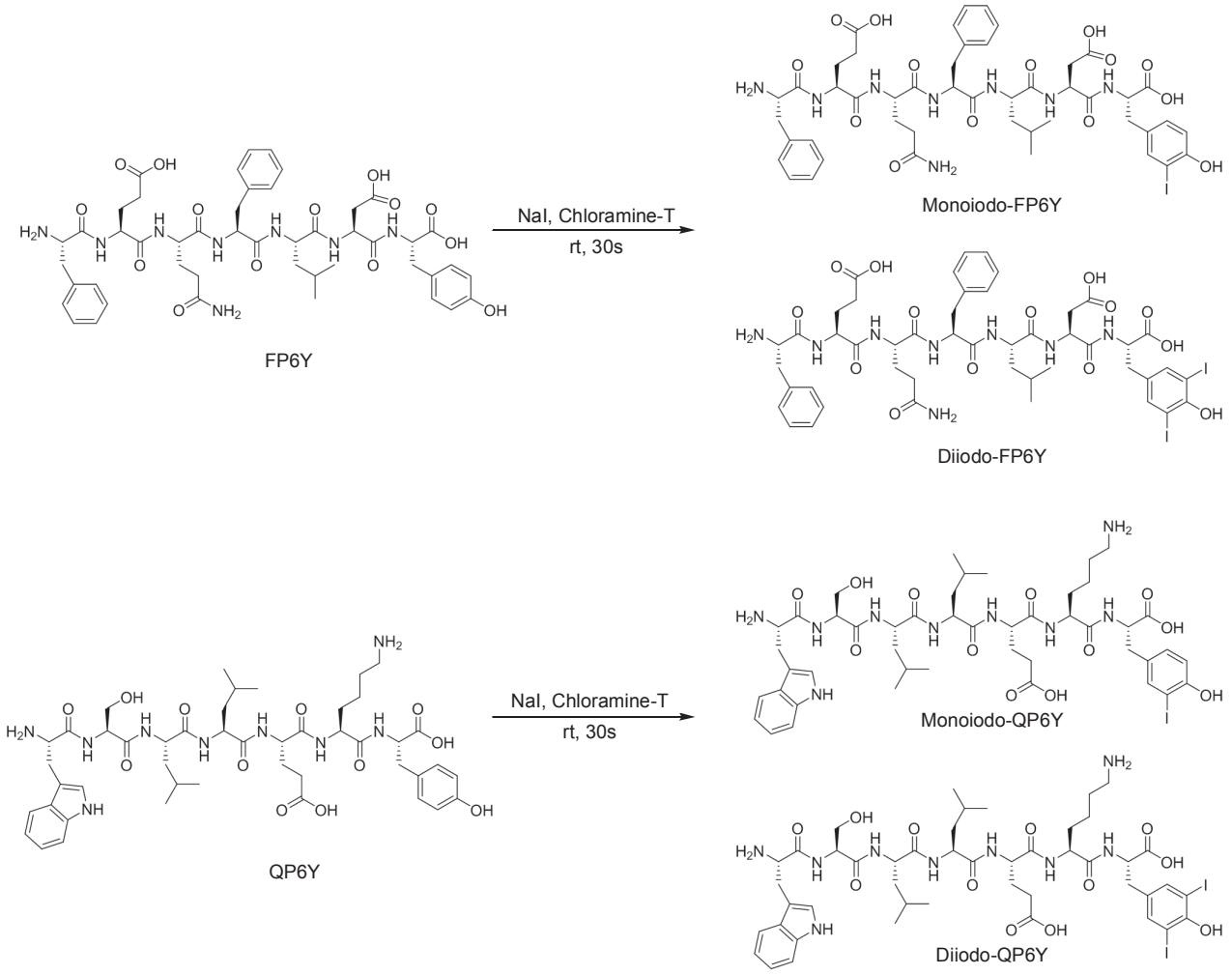


Figure 3. Representative LC mass elution profile of cold iodination reaction mixture (A) and LC mass/mass spectra of heptapeptide (B) and iodinated products (C, D). (1); FP6Y ([M-H], 959 *m/z*), (2); Monoiodo-FP6Y ([M+I-H], 1085 *m/z*), (3); Diiodo-FP6Y ([M+2I-H], 1211 *m/z*), (4); Chloramine-T. F-1; Fragment-1, 778 *m/z*, *TIC;* Total ion count, *UV*; UV absorbance.

(3) are the products newly formed after iodination reaction. Peak (1) is FP6Y. Figure 3B~3D show the mass and the mass/mass spectra of peak (1)~(3), respectively. The mass spectrum of peak (1), FP6Y (FEQFLDY, exact mass: 960.42), represents 959.41 *m/z* (M-H) (Figure 3B upper pane). The mass spectra of peak (2), monoiodo-FP6Y (FEQFLDY-iodine, exact mass: 1086.32) and peak (3), diiodo-FP6Y (FEQFLDY-2 iodine, exact mass: 1212.22) represent 1085.31 *m/z* (M+I-H) and 1211.20 *m/z* (M+2I-H), respectively (Figure 3C and 3D, upper pane). All the mass/mass spectra of FP6Y, monoiodo- and diiodo-FP6Y show the same fragment having 778.34 *m/z* ($[FEQFLD]^-$, exact mass: 778.34) (Figure 3B~3D, middle pane). These results show that iodine has been added to tyrosine moiety of heptapeptide after the iodination reaction (Scheme 1).

The ratio of monoiodo- and diiodo-heptapeptide production varied depending on the ratio of NaI and heptapeptide amount in the reaction mixture. Figure 4 shows HPLC elution profiles of reaction mixtures in which various molar ratios of NaI and heptapeptide were used. Smaller ratio of NaI/FP6Y amount was bigger ratio of monoiodo-/diiodo-FP6Y. When the ratio of NaI/FP6Y amount was a unit, the production amount of diiodo-FP6Y was approximately two times more than monoiodo-FP6Y. When the ratio of NaI/FP6Y amount was a tenth, the production amount of monoiodo-FP6Y was approximately three times more than diiodo-FP6Y (Table 1).

In the iodination reaction of peptides having a tyrosine residue using chloramine-T can be formed mono- and di-iodinated peptides.[22,23] We also found that monoiodo- and diiodo-heptapeptide products were formed by HPLC-UV/MS (Figure 3). The iodination reaction of peptides is affected by several reaction parameters, such as pH, reaction time and amount of iodide.[29] The amount of radioiodine (1 ~ 2 mCi) used in our labeling procedure was very small comparing to the substrate. The ratio of iodine and peptide amount was approximately less than 1/50.



Scheme 1

Table 1. Effect of the amount of iodine used in the iodination reaction of FP6Y on the production of Monoiodo- and Diiodo-FP6Y

Molar ratio (NaI/FP6Y)	HPLC peak area of products		Mean production ratio (Monoiodo-/Diiodo-FP6Y)
	Monoiodo-FP6Y	Diiodo-FP6Y	
1	471363 728913	1367037 1019511	0.503
0.5	555153 699196	628296 483122	1.13
0.1	183796 194875	69406 53065	3.09

The effect of different amount of iodine was evaluated using cold iodine (NaI) and FP6Y. The effect on the amounts of monoiodo- and diiodo-FP6Y formed were determined by HPLC-UV. The ratio of monoiodo-/diiodo-FP6Y formed was increased by decreasing the molar ratio of NaI/FP6Y (Figure 4, Table 1). It suggests that the major product from radioiodination of heptapeptide was monoiodo-heptapeptide.

Radiolabeling of the heptapeptide. To examine cellular uptake of peptides, FP6Y and QP6Y were labeled with iodine-125. The radio-labeling yields of [^{125}I]iodo-FP6Y and [^{125}I]iodo-QP6Y were approximately 97.4% and 86.5%, respectively. [^{125}I]iodo-FP6Y and [^{125}I]iodo-QP6Y were purified by preparative HPLC. Figure 5 shows the elution profiles of radioiodination mixture (Figure 5A) and purified product (Figure 5B).

Cellular uptakes of radio-iodinated heptapeptides in lung carcinoma cells. Immunoblotting results showed the different expression level of HSP27 protein in NCI-H1299 cells and NCI-H460 cells (Figure 6A). Because NCI-H1299 cells showed

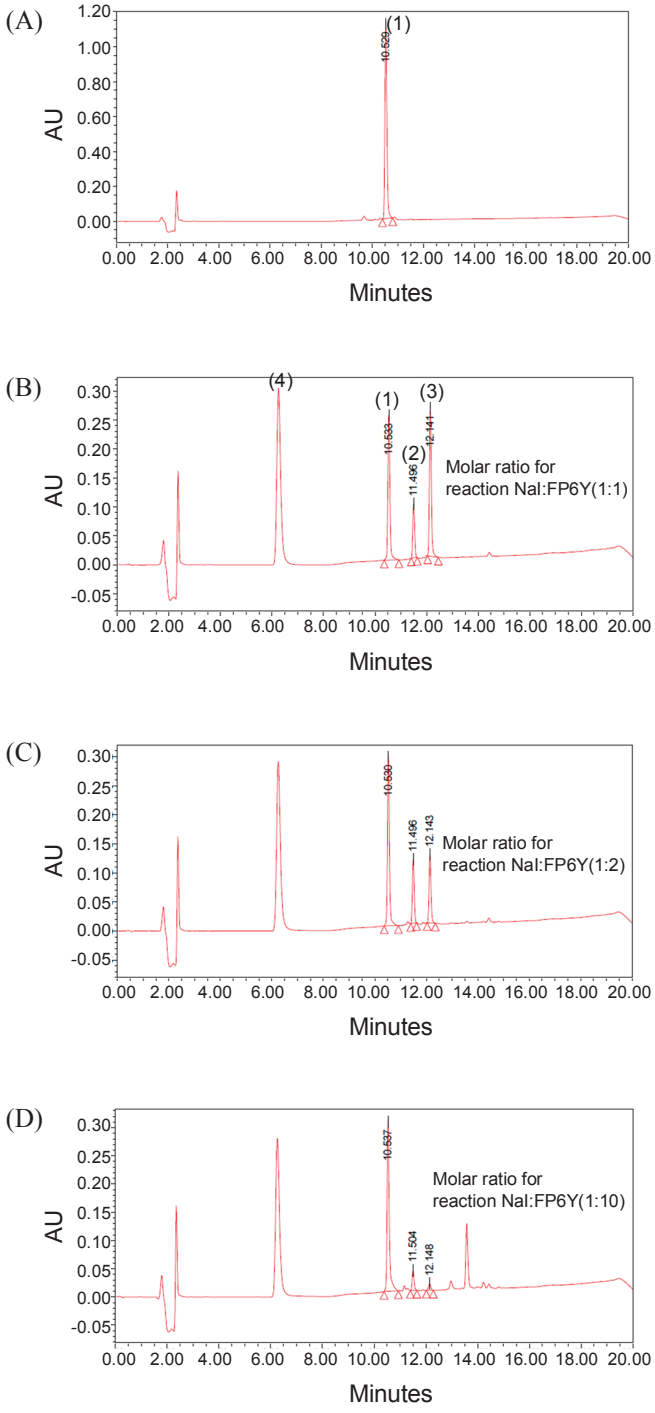


Figure 4. HPLC-UV elution profile of FP6Y (A) and cold iodination reaction mixtures (B-D). (1); FP6Y, (2); Monoiodo-FP6Y, (3); Diiodo-FP6Y, (4); Chloramine-T.

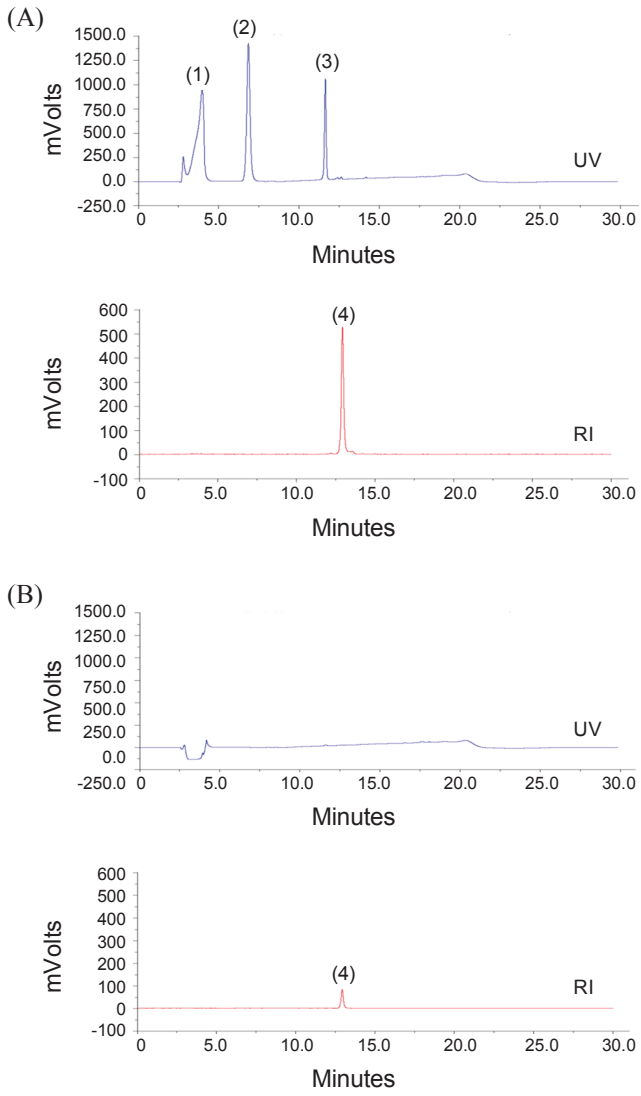


Figure 5. Representative HPLC elution profile of radiolabeling reaction mixture (A) and purified radio-iodinated heptapeptide (B). (1); Ascorbic acid, (2); Chloramine-T, (3); FP6Y, (4); [^{125}I]iodo-FP6Y. *UV;* UV absorbance, *RI;* radio-activity.

higher HSP27 protein expression than NCI-H460, we compared the uptakes of radio-labeled heptapeptides between two cell lines. Cellular uptake value of [^{125}I]iodo-FP6Y and [^{125}I]iodo-QP6Y were continuously increased according to the length of incubation time in both cells. Selective uptake of [^{125}I]iodo-FP6Y in NCI-H1299 cells was shown, compared with NCI-H460 cells at 24 hour incubation. But uptake of [^{125}I]iodo-QP6Y was not significantly different in both cells until 24 hour incubation (Figures 6B, 6C).

Peptides are rapidly degraded in the circulation, and their relatively large size and often charged nature make them impermeable cell membranes to cancer cell membranes. However, FP7 is relatively small and permeates cell membranes without other penetrating peptides as it contains hydrophobic amino acids such as 2 Phe and 1 Leu. Another possibility might be due to that the peptides can permeate cell membrane by transporters like PEPT1 and PEPT2.[30] In NCI-H1299 cells which showed HSP27 overexpression, [^{125}I]iodo-FP6Y accumulation was about triple higher than [^{125}I]iodo-QP6Y when it was examined at 24 hr of incubation. Although further studies are needed, this result suggests a possibility to use radio-iodinated heptapeptide as a tracer for HSP27 overexpressed tumors such as mostly cancer cells or tissues.

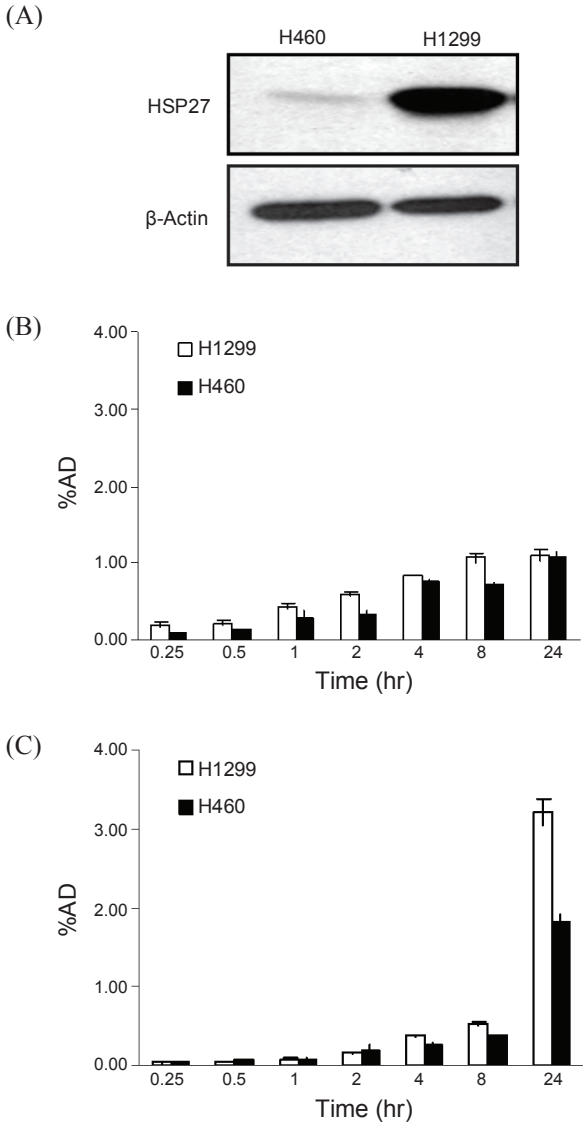


Figure 6. Cellular uptakes of [^{125}I]iodo-QP6Y and [^{125}I]iodo-FP6Y in NCI-H1299 and NCI-H460 cells. Uptakes of [^{125}I]iodo-QP6Y (B) and [^{125}I]iodo-FP6Y (C) in NCI-H1299 and NCI-H460 cells were measured after incubation for 0.25, 0.5, 1, 2, 4, 8, 24 h. Immunoblotting result (A) showed that NCI-H1299 cells express HSP27 protein much higher than NCI-H460. Uptake of [^{125}I]iodo-FP6Y in NCI-H1299 cells was higher than in NCI-H460 cells. %AD; percentage of added dose.

Conclusion

In this study, we examined cellular uptake of heptapeptide (FP7) which can interact with HSP27 and abrogate HSP27-mediated radio- or chemoresistance. Radio iodinated FP7 as a sensitive tracer showed high cellular uptake in NCI-H1299 cells with HSP27 overexpression than in NCI-H460 cells.

In summary, we successfully prepared a radio-iodinated heptapeptide targeting HSP27 and elucidated its selective cell uptake. Radio-iodinated heptapeptide might be a potential probe for detecting HSP27 protein with high expression of tumors.

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