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NOTE

Development of a molecule-recognized promoter DNA sequence for inhibition of *HER2* expression

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HER2 (also known as *ERBB2*, *NEU*) is one of the transmembrane tyrosine kinase receptor genes belonging to the *EGFR* family. Its expression is kept at a very low level in normal cells, but in tumors, over 30% of breast cancer is detected with extremely high levels of *HER2* mRNA. The overexpression of HER2 is frequently accompanied by tumor migration, low sensitivity to chemotherapy and an adverse prognosis.[1–5] Earlier studies have shown that HER2 protein overexpression and accumulation occurred mainly because of the resulting transcriptional deregulation, not because of the mRNA stabilization[6] and transcription-regulating sequences located in the upstream of the *HER2* coding region.[7] Thus, the inhibition of *HER2* transcription has been considered a useful method of cancer therapy. However, there is no report that a chemical reagent decreases *HER2* transcription in cancer cells. Hence, a compound that pre-transcriptionally deregulates *HER2* expression needs to be evaluated as a potentially useful *HER2* silencer for cancer therapy.

From studies of the double-stranded DNA minor groove recognition of naturally occurring antitumor/antiviral antibiotics, including duocarmycin A and distamycin A, pyrrole–imidazole polyamide (PI polyamide) has been discovered to be a designable DNA-recognition molecule in a sequence-dependent manner.[8–12] A PI polyamide compound composed of the aromatic amino acids, *N*-methylpyrrole (Py) and *N*-methylimidazole (Im), is able to recognize the complementary DNA and bind to the minor groove in a sequence-specific manner because the Py–Im pair recognizes the complementary cytosine–guanine (C–G) and the Im–Py combination will bind to guanine–cytosine (G–C), respectively.[13–17] A pairing of Py–Py or β-alanine–β-alanine (β–β) binds to adenine–thymine or thymine–adenine (A–T or T–A) base pairs.[14–18] The PI polyamide containing γ-aminobutyric acid and *N*, *N*-dimethylaminopropylamine as an internal guide residue was found to specifically bind as a hairpin to be designated a target site with ~300-fold enhancement relative to the binding affinities of the individual unlinked polyamide pair.[18] In addition, this compound has a different character from other gene-silencing tools, such as siRNA or antisense oligo nucleotides, because penetration in the living cells, cytosol import and nuclear transport of PI polyamide occur without any delivery system and may not be influenced by any catabolic enzymes or metabolic enzymes, such as nucleases and *P*450 enzymes, even in animals.[19–22] These findings highlight the advantages of PI polyamide as a suitable candidate compound for pre-transcriptional HER2 gene silencing. An earlier publication has also reported that PI polyamide compounds showed specific binding at the Ets-binding site of the HER2/neu promoter region and inhibition of HER2/neu promoter-driven transcription measured in a cell-free system using nuclear extract from a human breast cancer cell line, SKBR-3.[23] As far as we know, there was no report that showed inhibition of HER2/neu promoter-driven transcription and of cell growth in cancer cells using the PI polyamide compound. In this study, we designed and synthesized a novel PI polyamide compound targeting the HER2 transcription factor (HTF)-binding site, a relatively new activator protein-2-binding site that has been reported to contribute to HER2/c-erbB2 gene overexpression in tumor cell lines.[24,25]

First, we designed an eight-base-recognizing structure of PI polyamide (Figure 1a), which binds to the HTF-binding site inside the *HER2* promoter region (Figure 1b). Polyamide was synthesized, according to the method described earlier.[26]

Next, we performed a direct binding assay using Biacore2000 (GE Healthcare Ltd, Little Chalfont, UK) to see whether PI polyamide-*HER2* binds to a target sequence consisting of double-strand oligonucleotides containing the HTF-binding site. We confirmed that PI polyamide-*HER2* shows an approximately 10-times higher

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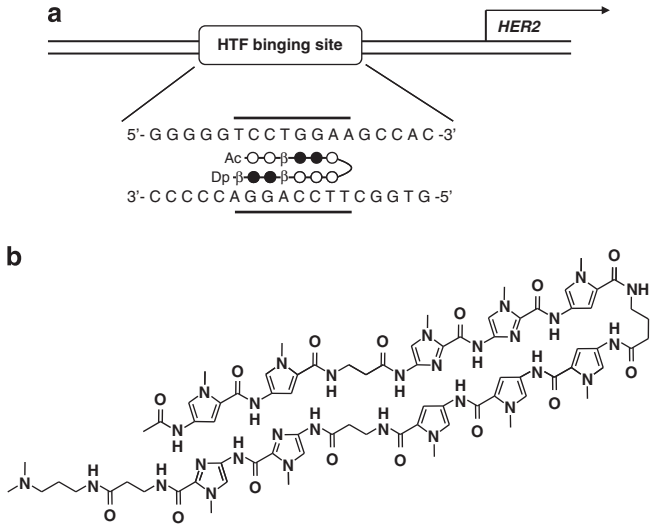

Figure 1 Designed pyrrole–imidazole polyamide targeting *HER2* promoter region. (**a**) Targeting *HER2* promoter region. Bold lines are targeting sequences. Open circles: Py (*N*-methylpyrrole); black circles: Im (*N*-methylimidazole); ß: beta-alanine; Ac: Acetyl; Dp: *N*, *N*-dimethylaminopropylamine); γ-aminobutyric. (**b**) Chemical structure of PI polyamide-*HER2*.

Table 1 Kinetic constants for binding between PI polyamide-*HER2* and the *HER2* promoter region in a Biacore assay

Analyte	*Ligand*	k_a *(1/Ms)*	k_d *(1/s)*	K_A *(1/M)*	K_D *(M)*
PI poryamide-*HER2*	Match	4.01×10^4	1.93×10^{-3}	2.08×10^7	4.82×10^{-8}
	Mismatch	2.47×10^4	1.03×10^{-2}	2.40×10^6	4.16×10^{-7}

The kinetic constants were calculated from the surface plasmon resonance sensorgrams for the interaction between PI polyamide-*HER2* and biotin-labeled double-strand oligonucleotides hairpin DNA (100 nM) on a sensor chip SA. Match: 5′-CGGGGGTCCTGGAAGCCACAATTTTTTGTG GCTTCCAGGACCCCCG-3′. Mismatch: 5′-CGGGGGTCCTGGGGGCCACAATTTTTTGTGGCCCCCAGG ACCCCCG-3′. The concentration of PI polyamide-*HER2* varies at 0, 50, 100, 250, 1000 and 2000 nM. k_a, association rate constant; k_d, dissociation rate constant; K_A, association equilibrium constant; K_D, dissociation equilibrium constant.

preference for binding to a complementary DNA sequence, including the HTF-binding sites than those for binding to mutated DNA (Table 1).

Furthermore, we examined PI polyamide-*HER2* efficiency for cell proliferation at human breast and colon cancer cell lines, COLO205 (human, Caucasian, colon, adenocarcinoma cell line), HT29 (human, Caucasian, colon, adenocarcinoma grade II cell line), MCF-7 (human, Caucasian, breast, adenocarcinoma cell line) and MDA-MB-231 (human, Caucasian, breast, adenocarcinoma cell line). Cell culture conditions: COLO205 (ATCC number: CCL-222), HT29 (ATCC number: HTB-38), MCF-7 (ATCC number: HTB-22) and MDA-MB-231 (ATCC number: HTB-26) were cultured in RPMI 1640 containing 10% fetal bovine serum and 50 U ml^{-1} Penicillin, 50 μg ml^{-1} Streptomycin in 5% CO_2 in an incubator at 37 °C. Cell proliferation assay condition: colo205, MCF-7 and MDA-MB-231 cells were seeded on 96-well microplates (1.0×10^3 cells per well). Test compound was dissolved in 50% DMSO at an appropriate concentration and was treated for 72 h at 5% CO_2, 37 °C atmosphere. Living cells were detected by WST-8 (NacalaiTesque, Kyoto, Japan) using the maker's manual. The absorbance (A450) of each sample was measured by a Wallac 1420 multilabel counter (Amersham Biosciences, Piscataway, NJ, USA).) Consequently, PI polyamide-*HER2* showed cytostatic activity for all four cancer cells and, most significantly, for MDA-MB-231 cells (Figure 2a). These results suggested that PI polyamide-*HER2* binds to a complementary DNA sequence of the *HER2* promoter region containing an HTF-binding site and inhibited cell proliferation in various cancer cells. We therefore analyzed the expression level of *HER2/ErbB2* mRNA in the most responsive of MDA-MB-231 cells. Quantitative measurements using real-time PCR experiments were performed to evaluate whether PI polyamide-*HER2* is able to decrease the expression of *HER2* mRNA in MDA-MB-231 cells. (qRT-PCR experiments: MDA-MB-231 cultured in six-well plates (1.5×10^4 cells per well) were incubated in the presence of

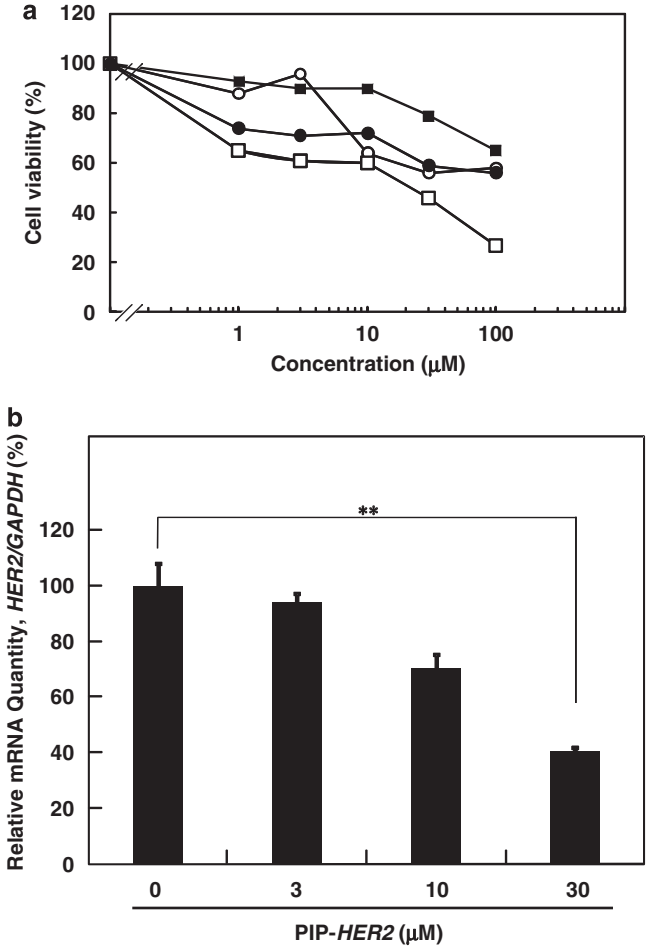

Figure 2 Effects of PI polyamide-*HER2* on cell growth and mRNA expression in MDA-MB-231 cell. (**a**) Effects of PI polyamide-*HER2* on the growth of COLO205, HT29, MCF-7 and MDA-MB-231 cells in a proliferation assay. Test compounds were dissolved in 50% DMSO at appropriate concentrations and treated for 72 h. Black squares: colo205 ($IC_{50}>100$ μM); black circles: HT29 ($IC_{50}<100$ μM); open circles: MCF-7 ($IC_{50}>100$ μM); open squares: MDA-MB-231 ($IC_{50}=30$ μM). (**b**) PI polyamide-*HER2* treatment decreased *HER2* expression in MDA-MB-231 cells. The cell had been treated with different concentrations of PI polyamide-*HER2* for 48 h, and *HER2* mRNA expression was determined by quantitative real-time PCR experiments. The *HER2* mRNA expression levels were expressed as a relative percentage to the control value. Data are expressed as the mean ± s.d. ($n=3$ for each group). The statistical significance of differences between control and experimental groups was determined by using a two-group two-tailed Student's *t*-test; $^{**}P<0.01$ was taken as the level of statistical significance.

some concentration of the test compound for 48 h in 37 °C in a 5% CO_2 atmosphere. After that, harvested cell lines were used for isolation of these total RNA with ISOGEN (NIPPON gene Co. Ltd, Tokyo, Japan) and cDNA made by reverse transcription PCR using Prime script RT reagent kit (Takara Bio Inc., Shiga, Japan). The abundance of *HER2* mRNA was determined by relative quantification that used 1% DMSO-treated cells as control, and GAPDH was measured as internal control with SYBR Premix EXTaq (Takara Bio Inc.) on Thermal Cycler Dice (Takara Bio Inc.). The results showed that PI polyamide-*HER2* induced dose-dependent suppression of *HER2* mRNA expression (Figure 2b). These findings provide a possible molecular basis to explain how PI polyamide-*HER2* inhibits cell proliferation in MDA-MB-231 cells.

It has been reported that the binding ability of PI polyamide for a target sequence depends on the recognition for linear combination of Watson–Crick base pairs.[16] PI polyamide-*HER2* should have approximately 13 000–50 000 capable target sites in a whole genome. Nevertheless, we think that the candidate-binding sites of PI polyamide-*HER2* must be more restricted because of the selective recognition of PI polyamide-*HER2* only at the non-histone binding to the minor groove region of double-helical DNA sequences. To overcome this off-target effect query, further comprehensive analysis is needed to investigate the global binding of PI polyamide-*HER2* using a Genome Precipitation assay in conjunction with microarray chip hybridization or with large-scale sequencing. A whole-genome gene-expression microarray may also help to track the off-target effect. Alternatively, improvement of specificity by a combinatorial modification of the PI polyamide-*HER2* compound may be needed.[27,28]

These observations indicate that PI polyamide-*HER2* induces the decrease of *HER2* mRNA expression and consequently inhibits cell proliferation in various cancer cells, including MDA-MB-231 cells. As far as we know, this is the first report that a chemical reagent decreases HER2 transcription in cancer cell and inhibits cell growth in various cancer cell lines. We, therefore, propose that *HER2* transcription can be controllable by means of PI polyamide-*HER2* administration and that PI polyamide-*HER2* may be a new type of chemical inhibitor for *HER2* silencing.

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