Antitumor activity of HM781-36B, a pan-HER tyrosine kinase inhibitor, in HER2-amplified breast cancer cells

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HM781-36B is an orally administered pan-human epidermal growth factor receptor (HER) inhibitor. To explore the role of pan-HER inhibitor in breast cancer, we investigated the antitumor effect and mechanisms of HM781-36B in breast cancer cell lines. Six breast cancer cell lines (BT474, MDA-MB-453, SK-BR-3, T47D, MCF-7, and MDA-MB-231) were tested. The growth inhibitory effect was assessed using the tetrazolium bromide [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide] assay. The cell cycle at various concentrations of HM781-36B was analyzed by flow cytometry, and analysis of downstream molecules was performed by western blot analysis. Interaction of HM781-36B with cytotoxic chemotherapeutic agents was analyzed by combination index using CalcuSyn. The HER2-amplified cells (SK-BR-3, BT474, and MDA-MB-453) were sensitive to HM781-36B (IC_{50} = 0.001 μmol/l, 0.0012 μmol/l, and 0.0095 μmol/l, respectively). HM781-36B induced G1 arrest and resulted in apoptosis. It reduced the level of p-HER2, p-AKT, p-ERK, and p-STAT3. HM781-36B combined with 5-fluorouracil, cisplatin, paclitaxel, or gemcitabine showed a synergistic inhibitory effect on the HER2-amplified and on some of the HER2-nonamplified breast cancer cells. HM781-36B could be a promising treatment for HER2-amplified breast cancer as a single agent or in combination with cytotoxic agents and can be a candidate for treatment of HER2-nonamplified breast cancer in combination with cytotoxic agents. *Anti-Cancer Drugs* 23:288–297 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The human epidermal growth factor receptor (HER) family consists of four members: epidermal growth factor receptor (EGFR), HER2, HER3, and HER4, and these proteins regulate the growth and differentiation of malignant cells [1,2]. These receptors are inactive as monomers, but the formation of a homodimer or a heterodimer induces phosphorylation of tyrosine kinase, which controls such cellular processes as apoptosis, migration, growth, adhesion, and differentiation by triggering a complex, multilayered network of interrelated signaling pathways [3,4].

Numerous tyrosine kinase inhibitors are currently being evaluated and developed. For targeting HER1, a number of antibodies and small-molecule tyrosine kinase inhibitors are being clinically developed. Gefitinib and erlotinib are synthetic inhibitors of HER1 kinase activity [5,6]. Cetuximab is a monoclonal antibody that specially targets HER1, and it has been approved for the treatment of advanced colorectal cancer [7]. Trastuzumab is a humanized monoclonal antibody that targets the extracellular domain of HER2, and it has been established as a form of treatment in a metastatic and adjuvant setting of HER2-amplified breast cancer [8]. Lapatinib is a synthetic small-molecule inhibitor of both HER2 and EGFR tyrosine kinase. It also has the advantage of having dual targets and of being a small-molecule inhibitor, and it inactivates EGFR/HER2 or HER2/HER3 heterodimers [9]. Clinically, lapatinib has already been proven to be effective as a single agent or in combination treatment with capecitabine for trastuzumab-resistant HER2-amplified breast cancer [10–12].

A pan-HER inhibitor has now been developed. CI-1033 is an irreversible pan-HER tyrosine kinase inhibitor, and it binds to the ATP binding site in the cytoplasmic domain of EGFR. This prevents autophosphorylation and blocks downstream signaling [4,13]. *In vitro*, CI-1033 has significantly reduced the activated AKT and MAP kinases in breast cancer cell lines [14], and it has been evaluated in a phase I clinical study for treating patients with advanced solid tumor [15–17]. PF-00299804 has been developed as a second-generation irreversible pan-HER tyrosine kinase inhibitor and it showed significant antitumor activity in a lung cancer xenograft model that was resistant to gefitinib (L858R/T790M) [18]. HKI-272 also showed inhibited activity in cell lines with high levels of HER2 *in vivo* and had an acceptable efficacy and safety profile in phase I and II studies [19–21].

The activity of the pan-HER inhibitor and the synergism with cytotoxic chemotherapeutic agents have not yet

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been fully tested in breast cancer cells. Even though trastuzumab showed a synergistic or additive effect with cisplatin, paclitaxel, or gemcitabine in breast cancer cell lines [22,23], enhanced chemosensitivity of pan-HER inhibitor has been reported only with CI-1033 and cisplatin in HER1 receptor-overexpressing cell lines [24].

The studies described herein were therefore designed to investigate the cytotoxic effects of HM781-36B, a quinazoline-based irreversible pan-HER inhibitor, on breast cancer cells when it was administered alone or in combination with clinically relevant cytotoxic chemotherapeutic agents [5-fluorouracil (5-FU), cisplatin, paclitaxel, and gemcitabine].

Materials and methods

Cell lines and culture

Human breast cancer cell lines (MDA-MB-453, SK-BR-3, T47D, MCF-7, MDA-MB-231, and BT474) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). MDA-MB-453, SK-BR-3, and BT474 are cell lines in which HER2 amplification has been demonstrated [10]. All the cell lines except BT474 were cultured in RPMI-1640 medium (WelGENE Inc. Daegu, Korea) supplemented with 10% fetal bovine serum and gentamicin (10 μg/ml). BT474 was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and gentamicin (10 μg/ml). They were incubated under standard culture conditions (20% O_2 and 5% CO_2; 37°C).

Drugs and reagents

HM781-36B was provided by Hanmi Pharm. Co., Ltd (Seoul, Korea). It was initially dissolved in dimethylsulfoxide (Sigma Chemical Co., St. Louis, Missouri, USA) at a concentration of 10 mmol/l and stored in small aliquots at −20°C.

Cell growth inhibition assays

Viable cell growth was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay. All the cell lines were plated into 96-well plates at a density of 3×10^3 to 6×10^3 and cultured in an incubator for 24 h. The cells were then treated with increasing concentrations of HM781-36B (range: 0.0001–10 μmol/l) for 72 h at 37°C. Tetrazolium dye (MTT; Sigma Chemical) solution was added to each well and these were then incubated for 4 h at 37°C before removing the medium. After treatment with dimethylsulfoxide (Sigma Chemical), cell viabilities were determined by measuring absorbance at 540 nm in a microplate reader (Spectra Classic, Tecan Co., Austria).

To evaluate the effect of concurrent exposure of HM781-36B with other cytotoxic chemotherapeutic agents, the cells were treated with serial dilutions of each drug individually by a calculation on the basis of the IC_{50} and with both drugs simultaneously. After 72 h of exposure to the drugs, growth inhibition was measured using the MTT assay.

Cell cycle analysis

The cells were plated in each culture dish, and they were treated with the indicated concentration of HM781-36B for 48 h. The cells were then centrifuged at 1600 rpm for 5 min, fixed in 70% ethanol, and stored at −20°C. Before analysis, the samples were digested with RNAse (100 g/ml) for 10 min at 37°C and then stained with propidium iodide (50 μg/ml). The relative DNA content per cell was determined using a fluorescence-activated cell sorting analysis Calibur flow cytometer (Becton Dickinson Biosciences, San Jose, California, USA) equipped with a ModFit LT program (Verity Software House Inc., Topsham, Maine, USA).

Annexin V staining

The SK-BR-3, BT474, and MDA-MB-453 cells were treated with increasing doses of HM781-36B (0.001, 0.01, and 0.1 μmol/l) for 48 h, followed by staining with Annexin V–phycoerythrin and 7-aminoactinomycin D (Becton Dickinson Biosciences). Apoptotic cell death was measured by counting the number of cells that stained for Annexin V–phycoerythrin by fluorescence-activated cell sorting analysis.

Western blot analysis

After treatment with HM78-136B as indicated, the cells were washed with ice-cold PBS and suspended in lysis buffer [50 mmol/l Tris-HCl (pH 7.5), 1% NP-40, 0.1% sodium deoxycholate, 150 mmol/l NaCl, 50 mmol/l NaF, 1 mmol/l sodium pyrophosphate, 1 mmol/l sodium vanadate, 1 mmol/l nitrophenylphosphate, 5 mmol/l benzamidine, 2 mmol/l phenylmethanesulfonylfluoride, 0.1 mmol/l aprotinin, 0.1 mmol/l leupeptin, and 0.1 mmol/l pepstatin A] at 4°C. After centrifugation, the samples containing equal amounts of total protein were resolved on SDS-polyacrylamide denaturing gel, and the proteins were transferred to nitrocellulose membranes. After blocking, the membrane was incubated with primary antibodies at 4°C overnight. Antibodies against p-EGFR (pY858, pY1068), p-HER2, p-HER3, p-AKT (pS473), p-ERK (p44/p42), p-STAT3 (pY705), $p27^{kip1}$, cyclin D, cyclin E, HER2, HER3, AKT, ERK, STAT3, caspase 3, caspase 7, caspse 8, caspase 9, cytochrome c, PARP, Bcl-2, Bcl-xL, Mcl-1, Bim, and Bax were purchased from Cell Signaling Technology (Beverley, Massachusetts, USA). Anti-EGFR antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Detection was performed using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Analysis of cytochrome c release

For detecting mitochondrial cytochrome c release into the cytosol, the cells were harvested at each time point and resuspended using a Qproteome cell compartment kit (Qiagen, Germantown, Maryland, USA) according to the instructions. After incubation for 10 min at

4°C, the cells were centrifuged at $1000 \times g$ for 10 min, and the cytosolic protein extracts were collected.

Determination of synergism and antagonism

To evaluate the effects of HM781-36B administered in conjunction with chemotherapeutic agents (5-FU, paclitaxel, cisplatin, or gemcitabine), cells were treated with serial dilutions of each drug individually and with both drugs simultaneously at a fixed ratio of doses that corresponded to the individual IC_{50}. Some HER2-amplified breast cancer cell lines (SK-BR-3 and BT474) were exposed to various concentrations of HM781-36B (0.0000625, 0.000125, 0.00025, 0.0005, and 0.001 μmol/l) and chemotherapeutic agents such as 5-FU, paclitaxel, cisplatin, or gemcitabine at a ratio of 1 : 5000, 1 : 50, 1 : 5000, or 1 : 10. Another HER2-amplified cell (MDA-MB-453) was exposed to various concentrations of HM78-136B (0.000625, 0.00125, 0.0025, 0.005, and 0.01 μmol/l/l) and 5-FU, paclitaxel, or gemcitabine at a ratio of 1 : 500, 1 : 5, or 1 : 10 and to various concentrations of HM781-36B (0.003125, 0.00625, 0.0125, 0.025, and 0.05 μmol/l/l) and cisplatin at a ratio of 1:1000. In combination with 5-FU, MCF-7, T47D, and MDA-MB-231 were exposed to concentrations of HM781-36B (0.625, 1.25, 2.5, 5, and 10 μmol/l/l) at a ratio of 1 : 1. In combination with paclitaxel, MDA-MB-231 and T47D were exposed to concentrations of HM781-36B (0.05, 0.25, 0.5, 2.5, and 5 μmol/l/l) at a ratio of 200 : 1, whereas MCF-7 was exposed to concentrations of HM781-36B (0.03125, 0.0625, 0.125, 0.25, and 0.5 μmol/l/l) at a ratio of 1 : 1. Cisplatin or gemcitabine were exposed to concentrations of HM781-36B (0.03125, 0.0625, 0.125, 0.25, and 0.5 μmol/l/l) at a ratio of 1 : 1. After 72 h of exposure, cell viability was measured using the MTT assay.

A commercially available software package (CalcuSyn: Biosoft, Cambridge, UK) was used for determining the presence of synergism. Data can be processed both for individual drugs and for constant-ratio combinations of drugs. CalcuSyn automatically graphs the data and produces reports with summary statistics on all the drugs, plus a detailed analysis of drug interactions, including the combination index (CI) and the effective dose for any value of x. Estimates of the accuracy of the effective dose for any value of x and CI can be calculated with Monte Carlo simulations or by using a highly accurate algebraic estimation algorithm. The plots drawn by CalcuSyn include the dose effect, the median effect, the CI effect, and isobolograms. This is calculated by using the Median Effect methods described by Chou and Talalay [25]. $CI < 1$, $CI = 1$, or $CI > 1$ indicates synergistic, additive, or antagonistic effects, respectively.

Data treatment and statistical analysis

All experiments were conducted in duplicate or triplicate and repeated at least twice. All data are reported as means ± standard error. The statistical significance of the results was calculated using an unpaired Student's *t* test, and *P* values of less than 0.05 were considered to be statistically significant.

Results

Growth inhibitory activity of HM781-36B in the HER2-amplified breast cancer cell lines

To investigate the growth inhibitory activity of HM781-36B, each cell line was treated with increasing doses of HM781-36B (0.0001, 0.001, 0.01, 0.1, 1, and 10 μmol/l) for 72 h (Fig. 1).

HER2-amplified breast cancer cell lines such as SK-BR-3, BT474, and MDA-MB-453 showed an IC_{50} of less than 1 μmol/l (0.001, 0.0012, and 0.0095 μmol/l), whereas the other nonamplified cell lines showed an IC_{50} of more than 10 μmol/l. From these results, we have proven that HM78-136B has a growth inhibitory activity in HER2-amplified breast cancer.

Apoptotic effect and cell cycle analysis of HM781-36B in the breast cancer cell lines

The HER2-amplified cell lines (MDA-MB-453, SK-BR-3, and BT474) and MDA-MB-231 cells were treated with HM781-36B at various doses (0.001, 0.01, and 0.1 μmol/l) for 48 h. As measured by flow cytometry, HM781-36B increased the sub-G1 phase and induced G1 arrest in SK-BR-3 and BT-474 cells. G1 arrest was also observed in MDA-MB-453 cells, but an increased sub-G1 phase was not detected (Fig. 2a).

Apoptosis was detected by Annexin V staining in the SK-BR-3, BT-474, and MDA-MB-453 cells (Fig. 2b).

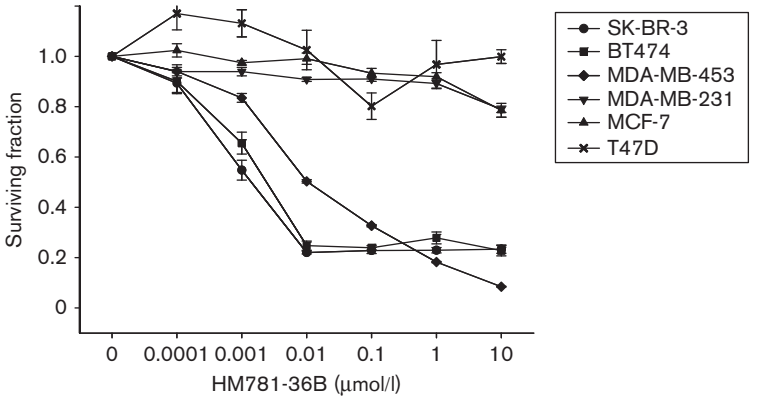
G1 arrest and apoptosis were also demonstrated by western blot analysis in the SK-BR-3, BT474, MDA-MB-453, and MDA-MB-231 cells with various doses of HM781-36B (0.001, 0.01, and 0.1 μmol/l) (Fig. 3a and b). Cyclin D and cyclin E are proteins that are usually induced in the S phase. These proteins were reduced with an increasing dose of HM781-36B in the SK-BR-3, BT474, and MDA-MB-453 cells, and $p27^{kip}$, which is a member of the cyclin-dependent kinase inhibitory proteins, was also induced. Therefore, the G1 arrest effect of HM781-36B could be observed from this result. The amount of cleaved form of caspase 3, caspase 7, and PARP was increased in the SKBR-3, BT474, and MDA-MB-453 cells.

Action of HM781-36B on the mitochondrial pathway during apoptosis

Apoptosis can be induced through the extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) signaling pathway. In the mitochondrial pathway, the release of mitochondrial cytochrome c activates caspase-9 as an initial caspase, and this subsequently induces the activation of caspases-3, which cleaves target proteins, leading to apoptosis [26–28]. We tested cytochrome c, caspase 8, and caspase 9 to demonstrate the action of

Fig. 1

Cell	SK-BR-3	BT474	MDA-MB-453	MDA-MB- 231	MCF-7	T47D
IC_{50} (μmol/l)	0.001	0.0012	0.0095	>10	>10	>10



Growth inhibitory effect of HM781-36B. After treatment with increasing doses of HM781-36B (0.0001, 0.001, 0.01, 0.1, 1, and 10 μmol/l) for 72 h, the IC_{50} (micromole per liter), a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide assay was evaluated in BT474, SK-BR-3, MCF-7, MDA-MB-231, MDA-MB-453, and T47D cell lines. The expressed data are representative of three independent experiments.

HM781-36B on the mitochondrial pathway in breast cancer cell lines. On western blotting, induction of cytochrome c and cleavage of caspase 9 were observed on SK-BR-3 and BT474 cells, and we showed that HM781-36B influenced the intrinsic pathway during apoptosis (Fig. 3c).

This death pathway is largely controlled by proapoptotic and antiapoptotic proteins. Therefore, the expressions of proapoptotic (Bim and Bax) and antiapoptotic (Bcl-2, Bcl-xL, and Mcl-1) proteins were evaluated in SK-BR-3, BT474, MDA-MB-453, and MDA-MB-231 cells after treatment with HM781-36B (Fig. 3d). The expression of Bim was increased in SK-BR-3, BT474, and MDA-MB-453 cells in a dose-dependent manner, but Bax was not observed in the control cells. HM781-36B reduced the expressions of Bcl-2, Bcl-xL, and Mcl-1, which are antiapoptotic molecules in the SK-BR-3, MDA-MB-453, and BT474 cells. However, these results were not observed in MDA-MB-231 cells.

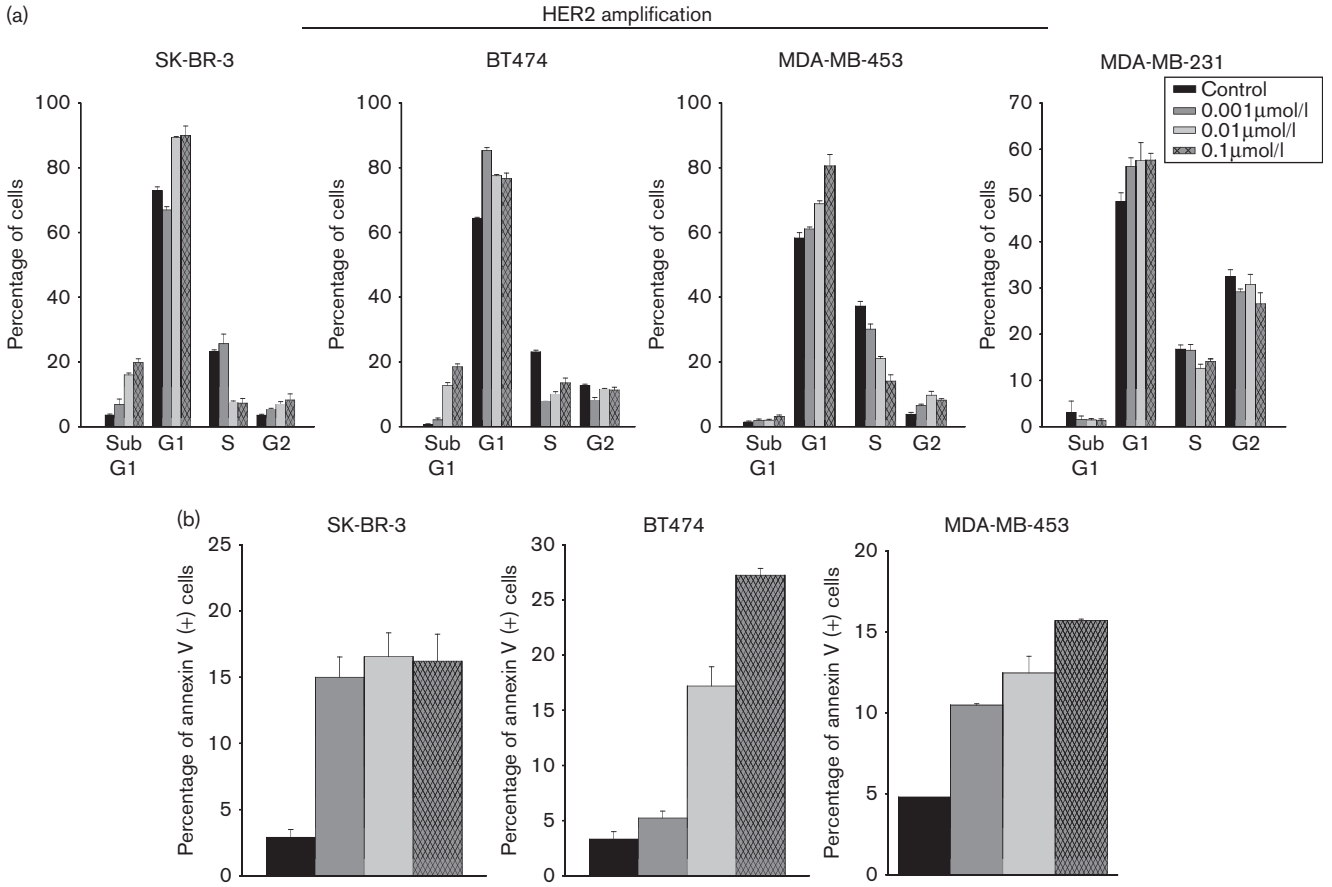
Effect of HM781-36B on the HER family and on downstream signaling

To establish the mechanism of antitumor activity of HM781-36B, we evaluated the changes in the protein expressions of the HER family and in downstream signaling (Fig. 4). SK-BR-3, BT474, MDA-MB-231, and T47D cells were treated with various doses (0.001, 0.01, and 0.1 μmol/l) for 48 h. In the SK-BR-3 and BT474 cells, p-HER2 and p-EGFR were reduced in a dose-dependent manner. Downstream signaling was downregulated in SK-BR-3 and BT474 cells, as shown by the reduced protein levels of p-AKT, p-ERK, and p-STAT3. In the case of MDA-MB-231 cells, reduced p-EGFR was also observed, but the downstream signaling was not changed. In the case of T47D cells, which are HER2-nonamplified cells, the protein levels of p-EGFR, p-HER2, p-AKT, p-ERK, and p-STAT3 were not changed after treatment with HM781-36B.

Synergistic effect of HM781-36B combined with cytotoxic chemotherapeutic agents

We examined the effect of HM781-36B combined with cytotoxic drugs (5-FU, paclitaxel, cisplatin, and gemcitabine) on breast cancer cell lines. Synergistic or additive effects were evaluated after simultaneous exposure to HM781-36B and cytotoxic drugs for 72 h and were indicated according to the CI (Fig. 5). In the SK-BR-3, BT474, and MDA-MB-453 cells, HM781-36B combined with 5-FU showed a synergistic effect, and this was also observed in MCF-7 cells, all of which were resistant to HM781-36B and 5-FU separately (Fig. 5a). The synergistic activity of HM781-36B combined with paclitaxel was seen in the HER2-amplified breast cell lines, and this synergistic effect was also observed in the HER2-nonamplified breast cell lines, including MCF-7, all of which were resistant to HM781-36B and paclitaxel separately (Fig. 5b). In the SK-BR-3 cells, HM781-36B combined with cisplatin showed a synergistic effect, and the MDA-MB-231 and T47D cells, which were not sensitive to HM781-36B and cisplatin separately, also showed this synergistic effect (Fig. 5c). HM781-36B combined with gemcitabine showed a synergistic effect in all cell lines, including the MDA-MB-231, T47D, and MCF-7 cells, all of which were resistant to HM781-36B and gemcitabine separately (Fig. 4d).

Fig. 2



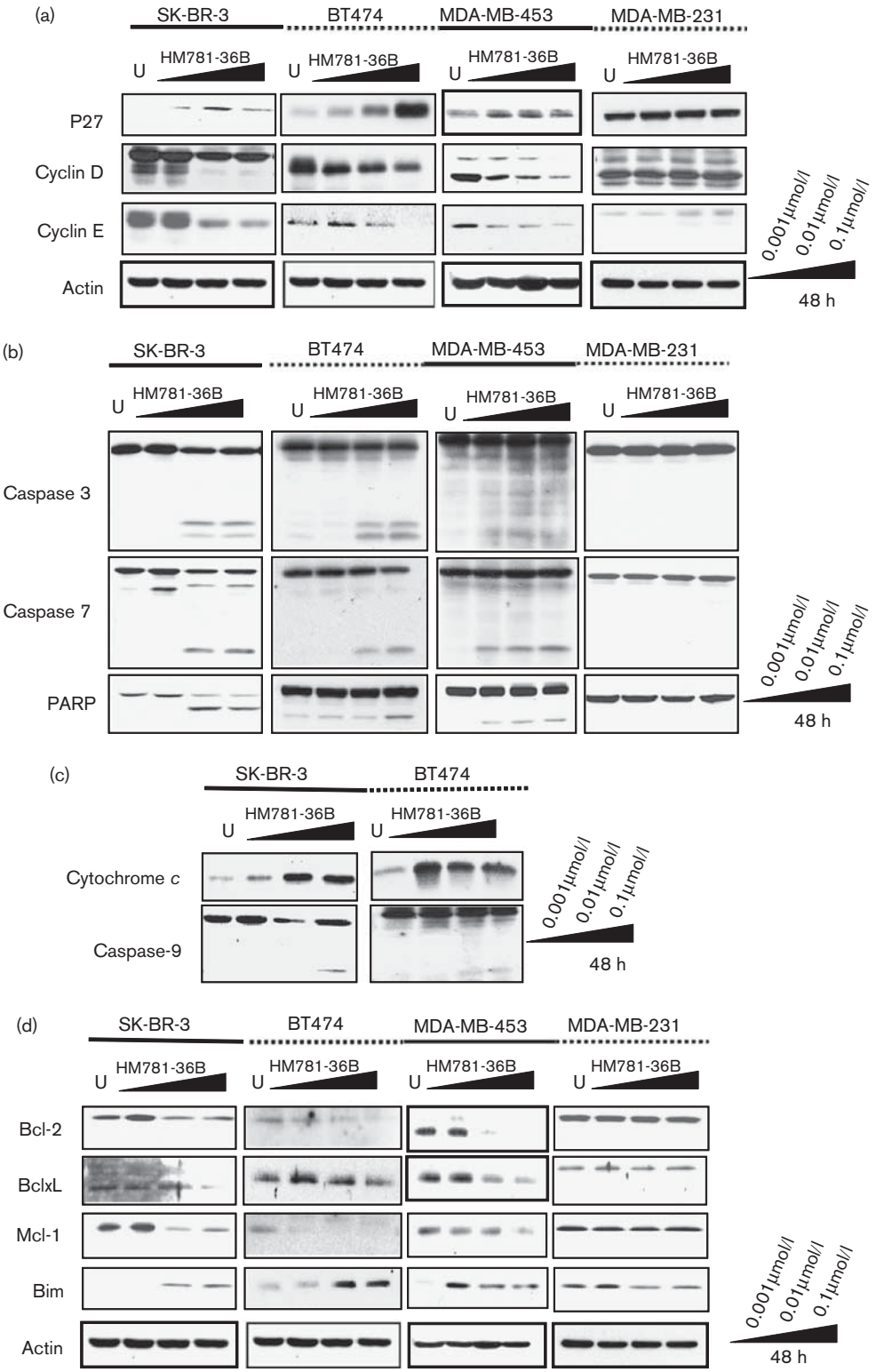
Effects of HM781-36B on cell cycle distributions. (a) The human epidermal growth factor receptor 2 (HER2)-amplified cell lines (SK-BR-3, MDA-MB-453, BT474) and the HER2-nonamplified cell lines (MDA-MB-231) were treated with HM781-36B (0.001, 0.01, and 0.1 μmol/l) and then collected for analysis. After the indicated treatment times, the cells were fixed with 70% ethanol, stained with propidium iodide, and subjected to flow cytometric analysis. Proportions of cells in the G1, S, and G2-M phase were quantified using the ModFit LT program (Verity Software House); total percentages of G1, S, and G2-M phases are 100% in our data. The fraction of sub-G1 content was separately calculated as a percentage of total gated events. (b) For assessing the apoptosis of SK-BR-3, BT474, and MDA-MB-453 cells, the staining of Annexin V-phycoerythrin by fluorescence-activated cell sorting analysis Caliber was performed to confirm the apoptosis. The expressed data are representative of three independent experiments.

Discussion

This study provides the first data to prove the growth inhibitory effect of pan-HER tyrosine kinase inhibitor, HM781-36B, and its mechanism of action in breast cancer cell lines. HM781-36B showed growth inhibitory effects in the HER2-amplified breast cancer cell lines, including MDA-MB-453, SK-BR-3, and BT474 ($IC_{50} = 0.0095$ μmol/l, 0.001 μmol/l, and 0.0012 μmol/l, respectively), compared with HER2-nonamplified cell lines, including high levels of EGFR expression. p-AKT, p-ERK, and p-STAT3 were significantly reduced in HER2-amplified cells. $p27^{kip}$ was upregulated and cyclins (cyclin D and cyclin E) were downregulated after the treatment of HM781-36B in SK-BR-3 and BT474 cells. And the amount of cleaved form of caspase 3, caspase 7, and PARP were increased in those sensitive cell lines. Thus, G1 arrest and apoptosis was proven after treatment with HM781-36B. This apoptosis was affected by the intrinsic (mitochondria-mediated) signaling pathway as shown by increased cytochrome c and cleavage of caspase 9, and it was confirmed by the change in downstream molecules such as antiapoptotic Bcl-2, Bcl-xL, and Mcl-1 and pro-apoptotic Bim. Moreover, a combination therapy of HM781-36B with a clinically relevant cytotoxic agent such as 5-FU, cisplatin, paclitaxel, or gemcitabine resulted in synergistic activity on the HER2-amplified cells and on some of the HER2-nonamplified breast cancer cell lines.

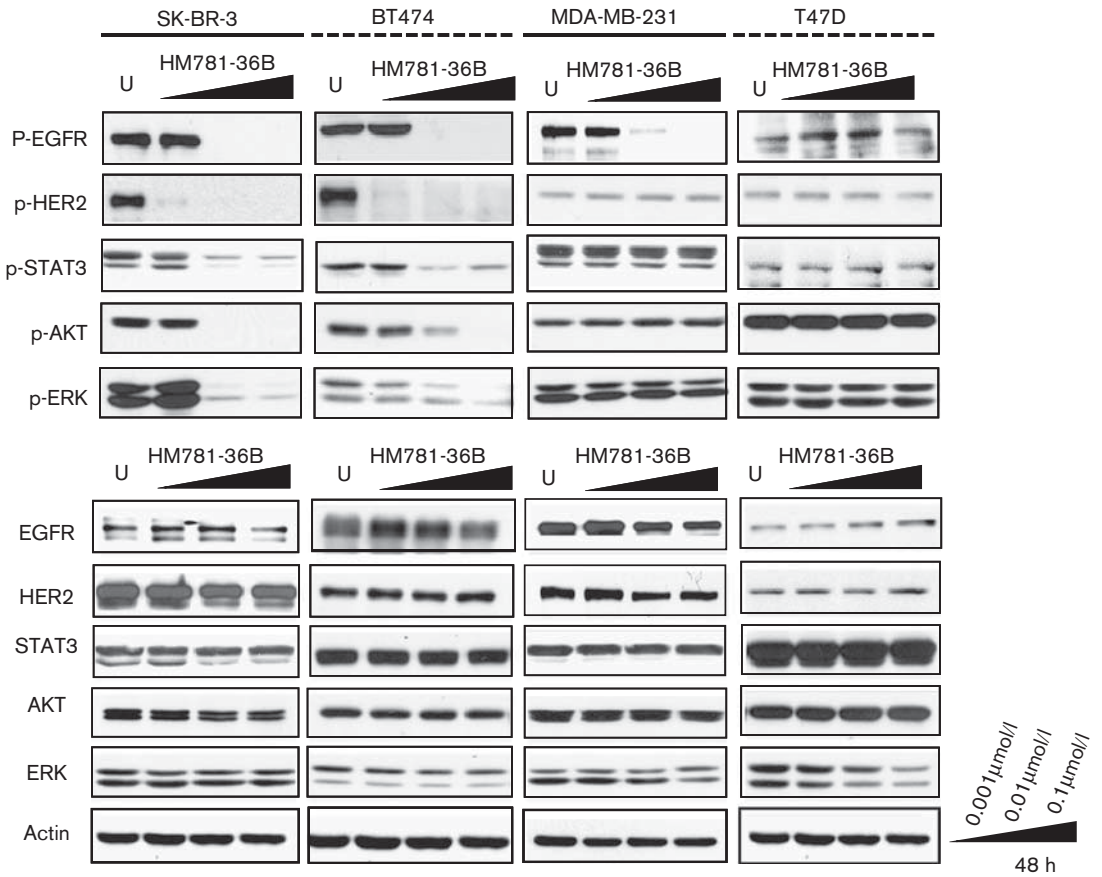
HER2 is overexpressed in 20–30% of breast cancer cases and plays a role in promoting proliferation, survival, motility, and angiogenesis of breast cancer cells. Trastuzumab (herceptin) is the first HER2-targeted therapy approved for metastatic breast cancer by the United States of FDA in 1998. As the single agent, it showed a response rate of 15–35% in metastatic breast cancer [8,29], a reduced rate

Fig. 3



G1 arrest and the apoptotic effects of HM781-36B. (a) Cell cycle-dependent molecules ($p27^{kip}$, cyclin D, and cyclin E) were checked by western blotting to identify the G1 arrest with 48 h of treatment with HM78-136B (0.001, 0.01, and 0.1 μmol/l) in the SK-BR-3, BT474, MDA-MB-453, and MDA-MB-231 cells. (b) For confirmation of apoptosis, the apoptotic molecules [poly (ADP-ribose) polymerase (PARP), caspase 3, and caspase 7] were tested in the same manner, and the cleavage of PARP, caspase 3, and caspase 7 was observed. (c) For determining the mitochondrial pathway (the intrinsic or extrinsic pathway), caspase 8, caspase 9, and cytochrome c were assessed by western blotting in SK-BR-3 and MDA-MB-453 cells after treatment with HM78-136B. (d) Changes in the Bcl-2 protein family (Bcl-2, Bcl-xL, and Mcl-1) and in proapoptotic proteins (Bim and Bax) were observed by western blotting after treatment with HM781-36B. The expressed data are representative of three independent experiments.

Fig. 4



Effect of HM781-36B on the human epidermal growth factor receptor (HER) family and downstream signaling. SK-BR-3, BT474, T47D, and MDA-MB-231 cells were treated with HM781-36B (0.001, 0.01, and 0.1 μmol/l) for 48 h. The HER family (HER1, HER2) and the phosphorylated forms of each receptor were observed with western blotting. Downstream signaling molecules (AKT, ERK, and STAT3) and the phosphorylated forms of each protein were also observed with the same treatments. p-HER2 was downregulated without changing the total forms in the SK-BR-3 and BT474 cells and reductions of p-AKT, p-ERK, and p-STAT3 were also detected. The expressed data are representative of three independent experiments. EGFR, epidermal growth factor receptor.

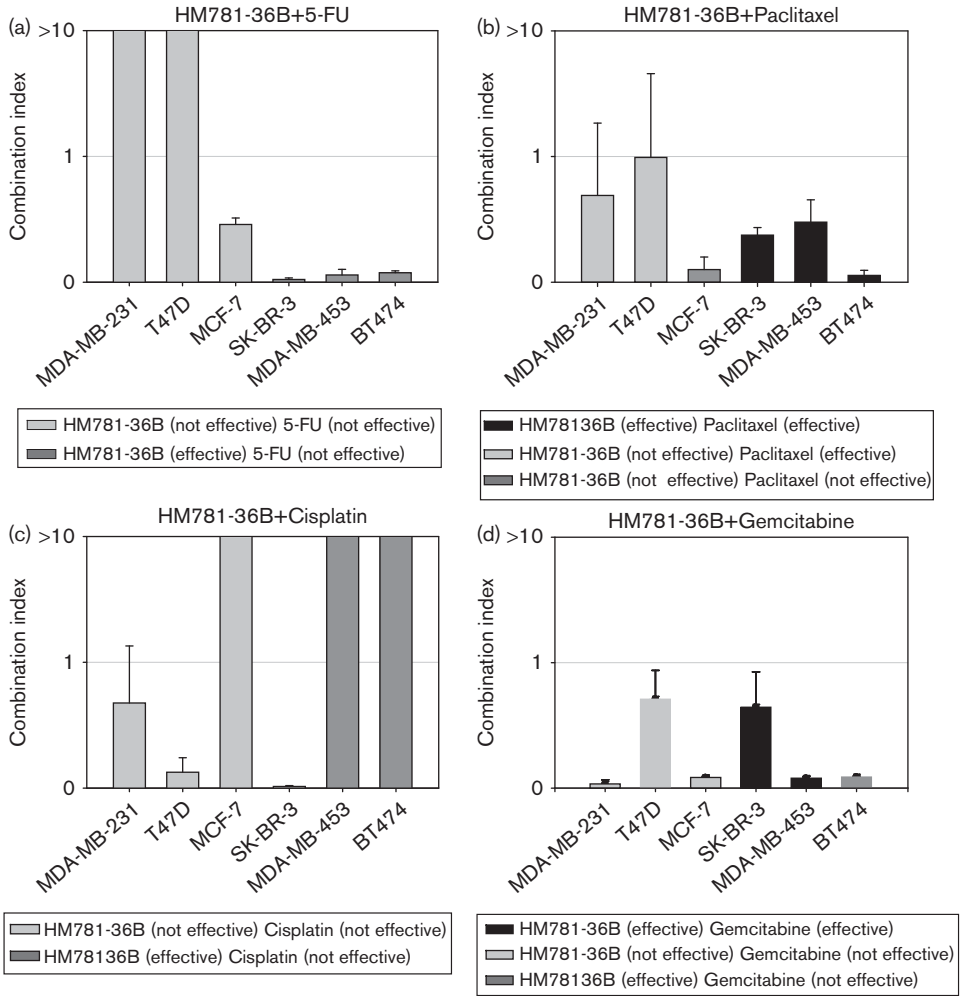
of relapse by 50%, and improved survival by 33% in an adjuvant setting [30–33]. However, trastuzumab-refractory or resistant patients with breast cancer are emerging, and this may be demonstrated through various mechanisms including altered antibody interaction, phosphatase and tensin homolog loss and enhanced AKT signaling, p27 loss, signaling through other EGFR family receptors, and signaling through other growth factor receptors [34–42].

Recently, preclinical data suggested that lapatinib may retain activity in trastuzumab-resistant phosphatase and tensin homolog-deficient and trastuzumab-resistant p95HER2 breast cancer cells [43,44]. In clinical studies, single lapatinib demonstrated a clinical benefit rate of 14–22% in patients with trastuzumab refractory disease [45,46], and the combination of lapatinib with capecitabine showed an increased time to progression after trastuzumab-based chemotherapy was increased to 6.2 months when compared with 4.3 months for capecitabine alone in the EGF10151 study [47]. Although lapatinib showed a clinical activity in trastuzumab refractory disease, resistance to lapatinib develops in some breast cancer cases with HER2 positivity during exposure to lapatinib. The mechanisms of resistance to lapatinib are not well characterized, but several hypotheses are emerging, including increased AXL expression, hyperactivation of PI3K signaling, elevated MCL-1 expression, decreased BAK activation, and HER2 mutations [48–51].

For overcoming this resistance to trastuzumab or lapatinib, the use of a pan-HER tyrosine kinase inhibitor with compensatory signaling by other HER receptor represents a promising therapeutic strategy for patients with resistance to trastuzumab or lapatinib.

CI-1033, PF-00299804, and HKI-272 are orally available pan-HER tyrosine kinase inhibitors. The effect of CI-1033 and PF-00299804 in refractory disease for trastuzumab or lapatinib has not been shown yet. However, data from a phase I study with neratinib (HKI-272), an

Fig. 5



HM781-36B administered together with cytotoxic drugs results in synergistic activity. For determining the synergism or antagonism of HM781-36B with cytotoxic drugs, MDA-MB-231, T47D, MCF-7, SK-BR-3, BT474, and MDA-MB-453 cells were observed after a combination treatment of HM781-36B with cytotoxic agents [5-fluorouracil (5-FU), paclitaxel, cisplatin, or gemcitabine]. All the cell lines were treated with serial dilutions of each drug individually as calculated on the basis of IC_{50} and with both drugs simultaneously. The combination index (CI) was calculated using CalcuSyn and it showed a survival fraction of 50%. CI$<$1, CI$=$1, or CI$>$1 indicates synergistic, additive, or antagonistic effects, respectively. The expressed data are representative of three independent experiments. (a) Combination of HM78-136B with 5-FU. (b) Combination of HM78-136B with paclitaxel. (c) Combination of HM78-136B with cisplatin. (d) Combination of HM78-136B with gemcitabine.

irreversible pan-ErbB receptor tyrosine kinase inhibitor, in patients with solid tumors showed efficacy in trastuzumab-treated patients [19], and a phase II study with HKI-272 involving primarily trastuzumab-refractory HER2-positive metastatic breast cancer patients demonstrated a response rate of 24% and a 16-week progression-free survival rate of 59% [21]. A phase I/II study evaluating the combination of paclitaxel and HKI-272 in HER-2 positive disease (NCT00445458) is ongoing, and a phase II study of CI-1033 in metastatic breast cancer (NCT00051051) has recently reached its accrual goal; however, results are yet to be presented. If there are promising results of these studies, a pan-HER tyrosine kinase inhibitor in pretreated breast cancer with HER2 positivity can be expected.

HM781-36B is a quinazoline-based irreversible pan-HER tyrosine kinase inhibitor. It has already shown potent antitumor activity in HER2-amplified gastric cancer cells [52]. Furthermore, it exerted synergistic effects with chemotherapeutic agents on the HER2-amplified and on some of the HER2-nonamplified gastric cancer cells. In our study, we could show that HM781-36B had growth inhibitory activity in HER2-amplified breast cancer cell lines, and there was a synergistic effect of HM781-36B combined with several cytotoxic chemotherapeutic agents. HM781-36B in combination with 5-FU exerted synergistic effects in HER2-amplified cells and in HER2-nonamplified cells (MCF-7), although HM781-36B alone did not inhibit the growth of this HER2-nonamplified cell. We believe that thymidylate synthase (TS) downre-

gulation can be one of the mechanisms to explain synergism between HM781-36B and 5-FU with, because TS downregulation by treatment of lapatinib has been shown in a previous study [53,54].

Furthermore, TS downregulation by gefitinib was detected in non-small-cell lung cancer cells with met proto-oncogene (hepatocyte growth factor receptor) amplification, and combination of 5-FU and gefitinib synergistically inhibited the proliferation of cells with met proto-oncogene (hepatocyte growth factor receptor) amplification [55]. The combination of HM781-36B with cisplatin also showed synergistic effect in several HER2-nonamplified cells. One report indicated that resistance to gefitinib, an EGFR TKI, can be overcome by cisplatin treatment [56]. Furthermore, as ERCC1, XRCC1, and XRCC3 gene polymorphisms were identified as useful candidate markers for predicting better survival in non-small-cell lung cancer patients after treatment with platinum-based chemotherapy (cisplatin) and as inhibition of HER2 signaling was associated with DNA repair processes after cisplatin treatment, enhanced cytotoxicity through DNA repair processes can be one of the possible mechanisms to explain the synergistic effect of HM781-36B and cisplatin [57,58]. In particular, the combination of HM781-36B with paclitaxel or gemcitabine showed a synergistic effect in MCF-7. This could be explained by in-vitro data showing that the combination of trastuzumab with paclitaxel or gemcitabine had a synergistic growth inhibitory effect in MCF-7 [59,60]. These findings suggest a rationale for administering HM781-36B as a single agent or in combination with several cytotoxic agents for treating breast cancer. Further, an in-vivo study of HM781-36B alone or in combination with cytotoxic agents in breast cancer and in refractory breast cancer to trastuzumab or lapatinib will also be needed with HM781-36B.

Acknowledgements

Conflicts of interest

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