HER2-Targeted Hybrid Peptide That Blocks HER2 Tyrosine Kinase Disintegrates Cancer Cell Membrane and Inhibits Tumor Growth In Vivo

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Abstract

HER2 is a transmembrane oncoprotein encoded by the HER2/neu gene and is overexpressed in approximately 20% to 30% of breast cancers. We have recently designed a novel class of drug, the hybrid peptide, which is chemically synthesized and is composed of a target-binding peptide and a lytic peptide containing cationic-rich amino acid components that disintegrate the cell membrane, leading to cancer cell death via membrane lysis. In this study, we designed a HER2-binding peptide linked to this novel lytic peptide, which we termed the HER2-lytic hybrid peptide and assessed the cytotoxic activity of this hybrid peptide in vitro and in vivo. The HER2-lytic hybrid peptide showed high cytotoxic activity against all ovarian and breast cancer cell lines, even trastuzumab- and/or lapatinib-resistant cells, but not against normal cells. Competition assays using anti-HER2 antibody and knockdown of this receptor by siRNA confirmed the specificity of the HER2-lytic hybrid peptide. In addition, it was shown that the HER2-lytic hybrid peptide can disintegrate the cancer cell membrane of HER2-overexpressing SK-BR-3 cancer cells in only 5 minutes, but not normal cells, and block HER2 signaling. Intravenous administration of the HER2-lytic peptide in the athymic mouse implanted with BT-474 and MDA-MB-453 cells significantly inhibited tumor progression. The HER2-lytic hybrid peptide was effective even in breast cancer cell lines that are resistant to trastuzumab and/or lapatinib in vitro and in vivo. Therefore, this hybrid peptide may provide a potent treatment option for patients with cancer.

Introduction

The human epidermal growth receptor 2 (HER2; ErbB2/neu) oncogene encodes a receptor tyrosine kinase that is overexpressed in 20% to 30% of human breast cancers and is correlated with poor prognosis (1, 2). As HER2 is overexpressed at the cell surface of tumor cells, the accessibility of the receptor makes it a suitable candidate for molecular targeted therapies. Over the past few years, monoclonal antibodies (mAbs) as well as tyrosine kinase inhibitors that target HER2 have been developed.

Trastuzumab, a humanized recombinant monoclonal antibody against HER2, is currently used for the treatment of HER2-positive breast cancers (3). Prognosis was improved in patients with HER2/neu-positive disease who received trastuzumab (4). Nevertheless, a significant number of patients with HER2-overexpressing breast cancer will be resistant, either initially or eventually, to anti-HER2-based therapy with trastuzumab (5, 6). After the development of trastuzumab, several other drugs targeting the HER receptor family have also been developed, including the small molecule kinase inhibitor lapatinib, which has proven effective in clinical trials (7–9). A recently reported clinical trial of single-agent lapatinib in trastuzumab-refractory advanced breast cancer showed a 12.8% response rate, consistent with the previous trial; median time to progression in the single-agent trial was 15.3 weeks (10). Thus, lapatinib is an important and effective therapy for a subset of patients with HER2-overexpressing metastatic breast cancers that have progressed on trastuzumab. However, similar to trastuzumab, the median duration of response to lapatinib was less than 1 year, and the majority of trastuzumab-pretreated patients (almost 80%) failed to respond to lapatinib.

To overcome drug resistance, we have designed a novel molecular-targeted drug termed a "hybrid peptide," which is chemically synthesized and is composed of a target-binding peptide and a lytic peptide containing cationic-rich amino acid components that disintegrates the cancer cell membrane selectively (11–13). We have reported that epidermal growth factor receptor (EGFR)-lytic peptide, which is composed of EGFR-binding...
peptide and the lytic peptide, successfully induced sufficient cytotoxic activity against cancer cell lines resistant to a tyrosine kinase inhibitor, such as erlotinib, gefinitib, or PD153035 (11).

To use this concept, we have designed a new peptide that targets HER2, termed HER2-lytic hybrid peptide, which is composed of a HER2-binding moiety and a cellular membrane-lytic moiety, using the previous identification of HER2-binding peptide sequences (14). Here we show the selective cytotoxic activity and characterize the cancer cell-killing mechanisms of HER2-lytic hybrid peptide as well as report the antitumor activity of this molecule in an *in vivo* xenograft model.

Materials and Methods

**Cell lines**

Human breast cancer cell lines (SK-OV-3, SK-BR-3, BT-474, T-47D, ZR75-1, BT-20, MCF-7, MDA-MB-361, MDA-MB-453, MDA-MB-231, and UACC-893) were purchased from the American Type Culture Collection. Rat heart-derivered embryonic myocytes (H9c2) was purchased from the European Collection of Cell Culture. Primary hepatocyte cell (HC; ACBRI 3716) and pancreatic epithelial cell (PE; ACBRI 515) were purchased from DS Pharma Biomedical. No authentication of cell lines was done by the authors. Cells were cultured in RPMI-1640 (BT-474, T-47D, ZR75-1, BT-20), MDA-MB-361, MDA-MB-453, MDA-MB-231, and UACC-893), CS-C (HC and PE cells), MEM (MCF-7), D-MEM (H9c2), or McCoy's (SK-OV-3 and SK-BR-3) culture medium with 10% fetal bovine serum (BioWest), 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque) under 5% CO2.

**Preparation and synthesis of peptides**

The following peptides were produced from Invitrogen or Sigma. Bold and underlined letters indicate D-amino acids.

1. HER2-binding moiety peptide: KCCYSL
2. Lytic peptide: KLKKLKKK
3. HER2-lytic hybrid peptide: KCCYSLGGGKLKKLKKK

All peptides were synthesized using solid-phase chemistry, purified to homogeneity (i.e., >80%) by reverse-phase high-pressure liquid chromatography, and assessed by mass spectrometry. Peptides were dissolved in water.

**Antibodies**

Antibodies against phospho-HER2 (Tyr1248), Akt monoclonal antibody, phospho-Akt (Ser473) monoclonal antibody, p44/42 mitogen-activated protein kinase (MAPK; Erk1/2) mouse monoclonal antibody (MAPK), and Phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204) rabbit polyclonal antibody were obtained from Cell Signaling. Anti-HER2 antibody was purchased from BD Transduction Laboratories. Anti-Src antibody was obtained from Enabling Discovery. Anti-Phosphor-Src (pY418) antibody was purchased from Enabling Discovery in Life Sciences.

**Cell viability assay**

Cell viability assay was done as described previously (15). Briefly, cells were seeded in 96-well plates at 3 × 10^3 cells per well and incubated for 24 hours. The cells were then incubated with increasing concentrations of lytic peptide or the HER2-lytic peptide for 72 hours. Cell viability was measured using WST-8 solution (Cell Count Reagent SF, Nacalai Tesque). For competition assays, the cells were incubated with anti-HER2 monoclonal antibody (BD Biosciences) or mouse IgG isotype control for 3 hours then incubated with HER2-lytic hybrid peptide for 24 hours.

**Immunofluorescence staining**

HER2 expression was determined using flow cytometry by incubating 1 × 10^6 cells with an FITC-conjugated human monoclonal antibody against HER2 (BD Biosciences). All staining was conducted at room temperature for 30 minutes. Cells were washed with phcytometry (FACS Calibur; BD Biosciences). The mean fluorescence intensity (MFI) was determined using Win MDI version 2.9 software (The Scripps Research Institute).

**siRNA transfection**

siRNA transfection was conducted as previously described (15). The after stealth RNA duplexes were synthesized by Invitrogen:

HER2 sense,
5'-GUCACAGGGGCCCCUCCCCAGAGGCCC-3';
HER2 antisense,
5'-GGGCCCUCUGGGAGGCCCCUUGAC-3';
scramble sense,
5'-GCAUCGACAGACAAUCUCAGUUU-3'; and
scramble antisense,
5'-AAACTGAGAUGUGUCUGACAGC-3'.

SKBR3 and SKOV3 cells were grown to 40% confluence on a 6-well plate, and then transfection of these cells with siRNAs (100 pmol/mL) was done using Lipofectamine RNAi Max (Invitrogen), according to the manufacturer’s protocol.

**Confocal fluorescence microscopy**

HC and SK-BR-3 cells were grown to 20% confluence on a chamber slide for 24 hours and HER2-lytic peptide labeled with CF488A (Biotium) was added to the medium at a final concentration of 15 μmol/L. Confocal images were taken 5, 15, and 30 minutes after addition of HER2-lytic peptide using an Olympus FV1000 confocal laser scanning microscope (Tokyo, Japan).
Binding assay
HER2-lytic peptide labeled with CF488A (Biotium) was incubated with SK-BR-3 and PE cells. After incubation for 5, 15, or 30 minutes, cells were washed twice with phosphate-buffered saline (PBS), and binding peptides were detected using flow cytometer (FACS Calibur; BD Biosciences) in FL-1 channel. Binding activity was calculated from the value of MFI.

Immunoblotting
Cells were washed with PBS and lysed in lysis buffer (Promega). Protein lysates were resolved by SDS-PAGE and transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare) in glycine-Tris buffer. Membranes were blocked with 1% bovine serum albumin in PBS and incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Membranes were subjected to chemiluminescence detection using an LAS-3000 LuminoImage analyzer (Fujifilm).

In vivo efficacy in xenograft models
Animal experiments were carried out in accordance with regulations on Animal Experiments at Kyoto University, Kyoto, Japan. Breast cancer cell line BT474 and MDA-MB-453 cells were resuspended to 1 x 10^7 cells/100 μL in 50% PBS/50% Matrigel (BD Biosciences) and implanted subcutaneously into the flank region of 7-week-old athymic female nude mice weighing 17 to 21 g. When tumors reached 20 to 60 mm^3 in volume, animals were randomly assigned to 2 groups, receiving either saline (control) or HER2-lytic peptide (3 mg/kg) as intravenous injections (50 μL/injection) 3 times a week for a total of 9 doses. Tumors were measured with a caliper and tumor volume (in mm^3) was calculated using the following formula: length x width^2 x 0.5. All values are expressed as the mean ± SD.

Statistical analysis
Data are expressed as the mean ± SD of triplicate determinations. Statistical difference was determined using Student t test, and P-values less than 0.05 were considered statistically significant.

Results
Enhancement of cytotoxic activity of Her2-lytic hybrid peptide compared with the lytic peptide alone is dependent on cell-surface HER2 expression levels
First we examined the correlation between expression levels of HER2 on the cell surface and cytotoxic activity using 13 cell lines (1 ovarian cancer, 10 breast cancer, and 2 normal). As shown in Fig. 1A, treatment with the lytic peptide alone induced dose-dependent cytotoxic cell killing in all cancer cell lines. However, molecular-targeted HER2-lytic hybrid peptide was superior to lytic peptide alone in inducing cytotoxic activity in all the cell lines. As shown in Table 1, the range of IC50 values (the peptide concentration that induces 50% inhibition of control cell growth) was 3.5 to 20.7 μmol/L for the HER2-lytic hybrid peptide, whereas lytic peptide alone induced modest cytotoxic activity with the IC50 values ranging from 14.1 to 65.9 μmol/L. These data suggest that the cancer cells are more sensitive (2.2- to 5.2-fold) to the HER2-lytic hybrid peptide than to the lytic peptide alone.

We then assessed the cytotoxic activity of these peptides in the 2 normal cell lines. As shown in Fig. 1B, HC or PE cells showed only minimal cell death at a peptide concentration of 30 or 40 μmol/L, indicating that these normal cells were less sensitive to the peptides than cancer cell lines.

The correlation between the IC50 values for these peptides and HER2 expression in both cancer and normal cells was also assessed. A total of 6 of 11 established ovarian and breast cancer cell lines (SK-OV-3, SK-BR-3, BT-474, MDA-MB-361, MDA-MB-453, and UACC-893) are known to have HER2 gene amplification and overexpress HER2 protein (16–18). In this study, a similar result was provided. As shown in Table 1, the MFI values for these 6 cell lines ranged from 52.8 to 91.4, whereas the MFI for the remaining 5 cell lines ranged from 5.7 to 18.9. Furthermore, the MFI of the 2 normal cell lines was even lower (2.6 and 4.1).

As shown in Fig. 1C, the MFI value was not correlated with the IC50 values of the HER2-lytic hybrid peptide (r = −0.50; left) or lytic peptide alone (r = −0.21; middle). By contrast, MFI correlated well with the ratio of the IC50 values of lytic peptide alone/HER2-lytic peptide in each cell line (r = 0.81; Fig. 1C, right). These results suggest that the increase in cytotoxicity that occurs when the HER2 moiety is added to the lytic peptide is dependent on cell-surface HER2 expression levels.

Binding of the HER2-lytic hybrid peptide to HER2 protein on the cell surface is critically required for induction of target-specific cell killing
To confirm the specificity of the HER2-lytic hybrid peptide for HER2, we conducted a competition assay using anti-HER2 monoclonal antibody and siRNAs specific to HER2. As shown in Supplementary Fig. S1A, the anti-HER2 antibody used in this study did not show any cytotoxic or proliferative effects on the BT-474 and MDA-MB-231 cancer cells. Either anti-HER2 monoclonal antibody or mouse IgG isotype control was added to the BT-474 or MDA-MB-231 cultures 3 hours before exposure to the HER2-lytic hybrid peptide to assess the effect of this peptide on cytotoxic activity. As shown in Fig. 2A (left), the inhibition of cytotoxic activity of the HER2-lytic peptide in BT-474 cells by HER2 monoclonal antibody was dose dependent, whereas no significant difference in cytotoxic activity with regard to antibody concentration was found with the mouse IgG isotype control. By contrast, no inhibition of cytotoxic activity by anti-HER2 or control antibody was observed in MDA-MB-231 (Fig. 2A, right).

We also assessed whether cancer cells became less sensitive to the HER2-lytic hybrid peptide on reduction of HER2 expression by HER2 siRNA. The levels of target
Figure 1. Cytotoxic activity of HER2-lytic hybrid peptide. A, eleven cancer cell lines were cultured with various concentrations of HER2-lytic hybrid peptide or lytic peptide (0–90 μmol/L) for 72 hours, and cytotoxic activity was assessed using WST-8 reagent. Black and white squares indicate HER2-lytic hybrid peptide and lytic peptide, respectively. B, two normal cell lines were cultured with various concentrations of peptides (0–60 μmol/L) for 72 hours and cytotoxic activity was assessed. Absorbance values obtained with untreated cells were set at 100%. Black and white squares indicate HER2-lytic hybrid peptide and lytic peptide, respectively. Data are represented by mean ± SD (error bars) from triplicate determinations. C, IC50 values of HER2-lytic hybrid peptide (left) and lytic peptide alone (middle) in normal and cancer cells, and the IC50 ratio of lytic/HER2-lytic hybrid peptide (right) are shown plotted against the MFI of HER2 expression.
protein in the cells were confirmed by immunoblotting (Fig. 2B, inset). After transfection of HER2 siRNA into SK-BR-3 or SK-OV-3 cells, the cells became less sensitive to the cytotoxic effect of the HER2-lytic hybrid peptide (5 μmol/L for SK-BR-3 cells and 10 μmol/L for SK-OV-3 cells) compared with that of cells transfected with scramble siRNA as a control (Fig. 2B). We also confirmed that siRNA used in this study was not cytotoxic to cancer cell lines or normal cell line (SK-BR-3, SK-OV-3, and PE) as shown in Supplementary Fig. S1B. These results suggest that the binding of the HER2-lytic hybrid peptide to HER2 expressed on the cell surface is critically required for the induction of target-specific cell killing.

**HER2-lytic hybrid peptide penetrates the cancer cell membrane, allowing rapid killing of cancer cells**

Figure 3A shows representative confocal images of HER2-overexpressing breast cell line SK-BR-3 and normal HC cells treated with CF488A-labeled HER2-lytic hybrid peptide at 15 μmol/L for 30 minutes. The peptide was localized to the membrane of SK-BR-3 cells, accumulated in the membrane after 5 to 15 minutes (Fig. 3A, arrows), and then penetrated the cell membrane and was incorporated into the cytoplasm (Fig. 3A, arrowheads). By contrast, the HER2-lytic peptide did not accumulate in the membrane of normal HC cells (Fig. 3A).

Next, we examined both the binding activity of CF488A-labeled HER2-lytic peptide (15 μmol/L) to SK-BR-3 and HC cells by FACS (Fig. 3B) and the cytotoxicity (Fig. 3C) of SK-BR-3 and HC cells treated with HER2-lytic peptide (15 μmol/L) for 5, 15, or 30 minutes. These results revealed a direct correlation between binding activity and cytotoxicity. Exposure to HER2-lytic hybrid peptide (15 μmol/L) resulted in a time-dependent loss of viability in SK-BR-3 cells; exposure of SK-BR-3 cells to the peptide for only 5 minutes was sufficient to kill approximately 50% of cancer cells, and >80% of cells lost viability after 30 minutes. By contrast, in normal HC cells we did not observe a time-dependent increase of binding activity or cytotoxicity by the HER2-lytic peptide.

**HER2-lytic hybrid peptide inhibits HER2 signaling**

Although the HER2-binding moiety (KCCYSL) was selected from a random peptide bacteriophage library and exhibited specific binding activity to the recombinant extracellular domain of ErbB2 and to human cancer cells expressing the HER2 receptor (14), the effect of the peptide on HER2 signaling remains unclear. Therefore, we examined the effects of both the HER2-binding moiety and the HER2-lytic hybrid peptide on HER2 and on 3 major signaling transduction pathways activated by this tyrosine kinase receptor. Treatment of HER2-binding peptide for 3 hours did not result in any proliferative or cytotoxic effects on SK-BR-3, BT-474, MDA-MB-361, MDA-MB-453, or MDA-MB-231 cells (Supplementary Fig. S2).

It has been reported that SK-OV-3, SK-BR-3, and BT-474 are sensitive to trastuzumab and lapatinib, MDA-MB-361 cells are sensitive to trastuzumab and resistant to lapatinib, MDA-MB-453 cells are sensitive to both trastuzumab and lapatinib, and that UACC-893 and MDA-MB-453 are resistant to both trastuzumab and lapatinib (16–18).

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**Table 1. Cytotoxic activity of peptides in various cell lines and HER2 expression**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Organ</th>
<th>IC50 (μmol/L)a</th>
<th>Lytic peptide alone</th>
<th>HER2-lytic peptide</th>
<th>IC50 Ratio</th>
<th>MFIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer cells</td>
<td></td>
<td>Lytic peptide alone</td>
<td>HER2-lytic peptide</td>
<td>IC50 Ratio</td>
<td>MFIb</td>
<td></td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>Ovary</td>
<td>65.9 ± 1.4</td>
<td>17.1 ± 1.2</td>
<td>3.9</td>
<td>56.9 ± 14.1</td>
<td></td>
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<tr>
<td>SK-BR-3</td>
<td>Breast</td>
<td>25.7 ± 0.6</td>
<td>5.9 ± 0.2</td>
<td>4.4</td>
<td>52.8 ± 14.7</td>
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</tr>
<tr>
<td>BT-474</td>
<td>Breast</td>
<td>34.5 ± 3.8</td>
<td>7.6 ± 0.7</td>
<td>3.4</td>
<td>56.9 ± 12.7</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>Breast</td>
<td>40.1 ± 8.6</td>
<td>7.7 ± 0.7</td>
<td>3.4</td>
<td>91.4 ± 5.3</td>
<td></td>
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<tr>
<td>MDA-MB-453</td>
<td>Breast</td>
<td>16.1 ± 0.9</td>
<td>4.2 ± 0.3</td>
<td>2.5</td>
<td>82.5 ± 10.8</td>
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<tr>
<td>UACC-893</td>
<td>Breast</td>
<td>17.5 ± 2.2</td>
<td>3.5 ± 0.7</td>
<td>5.0</td>
<td>53.6 ± 3.5</td>
<td></td>
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<tr>
<td>T-47D</td>
<td>Breast</td>
<td>14.1 ± 3.6</td>
<td>5.7 ± 2.6</td>
<td>3.0</td>
<td>10.4 ± 0.9</td>
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<tr>
<td>ZR75-1</td>
<td>Breast</td>
<td>19.4 ± 1.7</td>
<td>8.9 ± 2.5</td>
<td>2.0</td>
<td>11.2 ± 6.6</td>
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<tr>
<td>BT-20</td>
<td>Breast</td>
<td>20.0 ± 2.9</td>
<td>6.3 ± 1.0</td>
<td>3.2</td>
<td>18.9 ± 2.2</td>
<td></td>
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<tr>
<td>MCF7</td>
<td>Breast</td>
<td>75.9 ± 5.8</td>
<td>20.7 ± 4.6</td>
<td>3.7</td>
<td>8.7 ± 3.0</td>
<td></td>
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<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>27.0 ± 1.5</td>
<td>9.0 ± 0.6</td>
<td>3.0</td>
<td>5.7 ± 1.3</td>
<td></td>
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<tr>
<td>Normal cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HC</td>
<td>Liver</td>
<td>59.2 ± 5.0</td>
<td>42.9 ± 4.5</td>
<td>1.4</td>
<td>4.1 ± 1.2</td>
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<tr>
<td>PE</td>
<td>Pancreas</td>
<td>59.6 ± 7.2</td>
<td>51.7 ± 0.7</td>
<td>1.2</td>
<td>2.6 ± 2.7</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HC, hepatocyte; PE, pancreatic epithelial cell.

aThe value of IC50 is represented by mean ± SD from triplicate determinations.

bMFI, mean fluorescent intensity; this is the extent of binding of the phycoerythrin-conjugated anti-HER2 monoclonal antibody to cells.

The value of MFI is represented by mean ± SD from 3 independent assays.

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Molecular Cancer Therapeutics
Exposure of BT-474, SK-BR-3, MDA-MB-361, MDA-MB-453, and MDA-MB-231 cells to HER2-lytic hybrid peptide (at half the IC50 value for each cell line for 3 hours) had almost no effect on the viability of these cells (Supplementary Fig. S3). SK-BR-3 and BT-474 treated with HER2-lytic hybrid peptide resulted in a decrease in HER2 protein and phosphorylation-HER2, -AKT, -MAPK, and -Src proteins, and exposure of HER2-binding moiety peptide of BT-474 and SK-BR-3 also resulted in a minor decrease in the same phosphorylation proteins (Fig. 4). However, exposure of MDA-MB-361, MDA-MB-453, and MDA-MB-231 cells to both peptides did not have any effect on HER2 and on the 3 signal transduction pathways activated by this tyrosine kinase receptor. Although MDA-MB-361 and MDA-MB-453 are HER2-overexpressing cell lines (Fig. 4); as previously suggested, they do not solely rely on HER2 signaling for proliferation because of, for instance, growth factor receptor signaling redundancy (19–22). However, the HER2-lytic hybrid peptide was effective in trastuzumab- and/or lapatinib-resistant MDA-MB-453 and MDA-MB-361 cells (Fig. 1A and Table 1).

**Antitumor activity of HER2-lytic peptide in vivo**

To assess the antitumor effect of HER2-lytic peptide in a xenograft model of HER2-overexpressing breast cancer, BT-474 (both trastuzumab and lapatinib sensitive) and MDA-MB-453 (both trastuzumab and lapatinib resistant) cells were implanted subcutaneously into athymic mice. Subsequently, HER2-lytic peptide was injected intravenously at a dose of 3 mg/kg, 3 times a week for a total of 9 doses. The tumor volume was inhibited significantly \( P < 0.05 \); Fig. 5A). As shown in Fig. 5A and B, the tumor volumes of BT-474 on day 41 and of MDA-MB-453 on day 47 in the 3 mg/kg dosage group were reduced to 47% and 37%, respectively, of the control saline group. No abnormalities were observed in peripheral organs such as liver, kidney, and spleen on histological examination in the Her2-lytic-treated group (Fig. 5C). There were no differences in body weight or biochemical and hematology test results between the saline-treated and the HER2-lytic peptide-treated groups (Supplementary Table S1). To determine the lethal dose, we showed a single i.v. injection of HER2-lytic peptide. There was no lethal toxicity in the mice, which were administrated with 10 mg/kg. Although, 3 of 5 mice, which were administrated with 20 mg/kg died, the significant toxicity was not observed in the survived 2 mice. Contrary, all 5 mice, which were administrated with 40 mg/kg died after the injection. Thus, the lethal dose in 50% was estimated between 10 and 20 mg/kg (Supplementary Table S2).

**Discussion**

Therapeutic peptides are gaining increasing popularity for medicinal use in a variety of applications (23),
including as tumor vaccines (24), for antimicrobial therapy (25), and for nucleic acid delivery (26). The HER2-binding peptide was developed both as a cancer imaging tool for diagnosis and as a therapeutic agent to target HER2-overexpressing cancer cells (14). Several peptides derived from screening phage-displayed peptide libraries have been developed as drug candidates and tested in clinical trials, thus validating their peptide-targeting potential (27). It is also known that peptide therapeutic agents are relatively easily generated using solid-phase chemical synthesis techniques and are generally less expensive than antibody-based therapeutics. In addition, in contrast to immunotoxins, because these peptides have lower molecular weights than proteins they may be less immunogenic and toxic.

In this study we linked 2 functional domains to produce a novel bifunctional peptide that binds to HER2 to cause cell death via lysis. It has been shown that the lytic sequence used in this study has higher selectivity than other sequences tested to date (28), with regard to discrimination between membranes of normal and cancer cells, and is suitable for chimerization with a targeting sequence. As a result, we have confirmed that HER2-lytic hybrid peptide designed in this study could have both high selectivity for normal and cancer cell lines and specificity for HER2 (Table 1, and Figs. 1 and 2). We considered the reason that HER2-lytic was less cytotoxicity for normal cells both effects that HER2 expression in normal cells are negative and the lytic peptide is less sensitive for normal cells. As shown in Fig. 1C, because the increase in cytotoxicity of HER2-lytic depends on the cell-surface HER2 expression levels, we can consider it is less sensitivity for normal cells which do not express HER2. In addition, Table 1 showed that lytic peptide has...
higher sensitivity for most cancer cells than normal cells except for MCF 7 and SKOV3. We have already shown that selectivity of hybrid peptide is enhanced by both effects the target binding peptide and the lytic peptide which has selective binding for components of cancer cell membrane (11, 15). There are some kinds of cancer cell lines whose sensitivity against the lytic peptide is low, like MCF7 and SK-OV-3, the lytic peptide sensitivity is currently under investigation. However, targeting HER-2 with Trastuzumab has been associated with development of cardiac toxicity. So, we measured cytotoxicity using H9c2 cells (rat heart-derived embryonic myocytes) are normal cells of the Her2 expression (29). The CCY sequence of HER2-binding peptide mimics a CCY motif in the structure of the EGF-like domain of ErbB ligands (14). Because the CCY motif is perfectly conserved among human, mouse, and rat, it is thought that HER2-binding peptide binds to HER2 of rat. The remarkable toxicity was not found at the high concentration of 60 μmol/L in H9c2 cells (Supplementary Fig. S4). Therefore, it is suggested that the toxicity to the heart of human is low.

Exposure of HER2-overexpressing cell lines (SK-BR-3 and BT-474) to the HER2-binding moiety slightly inhibited 3 major signaling transduction pathways activated by the tyrosine kinase receptor. These results suggest that inhibitory activity against HER2 signaling was enhanced...
by hybridization with the lytic peptide. Downregulation of HER2 signaling may contribute to its antitumor effect; however, the efficacy of the lytic peptide is most likely a consequence of membrane damage in the target cells as evidenced by the rapid cell killing shown in Fig. 3C. Because the HER2-binding sequence peptide itself does not have a cytotoxic effect (Supplementary Fig. S2) and is only slightly internalized (30), HER2-lytic peptide accumulates on the cell surface and, after reaching a threshold concentration, may penetrate the membrane leading to cell death. As shown in Fig. 3A (at 15 minutes for SK-BR-3 cells) and Fig. 3C, the cells died as a result of acute injury, swelling, and bursting. These findings suggest a necrotic mode of action rather than apoptosis; however, the detailed mechanisms by which the lytic peptide acts are still not fully understood.

Furthermore, the HER2-lytic hybrid peptide was effective against trastuzumab- and/or lapatinib-resistant MDA-MB-453 and MDA-MB-361 cells (Fig. 1 and Table 1). The lytic peptide kills cancer cells because of a unique mechanism involving membrane disruption. The fact that there is a difference between membranes of cancer and normal cells, which makes it possible to develop peptides with selectivity for tumor cells; whereas normal mammary cells possess zwitterionic membranes (31), cancer cells have a more negatively charged membrane because of 3% to 9% phosphatidylserine or glycosaminoglycans (32, 33). Because of the strong lytic activity to membrane of cancer cell, the lytic peptide probably is not capable of developing resistance.

Although it seems that most peptides are relatively easily inactivated by serum components in the human body, it has been shown that diastereomeric peptides are relatively free from inactivation in serum (34), and that a lytic diastereomeric peptide administered intravenously at a dose of 9 mg/kg reduces tumor growth in an animal model of human prostate cancer without rapid degradation of the peptide in blood (35). We conducted ex vivo examination to investigate the change of cytotoxic activity in serum protein solution. The HER2-lytic peptide was mixed with serum sample, which was prepared from FBS in vitro, and incubated it for 0, 3, 6, and 24 hours and conducted WST-8 assay. The cytotoxicity 24 hours later was still 21.3% when the cytotoxic activity of 0 hour set as 100% (Supplementary Fig. S5). From this result, it may be said that a schedule of 3 times a week was rational. In addition, in our previous studies (11, 12, 15), we showed significant effects without remarkable toxicity in the schedule of 3 times a week. Furthermore, it has been found that the HER2-binding sequence could be targeted to tumor sites in vivo (30). IC50 of the HER2-lytic peptide for breast cancer cells is 5 to 10 μmol/L in vitro, which is approximately 14.7 to 29.3 mg/L. Given that the total blood volume of the nude mice (body weight approximately 20 g) is 1.5 mL, we expected that the HER2-lytic peptide would exert antitumor effects at a dose of approximately 1.1 to 2.2 mg/kg. In the in vivo study, the HER2-lytic peptide-treated group showed between 47% (BT-474) and 37% (MDA-MB-453) tumor growth inhibition at a dose of 3 mg/kg. We showed that this dose coincide with that of expected from in vitro data.

In this study we have showed that HER2-lytic hybrid peptide binds specifically to HER2 and selectively kills cancer cells, including cells resistant to trastuzumab and lapatinib, and inhibits HER2 signaling. The results also suggested that HER2-lytic hybrid peptide penetrates the cancer cell membrane, and induces rapid killing and necrotic cell death. Furthermore, intravenous administration of the HER2-lytic peptide in BT-474 and MDA-MB-453 cells in the xenograft model significantly inhibited tumor progression. The HER2-lytic hybrid peptide might provide a new therapeutic option for patients with cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Nana Kawaguchi, Kumi Kodama, Aya Torisawa, Keiko Shimoura, and Maiko Yamada of the Department of Pharmacoepidemiology, Kyoto University, for technical assistance with cell culturing and animal care.

Grant Support
Research in the laboratory of K. Kawakami is supported by a grant-in-aid for Young Scientists (A; grant No. 23680089) from the Japan Society for the Promotion of Science (JSPS) and by a Grant for Scientific Research from the Sagawa Foundation for Promotion of Cancer Research.

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Received August 22, 2012; revised January 2, 2013; accepted January 22, 2013; published OnlineFirst January 28, 2013.

References


Molecular Cancer Therapeutics

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