

High Levels of HER-2 Expression Alter the Ability of Epidermal Growth Factor Receptor (EGFR) Family Tyrosine Kinase Inhibitors to Inhibit EGFR Phosphorylation *in Vivo*

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ABSTRACT

The epidermal growth factor receptor (EGFR) and HER-2 tyrosine kinases have been implicated in the development, progression, and severity of several human cancers and are attractive targets for therapeutic intervention. SU11925 was developed as a small molecule inhibitor of the tyrosine kinase activity of both EGFR and HER-2. In cellular assays, SU11925 exhibited similar potency against EGFR and HER-2, inhibiting EGF-stimulated EGFR autophosphorylation in A431 (human epidermoid carcinoma) cells with an IC_{50} of 30 nM and HER-2 phosphorylation in SK-OV-3TTP5 (human ovarian carcinoma) cells with an IC_{50} of 38 nM. In contrast to its similar activity against the two targets in cellular assays, ~10-fold higher plasma concentrations of SU11925 were required to inhibit HER-2 phosphorylation in HER-2-overexpressing tumors compared with EGFR phosphorylation in EGFR-overexpressing tumors *in vivo*. Consistent with the proposed mechanism of action of this inhibitor, SU11925 inhibited the s.c. growth of EGFR- and HER-2-dependent tumors in athymic mice at doses that produced substantial inhibition of target receptor phosphorylation *in vivo*. An unexpected finding from these studies was that higher plasma concentrations of SU11925 were required to inhibit EGFR phosphorylation *in vivo* in tumors that also express high levels of HER-2 than in tumors that express EGFR alone. This observation, which suggests that it is more difficult to inhibit EGFR phosphorylation *in vivo* in cells that express high levels of HER-2, was confirmed with ZD1839 (Iressa), a selective EGFR inhibitor that also targets the tyrosine kinase catalytic site. The potential clinical implications of this observation are discussed.

INTRODUCTION

RTKs,² which initiate specific intracellular signaling pathways in response to binding of extracellular growth factors, are known to play important roles in regulating normal cell growth. The four members of the ErbB (or EGFR) family of RTKs play important roles in regulating a wide variety of cellular functions, including regulation of mitogenesis, cell death, angiogenesis, and cell differentiation (1, 2). In addition, members of the ErbB family have been shown to be overexpressed or altered in a number of human cancers, including mammary, ovarian, non-small cell lung, glioblastoma, prostate, pancreas, head and neck, and other cancers (3–5). Many studies have indicated that overexpression of these receptors correlates with the development and progression of several human cancers, as well as with poor prognosis (6–13). Consequently, members of this family of RTKs have been identified as attractive candidates for targeted cancer therapy (reviewed in Refs. 1 and 5).

The ErbB family members exist as monomers spanning the plasma membrane of the cell. After binding of the appropriate soluble extracellular ligand, the monomeric receptors dimerize and become functionally active. The diverse effects of EGFR family RTKs on cell function and signaling pathways are mediated via activation of their RTK activity by receptor-selective ligands (*e.g.*, EGF, transforming growth factor- α , heregulins, betacellulin; Refs. 14–16). Regulation of receptor signal transduction is also mediated by the formation of a variety of homo- and heterodimers with other ErbB family members, depending on the relative abundance of the different receptors and ligands (15–18).

Several small molecule inhibitors targeting the tyrosine kinase domains of specific RTKs are under development as therapeutic agents to treat a variety of cancers. In the case of the ErbB family, the preclinical efficacy of EGFR-selective, small molecule tyrosine kinase inhibitors [*i.e.*, ZD1839 (Iressa, Astra-Zeneca) and OSI-774] in EGFR-dependent tumor models has been well characterized (19–21). In clinical trials, these compounds have shown evidence of antitumor activity against cancers for which EGFR commonly has been shown to play a significant role in the molecular pathogenesis (22–26). However, little is known about the effects of small molecule inhibitors on EGFR and HER-2 in HER-2-dependent tumor models and cancers. This is of particular interest because the gene for HER-2 has been shown to be amplified and overexpressed in a

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² The abbreviations used are: RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; ZD1839, (3-chloro-4-fluoro-phenyl)-{7-methoxy-6-(3-morpholin-4-yl-propoxy)-quinazolin-4-yl}-amine; SU11925, (3-chloro-4-fluoro-phenylamino)-5-{1-[5-((3R,5S)-3,5-dimethyl-piperazine-1-carbonyl)-3-methyl-1H-pyrrol-2-yl]-meth-(Z)-ylidene}-5,7-dihydro-pyrrolo{2,3-d}pyrimidin-6-one.

significant portion of human breast and ovarian cancers, resulting in high levels of expression of the *HER-2* gene product (12, 27). Because the *HER-2* receptor is the preferred dimerization partner for other ErbB/EGFR family members (15, 16), its overexpression results in a shift in the equilibrium of receptor dimerization patterns toward the formation of *HER-2* homodimers and heterodimers. In addition, because the *HER-2* receptor has no known direct-binding, soluble ligand and is often constitutively tyrosine phosphorylated *in vivo*, this shift in equilibrium would also favor signal transduction mediated through *HER-2* homo- and heterodimers (16).

Amplification and overexpression of the *HER-2* gene has been shown to correlate with poor prognosis in breast and ovarian cancer (12, 27). This demonstration, combined with the knowledge that *HER-2* is the preferred dimerization partner for the other ErbB family members and that EGFR/*HER-2* heterodimer formation mediates sustained receptor signaling (15), has led to the suggestion that an inhibitor targeting both EGFR and *HER-2* would have potential advantages over a selective inhibitor targeting either receptor alone. SU11925 was developed as a pyrrolo-pyrimidinone-based small molecule inhibitor with equipotent inhibition of the tyrosine kinase activity of both EGFR and *HER-2*. The primary goal of the present study was to determine the ability of this compound, as a prototype, dual-specificity EGFR/*HER-2* inhibitor, to inhibit *in vivo* receptor phosphorylation and tumor growth in EGFR- and *HER-2*-dependent cancer models.

MATERIALS AND METHODS

Compounds

SU11925 and ZD1839 (Fig. 1; Ref. 28) were synthesized at SUGEN, Inc.³

Cells

A431 human epidermoid carcinoma and SK-OV-3 human ovarian carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). SK-OV-3TP5 cells were derived from SK-OV-3 cells passed five times as s.c. tumors in athymic mice. NIH3T3/*HER-2* cells were derived from NIH3T3 mouse fibroblasts that were engineered to overexpress full-length human *HER-2* protein (29). Cells were propagated as monolayers in DMEM (A431 and NIH3T3/*HER-2* cells) or McCoy's 5A medium (SK-OV-3 and SK-OV-3TP5 cells) supplemented with 2% fetal bovine serum (Life Technologies, Inc., Bethesda, MD).

Cell Kinase Assay

A431 or SK-OV-3TP5 cells were seeded at low density and grown to 80% confluence in six-well plates containing the appropriate growth medium (see above). When the cells reached 80% confluence, the growth medium was replaced with the corresponding serum-free medium containing 0.1% BSA. Twenty-four h later, serial dilutions of the inhibitor (SU11925 or ZD1839) were added to individual wells to produce the desired

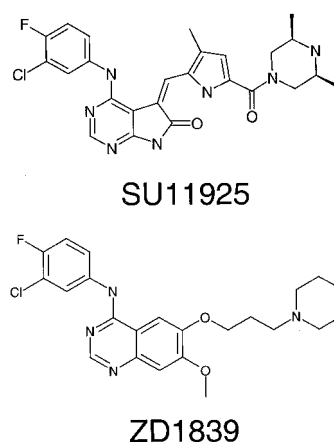


Fig. 1 Structures of SU11925 and ZD1839 (Iressa).

final inhibitor concentration. After the addition of the inhibitor 2.5 h later, A431 cells were stimulated with EGF (20 ng/ml; R & D Systems, Minneapolis, MN) for 5 min; SK-OV-3TP5 and NIH3T3/*HER-2* cells were not stimulated. Cells were then washed with cold PBS, lysed in 0.2 ml/well of HNTG buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM magnesium chloride, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 0.5 mM sodium fluoride, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride] for 10 min, and the lysates were collected as the supernatant fraction after a 12-min centrifugation at 15,000 \times g. The amount of protein in each lysate was determined with the bicinchoninic acid procedure (Pierce, Rockford, IL) according to the manufacturer's instructions with BSA as the standard.

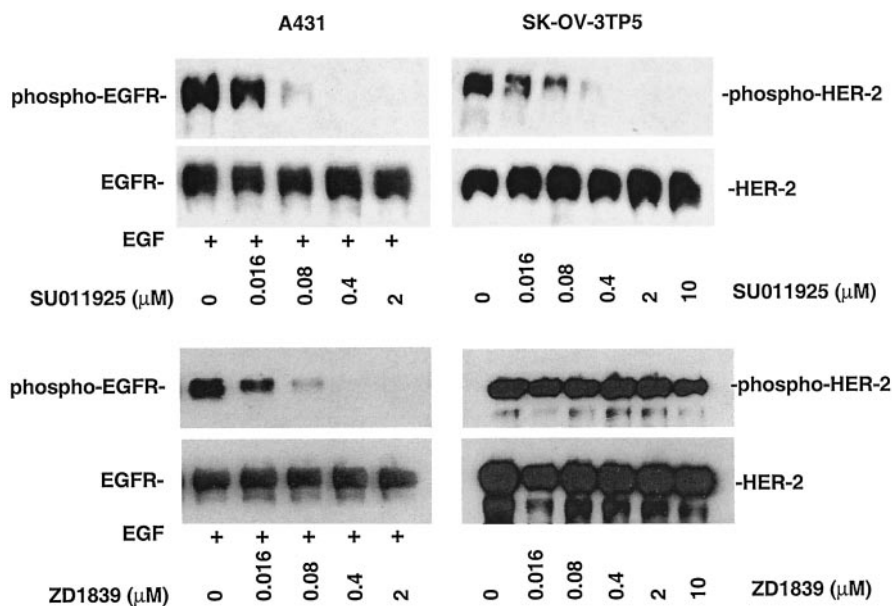
Total EGFR or *HER-2* was immunoprecipitated from 0.5 mg of cell lysate protein with the addition of an antibody directed against EGFR (SUMO1, SUGEN reagent) or *HER-2* (SUMO2, SUGEN reagent) for 1 h at 4°C, followed by capture of the antibody/receptor complexes on protein G-agarose (United Biomedical Inc., Lake Placid, NY). Immunoprecipitated protein was resolved by SDS-PAGE electrophoresis and electrotransferred to a nitrocellulose membrane. The membrane was blocked for 1 h in TBST [10 mM Tris (pH 7.4), 150 mM NaCl, and 1% Triton X-100] containing 5% nonfat dry milk and incubated overnight at 4°C with antibodies specific for EGFR (sc-03; Santa Cruz Biotechnology, Santa Cruz, CA), EGFR activation loop phosphorylation site Y1173 (05-483; United Biomedical, Inc.), *HER-2* (OP15; Calbiochem, San Diego, CA), or *HER-2* activation loop phosphorylation site Y1248 (clone PN2A; NeoMarkers, Lab Vision Corp., Fremont, CA). After washing, the membranes were incubated with antimouse or antirabbit horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed again, and developed by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham Pharmacia Biotechnology, Piscataway, NJ).

In Vivo Studies

Animals. Female athymic mice (Balb/c, *nu/nu*) were obtained from Charles River Breeding Laboratories (Wilmington,

³ L. Sun, manuscript in preparation.

Fig. 2 Effect of SU11925 and ZD1839 on EGF-induced EGFR autophosphorylation in A431 cells or HER-2 phosphorylation in SK-OV-3TP5 cells. Cells were exposed to the indicated concentrations of SU11925 or ZD1839 for 2.5 h. A431 cells were then stimulated with EGF (20 ng/ml, denoted by +), for 5 min; SK-OV-3TP5 cells were left unstimulated. EGFR and HER-2 protein expression and phosphorylation state were determined after immunoprecipitation of the indicated receptor from tumor lysate and subsequent immunoblot analysis as described in "Materials and Methods." Results shown are representative of two independent experiments.



MA). Animals were housed on a 12-h light/dark cycle with food and water provided *ad libitum* in a barrier facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Animals were examined before the initiation of experiments to ensure that they were healthy and acclimated to the laboratory environment. All procedures were conducted in accordance with the Institute for Laboratory Animal Research *Guide for the Care and Use of Laboratory Animals* and with SUGEN Animal Care and Use Committee guidelines.

Efficacy Studies. Efficacy studies in mice bearing A431, SK-OV-3TP5, or NIH3T3/HER-2 tumor models were performed as described previously (30). Tumor cells ($5\text{--}10 \times 10^6$ cells/animal) were implanted *s.c.* into the hindflank region of mice on day 0. Daily treatment with inhibitor was initiated when tumors were $\sim 50 \text{ mm}^3$ in size. Test substances were administered by oral gavage (100 μl) in a Cremaphor-based vehicle or by *i.p.* injection (50 μl) in DMSO with doses based on group average weight. Tumors were measured twice weekly with Vernier calipers, and tumor volumes were calculated as the product of length \times width \times height.

***In Vivo* Target Modulation Studies.** For *in vivo* target modulation studies, *s.c.* tumors were allowed to grow untreated to a size of $\sim 500 \text{ mm}^3$. Mice were then administered a single dose of inhibitor. At the indicated times after dosing, individual mice were sacrificed, their tumors were resected, and a blood sample was taken from the cardiac left ventricle with a heparin sulfate-primed syringe. In experiments using A431 tumor-bearing mice, EGF (50 μg) was injected into the tail vein of each mouse 30 min before the animal was sacrificed.

Resected tumors were immediately frozen and pulverized with a liquid nitrogen-cooled cryomortar and pestle. Cold HNTG buffer was used to extract protein from individual powdered tumors, and the amount of phosphorylated and total EGFR or HER-2 in each sample was determined by Western blot analysis of immunoprecipitated RTK as described above.

Plasma was obtained by centrifuging individual blood samples at $3000 \times g$ for 10 min at 4°C in an Eppendorf 5417R refrigerated centrifuge. Plasma samples were stored at -80°C until they could be analyzed for drug concentration by liquid chromatography-tandem mass spectrometry. Briefly, standards for SU11925 or ZD1839 were prepared in blank mouse plasma. Plasma samples (100 μl) or standards in mouse plasma were added into 400 μl of acetonitrile. The mixture was then filtered with a 3M Empore 96-Well PPT Filter Plate (3M, Minneapolis, MN). The amount of compound in each filtrate sample was quantified by liquid chromatography-tandem mass spectrometry based on standard curves generated with known amounts of the compounds.

RESULTS

Effect of SU11925 on Cellular EGFR and HER-2 RTK Activity *in Vitro*. Inhibition of EGFR tyrosine kinase activity was evaluated in human A431 cells, which express high levels of EGFR protein with little or no detectable HER-2 in culture (data not shown; Ref. 31). Human SK-OV-3TP5 cells, which express high levels of HER-2 and low but detectable levels of EGFR (data not shown; Ref. 32) and NIH3T3/HER-2 cells, which normally express EGFR and were engineered to express high levels of human HER-2, were used to evaluate inhibition of HER-2 phosphorylation.

As shown in Fig. 2, SU11925 (structure shown in Fig. 1) was a potent inhibitor of both EGFR and HER-2 tyrosine kinase activity in the cell-based assay. The activity of this compound was similar against the two targets, with IC_{50} s of 30 and 38 nM against EGF-stimulated EGFR tyrosine autophosphorylation (Y1173) in A431 cells and HER-2 tyrosine phosphorylation (Y1248) in SK-OV-3TP5 cells, respectively (Table 1). The activity of SU11925 against HER-2 phosphorylation was confirmed using NIH3T3/HER-2 cells. In these cells, SU11925 inhib-

Table 1 Summary of the *in vitro* activities of SU11925 and ZD1839

Compound and cell line	EGFR	
	IC ₅₀ (cell)	HER-2 IC ₅₀ (cell)
SU11925		
A431	30 nM	NA ^a
NIH3T3/HER-2	NA	75 nM
SK-OV-3TP5	NA	38 nM
ZD1839		
A431	30 nM	NA
NIH3T3/HER-2	NA	ND
SK-OV-3TP5	NA	>2000 nM

^a NA, not applicable; cell lines did not express receptor in sufficient quantity for analysis. ND, not determined.

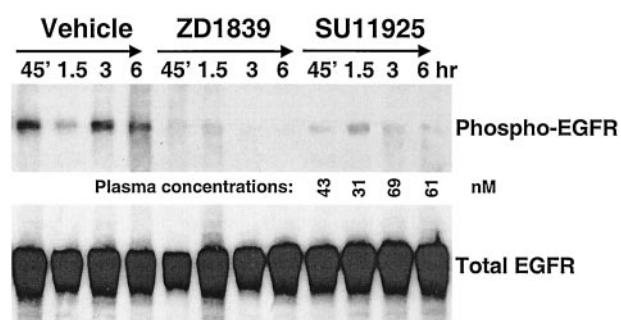


Fig. 3 Effects of oral administration of SU11925 or ZD1839 on EGF-induced EGFR autophosphorylation in A431 tumors *in vivo*. Athymic mice bearing A431 tumors were given a single p.o. dose (100 mg/kg) of SU11925, ZD1839, or vehicle alone. EGF (50 µg) was injected into the tail vein of each mouse 30 min before sacrifice. At the time of sacrifice (45 min and 1.5, 3, and 6 h postdose), plasma was collected from each mouse, and tumor lysates were prepared as described in "Materials and Methods." The relative abundance of phospho-EGFR and total EGFR in the tumor lysates was determined by immunoblot analysis. The plasma SU11925 concentration determined for each animal at the time of tumor harvest is listed below the corresponding lane. Results shown are representative of two independent experiments.

ited HER-2 phosphorylation with an IC₅₀ of 75 nM, which was similar to the value obtained in SK-OV-3TP5 cells (Table 1).

For comparison, the activity of ZD1839 (structure shown in Fig. 1), a selective EGFR tyrosine kinase inhibitor, was also tested in these cells. Consistent with previous data (23), ZD1839 was a potent inhibitor of EGF-stimulated EGFR tyrosine autophosphorylation in A431 cells (IC₅₀, 30 nM), but exhibited weak activity against HER-2 phosphorylation in SK-OV-3TP5 cells (IC₅₀, >2000 nM; Fig. 2; Table 1) and NIH3T3/HER-2 cells (IC₅₀, >5000 nM; Table 1).

Effect of SU11925 and ZD1839 on EGFR Phosphorylation *in Vivo* in an EGFR-dependent Tumor Xenograft.

Because both SU11925 and ZD1839 were able to inhibit EGFR phosphorylation in cells, the ability of these compounds to inhibit EGFR phosphorylation *in vivo* was investigated in mice bearing A431 tumors. As shown in Fig. 3, oral administration of a single dose of either SU11925 or ZD1839 at 100 mg/kg resulted in a substantial degree of inhibition of EGF-stimulated EGFR autophosphorylation in A431 tumors through 6 h. On the basis of measurements of the amount of inhibitor in each of the plasma samples, it was determined that plasma concentrations of

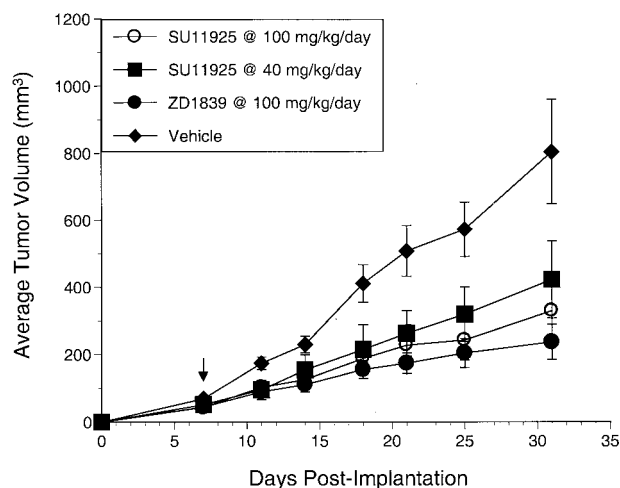


Fig. 4 Effect of SU11925 and ZD1839 on growth of established A431 xenografts. Athymic mice bearing established (~50 mm³) A431 tumors were given daily oral doses of SU11925 (40 or 100 mg/kg/day), ZD1839 (100 mg/kg/day), or vehicle alone starting on day 7 and continuing for the duration of the experiment. At least eight mice were included in each group. Results shown indicate mean tumor volumes and are representative of two independent experiments; bars, SE.

SU11925 between 31 and 67 nM (16–35 ng/ml) were sufficient to effect a substantial inhibition of EGFR phosphorylation *in vivo*. A slightly lower degree of inhibition was observed in the animal model with the lowest plasma concentration (31 nM), suggesting that the plasma concentrations of SU11925 in this experiment were near the minimal plasma levels required to inhibit EGFR phosphorylation *in vivo*. Plasma concentrations of ZD1839 were not determined in this experiment, although plasma concentrations of ZD1839 in excess of 2500 nM (1000 ng/ml) were measured for >8 h after administration of a 100 mg/kg oral dose in several other experiments (compare Fig. 5B). However, lower doses of ZD1839 were not tested; therefore, the lowest plasma concentration of ZD1839 required to inhibit EGFR phosphorylation *in vivo* could not be determined.

The ability of SU11925 and ZD1839 to inhibit EGFR phosphorylation *in vivo* correlated with inhibition of tumor growth. As shown in Fig. 4, daily p.o. administration of SU11925 and ZD1839 effectively inhibited the growth of A431 tumors. Antitumor efficacy calculations were expressed as the percentage of tumor growth inhibition ($1 - T/C \times 100$, where T and C are the mean treated tumor mass and the mean control tumor mass, respectively) on the final treatment day ($n \geq 8$ animals/group). Consistent with the data from the target modulation studies described above, SU11925 and ZD1839 at 100 mg/kg/day resulted in a similar degree of inhibition, with ZD1839 and SU11925 causing 71 and 59% inhibition of tumor growth, respectively. A slight dose-response relationship was observed for SU11925 with the 100 and 40 mg/kg/day dosages causing 59 and 48% inhibition of tumor growth, respectively. Collectively, the results of these experiments indicated that both SU11925 and ZD1839 inhibited tumor EGFR phosphorylation *in vivo*, which correlated with the inhibition of growth of EGFR-dependent A431 tumors in athymic mice.

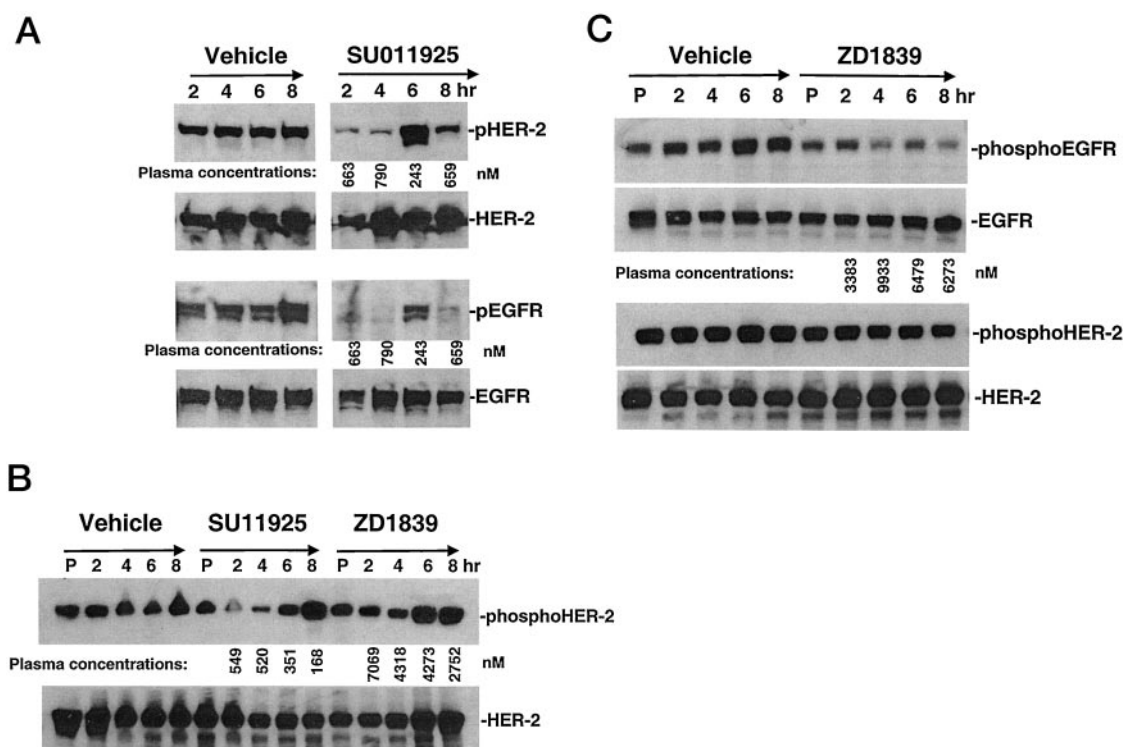


Fig. 5 Effects of SU11925 (A) or ZD1839 (B) on EGFR and HER-2 phosphorylation in NIH3T3/HER-2 or SK-OV-3 (C) tumors *in vivo*. A, athymic mice bearing NIH3T3/HER-2 tumors were administered SU11925 i.p. at 80 mg/kg/day or DMSO vehicle alone for 4 days. B, athymic mice bearing NIH3T3/HER-2 tumors were orally administered ZD1839 at 100 mg/kg or Cremaphor-based vehicle for 2 days. C, athymic mice bearing human SK-OV-3 tumors were orally administered SU11925 i.p. at 80 mg/kg/day for 4 days or ZD1839 at 100 mg/kg for 2 days, or DMSO-based vehicle i.p. for 4 days. Before dosing (P) and at 2, 4, 6, and 8 h after the last dose, mice were euthanized, plasma was collected from each mouse, and tumor lysates were prepared as described in "Materials and Methods." The relative abundance of phospho-HER-2 and total HER-2 or phospho-EGFR and total EGFR in the tumor lysates was determined by immunoblot analysis. The plasma SU11925 or ZD1839 concentration determined for each animal at the time of tumor harvest is listed below the corresponding lane. Results shown are representative of at least two independent experiments.

Effect of SU11925 and ZD1839 on EGFR and HER-2 Receptor Phosphorylation *in Vivo* in HER-2-dependent Tumor Xenografts. The *in vivo* activities of SU11925 and ZD1839 were also evaluated in the HER-2-dependent NIH3T3/HER-2 and SK-OV-3TP5 tumor models. Each of these tumor models were shown to express high levels of HER-2 and their growth *in vivo* was effectively inhibited by Herceptin, a monoclonal antibody targeting the HER-2 protein, suggesting that HER-2 plays a role in the growth of these tumors (data not shown).

As described above, SU11925 exhibited similar inhibitory activity against EGFR and HER-2 RTKs in cell-based assays. Therefore, the 100 mg/kg oral dose of SU11925 was tested for its ability to inhibit HER-2 receptor phosphorylation *in vivo* in mice bearing NIH3T3/HER-2 tumors. Although the 100 mg/kg dose of SU11925 caused substantial inhibition of EGFR phosphorylation in A431 tumors (Fig. 3), it had no effect on HER-2 phosphorylation or growth of NIH3T3/HER-2 tumors (data not shown).

To determine whether higher plasma levels of SU11925 were required to inhibit HER-2 phosphorylation in NIH3T3/HER-2 tumors, SU11925 was administered to tumor-bearing mice by i.p. injection. As shown in Fig. 5A, i.p. administration of SU11925 at 80 mg/kg/day for 4 days resulted in the inhibition

of EGFR and HER-2 phosphorylation in NIH3T3/HER-2 tumors at three of the four time points after the last dose. Evaluation of the SU11925 plasma concentrations of mice given SU11925 by i.p. injections confirmed that higher levels of SU11925 (mean, 600 nM) were achieved with i.p. administration compared with oral administration (mean, 50 nM). Also, higher SU11925 plasma levels (663, 790, and 659 nM) were detected in the three mice with evidence of inhibition of EGFR and HER-2 receptor phosphorylation than in the one mouse (243 nM) in which phosphorylation was not inhibited (Fig. 5A). The effect of SU11925 on HER-2 tyrosine phosphorylation was also evaluated in SK-OV-3TP5 xenografts. In this model system, i.p. administration of SU11925 at 80 mg/kg resulted in partial inhibition of HER-2 receptor phosphorylation in mice with SU11925 mouse plasma concentrations of 549 and 520 nM, but no evidence of inhibition was observed in mice with plasma concentrations of 351 or 168 nM (Fig. 5B). Taken together, these data suggested that plasma SU11925 concentrations >400 nM (~200 ng/ml) were required to inhibit EGFR and HER-2 receptor phosphorylation in tumors that express high levels of HER-2 receptor *in vivo*. Thus, although SU11925 exhibited similar activity against EGFR and HER-2 receptors in cell-based assays, these results suggested that roughly 10-fold higher concentrations of SU11925 were required to inhibit EGFR and

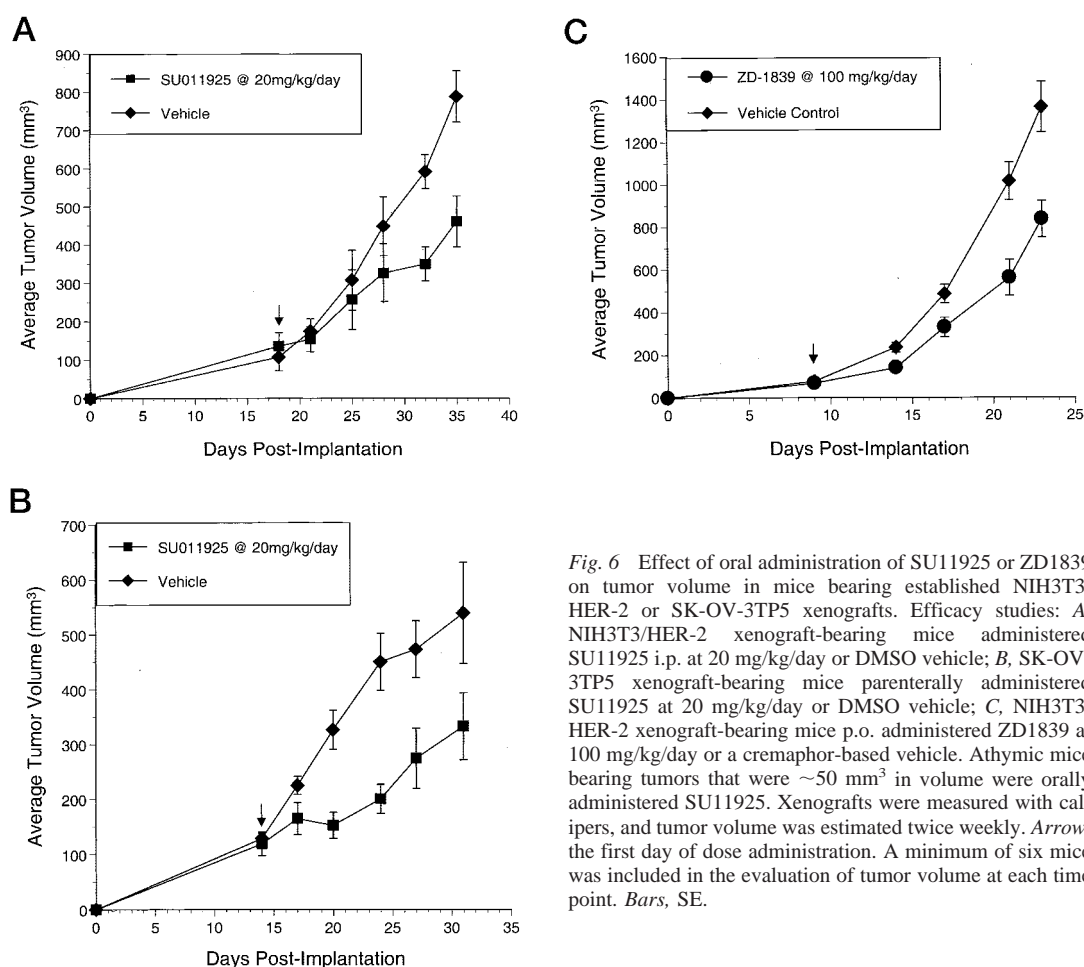


Fig. 6 Effect of oral administration of SU11925 or ZD1839 on tumor volume in mice bearing established NIH3T3/HER-2 or SK-OV-3TP5 xenografts. Efficacy studies: **A**, NIH3T3/HER-2 xenograft-bearing mice administered SU11925 i.p. at 20 mg/kg/day or DMSO vehicle; **B**, SK-OV-3TP5 xenograft-bearing mice parenterally administered SU11925 at 20 mg/kg/day or DMSO vehicle; **C**, NIH3T3/HER-2 xenograft-bearing mice p.o. administered ZD1839 at 100 mg/kg/day or a cremaphor-based vehicle. Athymic mice bearing tumors that were ~ 50 mm³ in volume were orally administered SU11925. Xenografts were measured with calipers, and tumor volume was estimated twice weekly. *Arrow*, the first day of dose administration. A minimum of six mice was included in the evaluation of tumor volume at each time point. *Bars*, SE.

HER-2 phosphorylation in HER-2-dependent xenografts compared with an EGFR-dependent xenograft *in vivo*.

To test whether the reduced sensitivity of EGFR to pharmacological inhibition of phosphorylation was specific to SU11925, the ability of ZD1839 to inhibit EGFR phosphorylation in the NIH3T3/HER-2 tumors was investigated. As shown in Fig. 5C, p.o. administration of ZD1839 at 100 mg/kg resulted in partial inhibition of EGFR phosphorylation in the NIH3T3/HER-2 tumors. However, the inhibition observed was to a much lesser extent compared with the complete inhibition of EGFR phosphorylation observed in A431 tumors at the same dose (Fig. 3). Thus, the specific EGFR inhibitor ZD1839 also showed a marked decrease in its ability to inhibit EGFR phosphorylation *in vivo* in tumors expressing high amounts of HER-2. As expected, ZD1839 had little if any effect on HER-2 phosphorylation in NIH3T3/HER-2 (Fig. 5C) or SK-OV-3TP5 (Fig. 5B) tumors.

The ability of SU11925 to inhibit the growth of HER-2-overexpressing tumors was evaluated in mice bearing small established (~ 50 – 100 mm³) NIH3T3/HER-2 or SK-OV-3TP5 xenografts. Daily i.p. administration of SU11925 at 20 mg/kg/day to athymic mice bearing established NIH3T3/HER-2 and SK-OV-3TP5 tumors resulted in 41 and 55% inhibition of tumor

growth, respectively (Fig. 6, A and B). Analysis of plasma from mice dosed i.p. at 20 mg/kg indicated that SU11925 plasma levels were comparable with those measured in mice dosed i.p. at 80 mg/kg (compare Fig. 5A). Thus, the antitumor efficacy observed in these HER-2-dependent xenograft models was consistent with the *in vivo* inhibition of HER-2-receptor phosphorylation.

The ability of ZD1839 to inhibit the growth of HER-2-dependent tumors was also evaluated in mice bearing established NIH3T3/HER-2 or SK-OV-3TP5 xenografts. Daily p.o. administration of ZD1839 at 100 mg/kg/day to mice bearing established NIH3T3/HER-2 tumors resulted in a 29% inhibition of tumor growth (Fig. 6C), whereas no effect of ZD1839 treatment was observed in the SK-OV-3TP5 model (data not shown). The ability of ZD1839 to partially inhibit the growth of the NIH3T3/HER-2 tumors is likely attributable to its effect on EGFR rather than any effect on HER-2 (compare Fig. 5C).

DISCUSSION

Several mechanism-based targeted therapies are currently being developed for the treatment of specific cancers. Among these are agents that target the ErbB (or EGFR) family of RTKs.

Herceptin, a monoclonal antibody directed against the extracellular domain of HER-2, has been approved for the treatment of breast tumors that express high levels of HER-2. In addition, EGFR-selective agents such as the tyrosine kinase inhibitors ZD1839, OSI-774, and the monoclonal antibody C225 (cetuximab) are in clinical trials for the treatment of patients with tumors commonly expressing EGFR. These agents have shown encouraging clinical results (22, 24–26, 33, 34). However, some patients have not responded to these targeted therapies despite demonstration of the expression of the appropriate receptor (22, 24–26, 33, 34), suggesting that there continues to be a need to more fully understand the ability of these agents to inhibit their target in the context of genetically diverse cancers.

Although there is an increasing amount of information describing the genetic heterogeneity of human cancers, the factors that specifically influence their responsiveness to targeted therapies are not understood. This is particularly true in the case of the ErbB family of RTKs. Numerous studies indicate that ErbB family members form homo- and heterodimers with each other, that they respond to a number of receptor-selective soluble ligands, and that they interact with a large number of intracellular signaling proteins (15, 16). As a result of these studies, an effort to develop bifunctional inhibitors capable of inhibiting the tyrosine kinase activity of both the EGFR and HER-2 receptors has been initiated. The rationale for choosing these two targets is based on three points: (a) EGFR and HER-2 are commonly overexpressed in human cancers (3–5); (b) HER-2 is the preferred dimerization partner for other members of the ErbB family and, therefore, most likely to be included in functional dimers when it is expressed at high levels (16); and (c) EGFR/HER-2 heterodimers are a functionally potent signaling combination (15).

In this study, we have evaluated SU11925, a small molecule inhibitor with equivalent activity against EGFR and HER-2, the EGFR-selective inhibitor ZD1839 (Iressa), in s.c. xenograft tumor models that express high levels of EGFR in the absence of HER-2 (A431) or that express high levels of HER-2 (NIH3T3/HER-2 or SK-OV-3TP5) in the presence of EGFR.

In A431 tumors, which overexpress EGFR in the absence of HER-2, the dual-function inhibitor and selective inhibitor each effectively inhibited the growth of A431 tumors at dosages that produced substantial inhibition of EGFR phosphorylation *in vivo*. In NIH3T3/HER-2 and SK-OV-3TP5 tumors, which express high levels of HER-2, only the dual-function inhibitor SU11925 substantially inhibited HER-2 phosphorylation *in vivo*. Consequently, SU11925 inhibited growth to a greater extent in these tumors, although ZD1839 partially inhibited growth of NIH3T3/HER-2 tumors, perhaps because of its ability to partially inhibit EGFR phosphorylation in this tumor.

Although the results summarized thus far would have been predicted based on the activity profile of the two inhibitors determined in cell-based assays, there were two unanticipated findings from these experiments. The first was that the plasma concentration of SU11925 required to inhibit HER-2 phosphorylation in HER-2-dependent tumors *in vivo* (~600 nM) was ~10-fold higher than that required to inhibit EGFR phosphorylation (~45 nM), although the two RTKs exhibited similar sensitivity to the inhibitor in cell-based assays (Table 1). This observation suggests a fundamental difference in EGFR/HER-2

signaling in tumors. One possible explanation for this difference is that although the majority of EGFR phosphorylation in the A431 model was EGF stimulated in both the cell-based assay and *in vivo*, HER-2 is either constitutively phosphorylated or phosphorylated in response to binding of an unknown endogenous ligand *in vivo*. Factors that could also influence HER-2 signal transduction *in vivo* also include cell-cell contact, cell-extracellular matrix interaction, and direct interaction of the receptor with components in the extracellular matrix. HER-2 has been shown to physically interact with catenins linking the receptor with cadherin-mediated cell-cell adhesion (35, 36). HER-2 has also been shown to interact with integrins and influence integrin-mediated signal transduction linking HER-2 with cell-extracellular matrix interactions (37–40). Direct interaction of the extracellular domain of HER-2 with components of the extracellular matrix has also been reported, although the consequence of this interaction is not known (41). Thus, there are several factors that could potentially account for difficulty in the pharmacological inhibition of HER-2 phosphorylation in its natural setting (*i.e.*, *in vivo*) compared with a cell culture setting.

The second unexpected finding was that higher plasma concentrations of SU11925 (~500 nM) and ZD1839 (~7500 nM) were required to inhibit EGFR phosphorylation in NIH3T3/HER-2 tumors, which express high levels of HER-2 relative to EGFR, compared with A431 tumors, which express primarily EGFR (~45 nM for SU11925; <2500 nM for ZD1839). In the case of SU11925, the dual-specificity inhibitor, there was a difference ~10-fold in sensitivity of EGFR under the two conditions. We were not able to make a similar quantitative determination for the EGFR-specific inhibitor ZD1839 because the lowest plasma concentration of inhibitor required to block EGFR phosphorylation in A431 tumors *in vivo* was not determined. However, it was apparent that plasma ZD1839 concentrations that completely inhibited EGFR phosphorylation in A431 tumors *in vivo* had only a partial effect on EGFR phosphorylation in NIH3T3/HER-2 tumors (compare the results in Fig. 2 with those of Fig. 4B).

The exact reason for the effect of high HER-2 expression on the reduced susceptibility of EGFR phosphorylation to inhibition in these models is not clear. Because HER-2 is the preferred dimerization partner for ErbB family members and is expressed at high levels in NIH3T3/HER-2 tumors, the equilibrium of dimerization patterns of EGFR family members would shift primarily from EGFR-containing dimers in A431 xenografts to dimers containing the HER-2 receptor in NIH3T3/HER-2 tumors. Although the effects of SU11925 and ZD1839 on the homo- and heterodimerized ErbB family members are not known, the results from the present study indicate that pharmacological inhibition of EGFR phosphorylation *in vivo* does depend on the relative levels of expression of EGFR and HER-2, perhaps because HER-2 may be at least partially responsible for EGFR phosphorylation and activation under these conditions (15, 16). The 10-fold difference in sensitivity of EGFR to pharmacological inhibition in the presence of high levels of HER-2 in these models suggests that higher drug exposures would be required to achieve efficacy under conditions in which HER-2 is the primary ErbB family member. In this respect, the difference in sensitivity in treating tumors expressing high levels of HER-2 could prove to be an issue attributable to the

demonstration of the potential for mechanism-based, dose-limiting toxicities observed for EGFR-selective inhibitors (*i.e.*, EGFR-dependent skin rash and gastrointestinal toxicities). However, additional studies using other tumor models with intermediate receptor expression patterns and heterogeneous genetic profiles would be useful in the further understanding and clinical translation of these findings.

In conclusion, the results of this study, which is the first to compare the *in vivo* activities of an EGFR-specific and a dual function EGFR/HER-2 inhibitor in EGFR- and HER-2-dependent tumors models, indicate that both types of inhibitors can effectively inhibit the growth of tumors that express the targeted receptor. In this respect, these findings support the continued optimism that this class of inhibitors will have a very significant impact for the patients suffering from cancers that rely on signaling via the EGFR and HER-2 receptors. However, another conclusion of this study is that higher concentrations of inhibitor may be required to inhibit EGFR phosphorylation *in vivo* in tumors expressing high levels of HER-2 than in tumors that express high levels of EGFR alone. This finding underscores the need to know the receptor expression pattern in the tumor of an individual patient to determine the appropriate dose of these inhibitors and to identify patient populations most likely to benefit from treatment. It also suggests the necessity of evaluating the activity of these promising compounds in models that represent the full range of genetic heterogeneity in which these inhibitors are likely to be used in clinical practice.

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High Levels of HER-2 Expression Alter the Ability of Epidermal Growth Factor Receptor (EGFR) Family Tyrosine Kinase Inhibitors to Inhibit EGFR Phosphorylation *in Vivo*

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