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Preclinical and phase I clinical studies of KW-2450, a dual IGF-1R/IR tyrosine kinase inhibitor, in combination with lapatinib and letrozole

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Abstract

Background: KW-2450 is an oral dual insulin-like growth factor-1 receptor/insulin receptor tyrosine kinase inhibitor. We investigated the *in vitro* and *in vivo* preclinical activity of KW-2450 plus lapatinib and letrozole and conducted a phase I trial of the triple-drug combination in one male and 10 postmenopausal female patients with advanced/metastatic hormone receptor-positive, human epidermal growth factor receptor 2 (HER2)-positive breast cancer.

Methods: A series of *in vitro* and *in vivo* animal studies was undertaken of KW-2450 in combination with lapatinib and hormonal agents. The phase I trial was conducted to establish the safety, tolerability, and recommended phase II dose (RP2D) of KW-2450 administered in combination with lapatinib and letrozole.

Results: Preclinical studies showed KW-2450 and lapatinib act synergistically to induce *in vitro* apoptosis and inhibit growth of HER2-positive MDA-MB-361 and BT-474 breast cancer cell lines. This combined effect was confirmed *in vivo* using the MDA-MB-361 xenograft model. KW-2450 showed synergistic *in vitro* growth inhibition with letrozole and 4-hydroxytamoxifen in ER-positive MCF-7 breast cancer cells and MCF-7-Ac1 aromatase-transfected MCF-7 cells. In the phase I study, dose-limiting toxicity (DLT; grade 3 rash and grade 3 hyperglycemia, respectively) occurred in two of three patients at the dose of KW-2450 25 mg/day plus lapatinib 1500 mg/day and letrozole 2.5 mg/day. The RP2D of the triple-drug combination was established as KW-2450 25 mg/day, lapatinib 1250 mg/day, and letrozole 2.5 mg/day with no DLT at this dose level.

Conclusions: The proposed phase II study of the RP2D for the triple-drug combination did not progress because of anticipated difficulty in patient enrollment and further clinical development of KW-2450 was terminated.

Keywords: breast cancer, estrogen receptor-positive, HER2-positive, KW-2450, lapatinib, letrozole

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Introduction

Breast cancer is the most common malignancy and second highest cause of death from malignancy in women in the United States (US), with an estimated 249,260 new cases in 2016.[1] Despite improvements in the prognosis of breast cancer seen in recent decades,[1] additional therapeutic advances are needed, particularly for patients with metastatic/advanced disease. One of the more recent breakthroughs in the treatment of breast cancer was the identification of the human epidermal growth factor receptor 2 (HER2) and its role in the development of aggressive breast

tumors. HER2 overexpression, which is detected in 20–25% of invasive breast cancers, is associated with poorer prognosis, more aggressive disease, and higher risk of recurrence.[2–5] The use of agents such as trastuzumab, a monoclonal antibody to HER2, or lapatinib, a tyrosine kinase inhibitor of the HER2 receptor, has led to improved outcome for patients with HER2-positive breast cancer.[6]

Molecular crosstalk between the estrogen receptor (ER) and HER2 may be responsible for hormone resistance in some patients with ER-positive breast cancer. Activation of the HER2 pathway results in downstream activation of the phosphatidylinositol-3-kinase (PI3K)/Akt and MAP kinase (MAPK) pathways that mediate the effect of HER2 on cell proliferation and survival, and these activated pathways can, in turn, phosphorylate and activate the ER.[7] Similarly, ER activation can result in HER2 activation, and inhibition of HER2 can block estrogen-induced activation of MAPK by the ER.[8] Indeed, the addition of lapatinib to the nonsteroidal aromatase inhibitor letrozole significantly improves progression-free survival (PFS) among postmenopausal women with ER-positive HER2-positive metastatic breast cancer.[9] This combination is an approved first-line therapy in this population.

Insulin-like growth factor-1 receptor (IGF-1R) is a tyrosine kinase receptor whose pathway has a significant effect on cell proliferation, differentiation, survival, and angiogenesis,[10,11] and its dysregulation is implicated in breast cancer.[12–15] Molecular crosstalk also appears to occur between IGF-1R and both ER and HER2: estrogen can increase IGF-1R expression in breast cancer cells;[16] IGF-1R activates the same PI3K/Akt and MAPK pathways as HER2 and also activates ER *via* these pathways;[17] the combination of aromatase and IGF-1R inhibition synergistically inhibits breast cancer cellular proliferation;[18] trastuzumab resistance may be associated with IGF-1R overexpression;[19] and IGF-1R can dimerize with HER2 and induce phosphorylation of HER2 in trastuzumab-resistant but not trastuzumab-sensitive cells.[20] Clinically, everolimus, an inhibitor of the mammalian target of rapamycin (mTOR), which is a known downstream target of IGF-1R, in combination with an aromatase inhibitor improved PFS in patients with hormone receptor-positive advanced breast cancer who had been previously treated with a nonsteroidal aromatase inhibitor.[21]

Given the potential for crosstalk between IGF-1R, HER2, and ER, and that this crosstalk may be related to resistance to the effects of drugs targeted to these receptors, a combination of agents inhibiting each of these three receptors may show therapeutic synergy in the treatment of breast cancer. KW-2450 (Kyowa Kirin Pharmaceutical Development, Inc., Princeton, NJ, USA) is an investigational, orally active, dual IGF-1R/insulin receptor (IR) tyrosine kinase inhibitor.[22–24] We describe *in vitro* and *in vivo* preclinical studies of KW-2450 plus lapatinib and letrozole and a phase I trial of the triple combination in postmenopausal patients with advanced/metastatic hormone receptor-positive, HER2-positive breast cancer.

Methods

All animal studies were approved and conducted in accordance with company policy on the care and use of laboratory animals (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan).

Cell lines

BT-474, MDA-MB-361, and MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7-Ac1, a human breast cancer cell line that stably expresses aromatase, was kindly provided from Professor A. Brodie (University of Maryland, Baltimore, MD, USA). BT-474 cells were maintained in culture medium [Dulbecco's modified Eagle medium (DMEM; high glucose, Invitrogen, Grand Island, NY, USA), 10% NCTC-135 medium (Sigma-Aldrich, St. Louis, MO, USA), 0.01 mg/ml bovine insulin (Sigma-Aldrich), 1.2 mmol/l oxaloacetic acid (Sigma-Aldrich), and 10% heat-inactivated fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA)]. MDA-MB-361 cells were maintained in Leibovit's L-15 medium (Invitrogen) supplemented with 20% heat-inactivated FBS. MCF-7 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 1% nonessential amino acids solution (Nacalai Tesque, Kyoto, Japan), 1 mmol/l sodium pyruvate, and 0.01 mg/ml bovine insulin. MCF-7-Ac1 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% nonessential amino acids solution, 1 mmol/l sodium pyruvate, 0.01 mg/ml bovine insulin, and 800 μg/ml Geneticin® (Invitrogen).

Apoptosis analysis

BT-474 cells were resuspended with assay medium (DMEM high glucose, 10% NCTC-135 medium, 0.01 mg/ml bovine insulin, 1.2 mmol/l oxaloacetic acid, and 1% heat-inactivated FBS). MDA-MB-361 cells were resuspended with assay medium (Leibovit's L-15 medium supplemented with 10% heat-inactivated FBS). BT-474 cells (3×10^3) or MDA-MB-361 cells (4×10^3) were seeded into 96-well F-bottom half area plates. The plates were preincubated in a CO_2 incubator for 24 h at 37°C. KW-2450 or lapatinib (at increasing concentrations in assay medium) were added and incubated for an additional 24 h. Caspase-3/7 activity was measured by using the Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luminescence was measured with a Topcount® NXT™ (Hewlett Packard, Meriden, CT, USA) counter.

Western blotting

BT-474 or MDA-MB-361 cells (2×10^5 cells/ml) were seeded into 10-cm dishes with assay medium and treated with KW-2450 or lapatinib (each 100 nmol/l) for 48 h. Harvested cells were suspended in NP40 Cell Lysis Buffer (Invitrogen) containing 1 mmol/l phenylmethanesulfonyl fluoride (Sigma-Aldrich) and 1% protease inhibitor cocktail for mammalian tissues (Sigma-Aldrich) and then settled on ice for 30 min. Clarified cell lysates were obtained by centrifugation and the protein concentration of each lysate was measured by a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For preparation of SDS-PAGE samples, each lysate was mixed with 5× sample buffer (Thermo Fisher Scientific, Rockford, IL, USA) and heated for 5 min at 95°C. The protein in each sample was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were incubated with each primary antibody against P-HER2, P-IGF-1R/IR, P-Akt, HER2, IGF-1R, Akt (Cell Signaling Technologies, Beverly, MA, USA), or β-actin (Sigma-Aldrich). After the membranes were washed three times with tris-buffered saline-Tween 20 (TBS-T) buffer for 10 min, they were incubated for 1 h with horseradish peroxidase-linked antirabbit antibody or antimouse antibody (GE Healthcare, Buckinghamshire, UK) depending on the species of the primary antibody. After washing, the membranes were soaked in SuperSignal® WestPico Chemiluminescent Substrate (Thermo Fisher Scientific) and then exposed to X-ray film (Fujifilm, Tokyo, Japan).

Gene expression analysis

MDA-MB-361 cells (2×10^5 cells/ml) were seeded into 15-cm dishes with assay medium and treated with KW-2450 or lapatinib (each 100 nmol/l) for 48 h. RNA was extracted by using TRIzol (Invitrogen) according to the manufacturer's protocol. All samples were quantitated using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and RNA quality was analyzed using Agilent Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). Total RNA (500 ng) was labeled using a Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The labeled cRNA were hybridized onto a 4 × 44K Human Whole Genome Microarray Kit G4112F (Agilent Technologies, Santa Clara, CA, USA) followed by purification with an RNeasy Mini Kit (Qiagen, Tokyo, Japan). Hybridization was carried out at 65°C for 17 h in a hybridization oven rotator. The arrays were washed using GENE Expression Wash Buffer Kit (Agilent Technologies, Santa Clara, CA, USA). Slides were scanned on a microarray scanner model G2565C (Agilent Technologies, Santa Clara, CA, USA) with Agilent Feature Extraction software version 10.7 (Agilent Technologies) used for image analysis. The data were imported into the GeneSpring GX11.02 multi-omics platform (Agilent Technologies, Santa Clara, CA, USA). Raw signals were liner-shifted with the 65th percentile value of each array as a reference, followed by the baseline transformation for dimethyl sulfoxide (DMSO) treatment in each probe and the selected expression changed probes. Microarray data were submitted to the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE82161.

Combination index in HER2/IGF-1R double-positive breast cancer cell lines

BT-474 (8×10^3) or MDA-MB-361 (2.4×10^3) cells were seeded into 96-well F-bottom plates with assay buffer and treated with increasing concentrations of KW-2450 or lapatinib for 72 h. Cell viability was determined by using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The absorbance (450 nm) corresponding to viable cells was calculated by

subtracting the absorbance (690 nm) of the blank from that of each sample. These values were designated as a percent of control (cell viability).

The interaction of KW-2450 and lapatinib was determined by combination index (CI) methods using the CompuSyn software program (ComboSyn, Inc., New York, NY, USA). A CI value at 50% inhibition (fraction affected, Fa = 0.5) based on the CI isobologram equation was also calculated. The results obtained from the combination ratio, which showed that the most potent Fa value was 0.5, were defined in this report as combination effects on antiproliferative activity. If all relative growth values obtained from a combination ratio were above or below 50%, the Fa = 0.5 value obtained from that combination ratio was represented as a reference.

CI under hormone-dependent conditions. A β-estradiol-induced cell proliferation assay was performed with steroid-depleted medium, in which RPMI 1640 medium without phenol red (Invitrogen) was supplemented with 5% charcoal-stripped bovine calf serum (Thermo Scientific), 1% nonessential amino acids solution, 1 mmol/l sodium pyruvate, and 0.01 mg/ml bovine insulin. MCF-7 cells were preincubated with steroid-depleted medium for 6 days, and 2×10^3 of these cells were re-seeded into 96-well F-bottom plates and incubated for 24 h at 37°C. β-estradiol (Sigma-Aldrich, final concentration 1 nmol/l) was added followed by increasing concentrations of KW-2450 or 4-hydroxytamoxifen (Sigma-Aldrich) and then incubated for 7 days at 37°C. For the aromatase-dependent cell proliferation assay, MCF-7-Ac1 cells were preincubated, re-seeded, and treated with steroid-depleted medium according to the same method as for MCF-7 cells. However, androstenedione (Sigma-Aldrich, final concentration of 10 nmol/l) was then added followed by increasing concentrations of KW-2450 or letrozole (Kemprotec, Middlesbrough, UK) and then incubated for 7 days at 37°C. Cell viability was determined using Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany). The CI and combination effect were determined as previously described.

Preclinical in vivo *study*

The 6-week-old female NOD-SCID mice (NOD/ShiJic-scidJcl, CLEA Japan, Tokyo, Japan) were maintained with free access to autoclaved tap water and irradiated feed (CL-2, CLEA Japan). After 47 days of laboratory housing, each mouse received 3 mg of intraperitoneal cyclophosphamide (Shionogi, Osaka, Japan) and a 0.36-mg subcutaneous pellet of 17β-estradiol (60-day release, Innovative Research of America, Sarasota, FL, USA). On the next day, MDA-MB-361 cells (9×10^6 cells in 0.1 ml/mouse) were subcutaneously implanted into the mice. After 14 days from inoculation, mice whose tumor volume reached 101.42–154.90 mm^3 were selected for the experiment. The day of group allocation was designated as Day 0. Methylcellulose 400 (vehicle control) or KW-2450 (40 mg/kg), lapatinib (60 mg/kg), or their combination in vehicle were orally administered once daily for 14 days. Tumor volume was calculated by the antitumor test system II as follows: $V = DL \times DS \times DS \times 1/2$ (DL, long diameter; DS, short diameter). The relative tumor volume in each mouse was represented as V/V_0, where V_0 was V on Day 0. The relative V/V_0 on each day was represented as T/C, where C and T were the mean V/V_0 in the vehicle group and the treated group, respectively. Statistical analysis was performed using SAS software (Release 9.4, SAS Institute Inc., Cary, NC, USA), in which V on Day 14 was used as a parameter. The difference in V comparing the KW-2450 monotherapy or lapatinib monotherapy group with the combination group was analyzed by the Dunnett test with $p < 0.05$ considered significant.

Phase I study

The study was initially planned as a phase I/II study but phase II was terminated prematurely (see Discussion). The phase I design was a sequential, ascending, multi-dose, open-label study incorporating a standard 3 + 3 dose escalation schedule in patients with advanced/metastatic hormone receptor-positive HER2-positive breast cancer. The study was conducted at four institutions in the US (Breastlink Medical Group, Long Beach, CA; MD Anderson Cancer Center, Houston, TX; Detroit Clinical Research Center, Farmington Hills, MI; and Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL). The protocol, informed consent form, and amendments were reviewed and approved by the Institutional Review Board at each study center. This study was conducted in accordance with the Declaration of Helsinki and International Conference for Harmonisation Good Clinical Practice guidelines. All patients provided written informed consent. The study was registered at ClinicalTrials.gov [ClinicalTrials.gov identifier: NCT01199367].

The primary objective was to establish the safety, tolerability, and a recommended phase II dose (RP2D) of KW-2450 administered in combination with lapatinib and letrozole. The secondary objective was to determine the pharmacokinetic and pharmacodynamic profile of KW-2450, lapatinib, and letrozole when administered in combination. The recommended, approved doses of lapatinib and letrozole during their co-administration were 1500 and 2.5 mg once daily, respectively. The maximum tolerated dose (MTD) of KW-2450 was 37.5 mg once daily in patients with advanced/metastatic solid tumors.[22–24] Since *in vitro* drug interaction studies suggested a potential for increased plasma KW-2450 concentrations during co-administration of KW-2450 with lapatinib (data on file, Kyowa Kirin Pharmaceutical Development, Inc.), it was decided to reduce initial doses to ~70% of the single-agent MTD for KW-2450 and to ~70% of the approved lapatinib dose. Starting dose levels were selected as KW-2450 25 mg once daily plus lapatinib 1250 mg once daily and letrozole 2.5 mg once daily in Cohort 1. The dose of lapatinib alone was increased to 1500 mg once daily for the triple-drug combination in Cohort 2. No patients were recruited to subsequent escalation cohorts because the MTD had been reached. Details of patient selection and exclusion, and efficacy, safety, and pharmacokinetic/pharmacodynamic assessments are summarized in additional file 1 (Data S1).

Results

In vitro *and* in vivo *combined effect of KW-2450 and lapatinib in HER2-positive breast cancer cells*

The combined effect of KW-2450 and lapatinib for apoptosis induction measured by caspase-3/7 activation was examined for both HER2-positive BT-474 and MDA-MB-361 breast cancer cells. In BT-474 cells, KW-2450 alone induced relatively minor apoptosis (~225% at 400 nmol/l), whereas lapatinib alone induced relatively greater apoptosis (~400% at 200 nmol/l). The addition of KW-2450 enhanced apoptosis induced by lapatinib in a dose-dependent manner [Figure 1(a)]. In MDA-MB-361 cells, KW-2450 alone induced apoptosis at concentrations ranging from 25 to 400 nmol/l, whereas lapatinib alone did not. The combination of KW-2450 and lapatinib showed remarkable synergistic induction of apoptosis [Figure 1(b)]. This suggests that MDA-MB-361 is relatively resistant to lapatinib alone but co-treatment with KW-2450 overcomes this intrinsic resistance.

We then determined the effect on downstream signaling of HER2 and IGF-1R [Figure 2(a)]. In both BT-474 and MDA-MB-361 cells, KW-2450 alone inhibited phosphorylation of IGF-1R/IR and lapatinib alone inhibited that of HER2 as expected from their mechanisms of action. In MDA-MB-361 cells, which are resistant to lapatinib-induced apoptosis [Figure 1(b)], lapatinib alone did not inhibit phosphorylation of Akt whereas the combination of KW-2450 and lapatinib induced its strong inhibition. DNA microarray studies revealed that the combination of KW-2450 and lapatinib inhibited Ki67 (MKI67, probe A_24_P346855) and survivin (BIRC5, probe A_23_P118815) expression and increased tissue inhibitor of matrix metalloproteinase 3 (TIMP3, probe A_23_P399078) expression in MDA-MB-361 cells [Figure 2(b)]. TIMP3 increased approximately 5-fold with KW-2450 alone and more than 10-fold with a combination of KW-2450 and lapatinib.

We then assessed the *in vivo* antitumor activity of the combination of KW-2450 and lapatinib in an immunodeficient NOD-SCID mouse model subcutaneously inoculated with MDA-MB-361 cells. On Day 14, treatment with KW-2450 plus lapatinib showed significantly greater inhibition compared with KW-2450 alone ($p = 0.0189$) or lapatinib alone ($p = 0.0007$; Figure 3). Maximum body weight loss was observed in the combination group. However, the body weight loss was within 10% of initial body weight. No severe toxicity was observed in any treatment groups.

In vitro *effect of KW-2450 combination with lapatinib, an anti-estrogen, or an aromatase inhibitor in breast cancer*

CI values for KW-2450 and lapatinib were determined in accordance with Chou and Talalay's method.[25] The combination effect of KW-2450 and lapatinib was evaluated from the combination index plot using the fraction of antiproliferative activity (Fa) and the most potent CI (Fa = 0.5) values were determined. In BT-474 cells, the CI value was 0.69 for the combination ratio of 8:1 (KW-2450:lapatinib), suggesting synergism. In MDA-MB-361 cells, the CI value was 0.064 for the combination ratio of 1:1 (KW-2450:lapatinib), suggesting very strong synergism (Table 1).

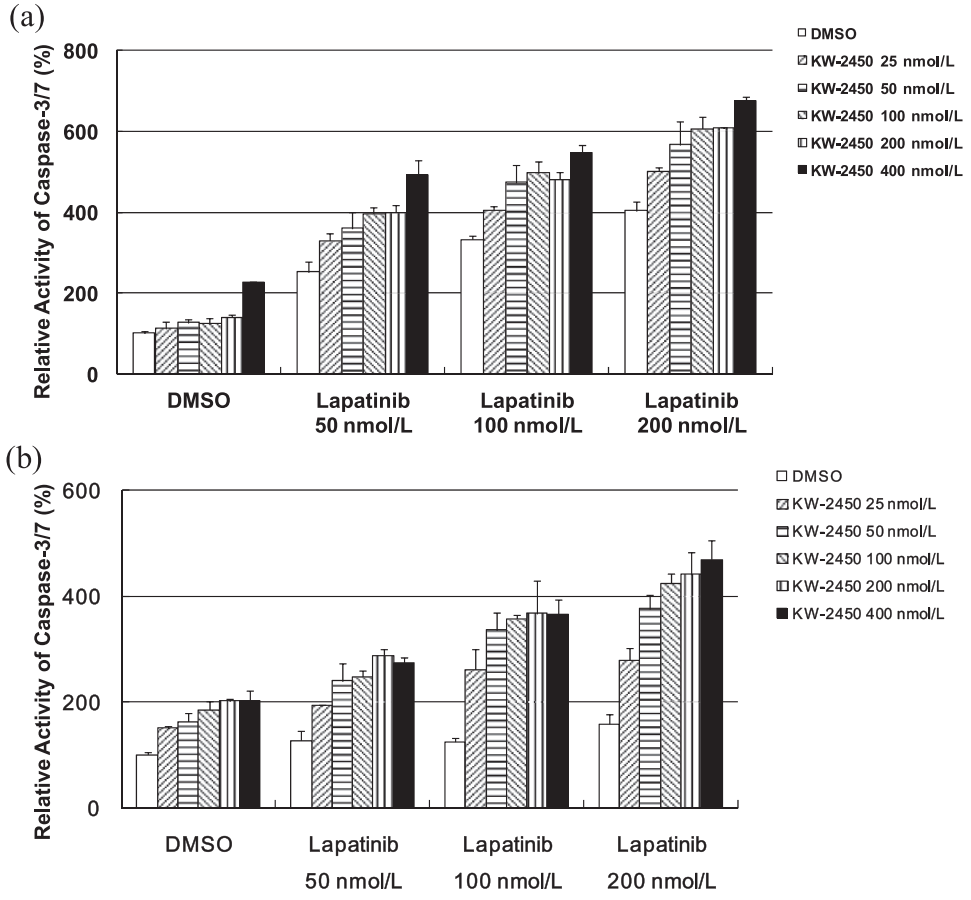

Figure 1. Effect of KW-2450, lapatinib, and their combination on caspase-3/7 activation in BT-474 (a) or MDA-MB-361(b) cells. Each column represents the mean and standard deviation of three experiments.

Next, CI values for KW-2450 and an anti-estrogen or an aromatase inhibitor were determined by the same method. The combination effect of KW-2450 and 4-hydroxytamoxifen was evaluated in MCF-7 cells in a β-estradiol-dependent condition. The CI value was 0.54 for the combination ratio of 1:4 (KW-2450:4-hydroxytamoxifen), suggesting synergism in ER-positive cells. Finally, the combination effect of KW-2450 and letrozole was evaluated in aromatase-transfected MCF-7 cells (MCF-7-Ac1 cells) in an androstenedione-dependent condition. The CI value was 0.29 for the combination ratio of 2:1 (KW-2450:letrozole), suggesting strong synergism in aromatase-driven ER-positive cells (Table 1).

Phase I study of KW-2450 in combination with lapatinib and letrozole

The patients were treated between July 26, 2011 and December 12, 2012. Their baseline demographic and clinical characteristics are summarized in Table 2. All patients (10 women and 1 man) were White and had stage IV, HER2-positive, and hormone (estrogen or progesterone) receptor-positive breast cancer. All 10 women were postmenopausal. All patients had received prior chemotherapy, trastuzumab therapy, and surgery, and the majority had received prior radiotherapy (90.9%) and hormonal therapy (81.8%). The mean (± SD) treatment duration for Cohorts 1 and 2 was 78.6 ± 58.38 days (range 16–197) and 125.3 ± 21.36 days (range 113–150), respectively.

Dose-limiting toxicities (DLTs), treatment duration, and reason for discontinuation among the 11 patients enrolled in the study (8 in Cohort 1, and 3 in Cohort 2) are summarized in Table 3. There were three patients initially enrolled in Cohort 1 but one (Patient 1) withdrew before completing the 30-day observation period for reasons other than DLT (grade 3 convulsion unrelated to treatment) and was replaced with Patient 4; none experienced DLT. There were three

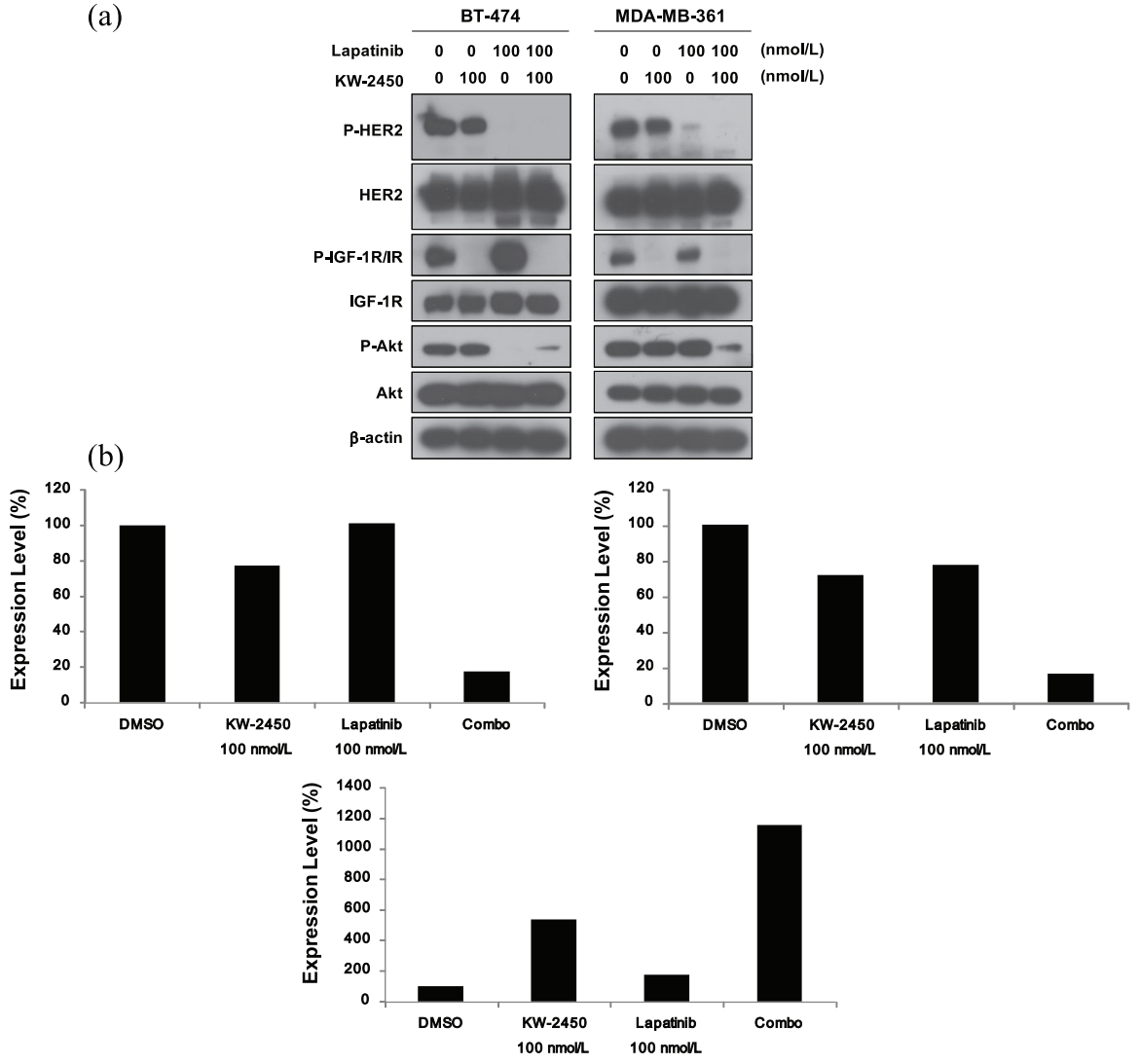

Figure 2. (a) Combined effect of KW-2450 and lapatinib on downstream signaling of IGF-1R and HER2 in BT-474 (*left panel*) or MDA-MB-361 (*right panel*) cells. (b) Combined effect of KW-2450 and lapatinib on expression of Ki67 (*upper left panel*), survivin (*upper right panel*), and TIMP3 (*lower panel*) in MDA-MB-361 cells.
HER2, human epidermal growth factor receptor 2; IGF-1R, insulin-like growth factor-1 receptor.

patients enrolled in Cohort 2 and two experienced DLTs. In one, a grade 3 rash occurred on Day 12, dosing with KW-2450 and lapatinib was interrupted (lapatinib dose was reduced to 1250 mg/day once the rash had resolved) and the patient continued treatment with KW-2450, letrozole, and reduced dose lapatinib until disease progression without recurrence of rash. The other patient in Cohort 2 with a DLT experienced grade 3 hyperglycemia on Day 30. The patient started taking metformin and continued with moderate hyperglycemia until disease progression. Cohort 1 was then expanded to enroll an additional three patients. Patient 10 had a dose interruption due to a nondrug-related adverse event (AE; grade 3 elevated bilirubin related to acyclovir), did not complete the 30-day observation period, and was replaced with Patient 11. None experienced DLT. Concomitant KW-2450 25 mg/day, lapatinib 1250 mg/day, and letrozole 2.5 mg/day was therefore established as the MTD according to the protocol.

Treatment-emergent AEs are summarized in Table 4. All 11 patients (100%) experienced treatment-emergent AEs. The most common treatment-emergent AEs were diarrhea ($n = 6$), dyspnea ($n = 3$), rash ($n = 3$), and hyperglycemia

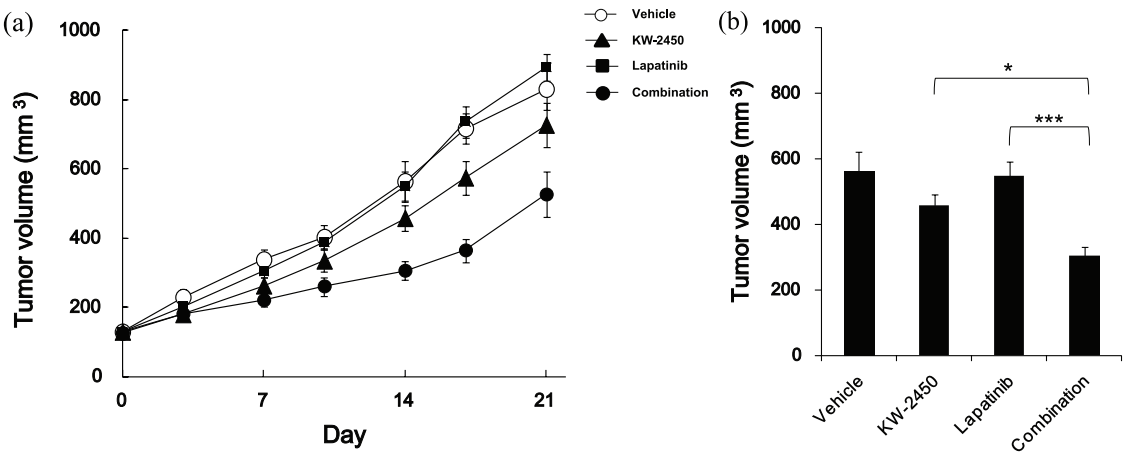

Figure 3. (a) Antitumor activity of KW-2450, lapatinib, and their combination in MDA-MB-361-implanted NOD-SCID mice. Each plot represents the mean ± standard error of the mean of tumor volume ($n = 5$). (b) Tumor volume (mm^3) on Day 14. Each plot represents the mean ± standard error of the mean of tumor volume ($n = 5$). Statistical significance (Dunnett test): *$p = 0.0189$ (KW-2450 group *versus* combination group), ***$p = 0.0007$ (lapatinib group *versus* combination group).

Table 1. Combination index.

Combination of drugs	Ratio	Cell line	CI	Combination effect
KW-2450:lapatinib	8:1	BT-474	0.69	Synergism
KW-2450:lapatinib	1:1	MDA-MB-361	0.064	Very strong synergism
KW-2450:4-hydroxytamoxifen	1:4	MCF-7	0.54	Synergism
KW-2450:letrozole	2:1	MCF-7-Ac1	0.29	Strong synergism

CI, combination index.

and fatigue (each $n = 2$). Other treatment-emergent AEs occurred in only one patient. There were no grade 4 or 5 AEs. Overall, 4 of the 11 patients (36.4%) experienced serious AEs: 2 of 8 patients (25%) in Cohort 1 (1 with a grade 3 catheter site infection and 1 with a grade 3 convulsion, both unrelated to study drugs) and 2 of 3 patients (66.7%) in Cohort 2 (1 with diarrhea and dehydration definitely related to lapatinib and 1 with hyperglycemia probably related to KW-2450). Overall, two patients discontinued treatment due to AEs, which were unrelated to study medication (convulsion in Cohort 1 and central nervous system metastasis in Cohort 2). Of the 11 patients with AEs, 7 (63.6%), 8 (72.7%), and 4 (36.4%) experienced AEs related to KW-2450, lapatinib, and letrozole, respectively. With the exception of hyperglycemia, there were no clinically meaningful shifts from baseline for vital signs, laboratory measurements, cardiac measurements, or electrocardiogram parameters.

Of the 11 patients, 6 (54.5%) maintained stable disease as best response, 4 (36.4 %) had progressive disease, and 1 was not evaluable (discontinued on Day 17 due to AE prior to tumor evaluation). The duration of stable disease was 5, 7, >7, 8, 8, and 15 weeks, respectively.

Pharmacokinetic parameters for KW-2450, lapatinib, and letrozole are summarized in an additional file (Table S1). The mean terminal half-life of KW-2450 was 10.5–14.7 h and did not appear to be affected by repeated dosing or when the lapatinib dose increased from 1250 to 1500 mg/day. There was negligible accumulation of KW-2450 in plasma on multiple daily dosing. Exposure to lapatinib (assessed by mean maximum plasma concentrations and area under the plasma concentration–time curves) increased in a more than dose-proportional manner (a 1.7 to 2.1-fold increase in exposure with a 1.2-fold increase in dose) comparing 1250 and 1500 mg/day doses.

Table 2. Baseline demographic and clinical characteristics.

Characteristics	Total ($N = 11$)
Mean ± SD age, years (range)	49.5 ± 10.0 (32–64)
Mean ± SD height, cm (range)	161.7 ± 8.8 (147.5–177.8)
Mean ± SD weight, kg (range)	68.5 ± 12.1 (52.2–90.9)
Sex, *n* (%)	
Female	10 (90.9)
Male	1 (9.1)
Race, *n* (%)	
White	11 (100)
Stage, *n* (%)	
IV	11 (100)
ECOG performance status	
0	5 (45.5)
1	6 (54.5)
HER2 status, *n* (%)	
IHC 3+	7 (63.6)
FISH-positive	4 (36.4)
Hormone receptor status, *n* (%)	
ER-positive	10 (90.9)
PR-positive	5 (9.1)
Previous therapy, *n* (%)	
Chemotherapy	11 (100)
Trastuzumab	11 (100)
Surgery	11 (100)
Radiotherapy	10 (90.9)
Hormonal therapy	9 (81.8)
No. of previous chemotherapies, *n* (%)	
1	4 (36.4)
2	0
3	4 (36.4)
⩾4	3 (27.3)
No. of hormonal therapies, *n* (%)	
0	2 (18.2)
1	2 (18.2)
2	6 (54.5)
3	1 (9.1)

ECOG, Eastern Cooperative Oncology Group; ER, estrogen; FISH, fluorescence *in situ* hybridization; HER2, human epidermal growth factor receptor-2; IHC, immunohistochemistry; PR, progesterone; SD, standard deviation.

Table 3. Dose escalation, DLTs, treatment duration, and reason for discontinuation.

Cohort (dose level[a])/Patient no.	DLT	Therapy duration (days)	Reason for discontinuation
Cohort 1 (25/1250/2.5)			
Patient 1	No	17	AE (non-DLT)[b]
Patient 2	No	50	PD
Patient 3	No	50	PD
Patient 4[c]	No	30	PD
Cohort 2 (25/1500/2.5)			
Patient 5	No	114	PD
Patient 6[d]	Grade 3 rash	114	PD
Patient 7[e]	Grade 3 hyperglycemia	150	AE (DLT-related)
Expansion Cohort 1 (25/1250/2.5)			
Patient 8	No	110	PD
Patient 9	No	225	PD
Patient 10	No	86	PD
Patient 11[f]	No	98	PD

[a]Dose level in mg/day for KW-2450, lapatinib, and letrozole, respectively.
[b]Grade 3 convulsion unrelated to treatment.
[c]Replaced Patient 1.
[d]Dose interrupted for all therapies, lapatinib reduced to 1250 mg/day on rash resolution, and patient remained on therapy with KW-2450, letrozole, and reduced dose lapatinib.
[e]Hyperglycemia controlled by metformin and patient continued therapy with moderate hyperglycemia.
[f]Replaced Patient 10 who experienced grade 3 elevated bilirubin related to acyclovir causing dose interruption.
AE, adverse event; DLT, dose-limiting toxicity; PD, progressive disease.

In addition, there was moderate accumulation of lapatinib in plasma (1.23 to 2.27-fold) on multiple daily dosing. Letrozole showed high accumulation in plasma (4.7 to 6.3-fold) on multiple daily dosing.

There were no meaningful changes in IGF-1, IGF-binding protein-3, or growth hormone with treatment (data not shown). Insulin and glucose levels increased with treatment (peak values at 2 h after meal intake and 4 h after drug intake) and returned to pre-dose levels within 24 h in most patients in both cohorts (data not shown).

Discussion

The combination of KW-2450 and lapatinib showed *in vitro* apoptotic synergism in the double-positive HER2/IGF-1R cell line, MDA-MB-361, overcoming resistance toward lapatinib alone. Investigation of downstream signaling indicated that phosphorylation of Akt, decreased Ki67 and survivin expression, and increased TIMP3 expression might be useful pharmacodynamic biomarkers associated with KW-2450 plus lapatinib combination treatment. High TIMP3 tumor levels have been associated with successful tamoxifen treatment in patients with breast cancer.[26] KW-2450 plus lapatinib showed *in vivo* synergistic antitumor activity in the double-positive HER2/IGF-1R tumor-bearing murine model, which was resistant to lapatinib alone. Furthermore, KW-2450 showed *in vitro* synergism with hormonal agents such as 4-hydroxytamoxifen and letrozole in estrogen- and aromatase-dependent cell lines, respectively. These preclinical results supported the hypotheses concerning the interaction between IGF-1R,

Table 4. Treatment-emergent AEs.

	No. of patients (%)		
	Cohort 1 (25/1250/2.5)[a] [*n* = 8]	**Cohort 2 (25/1500/2.5)[a] [*n* = 3]**	**Total [*N* = 11]**
Any AE	8 (100.0)	3 (100.0)	11 (100.0)
Any AE by preferred term[b]			
Diarrhea	3 (37.5)	3 (100)	6 (54.5)
Dyspnea	3 (37.5)	0	3 (27.3)
Rash	1 (12.5)	2 (66.7)	3 (27.3)
Fatigue	1 (12.5)	1 (33.3)	2 (18.2)
Hyperglycemia	1 (12.5)	1 (33.3)	2 (18.2)
Any serious AE	2 (25.0)	2 (66.7)	4 (36.4)
Any AE leading to discontinuation	1 (12.5)	1 (33.3)	2 (18.2)
Any grade 4/5 AE	0	0	0
Grade 3 AE related to KW-2450 by preferred term[b]			
Hyperglycemia	1 (12.5)	1 (33.3)	2 (18.2)
Liver function test abnormality	1 (12.5)	0	1 (9.1)
Diarrhea	1 (12.5)	0	1 (9.1)
Rash	0	1 (33.3)	1 (9.1)
Grade 3 AE related to lapatinib by preferred term[b]			
Liver function test abnormality	1 (12.5)	0	1 (9.1)
Diarrhea	1 (12.5)	0	1 (9.1)
Diarrhea + dehydration	0	1 (33.3)	1 (9.1)
Rash	0	1 (33.3)	1 (9.1)
Grade 3 AE related to letrozole by preferred term[b]			
Liver function test abnormality	1 (12.5)	0	1 (9.1)
Diarrhea	1 (12.5)	0	1 (9.1)

[a]Dose level in mg/day for KW-2450, lapatinib, and letrozole, respectively.
[b]Occurring in more than one patient overall according to MedRA v.12.1.
AE, adverse event; DLT, dose-limiting toxicity.

HER2, and ER, providing further justification for clinical investigation of the triple-drug combination of KW-2450, lapatinib, and letrozole in postmenopausal patients with ER-positive, HER2-positive advanced or metastatic breast cancer.

In the phase I study, the MTD was established in Cohort 1 with the combination of KW-2450 25 mg/day, lapatinib 1250 mg/day, and letrozole 2.5 mg/day. There were no DLTs among the eight patients in expanded Cohort 1. A total of two of three patients in Cohort 2 experienced DLTs (grade 3 hyperglycemia and grade 3 rash, respectively). Grade 3 hyperglycemia and grade 3 rash have been previously reported as DLTs during a phase I study of single-agent KW-2450 among patients with previously treated advanced solid tumors at doses of 37.5 mg/day (two of six patients) and 50 mg/day (one of seven patients), respectively.[23,24]

The safety signals seen with the combination of KW-2450, lapatinib, and letrozole were as expected. The most common AEs reported in more than one patient were diarrhea ($n = 6$), dyspnea ($n = 3$), rash ($n = 3$), and hyperglycemia and fatigue (each $n = 2$). Overall, two patients discontinued treatment due to AEs but the AEs were considered to be related to the underlying disease process. No patients died and none had grade 4 AEs. Grade 3 treatment-related AEs were: hyperglycemia ($n = 2$), abnormal liver function test ($n = 1$), and diarrhea ($n = 1$) for KW-2450; abnormal liver function test, diarrhea, diarrhea plus dehydration, and rash (each $n = 1$) for lapatinib; and abnormal liver function test and diarrhea (each $n = 1$) for letrozole. These severe AEs associated with lapatinib and letrozole have been reported infrequently with these agents given alone or during combination therapy for HER2-positive metastatic breast cancer.[27,28]

With respect to pharmacokinetics, KW-2450 appeared to show negligible accumulation in plasma after continuous administration of 25 mg/day for 4 weeks. Exposure to lapatinib assessed by maximum plasma concentrations and area under the plasma concentration–time curves increased in a more than dose-proportional manner (1.7 to 2.1-fold increase in exposure with a 1.2-fold increase in dose) comparing 1250 and 1500 mg/day doses. Lapatinib accumulated moderately (1.23 to 2.27-fold) in plasma after continuous administration of 1250 or 1500 mg/day doses for 4 weeks. This accumulation is in line with the previously reported pharmacokinetics of lapatinib.[27] Letrozole showed a high degree of accumulation (4.7 to 6.3-fold) in plasma after continuous administration of 2.5 mg/day for 4 weeks. Some degree of accumulation of letrozole is in line with the previously reported pharmacokinetics of letrozole, which has a long terminal half-life (42 h) and requires several weeks to reach steady-state plasma concentrations,[29] although precise accumulation data do not appear to have been reported in the literature.

With respect to antitumor response, 6 of the 11 patients (54.5%) maintained stable disease as a best response. This compares favorably with the 44% clinical benefit rate reported during first-line treatment with letrozole 2.5 mg/day plus lapatinib 1500 mg/day in the phase III study of postmenopausal hormone receptor-positive HER2-positive metastatic breast cancer.[8] However, stable disease had to be $\geqslant$6 months in the previous study to be included as clinical benefit and the duration of treatment was relatively short in our study, ranging from 5 to 15 weeks.

The original study plan included a phase II portion for the trial after completion of the phase I portion. The randomized phase II study was originally planned to test the additional effect of KW-2450 on the lapatinib/letrozole combination at their approved doses (1500/2.5 mg/day) in the first-line setting. The RP2D in the phase I study for the three-drug combination was established as KW-2450 25 mg/day, lapatinib 1250 mg/day, and letrozole 2.5 mg/day, which rendered this randomization impossible according to the original protocol. Furthermore, the combination of lapatinib and letrozole has not been as widely accepted as a standard of care in the first-line setting,[30] resulting in more difficult enrollment of patients into the proposed phase II study than was previously anticipated. It was therefore decided to terminate the study at completion of phase I. Patients who were already receiving treatment in the phase I study when this decision was made were allowed to continue receiving study medication until disease progression or other discontinuation criteria were met. The clinical development of KW-2450 was consequently terminated.

In conclusion, the *in vitro* and *in vivo* preclinical studies supported the clinical investigation of the triple-drug combination of KW-2450, lapatinib, and letrozole in postmenopausal patients with advanced/metastatic hormone receptor-positive, HER2-positive breast cancer. The RP2D was established in this population as KW-2450 25 mg/day, lapatinib 1250 mg/day, and letrozole 2.5 mg/day. The proposed phase II study of the RP2D for the triple-drug combination according

to the protocol did not progress because of anticipated difficulty in patient enrollment and further clinical development of KW-2450 was terminated.

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Conflict of interest statement

HU, YM, FK, MS, TO, HK, NS, DN, and SA are employees of Kyowa Hakko Kirin Co., Ltd. NTU has a speaker's agreement with Kyowa Kirin Pharmaceutical Development, Inc. TF has no conflicts of interest. All preclinical tests were performed by Kyowa Hakko Kirin Co., Ltd, and funding for the trial was provided by Kyowa Hakko Kirin Co., Ltd. Medical writing assistance was funded by Kyowa Kirin Pharmaceutical Development, Inc.

Supplemental Material

Supplemental material for this article is available online.

References

1. Siegel RL, Miller KD and Jemal A. Cancer statistics. *CA Cancer J Clin* 2016; 66: 7–30.
2. Slamon DJ, Clark GM, Wong SG, *et al*. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235: 177–182.
3. Slamon DJ, Godolphin W, Jones LA, *et al*. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244: 707–712.
4. Ross JS and Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. *Oncologist* 1998; 3: 237–252.
5. Hudis CA. Trastuzumab – mechanism of action and use in clinical practice. *N Engl J Med* 2007; 357: 39–51.
6. Morrow PKH, Zambrana F and Esteve FJ. Advances in systemic therapy for HER2-positive metastatic breast cancer. *Breast Can Res* 2009; 11: 207–216.
7. Osborne CK, Shou J, Massarweh S, *et al*. Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clin Cancer Res* 2005; 11: 865S–870S.
8. Song RX, Chen Y, Zhang Z, *et al*. Estrogen utilization of IGF-1R and EGF-R to signal in breast cancer cells. *J Steroid Biochem Mol Biol* 2010; 118: 219–230.
9. Johnston S, Pippen J, Pivot X, *et al*. Lapatinib combined with letrozole versus letrozole and placebo a first-line therapy for postmenopausal hormone-receptor positive metastatic breast cancer. *J Clin Oncol* 2009; 27: 5538–5546.
10. LeRoith D, Werner H, Beitner-Johnson D, *et al*. Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev* 1995; 16: 143–163.
11. Khandwala HM, McCutcheon IE, Flyvbjerg A, *et al*. The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocr Rev* 2000; 21: 215–244.
12. Rubin R and Baserga R. Insulin-like growth factor-I receptor. Its role in cell proliferation, apoptosis, and tumorigenicity. *Lab Invest* 1995; 73: 311–331.
13. Resnik JL, Reichart D, Huey K, *et al*. Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. *Cancer Res* 1998; 58: 1159–1164.
14. Hankinson SE, Willett WC, Colditz GA, *et al*. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* 1998; 351: 1393–1396.

15. Hasdell DL, Murphy K, Bonntete SG, *et al*. Cooperative interaction between mutant p53 and des(1-3)IGF-I accelerates mammary tumorigenesis. *Oncogene* 2000; 19: 889–898.

16. Lee AV, Jackson JG, Gooch JL, *et al*. Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo. *Mol Endocrinol* 1999; 13: 787–796.

17. Simoncini T, Hafezi-Moghadan AM, Brazil DP, *et al*. Interaction of oestrogen receptor with the regulatory subunit of phophatidylinositol-3-OH kinase. *Nature* 2000; 407: 538–541.

18. Lisztwan J, Pornon A, Chen B, *et al*. The aromatase inhibitor letrozole and inhibitors of insulin-like growth factor I receptor synergistically induce apoptosis in in vitro models of estrogen-dependent breast cancer. *Breast Cancer Res* 2008; 10: R56.

19. Lu YH, Zi XL, Zhao YH, *et al*. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab. *J Natl Cancer Inst* 2001; 93: 1852–1857.

20. Nahta R, Yuan LXH, Zhang B, *et al*. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Res* 2005; 65: 11118–11128.

21. Baselga J, Campone M, Piccart M, *et al*. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N Engl J Med* 2012; 366: 520–529.

22. Maekawa Y, Koizumi F, Kashima N, *et al*. KW-2450, a novel orally-active dual IGF1R/IR inhibitor: Antitumor effects in vitro and in vivo. *Cancer Res* 2011: 71(Suppl. 1): abstract 3605.

23. Schwartz GK, Dickson MA, LoRusso PM, *et al*. Preclinical and first-in-human phase I studies of KW-2450, an oral tyrosine kinase inhibitor with insulin-like growth factor receptor-1/insulin receptor selectivity. *Cancer Sci* 2016; 107: 499–506.

24. Dickson MA, LoRusso P, Sausville EA, *et al*. Open-label, sequential, ascending, multi-dose, phase I study of KW-2450 as monotherapy in subjects with previously treated advanced solid tumors. *J Clin Oncol* 2011; 29(Suppl.): abstract 3078.

25. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006; 58: 621–681.

26. Span PN, Lindberg RL, Manders P, *et al*. Tissue inhibitors of metalloproteinase expression in human breast cancer: TIMP-3 is associated with adjuvant endocrine therapy success. *J Pathol* 2004; 202: 395–402.

27. Oakman C, Pestrin M, Zafarana E, *et al*. Role of lapatinib in the first-line treatment of patients with metastatic breast cancer. *Cancer Manag Res* 2010; 2: 13–25.

28. Schwartzberg LS, Franco SX, Florance A, *et al*. Lapatinib plus letrozole as first-line therapy for HER-2+ hormone receptor-positive metastatic breast cancer. *Oncologist* 2010; 15: 122–129.

29. Bhatnagar AS. The discovery and mechanism of action of letrozole. *Breast Cancer Res Treat* 2007; 105(Suppl. 1): 7–17.

30. National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology. Breast Cancer, version 3.2015. www.nccn.org/professionals/physician_gls/pdf/breast.pdf (published on 16 July 2015) (accessed 24 July 2015).