[CANCER RESEARCH 64, 3958–3965, June 1, 2004]

Antitumor Activity of HKI-272, an Orally Active, Irreversible Inhibitor of the HER-2 Tyrosine Kinase

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ABSTRACT

HER-2 belongs to the ErbB family of receptor tyrosine kinases, which has been implicated in a variety of cancers. Overexpression of HER-2 is seen in 25–30% of breast cancer patients and predicts a poor outcome in patients with primary disease. Trastuzumab (Herceptin), a monoclonal antibody to HER-2, is specifically approved for HER-2-positive breast cancer but is active only in a subset of these tumors. Blocking HER-2 function by a small molecule kinase inhibitor, therefore, represents an attractive alternate strategy to inhibit the growth of HER-2-positive tumors. HKI-272 is a potent inhibitor of HER-2 and is highly active against HER-2-overexpressing human breast cancer cell lines *in vitro*. It also inhibits the epidermal growth factor receptor (EGFR) kinase and the proliferation of EGFR-dependent cells. HKI-272 reduces HER-2 receptor autophosphorylation in cells at doses consistent with inhibition of cell proliferation and functions as an irreversible binding inhibitor, most likely by targeting a cysteine residue in the ATP-binding pocket of the receptor. In agreement with the predicted effects of HER-2 inactivation, HKI-272 treatment of cells results in inhibition of downstream signal transduction events and cell cycle regulatory pathways. This leads to arrest at the G_1-S (Gap 1/DNA synthesis)-phase transition of the cell division cycle, ultimately resulting in decreased cell proliferation. *In vivo*, HKI-272 is active in HER-2- and EGFR-dependent tumor xenograft models when dosed orally on a once daily schedule. On the basis of its favorable preclinical pharmacological profile, HKI-272 has been selected as a candidate for additional development as an antitumor agent in breast and other HER-2-dependent cancers.

INTRODUCTION

The ErbB family of receptor tyrosine kinases consists of four members: epidermal growth factor receptor (EGFR; also called HER-1, ErbB-1); HER-2 (*neu*, ErbB-2); HER-3 (ErbB-3); and HER-4 (ErbB-4) [reviewed in Refs. 1, 2]. All four receptors are tyrosine-protein kinases and consist of an extracellular ligand-binding domain, a single membrane-spanning region, and intracellular tyrosine kinase and regulatory domains. A variety of peptide ligands bind to the receptors (except HER-2) with different affinities and overlapping specificity. Ligand-dependent receptor oligomerization (homo- or heterodimerization) results in autophosphorylation at the regulatory domain and intracellular signal transduction, ultimately leading to increased cell proliferation. No ligand has been identified for HER-2; instead, this protein functions as a coreceptor by binding to other receptors in the family.

Deregulation of growth-factor signaling due to hyperactivation of the ErbB receptors (primarily EGFR and HER-2) is seen in several cancer types (2, 3). Activation of EGFR may be because of overexpression, mutations resulting in constitutive activation, or autocrine expression of ligand. In contrast, activation of HER-2 occurs mainly by overexpression, which leads to spontaneous homodimerization and activation of downstream signaling events in a ligand-independent manner (2–4). The role of HER-2 has been most thoroughly studied in breast cancer, where it is overexpressed in 25–30% of cases and is correlated with a poor prognosis (3–6). Overexpression occurs primarily as a result of gene amplification. HER-2 overexpression is also seen in ovarian cancer (6), lung cancer (especially lung adenocarcinomas; Refs. 7–9), and in hormone-refractory prostate cancer (10, 11).

The only agent currently approved for HER-2-positive breast cancer treatment is trastuzumab (Herceptin), a humanized monoclonal antibody against the extracellular domain of HER-2. Although trastuzumab treatment has rapidly become the standard of care for HER-2-positive breast cancer, it shows only a 15% response as monotherapy and a 49% response in combination with paclitaxel (12, 13). Optimal responses are observed in patients with HER-2 gene amplification (14). Trastuzumab appears to exert its antitumor effect by accelerating the internalization and degradation of the HER-2 receptor (15), antibody-dependent cell-mediated cytotoxicity (15), and antiangiogenic activity (16, 17). The goal of this study was to identify a small molecule HER-2 antagonist that, unlike trastuzumab, directly blocks HER-2 kinase activity and would offer an alternative approach to the treatment of HER-2-positive cancers. The ErbB family has been the target of drug discovery efforts at Wyeth and has resulted in the development of EKB-569, an irreversible-binding inhibitor of EGFR, currently in clinical trials for EGFR-dependent tumors. This compound is predicted to covalently modify a cysteine residue (cysteine-773) within the ATP binding site of the kinase (18). EKB-569 shows poorer efficacy in HER-2-dependent tumor models than in EGFR-dependent models (19). Therefore, a compound that is more potent than EKB-569 in HER-2-expressing tumors will complement the activity of this compound in the clinic. Because HER-2 is highly homologous to EGFR in the catalytic domain, with conservation of the targeted cysteine residue (as cysteine-805; Ref. 20), synthetic efforts were focused on the chemical scaffold of EKB-569 (4-anilinoquinoline-3-carbonitrile), which was modified to improve the inhibitory activity against HER-2. The biological properties of HKI-272, the lead compound from this effort, are described.

MATERIALS AND METHODS

Cell Culture. Cells were maintained in RPMI 1640 (Invitrogen-Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS (Invitrogen) and 50 μg/ml gentamicin (Invitrogen) at 37°C in a humidified incubator, under 5–10% CO_2. A431, BT474, SK-Br-3, MDA-MB-435, and SW480 cells were obtained from the American Type Culture Collection (Manassas, VA); 3T3 and 3T3/*neu* cells were provided by Dr. Mary L. Disis (University of Washington, Seattle, WA).

Purification of HER-2 and EGFR Cytoplasmic Domains. The cytoplasmic domain of HER-2 (amino acids 676-1255) and EGFR (amino acids 645-1186) was cloned into baculovirus expression vectors [EGFR: pCRBac/Bac-N-Blue (Invitrogen); HER-2: pFastBacHTc (Invitrogen)]. Both constructs contain an NH_2-terminal 6×-histidine tag to facilitate purification. Sf9 insect cells (Invitrogen) were cultured in Grace's insect medium (Invitrogen) sup-

Received 9/10/03; revised 2/23/04; accepted 3/25/04.

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plemented with 10% FBS and 50 μg/ml gentamicin. For protein production, recombinant viral stock was used to infect Sf9 cells (multiplicity of infection = 10) for 3 days. Infected cells were lysed in 50 mM HEPES (pH 7.5), 10 mM NaCl, 1% Triton X-100, 10 μM ammonium molybdate, 100 μM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Pefabloc SC, and 16 μg/ml benzamidine HCl (all chemical reagents obtained from Sigma-Aldrich, St. Louis, MO). Lysates were clarified by centrifugation and fractionated by nickel affinity chromatography (Nickel-NTA superflow; Qiagen, Valencia, CA). In brief, lysates were incubated with affinity resin in 50 mM sodium phosphate (pH 8), 300 mM NaCl, and 5 mM imidazole for 25 min at 4°C and packed into a column for washing and elution. Nonspecific proteins were removed by washing the column with buffer containing 100 mM imidazole. Specifically bound proteins were eluted with buffer containing 250 mM imidazole. Purified proteins were dialyzed against 20 mM HEPES (pH 7.5), 50 mM NaCl, 10% glycerol, and 1 μg/ml protease inhibitors and stored at −80°C in single-use aliquots. Purity was determined by SDS-PAGE, followed by staining with Coomassie Blue R250 (Bio-Rad, Hercules, CA). Purity was estimated to be >80%.

Kinase Assays. Activity of HER-2 and EGFR cytoplasmic domains was measured by an autophosphorylation assay using time-resolved fluorometry (DELPHIA; Wallac/Perkin-Elmer Life Sciences, Boston, MA). Compounds were prepared as 10 mg/ml stocks in DMSO and diluted in 25 mM HEPES (pH 7.5; 0.002 ng/ml–20 μg/ml). Enzyme [diluted in 100 mM HEPES (pH 7.5) and 50% glycerol] was incubated with inhibitor in 4 mM HEPES (pH 7.5), 0.4 mM $MnCl_2$, 20 μM sodium vanadate, and 0.2 mM DTT for 15 min at room temperature in 96-well ELISA plates (Maxisorb; Nalge Nunc, Rochester, NY). The kinase reaction was initiated by the addition of 40 μM ATP and 20 mM $MgCl_2$ and allowed to proceed for 1 h at room temperature. Plates were washed, and phosphorylation was detected using Europium-labeled anti-phospho-tyrosine antibodies (15 ng/well; Wallac). After washing and enhancement steps according to the manufacturer's recommendations, signal was detected using a Victor^2 fluorescence reader (Wallac; excitation wavelength 340 nm, emission wavelength 615 nm). The concentration of compound that inhibited receptor phosphorylation by 50% (IC_{50}) was calculated from inhibition curves.

Assays for other kinases were performed using recombinant enzymes expressed in bacterial, insect, or human cell lines. All enzymes used were serine-threonine kinases, except c-Met, KDR, src (tyrosine kinases), and MEK1 (dual specificity). Substrates used were peptides (Akt, IKK-2, MK2, PDK1, src, and Tpl2), proteins (cyclin D1/CDK4, cyclin E/CDK2, cyclin B1/CDK1, and c-Raf), poly(glutamic $acid_4$-tyrosine) (KDR), or the kinase itself (autophosphorylation; c-met). Phosphorylation was measured using TMB peroxidase substrate (Pierce, Rockford, IL) for cyclin/cyclin-dependent kinase (cdk), LabChip (Caliper Technologies, Mountain View, CA) for MK-2, or DELPHIA/LANCE (Wallac) for all others.

Cell Proliferation Assays. Cells were plated in 96-well tissue culture plates (3T3, 3T3/*neu*, 5000 cells/well; A431, SK-Br-3, BT474, MDA-MB-435, and SW620, 10,000 cells/well). The following day, dilutions of compound (0.5 ng/ml–5 μg/ml) were added, and cells were cultured for 2 days (6 days for BT474). Cell proliferation was determined using sulforhodamine B, a protein binding dye. Briefly, cells were fixed with 10% trichloroacetic acid and washed extensively with water. Cells were then stained with 0.1% sulforhodamine B (Sigma-Aldrich) and washed in 5% acetic acid. Protein-associated dye was solubilized in 10 mM Tris, and absorbance was measured at 450 nM (Victor^2). Inhibition of cell proliferation was calculated using the formula: percentage of inhibition = $100 - 100\ (T_d - T_o/T_c - T_o)$, where T_d is the absorbance of drug treated cells, T_c is the absorbance of untreated cells, and T_o is the absorbance at the time of drug addition. T_o values were determined by plating cells separately and fixing them at the time of drug addition. The concentration of compound which inhibits cell proliferation by 50% (IC_{50}) was determined from inhibition curves.

Protein Immunoblotting. For analysis of receptor phosphorylation, cells (BT474 or A431 cells) were incubated with various concentrations of HKI-272 for 3 h. For A431 cells, EGF (100 ng/ml; R&D Systems, Minneapolis, MN) was added to the cells during the last 15 min. In experiments designed to evaluate reversibility of inhibition, compound-containing medium was removed after 3 h and replaced with fresh medium without compound. Incubation was then continued for 5 h, with two additional changes of medium during the incubation. For analysis of signal transduction and cell cycle regulatory pathways, cells were incubated with HKI-272 overnight. After treatment, cells were washed twice in PBS (Invitrogen) and lysed in 50 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 0.2 mM sodium vanadate. Alternatively, cells were lysed directly in NuPAGE sample buffer (Invitrogen). Lysates were clarified by centrifugation, and protein concentration was estimated using the Bio-Rad or Bio-Rad D_C protein assay. Proteins were separated by SDS-PAGE (Ready Gels; Bio-Rad or Novex; Invitrogen) or by NuPAGE (Invitrogen) and transferred to nitrocellulose (Bio-Rad). Nonspecific sites were blocked, and blots were incubated with antibody solutions. After washing and incubation with secondary antibodies, blots were developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The following antibodies/conjugates were used: phospho-tyrosine-horseradish peroxidase conjugate (BD Biosciences PharMingen, San Diego, CA), EGFR, HER-2, retinoblastoma gene product (Rb), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-mitogen-activated protein kinase (MAPK) and MAPK (Biosource, Camarillo, CA), phospho-Akt and Akt (Cell Signaling Technologies, Beverly, MA). Blots were scanned and quantified using FluorS Multi-Image analyzer (Bio-Rad).

Binding Studies with [^{14}C]HKI-272. Recombinant HER-2 cytoplasmic domain (100 ng) was incubated with 1 μM [^{14}C]HKI-272 (specific activity 55 mCi/mmol; Amersham) in the absence or presence of 5 μM unlabeled HKI-272 for 15 min on ice. Samples were boiled in SDS sample buffer, and proteins were separated by SDS-PAGE. Gels were treated with autoradiographic enhancer (En^{3}Hance; NEN, Boston, MA), dried, and exposed to film. For labeling cells, 5×10^6 BT474 cells were treated with 1 μM [^{14}C]HKI-272 for 2 h at 37°C or pretreated with unlabeled HKI-272 (5 μM) for 1 h at 37°C before addition of label. Cells were washed with PBS, boiled in SDS sample buffer, and analyzed by SDS-PAGE and fluorography. A separate sample of identically labeled cells was lysed in 10 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM sodium vanadate, and 100 mM sodium fluoride and immunoprecipitated with 4 μg of anti-HER-2 antibodies (Santa Cruz Biotechnology) for 1 h at 4°C. Immune complexes were collected on protein A-agarose beads (Invitrogen), boiled in SDS sample buffer, separated by SDS-PAGE, and detected by fluorography.

Cell Cycle Analysis. BT474 cells were incubated with HKI-272 (0–2 nM) in 6-well clusters overnight. Immediately before collection, cells were pulse labeled for 30 min with 10 μM bromodeoxyuridine (Sigma-Aldrich). Cells were collected by trypsinization, washed, and fixed in 80% ethanol for 15 min at −20°C. After acid denaturation, permeabilization, and neutralization, cells were stained with anti- bromodeoxyuridine-FITC conjugates (BD Biosciences, San Jose, CA), counterstained with propidium iodide (Sigma-Aldrich), and analyzed by flow cytometry (FACSort; BD Biosciences). Data were analyzed using CELLQuest software (BD Biosciences).

Tumor Xenograft Studies. Tumor cells (maintained in tissue culture) or tumor fragments were implanted s.c. in the flanks of female athymic (nude) mice (Charles River Laboratories, Wilmington, MA). For estrogen-dependent cell lines (BT474, MCF-7, and SK-OV-3), animals were implanted with hormone pellets (0.72 mg of 17-β estradiol, 60-day release; Innovative Research of America, Sarasota, FL) 1 week before implantation of tumors. Additionally, SK-OV-3 cells were suspended in Matrigel basement membrane matrix (BD Biosciences, Billerica, MA) for implantation. Treatment was initiated after tumors had reached a size of 90–200 mg, following random assignment of the animals to different treatment groups (staging, day 0). For 3T3/*neu* xenografts, treatment was initiated the day after tumor implantation (day 0). HKI-272 was formulated in 0.5% methocellulose-0.4% polysorbate-80 (Tween 80) and administered daily, p.o., by gavage. Tumor mass [(length × width^2)/2] was determined every 7 days. Tumor outgrowth in all xenograft studies, except 3T3/*neu*, was expressed as relative tumor growth: the ratio of the mean tumor mass to the mean tumor mass on day 0. Inhibition of tumor growth was calculated relative to vehicle-treated controls. Statistical significance of inhibition was demonstrated using one-tailed Student's *t* test (equal variance) after log transformation of the data.

HER-2 Phosphorylation in Xenografts. Athymic female nude mice (5 animals/group) were implanted s.c. with BT474 tumor fragments (~30 mm^3). When tumors reached 200–300 mg, animals were given a single oral dose (40 mg/kg) of HKI-272 in pH 2.0 water. Tumors from control and treated animals were excised at 1, 3, 6, and 24 h and minced. Tumor fragments were suspended

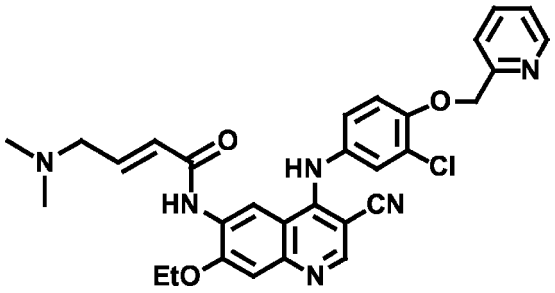

Fig. 1. Structure of HKI-272 (M_r 557.05).

Table 1 *Inhibition of ERBB kinases by HKI-272*[a]

Enzyme	IC_{50} (nM)[b]
HER-2	59 ± 13 (n = 5)
Epidermal growth factor receptor	92 ± 17 (n = 5)

[a] Purified recombinant COOH-terminal fragments of HER-2 and epidermal growth factor receptor were incubated with ATP in the absence or presence of a range of compound concentrations. Autophosphorylation of the receptors was determined using phospho-tyrosine antibodies. The concentration of compound that inhibits phosphorylation by 50% is shown.

[b] Mean and SE with the number of independent determinations in parentheses.

in 10 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM sodium vanadate, and 100 mM sodium fluoride and lysed by homogenization on ice with a polytron. After clarification by centrifugation, protein concentration in lysates was estimated using the Bio-Rad D_C protein assay. Sixty μg of lysate pooled from each group were analyzed by SDS-PAGE and immunoblotting with phospho-tyrosine-specific antibodies. Pooled extracts were also immunoprecipitated using 4 μg of anti-HER-2 antibodies for 1 h at 4°C. Immune complexes were collected on protein A-agarose, washed, and analyzed by immunoblotting using phospho-tyrosine-specific antibodies. Extracts from individual tumors were analyzed to determine variability between animals.

RESULTS

Kinase Assays. In a cell-free autophosphorylation assay using the recombinant cytoplasmic domain of HER-2, HKI-272 reduced kinase activity by 50% (IC_{50}) at a concentration of 59 nM (Fig. 1 and Table 1). It also inhibited the kinase activity of the EGFR cytoplasmic domain under similar assay conditions at 92 nM. HKI-272 did not significantly inhibit several serine-threonine kinases tested (Akt, cyclin D1/cdk4, cyclin E/cdk2, cyclin B1/cdk1, IKK-2, MK-2, PDK1, c-Raf, and Tpl-2) or the tyrosine kinase, c-met (Table 2). It weakly inhibited two other tyrosine kinases tested, KDR and src (IC_{50} 800 and 1400 nM, respectively; Table 2), but was 14- and 24-fold less active against these kinases, compared with HER-2. HKI-272 is, therefore, a highly selective inhibitor of HER-2 and EGFR.

Cell Proliferation Assays. The activity of HKI-272 was evaluated in a panel of cell lines with varying levels of expression of EGFR and HER-2 (Table 3). HKI-272 repressed the proliferation of a mouse fibroblast cell line (3T3) transfected with the HER-2 oncogene (3T3/*neu*) by 50% (IC_{50}) at 3 nM. This value was 230-fold lower than that obtained with the isogenic untransfected cells (IC_{50} 700 nM), demonstrating that HKI-272 has a high degree of selectivity for this oncogenic pathway. HKI-272 also inhibited two other HER-2-overexpressing breast cancer cell lines, SK-Br-3 and BT474 (IC_{50} 2 nM), but was much less active on MDA-MB-435 and SW620 (a breast and a colon cancer cell line, respectively) that are EGFR- and HER-2-negative. Consistent with its activity against EGFR, HKI-272 inhibited proliferation of the epidermal carcinoma cell line, A431, that overexpresses EGFR (IC_{50} 81 nM).

Receptor Phosphorylation. The ability of HKI-272 to inhibit the function of the target receptor was determined by measuring receptor autophosphorylation in intact cells exposed to the compound (Fig. 2 and Table 4). HKI-272 decreased ligand-independent receptor phosphorylation by 50% (IC_{50}) at 5 nM in BT474 cells, consistent with its effects on cell proliferation. It also repressed EGF-dependent phosphorylation of EGFR in A431 cells at a comparable dose (IC_{50} 3 nM), which is lower than the IC_{50} value in the cell proliferation assay (81 nM; compare Table 4 with Table 3).

Irreversible Binding to HER-2. EKB-569 has been previously shown to form a covalent adduct with EGFR (19). This is most likely because of the interaction between the Michael acceptor functional group of EKB-569 with cysteine-773 within the ATP binding site of EGFR. On the basis of the presence of the same Michael acceptor

Table 2 *Inhibition of kinases by HKI-272*

Kinase	IC_{50} (nM)[a]
Akt	>20,000
Cyclin D1/CDK4	>50,000
Cyclin E/CDK2	>18,000
CyclinB1/CDK1	>18,000
IKK-2	>9,000
KDR	800
c-Met	>35,000
MK2	>45,000
PDK1	>5000
c-Raf/MEK1	>18,000
src	1,400
Tpl-2	>18,000

[a] The concentration of HKI-272 that inhibits activity by 50% is shown.

Table 3 *Effect of HKI-272 on cell proliferation*[a]

Cell line	HER-2	Epidermal growth factor receptor	IC_{50} (nM)[b]
3T3	−	−	700 ± 78 (n = 5)
3T3/neu	+++	−	3 ± 0.14 (n = 5)
SK-Br-3	+++	+	2 ± 0.18 (n = 4)
BT 474	+++	+	2 ± 0.06 (n = 4)
A431	+	+++	81 ± 9 (n = 5)
MDA-MB-435	−	−	960 ± 165 (n = 2)
SW620	−	−	690 ± 84 (n = 5)

[a] Cells were incubated with various concentrations of compound for 2 days (6 days for BT474). Cell survival was determined using a protein binding dye assay. The concentration of drug that inhibits cell proliferation by 50% is shown. Relative expression of and epidermal growth factor receptor and HER-2 in the cell lines are indicated.

[b] Mean and SE are shown with the number of independent determinations in parentheses.

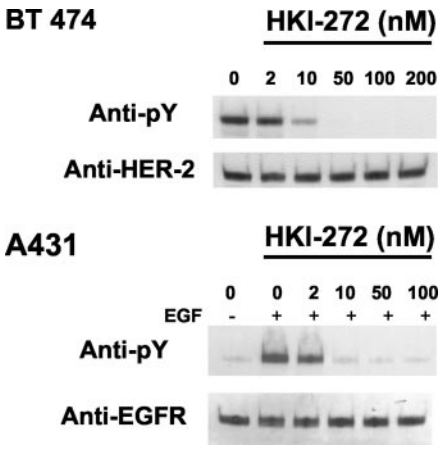

Fig. 2. Effect of HKI-272 on receptor phosphorylation. BT474 and A431 cells were incubated with the indicated concentrations of HKI-272 for 3 h at 37°C. For A431 cells, epidermal growth factor (EGF; 100 ng/ml) was added to the cultures during the last 15 min. Protein extracts were analyzed by SDS-PAGE and immunoblotting using phospho-tyrosine antibodies. Identical parallel blots were incubated with antibodies to HER-2 and EGF receptor (EGFR) to demonstrate equal protein loading.

Table 4 *Effect of HKI-272 on HER-2-signaling pathways and cell cycle regulation*[a]

Signaling marker	IC_{50} (nM)
Phospho-HER-2	5
Phospho-EGFR[b]	3
Phospho-MAPK	2
Phospho-Akt	2
Cyclin D1	9
% cells in S phase	2

[a] In HER-2 and EGFR phosphorylation assays, cells (BT474 and A431, respectively) were incubated with various concentrations of HKI-272 for 3 h at 37°C. For evaluation of downstream signaling markers, BT474 cells were incubated with HKI-272 for 12–16 h at 37°C. Protein extracts were analyzed by immunoblotting using phospho-tyrosine, phospho-MAPK, phospho-Akt, or cyclin D1 antibodies. Antibodies to HER-2, EGFR, MAPK, and Akt were used to correct for protein loading. Actin antibodies were used to normalize loading for cyclin D1. Blots were quantified by densitometric scanning. Concentration of HKI-272 (nM), which inhibits phosphorylation (protein levels for cyclin D1) by 50%, is given. For cell cycle analysis, BT474 cells were treated with HKI-272 for 12–16 h. Untreated and treated cells were pulse labeled for 30 min with bromodeoxyuridine to label cells in S phase. Uptake of label was quantified using bromodeoxyuridine antibodies/FITC conjugates by flow cytometry. The concentration of HKI-272, which decreased the number of cells in S phase by 50%, is given.

[b] EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase.

group in HKI-272 and conservation of a cysteine residue at an analogous position in HER-2 (cysteine-805; Ref. 20), it is expected that HKI-272 will irreversibly inhibit HER-2. To examine this, receptor phosphorylation in cells was measured immediately after or 5 h after removal of HKI-272 from the medium (Fig. 3*A*). If binding is irreversible, the inhibitor will remain bound to the kinase and will continue to block phosphorylation of the receptor after withdrawal of drug. In BT474 cells, HKI-272 (1 μM) inhibited HER-2 receptor phosphorylation by 98% compared with untreated controls, and no increase in phosphorylation was observed 5 h after removal of compound from the medium (97% inhibition). Similar results were seen in A431 cells, where HKI-272 inhibited receptor phosphorylation by >99%, and no recovery of phosphorylation was detected after withdrawal of the compound (105% inhibition). In contrast, a related EGFR inhibitor that lacks the Michael acceptor group (control) showed almost complete reversibility of inhibition (63 to 28% in BT474; 95 to 20% in A431 cells) within the same time period.

To directly demonstrate covalent interaction between HKI-272 and HER-2, [^{14}C]HKI-272 was incubated with the purified cytoplasmic domain of HER-2. After complete denaturation, the sample was analyzed by SDS-PAGE and fluorography (Fig. 3*B*). A single-labeled species, M_r ~95,000, corresponding to the HER-2 cytoplasmic domain was observed, which was decreased in the presence of a 5-fold excess of unlabeled compound. The reduction in signal occurred whether the labeled and unlabeled compounds were added together or protein was preincubated with unlabeled compound before addition of radiolabeled drug. In similar experiments with BT474 cells incubated with [^{14}C]HKI-272, a prominent band, M_r ~185,000, was labeled, and the extent of labeling was decreased when cells were treated with 5-fold excess of unlabeled compound before exposure to the label (Fig. 3*B*). Immunoprecipitation of the lysate with HER-2 antibodies produced a single species, M_r ~185,000, consistent with the expected size of HER-2 (Fig. 3*B*).

HER-2-Mediated Signal Transduction. The phosphorylation of HER-2 leads to activation of the MAPK pathway and the Akt signal transduction pathways. HKI-272 effectively repressed phosphorylation of MAPK and Akt in BT474 cells at concentrations consistent with the inhibition of cell proliferation (IC_{50} 2 nM; Fig. 4*A* and Table 4). In contrast, although trastuzumab inhibited the growth of BT474 cells *in vitro* (IC_{50} 0.4 μg/ml; unpublished observations), it failed to completely inhibit HER-2 receptor phosphorylation or downstream signaling events at concentrations up to 30 μg/ml (Fig. 4*B*).

Cell Cycle Progression. Activation of growth factor receptor signaling pathways ultimately result in cell proliferation because of increased progression through the G_1-S-phase boundary of the cell cycle. The effect of HKI-272 on cell cycle regulatory proteins (Fig. 5) and cell cycle phase transitions (Tables 4 and 5) was examined. HKI-272 repressed cyclin D1 expression by 50% at 9 nM in BT474 cells (Table 4). HKI-272 also reduced the phosphorylation of the Rb-susceptibility gene product at a similar dose, as indicated by a shift from the slower migrating phosphorylated forms to the faster-migrating unphosphorylated forms in SDS-PAGE (Fig. 5). The total amount of Rb was also reduced by HKI-272 treatment. A concomitant in-

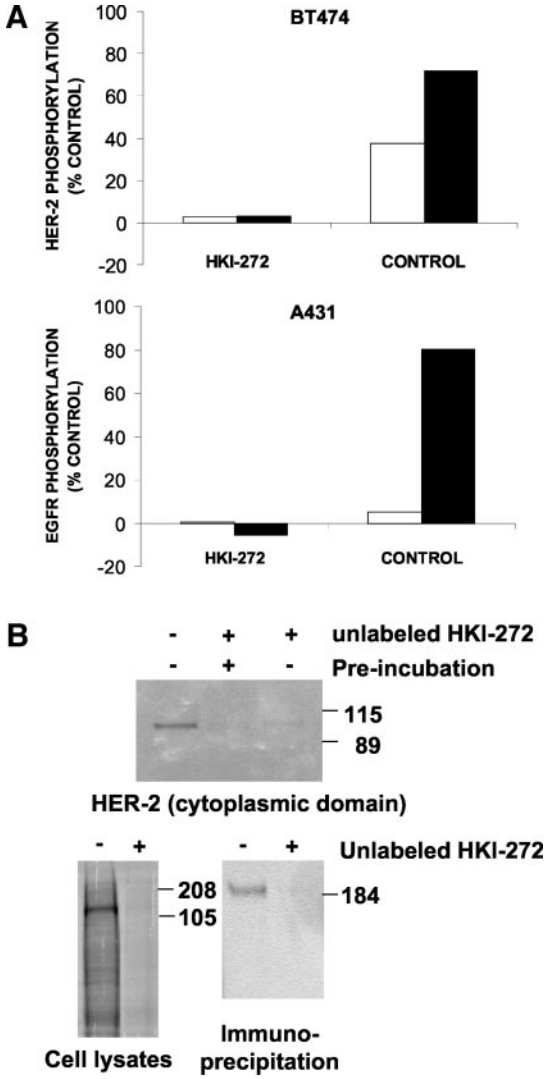

Fig. 3. Irreversible binding of HKI-272. *A*, irreversibility of phosphorylation inhibition. BT474 and A431 cells were incubated with 1 μM of the indicated compound for 3 h, and protein extracts were prepared. In separate parallel cultures, treated cells were washed and incubated in the absence of compound for 5 h. Protein extracts were analyzed by SDS-PAGE and immunoblotting using anti-phospho-tyrosine antibodies. Blots were quantified by densitometric scanning. Results were normalized for protein loading using anti HER-2 (for BT474) and anti-epidermal growth factor receptor (EGFR; for A431) antibodies. The control compound lacks the Michael acceptor functional group and is a reversible binding inhibitor. □, phosphorylation of receptor at the end of incubation period with compound; ■, phosphorylation levels 5 h after withdrawal of compound. *B*, binding of [^{14}C]HKI-272 to HER-2. *Top*, [^{14}C]HKI-272 was incubated with HER-2 (cytoplasmic domain) in the absence (−) or presence (+) of excess unlabeled compound. Compounds were either added together, or labeled HKI-272 was added after a 10-min preincubation with unlabeled compound, as indicated. Samples were denatured by boiling in SDS sample buffer and analyzed by SDS-PAGE and fluorography. *Bottom left*, [^{14}C]HKI-272 was incubated with BT474 cells in the absence (−) or presence (+) of excess unlabeled compound. Samples were denatured in SDS sample buffer and directly analyzed by SDS-PAGE and fluorography. *Bottom right*, a second sample of labeled cells was lysed and immunoprecipitated with HER-2 antibodies. The position of molecular weight markers (M_r, in thousands) are shown on the right.

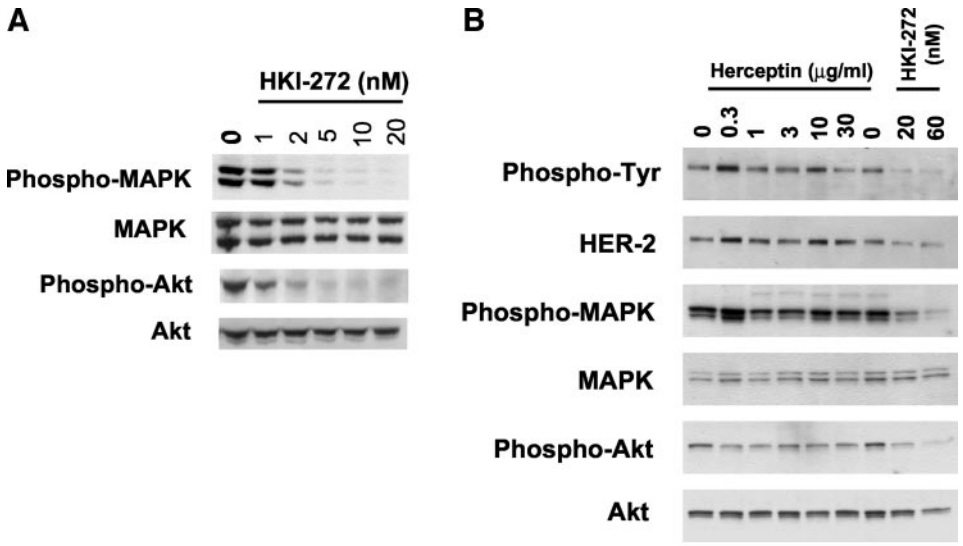

Fig. 4. Effect of HKI-272 and trastuzumab on signal transduction. BT474 cells were incubated with HKI-272 (*A*) or Herceptin (MedWorld Pharmacy, Valley Cottage, NY; *B*) for 12–16 h at 37°C. Protein extracts were analyzed by immunoblotting using phospho-tyrosine, phospho-mitogen-activated protein kinase (MAPK), or phospho-Akt antibodies. Antibodies to HER-2, MAPK, and Akt were used to demonstrate equal protein loading.

crease in p27, an inhibitor of cell cycle progression, was observed. Consistent with the effects on cyclin D1, p27, and Rb phosphorylation, HKI-272 blocked cell cycle progression, causing a G_1-S arrest (Tables 4 and 5). A 50% decrease in the number of cells in the S (DNA synthesis) phase of the cell cycle was observed at a concentration of 2 nM (Table 4), consistent with the effects on cell proliferation. This correlated with an increase in the number of cells in G_1 (Table 5). An increase in the number of cells with sub-G_1 DNA content, indicative of apoptosis, was observed at concentrations > 2 nM (data not shown). No changes were observed at the G_2-M (Gap2/mitosis) transition (Table 5).

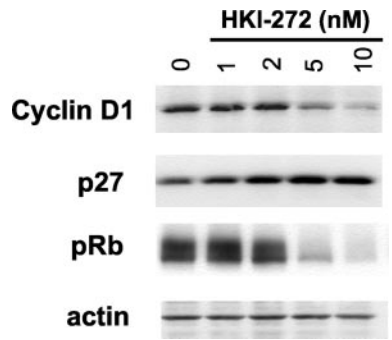

Fig. 5. Effect of HKI-272 on cell cycle regulatory proteins. BT474 cells were incubated with HKI-272 for 12–16 h at 37°C. Protein extracts were analyzed by immunoblotting using cyclin D1, p27, or retinoblastoma (Rb)-specific antibodies. Actin antibodies were used as control.

Table 5 *Effect of HKI-272 on cell cycle progression*[a]

HKI-272 (nM)	Sub-G_1	G_0-G_1	S	S (BrdUrd)*	G_2-M
0	8	61	21	26	11
0.2	9	63	16	18	13
0.4	11	65	12	14	13
1	7	69	10	12	14
2	11	66	12	10	11

[a] BT474 cells were treated with the indicated concentrations of HKI-272 for 12–16 h at 37°C. Untreated and treated cells were pulse labeled for 30 min with bromodeoxyuridine (BrdUrd). Cells were stained with BrdUrd antibodies/FITC conjugates, counterstained with propidium iodide, and analyzed by flow cytometry. The percentage of cells in sub-G1, G_1, S, and G_2 phases of the cell cycle are shown, as determined from the histogram of propidium iodide-stained cells. Additionally, the percentage of cells in S phase was confirmed by BrdUrd uptake (*). This data was use to calculate the IC_{50} shown in Table 4.

Activity in Tumor Xenografts. The *in vivo* activity of HKI-272 was studied in cells growing as xenografts in athymic (nude) mice. The activity of HKI-272 was first determined in xenografts of 3T3/*neu* cells. Treatment was initiated the day after tumor implantation in this model because of the rapid outgrowth of the tumors. HKI-272 reduced tumor growth in a dose-dependent manner when administered orally between 20 mg/kg/day (53% inhibition, day 21) and 80 mg/kg/day (98% inhibition; Fig. 6*A*). In a second independent test, HKI-272 showed antitumor effects between 10 mg/kg/day (34% inhibition, day 14) and 40 mg/kg/day (98% inhibition).

The effect of HKI-272 was next studied in xenografts of BT474 (Fig. 6*B*). HKI-272 treatment repressed tumor growth when administered to animals between 10 mg/kg/day (67% inhibition, day 28) and 40 mg/kg/day (93% inhibition). In three other independent experiments, antitumor effects were observed between 5 mg/kg/day (70–82% inhibition, day 21) and 40 mg/kg/day (88–90% inhibition). To determine whether inhibition of tumor growth was correlated with inhibition of the target receptor, HER-2 phosphorylation was studied after administering a single oral dose (40 mg/kg) of HKI-272 to mice bearing BT474 xenografts (Fig. 7). Phosphorylation of HER-2 was inhibited by 84% within 1 h of administration. Inhibition was sustained at 6 h (97%) and decreased to 43% over 24 h.

HKI-272 was also effective against xenografts of SK-OV-3, a HER-2-overexpressing human ovarian carcinoma cell line (Fig. 6*C*). HKI-272 repressed tumor growth in a dose-dependent manner, with maximum inhibition observed on day 21. Here, statistically significant inhibition of tumor growth was observed at doses between 5 mg/kg/day (31% inhibition) and 60 mg/kg/day (85% inhibition). Similar results were observed in two other independent experiments.

Because HKI-272 inhibits EGFR kinase and the growth of EGFR-overexpressing cells *in vitro*, the effect of the compound in A431 xenografts was evaluated (Fig. 6*D*). Maximum inhibition of tumor growth was observed at the 40 mg/kg/day dose (76% inhibition on day 15). Inhibition was also observed at lower doses from 5 mg/kg/day (32%) to 20 mg/kg/day (44%). However, repression of growth in this tumor model was less than that seen with comparable doses in HER-2-dependent tumors. Therefore, HKI-272 is less potent against EGFR-dependent tumors than HER-2-dependent tumors *in vivo*, although it has equivalent activity against the two kinases *in vitro*.

To determine the specificity of the antitumor effects of HKI-272 for cells expressing HER-2 or EGFR, the effect of the compound was

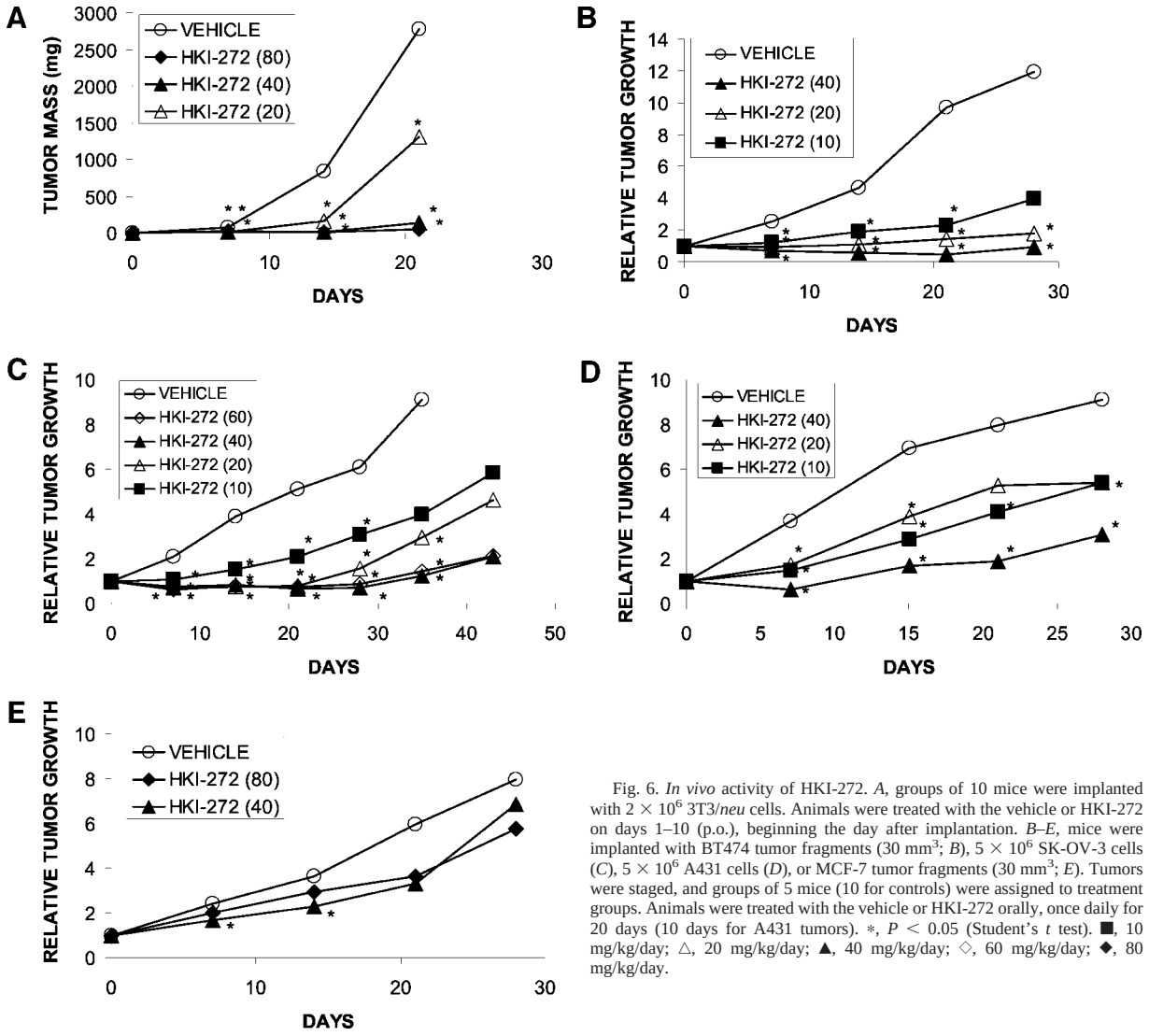

Fig. 6. *In vivo* activity of HKI-272. *A*, groups of 10 mice were implanted with 2×10^6 3T3/*neu* cells. Animals were treated with the vehicle or HKI-272 on days 1–10 (p.o.), beginning the day after implantation. *B–E*, mice were implanted with BT474 tumor fragments (30 mm^3; *B*), 5×10^6 SK-OV-3 cells (*C*), 5×10^6 A431 cells (*D*), or MCF-7 tumor fragments (30 mm^3; *E*). Tumors were staged, and groups of 5 mice (10 for controls) were assigned to treatment groups. Animals were treated with the vehicle or HKI-272 orally, once daily for 20 days (10 days for A431 tumors). ∗, $P < 0.05$ (Student's *t* test). ■, 10 mg/kg/day; △, 20 mg/kg/day; ▲, 40 mg/kg/day; ◇, 60 mg/kg/day; ◆, 80 mg/kg/day.

studied in MCF-7 xenografts (Fig. 6*E*). This human breast cancer cell line expresses low levels of both receptors and is resistant to HKI-272 *in vitro* (IC_{50} 5 μM). Treatment of animals with up to 80 mg/kg/day had only a marginal effect on tumor growth (28% inhibition, day 28), and no dose response was observed. HKI-272 was also inactive in xenografts of MX-1, a second human breast cancer cell line that expresses low levels of HER-2 and EGFR (data not shown).

DISCUSSION

HKI-272 is a potent inhibitor of the HER-2 and EGFR kinases *in vitro*. It also blocks activity of HER-4 (data not shown). In contrast, the structurally related molecule, EKB-569, is a potent inhibitor of EGFR (IC_{50} 39 nM in an autophosphorylation assay), whereas it is substantially less active toward HER-2 (IC_{50} 1255 nM; Ref. 19). HKI-272 was designed using a homology model for the catalytic domain of HER-2 kinase that was built using a combination of two closely related crystal structures as templates, fibroblast growth factor receptor-1 kinase for the NH_2-terminal lobe and hematopoietic cell kinase for the COOH-terminal lobe (18, 21). The core structure of HKI-272 is similar to the structure of EKB-569 but possesses a different anilino headpiece (compare Fig. 1 with Ref. 19). The binding

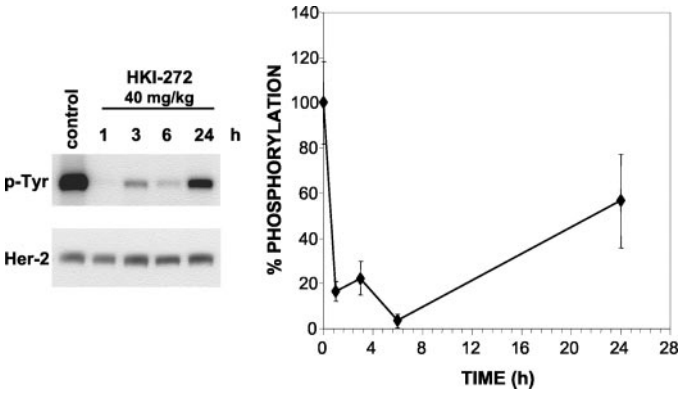

Fig. 7. Inhibition of HER-2 phosphorylation in xenografts. BT474 tumor-bearing mice (5 animals/group) were treated with vehicle or a single dose of HKI-272. Tumors from control and treated mice were dissected at various times after compound administration. Protein extracts from each time point were pooled and analyzed by SDS-PAGE and immunoblotting. *Left*, protein immunoblots. Phosphorylation of HER-2 was detected using phospho-tyrosine antibodies. HER-2-specific antibodies were used as controls for protein loading. *Right*, graphical representation of the immunoblot data. Blots were quantified by scanning densitometry. Mean and SDs are shown. SDs were obtained by quantifying phosphorylation of HER-2 in the individual tumors. Identical results were obtained when lysates were immunoprecipitated with anti-HER-2 antibodies.

model for HKI-272 at the ATP site in HER-2 indicates that the aniline portion of the molecule fits into a long lipophilic pocket. Therefore, a lipophilic 2-pyridinylmethyl moiety was placed at the *para*-position of the aniline and a lipophilic chlorine atom at the *meta*-position. The nature and placement of these groups most likely gives this compound its improved HER-2 activity compared with EKB-569.

HKI-272 interacts directly with its target enzyme and forms a covalent complex. This was suggested by the sustained inhibition of phosphorylation after withdrawal of drug from the medium and confirmed by direct labeling of HER-2 by [^{14}C]HKI-272. This feature is expected to allow the compound to compete effectively with the high concentrations of cellular ATP and inhibit kinase activity for the extended periods of time needed to affect tumor growth. HKI-272 has a Michael acceptor functional group at the 6-position (Fig. 1). Such groups are known to be reactive toward sulfhydryl groups. On the basis of the binding model, cysteine-805, located within the catalytic cleft of HER-2, is ideally positioned for covalent interaction with HKI-272 docked in the binding site (18). The binding model, as well as reactivity studies, suggest that the dimethylamino group present at the end of the Michael acceptor group can serve as an intramolecular catalyst for the addition of HKI-272 to the protein, which may accelerate the reaction between the bound drug and the protein. This entropic effect probably gives HKI-272 its ability to selectively react with the target enzyme (18). Using peptide mapping and mass spectroscopic analyses, PD 168393, a structurally related irreversible-binding ErbB inhibitor, has been shown to covalently bind to cysteine-773 of EGFR (22).

HKI-272 inhibits the proliferation of cell lines that show high levels of HER-2 (3T3/*neu*, SK-Br-3, and BT474) and is much less active in cell lines that express neither HER-2 nor EGFR (3T3, MDA-MB-435, and SW620). However, not all cells which show high levels of HER-2 are sensitive to this drug. MDA-MB-361 expresses ~40% of the receptors expressed by SK-Br-3 and BT474 cells (Refs. 23, 24 and unpublished observations). These cells are ~85-fold less sensitive to inhibition by HKI-272 relative to sensitive lines (190 nM IC_{50}, compared with 2 nM in BT474). Although the mechanism underlying this lack of sensitivity is unknown, it could be because of coexpression of other ErbB receptors or their ligands, which can modulate the sensitivity of cells to kinase inhibitors (25) or the expression of insulin-like growth factor I receptor, which has been reported to change the sensitivity of cells to trastuzumab (26). HKI-272 inhibits ligand-independent HER-2 phosphorylation in BT474 cells at a dose similar to that required to inhibit cell proliferation. However, EGF-dependent EGFR phosphorylation was inhibited at a lower dose (IC_{50} 3 nM), compared with the effects on cell proliferation (IC_{50} 81 nM). This may be because of a requirement for more complete inhibition of EGFR phosphorylation in A431 cells to affect cell proliferation or lateral signaling from other members of the EGFR family or their ligands, as discussed above.

HKI-272 treatment of BT474 cells results in the inhibition of MAPK and Akt phosphorylation, down-regulation of cyclin D1 levels, and induction of p27. The ras-Raf-MAPK pathway and the phosphotidylinositol-3 kinase/Akt pathway are the two major downstream signaling pathways initiated as a consequence of HER-2 receptor activation (27, 28). The two pathways collaborate in regulating the cell cycle. Specifically, they are involved in transcriptional induction and stabilization of a key cell cycle regulator, cyclin D1 (29). Recruitment and activation of CDK4 by cyclin D1 results in phosphorylation and inactivation of Rb, a negative regulator of the cell cycle (30). This process is antagonized by $p27^{kip1}$, a cyclin-dependent kinase inhibitor (31) the transcription and stability of which are negatively regulated by both pathways (32). Ultimately, modulation of these critical regulatory circuits by HKI-272 results in a cell cycle arrest. The inhibition by HKI-272 of the phosphotidylinositol-3 kinase/Akt pathway, which also controls cell survival (33), may be responsible for the apoptosis observed at higher doses of the compound. HKI-272 treatment of BT474 cells completely inhibits phosphorylation of HER-2, MAPK, and Akt, whereas trastuzumab treatment causes only a weak, partial inhibition over a wide range of concentrations (0–30 μg/ml). This is most likely caused by the different mechanisms of action of the two agents. Trastuzumab-mediated inhibition of phosphorylation and signal transduction are most likely caused by the internalization of the HER-2 receptor, followed by degradation (15). The duration and extent of down-modulation depend on the balance between removal of the cell surface receptor due to antibody-mediated internalization and the synthesis of new receptor. In contrast, the effects of HKI-272 are caused by the immediate inhibitory effect on the kinase activity of the receptor. Furthermore, because antibody-mediated receptor internalization is influenced by the density of antigens at the cell surface, HER-2 kinase inhibitors may be more effective than trastuzumab at inhibiting tumors with lower levels of HER-2.

HKI-272 inhibits the growth of HER-2-dependent tumors *in vivo*. The minimum dose, which causes a statistically significant inhibition of tumor growth, is estimated to be 5–10 mg/kg/day. In these xenograft studies, HKI-272 was well tolerated by the animals, and no weight loss or other compound-related toxicity was observed. In agreement with the *in vivo* antitumor effects of HKI-272, inhibition of HER-2 phosphorylation was also detected in xenografts. The sustained inhibition of phosphorylation seen in the xenograft studies is consistent with irreversible inhibition of the target because the terminal half-life of HKI-272 after a single oral dose (20 mg/kg) in nude mice is ~4 h (data not shown). The prolonged duration of inhibition supports the once-daily oral dosing used in the xenograft models. Although HKI-272 blocks the kinase activity of EGFR and inhibits the proliferation of EGFR-dependent cell lines, it is less potent in A431 tumor xenograft models than EKB-569, the EGFR inhibitor (19). This is unlikely to be due to the pharmacokinetic properties of the compound because these are similar to EKB-569 (data not shown), and it is quite effective at inhibiting the growth of HER-2-dependent tumors. This suggests that the sensitivity to kinase inhibitors, of A431 cells growing as monolayers, is different from the sensitivity when grown as xenografts.

Several other small molecule inhibitors targeting ErbB receptors are in preclinical or clinical development (34–36). As with HKI-272 and EKB-569, all compounds are designed to block kinase activity by binding to the ATP site of the enzyme, an approach that has been validated by Gleevec (imatinib mesylate), an inhibitor of the bcr-abl tyrosine kinase for the treatment of chronic myelogenous leukemia (37). Iressa (gefitinib; Ref. 38), a reversible EGFR inhibitor, was recently approved for lung cancer treatment. Although this compound is much less active against HER-2 in cell-free systems (38), it has been reported to inhibit the growth of HER-2-expressing cells *in vitro* and *in vivo* (24, 39). This may be because of cross-inhibition of HER-2 by EGFR in cells expressing both receptors. Tarceva (erlotinib) is a second reversible-binding EGFR inhibitor in clinical trials (35, 40). As a consequence of the high degree of homology between the kinase domains of HER-2 and EGFR, some ErbB antagonists directly inhibit both EGFR and HER-2 function, similar to HKI-272. Among these are CI-1033 (41), GW2016 (42), and PKI-166 (43). Of these, only CI-1033 has been shown to bind irreversibly to EGFR and HER-2 (22). CP-654577 has recently been reported as a selective HER-2 inhibitor (44).

In conclusion, HKI-272 is a promising new anticancer drug candidate for the treatment of breast cancers and other HER-2-dependent cancers. Because it also inhibits the EGFR kinase with similar po-

tency, it may be useful to treat tumors that overexpress both HER-2 and EGFR and be more efficacious than a specific EGFR or HER-2 antagonist. The observed modulation of downstream signaling pathways and cell cycle regulatory proteins by HKI-272 identifies additional drug targets in the growth factor response pathways (MAPK, Akt, and cyclin D1/CDK4). Inhibitors of these proteins may be useful in combination therapy with a HER-2 inhibitor. These targets also provide convenient pharmacodynamic markers (biomarkers) to monitor HKI-272 activity in clinical trials.

ACKNOWLEDGMENTS

We thank Drs. Philip Frost, Tarek Mansour, and Janis Upeslacis for their support and Joan Samson for technical assistance. S. Rabindran dedicates this article to the memory of Annapurna Rabindran.

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Antitumor Activity of HKI-272, an Orally Active, Irreversible Inhibitor of the HER-2 Tyrosine Kinase

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Cancer Res 2004;64:3958-3965.