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Cellular and in Vivo Activity of JNJ-28871063, A Nonquinazoline Pan-ErbB Kinase Inhibitor That Crosses the Blood-Brain Barrier and Displays Efficacy against Intracranial Tumors

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ABSTRACT

JNJ-28871063 is a potent and highly selective pan-ErbB kinase inhibitor from a novel aminopyrimidine oxime structural class that blocks the proliferation of epidermal growth factor receptor (EGFR; ErbB1)- and ErbB2-overexpressing cells but does not affect the growth of non–ErbB-overexpressing cells. Treatment of human cancer cells with JNJ-28871063 inhibited phosphorylation of functionally important tyrosine residues in both EGFR and ErbB2 and blocked downstream signal transduction pathways responsible for proliferation and survival. A single dose of compound reduced phosphorylation of ErbB2 receptors in tumor-bearing mice, demonstrating target suppression in vivo. Tissue distribution studies show that JNJ-28871063 crosses the blood-brain barrier and penetrates into tumors, where it is able to accumulate to higher levels than those found in the plasma. JNJ-28871063 showed oral antitumor activity in human tumor xenograft models that overexpress EGFR and ErbB2. In an intracranial ErbB2-overexpressing tumor model, JNJ-28871063 extended survival relative to untreated animals. The brain is a primary site of metastasis for EGFR-overexpressing lung cancers and ErbB2-overexpressing breast cancers. Therefore, the ability to penetrate into the brain could be an advantage over existing therapies such as trastuzumab (Herceptin) and cetuximab (Erbitux), which are antibodies and do not cross the blood-brain barrier. These results show that JNJ-28871063 is orally bioavailable, has activity against EGFR and ErbB2-dependent tumor xenografts, and can penetrate into the brain and inhibit ErbB2-overexpressing tumor growth.

The epidermal growth factor receptor (EGFR; HER1; ErbB1) and ErbB2 (HER2; neu) are prototypic members of the ErbB family of tyrosine kinase receptors that also includes ErbB3 (HER3) and ErbB4 (HER4). Multiple ligands can bind to and activate ErbB receptors, inducing autophosphorylation, dimerization, and the phosphorylation of cellular substrates that mediate increased DNA synthesis, cell proliferation, angiogenesis, metastasis, and resistance to apoptosis (reviewed in Hynes and Lane, 2005). A soluble ligand for ErbB2 has not been identified. However, all ErbB family members are able to homodimerize with themselves and heterodimerize with other members of the ErbB family.

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ABBREVIATIONS: EGFR, human epidermal growth factor receptor; EGF, epidermal growth factor; ErbB2, human epidermal growth factor receptor-2; HER, human epidermal growth factor receptor; JNJ-28871063, 4-amino-6-(4-benzyloxy-3-chloro-phenylamino)-pyrimidine-5-carbaldehyde *O*-(2-morpholin-4-yl-ethyl)-oxime; MAPK, mitogen activated protein kinase; PLC, phospholipase C; PBS, phosphate-buffered salineAKT, protein kinase B; TGI, tumor growth inhibition; TTE, time to endpoint; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal-regulated kinase; CDK, cyclin dependent kinase; BBB, blood-brain barrier.

In this way, the ErbB2 tyrosine kinase, which is the most potent of all kinase domains in the ErbB family (Di Fiore et al., 1990), can be activated without ligand association. Therefore, extensive cross-talk and transactivation occurs between the ErbB family isoforms, and inhibition of multiple isoforms may be desirable. EGFR or ErbB2 are overexpressed in many cancer types, including breast, ovarian, head and neck, brain, and non–small-cell lung cancer through either increased transcription or gene amplification (Libermann et al., 1985). Increased expression is associated with shortened survival, pointing to involvement in growth regulation of the tumors (Slamon et al., 1987). Overactivity of downstream signal transduction pathways are thought to be involved in uncontrolled cell growth and oncogenesis (Libermann et al., 1985). Many tumor types, including glioblastoma, breast, ovary, and non–small-cell lung cancer, express truncated EGFR (Moscatello et al., 1995), resulting in tumors with a more aggressive phenotype; mutations resulting in constitutive activation also occur (Nishikawa et al., 1994). ErbB2 is overexpressed in 30% of breast cancers and in many ovarian, endometrial, and gastric cancers, and overexpression correlates with a poor prognosis, increased metastasis, early relapse, and a shortened survival (Hudziak et al., 1989). ErbB2 overexpression can occur from gene amplification, increased mRNA levels, or overproduction of ErbB2 protein (Dalifard et al., 1998) and can promote estrogen independent growth thus releasing cells from the hormonal control of estrogen (Pietras et al., 1995). When overexpressed, ErbB2 may be constitutively active, resulting in cellular transformation (Hudziak et al., 1989).

Modulation of the ErbB pathway with specific antibodies and small molecules has demonstrated therapeutic benefit. Trastuzumab (Herceptin) and cetuximab (Erbitux) are antibodies targeting ErbB2 and EGFR, respectively, and gefitinib (Iressa) and erlotinib (Tarceva) are small-molecule tyrosine kinase inhibitors of EGFR. These agents exhibit antiproliferative effects on EGFR- or ErbB2-overexpressing tumor cells in vitro (Hudziak et al., 1989; Moyer et al., 1997; Inoue et al., 2000; Moulder et al., 2001) in experimental animals (Park et al., 1995; Pollack et al., 1999; Inoue et al., 2000; Wakeling et al., 2002) and in human clinical trials (Baselga et al., 1998; Cunningham et al., 2004; DiMaio et al., 2005).

Because ErbB2 can transactivate other ErbB receptors and is the preferred heterodimerization partner among all the ErbB receptors (Olayioye et al., 1998), simultaneous suppression of ErbB2 and EGFR may provide superior inhibition of signaling that drives tumor proliferation. Indeed, a combination of EGFR and ErbB2 kinase inhibitors was shown to be more effective at inhibiting proliferation and inducing apoptosis over either agent alone (Zhou et al., 2006). This strategy is reflected in the development of lapatinib (Tykerb, GW572016; GlaxoSmithKline), a small-molecule kinase inhibitor of EGFR and ErbB2 that has shown promising activity in the clinic and was recently approved for use in combination with capecitabine for the treatment of ErbB2-overexpressing advanced or metastatic breast cancer after prior therapy with an anthracycline, a taxane, and trastuzumab (Moy et al., 2007).

ErbB2-overexpressing tumors have a greater incidence of CNS metastasis, possibly resulting from an enhanced metastatic potential (Yu et al., 1994). In addition, the success of trastuzumab has increased the survival of patients with ErbB2-positive breast cancer and created a larger patient population in which brain metastases have time to develop because of an increased life span and the inability of trastuzumab to penetrate the blood-brain barrier (BBB) (Clayton et al., 2004). After diagnosis of metastatic lesions in the brain, less than 20% of patients survive for more than 1 year and less than 2% survive for 2 years or longer (Shaffrey et al., 2004). JNJ-28871063 is a potent inhibitor of the EGFR, ErbB2, and ErbB4 receptor kinases and showed efficacy against EGFR- and ErbB2-dependent tumor xenografts. Furthermore, this compound crosses the blood-brain barrier and showed activity in an ErbB2-overexpressing brain tumor model. Because few effective treatment options exist for ErbB-overexpressing tumors that have metastasized to the brain, these results support further development of JNJ-28871063 to determine whether it will provide therapeutic benefit for these cases.

Materials and Methods

Reagents. JNJ-28871063 (Fig. 1A) was synthesized by the Cancer Therapeutics Research and High Output Synthesis teams (J&J Pharmaceutical Research and Development, L.L.C.). All studies were carried out with the free base form of the compound. EGF (R&D Systems, Minneapolis, MN) was used to stimulate A431 cells. Trastuzumab was obtained from Genentech (South San Francisco, CA); gefitinib and erlotinib were obtained as marketed drug (Hanna's Pharmaceutical Supply Company, Wilmington, DE).

Cell Culture. HN5 cells were obtained from the Ludwig Institute for Cancer Research (Zurich, Switzerland). All other cells were from the American Type Culture Collection (Manassas, VA). The following human cell lines were used: BT474 and SKBR3 (breast carcinoma), N87 (gastric carcinoma), HeLa (cervical adenocarcinoma), HCT116 and HT29 (colon carcinoma), HN5 (head and neck carcinoma), A375 (melanoma), and MRC-5 (lung fibroblasts). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Cells were maintained at 37°C plus 5% CO_2 as exponentially growing monolayers in the following media supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 2 mM L-glutamine: HeLa in minimal essential medium with 0.1 mM nonessential amino acids and 1 mM sodium pyruvate; SKBR3, HCT116, and HT29 in McCoys 5a medium; MRC-5 in Eagle's minimal essential medium; A431, HN5, and A375 in Dulbecco's modified Eagle's medium with 4 mM L-glutamine and 1.5 g/l sodium bicarbonate; and N87 in RPMI 1640 medium with 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate.

ErbB Receptor Kinase Assays and Kinase Profiling. Inhibition of ErbB family members was measured in streptavidin coated 96-well scintillating microplates (PerkinElmer Life and Analytical Sciences, Waltham, MA) using N-terminal glutathione transferase-tagged soluble ErbB constructs expressed and purified from baculovirus. The reaction was incubated for 60 min at 30°C in 60 mM HEPES, pH 7.5, 3 mM magnesium chloride, 3 mM manganese chloride, 0.003 mM sodium vanadate, 1.2 mM dithiothreitol, 50 μg/ml polyethylene glycol 20,000, 0.001 mM ATP, 1.5 ng/ml biotinylated poly-GluTyr, and 0.2 μCi of [^{33}P]γ-ATP and for EGFR in 50 mM Tris, pH 8.0, 10 mM magnesium chloride, 0.1 mM sodium vanadate, 1 mM dithiothreitol, 0.005 mM ATP, 1.5 ng/ml biotinylated polyGluTyr, and 0.2 μCi of [^{33}P]γ-ATP). Plates were sealed and read on a TopCount scintillation counter (PerkinElmer Life and Analytical Sciences). Inhibition was measured by quantifying the amount of [^{33}P]γ-ATP incorporated into the immobilized peptide in the presence of various concentrations of test compound. Linear regression analysis of the percentage of inhibition by test compound was used to determine IC_{50} values (Prism 4; GraphPad Software, San Diego, CA).

Each measurement was performed at least in duplicate, and the IC_{50} values were calculated with standard deviation from two to eight separate experiments. Assays for inhibition of CDK1, CDK2, insulin receptor kinase-β, platelet-derived growth factor receptor-Rβ kinase, and vascular endothelial growth factor receptor-2 kinase were performed as described previously (Emanuel et al., 2004). Kinase selectivity profiling was carried out at Upstate Biotechnology (Charlottesville, VA) and Invitrogen.

Measurement of ErbB Tyrosine Phosphorylation in Cells and Tumors. ELISA assays were developed to measure total ErbB2 and EGFR receptor phosphorylation levels. Inhibition of ErbB2 phosphorylation was determined by treating 1×10^7 SKBR3 cells for 24 h with serial dilutions of test compound or vehicle and preparing cell lysates in buffer A [50 mM HEPES, 150 mM NaCl, 0.5% Triton X-100, 8% glycerol, 2 mM Na_3VO_4, 1.5 mM $MgCl_2$, and 1 mM EDTA containing the protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin-A and E64]. Lysates were transferred to a capture plate coated with a primary antibody specific for the human extracellular domain of the ErbB2 receptor. The phospho-ErbB2 ELISA was developed by using the precoated capture plate from the research use only version of the FDA-approved in vitro diagnostic ELISA for HER-2/neu (Oncogene Science/Bayer Co., Cambridge, MA) to immobilize total ErbB2 in the sample. The detection antibody was replaced by a mouse monoclonal antiphosphotyrosine antibody conjugated to horseradish peroxidase (Upstate Biotechnology) to determine the extent of phosphorylation of the immobilized ErbB2 receptor. A similar strategy using the same capture plate but substituting a different antiphosphotyrosine detection antibody has been described previously (Barbacci et al., 2003). The chromogenic substrate, tetramethylbenzidine, was used to measure the absorbance on a spectrophotometer at 450 nm. IC_{50} values were determined by subtracting background and calculating percent inhibition of total maximum signal in each assay. Samples were tested in duplicate or triplicate on two to eight separate occasions and the IC_{50} values are reported as mean values with standard deviation. To measure ErbB2 phosphorylation in tumors, mice bearing N87 xenografts were dosed orally with JNJ-28871063, and tumors were excised at various times after administration of compound. Tumors were minced and homogenized in cell lysis buffer. Protein concentration of the clarified cell lysates was determined with the bicinchoninic acid reagent (Pierce, Rockford, IL) and equal amounts of protein were evaluated. Inhibition of EGF-stimulated EGFR phosphorylation was determined by plating 1×10^7 A431 cells and allowing them to adhere overnight. The next day, media was replaced with media containing 1% fetal bovine serum, and cells were starved for 16 h in the presence of test compound or vehicle. Cells were then stimulated with 20 ng/ml EGF for 5 min, and cell lysates were prepared. Lysates were transferred to a capture plate coated with a primary antibody specific for the human extracellular domain of the EGF receptor. The phospho-EGFR ELISA used the precoated capture plate from a total EGFR ELISA (Calbiochem, San Diego, CA) to immobilize EGFR in the sample and the detection antibody was replaced with a mouse monoclonal antiphosphotyrosine antibody conjugated to horseradish peroxidase (Upstate Biotechnology) to determine the extent of phosphorylation of the immobilized EGFR as for the ErbB2 ELISA.

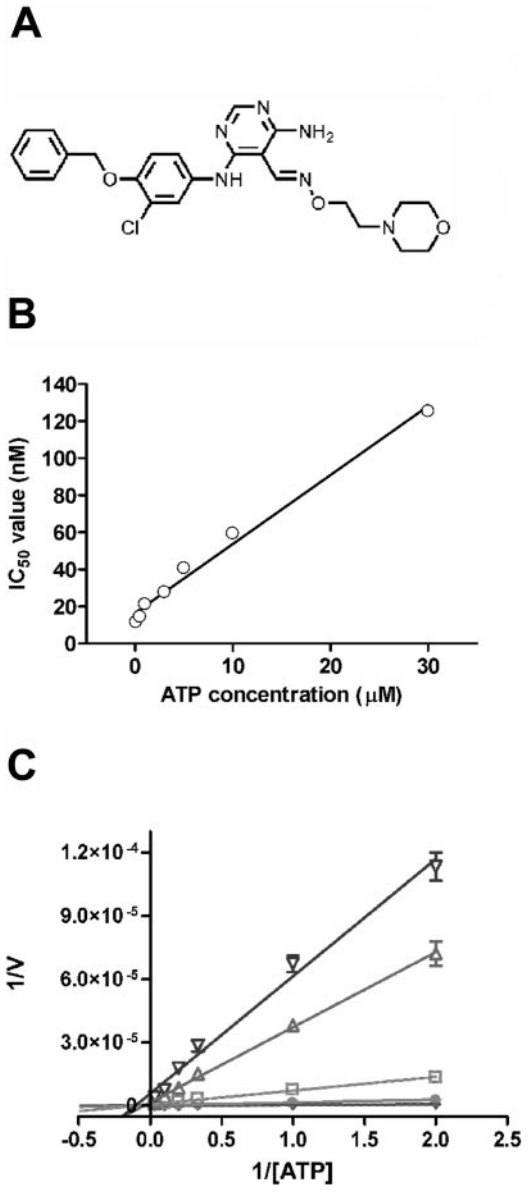

Fig. 1. A, the chemical structure of JNJ-28871063. B, the IC_{50} value for inhibition of recombinant human ErbB2 by JNJ-28871063 was calculated at increasing concentrations of ATP and produced a linear relationship relative to total ATP concentration. All groups were set up with $n = 3$; bars, S.D. C, double reciprocal plots of kinetic data from ErbB2 kinase assay shows the activity at various concentrations of JNJ-28871063 including 0.001 (▼), 0.01 (♦), 0.1 (●), 1 (□), 10 (△), and 100 μM (▽), plotted at increasing concentrations of ATP (0.5–30 μM) with enzyme and substrate concentrations held constant. Each concentration was analyzed in triplicate and representative data are shown.

Inhibition of Signal Transduction in ErbB-Overexpressing Cells. To measure the ability of compounds to inhibit EGF-stimulated receptor phosphorylation and downstream signaling in A431 cells or unstimulated ErbB2 receptor phosphorylation and downstream signaling in SKBR3 cells, approximately 10^7 cells were seeded and allowed to adhere overnight. Before stimulation with EGF, cells were incubated in serum-free media for an additional 24 h. Cells were then incubated with drug or DMSO vehicle alone for 16 h at a final DMSO concentration of 1%. A431 cells were stimulated with 100 ng/ml human recombinant EGF for 5 min, lysed in buffer A, and total protein was quantified with the bicinchoninic acid protein assay (Pierce). SKBR3 cells were not stimulated. Equal amounts of total protein of cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The phosphorylation state of the EGFR or ErbB2 receptors and activation of signaling pathways was detected by immunoblotting with phospho-specific antibodies (Cell Signaling Technology, Danvers, MA). The phospho-EGFR Y1068 antibody was from BioSource (Camarillo, CA). Total protein level was also determined for each receptor or signaling component by probing with specific antibodies to EGFR (Sigma, St. Louis, MO), ERK (Promega, Madison WI), ErbB2, PLCγ or AKT (Cell Signaling Technology) and each blot was also probed with β actin (Sigma, St. Louis, MO) as an internal loading control. Secondary antibodies and enhanced chemiluminescence detection reagents were from GE Healthcare (Chalfont St.

Giles, Buckinghamshire, UK). All immunoblots were analyzed and quantified by scanning on a LumiImager F1 (Roche Diagnostics Corporation, Indianapolis, IN).

Cell Proliferation Assays. Antiproliferative activity of ErbB kinase inhibitors was assessed in monolayer cultures of cells in logarithmic growth by [^{14}C]thymidine incorporation into cellular DNA as described previously (Emanuel et al., 2004) except that the total time that cells were exposed to drug was 96 h.

Tumor Xenograft Models. Female nu/nu mice (Charles River Laboratories, Wilmington, MA), 8 to 9 weeks of age, were implanted subcutaneously with 5×10^6 N87 gastric carcinoma cells or 4×10^6 A431 cells in the flank. For the BT474 model, CB.17 SCID mice (Charles River Laboratories) were implanted with 1 mm^3 BT474 tumor fragments. For the N87 and A431 models, each group contained eight mice; for the BT474 model, 10 mice were in each group. Tumors were monitored twice weekly and then daily as the neoplasms reached the desired size range (approximately 75 mg). Animals were pair-matched when their tumors were in the 62- to 126-mg range. Estimated tumor weight was calculated using the following formula: tumor weight = $w^2(l)/2$ where w = width and l = length in millimeters. The tumor growth delay method was used where an animal would be euthanized if its neoplasm reached a size of 2.0 g. However, this never occurred with the xenografts used in these studies. Animals were weighed twice weekly during the study and examined frequently for clinical signs of any adverse, drug-related side effects. Acceptable toxicity for cancer drugs in mice is defined by the NCI as no mean group weight loss of over 20% during the test, and not more than one toxic death among 10 treated animals. Compounds were formulated in 0.5% methylcellulose and administered p.o. in a volume of 10 ml/kg, excepto for trastuzumab, which was formulated in PBS and administered i.p. For statistical analysis, the unpaired *t* test was used to determine the statistical significance of any difference in tumor growth inhibition between a treatment group and the control group. All statistical analyses were conducted at *p* level of 0.05 (two-tailed). Tumor growth inhibition (TGI) values were calculated for all groups. Prism version 4 (GraphPad Software Inc., San Diego, CA) was used for all statistical analysis and for graphic presentation.

Intracranial Survival Model. For the intracranial study, N87 cells were harvested during logarithmic growth phase, resuspended in PBS, and 5×10^4 cells were injected intracranially in a total volume of 20 μl into female nu/nu mice (Charles River Laboratories), 8 to 9 weeks of age. Animals were monitored for clinical manifestations as a result of tumor progression. The efficacy measurement for the model was survival, which was recorded as time to endpoint (TTE), or death. Moribund animals were euthanized and included in the calculations. Increase in life span was calculated as a percentage based on [(median TTE of treated animals − median TTE of control)/median TTE of control]. In preliminary studies with up to 1×10^6 N87 cells implanted, mice exhibited clinical signs, and animals began to exit the study due to large intracranial tumor burden on day 12. Therefore, we reduced the number of cells implanted and initiated dosing the day after implant. Cells were implanted and animals were observed for any clinical signs for 24 h before treatment began. Dosing continued for 50 days and the study was terminated on day 58.

Results

Inhibition of ErbB Kinase Activity in Vitro. JNJ-28871063 (Fig. 1A) represents a novel class of aminopyrimidine oxime kinase inhibitor that has structural overlap with the aminoquinazoline pharmacophore known to inhibit various biologically relevant kinases. IC_{50} values of 22, 38, and 21 nM, respectively, were obtained for inhibition of the ErbB1, ErbB2, and ErbB4 isoforms. In these in vitro kinase assays, JNJ-28871063 exhibited a broader ErbB inhibitory profile than the comparator compounds gefitinib and erlotinib, which are focused primarily on ErbB1 inhibition, and has pan-Erb inhibitory activity similar to that of lapatinib (Table 1). The IC_{50} value for inhibition of ErbB2 by JNJ-28871063 at increasing concentrations of ATP exhibited a linear relationship relative to the concentration of ATP present in the reaction (Fig. 1B). Double-reciprocal plots of serial dilutions of JNJ-28871063 assayed at various total ATP concentrations also demonstrated an ATP competitive pattern of inhibition of the ErbB2 kinase, with each line intersecting the *y*-axis at approximately the same point (Bohinski, 1983) (Fig. 1C).

In Vitro Kinase Selectivity Profile. To characterize the selectivity of inhibition, compounds were tested in duplicate at a single concentration of 1 or 3 μM for activity against a representative panel of 210 tyrosine and serine/threonine kinases in the presence of 10 μM ATP (Upstate Biotechnology; Invitrogen). The percentage inhibition of control is presented as a heat map in Fig. 2. Lapatinib was the most selective inhibitor, with two off-target kinases inhibited between 50 and 80% and two off target kinases inhibited greater than 80%. JNJ-28871063 inhibited six off-target kinases between 50 and 80% and 1 off target kinase greater than 80%. Gefitinib and erlotinib were less selective, with 24 off-target kinases inhibited greater than 50% and 12 off-target kinases inhibited greater than 80%. The specific off-

TABLE 1

In vitro activity of JNJ-28871063 and comparators

Inhibition of kinase activity was determined in vitro with human recombinant enzymes. Each measurement was performed at least in duplicate and IC_{50} values were calculated in 2 – 8 separate experiments

ErbB family kinase	IC_{50}			
	JNJ-28871063	Lapatinib	Gefitinib	Erlotinib
	nM			
Inhibition of ErbB kinase activity				
ErbB1 (EGFR)	22 ± 10	11 ± 0.5[a]	11 ± 5	16 ± 1
ErbB2 (HER2)	38 ± 28	9 ± 0.75[a]	85 ± 3	281 ± 18
ErbB4 (HER4)	21 ± 4	367 ± 4[a]	210 ± 8	472 ± 32
Inhibition of ErbB receptor phosphorylation in cells				
SKBR3[b]	223 ± 34	32 ± 1	4311 ± 6	>100,000
A431[c]	107 ± 46	63 ± 13	9 ± 2	5 ± 2

[a] Rusnak et al. (2001).
[b] Inhibition of unstimulated HER2 receptor phosphorylation.
[c] Inhibition of EGF-stimulated EGFR receptor phosphorylation.

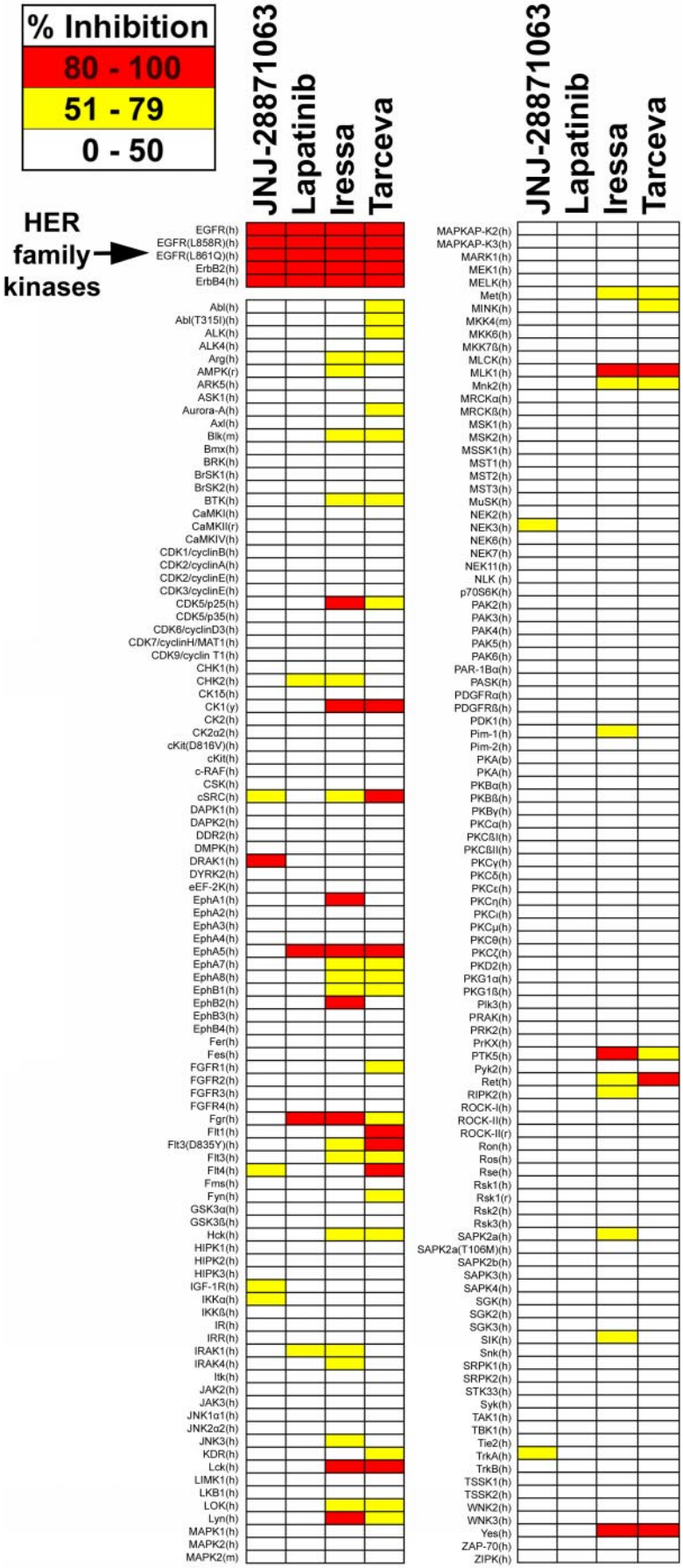

Fig. 2. Compounds were evaluated at a single concentration (3 μM) in duplicate in the presence of 10 μM ATP for inhibition of tyrosine and serine/threonine kinases to determine their selectivity profile. Kinases that were inhibited by 80% or more were as follows: JNJ-28871063: DRAK1; lapatinib: EphA5, Fgr; gefitinib: CDK5/p25, CK1, EphA1, EphA5, EphB2, Fgr, Lck, Lyn, MLK1, PTK5, Yes; erlotinib: CK1, cSrc, Flt1, Flt3(D835Y), Flt4, Lck, MLK1, Ret, Yes.

target kinases inhibited greater than 80% are listed in the legend to Fig. 2.

JNJ-28871063 Inhibited Proliferation of ErbB-Overexpressing Cells. The ability of kinase inhibitors to repress the growth of cell lines expressing various levels of EGFR and ErbB2 was evaluated. JNJ-28871063 demonstrated potent growth inhibition in vitro of human cancer cell lines overexpressing the ErbB2 receptor with IC_{50} values in the range of 60 to 168 nM (Table 2). JNJ-28871063 also inhibited growth of the EGFR-overexpressing cell line A431 in the same range as other EGFR kinase inhibitors but was less potent on the HN5 line than the other inhibitors. JNJ-28871063 and lapatinib showed greater inhibition of ErbB2-overexpressing cell lines than gefitinib and erlotinib but were nearly as potent in the EGFR-overexpressing A431 cells. Antiproliferative activity of specific ErbB kinase inhibitors on non-ErbB2/EGFR-overexpressing cells is expected be much lower and probably operates through a mechanism other than ErbB2/EGFR inhibition. JNJ-28871063 exhibited the least effect at inhibiting growth of non–ErbB-overexpressing cell lines whose growth is not driven by ErbB signaling (Table 2). Expression levels of ErbB2 and EGFR in all cell lines were confirmed by immunoblotting (data not shown).

Inhibition of ErbB Phosphorylation in Cells. To determine whether JNJ-28871063 or comparator compounds were able to penetrate into cells and reduce phosphorylation levels of the ErbB2 receptor, a sandwich ELISA was developed in a 96-well format. JNJ-28871063 was able to reduce the basal level of ErbB2 phosphorylation in SKBR3 cells, indicating that the compound effectively reaches its intracellular target and interferes with activation of the ErbB2 receptor. In this assay, JNJ-28871063 and lapatinib were 19 ± 3- and 135 ± 4-fold more potent, respectively, than gefitinib in blocking total ErbB2 receptor phosphorylation in SKBR3 cells, whereas erlotinib showed no effect on ErbB2 phosphorylation (Table 1). An ELISA assay was also developed to measure the ability of compounds to block EGF-stimulated EGFR phosphorylation in the A431 cell line. JNJ-28871063 and lapatinib were both able to potently inhibit ligand induced EGFR phosphorylation in EGFR-overexpressing cells but were less effective than gefitinib and erlotinib (Table 1).

ErbB Receptor Phosphorylation and Signaling. The ability of compounds to inhibit activation of individual phosphotyrosine residues was examined in both EGFR- and ErbB2-overexpressing cells and is presented in Fig. 3. To activate EGFR receptors and signaling in EGFR-overexpressing A431 cells, the cells were serum-starved and treated with compound before stimulation with 100 ng/ml EGF. ErbB2-overexpressing BT474 cells were not stimulated because they maintain a high level of ErbB2 activation and signaling as a result of receptor overexpression (Yarden and Sliwkowski, 2001). Cells were treated with a single concentration of drug (3 μM) representative of maximum efficacious plasma levels achieved in nude mouse tumor models. In BT474 cells, gefitinib, JNJ-28871063, and lapatinib inhibited phosphorylation on Tyr877 to a greater extent than erlotinib. JNJ-28871063 and lapatinib were more potent in blocking Tyr1221 phosphorylation than the EGFR inhibitors gefitinib and erlotinib, whereas erlotinib was the only compound that could not completely inhibit Tyr1248 phosphorylation at this concentration. All compounds blocked MAPK phosphorylation on Thr202/Tyr204 and Thr218/Tyr220 residues. The upper band in the phospho-ERK and total ERK blots is probably a nonspecific band that was observed in all samples in this cell line. JNJ-28871063, lapatinib, and gefitinib inhibited phosphorylation of AKT on Ser473 and PLCγ on Tyr783, whereas erlotinib showed some reduction of phosphorylation on these proteins but was less effective. In the EGFR-overexpressing cell line A431, lapatinib and gefitinib were equally effective at blocking EGF-stimulated phosphorylation of the EGFR on Tyr845, Tyr1068, Tyr1148 and Tyr1173. JNJ-28871063 and erlotinib were also equipotent but less effective than lapatinib and gefitinib at inhibiting phosphorylation on these residues. All compounds reduced EGFR phosphorylation on Tyr992 to basal (unstimulated) levels; however, this residue remained highly phosphorylated even after starvation and very little induction was achieved after EGF treatment. All compounds reduced AKT phosphorylation on AKT S473 to unstimulated levels; only gefitinib significantly reduced MAPK phosphorylation. The effects on MAPK activation observed in the immunoblots resulted from a 16-h exposure to compound and do not parallel the growth inhibition obtained from a 96-h treatment period seen in the A431 cell proliferation assays (Table 2).

JNJ-28871063 Penetrates Tumors and Crosses the Blood Brain Barrier. The tissue distribution of JNJ-28871063 and lapatinib was examined in nude mice bearing A431 tumor xenografts by measuring the amount of drug present in plasma, tumor, and brain after a single oral dose of 100 mg/kg. The 1- and 2-h time points were selected for analysis of drug levels in the brain because preliminary pharmacokinetic studies showed that peak drug plasma levels were present at 1 h after oral dosing for JNJ-28871063 and lapatinib in both mice and rats. After 1 h, both JNJ-28871063 and lapatinib produced micromolar drug plasma concentra-

TABLE 2
Inhibition of cell proliferation by ErbB inhibitors

Cell Line (Receptor Overexpressed)	IC_{50}			
	JNJ-28871063	Lapatinib	Gefitinib	Erlotinib
	nM			
N87 (HER2)	159 ± 23	13 ± 5	377 ± 125	2013 ± 102
BT474 (HER2)	60 ± 6	14 ± 5	487 ± 218	4890 ± 1039
SKBR3 (HER2)	168 ± 20	9 ± 1	1172 ± 257	5807 ± 847
A431 (EGFR)	702 ± 153	395 ± 156	825 ± 106	650 ± 118
HN5 (EGFR)	516 ± 76	42 ± 3	32 ± 5	41 ± 1
HeLa	>10,000	>10,000	>10,000	>10,000
A375	>10,000	>10,000	>10,000	>10,000
HCT116	>10,000	>10,000	>10,000	>10,000
HT29	>10,000	2035 ± 271	4220 ± 459	9917 ± 61
MRC5 primary fibroblasts	>10,000	5671 ± 401	8795 ± 800	1864 ± 218

tions. JNJ-28871063 accumulated to slightly higher drug levels in tumors than found in plasma, whereas there were lower levels of lapatinib in tumors than found in plasma. JNJ-28871063 penetrated into the brain and was present at concentrations double those found in plasma, whereas lapatinib showed very little penetration into the brain (Fig. 4). Drug concentrations were also measured in this experiment after 2 h, and brain/tumor/plasma drug levels were all slightly lower than at 1 h, but the trend was the same as that seen at the 1-h time point (data not shown). In a separate experiment, drug levels in plasma and tumor were measured in mice bearing N87 xenografts and JNJ-28871063 accumulated to higher levels in tumor tissue (data not shown). After single-dose i.v. administration in rats, JNJ-28871063 was also able to penetrate into the brain and accumulate to 2.5-fold higher levels than present in plasma, whereas the ability of lapatinib to penetrate into the brain was extremely low (data not shown).

Inhibition of Human Tumor Xenograft Growth. The antitumor activity of JNJ-28871063 was evaluated in several human tumor xenograft models. Before initiating treatment, tumors were implanted and allowed to establish growth for 7 to 14 days. Results of these studies are summarized in Table 3 for the various tumor types.

The N87 gastric carcinoma overexpresses ErbB2 as a result of gene amplification (Kasprzyk et al., 1992). In both N87 models, JNJ-28871063 was formulated as a suspension in 0.5% methylcellulose and administered p.o. at 100 mg/kg daily for 30 days. In the first N87 model, trastuzumab was reconstituted in PBS as recommended by the manufacturer and administered i.p. at 20 mg/kg twice a week for 4 weeks.

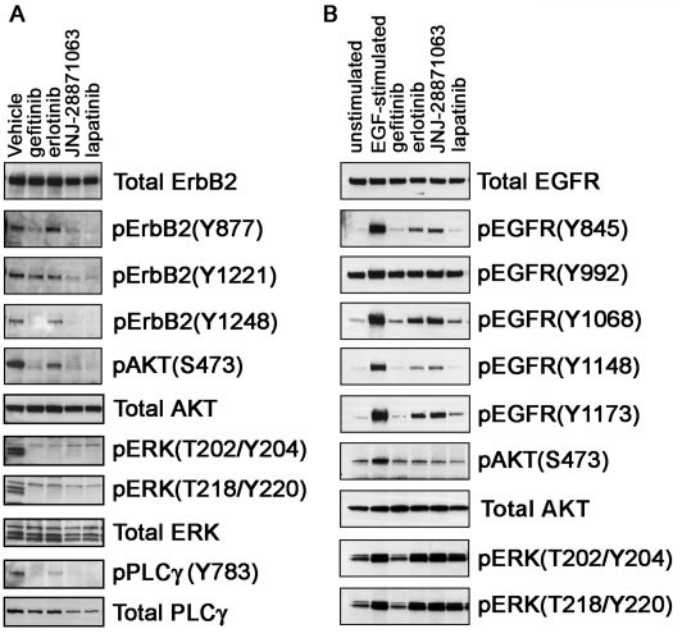

Fig. 3. Inhibition of ErbB receptor phosphorylation and signal transduction. The ability of inhibitors to block ErbB2 phosphorylation in BT474 cells (A) and EGF-stimulated EGFR phosphorylation in A431 cells (B) was determined by immunoblotting. A431 cells were serum-starved before stimulation with EGF; BT474 cells were not starved. Cells were treated with vehicle alone or 3 μM compound for 16 h. Equal amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Each membrane was probed with β-actin as an internal control (data not shown). Representative data are shown.

JNJ-28871063 showed significant inhibition of tumor growth (TGI = 71%, $p = 0.006$), whereas trastuzumab showed slight but not significant inhibition of tumor growth (TGI = 11.4%, $p = 0.486$) (Fig. 5A and Table 3). In a second N87 model, JNJ-28871063 again showed a significant TGI of 69.4% ($p = 0.003$); lapatinib also resulted in significant tumor inhibition (TGI = 70.1%, $p = 0.029$), whereas gefitinib (Iressa, ZD1839; AstraZeneca) did not significantly inhibit tumor growth (Fig. 5B and Table 3). JNJ-28871063 was also evaluated in an A431 human tumor xenograft model to assess its inhibitory effects on EGFR-overexpressing tumor growth. The A431 epidermoid carcinoma exhibits gene amplification for the EGFR, and in this cell line, EGFR activates signaling pathways responsible for driving proliferation, invasion, and survival of cancer cells (Kawamoto et al., 1983). JNJ-28871063 was formulated as a suspension in 0.5% methylcellulose and administered p.o. at 100 mg/kg daily for 30 days. Gefitinib and lapatinib were used as comparators in this study and were prepared and dosed p.o. at 100 mg/kg in the same vehicle and on the same schedule as JNJ-28871063. The experiment started dosing on day 10 (10 days after tumor inoculation) and was terminated on day 35. Inhibition of tumor growth is shown graphically in Fig. 5C, and the summary of the TGI values and percent body weight loss for this study are presented in Table 3. JNJ-28871063 produced a significant inhibition of tumor growth at 100 mg/kg (TGI = 66.8%; $p = 0.025$). Gefitinib caused regression of established tumors and completely inhibited tumor growth at 100 mg/kg (TGI = 94.5%; $p = 0.002$). Lapatinib resulted in a 9.4% TGI, which was not significant ($p = 0.596$). All groups experienced a net weight gain over the course of the study, and no treatment-related deaths were recorded in any group. JNJ-28871063 was evaluated in an estrogen-dependent BT474 human breast carcinoma tumor xenograft model in CB.17 SCID mice. Trastuzumab was formulated as above and administered i.p. at 12.5 mg/kg twice a week for 4 weeks. JNJ-28871063 and lapatinib were formulated in 0.5% methylcellulose as suspensions and administered p.o. twice per day for 30 days. In CB.17 SCID mice, JNJ-28871063 dosed at 200 mg/kg produced equivalent plasma levels to lapatinib dosed at 100 mg/kg. In nude mice, both compounds resulted in equivalent drug plasma levels from a 100 mg/kg dose (data not shown). Because of the pharmacokinetic differences in the two mouse strains, JNJ-28871063 was dosed at 200 mg/kg in the BT474 model to keep exposure levels equal. Both JNJ-28871063 and lapatinib produced significant tumor growth inhibition of 61.8 and 71.9% TGI relative to control. Trastuzumab produced a 65.8% TGI, but this was not significant because of the wide range of tumor sizes in this group ($p = 0.06$) (Fig. 5D and Table 3).

HER2 Pharmacodynamic Assay. A pharmacodynamic assay was used to assess the effects of drug treatments on ErbB2 phosphorylation levels in athymic mice bearing N87 tumor xenografts. Mice were implanted with N87 tumors that were allowed to establish to a size of 300 to 600 mg. A single oral dose of JNJ-28871063 was administered p.o., tumors were excised at various times after dosing, and equal amounts of protein were evaluated in the ErbB2 phosphorylation ELISA. JNJ-28871063 significantly reduced the phosphorylation level of ErbB2 in nude mice bearing N87 human tumor xenografts after oral administration. Maximal reduction of ErbB2 phosphorylation (63.1%) was observed in the

tumors sampled 1 h after administration of 100 mg/kg compound, but a reduction of 44.9% in ErbB2 phosphorylation was still apparent 4 h after dosing (Fig. 5E).

Efficacy in an ErbB2-Overexpressing Intracranial Model. To determine whether the ability of JNJ-28871063 to penetrate into the brain would translate to efficacy against ErbB2-overexpressing tumors within the brain, an N87 intracranial model was carried out. Intracranial injection of N87 cells in control mice produced clinical signs by day 19 and the first death as a result of tumor progression on day 32. The median TTE of untreated control animals was 41.5 days. Twice daily treatment beginning on day 1 with 200 mg/kg JNJ-28871063 resulted in a median TTE of 54.5 days and significantly extended survival by 31.3% ($p = 0.002$). In the JNJ-28871063–treated group the first death as a result of tumor progression did not occur until day 52 (Fig. 5F).

Discussion

JNJ-28871063 is a pan-ErbB kinase inhibitor from a novel structural class that is equipotent against the three recombinant erbB kinases with active catalytic domains (ErbB1, -2, and -4; IC_{50} = 21–38 nM). Kinetic studies indicate that the mechanism of inhibition is by an ATP-competitive mode. The compound did not inhibit more than 200 other kinases greater than 50% at concentrations of 1 or 3 μM and has a unique inhibitory profile compared with quinazoline ErbB kinase inhibitors, including gefitinib and erlotinib, which selectively target EGFR kinase, or lapatinib, a pan-ErbB kinase inhibitor. JNJ-28871063 shows superior inhibition of the ErbB4 kinase relative to lapatinib and the other comparators in this study. Although the contribution of ErbB4 in driving proliferation of tumors is not clear, ligands have been identified that bind only to ErbB4, which can activate ErbB2 and EGFR through heterodimer formation (Carraway and Cantley, 1994). JNJ-28871063 blocked the growth of cells that overexpress ErbB2 and reduced phosphorylation of the ErbB2 receptor in such cells. JNJ-28871063 and lapatinib were at least 10-fold more potent inhibitors of ErbB2-overexpressing cell growth than the EGFR-targeted compound erlotinib and were also more potent than gefitinib, which showed some activity toward slowing ErbB2-overexpressing cell proliferation. All compounds were in the same potency range for slowing EGFR-overexpressing cell growth and reducing EGF-stimulated EGFR phosphorylation in the A431 cell line, but JNJ-28871063 was 10-fold less efficacious than the comparators at inhibiting growth of the HN5 cell line. This difference cannot be explained by potency against EGFR kinase alone and may be due to genetic factors unique to the HN5 cell line. Treatment of ErbB-overexpressing cells with tyrosine kinase inhibitors that block ErbB phosphorylation prevents growth by enhancing internalization, ubiquitination and proteasomal degradation of the receptor (Citri et al., 2002). Growth inhibition was specific for cells overexpressing ErbB receptors, because greater than 14-fold higher concentrations of JNJ-28871063 failed to inhibit the growth of cells that do not overexpress ErbB2 or EGFR. JNJ-28871063 was the most specific for ErbB-overexpressing cells and showed no antiproliferative effects at the highest concentration tested on cell types not dependent on ErbB signaling for growth. In a pharmacodynamic assay with animals bearing ErbB2-overexpressing tumors, a single dose of JNJ-28871063 was able to reduce the level of ErbB2 phosphorylation in the tumors for more than 4 h after dosing. The cellular activity in EGFR- and ErbB2-overexpressing tumor lines revealed distinct profiles of activity for the EGFR- and pan-ErbB–targeted compounds. Both JNJ-28871063 and lapatinib equally inhibited phosphorylation of the ErbB2 receptor in BT474 breast carcinoma cells on tyrosine residues responsible for activating downstream

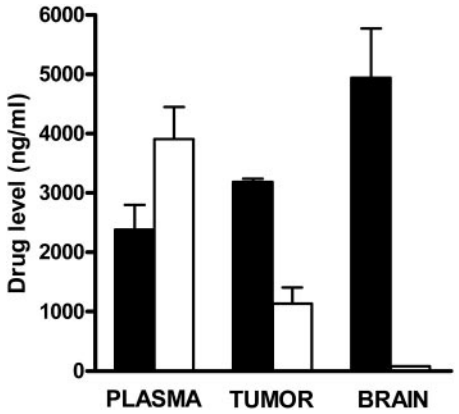

Fig. 4. Drug plasma and tissue distribution of JNJ-28871063 and lapatinib in nude mice 1 h after 100 mg/kg p.o. Nude mice bearing A431 xenografts were given a single oral dose of compound. Drug levels were determined in plasma, tumor and brain 1 h after dosing. Filled bars represent drug levels for JNJ-28871063, and open bars represent lapatinib. All groups were set up with $n = 4$ and the data presented are means; bars, S.E.

TABLE 3

In vivo activity of JNJ-28871063 in human tumor xenograft models

All dosing was carried out in groups of 8 mice. TGI was calculated as the difference between the change in control and drug-treated tumor volumes on the last day of the study. Statistical significance was evaluated by comparing the mean tumor size of control groups with drug-treated groups using a two-tailed Student's *t* test.

Graph	Tumor Type	Compound	Dose	Route	Schedule	TGI	P Value
			mg/kg			%	
A	N87	JNJ-28871063	100	p.o.	qd × 35	71.0	0.006
A	N87	Trastuzumab	20	i.p.	biw × 4	11.41	0.486
B	N87	JNJ-28871063	100	p.o.	qd × 30	69.4	0.003
B	N87	Gefitinib	100	p.o.	qd × 30	36.35	0.403
B	N87	Lapatinib	100	p.o.	qd × 30	70.1	0.029
C	A431	JNJ-28871063	100	p.o.	qd × 25	66.8	0.025
C	A431	Gefitinib	100	p.o.	qd × 25	94.5	0.002
C	A431	Lapatinib	100	p.o.	qd × 25	9.4	0.596
D	BT474	JNJ-28871063	200	p.o.	bid × 30	61.8	0.035
D	BT474	Lapatinib	100	p.o.	bid × 30	76.9	0.049
D	BT474	Trastuzumab	12.5	i.p.	biw × 4	65.8	0.06

p.o., oral gavage; i.p., intraperitoneal; qd, every day; bid, twice per day; biw, twice per week.

growth pathways at the single concentration examined (3 μM). In ErbB2-overexpressing BT474 cells, JNJ-28871063 and lapatinib strongly inhibited the Tyr1221 and Tyr1248 residues that are the major autophosphorylation sites on ErbB2 and activate the MAPK pathway (Hazan et al., 1990) or, in the case of Tyr1248, may mediate transforming potential of ErbB2 (Ben-Levy et al., 1994). Gefitinib showed some reduction of ErbB2 receptor phosphorylation on Tyr877 and Tyr1248 but had less effect on Tyr1221 than JNJ-28871063 and lapatinib, whereas erlotinib had no effect, which may be explained by the slightly higher in vitro potency of gefitinib against the ErbB2 kinase. The Tyr877 residue couples ErbB2 to Src but does not modulate ErbB2 catalytic function or mitogenic activity (Segatto et al., 1990). Erlotinib was less effective than the other compounds at inhibiting AKT phosphorylation on Ser473, which is required for mediating cell survival and blocking proapoptotic factors such as Bad, caspase-9, and forkhead (Burgering and Coffer, 1995). We were surprised to find that erlotinib inhibited MAPK and PLCγ activation in BT474 cells, although it showed no effect on ErbB2 receptor phosphorylation. These effects may be mediated through inhibition of EGFR transactivation although BT474 cells express low levels of EGFR. In EGFR-overexpressing A431 cells, JNJ-28871063 inhibited EGF-stimulated phosphorylation of all residues examined, including the major autophosphorylation sites Tyr1068, Tyr1148, and Tyr1173 as well as Tyr845 and Tyr992 that serve as minor sites activating pathways leading to DNA synthesis and cell division (Biscardi et al., 1999). The Tyr992 residue is a binding site for PLCγ (Emlet et al., 1997), whereas Tyr1068 serves as a binding site for the Grb2/SH2

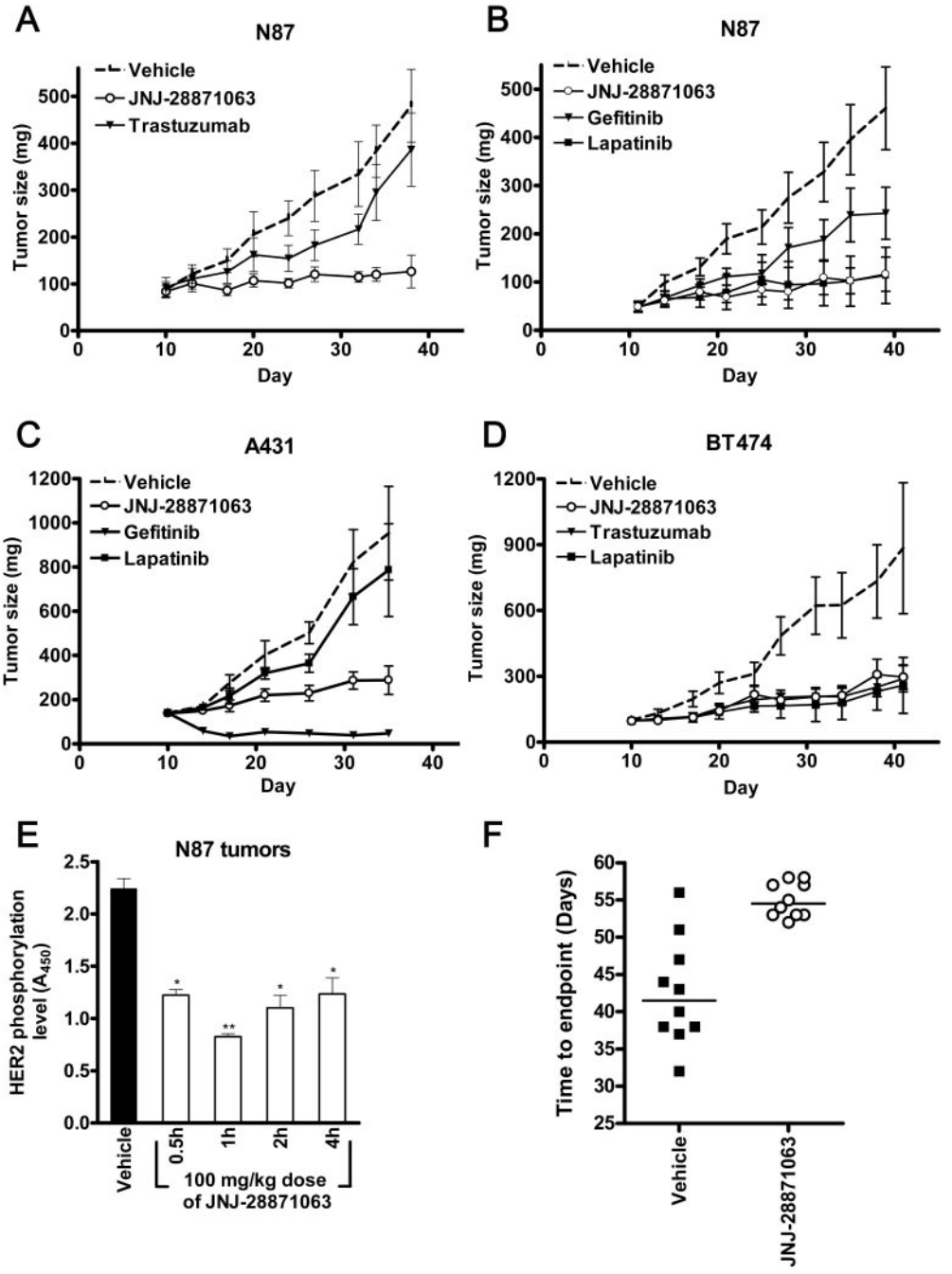

Fig. 5. In vivo effects of ErbB inhibitors. A, N87 ErbB2-overexpressing gastric carcinoma model with trastuzumab. JNJ-28871063 was formulated in 0.5% methylcellulose and administered p.o. at 100 mg/kg daily for 30 days schedule. Trastuzumab (Genentech, San Francisco, CA) was resuspended in PBS and dosed i.p. at 20 mg/kg twice a week for 4 weeks. B, N87 ErbB2-overexpressing gastric carcinoma model with gefitinib and lapatinib. All compounds were formulated in 0.5% methylcellulose and administered p.o. at 100 mg/kg daily for 30 days. C, A431 EGFR-overexpressing epidermoid carcinoma. All compounds were formulated in 0.5% methylcellulose and administered p.o. at 100 mg/kg daily for 25 days. D, BT474 ErbB2-overexpressing breast carcinoma model. JNJ-28871063 was formulated in 0.5% methylcellulose and administered p.o. at 200 mg/kg twice daily for 30 days. Lapatinib was formulated in 0.5% methylcellulose and administered p.o. at 100 mg/kg twice daily for 30 days. Drug plasma levels for JNJ-28871063 and lapatinib were equivalent under this dosing regimen in CB.17 SCID mice as measured over a 4-h period after a single dose (data not shown). Trastuzumab was administered i.p. in PBS at 12.5 mg/kg twice a week for 4 weeks. Data points represent means for eight mice in all treatment groups for N87 and A431 models and 10 mice for BT474 model. Bars, S.E. E, nude mice bearing N87 tumor xenografts were administered a single 100 mg/kg dose of JNJ-28871063 and tumors were removed at 0.5 to 4 h after dosing. The total phosphorylation level of the ErbB2 receptor was determined from three mice at each time point in an ELISA assay as described under *Materials and Methods*. The ErbB2 phosphorylation level for vehicle treated mice did not change over time. F, N87 intracranial model. 5×10^4 cells were injected intracranially, and animals ($n = 10$) were treated with vehicle or 200 mg/kg JNJ-28871063 daily for 50 days. The TTE is indicated for each animal, and the horizontal line denotes the median for each group.

domain and is essential for MAPK activation (Rojas et al., 1996). The Tyr1173 site is important for EGF-mediated signal transduction and is also required for the enhanced tumorigenicity of the EGFRvIII mutant receptor (Su Huang et al., 1997). JNJ-28871063 demonstrated activity equivalent to erlotinib at blocking receptor activation but was less effective than gefitinib and lapatinib. All compounds inhibited AKT phosphorylation equally, but gefitinib was the only compound able to reduce MAP kinase phosphorylation to basal levels in this cell line. It is noteworthy that lapatinib was more active than erlotinib and as effective as gefitinib, although the in vitro potency on purified EGFR kinase is quite similar for all the compounds examined. A431 cells express very low levels of ErbB2, so the differences in activity may result from other factors, such as cell permeability, stability of the compounds, or off-target effects. JNJ-28871063 showed a activity profile similar to that of erlotinib in reducing EGFR phosphorylation in A431 cells but, as mentioned above, was more effective than erlotinib at reducing ErbB2 phosphorylation in BT474 cells.

JNJ-28871063 was highly efficacious in human tumor xenograft tumor models that overexpress EGFR or ErbB2. In particular, JNJ-28871063 potently inhibited the growth of the N87 gastric carcinoma that expresses high levels of ErbB2. In this model, JNJ-28871063 was more effective than the anti-ErbB2 antibody trastuzumab and the small molecule gefitinib (which targets the EGFR), and was equally effective compared with the pan-ErbB kinase inhibitor lapatinib. In xenograft models with the EGFR-overexpressing cell line A431, JNJ-28871063 inhibited growth by >80% and was more efficacious than lapatinib, but neither compound caused tumor regression that can be obtained with gefitinib.

Studies in nude mice indicated that JNJ-28871063 accumulates into tumor tissue at higher levels than found in plasma, giving this compound favorable pharmacokinetic properties for suppressing ErbB receptor phosphorylation in tumors. The ability to penetrate and accumulate into the brain in nude mice after oral administration and rats after i.v. administration (data not shown) also suggests that JNJ-28871063 may be efficacious against ErbB2-overexpressing intracranial metastases. In an N87 ErbB2-overexpressing intracranial model, JNJ-28871063 showed a statistically significant increase in survival over untreated mice after oral administration. The ability to penetrate into brain tissue could have therapeutic advantage because EGFR is often overexpressed in primary brain tumors and also in breast and non–small-cell lung carcinomas that frequently metastasize to the brain (Wikstrand et al., 1995). Furthermore, patients with ErbB2-overexpressing breast cancer frequently experience metastases to the brain (Kirsch and Hochberg, 2003). These patients have an extremely poor prognosis, and intracerebral tumors are often the cause of death. Autopsies revealed that 20 to 30% of patients who die of breast cancer have brain metastases (Grossi et al., 2003). Although there are preliminary reports of clinical activity against ErbB2-overexpressing brain metastases (Lin et al., 2006), lapatinib was unable to penetrate into the brain of nude mice after oral administration. Whether lapatinib can cross the BBB in humans has yet to be published. However, the ability of a compound to cross the BBB in rodents does not predict for the human situation. In addition, the BBB is often compromised in cancer patients, and the blood supply to intracranial tumors is abnormal, with vasculature developing a tortuous and leaky conformation.

JNJ-28871063 is an orally available pan-ErbB inhibitor with a unique activity profile in cellular and animal models. Its ability to penetrate into the brain may result in enhanced activity against ErbB-overexpressing tumors that find refuge in the brain and provide a therapeutic benefit over many conventional therapies. Furthermore, small molecule kinase inhibitors may retain activity against tumors expressing the EGFRvIII mutant receptor that contains a truncated extracellular domain (Nishikawa et al., 1994), whereas binding of antibodies targeting the EGFR wild-type extracellular domain may be compromised.

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