Preclinical Antitumor Activity of BMS-599626, a pan-HER Kinase Inhibitor That Inhibits HER1/HER2 Homodimer and Heterodimer Signaling

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Abstract

Purpose: The studies described here are intended to characterize the ability of BMS-599626, a small-molecule inhibitor of the human epidermal growth factor receptor (HER) kinase family, to modulate signaling and growth of tumor cells that depend on HER1 and/or HER2.

Experimental Design: The potency and selectivity of BMS-599626 were assessed in biochemical assays using recombinant protein kinases, as well as in cell proliferation assays using tumor cell lines with varying degrees of dependence on HER1 or HER2 signaling. Modulation of receptor signaling was determined in cell assays by Western blot analyses of receptor autophosphorylation and downstream signaling. The ability of BMS-599626 to inhibit receptor heterodimer signaling in tumor cells was studied by receptor coimmunoprecipitation. Antitumor activity of BMS-599626 was evaluated using a number of different xenograft models that represent a spectrum of human tumors with HER1 or HER2 overexpression.

Results: BMS-599626 inhibited HER1 and HER2 with IC_{50} of 20 and 30 nmol/L, respectively, and was highly selective when tested against a broad panel of diverse protein kinases. Biochemical studies suggested that BMS-599626 inhibited HER1 and HER2 through distinct mechanisms. BMS-599626 abrogated HER1 and HER2 signaling and inhibited the proliferation of tumor cell lines that are dependent on these receptors, with IC_{50} in the range of 0.24 to 1 μmol/L. BMS-599626 was highly selective for tumor cells that depend on HER1/HER2 and had no effect on the proliferation of cell lines that do not express these receptors. In tumor cells that are capable of forming HER1/HER2 heterodimers, BMS-599626 inhibited heterodimerization and downstream signaling. BMS-599626 had antitumor activity in models that overexpress HER1 (GEO), as well as in models that have *HER2* gene amplification (KPL4) or overexpression (Sal2), and there was good correlation between the inhibition of receptor signaling and antitumor activity.

Conclusions: BMS-599626 is a highly selective and potent inhibitor of HER1 and HER2 kinases and inhibits tumor cell proliferation through modulation of receptor signaling. BMS-599626 inhibits HER1/HER2 receptor heterodimerization and provides an additional mechanism of inhibiting tumors in which receptor coexpression and heterodimerization play a major role in driving tumor growth. The preclinical data support the advancement of BMS-599626 into clinical development for the treatment of cancer.

The human epidermal growth factor (EGF) receptor (HER) family consists of four distinct polypeptides, each with a cytoplasmic sequence that is homologous to other protein tyrosine kinases (1). HER1 (also referred to as the EGF receptor) has been shown to be overexpressed in the majority of solid tumors and can be activated by either autocrine expression of ligand(s) or paracrine ligand expression by stromal elements (2). HER1 expression has been shown in as much as 80% to 90% of colon and non–small-cell lung cancer (3). Data on the expression of ligands in tumors are rather limited, but at least one ligand (transforming growth factor α) has been shown to be coexpressed with HER1 in some tumors (4–6). HER2 is encoded by a single gene on human chromosome 17, which is a frequent site of gene amplification in breast cancer. *HER2* gene amplification and overexpression of the protein have been shown to occur in ~30% of all breast cancer (7). Clinical studies have shown unequivocally that *HER2* gene amplification is a prognostic indicator for poor outcome in breast cancer

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(8, 9). In addition to breast cancer, there is good correlation between *HER2* gene amplification and overexpression in gastric, salivary gland, and bladder cancer (10–13). However, recent studies have shown that many tumor cells that do not show gene amplification nevertheless overexpress HER2 protein, albeit to levels that are below those found in cells with gene amplification. A number of pharmacologic agents have been evaluated for the therapeutic treatment of tumors that overexpress HER1 or HER2. Trastuzumab is a humanized monoclonal antibody that binds specifically to the extracellular juxtamembrane sequence of HER2 (14). Trastuzumab was approved in 1999 by the Food and Drug Administration for the treatment of metastatic breast cancer in patients with tumors that have *HER2* gene amplification. Despite the clinical efficacy of trastuzumab, there remains a significant unmet medical need for patients with tumors that express HER2. As a single agent, trastuzumab is efficacious in 35% of patients with high HER2 overexpression (3+), but not in those with intermediate or low level of receptor expression (15, 16). In addition, trastuzumab use is associated with a significant incidence of cardiac toxicity in patients who had prior treatment with anthracyclins (17). Although neuregulin/HER signaling has been shown to be indispensable for cardiac development, the molecular mechanism of the cardiotoxicity of trastuzumab has yet to be fully established (18). It therefore remains plausible that inhibition of HER2 signaling by kinase inhibitors may have a toxicity profile distinct from that of trastuzumab. HER1 is the target of a number of antibodies as well as small-molecule kinase inhibitors that have been in clinical development. Gefitinib and erlotinib are synthetic inhibitors of the HER1 kinase activity and were approved in the United States for the treatment of advanced non–small-cell lung cancer (19, 20). Cetuximab is a chimeric monoclonal antibody that specifically targets HER1 and has been approved for the treatment of advanced colorectal cancer (21). The anti-HER1 antibodies are highly selective for HER1, whereas the HER1 kinase inhibitors also possess weak inhibitory activity towards HER2.

In cultured cells, biological signaling and transformation by HER1 requires the dimerization and activation of the receptor by a ligand. By contrast, overexpresson of HER2 is sufficient to result in receptor activation and cell transformation. Coexpression of HER1 and HER2 has been shown in breast, ovarian, and bladder tumors, as well as in squamous cell carcinomas of the head and neck (22–24). Because HER1 and HER2 form heterodimers that are activated by EGF and related ligands, heterodimer signaling is believed to play a significant role in the pathobiology of these tumors. Receptor coexpression in tumor cells suggests that targeting both HER1 and HER2 will be more effective in modulating proliferation than inhibiting either receptor alone (25). In support of this concept, treatment of ovarian tumor cells, using a combination of antibodies to HER1 and HER2, resulted in synergistic efficacy compared with treatment with either antibody alone (26). The epidemiology of HER1 and HER2 expression (both homo- and heterodimers) and the fact that Herceptin has provided effective treatment for only a subpopulation of HER2-expressing tumors have provided a rationale for identifying small-molecule kinase modulators that inhibit both receptors (25). Although early examples of HER1 kinase inhibitors had only weak affinity for HER2, extensive sequence homology (83% identity) between the two kinase domains suggests that it would be feasible to identify compounds that inhibit both receptors with comparable potency. Recently, quinazoline and pyrrolotriazine analogues that are dual inhibitors of HER1 and HER2 kinases have been identified (27, 28). Here we describe the properties of a novel pan-HER kinase inhibitor with broad spectrum antitumor activity, which has advanced into clinical testing.

Materials and Methods

Protein kinase assays. Unless specified otherwise, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). The entire cytoplasmic sequences of HER1, HER2, and HER4 were expressed as recombinant proteins in Sf9 insect cells. HER1 and HER4 were expressed as fusion proteins with glutathione-*S*-transferase and were purified by affinity chromatography on glutathione-*S*-Sepharose (Amersham Biosciences, Piscataway, NJ). HER2 was subcloned into the pBlueBac4 vector (Invitrogen, Carlsbad, CA) and expressed as an untagged protein using an internal methionine codon (M687) for translation initiation. The truncated HER2 protein was isolated by chromatography on a column of DEAE-Sepharose (Amersham Biosciences) equilibrated in a buffer that contained 0.1 mol/L NaCl, and the recombinant protein was eluted with a buffer containing 0.3 mol/L NaCl. For the HER kinase assays, reaction volumes were 50 μL and contained 10 ng of glutathione-*S*-transferase fusion protein or 150 ng of partially purified HER2. The mixtures also contained 1.5 μmol/L poly(Glu/Tyr) (4:1), 1 μmol/L ATP, 0.15 μCi [γ-^{33}P]ATP (Perkin-Elmer, Boston, MA), 50 mmol/L Tris-HCl (pH 7.7), 2 mmol/L DTT, 0.1 mg/mL bovine serum albumin, and 10 mmol/L $MnCl_2$. Reactions were allowed to proceed at 27°C for 1 hour and were terminated by the addition of 10 μL of a stop buffer (2.5 mg/mL bovine serum albumin and 0.3 mol/L EDTA), followed by a 108-μL mixture of 3.5 mmol/L ATP and 5% trichloroacetic acid. Acid-insoluble proteins were recovered on GF/C Unifilter plates with a Filtermate harvester (both from Packard Instrument Co., Meriden, CT). Incorporation of radioactive phosphate into the poly(Glu/Tyr) substrate was determined by liquid scintillation counting. Percent inhibition of kinase activity was determined by nonlinear regression analyses and data were reported as the inhibitory concentration required to achieve 50% inhibition relative to control reactions (IC_{50}). Data are the averages of triplicate determinations. All other tyrosine kinases were also assayed using poly(Glu/Tyr) as a substrate. The remainder of the protein kinases listed in Table 1 were obtained from Upstate Biotech (Charlottesville, VA) or Invitrogen and were assayed under conditions recommended by the suppliers. Kinetics of HER1 and HER2 inhibition were determined in reaction mixtures that contained varying concentrations of ATP and BMS-599626. The intercepts and slopes of double-reciprocal plots were replotted to yield K_i as described (29).

Cell assays. Sal2 mouse salivary gland tumor cells were previously described (30). KPL-4 cells were obtained from Dr. J. Kurebayashi (Kawasaki Medical School, Kurashiki,Okayama, Japan) and PC9 cells were kindly provided by Dr. K. Nishio (National Cancer Center of Japan, Tokyo, Japan; refs. 31, 32). All other cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were plated at 1,000 per well in 96-well plates and were cultured for 24 hours before test compounds were added. Compounds were diluted in culture medium such that the final concentration of DMSO never exceeded 1%. Following the addition of compounds, the cells were cultured for an additional 72 hours before cell viability was determined by measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye with the CellTiter96 kit (Promega, Madison, WI). For some cell lines, there was a lack of a correlation between 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye metabolism and cell number, and a thymidine

uptake assay was used to measure proliferation of these cell lines. Cells were plated in 96-well plates and treated with compounds as above. At the end of the 72-hour incubation, cells were pulsed with [^{3}H]thymidine (0.4 μCi/well, Perkin-Elmer) for 3 hours before they were harvested. Cells were digested with 2.5% trypsin for 10 minutes at 37°C and were harvested by filtration using a Packard Filtermate Harvester and GF/C Unifilter plates as described above. Incorporation of radioactive thymidine into nucleic acids was determined by liquid scintillation counting.

Immunoprecipitation and Western blot analyses. Cultured cells that had reached ~70% to 80% confluence were used for protein analyses. For analysis of EGF-dependent HER1 signaling, GEO cells were cultured overnight (~16 hours) in low-serum medium (RPMI 1640 supplemented with 0.5% fetal bovine serum). The cultures were then treated with compound for 1 hour, followed by stimulation with EGF (100 ng/mL; Invitrogen) for 5 minutes. Cell lysates were prepared by scraping cells into a lysis buffer that contained 10% glycerol, 1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.7), 1 mmol/L EDTA, 0.15 mol/L NaCl, 1 mmol/L sodium orthovanadate, 40 μmol/L ammonium molybdate, and 1% Complete protease inhibitors cocktail (Roche Biochemicals, Indianapolis, IN). The cell lysates were incubated on ice for 5 minutes and were centrifuged at 10,000 rpm for 10 minutes in a refrigerated tabletop microfuge to remove detergent-insoluble materials. The supernatants were then recovered and used for Western blot analyses or immunoprecipitation. Tumor lysates were prepared in a similar manner, except that tumors were homogenized in lysis buffer using a glass Dounce homogenizer. Protein concentrations in cell and tumor lysates were determined with the MicroBCA kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a standard. Immunoprecipitation was done with 4 μg of monoclonal anti-CD8 antibody (Biosource International, Camarillo, CA) or 4 μL of a polyclonal anti-HER1 antiserum. The following antibodies used in Western blotting were obtained from Cell Signaling (Danvers, MA): anti–phospho-HER1(Y1068), anti–phospho-HER2(Y1248), anti–phospho-mitogen-activated protein kinase (MAPK), anti-MAPK, anti–phospho-AKT, and anti-AKT. Monoclonal anti-phosphotyrosine antibody (PY20) was obtained from Invitrogen. Antibodies to HER1 and HER2 were previously described (30). Inhibition of protein phosphorylation was quantitated by densitometry analyses of the autoradiograms.

Table 1. Potency of enzymatic inhibition by BMS-599626

Enzyme	IC_{50} (nmol/L)
HER1	22
HER2	32
HER4	190
VEGFR-2	8,800
c-KIT	38,000
Lck	4,000
MEK	2,500
IGF-IR	>50,000
FGFR1	>50,000
Met	>40,000
Emt	>50,000
Syk	>50,000
Cdk2/cyclin E	>50,000
IKK	>10,000
PKA	>50,000
PKCα	>50,000
PKCδ	>50,000
PKCθ	>50,000
PKCξ	>50,000
P38α	>50,000
CaMKII	>50,000
AKT1	>50,000
GSK3β	>50,000
MAPKAP K2	>50,000

Abbreviations: VEGFR, vascular endothelial growth factor receptor; MEK, MAPK/extracellular signal–regulated kinase kinase; IGF-IR, insulin-like growth factor type I receptor; FGFR1, fibroblast growth factor receptor 1; Cdk2, cyclin-dependent kinase 2; IKK, IκB kinase; PKA, protein kinase A; PKC, protein kinase C; GSK3β, glycogen synthase kinase 3β; MAPKAP K2, MAPK-activated protein kinase 2.

Efficacy evaluation in tumor models. The following murine and human tumor models were employed in the evaluation of BMS-599626: SAL2 murine salivary gland tumor, N87 human gastric carcinoma, BT474 human breast tumor, A549 human non–small-cell lung tumor, and GEO human colon tumor. All tumors were maintained and passaged in athymic female nude mice (*nu/nu*, HSD; Harlan, Indianapolis, IN). Tumors were propagated as s.c. transplants using tumor fragments obtained from donor mice. Trastuzumab was obtained from Genentech, Inc. (South San Francisco, CA). For oral administration to mice, BMS-599626 was dissolved in a mixture of propylene glycol/water (50:50). The volume of all compounds administered was 0.01 mL/g body weight. Each nude mouse was given a s.c. implant of a tumor fragment (~20 mg) with a 13-gauge trocar. Tumors were allowed to grow to ~100 to 200 mm^3 and animals were evenly distributed to various treatment and control groups of eight mice each. Tumor response was determined by measurement of tumors with a caliper twice a week until the tumors reached a predetermined "target" size of 0.5 to 1.0 g. Tumor weights (mg) were estimated from the following formula: tumor weight = (length × width^2) / 2. Tumor growth inhibition was calculated using the formula

$$\%\ \text{Tumor growth inhibition} = \left(1-\frac{T_t}{C_t}\right)\left(1-\frac{C_0}{C_t}\right) \text{ or } \frac{(C_t - T_t)}{(C_t - C_0)}$$

where C_t is median control tumor size at the end of treatment, C_0 is median control tumor size at treatment initiation, and T_t is median tumor size of treated group at the end of treatment.

Activity is defined as the achievement of durable tumor growth inhibition of ≥50% for a period equivalent to at least 1 tumor volume doubling time and drug treatment must be for a period equivalent to at least 3 tumor volume doubling time.

For pharmacokinetic analyses, plasma samples were deproteinized by extraction with acetonitrile and analyzed by high-performance liquid chromatography/tandem mass spectrometry (MS/MS). The high-performance liquid chromatography system consisted of a high-performance liquid chromatography/autosampler combination (model 1100, Hewlett Packard, Palo Alto, CA). The column used was a Prodigy C18-ODS3 (2 × 50 mm, with 3-μm particles; Phenomenex, Torrance, CA) maintained at 60°C and a flow rate of 0.5 mL/min. The mobile phase consisted of 5 mmol/L ammonium acetate in 90% water/10% acetonitrile (A) and 5 mmol/L ammonium acetate in 10% water/90% acetonitrile (B) (pH 5.0). The mobile phase composition was held constant at 60% A:40% B until all components were eluted. The high-performance liquid chromatography was interfaced to a LCQ Advantage ion-trap mass spectrometer (Finnigan, Waltham, MA) operated in the positive ion electrospray, full MS/MS mode. For BMS-599626, fragmentation of *m/z* 531 yielded daughter ions for quantitation at *m/z* 432. For the internal standard, *m/z* 545 was fragmented to yield daughters at *m/z* 431. Helium was used as the collision gas. The standard curve ranged from 9 nmol/L to 35 μmol/L and was fitted with a quadratic regression weighted by reciprocal concentration (1 / *x*).

Results

BMS-599626 inhibits HER1 and HER2 kinase activity and signaling. BMS-599626 (Fig. 1A) was identified by optimizing a series of pyrrolotriazine analogues for their ability to inhibit

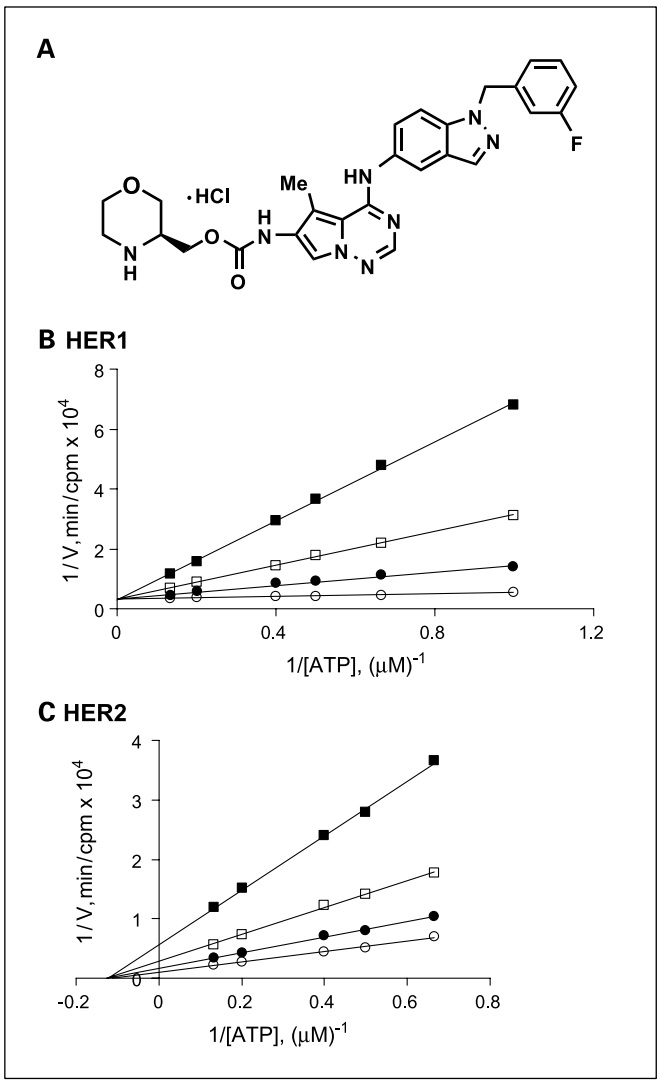

Fig. 1. *A,* structure of BMS-599626. *B* and *C,* kinetics of enzyme inhibition by BMS-599626. Double reciprocal plots of reaction rate (*V*) and ATP concentrations were used to determine the mechanism of inhibition of HER1 (*B*) and HER2 (*C*). Reaction mixtures contained no inhibitor (○), or BMS-599626 at 3 (●), 10 (□), and 30 (■) nmol/L. Points, averages of triplicates, with SDs no more than 15%. Each experiment has been repeated at least thrice.

both HER1 and HER2 kinases. It inhibited the enzymatic activity of recombinant kinases consisting of the entire cytoplasmic sequences of the receptors, with comparable potency (IC_{50} of 20 and 30 nmol/L; Table 1). BMS-599626 also inhibited the related receptor HER4, but with reduced potency. BMS-599626 was found to be a relatively selective kinase inhibitor when subjected to analyses involving 21 diverse protein kinases. Among the protein kinases tested, only MAPK/extracellular signal–regulated kinase kinase and Lck were inhibited with ~100-fold less potency than the inhibition of HER1 and HER2. BMS-599626 was also evaluated in a competitive binding assay and was found to have no significant affinity for any one of a panel of 110 protein kinases other than HER1 (data not shown; ref. 33). Kinetic analyses of HER1 inhibition revealed that BMS-599626 behaved as an ATP-competitive inhibitor (Fig. 1B). By contrast, inhibition of HER2 by BMS-599626 involved an ATP noncompetitive mechanism (Fig. 1C). The slopes and intercepts were replotted to yield K_i of 2 and 5 nmol/L, respectively, for HER1 and HER2. For both HER1 and HER2, BMS-599626 behaved as a mixed-type inhibitor relative to the phosphoacceptor substrate (data not shown).

BMS-599626 inhibited the proliferation of tumor cells that are dependent on HER1/HER2 signaling (Table 2). It was most effective in inhibiting the proliferation of Sal2 cells, which were derived from a transgenic mouse tumor in animals expressing a constitutively active CD8HER2 chimeric receptor (30). Significant inhibition was also observed with the breast tumor and gastric carcinoma cell lines that were previously shown to have *HER2* gene amplification and protein overexpression (34–37). Breast tumor cell lines such as HCC202, HCC1954, and AU565, which express high levels of both HER1 and HER2 but without *HER2* gene amplification, were inhibited by BMS-599626. BMS-599626 was also effective in inhibiting the proliferation of the GEO colon tumor cells, which express a high level of wild-type HER1 (38). PC9, a non–small-cell lung tumor cell line previously found to express HER1 with an exon 19 deletion, was inhibited by BMS-599626 with potency that is comparable to that for GEO cells (32). There was no significant effect on the proliferation of the ovarian tumor cell line A2780 and MRC5 fibroblasts, neither of which expresses HER1 or HER2.

Sal2 cells express a CD8HER2 fusion protein that is constitutively phosphorylated (30). Treatment of Sal2 cells with BMS-599626 resulted in the inhibition of receptor autophosphorylation, as well as MAPK phosphorylation, with IC_{50} of 0.3 and 0.22 μmol/L, respectively (Fig. 2A). In Sal2 cells, CD8HER2 signals as a homodimer and activates downstream signaling through the MAPK pathway exclusively (30).[4] As a result, there was no apparent inhibition of AKT phosphorylation in Sal2 cells on inhibition of CD8HER2 phosphorylation. In GEO cells, HER1 phosphorylation was stimulated by treatment with EGF and was inhibited by BMS-599626 (Fig. 2B; IC_{50}, 0.75 μmol/L). There was also nearly complete inhibition of EGF-dependent MAPK (IC_{50}, 0.8 μmol/L) but only partial inhibition of AKT signaling. The latter likely reflects the activation of AKT by multiple upstream signals. Treatment of N87 cells with BMS-599626 led to the inhibition of HER2 (IC_{50}, 0.38 μmol/L), which is expressed to a high level because of gene amplification, as well as MAPK and AKT phosphorylation (IC_{50} of 0.35 μmol/L for both; Fig. 2C). In all instances, the inhibition of kinase signaling was dose dependent and the potency correlated with the potency in the inhibition of cell proliferation. These results support the conclusion that BMS-599626 possesses the potency to inhibit both HER1 and HER2 kinase activities and that the compound inhibits the proliferation of tumor cell lines that are dependent on signaling from either receptor. The data in Fig. 2 also revealed some subtle differences in the extent to which the intracellular signaling by AKT may be dependent on the receptor kinases and cell context. The ability of BMS-599626 to modulate heterodimer formation and signaling was also examined in AU565 breast tumor cells, which express both HER1 and HER2. As shown in Table 2, the proliferation of AU565 cells was inhibited by BMS-599626.

[4] Unpublished results.

Table 2. Potency of BMS-599626 in cell proliferation assays

Cell line	Receptor expression and mutation status	IC_{50} (μmol/L)
Sal2*	CD8HER2	0.24
BT474*	HER2+++	0.31
N87*	HER2+++	0.45
KPL-4†	HER2+++	0.38
HCC202†	HER2++	0.94
HCC1954†	HER2++	0.34
HCC1419†	HER2+++	0.75
AU565†	HER2++/HER1++	0.63
ZR-75-30†	HER2+++	0.51
MDA-MB-175†	HER2+	0.84
GEO†	HER1+++	0.90
PC9†	HER1+++; exon 19 del(746-750)	0.34
A2780*	HER1-/HER2-	>10
MRC5*	HER1-/HER2-	>10

*Cell lines assayed with CellTiter96.
†Proliferation assayed by measuring thymidine incorporation.

Treatment of AU565 cells with EGF resulted in HER1 and HER2 receptor phosphorylation and the activation of both MAPK and AKT signaling (Fig. 3A). Treatment with BMS-599626 inhibited receptor phosphorylation and downstream signaling. EGF treatment resulted in the formation of HER1/HER2 heterodimers, as measured by the coimmunoprecipitation of HER2 by an anti-HER1 antibody (Fig. 3B, *middle*). The EGF-dependent heterodimer formation was inhibited by BMS-599626 (Fig. 3B). It is therefore evident that BMS-599626 was effective in inhibiting not only the enzymatic activity of individual receptors but also their ability to form heterodimers. Similar extent of inhibition was also observed in other tumor cells stimulated with EGF or transforming growth factor α (data not shown).

Antitumor activity of BMS-599626. BMS-599626 was evaluated for its ability to inhibit the growth of tumors that depend on HER kinase signaling. When implanted in nude mice, the Sal2 tumors exhibited a rapid growth rate. Oral administration of BMS-599626 resulted in a dose-dependent inhibition of Sal2 tumor growth (Fig. 4A). At 60 mg/kg, BMS-599626 achieved a significant delay of tumor growth in the course of treatment, but tumor growth resumed following the cessation of treatment. Higher doses of BMS-599626 provided more sustained inhibition of tumor growth, but in all cases tumor growth resumed on treatment cessation. In a once-daily regimen, the maximum tolerated dose for 14 days of dosing of BMS-599626 was 240 mg/kg. The Sal2 tumor model was previously shown to be dependent on signaling from a CD8HER2 homodimer (30). These data show that BMS-599626, because of its ability to inhibit HER2 kinase, had antitumor efficacy in a model that is dependent on HER2. BMS-599626 treatment resulted in the inhibition of GEO xenograft tumor growth when given once daily for 14 days (Fig. 4B). Because GEO tumor cells overexpress HER1 and are dependent on HER1 signaling, the ability of BMS-599626 to inhibit GEO tumor growth may be attributable to its intrinsic potency in inhibiting HER1 kinase. BMS-599626 was also evaluated in a human breast tumor xenograft, KPL-4, which had previously been shown to have *HER2* gene amplification and protein overexpression. The anti-HER2 monoclonal antibody trastuzumab was efficacious in this model, as was BMS-599626 (Fig. 4C). At its maximum tolerated dose of 180 mg/kg in a 21-day dosing regimen, BMS-599626 yielded comparable antitumor activity to that achieved with trastuzumab. In addition to efficacy in the Sal2, GEO, and KPL4 models, BMS-599626 had similar antitumor activity in other HER2 amplified xenograft models including the BT474 breast and N87 gastric tumors, as well as other HER1-overexpressing non–small-cell lung tumors (A549 and L2987; data not shown). The dynamic range in dose response observed with BMS-599626 in the Sal2 allografts suggests that the tumor model may be suited for examining the relationship between pharmacokinetics and target inhibition. Tumor-bearing mice were treated with a single dose of BMS-599626 and tumors were excised for an analysis of receptor phosphorylation. An efficacious regimen, at 120 mg/kg, resulted in a marked inhibition of CD8HER2 receptor phosphorylation for at least 7 hours after dosing, with receptor phosphorylation fully

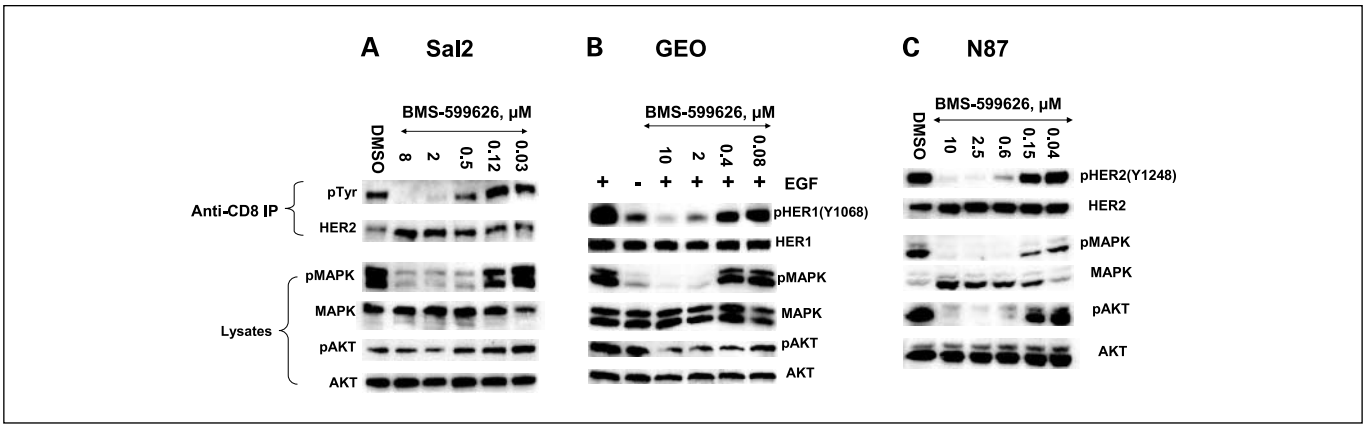

Fig. 2. BMS-599626 inhibits receptor phosphorylation and downstream signaling in tumor cells with activated receptors. *A,* Sal2 salivary gland tumor cells were treated with BMS-599626 at concentrations indicated and cell lysates were immunoprecipitated with an anti-CD8 antibody. The immune complexes were analyzed by Western blots with anti-phosphotyrosine antibodies, followed by anti-HER2 antibodies. The cell lysates were also analyzed by Western blotting with antibodies to phospho-MAPK and phospho-AKT. In each case, the filter was reprobed with antibodies to MAPK and AKT. *B,* GEO colon tumor cells were cultured overnight in culture medium containing 0.5% serum and were treated with BMS-599626 for 1 hour before they were stimulated with EGF (100 ng/mL). Whole-cell lysates were prepared and analyzed by Western blotting with antibodies indicated on the right. *C,* lysates of N87 cells were analyzed by Western blotting with antibodies indicated on the right.

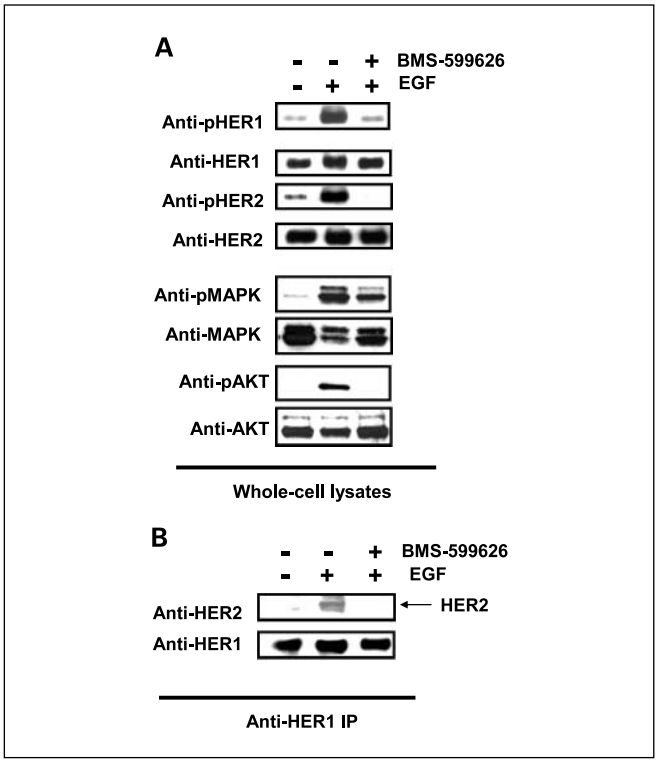

Fig. 3. BMS-599626 inhibits HER1/HER2 heterodimer formation. AU565 breast tumor cells were cultured overnight in medium containing 0.5% serum and were treated for 1 hour with 1 μmol/L BMS-599626 before stimulation with EGF. *A,* whole-cell lysates were analyzed by Western blotting with antibodies indicated on the left side. *B,* lysates were immunoprecipitated with anti-HER1 antibodies and the immune complexes were analyzed by Western blotting with antibodies to HER2 to detect heterodimers. The filter was subsequently reprobed with antibodies to HER1.

recovered by 24 hours (Fig. 5A). In tumors exposed to a subefficacious dose of BMS-599626 (30 mg/kg), there was minimal decrease in receptor phosphorylation. Inhibition of CD8HER2 receptor phosphorylation in the tumors is associated with a parallel decrease in MAPK signaling, confirming the mechanistic link between antitumor activity and inhibition of receptor signaling. Pharmacokinetic analyses revealed that there was a dose-dependent increase in blood levels of BMS-599626 as the dose was increased from 30 to 120 mg/kg (Fig. 5B). The data also suggest that a blood level of between 1 and 5 μmol/L of BMS-599626 at 7 hours post-dose was required for inhibition of CD8HER2 receptor phosphorylation and tumor growth.

Discussion

The sequence similarity between HER1 and HER2 suggests that it should be feasible to identify small-molecule compounds that inhibit both receptor kinases. The pyrrolotriazine core was previously identified as a template for designing inhibitors of HER1 kinase (28). Further optimization of the chemical series led to the identification of a number of compounds that inhibit HER1 and HER2 with comparable potency. BMS-599626 was selected among these analogues for more extensive characterization on the basis of its activity in the Sal2 tumor allograft model. Sal2 is a mouse salivary gland

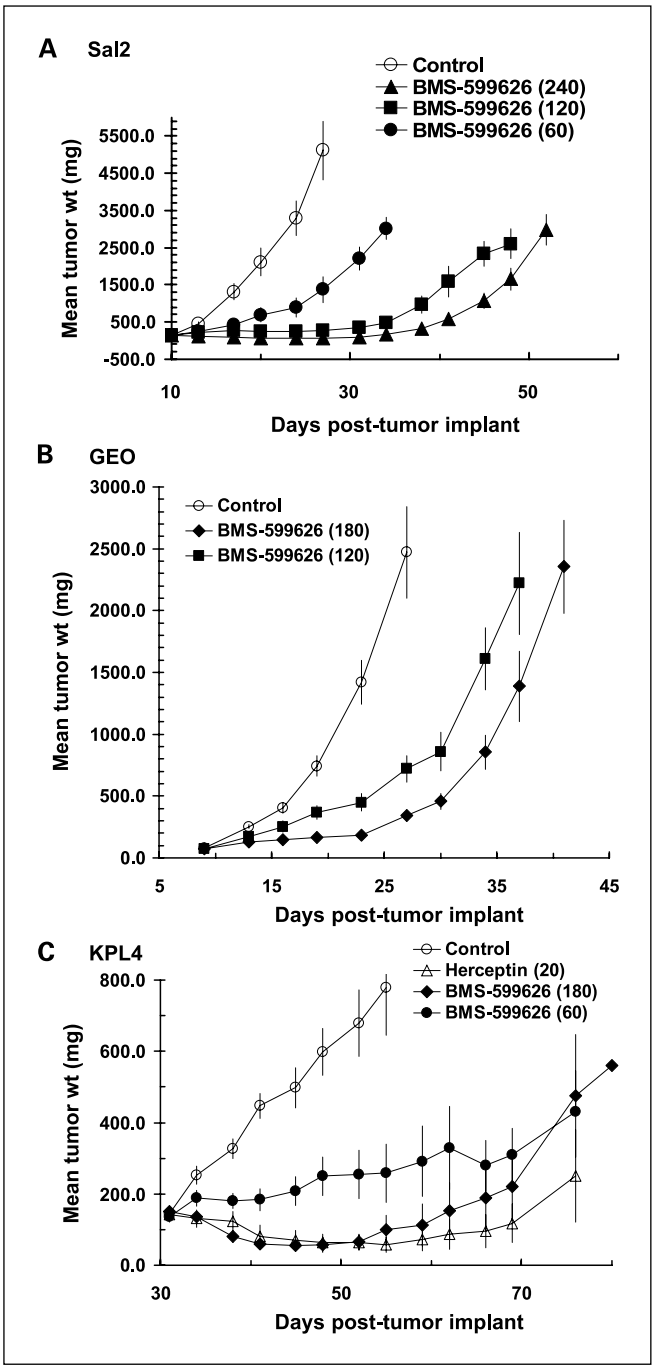

Fig. 4. Antitumor activity of BMS-599626. Nude mice implanted with tumors of predetermined sizes were treated and tumor measurements are shown for mice treated with vehicle control (○) or BMS-599626. *A,* mice with Sal2 mouse salivary gland tumors were treated for 14 days (days 10-24) with BMS-599626 at 60 (●), 120 (■), and 240 (▲) mg/kg. The *P* values for the regimens are 0.0013 (60 and 120 mg/kg) and 0.0004 (240 mg/kg). *B,* mice with GEO human colon tumors were treated with BMS-599626 daily for 14 days (days 10-24) with doses of 120 (■) and 180 (♦) mg/kg. The *P* values are 0.0014 and 0.0010 respectively. *C,* mice bearing KPL-4 human breast tumors were treated with trastuzumab (△; i.p. administration, once every 4 days for 20 days; $P = 0.0057$) or BMS-599626 (oral, once daily for 21 days) at 60 mg/kg (●; $P = 0.034$) and 180 mg/kg (♦; $P = 0.010$).

tumor that expresses a CD8HER2 transgene and is dependent on signaling from the CD8HER2 homodimer. Inhibition of Sal2 cell proliferation *in vitro* and tumor growth in nude mice confirmed the intrinsic potency of BMS-599626 in inhibiting

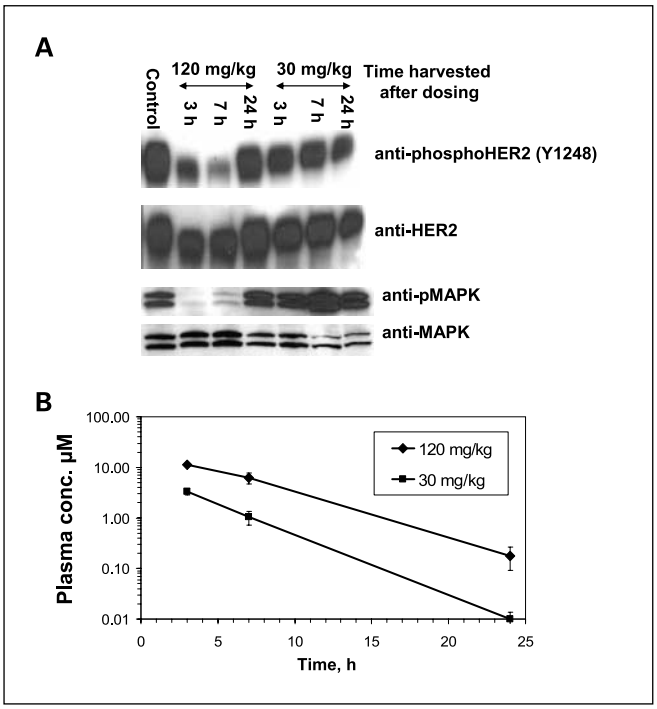

Fig. 5. Pharmacokinetics of BMS-599626 and pharmacodynamic assessment of Sal2 tumor inhibition by BMS-599626. Tumors were harvested at different times following treatment and receptor phosphorylation was determined by Western blot analyses (*top*). Mice were treated in groups of three animals each and the tumors from each group were pooled for lysate preparation and analyses. BMS-599626 was given in a single oral dose of 30 or 120 mg/kg. Bottom, plasma concentrations of BMS-599626 in each group of animals.

HER2 kinase activity and the feasibility of small-molecule inhibition of HER2 kinase for cancer treatment. In Sal2 tumors treated with BMS-599626, there was dose-dependent inhibition of receptor signaling, as measured by CD8HER2 and MAPK phosphorylation, which correlated with antitumor efficacy. Analyses of the pharmacokinetic and pharmacodynamic correlation also provided an estimate of the plasma concentration of BMS-599626 that was required for efficacy.

BMS-599626 inhibited the proliferation of a collection of diverse tumor cell lines that are dependent on signaling from either HER1 or HER2. The potencies with which BMS-599626 inhibited proliferation of cultured cells are in close agreement with those for inhibiting receptor phosphorylation, suggesting that modulation of receptor signaling is the principal mechanism of inhibiting cell proliferation. BMS-599626 is highly selective for HER1 and HER2. In enzymatic and binding assays, the compound did not have significant affinity or potency against other kinases. Kinetic analyses suggested that BMS-599626 competed with ATP for binding to HER1 but that its interaction with HER2 involved a distinct site. The apparent divergence in mechanism of inhibition between HER1 and HER2 is not without precedent. A small-molecule inhibitor of the cyclin-dependent kinases was previously shown to inhibit cyclin-dependent kinase 2 and cyclin-dependent kinase 4 by distinct mechanisms (39). Similarly, an inhibitor of the Src kinase families was found to exhibit distinct modes of inhibition of two highly related members within the family (40). Because a crystal structure of HER2 is not available, molecular modeling was attempted to provide a rationalization of the observed kinetic data. However, these attempts have not yielded definitive insights because of the highly dynamic nature of the active site structure. Further elucidation of the mechanism of HER2 inhibition by BMS-599626 will have to await future structure determination of the receptor/inhibitor complex. Among the tumor cell lines that were inhibited by BMS-599626 are those that express HER2, but at levels that are below those with *HER2* gene amplification. These cell lines are insensitive to trastuzumab, in agreement with previous observations that the antibody is effective only in tumor cells with *HER2* gene amplification. These cell lines invariably express both HER1 and HER2 and can be shown to form heterodimers in a ligand-dependent manner. In AU565 cells, BMS-599626 inhibited the formation of HER1/HER2 heterodimers. This is in contrast to the previous observations that quinazolines, such as gefitinib, stabilize the formation of inactive heterodimers (41, 42). The apparent difference in how these compounds affect heterodimer formation may be attributable to the structural dissimilarity in the side chains attached to the core templates and how these side chains may differentially influence the conformation of the receptor kinases once they are bound. This hypothesis is consistent with the distinctive mechanism of HER2 inhibition and may translate to efficacy and/or tolerability advantage in cancer patients, thereby differentiating BMS-599626 from other HER1/HER2 inhibitors in clinical development. The ability of BMS-599626 to inhibit proliferation of tumor cells expressing HER1 and HER2 supports the concept of targeting tumor cells that signal from HER1/HER2 heterodimers and the hypothesis that a pan-HER kinase inhibitor will have a broader spectrum of antitumor activity than agents that are selective for HER1 or HER2. The novel mechanism of action of BMS-599626 supports the clinical evaluation of BMS-599626 as a treatment for tumors that overexpress HER1 or HER2 kinase; clinical evaluation of BMS-599626 is under way. To date, a number of other pan-HER kinase inhibitors have entered clinical development and these molecules have distinct potency and selectivity profile in kinase inhibition as well as distinct mode of action (including irreversible binding; refs. 43–45). Differentiation among these molecules will require a comparison not only of preclinical properties but also of the pharmacokinetics and safety profiles in cancer patients.

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