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 (phase 3 or phase 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 131I, 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 Na131I (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 108 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 titering.

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 131I 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 Na131I (1.8×10^7 Bq) were added to the mixtures and incubated at room temperature for 30 min. YR13 was also labeled with stable 127I. The iodination was quenched after removing Iodogen. The MALDI-TOF mass spectrometry m/z for [M+H]+ was found: 1694.3 ([127I-YR13+H]+); and 1820.2 ([127I2-YR13+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 [131I] 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±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 10^7 PFU. Ten individual blue 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 EDIKPKTLSAFR, but the other four clones had no consistent residues. All 10 sequenced clones had the consistent residue EDIKPKTLSAFR 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

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>No. of hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDIKPKTLSAFR</td>
<td>6</td>
</tr>
<tr>
<td>TQPADLQTHNHN</td>
<td>1</td>
</tr>
<tr>
<td>FDHSSKWTRTSP</td>
<td>1</td>
</tr>
<tr>
<td>YSHNTTNLYFS</td>
<td>1</td>
</tr>
<tr>
<td>WPRYAEStLQLR</td>
<td>1</td>
</tr>
</tbody>
</table>

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 EDIKPKTLSAFR 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 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 radioactive 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 [\(^{131}\text{I}\)]iodo-YR13, [\(^{131}\text{I}\)]iodo-EY13NH\(_2\), [\(^{131}\text{I}\)]iodo-EY13 and [\(^{131}\text{I}\)]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 [\(^{131}\text{I}\)]iodo-YR13 and 90% of [\(^{131}\text{I}\)]iodo-EY13 remained after incubation at 37 °C for 24 h. Only approximately 5% of [\(^{131}\text{I}\)]EY13NH\(_2\) and [\(^{131}\text{I}\)]Fmoc-YR13 was degraded into traces of radioiodide at 24 h. The radioiodide might be produced directly from deiodination of [\(^{131}\text{I}\)]iodo-EY13NH\(_2\) and [\(^{131}\text{I}\)]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, EY13NH\(_2\) 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 EY13NH\(_2\) was more than 90% of the tracer in supernatant. Much radioiodine was lost from [\(^{131}\text{I}\)]iodo-YR13 and [\(^{131}\text{I}\)]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 [\(^{131}\text{I}\)]iodo-Fmoc-YR13 and [\(^{131}\text{I}\)]iodo-EY13NH\(_2\) at 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 [\(^{131}\text{I}\)]iodo-YR13 was [\(^{131}\text{I}\)]iodo-tyrosine (Rt 5.6 min) at 1 h post-injection. The metabolites were not detected in serum of mice injected with [\(^{131}\text{I}\)]iodo-EY13NH\(_2\) or [\(^{131}\text{I}\)]iodo-EY13. [\(^{131}\text{I}\)]iodo-tyrosine and [\(^{131}\text{I}\)]iodo-tyrosine-NH\(_2\) were not found. Two major metabolites were tested in serum of mice injected with [\(^{131}\text{I}\)]iodo-Fmoc-YR13 at 1 h. One was eluted with the same retention time as [\(^{131}\text{I}\)]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 [\(^{131}\text{I}\)]iodo-tyrosine or [\(^{131}\text{I}\)]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 [\(^{131}\text{I}\)]iodo-EY13 and [\(^{131}\text{I}\)]iodo-EY13-NH\(_2\), only radioiodine was found in the tracer if tyrosine or tyrosine-NH\(_2\) was added to the C-terminus, whereas [\(^{131}\text{I}\)]iodo-tyrosine and [\(^{131}\text{I}\)]iodo-tyrosine-NH\(_2\) were not detected. Amidation of C-terminal derivatized peptide with a tyrosine can not only block deiodination, but also inhibit the release of [\(^{131}\text{I}\)]-tyrosine-NH\(_2\).

The accumulation of the radioiodine from labeled Fmoc-YR13 and EY13NH\(_2\) 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/EY13NH\(_2\)). This result showed that, if blocked, the N- and C-termini of peptides...
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>
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<th>Organ or tissue</th>
<th>Time (min)</th>
<th>%ID/g</th>
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<tbody>
<tr>
<td></td>
<td>EY13NH₂</td>
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</tr>
<tr>
<td>Liver</td>
<td>30</td>
<td>3.37±0.48</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.47±0.41</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.72±0.07</td>
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<tr>
<td>Heart</td>
<td>30</td>
<td>2.23±0.23</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.75±0.12</td>
</tr>
<tr>
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<td>120</td>
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<tr>
<td>Kidney</td>
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<tr>
<td>Lung</td>
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<td>Brain</td>
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<tr>
<td></td>
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<tr>
<td>Muscle</td>
<td>30</td>
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</tr>
<tr>
<td></td>
<td>60</td>
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<td></td>
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<tr>
<td></td>
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<td></td>
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<tr>
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</tr>
<tr>
<td></td>
<td>120</td>
<td>1.89±0.42</td>
</tr>
</tbody>
</table>

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

http://www.abbs.info; www.blackwellpublishing.com/abbs
The 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

![Fig. 4 Tissue distribution of [1^31]I EY13NH2, [1^31]I EY13, [1^31]I YR13 and [1^31]I Fmoc-YR13 in mice bearing nasopharyngeal carcinoma tumor](image)

%ID/g, percent of injected dose per gram of tissue.
low radioactivity in intestine [30]. Therefore, in the case of \[^{131}I\]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 NH₃ 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 RLLDTNRPLLPY (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 (Po-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.

**References**


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