Non–Small-Cell Lung Cancer and Ba/F3 Transformed Cells Harboring the ERBB2 G776insV_G/C Mutation Are Sensitive to the Dual-Specific Epidermal Growth Factor Receptor and ERBB2 Inhibitor HKI-272

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Abstract

Mutation-specific cancer therapy has shown promising clinical efficacy. In non–small-cell lung cancer (NSCLC), the presence of mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase correlates with clinical response to small-molecule tyrosine kinase inhibitors. Here, we show that cells harboring the G776insV_G/C mutation in the related ERBB2 tyrosine kinase (also known as HER2 or Neu), present in a small percentage of NSCLCs, are sensitive to HKI-272, an irreversible dual-specific kinase inhibitor targeting both EGFR and ERBB2. In the ERBB2-mutant NCI-H1781 cell line, HKI-272 treatment inhibited proliferation by induction of G_1 arrest and apoptotic cell death. Furthermore, HKI-272 abrogated autophosphorylation of both ERBB2 and EGFR. Finally, Ba/F3 murine pro-B cells, engineered to express mutant ERBB2, became independent of interleukin-3 and sensitive to HKI-272. Thus, the subset of NSCLC patients with tumors carrying the ERBB2 G776insV_G/C mutation may benefit from treatment with HKI-272. (Cancer Res 2006; 66(13): 6487-91)

Introduction

Reversible anilinoquinazoline class tyrosine kinase inhibitors targeting the epidermal growth factor receptor (EGFR) tyrosine kinase, erlotinib and gefitinib, have shown clinical activity in patients with non–small-cell lung cancer (NSCLC; refs. 1, 2). Heterozygous mutations in the kinase domain of EGFR have been found to correlate with response to these tyrosine kinase inhibitors (3–5). Mutations fall into four major classes: single-base substitutions in exon 18; deletions in exon 19; insertion/duplications in exon 20; and a single-base substitution, L858R, in exon 21 (6). In contrast to the other mutations, insertions in exon 20 were not detected in tumors from patients responding to EGFR tyrosine kinase inhibitor treatment. In cell culture models, these mutants were found to be insensitive to gefitinib or erlotinib but were sensitive to an irreversible EGFR tyrosine kinase inhibitor, CL-387,785 (7).

Mutations in ERBB2 have also recently been described in NSCLC adenocarcinomas. In the first report, five mutations were found among a total of 120 primary NSCLCs, 51 of which were adenocarcinomas (8). In a larger series including 671 primary NSCLCs, 394 of which were adenocarcinomas, 11 adenocarcinomas were found to harbor an ERBB2 mutation (9). These mutations targeted residues that are homologous to the ones affected by the exon 20 insertions in EGFR. Among the 11 adenocarcinomas harboring ERBB2 mutation in the second series, 6 carried the YVMA 776-779ins mutation and 2 carried the G776insV_G/C mutation. The latter mutation was also found in the NCI-H1781 NSCLC adenocarcinoma cell line (9).

To date, trastuzumab (Herceptin), a humanized monoclonal antibody against the extracellular domain of ERBB2, has not shown significant activity in NSCLC clinical trials in unselected patients or in those whose tumors overexpress ERBB2 (10). Although tumors harboring ERBB2 mutations comprise only a small percentage of NSCLCs, they may represent a subset in which trastuzumab or other ERBB2 inhibitors should be clinically evaluated.

HKI-272, a novel, orally available, irreversible dual-specific inhibitor that targets both EGFR and ERBB2 with nanomolar potency, is currently undergoing clinical evaluation in lung and breast cancers (11). HKI-272 has shown preclinical activity against the A431 EGFR-dependent cell line (12). In addition, the drug is cytotoxic to NSCLC cells harboring EGFR kinase domain mutations that also carry a substitution mutation in exon 20 (T790M; ref. 13) known to confer resistance to gefitinib or erlotinib (14, 15). HKI-272 is particularly active against wild-type ERBB2 (HER2)-dependent cell lines, either engineered or derived from *ERBB2* (*HER2*)-amplified breast carcinomas. In these cell lines, HKI-272 inhibits ERBB2-mediated signal transduction pathways and cell cycle progression *in vitro* and causes tumor growth inhibition in xenograft models *in vivo* (12).

Here, we tested if the presence of lung cancer–derived ERBB2 mutation confers sensitivity to HKI-272 in both NCI-H1781 and Ba/F3 cells ectopically expressing ERBB2 harboring the G776insV_G/C mutation. These cells display expected resistance to erlotinib, but are indeed sensitive to HKI-272, suggesting a promising small-molecule tyrosine kinase inhibitor strategy for this group of NSCLCs.

Materials and Methods

Cells. NCI-H1781, NCI-H1975, and A549 NSCLC cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were maintained in the ATCC-specified growth medium. The HCC827 cell line

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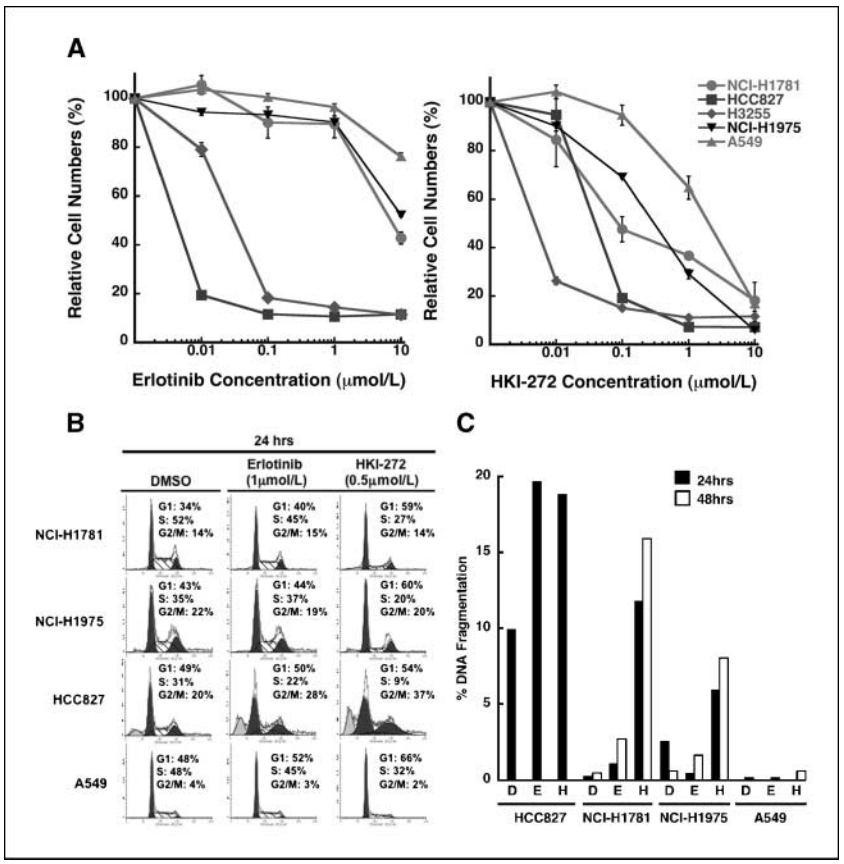

Figure 1. HKI-272 has antiproliferative activity against NSCLC harboring EGFR or ERBB2 mutation. *A,* dose-dependent growth inhibition of NSCLC cells treated with HKI-272 or erlotinib determined by CCK-8 assay. Only HCC827 and H3255 cells are sensitive to erlotinib whereas NCI-H1781 and NCI-H1975 cells are also sensitive to HKI-272. *Points,* average of two independent experiments, each done in triplicate; *bars*, SD. *B,* NSCLC cell lines were treated with DMSO, 1 μmol/L erlotinib, or 0.5 μmol/L HKI-272 for 24 hours and harvested for flow cytometry. At 24 hours, erlotinib treatment resulted in appearance of a sub-G_1 peak in HCC827 cells but had little effect on the other cell lines. HKI-272 treatment also resulted in HCC827 cells with sub-G_1 DNA content; the other cell lines showed G_1 arrest, with the weakest effects on A549 cells. *C,* NSCLC cell lines were treated as in (*B*). *D, E,* and *H,* treatment with DMSO, erlotinib, and HKI-272, respectively. Cells were harvested for flow cytometry at 24 and 48 hours and sub-G_1 DNA content was quantified.

was obtained from Dr. Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX; ref. 16). H3255 cells were maintained as previously described (17, 18).

Drug treatments. Erlotinib was purchased from commercial suppliers. HKI-272 was provided by Wyeth Pharmaceuticals (Pearl River, NY). Stock solutions of tyrosine kinase inhibitors were prepared in DMSO at a concentration of 10 mmol/L and maintained at −20°C. Drugs were diluted to 1 mmol/L in DMSO for a working solution and used at concentrations ranging from 0.001 to 10 μmol/L.

Retrovirus production and infection. The production and infection of retrovirus expressing mutant ERBB2 were carried out as previously described (7). Ba/F3 cells were cultured as previously described (19). Polyclonal cell lines stably expressing G776insV_G/C mutant ERBB2 were established by hygromycin selection and subsequently cultured in the absence of interleukin-3 (IL-3). Pooled stable cells that were transformed to IL-3 independence were used for drug-sensitivity experiments.

Site-directed mutagenesis. Mutations were introduced into full-length human mutant ERBB2 and subcloned into the pBABE-hygro vector using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and verified by sequencing.

Cell proliferation assays. NSCLC and Ba/F3 cells were cultured in the presence of drugs or vehicle for 72 hours and viability was determined using the CCK-8 colorimetric assay (Dojindo, Gaithersburg, MD) or the WST assay (Roche, Indianapolis, IN), respectively, as previously described (7, 18).

Western blot analysis. Whole-cell lysates were subjected to SDS-PAGE followed by Western blotting as previously described (17). Anti-EGFR, anti–phospho-EGFR (Tyr^{1068}), anti–phospho-ERBB2 ($Tyr^{1221/1222}$), anti-Akt, anti–phospho-Akt (Ser^{473}), anti–extracellular signal–regulated kinase (Erk) 1/2, and anti–phospho-Erk1/2 (Thr^{202}/Tyr^{204}) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-ERBB2 (3B5) was from EMD Biosciences (San Diego, CA). Antitubulin (clone DM 1A) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Anti-RasGAP was from Santa Cruz Biotechnology (Santa Cruz, CA).

Fluorescence-activated cell sorting analysis. For cell cycle analysis, adherent and nonadherent cells were pooled, fixed, and stained with propidium iodide, as previously described (17), and analyzed for DNA content by flow cytometry using the ModFit program (Verity Software House, Topsham, ME).

Detection of apoptosis by flow cytometry. Adherent and nonadherent cells were pooled. Apoptosis was assessed using an Annexin V-FLUOS Staining Kit (Roche) according to the instructions of the manufacturer.

Results and Discussion

NCI-H1781 cells harboring an ERBB2 mutation are resistant to erlotinib but sensitive to HKI-272. We first explored the effects of either erlotinib or HKI-272 on the proliferation of a panel of NSCLC cell lines (Fig. 1*A*). Cells were considered sensitive if the IC_{50} was <1 μmol/L. Consistent with previous reports, H3255 (EGFR L858R) and HCC827 (EGFR E746_A750del) were sensitive to erlotinib treatment (15, 16), with IC_{50} values of 0.031 and 0.003 μmol/L, respectively (Fig. 1*A*). Both cell lines were also extremely sensitive to HKI-272 treatment with IC_{50} values of 0.0049 and 0.039 μmol/L, respectively. In contrast, NCI-H1781 cells, known to carry wild-type EGFR and the G776insV_G/C mutation of ERBB2 (9), were resistant to erlotinib. However, these cells were highly sensitive to HKI-272 with an IC_{50} of 0.085 μmol/L. As expected, A549 cells, expressing wild-type EGFR and mutant K-RAS, were resistant to both erlotinib and HKI-272 (IC_{50} >1 μmol/L), whereas the erlotinib-resistant cell line NCI-H1975, carrying the T790M resistance mutation of EGFR, was sensitive to HKI-272 (IC_{50}: 0.29 μmol/L), consistent with a prior study (13).

To determine the mechanism of growth inhibition of these NSCLC cell lines by either erlotinib or HKI-272, we did flow

cytometric analysis to assess the cell cycle distribution of vehicle-treated and drug-treated cells (Fig. 1*B*). After exposure to 1 μmol/L erlotinib for 24 hours, HCC827 cells showed a sub-G_1 peak, consistent with the induction of apoptosis in this cell line. In the other cell lines, cell cycle distribution was largely unchanged, with only a slight increase in G_1 DNA content and small diminution in S-phase content noted in NCI-H1781 and A549 cells. In contrast, 0.5 μmol/L HKI-272 arrested all NSCLC cells tested within 24 hours.

Cell cycle analyses done after 48 hours of HKI-272 treatment showed a sub-G_1 population in all cell lines except A549, suggesting the induction of apoptosis (Fig. 1*C*). In HCC827 cells, distortion of entire cell cycle pattern at 48 hours also suggested substantial apoptosis (data not shown). To confirm and more accurately quantify the apoptotic response, untreated and treated cells were subjected to an Annexin V apoptosis assay (Fig. 2*A* and *B*). These data confirm the induction of robust concentration-dependent apoptosis by HKI-272 in both EGFR-mutant and ERBB2-mutant cell lines. In contrast, apoptosis was not detected in A549 cells. In addition, HKI-272 effectively kills ERBB2-mutant and EGFR kinase domain–mutant NSCLC cells to a similar extent. In summary, the inhibition of proliferation by HKI-272 in sensitive cells involves G_1 arrest and the induction of apoptosis.

Mutant ERBB2 is constitutively active independent of ligand in NCI-H1781 cells and is suppressed by HKI-272. To further characterize the mechanism of action of HKI-272 in ERBB2-mutant NSCLC cells, we examined the expression of EGFR and ERBB2 in the cell line panel. All of the cell lines expressed high levels of ERBB2, although levels of EGFR varied (Fig. 3*A*). Next, we determined if the activation of mutant ERBB2 could be inhibited by EGFR-targeted tyrosine kinase inhibitors. In NCI-H1781 cells, erlotinib failed to inhibit both EGFR and ERBB2 phosphorylation induced by EGF stimulation (Fig. 3*B*). Interestingly, in the presence of erlotinib, there was reduction of Akt and Erk phosphorylation at high concentration despite the absence of effects on cellular proliferation (Fig. 1). In contrast to erlotinib, HKI-272 blocked the EGF-stimulated phosphorylation of both EGFR and ERBB2 in a concentration-dependent manner, with complete suppression at concentrations of ≥0.1 μmol/L, paralleled by diminution of Akt and Erk phosphorylation (Fig. 3*B*).

We also examined receptor activation and downstream signaling after a more detailed time course of EGF stimulation. In the experiment shown in Fig. 3*C*, cells were starved overnight, exposed briefly to DMSO or tyrosine kinase inhibitors, and then stimulated with EGF for up to 4 hours in the continued presence of vehicle or drug treatment. In the absence of tyrosine kinase inhibitors, EGFR in NCI-H1781 cells was phosphorylated within 60 minutes and dephosphorylated by 120 minutes after EGF stimulation. In the presence of erlotinib during EGF stimulation, phosphorylation of EGFR progressed more slowly, peaking at 240

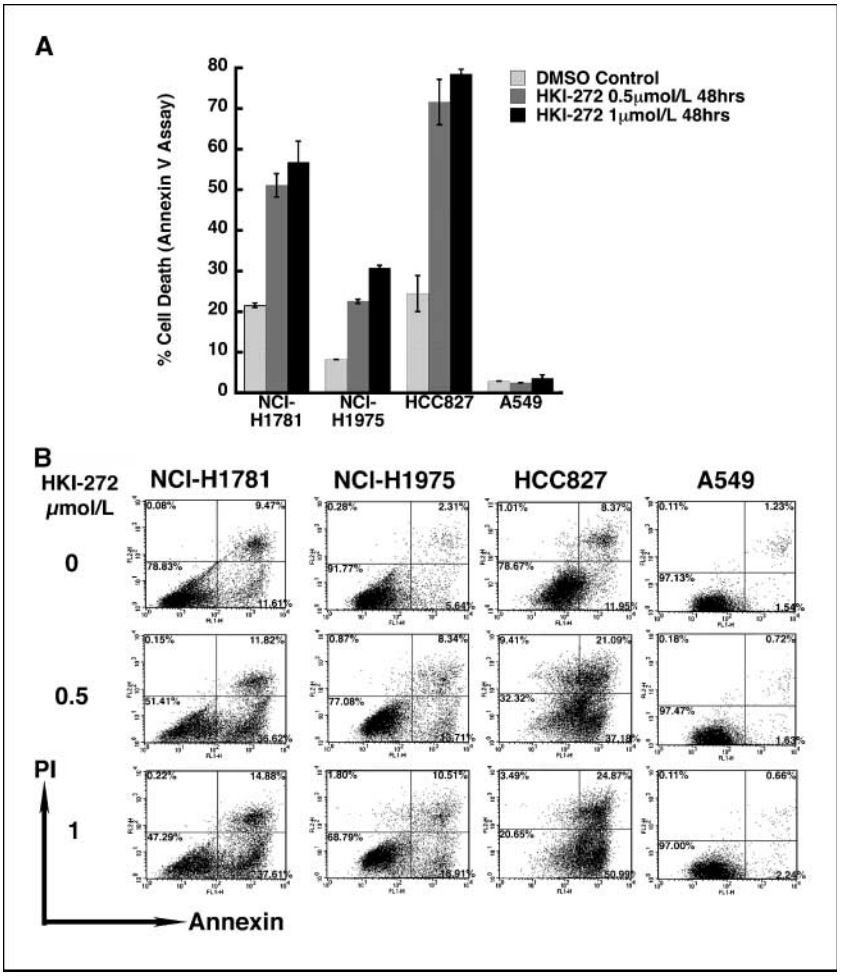

Figure 2. HKI-272 induces apoptosis in ERBB2 and EGFR-kinase domain mutant cells. *A,* quantification of apoptosis by Annexin V assay. NSCLC cell lines were grown in DMSO or HKI-272 at the indicated concentrations for 48 hours. *Columns,* average of three experiments; *bars*, SD. *B,* representative flow histograms of Annexin V apoptosis assays. NSCLC cell lines were grown in DMSO or the indicated concentrations of HKI-272 for 48 hours. The numbers indicate the percentage of total cells in the corresponding quadrant. *Bottom left quadrant,* viable cells; *bottom right quadrant,* early apoptotic cells; *top right quadrant,* late apoptotic cells; *top left quadrant,* nuclear debris.

minutes. In contrast, HKI-272 completely suppressed EGF-dependent phosphorylation of EGFR. The more complete suppression of EGFR phosphorylation by HKI-272 raises the possibility that both autophosphorylation and ERBB2-mediated phosphorylation contribute to EGFR activation in NCI-H1781 cells.

Phosphorylation of ERBB2 in NCI-H1781 cells was constitutive and independent of either EGF treatment or EGFR phosphorylation because neither overnight serum starvation nor preincubation with erlotinib failed to dephosphorylate ERBB2 (Fig. 3*C*; time 0 of EGF stimulation in DMSO- and erlotinib-treated cells). This was not the case in erlotinib-sensitive HCC827 cells, in which ERBB2 was completely dephosphorylated by either overnight serum starvation or preincubation with erlotinib (data not shown). Furthermore, in NCI-H1781 cells, baseline phosphorylation of Akt was relatively high and its phosphorylation on EGF stimulation sustained well beyond the time of complete dephosphorylation of EGFR; Erk signaling exhibited a similar pattern. In the presence of HKI-272, but not erlotinib, induction of both Akt and Erk phosphorylation by EGF stimulation was effectively inhibited. Taken together, these results suggest that the ERBB2 tyrosine kinase is constitutively active in a ligand-independent fashion in NCI-H1781 cells, resulting in activation of downstream signaling cascades. Constitutive activation of ERBB2 is sensitive to treatment with the EGFR/ERBB2 inhibitor HKI-272 but insensitive to the EGFR tyrosine kinase inhibitor erlotinib.

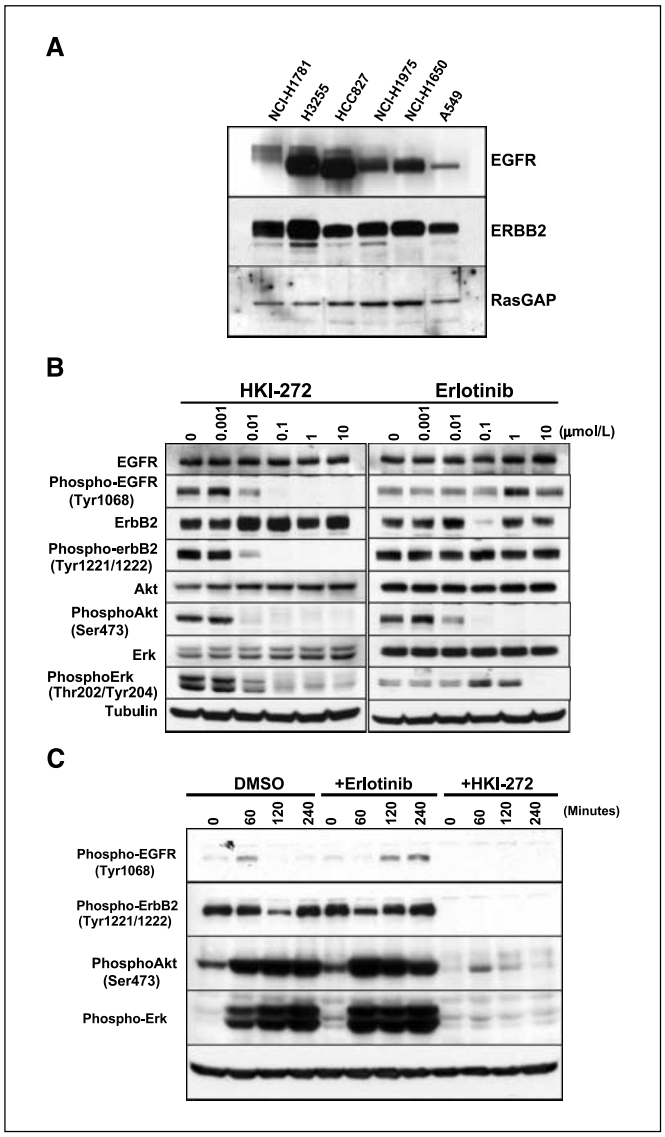

Figure 3. HKI-272 blocks constitutive activation of mutant ERBB2, EGFR, and downstream signaling pathways in NCI-H1781 cells. *A,* EGFR and ERBB2 expression in a panel of NSCLC cell lines. Radioimmunoprecipitation assay buffer lysates of indicated NSCLC cell lines were subjected to 6% SDS-PAGE and Western blotting to determine expression levels of EGFR and ERBB2. RasGAP was analyzed as a loading control. *B,* exponentially growing NCI-H1781 cells were starved for 2.5 hours in the presence of HKI-272 or erlotinib and then stimulated with 100 ng/mL EGF for 1 hour in the continued presence of drug. Lysates were subjected to Western blotting with the indicated antibodies. Tubulin was analyzed as a loading control. The data show the suppression of EGFR and ERBB2 phosphorylation by HKI-272, but not by erlotinib, in response to EGF. *C,* NCI-H1781 cells were starved overnight, then incubated with DMSO, 1 μmol/L erlotinib, or 0.5 μmol/L HKI-272 for 2.5 hours, followed by 100 ng/mL EGF stimulation for the indicated times. Mutant ERBB2 is constitutively phosphorylated in starved cells or erlotinib-treated cells. In contrast, mutant ERBB2 phosphorylation is completely suppressed by HKI-272.

Ectopic expression of mutant ERBB2 confers IL-3 independence and HKI-272 sensitivity on Ba/F3 cells. To test if cells harboring the lung cancer–derived ERBB2 G776insV_G/C mutation are sensitive to HKI-272 in an isogenic system, we stably transfected murine Ba/F3 pro-B cells with retroviral constructs encoding either wild-type or mutant ERBB2. Ba/F3 cells rely on continuous supplementation of IL-3 for proliferation but become IL-3 independent on transformation with activated oncogenes (20). After hygromycin selection, withdrawal of IL-3 revealed growth factor independence of the pooled stable cell lines carrying either wild-type or mutant ERBB2. These results confirm the known oncogenicity of wild-type ERBB2 (21, 22) and are similar to results described for Ba/F3 cell lines harboring the L858R EGFR (19).

As expected, Ba/F3 cells harboring the L858R EGFR mutation were most sensitive to erlotinib, with an IC_{50} of 0.009 μmol/L, whereas cells expressing wild-type ERBB2, mutant ERBB2, or the EGFR L858R/T790M mutation were more resistant, with IC_{50} values of 0.28, 0.31, and >10 μmol./L, respectively (Fig. 4*A*). In contrast, when treated with HKI-272, cells expressing mutant ERBB2 exhibited exquisite sensitivity with an IC_{50} of <0.001 μmol/L, similar to the IC_{50} of cells expressing wild-type ERBB2 (IC_{50} <0.001 μmol/L) and less than that of cells expressing EGFR L858R (IC_{50} 0.0028 μmol/L; Fig. 4*A*). Cells expressing the L858R/T790M double mutant of EGFR were also sensitive to this drug, although with a higher IC_{50} of 0.185 μmol/L, again consistent with results with dual-specific irreversible inhibitors (13). The sensitivity of Ba/F3 cells expressing mutant ERBB2 was recapitulated biochemically because HKI-272 caused concentration-dependent inhibition of ERBB2 phosphorylation in these cells (Fig. 4*B*), comparable to the inhibition of EGFR phosphorylation observed in L858R mutant cells (data not shown). These results indicate that cells dependent on ERBB2, conferred by expression of either the wild-type or the G776insV_G/C mutant protein, are highly sensitive to HKI-272. Furthermore, in this isogenic system, HKI-272 inhibits the proliferation of cell lines expressing either wild-type or mutant ERBB2 with similar potency.

In summary, we have shown that the lung cancer–derived G776insV_G/C mutation of ERBB2 is constitutively active and sensitive to the dual-specific EGFR/ERBB2 kinase inhibitor HKI-272. Results from both a naturally occurring ERBB2-mutant

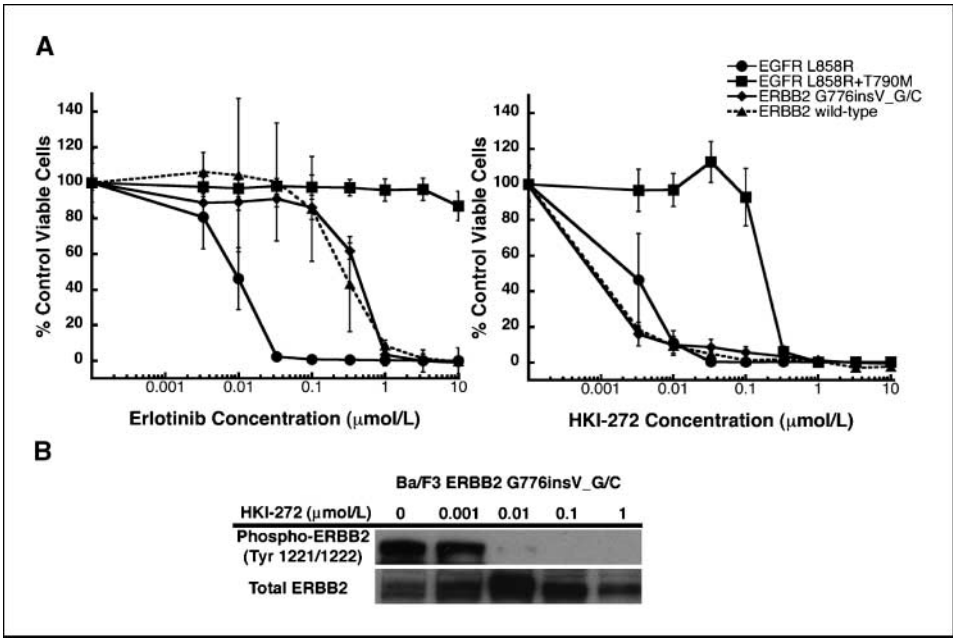

Figure 4. Tyrosine kinase inhibitor treatment of Ba/F3 cells expressing mutant EGFR, wild-type ERBB2, or mutant G776insV_G/C ERBB2. *A,* Ba/F3 cells exponentially growing in the absence of IL-3 were incubated with DMSO or the indicated concentrations of erlotinib or HKI-272 for 72 hours. As with NCI-H1781 cells, Ba/F3 cells expressing mutant ERBB2 are sensitive to HKI-272. *Points,* average of two experiments, each done in triplicate; *bars*, SD. *B,* Ba/F3 cells expressing mutant G776insV_G/C ERBB2 were treated with the indicated concentrations of HKI-272, showing dephosphorylation of the mutant receptor.

NSCLC cell line as well as Ba/F3 cells transformed to growth factor independence by ectopic expression of mutant ERBB2 show that G776insV_G/C mutant ERBB2 is constitutively active independent of ligand, suggesting a critical role for ERBB2 mutation in the transformation of a subset of NSCLCs. Growth inhibition of ERBB2-mutant cells by HKI-272 is due to G_1-phase arrest and induction of apoptosis. Further work will be necessary to determine whether HKI-272 is equipotent against other ERBB2 mutations, including the more prevalent YVMA 776-779ins mutation. Nonetheless, the small subset of patients with NSCLCs harboring the G776insV_G/C mutation may benefit from treatment with irreversible dual-specific EGFR/ERBB2 inhibitors such as HKI-272.

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