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Original Article

Novel HER2 selective tyrosine kinase inhibitor, TAK-165, inhibits bladder, kidney and androgen-independent prostate cancer *in vitro* and *in vivo*

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Purpose: TAK-165 is a new potent inhibitor of human epidermal growth factor receptor 2 (HER2) tyrosine kinase. Several reports suggest HER2 expression in bladder cancer, renal cell carcinoma (RCC) and androgen-independent prostate cancer. We therefore investigated the antitumor effect of TAK-165 on these urological cancer cells.
Materials and methods: Western blot analysis was performed to confirm HER2 expression in cell lines. To study *in vitro* efficacy, cells were treated with TAK-165 at various concentrations for 72 h and then counted using a hemocytometer. Then the IC_{50} value was calculated. In the xenograft model, after the tumor reached 200–300 mm^3 in volume, mice were orally administered TAK-165 10 mg/kg per day or 20 mg/kg per day or saline for 14 consecutive days ($n = 6$–8).
Results: HER2 expression was observed in HT1376, UMUC3, T24 (bladder), ACHN (kidney), DU145, LNCaP, LN-REC4 (prostate), although the expression level in these cells was weak compared with BT474 (a breast cancer cell line which expresses HER2 strongly). IC_{50} was varied from 0.09 to greater than 25 μmol/L in the bladder cancer cell line. ACHN cells were less sensitive *in vitro*. The prostate cancer cell lines studied were all sensitive (IC_{50} 0.053–4.62 μmol/L). In the xenograft model, treatment with TAK-165 significantly inhibited growth of UMUC-3, ACHN, and LN-REC4. The antitumor effect (T/C [%] = growth of TAK-165 treated tumor/average growth of control tumor × 100) after 14 days treatment were 22.9%, 26.0%, and 26.5% in UMUC3, ACHN and LN-REC4, respectively.
Conclusions: TAK-165 may be a hopeful new agent for bladder, kidney and androgen-independent prostate cancer.

Key words bladder cancer, HER2, tyrosine kinase inhibitor, prostate cancer, renal cell carcinoma, .

Introduction

Human epidermal growth factor receptor (EGFR; HER1, erbB1), human epidermal growth factor receptor 2 (HER2; erbB2), HER3 (erbB3) and HER4 (erbB4) are members of the ErbB family of transmembrane receptor tyrosine kinases. The ErbB receptors are expressed in a variety of tissues of epithelial, mesenchymal and neuronal origin, where they play fundamental roles in development, proliferation and differentiation.[1] Deregulated expression of receptors, in particular EGFR and HER2, has been implicated in the development and malignancy of numerous types of human cancers, and EGFR and HER2 have become a potential target for therapy. A humanized antibody against ErbB2 (Herceptin) is now in clinical use for advanced breast cancer,[2] and a tyrosine kinase inhibitor of ErbB1 (ZD1839) has been approved for therapy of advanced non-small cell lung cancer.[3]

In urological cancer, bladder cancer, prostate cancer and renal cell carcinoma are reported to express HER2.[4–6] In particular, bladder cancer and androgen-independent prostate cancer are reported to express HER2 frequently. In advanced cases, these cancers are untreatable by conventional therapy and new drugs are desired. So we studied the antitumor effect of a new HER2 tyrosine kinase inhibitor, TAK-165.[7,8]

In the present study, we confirmed TAK-165 efficacy on bladder cancer, prostate cancer and renal cell carcinoma *in vitro* and *in vivo*.

Materials and methods

Inhibitor, antibody, and growth factors

The HER2 tyrosine kinase selective inhibitor, TAK-165 was synthesized by Takeda Chemical Industries (Osaka, Japan). Anti-HER2 monoclonal antibody Trastzumab was purchased from Roche (Tokyo, Japan). The following antibodies were used for immunoblot analysis: anti-HER2 (CB11) from PROGEN (Heidelberg, Germany), EGFR

(1005), HER3 (C-17) and HER4 (C-18) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), monoclonal anti-phospho-tyrosine (clone 4G10) and antibodies against EGFR, PDGFR and FGFR from Upstate Biotechnology (Lake Placid, NY, USA) and phospho-HER2/erbB2 specific polyclonal antibody, anti-Akt, anti-phospho-Ser[473]-Akt, anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase from Cell Signaling Technology (Danvers, MA, USA).

Cells

The human bladder cancer cell line T24, renal cell carcinoma cell line ACHN, androgen-dependent prostate cancer cell line LNCaP, androgen-independent prostate cancer cell line DU-145, PC-3, and breast cancer cell line BT474 were obtained from the American Type Culture Collection (ATCC). Bladder cancer cell lines HT1376 and UMUC-3 were purchased from Dainippon Laboratories (Osaka, Japan). LN-REC4 cell line is a derivative of LNCaP cell line but which has grown well in the castrated SCID mouse after two passages in castrated mouse. BT474, LNCaP and LN-REC4 were cultured in RPMI1640 containing 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere and the other cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% FBS. Human embryonic normal fibroblasts, MRC-5, were obtained from the RIKEN cell bank (Wako, Japan) and cultured in Eagle's minimum essential medium (EMEM) with 10% FBS, glutamine, penicillin and streptomycin. A431, Jurkat, Namalwa and NIH3T3 cells were also procured from the ATCC.

Measurement of tyrosine kinase activity and downstream cascade of HER2

The activity of HER2/erbB2 tyrosine kinase and other receptor-type or non-receptor-type tyrosine kinase activities, and autophosphorylation were measured by western blotting methods. To determine HER2/erbB2 tyrosine kinase phosphorylation, BT-474 cells (2×10^5 cells/well) were seeded on 24-well plates and cultured overnight. TAK-165 was then added at various concentrations. After incubation for 2 h, the cells were harvested directly into sodium dodecyl sulfate (SDS)-sample buffer (200 µL). To determine other receptor-type tyrosine kinase inhibition, A-431 cells or NIH3T3 fibroblasts (2×10^5 cells/well) were seeded in medium containing 10% FBS and incubated overnight to allow the cells to adhere to the plates. The medium was replaced with serum-free medium, and the cells were allowed to grow for another day. TAK-165 was added 2 h before the addition of growth factors (A-431cells: EGF [20 ng/mL]; NIH3T3 fibroblasts: PDGF [20 ng/mL] or FGF [20 ng/mL]). After incubation for 5 min, the cells were lyzed in SDS-sample buffer. To determine non-receptor-type tyrosine kinase inhibition, A-431, Jurkat or Namalwa cells (5×10^5 cells/well) were used. After incubation with TAK-165 for 2 h, the cells were directly harvested into SDS-sample buffer. Aliquots containing equal amounts of total cell extract were run on 7.5% to 15% gradient SDS–polyacrylamide gel electrophoresis (PAGE). Following electrophoresis, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane, for western blot analysis using a relevant primary antibody. Detection of protein was accomplished by an enhanced chemiluminescent (ECL) detection method. The extent of tyrosine phosphorylation of HER2/erbB2, other tyrosine kinases, and phospho-protein were measured by the LAS-1000 plus lumino-image analyser (Fuji Film, Tokyo, Japan). The concentration of TAK-165 that inhibits HER2/erbB2 and other kinase phosphorylation by 50% (IC_{50}) was calculated from a dose–response curve generated by least-squares linear regression of the response using SAS software (SAS Institute, Cary, NC, USA). Effects of TAK-165 on Akt and MAPK activity, a well-known downstream pathway of HER2, were evaluated in BT474 using antibodies described above.

Analysis of HER family members expression in cell lines

To characterize the expression of HER families among cell lines, cells were washed twice with ice-cold phosphate-buffered saline (PBS), lyzed with RIPA buffer (10 mM sodium phosphate [pH 7.2], 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, and protease inhibitors), collected by scraping, and cleared by centrifugation. Fifty micrograms of protein were electrophoresed on a 7% SDS–PAGE gel, transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) and subjected to immunoblot analysis. Immunoreactive bands were detected using chemiluminescence (Pierce, Rockford, IL, USA).

Measurement of antiproliferative activity

Cells ($5–10 \times 10^6$) were seeded into 6-well plates and cultured overnight in a humidified 5% CO_2 atmosphere at 37°C. TAK-165 was then added at various concentrations, and the cells were treated continuously for 3 days. After the incubation period, cells were counted. The data is average of triplicates, and the IC_{50} value was calculated from a dose–response curve generated by least-squares linear regression of the response.

Xenograft studies in mice

These experiments used 5–9-week-old athymic nude mice (BALB/c nu/nµ) and SCID mice (C.B.-17 Scid/Scid). UMUC-3 and ACHN cells were implanted subcutaneously to nude mice. Castration was performed on SCID mice 1 week before implantation of LN-REC4 cells. UMUC-3 and LN-REC4 cells were implanted with 50% Matrigel solution (Becton Dickinson, Franklin Lakes, NJ, USA). After the tumor volume reached 200–300 mm³ in LN-REC4 and UMUC-3 cells and to 100–200 mm³ in ACHN, the mice were treated orally twice daily for 14 days with vehicle (control) or 10 or 20 mg/kg per day of TAK-165. In the Herceptin study against UMUC-3, treatments consisted of a twice weekly intraperitoneal injection of 20 mg/kg Herceptin in PBS for 2 weeks. Tumor growth was assessed by electronic caliper measurement of tumor diameter in two dimensions, and tumor volume was calculated using the equation: tumor volume = $A \times B^2 \times 1/2$ (mm³) where A is the largest diameter of the tumor and B is the smallest diameter of the tumor. Tumor size was measured

every 2 days throughout the study period. The antitumor effect was expressed as growth of each treated tumor/ average growth of control tumor × 100 [T/C (%)].

Results

TAK-165 inhibits HER2 phosphorylation and its down-stream Akt and MAPK in HER2 strongly expressing cells (BT474 breast cancer cell line) in vitro
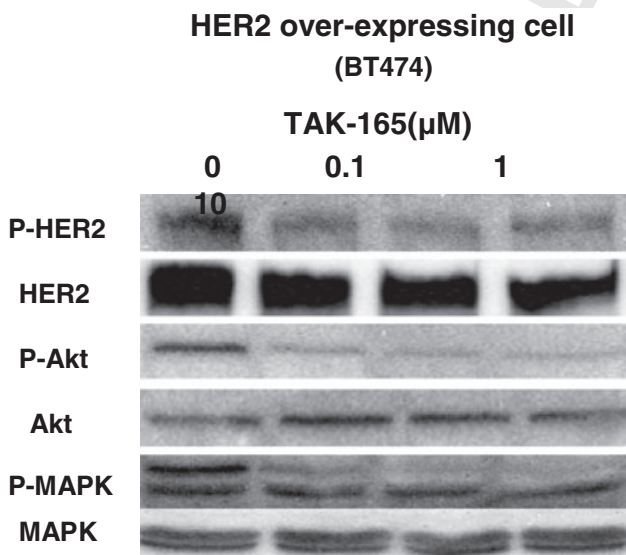
We first confirmed the effects of TAK-165 on HER2, Akt and MAPK phosphorylation using HER2 strongly expressing cell line (BT474). PI3K and MAPK pathway are well known to be activated by HER2.[9] PI3K-Akt pathway is associated with inhibition of apoptosis, and MAPK pathway is also associated with cell growth. TAK-165 inhibited HER2 phosphorylation sufficiently at 0.1 μmol/L, and phosphorylation of Akt and MAPK1 have also been sufficiently inhibited (Fig. 1).

TAK-165 selectively inhibits HER2 tyrosine kinase

To confirm TAK-165 selectivity, we examined influences of TAK-165 on the other tyrosine kinase activity. TAK-165 specifically inhibited HER2 tyrosine kinase IC_{50} 6 nmol/L and did not inhibit other types tyrosine kinase up to 25 000 nmol/L (Table 1).

Expression profiles of HER families in bladder, kidney and prostate cancer cell lines

In order to see the level of HER family protein expression, western blot analysis of HER family protein was carried out. As shown in Figure 2, three bladder cancer cell lines (HT1376, UMUC-3, and T24) expressed HER2 weakly. ACHN renal cell carcinoma cell line and four prostate cancer cell lines (DU145, PC-3, LNCaP, and LN-REC4) also expressed HER2. However, PC-3 cells expressed HER2 very faintly. There was no significant difference in HER2 expression between androgen-dependent LNCaP and its derivative androgen-independent cell line LN-REC4.
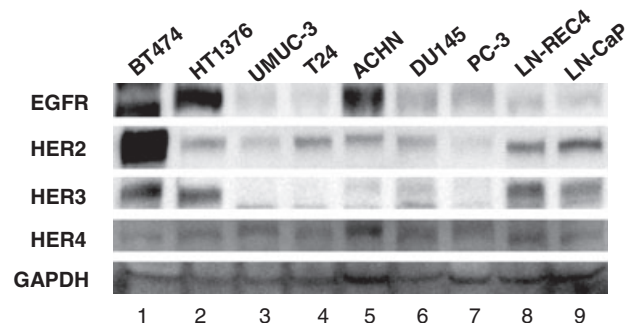
Sensitivity to TAK-165 in vitro

To evaluate TAK-165 sensitivity in vitro, after cells were treated with TAK-165 at various concentrations continuously for 3 days, cell numbers were counted and IC_{50} values of these cells were calculated. Most of cell lines studied this time indicated good TAK-165 sensitivity despite of weak HER2 expression (Table 2). Generally, TAK-165 sensitivity depends on HER2 levels of each cell line. Especially, BT474 cells which over-express HER2 strongly was highly sensitive (IC_{50} 0.005 μmol/L) and PC-3 cells which express HER2 very weakly was less sensitive (IC_{50} 4.62 μmol/L). But, HT1376 and ACHN cells that over-expressed EGFR showed high IC_{50} (IC_{50} > 25 μmol/L).

Table 1   Tyrosine-kinase-inhibitory activity of TAK-165

Tyrosine kinase	IC_{50} (nmol/L)
HER2/erbB2	6.0 (4.0–9.0)
EGFR	>25 000
FGFR	>25 000
PDGFR	>25 000
Jak1	>25 000
Src	>25 000
Blk	>25 000

The IC_{50} values (nmol/L) in the table were calculated from dose–response curves generated by least-squares linear regression of the response using SAS software. Values in parentheses represent the 95% confidence interval of the IC_{50}.

HER2 over-expressing cell
(BT474)

TAK-165(μM)

Fig. 1   Inhibition of HER2, Akt and MAPK phosphorylation by TAK-165 in BT474 (HER2 over-expressing cell line). BT474 cells were treated with TAK-165 for 2 h at indicated concentration. Then cell lysates were prepared and analysed for phosphorylation of HER2, Akt and MAPK by western blot with antibodies as described in Materials and methods.

Fig. 2   Expression profiles of HER family kinases in bladder, kidney and prostate cancer cell lines. Total protein were extracted from cell lysates and then western blots were performed (each lane 50 μg). Lane 1, BT474; Lane 2, HT1376; Lane 3, UMUC-3; Lane 4, T24; Lane 5, ACHN; Lane 6, DU145; Lane 7, PC-3; Lane 8, LN-REC4; Lane 9, LNCaP.

Table 2 Antiproliferative activity of TAK-165 *in vitro*

Lane (of Fig. 2)	Cell line	IC_{50} (µmol/L)
1	BT474 (Breast)	0.005
2	HT1376 (Bladder)	25<
3	UMUC-3 (″)	1.812
4	T24 (″)	0.091
5	ACHN (Kidney)	25<
6	DU145 (Prostate)	1.647
7	PC-3 (″)	4.620
8	LN-REC4 (″)	0.090
9	LNCaP (″)	0.053

Cells were treated with TAK-165 at various concentrations for 72 h. After the incubation period, the cells were counted. The IC_{50} value was calculated from a dose–response curve generated by least-squares linear regression of the response. The data were obtained from average of triplicates.

Xenograft growth inhibition

During our study of HER2 inhibitor on urological cancer, we first have studied the antitumor effect of Herceptin against UMUC-3 xenograft. After tumor volume reached 200–300 mm³ in mice, 20 mg/kg of Herceptin or vehicle were administered intraperitonealy twice per week for 2 weeks. Herceptin was ineffective to UMUC-3 tumor growth (Fig. 3a). Then next, we administered 10 mg/kg per day or 20 mg/kg per day TAK-165 twice daily for 2 weeks. TAK-165 significantly inhibited growth of UMUC-3 cells (Fig. 3b). Although ACHN cells had low sensitivity to TAK-165 *in vitro*, was significantly inhibited *in vivo* (Fig. 3c) and there were even some cases that tumor has disappeared and did not recur for several weeks after 14 days of treatment (data not shown). LN-REC4 was also significantly inhibited by TAK-165 (Fig. 3d). Treatment/
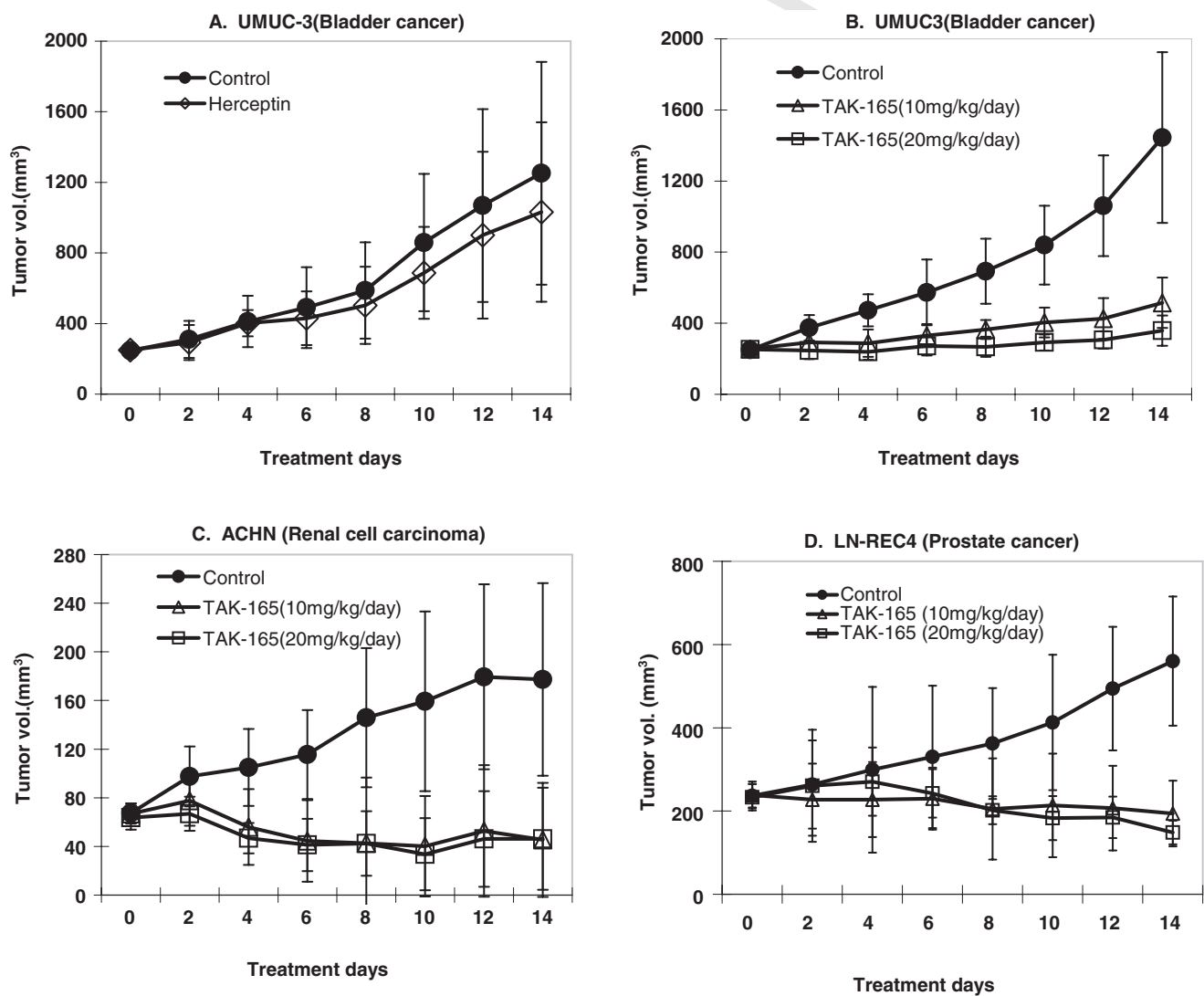
Fig. 3 Response of xenograft tumors to TAK-165 and Herceptin treatment. (a) Herceptin (20 mg/kg twice per week) or phosphate-buffered saline (control) was administered to mice bearing UMUC-3 (bladder cancer). (b–d) TAK-165 (10 mg/kg per day or 20 mg/kg per day) or vehicle was orally administered for continuously 14 days to mice bearing UMUC-3 (b), ACHN (c) (renal cell carcinoma), and LN-REC4 (d) (androgen independent prostate cancer) ($n = 6$–8 per group). Results are given as mean tumor volume ± SD.

control tumor volume ratio were 22.9%, 26%, 26.5% in UMUC-3, ACHN and LN-REC4, respectively. There was no statistical difference of antitumor effect between 10 mg/kg per day and 20 mg/kg per day TAK-165 treated groups. Concerned with side-effects, there were two deaths in TAK-165-treated groups (total 54 treated with TAK-165) and two deaths in the control group (total 22, treated with PBS). Weight loss was also observed, but it was less than 10% of treated animals and there was no statistical difference between TAK-165 and control group. These side-effects may have been caused by compulsory oral administration of compound. These data showed efficacy and safety of TAK-165.

Discussion

Recently, Herceptin was administered to patients with androgen-independent prostate cancer or with advanced bladder cancer as a single agent or in combination with chemotherapeutic agents.[10–12] In the reports, the efficacy of Herceptin alone was limited.[13] Because in urological cancers, HER2 is not expressed so strongly as breast cancer, such data might be natural. In this study, we compared the efficacy of TAK-165 with Herceptin in UMUC3 which express HER2 very weakly. Although the reason is unknown, TAK-165 significantly inhibited tumor growth as compared with Herceptin. One possible reason is that Herceptin did not show a quick effect over the 14-day observation period because it requires an immunological response. But another reason will also exist. In the ACHN study, TAK-165 was ineffective *in vitro* ($IC_{50} > 25\ \mu M$), but tumor growth was significantly inhibited *in vivo*. The degree of HER2 expression was not changed between *in vitro* and *in vivo* (data not shown). Although the high selectivity of TAK-165 for HER2 tyrosine kinase has been proved *in vitro*, we have to investigate *in vivo* selectivity and if there are other mechanisms of tumor suppression.

In the *in vivo* study, we could not observe dose-dependent effects of TAK-165 between a dose of 10 mg/kg per day and 20 mg/kg per day (Fig. 3). We think the 10 mg/kg per day of TAK-165 was enough to inhibit tumor growth. In the BT474 study, a dose-dependent effect was observed between 5 mg/kg per day and 10 mg/kg per day of TAK-165 (data not shown).

Because HER2 is not playing so crucial a role for survival or proliferation of cancer cells in most urological cancers compared with strongly expressed HER2 breast cancer, TAK-165 might need some combination to increase its efficacy. Combination with specific inhibitors that specifically targets oncoprotein is ideal for efficacy and safety. Combination with EGFR tyrosine kinase inhibitor or anti-EGFR antibody is one of the candidates. Zhou *et al.* reported a synergic effect of this combination in colon cancer.[14] Combination of Herceptin with a COX-2 inhibitor has also been reported, but the effect was limited.[15] Realistically, combination with conventional cytotoxic agents will be the first candidates for combination with TAK-165. Herceptin with paclitaxel or doxorubicin has proved synergic efficacy in experiments and has improved prognosis of patients with metastatic breast cancer.[16,17] We are now studying combinations with taxanes. Combination with radiation is also to be studied. Recently anti-HER2 monoclonal antibody has been proved to enhance radiosensitivity of esophageal cancer.[18]

In this study, we confirmed high selectivity of TAK-165 and high efficacy against bladder cancer, renal cell carcinoma and prostate cancer. Although there remains many to study about TAK-165, TAK-165 is a hope for urological cancer treatment.

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