

Radiolabeling and Biodistribution of a Nasopharyngeal Carcinoma-targeting Peptide Identified by *in vivo* Phage Display

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Abstract A dodecapeptide EDIKPKTSLAFR ligand targeting CEN-1 human nasopharyngeal carcinoma (NPC) was identified by *in vivo* phage display. Two tridecapeptides and their derivatives, named YR13 (YEDIKPKTSLAFR), EY13 (EDIKPKTSLAFRY), EY13-NH₂ (EDIKPKTSLAFRY-NH₂) and Fmoc-YR13 (Fmoc-YEDIKPKTSLAFR), were synthesized and radiolabeled with ¹³¹I. The stability *in vitro*, biodistribution and tissue distribution of selected phage particles in mice bearing NPC tumor were determined, and plasma metabolites analysis of radiolabeled peptides was carried out. Although Fmoc and NH₂ groups could protect the peptide from deiodination, only Fmoc group inhibited the binding of Fmoc-YR13 to NPC tumors. The compound EY13-NH₂, the C-terminal amide of peptide EY13, had the greatest serum stability, the least deiodination, and showed favorable tumor/blood ratios. The selected phage particles (phage 3 or phage 5) were more concentrated in NPC tumors than the control phage (initial phage display peptide library). EY13 could also inhibit the binding of selected phage particles to tumors. The results indicated that EDIKPKTSLAFR was a good candidate in diagnostic and therapeutic NPC.

Keywords *in vivo* phage display; biodistribution; radiolabeling; nasopharyngeal carcinoma; deiodination

Developing new drugs that target tumor cell or tumor-associated vasculature is crucial for the improvement of tumor diagnosis and therapy, especially in the early stage [1,2]. Phage display technology provides a powerful approach for the discovery of new tumor-specific molecules that could create noninvasive molecular imaging. Many peptides identified using phage display technology have shown clinical promise as cancer targeting agents due to higher specific tumor uptake, more rapid tumor penetration and less immunogenicity than monoclonal antibodies [3–6]. Some therapeutic drug-linked peptides selectively homing tumors after intravenous injection showed more marked therapeutic efficiency and less side-effects than the untargeted drugs [7,8].

Many researchers have devoted themselves to the study

of the causes inducing tumor formation. Although etiological factors were still not completely identified, one report [9] indicated that various genetic and epigenetic changes in carcinogenic cells and other tumor-associated tissues can result in changes in protein status, including tumor cell receptors and vascular receptors. The complete pictures of these receptors are not well known at present [10]. The application of *in vivo* phage display technology has an important advantage in that one can select ligands in the environment of the whole animal. This technology might also help us to map the landscape of receptors [11]. It was used by Pasqualini and Ruoslahti to select peptide ligands homing to normal organs, brain and kidneys [12]. The cyclic RGD, a small excellent angiogenic vasculature-targeting peptide was obtained in this way [7] by the same research group.

The rescued phage particles binding selectively to a specified normal tissue, a tumor or other angiogenesis-related receptors can be titered, amplified and sequenced [13–20]. Different peptide ligands have been selected from

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lung, skin, pancreas, intestine, uterus, retina, adrenal gland and tumor xenografts, as well as brain and kidney, using phage display technology [21–23]. However, many isolated peptides did not show high specificity or affinity to tumors or specific organs. Therefore, Landon *et al.* pioneered an *in vivo* selection scheme using a pre-cleared phage library [24,25], and some researchers prolonged circulative time *in vivo* so as to effectively yield phages that display uncommon peptides [19,20,24].

In this article, we selected 1 h as the circulation time of phage, in accordance with relevant reports [19,20,24], and identified a novel dodecapeptide EDIKPKTSLAFR specifically binding to nasopharyngeal carcinoma (NPC) using *in vivo* phage display. The 13 mer peptides and their derivatives YR13, EY13, EY13NH₂ and Fmoc-YR13 were synthesized and radiolabeled with ¹²⁵I, and the *in vitro* stability and biodistribution was determined. The plasma metabolites of four compounds were analyzed by HPLC. The tissue distribution of phage particles in mice bearing NPC tumor was detected. In addition, we also investigated whether modification of tyrosine modified with Fmoc or NH₂ impaired the affinity.

Materials and Methods

Reagents and equipment

All reagents were of analytical grade and were used without further purification. All water used was deionized. The Ph.D-12 phage display peptide library kit was purchased from NEB (New England Biolabs, Beijing, China). All other reagents were from Beijing Xin Jing Ke Biotechnology (Beijing, China). Polyethylene glycol/NaCl and LB medium (10 g bacto-tryptone, 5 g yeast extract and 5 g NaCl in 1 liter of distilled water) were prepared according to procedures recommended in the NEB product manual. No-carrier-added Na¹²⁵I (aqueous solution) was obtained from the China Institute of Atomic Energy (Beijing, China). DNA was sequenced by Sheng Gong Biotechnology (Shanghai, China). The peptides and their derivatives, YEDIKPKTSLAFR (YR13), EDIKPKTSLAFRY (EY13), EDIKPKTSLAFRY-NH₂ (EY13-NH₂) and Fmoc-YEDIKPKTSLAFR (Fmoc-YR13), were synthesized by GL Biochem (Shanghai, China). DMEM-P1 is a mixture containing the protease inhibitor (phenylmethylsulphonyl fluoride) (1 mM), aprotinin (20 µg/ml) and leupeptin (1 µg/ml) in Dulbecco's modified Eagle's medium [11]. Kunming mice (20–22 g, male) were purchased from the Breeding Center of Zoology at Peking University's Health

Science Center (Beijing, China). Male nude mice (BALB/c nu) bearing CNE-1 human NPC tumor were supplied by the Breeding Center of Zoology from the Chinese Academy of Medical Science (Beijing, China).

C₁₈ Sep-Pak cartridges were supplied by Waters Corporation (Milford, USA). A Symmetry C₁₈ column (5 µm, 3.9×150 mm; Waters) was used to analyze samples [26]. Analytical reverse phase (RP)-HPLC was carried out with the 600E solvent distribution system (Waters). A Packard 500 TR Series Flow Scintillation Analyzer (Ramsey, USA) was used to analyze the radioactivity of tissue samples. The radiochemical yield of labeled compounds was analyzed by a CRC-15R dose-calibrator (Capintec, Ramsey, USA). The matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were provided by the Beijing Mass Spectrometry Center, Chinese Academy of Sciences (Beijing, China).

Phage selection

Athymic nude mice at 4–6 weeks of age were injected subcutaneously in the right flank with CNE-1 human NPC cells (10⁷ cells in 200 µl Dulbecco's modified Eagle's medium) [20,27]. When the tumors reached approximately 0.5–1.0 cm in diameter (20–25 d), the mice bearing NPC tumor were used to carry out biopanning and biodistribution. Biopanning procedures were carried out according to the NEB product manual and the methods reported [7,12,20], with some modifications. Aliquots [200 µl, 10¹² plaque forming unit (PFU)/ml] of phage display peptide library diluted 1:10 in LB medium were injected into the tail vein of mice bearing NPC tumor. After the phage particles circulated for 1 h [19], the mice were killed by cervical dislocation and tumors were quickly removed and put to DMEM-P1 solution. The phage particles bound to NPC tumors were recovered by adding 1 ml of *Escherichia coli* 2738 bacterial culture and incubation at room temperature for 30 min [20,27]. The recovered phages were titered, amplified and purified [19,28]. The amplified and purified phage particles (10¹² PFU/ml) were again injected into mice as described above. For DNA sequence analysis, individual blue phag plaques were picked out from titer plates grown overnight after the third or fifth round of panning.

Tissue distribution of selected phage particles in organs and tumor xenografts

Mice bearing NPC tumor (three mice for each group) received a separate injection into the tail vein of 10⁹ PFU of the third round selected phage and the fifth round selected phage, the fifth round selected phage plus 200 µg

EY13 or YP13 [7,21,22] (a nonspecific random 13 peptide YSVSVGMLPSHAP as control peptide) and the control phage (initial phage display peptide library). Eight minutes after injection [20], the mice were killed by cervical dislocation. The tumor nodules and organs such as heart and brain were removed, washed and weighed prior to titrating.

In this report, abbreviations have been given to the different kinds of phages to simplify their description: the initial phage display peptide library is phage 1; the phage after the third round selection is phage 3; and the fifth round selected phage is phage 5.

Iodination and radioiodination of peptides and their derivatives

The peptides and their derivatives were labeled with ^{131}I using the Iodogen method [26]. YR13, EY13, Fmoc-YR13 and EY13NH₂ (30 μg) were added separately to a 0.5 ml polypropylene vial coated with 100 μg Iodogen. Phosphate-buffered saline (PBS; 50 μl , 0.1 M, pH 7.4) and Na ^{131}I (1.8×10^7 Bq) were added to the mixtures and incubated at room temperature for 30 min. YR13 was also labeled with stable ^{127}I . The iodination was quenched after removing Iodogen. The MALDI-TOF mass spectrometry m/z for $[\text{M}+\text{H}]^+$ was found: 1694.3 ($[\text{I}^{127}\text{I}-\text{YR13}+\text{H}]^+$); and 1820.2 ($[\text{I}^{127}\text{I}_2-\text{YR13}+\text{H}]^+$). The radiochemical yield of the labeled products was determined by RP-HPLC on a Waters Symmetry C₁₈ column (5 μm , 3.9×150 mm) at a flow rate of 1 ml/min, linear gradient from 90% solvent A (water with 0.1% trifluoroacetic acid) and 10% solvent B (acetonitrile with 0.1% trifluoroacetic acid) to 35% solvent A and 65% solvent B in 0–25 min. The labeled mixtures of peptides and derivatives were purified by C₁₈ Sep-Pak column or RP-HPLC. The radioiodinated products were diluted with PBS to obtain solutions with a radioactivity concentration of 1850 kBq/ml for use in animal experiments.

Octanol/water partition co-efficient

Approximately 10 kBq labeled peptide or derivative in 400 μl PBS was added to 400 μl n-octanol in a centrifugal tube [26]. The two-phase mixtures were vortexed at room temperature for 3 min and centrifuged at 4320 g for 15 min to separate the two phases. PBS and n-octanol (100 μl each) were separately pipetted for radioactivity counting in an automated gamma counter. The partition co-efficient was calculated from three parallel runs.

Stability of [^{131}I] peptides and their derivatives

RP-HPLC was used to evaluate the stability of labeled

YR13, EY13NH₂, EY13 and Fmoc-YR13 in PBS, separately [26,29]. To 200 μl of PBS was added 100 μl (20–30 μCi) of one of the labeled solutions. The mixtures were incubated at room temperature or 37 °C. At the incubation time points of 1 h and 24 h, the incubated solutions were analyzed by RP-HPLC with the method described above.

Biodistribution of radiolabeled peptides and their derivatives

Mice bearing NPC tumor were randomly allocated into groups, five mice bearing NPC tumor for each group. Labeled YR13, EY13, EY13NH₂ or Fmoc-YR13 (100 μl , 185 kBq) was injected into the tail vein of each mouse without anesthesia [26,29]. Mice were killed by cervical dislocation at 30 min, 1 h and 2 h post-injection. The organs or tissues were removed, washed and weighed prior to radioactivity counting. Injection solution (100 μl) was taken as a standard for calculating the percent of injected dose per gram of tissue, that is, %ID/g. The tumor to organ (tissue) ratio for each mouse was also calculated. The final results were expressed as the mean \pm SD.

In order to investigate what components resulted in the radioactivity in stomach contents, the washing fluid from the stomachs of mice at the 1 h time point was collected and washing mixtures were centrifuged to collect supernatants. The supernatants were passed through a 0.22 μm micropore filter membrane and analyzed by RP-HPLC.

Metabolism

Labeled YR13, EY13, EY13NH₂ or Fmoc-YR13 (100 μl , 7–10 MBq/20 μg) was injected for each normal mouse through the tail vein. Aliquots of blood samples taken at 10 min and 60 min after injection were centrifuged to collect serum (three mice at each time point). Serum was deproteinized by protein precipitation with acetonitrile [29]. The samples were then centrifuged at 4320 g for 15 min. Aliquots of plasma, serum and supernatant after acetonitrile precipitation were counted. The supernatants were concentrated and analyzed by RP-HPLC with the method already described.

Results

Identification of specific phage clones binding to tumor

Mean value for phage recovered from tumor was approximately 10^5 PFU. Ten individual blue phage plaques

were picked out for sequencing after three rounds, from which five different sequences were identified, as shown in **Table 1**. Of the 10 picked blue plaques, six had the same sequence EDIKPKTSLAFR, but the other four clones had no consistent residues. All 10 sequenced clones had the consistent residue EDIKPKTSLAFR after the fifth round. The N-terminal or C-terminal derivatized peptides with a tyrosine YR13 or EY13, and their derivatives Fmoc-YR13 and EY13NH₂, were subsequently synthesized.

Table 1 Peptide-consensus sequences of specific phage clones binding to nasopharyngeal carcinoma tumor after three rounds of biopanning

Amino acid sequence	No. of hits
EDIKPKTSLAFR	6
TQPADLQTHNHN	1
FDHSSKWTRTSP	1
YSHNTITNLYFS	1
WPRYAESTLQLR	1

Selected phages bind to NPC tumors

The biodistribution of selected phages (phage 3 and phage 5) and the control phage (initial phage library) was investigated in mice bearing NPC tumor. The phage particles were rescued from tumor, brain and heart after phages were injected into mice and circulated for 8 min. The selected phages showed more appreciable enrichment in tumor than control phage, and less phage particles were recovered from control organs (heart and brain). The recovery of phage 5 and phage 3 in tumor was approximately 102-fold and 63-fold higher than that of the control phage, respectively (**Fig. 1**, down). This is consistent with the percentage of peptide EDIKPKTSLAFR in phage solutions deduced from the DNA sequences (**Table 1**). The recovery of phage 5 and phage 3 in tumor was approximately 10-fold and 5-fold higher than that in heart, respectively. The selected phages and the control phage did not show any specific targeting to heart tissues (**Fig. 1**, up). Only less phage particles were recovered in brain of mice injected with selected phages and the control phage.

In order to determine whether the synthetic peptide and the selected phage clone competed for the same binding site, the peptide EY13 competitive inhibition assay was carried out (**Fig. 1**). The phage 5 and the cognate peptide EY13 or control peptide YP13 were simultaneously injected

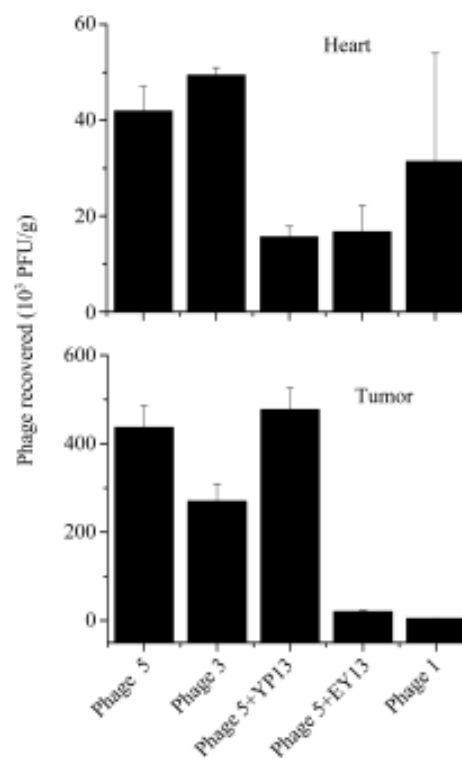


Fig. 1 Recovery of different kinds of phages from human nasopharyngeal carcinoma tumor xenografts

PFU/g, plaque forming unit per gram of tissue. YP13, a nonspecific random 13 peptide. Phage 1, control initial phage peptide library.

into mice bearing NPC tumor. The results indicated that binding of the selected phage (phage 5) was competitively inhibited by the cognate peptide EY13, but not inhibited by the control peptide. Both the targeting peptide (EY13) and non-specific control peptide (YP13) impaired the recovery of phages in heart (**Fig. 1**, up). The study showed that phage particles homing to NPC tumor could be accumulated after 3–5 rounds of panning *in vivo*, and the cognate peptide and selected phages possess the same binding site.

Radioiodination and characterization of ¹³¹I peptides and their derivatives

The RP-HPLC analysis results showed that the radiochemical purity of labeled peptides and their derivatives was higher than 90%. The retention times of ¹³¹I-, [¹³¹I] iodo-YR13, [¹³¹I]iodo-EY13NH₂, [¹³¹I]iodo-EY13 and [¹³¹I] iodo-Fmoc-YR13 were 2.9, 8.6, 9.4, 10.3 and 15.6 min, respectively. The n-octanol/PBS partition co-efficient (logP) of [¹³¹I]iodo-YR13, [¹³¹I]iodo-EY13NH₂, [¹³¹I]iodo-EY13 and [¹³¹I]iodo-Fmoc-YR13 was -1.00, -1.07, -1.07 and -0.49, respectively. MALDI-TOF mass spectrometry showed that the iodinated YR13 consisted of mixtures of

monoiodo peptide and diiodo peptide.

Stability of peptides and their derivatives

Stability of [^{131}I]iodo-YR13, [^{131}I]iodo-EY13NH₂, [^{131}I]iodo-EY13 and [^{131}I]iodo-Fmoc-YR13 in PBS was evaluated by incubation at room temperature and 37 °C for 24 h. The results of RP-HPLC analyses indicated that 80% of [^{131}I]iodo-YR13 and 90% of [^{131}I]iodo-EY13 remained after incubation at 37 °C for 24 h. Only approximately 5% of [^{131}I]iodo-EY13NH₂ and [^{131}I]iodo-Fmoc-YR13 was degraded into traces of radioiodide at 24 h. The radioiodide might be produced directly from deiodination of [^{131}I]iodo-EY13NH₂ and [^{131}I]iodo-Fmoc-YR13. The radiolabeled peptides were stable in PBS.

The activity in serum remained constant at 65%–75% of the one in plasma for labeled YR13, EY13, EY13NH₂ and Fmoc-YR13 after centrifugation. The amount of radioactivity in supernatant of serum deproteinized with acetonitrile was 75%–85% of the activity in serum for the four labeled compounds. Metabolites analysis showed that approximately 80% of the tracer remaining in the serum collected at 10 min post-injection was intact YR13 or EY13; intact Fmoc-YR13 or EY13NH₂ was more than 90% of the tracer in supernatant. Much radioiodine was lost from [^{131}I]iodo-YR13 and [^{131}I]iodo-EY13 at 1 h after injection and only approximately 30% of the activity was contributed by intact peptides (**Fig. 2**). However, a larger portion of intact [^{131}I]iodo-Fmoc-YR13 and [^{131}I]iodo-EY13NH₂ at

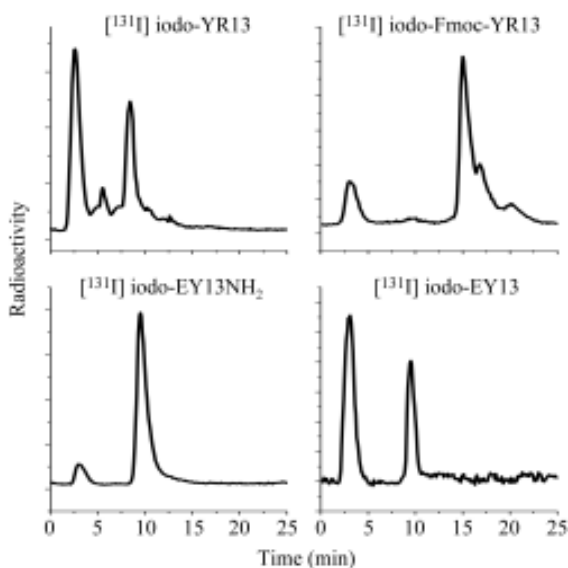


Fig. 2 Metabolites analysis of [^{131}I]iodo-YR13, [^{131}I]iodo-Fmoc-YR13, [^{131}I]iodo-EY13NH₂ and [^{131}I]iodo-EY13 by RP-HPLC in mouse serum collected 60 min after intravenous injection

1 h post-injection remained and only a small part of tracer was radioiodine.

RP-HPLC analysis showed that the major metabolite detected in serum of mice injected with [^{131}I]iodo-YR13 was [^{131}I]iodo-tyrosine (Rt 5.6 min) at 1 h post-injection. The metabolites were not detected in serum of mice injected with [^{131}I]iodo-EY13NH₂ or [^{131}I]iodo-EY13. [^{131}I]iodo-tyrosine and [^{131}I]iodo-tyrosine-NH₂ were not found. Two major metabolites were tested in serum of mice injected with [^{131}I]iodo-Fmoc-YR13 at 1 h. One was eluted with the same retention time as [^{131}I]iodo-Fmoc-tyrosine (Rt 20.1 min), the other was not determined.

The results suggested that much radioiodine was lost from labeled nude peptides (whether tyrosine was added to the N-terminal or C-terminal), and [^{131}I]iodo-tyrosine or [^{131}I]iodo-Fmoc-tyrosine was released by metabolic degradation when a tyrosine or Fmoc-tyrosine was added to the N-terminal. However, in the case of [^{131}I]iodo-EY13 and [^{131}I]iodo-EY13-NH₂, only radioiodine was found in the tracer if tyrosine or tyrosine-NH₂ was added to the C-terminus, whereas [^{131}I]iodo-tyrosine and [^{131}I]iodo-tyrosine-NH₂ were not detected. Amidation of C-terminal derivatized peptide with a tyrosine can not only block deiodination, but also inhibit the release of [^{131}I]iodo-tyrosine-NH₂.

The accumulation of the radioiodine from labeled Fmoc-YR13 and EY13NH₂ in thyroid of mice bearing NPC tumor was far less than that of labeled YR13 and EY13 (**Fig. 3**) at the same time points. At 30 min, activity in thyroid of mice was approximately 9.6-fold (YR13/Fmoc-YR13) and 9.2-fold (EY13/EY13NH₂). This result showed that, if blocked, the N- and C-termini of peptides

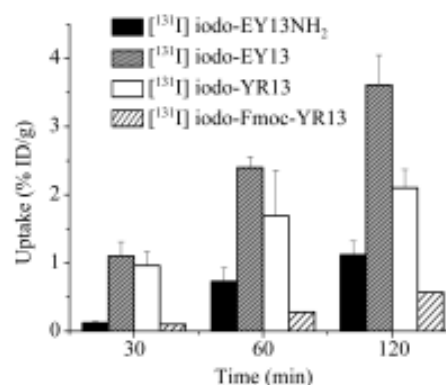


Fig. 3 Uptake of [^{131}I]iodo-YR13, [^{131}I]iodo-EY13, [^{131}I]iodo-EY13NH₂ and [^{131}I]iodo-Fmoc-YR13 in thyroid of mice bearing nasopharyngeal carcinoma tumor

%ID/g, percent of injected dose per gram of tissue.

are able to inhibit *in vivo* deiodination. The radioactivity in thyroid of mice injected with EY13 increased more rapidly than with YR13, indicating that radioiodine seems to be easily released by metabolic degradation when tyrosine is added to the C-terminus.

Biodistribution

The biodistribution of labeled EY13NH₂, YR13, EY13 and Fmoc-YR13 was determined (**Table 2**). The highest uptake of EY13NH₂, EY13 and YR13 in NPC tumors appeared at 30 min post-injection (3.72 %ID/g, 3.16 %ID/g and 3.81 %ID/g respectively), and the uptake of Fmoc-YR13 also reached its maximum 1.66 %ID/g at the same time point. It can also be seen that the uptake (%ID/g) of these four compounds in normal tissues was low, their

clearance was fast (stomach and intestine excluded) within 2 h and the tumor-to-tissue ratios steadily went up with time (**Table 3**). In blood, the concentration of radioactivity became low at 1 h and 2 h. The tumor/blood ratios of EY13NH₂, EY13 and YR13 increased continuously: from 0.57 at 30 min to 1.19 of EY13NH₂ at 2 h; from 0.54 at 30 min to 0.93 of EY13 at 2 h; and from 0.51 at 30 min to 1.00 of YR13 at 2 h. Although the uptake of Fmoc-YR13 in most organs (tissues) was similar to the other three compounds, the radioactivity of Fmoc-YR13 in tumor was much lower than that of YR13 so that tumor-to-blood ratios barely increased from 30 min to 120 min.

There were marked differences in gastrointestinal retention between [¹³¹I]iodo-EY13NH₂, [¹³¹I]iodo-EY13, [¹³¹I]iodo-YR13 and [¹³¹I]iodo-Fmoc-YR13 (**Fig. 4**). The

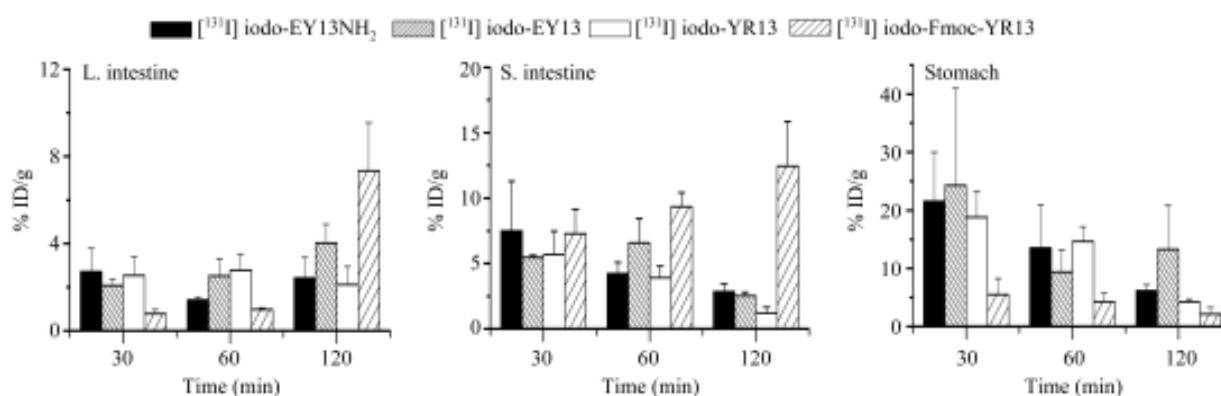
Table 2 Biodistribution of EY13NH₂, EY13, YR13 and Fmoc-YR13 in mice bearing nasopharyngeal carcinoma tumor

Organ or tissue	Time (min)	%ID/g			
		EY13NH ₂	EY13	YR13	Fmoc-YR13
Liver	30	3.37±0.48	2.88±0.68	3.21±0.69	2.48±0.39
	60	1.47±0.41	1.10±0.60	1.42±0.58	1.99±0.29
	120	0.72±0.07	1.08±0.02	0.56±0.12	1.51±0.35
Heart	30	2.23±0.23	2.22±0.54	2.49±0.58	1.23±0.30
	60	0.75±0.12	0.95±0.33	1.26±0.30	1.04±0.47
	120	0.60±0.11	0.71±0.35	0.49±0.04	0.55±0.05
Kidney	30	5.24±0.69	5.84±0.29	4.94±0.19	3.76±2.08
	60	1.54±0.24	2.36±1.18	2.88±1.26	2.33±0.81
	120	1.19±0.22	1.96±0.04	0.89±0.33	1.44±0.26
Lung	30	3.10±0.33	4.13±0.46	3.48±0.77	1.86±0.41
	60	1.42±0.14	3.68±0.74	2.20±0.87	1.27±0.41
	120	0.93±0.13	1.61±0.10	0.66±0.15	0.76±0.03
Spleen	30	1.72±0.16	2.28±0.60	2.24±0.86	1.15±0.26
	60	0.98±0.11	1.05±0.56	1.36±0.42	0.58±0.06
	120	0.61±0.10	1.03±0.11	0.55±0.06	0.34±0.05
Brain	30	0.67±0.36	0.42±0.08	0.51±0.10	0.25±0.11
	60	0.16±0.02	0.14±0.05	0.24±0.07	0.14±0.01
	120	0.14±0.10	0.18±0.04	0.09±0.02	0.10±0.02
Muscle	30	1.54±0.24	1.81±0.73	2.25±0.19	0.82±0.46
	60	0.52±0.01	1.52±0.37	0.79±0.22	0.41±0.04
	120	0.36±0.10	0.89±0.04	0.32±0.08	0.32±0.06
Blood	30	6.59±1.03	5.56±1.29	7.71±2.41	3.48±0.29
	60	2.28±0.33	3.80±0.54	3.42±1.36	3.41±0.21
	120	1.58±0.23	2.10±0.37	1.17±0.19	1.48±0.12
Tumor	30	3.72±0.64	3.16±0.69	3.81±0.72	1.66±0.11
	60	2.15±0.13	3.18±0.53	3.03±0.86	1.73±0.06
	120	1.89±0.42	1.95±0.02	1.17±0.30	0.73±0.07

%ID/g, percent injected dose per gram of tissue.

Table 3 Tumor-to-organ (tissue) ratios of peptides in mice bearing nasopharyngeal carcinoma tumor

Tumor/organ or tissue	Time (min)	Tumor/organ ratio			
		EY13NH ₂	EY13	YR13	Fmoc-YR13
Tumor-to-liver	30	1.09±0.26	0.81±0.03	1.24±0.49	0.68±0.14
	60	1.52±0.30	1.57±0.30	2.22±0.35	0.88±0.16
	120	2.65±0.74	1.79±0.05	2.10±0.40	0.54±0.16
Tumor-to-heart	30	1.64±0.43	1.26±0.29	1.61±0.66	1.42±0.48
	60	2.91±0.49	1.79±0.33	2.39±0.15	1.85±0.88
	120	3.20±0.83	2.18±0.78	2.36±0.58	1.41±0.09
Tumor-to-lung	30	1.18±0.31	0.76±0.08	1.14±0.46	0.93±0.26
	60	1.52±0.06	0.90±0.28	1.43±0.28	1.44±0.51
	120	2.10±0.81	1.22±0.09	1.75±0.08	1.02±0.05
Tumor-to-muscle	30	2.45±0.94	1.82±0.04	1.71±0.47	1.90±0.47
	60	4.14±0.37	2.08±0.26	3.98±0.34	4.28±0.58
	120	5.25±0.55	2.19±0.42	3.22±0.04	2.48±0.48
Tumor-to-blood	30	0.57±0.05	0.54±0.06	0.51±0.06	0.48±0.03
	60	0.95±0.12	0.84±0.05	0.91±0.09	0.51±0.05
	120	1.19±0.10	0.93±0.15	1.00±0.20	0.52±0.04

**Fig. 4** Tissue distribution of [¹³¹I]EY13NH₂, [¹³¹I]EY13, [¹³¹I]YR13 and [¹³¹I]Fmoc-YR13 in mice bearing nasopharyngeal carcinoma tumor

%ID/g, percent of injected dose per gram of tissue.

first three labeled compounds gave much higher uptake in stomach, for example, uptake for [¹³¹I]iodo-EY13 was 24.32%ID/g at 30 min, whereas the latter ([¹³¹I]iodo-Fmoc-YR13) gave rather low retention in stomach, only 5.47 %ID/g at 30 min. However, 4–8% of the tracer was found in stomach contents for [¹³¹I]iodo-EY13 and [¹³¹I]iodo-YR13 at 1 h post-injection. Less [¹³¹I]iodo-Fmoc-YR13 (<1.0 %ID/g) was detected, and approximately 20%–30% of the given dose for [¹³¹I]iodo-EY13NH₂ was determined. Therefore, we can conclude that a large amount of activity in stomach was from radioiodine lost from [¹³¹I]

iodo-EY13 and [¹³¹I]iodo-YR13. The activity in stomach of mice injected with [¹³¹I]iodo-EY13NH₂ was mainly provided with intact [¹³¹I]iodo-EY13NH₂ and a little radioiodine.

Perhaps due to different metabolic and excretion modes, the radiotracer of [¹³¹I]iodo-Fmoc-YR13 in small intestine remained largely and continually increased from 30 min to 2 h, followed by temporary suspension in large intestine. Radioiodide should not be the main chemical form in intestine [26]; if it is, Na⁺/I⁻ symporter (NIS) system could reabsorb it back into the circulatory system, resulting in

low radioactivity in intestine [30]. Therefore, in the case of [¹³¹I]iodo-Fmoc-YR13, a large part of the radioactivity in intestine could not be radioiodide.

The uptake in stomach of mice injected with labeled YR13, EY13, EY13NH₂ and Fmoc-YR13 showed significant difference. It is possible that YR13, EY13 and EY13NH₂, whose N-terminals are nude NH₂ are absorbed into stomach by higher acidity. They then go into small intestine and large intestine. Fmoc might hinder Fmoc-YR13 from going into stomach because the N-terminal of YR13 has been blocked. Therefore, the N-terminal state (blocked or not blocked) of peptides and their derivatives might result in their different biodistribution in stomach.

Discussion

Since phage display technology was reported 20 years ago by Smith [31], a central goal in the field of molecular targeting has been to find new cancer targeting peptides. One novel peptide EDIKPKTSLAFR that targets NPC tumor was identified by *in vivo* phage display. Lee *et al.* [20] isolated a 12 mer peptide RLLDTNRPLL PY (L-peptide) specifically binding to the NPC tumor cell *in vitro* with a phage display random peptide library. The L-peptide bound to the tumor cell surface of most NPC cell lines and biopsy specimens, but not normal nasal mucosal cells. L-peptide-linked liposomes carrying doxorubicin (L-peptide-Lipo-Dox) caused marked cytotoxicity in NPC cells and suppressed tumor growth better than Lipo-Dox. Because the biodistribution of L-peptide was not reported in the article and tumor-to-blood ratios were not obtained, it could not be concluded whether L-peptide could be used as an imaging agent. Other studies [6,24] found that peptides selected *in vitro* or *in situ* might not effectively target tumors. Therefore, new targeting peptides should be selected *in vivo*.

As the original dodecapeptide EDIKPKTSLAFR does not contain suitable structures for labeling, the N-terminal or C-terminal derivatized peptides with a tyrosine YR13 and EY13, and their derivatives Fmoc-YR13 and EY13NH₂, were synthesized. Labeled YR13, EY13 and EY13NH₂ possessed higher specific uptake and slower clearance rate in tumors. The results indicated that peptide EDIKPKTSLAFR has shown clinical promise as a cancer targeting agent.

Deiodination *in vivo* of directly radioiodinated peptide has been reported as an important phenomenon [32,33]. Alternative procedures have been studied to protect peptides from *in vivo* deiodination. The use of an acylation

agent derived from the prototypical *N*-succinimidyl 3-iodobenzoate for labeling proteins can decrease deiodination in mice [34–36]. Our laboratory found that a radioiodinated N-terminal tyrosine of a peptide that was protected with a *t*-butyloxycarbonyl group was quite resistant to *in vivo* deiodination and resulted in rapidly reducing radioactive background and negligible radioactivity accumulation in both thyroid and stomach [26]. In order to improve the quality of peptides and raise stability *in vivo*, Fmoc, another widely used α -amino-protecting group, was conjugated to YR13, and EY13 was amidated. The uptake of radioiodinated Fmoc-YR13 and EY13NH₂ in thyroid was far lower than that of radioiodinated YR13 and EY13 (**Fig. 3**). The RP-HPLC analysis of the serum collected 10 min and 60 min after injection showed that only a little radioiodine was lost from labeled Fmoc-YR13 and EY13NH₂ (**Fig. 2**). However, although Fmoc and NH₂ groups have the potency to protect peptides from deiodination, the larger Fmoc group might inhibit peptides from binding to NPC tumor with lower affinity. The amidation of peptide EY13 could not only block deiodination but also obtain a favorable tumor/blood ratio. It might be that the smaller NH₂- group increases the stability *in vivo* and does not affect affinity.

The low *n*-octanol/PBS partition coefficient (*P*_{o-w}) is often associated with the lower and shorter retention in background tissues and blood [37]. Therefore, the hydrophilic nature of peptides and their derivatives might result in lower uptake of these four compounds in the normal tissues and faster clearance so as to obtain favorable tumor-to-organ (tissue) ratios, except Fmoc-YR13. The synthetic peptide and the selected phage clone competed for the same binding site and illustrated the specificity of the tracer accumulation.

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