Evaluation of the antitumor effects and mechanisms of PF00299804, a pan-HER inhibitor, alone or in combination with chemotherapy or targeted agents in gastric cancer

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Abstract

Recently, HER2-directed treatment, such as trastuzumab, has shown clinical benefit in HER2-amplified gastric cancer. Based on recent studies about EGFR or HER2 targeting agents (including gefitinib, lapatinib, and trastuzumab) in gastric cancer, the potent effects of pan-HER inhibitors targeting the HER family are anticipated. In this study, we evaluated the activity and mechanisms of PF00299804, an irreversible pan-HER inhibitor, in gastric cancer in vitro and in vivo models. PF00299804 showed significant growth inhibitory effects in HER2-amplified gastric cancer cells (SNU216, N87), and it had lower 50% inhibitory concentration (IC50) values compared with other EGFR tyrosine kinase inhibitors, including gefitinib, lapatinib, BIBW-2992, and CI-1033. PF00299804 induced apoptosis and G1 arrest and inhibited phosphorylation of receptors in the HER family and downstream signaling pathways including STAT3, AKT, and ERK in HER2-amplified gastric cancer cells. PF00299804 also blocked EGFR/HER2, HER2/HER3, and HER3/HER4 heterodimer formation as well as the association of HER3 with p85α in SNU216 cells. The combination of PF00299804 with clinically relevant chemotherapeutic agents or molecular targeted agents including trastuzumab (an anti-HER2 monoclonal antibody), CP751871 (an IGF1R inhibitor), PD0325901 (an ERK1/2 inhibitor) and PF04691502 (a PI3K/mTOR inhibitor) produced synergistic effects. These findings suggest that PF00299804 can be used as a targeted therapy for the treatment of HER2-amplified gastric cancer through inhibition of HER family heterodimer formation and may augment antitumor efficacy of chemotherapeutic and/or molecular targeted agents.
Introduction

The epidermal growth factor receptors (EGFR/HER1/ErbB1, HER2/ErbB2/neu, HER3/ErbB3, and HER4/ErbB4) mediate several cell functions, including cell proliferation, migration, and survival (1). There is rich cross-talk among the EGFR family of receptors. EGF-ligands, including EGF, TGF-α, amphiregulin, heparin-binding EGF, β-cellulin, and epiregulin, bind to EGFRs. Heregulins bind directly to HER3 or HER4; and NRG2, NRG3, and β-cellulin bind to HER4 (2-3). After receptor-specific ligand binding, the receptors form homodimers or heterodimers with each other, which leads to the activation of downstream signaling (4). However, unlike other EGFR members, no ligand of HER2 has been identified (5). Nevertheless, HER2 is known to play a core role in EGFR signaling via preferred heterodimerization with EGFR, HER3, or HER4 (6-7). HER3 also forms heterodimers with EGFR, resulting in activation despite the absence of a known active kinase domain (8). Several studies have shown that these heterodimerizations within the EGFR family are accelerated by receptor specific ligands such as EGF or heregulins (9).

HER2 has emerged as an important therapeutic target in various types of cancer. In gastric cancer, the incidence of HER2 amplification by FISH or HER2 3+ expression by immunohistochemistry was reported to be 22%, and the HER2 positivity was found to be higher in intestinal compared with diffuse types of cancer. In a preclinical study, trastuzumab, an anti-HER2 monoclonal antibody, was found to have antiproliferative effects against gastric cancer cells and showed synergy with cytotoxic chemotherapeutic agents (10). Recently, a ToGA clinical trial comparing a combination of chemotherapy and trastuzumab with chemotherapy alone in HER2-positive advanced gastric cancer showed clinical benefits in terms of overall survival, progression-free survival, and response rate (11-12). The antitumor activity of lapatinib, an EGFR and HER2 dual tyrosine kinase inhibitor (TKI), has been examined in gastric cancer cells. Lapatinib induced selective and potent growth inhibition in HER2-amplified gastric cancer cells (SNU216 and N87) (13-15).
Currently, a phase III clinical trial comparing lapatinib plus chemotherapy versus chemotherapy alone in HER2-positive gastric cancer patients is ongoing (16).

Recent evidence has indicated that HER3 also plays a critical role in tumor resistance to therapeutic agents targeting EGFR or HER2 and is responsible for maintaining the proliferation of HER2-amplified cells owing to activation of the PI3K/Akt pathway. Furthermore, HER3 is becoming an important targeted molecule in cancer treatment (17-18). It is possible, therefore, that a pan-HER TKI, which targets all HER family members, may have more potent activity in HER-signal dominant tumors.

PF00299804 is an orally bioavailable, second-generation, irreversible pan-HER TKI currently under clinical development. When compared with the first-generation irreversible pan-HER inhibitor CI-1033, PF00299804 has more attractive properties including greater bioavailability, longer half-life, larger volume of distribution, and lower clearance. PF00299804 has been shown to induce objective responses during phase I/II trials in patients with EGFR TKI-refractory nonsmall cell lung cancer (NSCLC) (19-21).

Until now, there have been studies on the activity of pan-HER TKIs, such as BMS-599626 and HM781-36B (22-23), in gastric cancer, and a phase II study of PF00299804 as monotherapy in patients with HER2-positive advanced gastric cancer is currently ongoing (24). In the present study, we evaluated the activity of PF00299804 in a large panel of gastric cancer cell lines and sought to determine the mechanisms of selectivity of PF00299804 for gastric cancer cell lines through investigating alteration of HER family heterodimer formation during treatment. Furthermore, we described the combined effects of PF00299804 with chemotherapeutic and/or targeted agents including an anti-HER2 monoclonal antibody, and IGF1R, ERK1/2, and/or PI3K/mTOR inhibitors.
Materials and Methods

Reagents
PF00299804, trastuzumab, CP751871, PD0325901, and PF04691502 were provided by Pfizer Inc. (New York, NY, USA); other HER TKIs, including gefitinib, lapatinib, BIBW-2992, and CI-1033, were supplied by the Hanmi Pharmaceutical Company. The chemical structures of gefitinib, lapatinib, BIBW-2992, CI-1033, PF00299804, PD0325901, and PF04691502 are shown in supplemental Figure 1 (25-26). In addition, the other following chemotherapeutic agents were obtained: 5-fluorouracil (5-FU) from Ildong Pharmaceutical Co., Ltd. (Seoul, Korea) and cisplatin from Choongwoe Co., Ltd. (Seoul, Korea).

Cell Lines and Culture
Human gastric adenocarcinoma cell lines (SNU1, 5, 16, 216, 484, 601, 620, 638, 668, and 719) authenticated by DNA fingerprinting analysis were supplied by the Korean Cell Line Bank (27). Human gastric cancer cell lines (N87, AGS, KATOIII and HS746T) were purchased from the American Type Culture Collection (Rockville, MD, USA) that carries out cell line authentication by short tandem repeat analysis. Human gastric cancer cell lines (MKN74, MKN45, and MKN1) were obtained from Health Sciences Research Resource Bank (Osaka, Japan) that carries out cell line test by the specific isozyme pattern according to the standard operation protocol for quality control prescribed by The Japanese Tissue Culture Association. Human gastric cancer cell lines (HGC27, A3KAW, MCG803, IM95, BGC823, IM95M, SGC7901, GAM016, and FU97) that have been authenticated by short tandem repeat analysis were supplied by Crown Bioscience Laboratories (Taicang City, Jiangsu Province, China). Upon receipt, all cell lines were banked, and passaged for
less than 6 months before use in this study. N87 and SNU216 are cell lines in which HER2 gene amplification has been demonstrated (10, 28). All cell lines were maintained in RPMI-1640 or DMEM culture media (HyClone Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere under 5% carbon dioxide at 37°C.

**Cell Growth Inhibition Assay and Determination of Combination Index**

Tetrazolium dye (MTT; Sigma-Aldrich) assays were performed as described previously to evaluate the growth inhibitory effect of PF00299804 alone or in conjunction with chemotherapeutic agents (5-FU and cisplatin) on 11 gastric cancer cell lines (Table 1) (23).

For another panel of 18 gastric cancer cell lines (Figure 1B), a cell viability assay was conducted with either a single agent (PF00299804) or with a pair-wise combination of targeted agents (trastuzumab, CP751871, PD0325901, and PF04691502). Briefly, cells were seeded into 96-well plates and allowed to adhere overnight. The following day, cells were treated with either single or double agents (with equimolar ratio for combinations) in nine serially-diluted concentration points ranging from 10 µmol/L to 152 pmol/L (1 µmol/L to 15.2 pmol/L for trastuzumab and CP751871). After 72 hours, 0.1 mg/mL of resazurin salt dye (Sigma Aldrich, St. Louis, MO, USA) or 20% of the manufacturer’s recommended volume of Cell Titer Glo (Promega, Madison, WI, USA) was added. Readings from the Envision multi-reader (Perkin-Elmer, Waltham, MA, USA) were processed using the R package drug response curves (DRC) (29) to generate half maximal inhibitory concentration (IC₅₀) values. Cell counts were first adjusted by subtracting the average of the baseline cell counts from untreated cells assessed one day after cell seeding. The Cell Growth Inhibition (CGI) score for each compound concentration was calculated as:

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CGI = 1 - (\frac{\text{well-count} - \text{baseline}}{\text{plate control mean} - \text{baseline}}).
\]

The DRC package was then used to fit the adjusted CGI values versus the concentrations of the drugs. A four-parameter logistic model was used in the package to fit the DRCs and generate estimations and inferences of IC₅₀, slope, and upper and lower limits.
For the detection of combination effects, cells were treated with serial dilutions of each drug individually and with both drugs simultaneously at a fixed ratio of doses that corresponded to the individual IC₅₀. The methods described by Chou and Talalay were then used to determine if a synergistic effect existed (30-31). Analysis of the median effect was conducted using the CalcuSyn software (Biosoft) to determine a combination index (CI) value (CI > 1, antagonistic effect; CI = 1, additive effect; CI <1, synergistic effect).

Molecular Profiling

Molecular profiling was conducted using the 18 human gastric cancer cell lines shown in Figure 1B. Mutation data were obtained from the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Welcome Trust, Sanger Institute, http://www.sanger.ac.uk/genetics/CGP/cosmic/). CNV data were obtained from the COpy Number Analysis (CONAN) dataset (Welcome Trust, Sanger Institute, http://www.sanger.ac.uk/genetics/CGP/CopyNumberMapping/Affy_SNP6.shtml). Additional single nucleotide polymorphism (SNP) 6.0 arrays were profiled at Crown Bioscience Laboratories (Taicang City, Jiangsu Province, China). Arrays were processed using the R-based aroma.affymetrix methods. (http://www.aroma-project.org/)(32). Arrays were ratioed against a baseline profile of the average of the 128 female arrays from the International HapMap Project (http://www.affymetrix.com/estore/support/technical/sample_data/genomewide_snp6_data.affx).

HuPrime® Gastric Cancer Xenograft Models and Genotyping with Illumina System

HuPrime® gastric cancer xenograft models were established by Crown Bioscience Laboratories (Taicang City, Jiangsu Province, China) by transplanting new human tumor fragments (cryopreserved living tissue fragments) to immune-deficient mice. Female BALB/c nude mice (9 to 11 weeks old, 10 mice per group) with subcutaneous HuPrime® gastric cancer xenografts were treated with vehicle or PF00299804 (7.5 mg/kg orally daily for 21 days) when their mean tumor
volume reached 150 mm³. Tumor size was measured twice weekly using a caliper, and tumor volume was calculated with the formula: Volume (V) = 0.5 \times a \times b^2, where a and b are the long and short diameters (in millimeters) of the tumor, respectively. SNP analysis with human gastric cancer tissues using an Illumina cancer SNP panel was conducted by Illumina, Inc. (San Diego, CA, USA).

Cell Cycle Analysis

After incubation with PF00299804 under various concentrations (0.001, 0.01, and 0.1 μmol/L) for 48 hours, the cells were centrifuged at 1500 rpm for 5 minutes and then fixed in 70% alcohol and stored at -20°C. The samples were then dissolved in 10 μL RNAse (100 μg/mL) and subsequently incubated at 37°C for 10 minutes. Next, the samples were treated with propidium iodide, after which the DNA contents of the cells (10,000 cells per experimental group) were determined using an FACS Caliber flow cytometer (Becton Dickinson Biosciences) equipped with a ModFit LT program (Verity Software House, Inc.), as previously described (33).

Western blot and Immunoprecipitation

Cells were incubated with PF00299804 in 10% FBS media. After 48 hours, the cells were treated with a lysis buffer. The same amount of protein (15 μg) was then obtained from each suspension and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), after which it was transferred to nitrocellulose membranes. After blocking with a buffer, the membrane was incubated with primary antibodies at 4°C overnight. Antibodies against p-EGFR (pY1068), p-HER2 (pY1221/1222), p-HER3 (pY1289), p-STAT3 (pY705), p-AKT (pS-473), p-ERK (p44/p42), EGFR, HER2, HER3, HER4, STAT3, AKT, ERK, Caspase-3, Caspase-7, PARP, Bcl-2, Bim, cyclin D, p27kip1, and PI3-Kinase were purchased from Cell Signaling Technology (Beverley, MA, USA). Antibodies against MCL-1, cyclin E, CDK2, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-α-tubulin antibody was acquired from Sigma-Aldrich.

For immunoprecipitation, 1 mg of total protein from cell lysates using lysis buffer (1% NP-40, 5
mmol/L EDTA, 150 mmol/L NaCl, 50 mmol/L Tris-HCL, 10 mmol/L NaF, and 10 mmol/L Na₃P₂O₇ containing 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium vanadate) was incubated with anti-EGFR or anti-HER3 antibodies and Protein A/G plus agarose (Santa Cruz Biotechnology) and gently shaken. The precipitates were washed twice with ice-cold lysis buffer and resolved by SDS-PAGE, after which they were subjected to Western blot analysis.

**Xenograft Mouse Model**

To determine the *in vivo* activity of PF00199804, 7- to 8-week-old female SCID mice (Fox Chase SCID®, C.B-17/Scid-Prkdcscid, Charles River) were used. All studies were conducted in accordance with the recommendations of the Guide for Care and Use of Laboratory Animals. Mice were injected subcutaneously with N87 cells (1×10⁷ cells per 200 μL PBS), and tumor growth was monitored as tumor volumes approached 180 to 220 mm³. Tumors were measured using Vernier calipers twice weekly. Twelve days later (designated as day 1 [D1] of the study), mice were sorted into treatment groups with group mean tumor volumes of 190 to 193 mm³. Volume was calculated using the following formula: tumor volume (mm³) = width² × length/2 measured in millimeters for an N87 tumor.

Mice bearing subcutaneous N87 tumors of approximately 200 mm³ were orally administered PF00299804 daily at 5 mg/kg/day as a single agent or in combination with cisplatin administered intraperitoneally weekly × 3 at 4.5 mg/kg or 5-FU administered intraperitoneally weekly × 3 at 100 mg/kg. PF299804 dosing solutions were prepared in 50 mM sodium lactate buffer, pH 4. Dosing solutions of 5-FU were prepared fresh in 5% dextrose in deionized water (D5W). Cisplatin dosing solutions were prepared fresh in normal saline (0.9% NaCl) for each day of dosing.

Each animal reached the predetermined endpoint volume (800 mm³) or reached the end of the study. The time to endpoint (TTE, expressed in days) for each mouse was calculated using the following equation: \( TTE = \log_{10} (\text{endpoint volume}) - b / m \), where \( b \) is the intercept and \( m \) is the slope of
the line obtained by linear regression of a log-transformed tumor growth data set. The log rank test was used to assess the significance of the difference between the overall survival of the two groups. The log rank test analyzed the individual TTEs for all animals in a group, except those lost to the study due to nontreatment-related (NTR) deaths. The two-tailed statistical analyses were conducted with $P = 0.05$ for significance. This in vivo experiment was performed using a method similar to a previous study that evaluated the in vivo efficacy of PF00299804 in ovarian cancer and NSCLC models (20).

**Data Treatment and Statistical Analysis**

All experiments were conducted in at least triplicate. All data are reported as means (SE). The statistical significance of the results was calculated using an unpaired student t test, and P values of $< 0.05$ were considered to be statistically significant.

**Results**

**HER2 amplification is highly correlated with sensitivity to PF00299804**

The growth inhibitory effects of PF00299804 in a panel of 11 gastric cancer cell lines (SNU-1, 5, 16, 216, 484, 601, 620, 638, 668, 719 and N87), of which genetic status was previously reported, were examined (34). Interestingly, only HER2-amplified gastric cancer cells (SNU216 and N87) were sensitive to PF00299804 with a low range of IC$_{50}$ values (0.002 to 0.008 μmol/L). As HER2 amplification is a well-known sensitive marker for HER family TKIs, the efficacy of PF00299804 with other HER family TKIs, including gefitinib, lapatinib, BIBW-2992, and CI-1033, was compared. HER2-amplified gastric cancer cells were sensitive to all other HER family TKIs; however, in comparison, PF00299804 had 10- to 1000-fold lower IC$_{50}$ values and significantly inhibited the growth of N87 cells, even at the 0.001 μmol/L concentration (Table 1 and Figure 1A).
To confirm the growth inhibitory effects of PF00299804 in HER2-amplified cells and see if additional sensitive markers for PF00299804 exist, a cell viability assay was conducted in another panel of 18 gastric cancer cell lines, of which molecular profiling of about 27 genes was included (Figure 1B). As expected, HER2-amplified N87 cell lines were most sensitive to PF00299804.

In an effort to further identify predictive markers for drug response, the antitumor effects of PF00299804 was tested using primary human gastric cancer xenograft models. Significant tumor growth inhibition was observed in one (GA#110) out of four HuPrime® gastric cancer xenograft models evaluated. Then, an SNP array in human tissues was conducted to see if PF00299804 also had efficacy against other HER family proteins, such as EGFR-amplified gastric cancer. Interestingly, the signal intensity of two SNPs from model GA#110 was significantly higher than that of other models, suggesting a possible EGFR gene amplification in this model, and thereby suggesting EGFR amplification as a possible predictive marker for response to PF0029804 (Figures 1C and D). Collectively, these results indicated that HER2 amplification is a potent predictive marker for response to PF00299804 and provide the possibility for EGFR amplification as a potential predictive marker in addition to HER2 amplification.

**PF00299804 induces apoptosis and G1 arrest in HER2-amplified gastric cancer cells**

As HER2 amplification was found to be a potent predictive marker for response to PF00299804, the effects of PF00299804 on cell cycle progression were assessed to determine if antitumor effects of PF00299804 in HER2-amplified cell lines reflected any change in the cell cycle distribution. An increase of the portion of cells in the sub-G1 and G1 phases was detected in SNU216 and N87 cells. In contrast, no change in cell cycle distribution was detected in SNU668 cells (Figure 2A).

The effects of PF00299804 on apoptosis were further evaluated. The amount of the cleaved form of effector caspases (caspase-3 and caspase-7) and PARP was increased. The inhibition of anti-apoptotic Bcl-2 family protein (Mcl-1) and the induction of proapoptotic BH3-only Bcl-2 family
members (Bim) also occurred. Subsequently, changes in the expression of cell cycle regulatory molecules in response to PF00299804 were examined. Cyclin D, Cyclin E, and CDK2 were down-regulated, whereas P27kip1 was up-regulated (Figures 2B and C).

**PF00299804 blocks phosphorylation of HER family and downstream signaling pathways in HER2-amplified cells**

Because HER family activates a number of signal transduction pathways including the STAT3, PI3K/Akt and Ras/MAP kinase pathways, HER2-amplified cells (SNU216, N87) and HER2-nonamplified cells (SNU668) were compared based on the phosphorylation activity of HER family members and their key components of downstream signaling cascades. In SNU216 and N87 cells, PF00299804 decreased the phosphorylation activity of HER family members and STAT3, AKT and ERK. However, no change in the phosphorylation activity of HER2-nonamplified SNU668 cells was observed (Figure 3A).

**PF00299804 inhibits the formation of EGFR/HER2, HER2/HER3, and HER3/HER4 heterodimers and the association of HER3 with p85α in HER2-amplified cells**

It was then examined if PF00299804 affects the formation of HER family heterodimers. PF00299804 treatment abolished the formation of EGFR/HER2, HER3/HER2, and HER3/HER4 heterodimers and the association of HER3 with p85α induced by EGF or HRG in SNU 216 cells. Conversely, EGF or HRG did not induce formation of heterodimers, and PF00299804 treatment did not abolish the formation of heterodimers in SNU668 cells (Figures 3B and C).

Taken together, suppression of the formation of HER family heterodimers by PF00299804 treatment may be a possible mechanism to explain the efficacy of PF00299804 in HER2-amplified cancer cells on both HER family members and their downstream signaling molecules.

**PF00299804 enhances the growth inhibitory effects of chemotherapeutic agents in vitro and in**
vivo

PF00299804 was treated with 5-FU or cisplatin simultaneously in 11 gastric cancer cell lines as indicated in Table 1, to determine if interactions of PF00299804 with these chemotherapeutic agents commonly used to treat gastric cancer patients enhanced cytotoxicity. Although most cell lines were resistant to 5-FU or cisplatin, the combined treatment of PF00299804 with these chemotherapeutic agents produced synergistic effects in many gastric cancer cell lines, including HER2-nonamplified gastric cancer cells (Figure 4A and supplemental Table 1).

The *in vivo* efficacy was confirmed using an N87 human gastric cancer xenograft model. A significant growth delay was observed for PF00299804 with either 5-FU or cisplatin at the predetermined 800 mm³ tumor volume endpoint when compared with all relevant single agent controls (Figures 4B and C).

It was then tested whether PF00299804 can lead to any alteration of TS or DPD expression levels in PF00299804-treated cells, since these two enzymes are involved in the sensitivity to 5-FU (35). It was observed that PF00299804 suppressed TS or DPD in cell lines that are synergistic to a combination of PF00299804 and 5-FU (supplemental Figures 2A and B). In addition, it was found that PF00299804 down-regulated ERCC or XRCC1, which are useful candidate predictive markers for platinum-based chemotherapy as indicated in supplemental Figure 2C (36-37).

**PF00299804 exerts a synergistic effect when administered with targeted agents (trastuzumab, CP751871, PD0325901, and PF04691502)**

As recent studies have shown that there are benefits to targeting EGFR and other signaling pathways, including IGF1R, MEK and PI3K/mTOR, PF00299804 was combined with targeted agents (trastuzumab, CP751871, PD0325901, or PF04691502) to identify candidate markers for determination of synergistic effects between the two signaling pathways. A panel of 18 human gastric cancer cell lines was used as indicated in Figure 1B, for which genetic status data (mutation and CNV) have been reported at the Sanger Institute. PF00299804 demonstrated synergism with targeted
agents in various cell lines. In the case of the N87 cell line (the most sensitive cell line to PF00299804), PF00299804 exerted synergistic effects with four targeted agents (trastuzumab, CP751871, PD0325901, and PF04691502). Interestingly, the four targeted agents exerted synergism with PF00299804 in KRAS mutant cell lines (SNU1 and AGS). Three targeted agents (trastuzumab, CP751871, and PD0325901) demonstrated synergism with PF00299804 in FGFR2-amplified cell lines (KATO III and SNU16), for which genetic status was verified in a previous study (38). In MET-amplified cell lines (HS746T and MKN45), trastuzumab and CP751871 showed synergism with PF00299804, and CP751871 exerted synergism with PF00299804 in MYC-amplified cells (HGC27). In the case of APC mutant or deleted cell lines (HGC27, IM95M and MKN74), PD0325901 showed synergism with PF00299804 (Figure 1B and Table 2).

Discussion

Recently, EGFR and HER2 have arisen as effective therapeutic targets for various cancer types (39), and HER2 has been an important clinically relevant therapeutic target in gastric cancer. Trastuzumab and lapatinib have shown preclinical activity, and clinical trials of these compounds are ongoing (10, 13). In this study, PF00299804 demonstrated robust antiproliferative activity in HER2-amplified cancer cells. PF00299804 was a more potent inhibitor of the growth of HER2-amplified N87 cells than trastuzumab, which is currently being used clinically in HER2 gastric cancer (Figure
We also expect that PF00299804 would show better antitumor activity than trastuzumab in an N87 xenograft model. We did not perform an in vivo study for this comparison; however, in our study PF00299804 treatment led to tumor regression in an N87 xenograft model until day 14 (Figures 4B and C), while trastuzumab treatment did not. Trastuzumab did show enhanced antitumor efficacy compared to control group in a previous study (28).

Furthermore, it was previously reported that A431 NSCLC cells with EGFR amplification have shown sensitivity to gefitinib, suggesting a role for EGFR as well as HER2 as predictive markers (40). The results described in Figure 1C provide for the possibility that EGFR amplification can also act as a predictive marker for response to PF00299804.

Previous studies have revealed the mechanism of heterodimerization within the HER family. EGFR/HER2 heterodimerization is vital for initiation of the transduction of signal pathways such as PI3K/Akt and ERK. The monoclonal antibody cetuximab, targeting EGFR, completely inhibited the formation of EGFR/EGFR homodimers and EGFR/HER2 heterodimers in N87, HER2-amplified cells (41). Moreover, active heterodimerization of EGFR and HER2 was blocked by lapatinib, a reversible EGFR and HER2 TKI (14). Current studies suggest that HER2/HER3 heterodimer formation is important for activation of downstream pathways, particularly in HER2-positive cancers, because HER2 preferentially heterodimerizes with HER3 (17, 42). HER2 overexpression increased the sensitivity to gefitinib through inhibition of HER2/HER3 heterodimer formation (43). HER4 also participates in HER family signaling pathways, although its effects are not as well characterized as those of EGFR, HER2, and HER3 (44). The results of one preclinical study suggest that HER3/HER4 heterodimers may be responsible for activation of the MAPK pathway (45). The results of the present study indicate that PF00299804 not only reduced the formation of EGFR/HER2 heterodimers, but also inhibited the formation of HER2/HER3 and HER3/HER4 heterodimers in SNU216 cells (Figures 3B and C).

The effects of EGFR TKIs are limited by the drug resistance mechanism, and strategies for overcoming gefitinib resistance have been investigated. The combined treatment of lapatinib and
cetuximab was described as one such strategy for overcoming gefitinib resistance in NSCLC with the T790M mutation (14). Previously, PF00299804 potently inhibited the growth of H3255 and HCC827 cells engineered to express EGFR T790M mutation (19). The combination of PF00299804 and MET inhibitors could also overcome gefitinib resistance caused by MET amplification or autocrine HGF production (46). These studies indicate that PF00299804 has the potential for use in cancer treatment via mutations or overexpression/amplification of HER family members or their target molecules alone or in combination with chemotherapeutic and/or molecular targeted agents.

In the present study, we tested the combined effect of PF00299804 with clinically relevant chemotherapeutic agents (5-FU, cisplatin, paclitaxel, and docetaxel). Combinations of PF00299804 with these chemotherapeutic agents demonstrated synergistic effects in gastric cancer cells (Figures 4A-C and supplemental Tables 1 and 2). Interestingly, these synergistic effects were observed in some HER2-nonamplified cells in addition to HER2-amplified cells.

One possible mechanism of the synergistic effects between PF00299804 and 5-FU is the alteration of the expression level of thymidylate synthase (TS) or dihydropyrimidine dehydrogenase (DPD), which are 5-FU metabolizing enzymes and involved in cell sensitivity to 5-FU (35). TS was found to be down-regulated by lapatinib, and 5-FU resistance could be overcome by down-regulating TS (13, 15). As expected from previous results, PF00299804 suppressed expression of TS in HER2-amplified cell lines (SNU216 and N87) and in HER2-nonamplified cells (SNU601), which show synergistic effects of co-treatment of PF00299804 and 5-FU. However, PF00299804 did not suppress expression of TS in SNU668 cells that showed antagonistic effects of the co-treatment. In addition, we observed that PF00299804 suppressed expression of DPD as well as TS in SNU216 and N87 cell lines (supplemental Figures 2A and B).

As ERCC1, XRCC1, and XRCC3 gene polymorphisms were identified as useful candidate markers for predicting better survival of patients with NSCLC after treatment with platinum-based chemotherapy (cisplatin) and inhibition of HER2 signaling was associated with DNA repair processes after cisplatin treatment (36-37), we tested whether PF00299804 treatment induces any alteration of
ERCC1 or XRCC. We observed that PF00299804 suppressed expression of ERCC1 or XRCC1 in HER2-amplified cell lines (SNU216 and N87) and in HER2-nonamplified cells (SNU16), showing synergistic effects of co-treatment of PF00299804 and cisplatin. However, PF00299804 did not suppress expression of ERCC1 or XRCC1 in SNU668 cells that showed antagonistic effects of the co-treatment (supplemental Figure 2C).

HER2 overexpression is related to paclitaxel resistance, and combined treatment with trastuzumab and paclitaxel was found to make HER2-amplified breast cancers more sensitive to paclitaxel (47). In accordance with this study, paclitaxel exerted synergistic effects in HER2-amplified cells (SNU216, N87). These results may provide a preclinical rationale for the development of combination treatments composed of PF00299804 and cytotoxic chemotherapy in gastric cancer.

In a previous study, lapatinib plus trastuzumab showed preclinical or clinical synergistic effects (48). In addition, it has been demonstrated that acquired resistance to gefitinib, an EGFR TKI, is associated with the IGF-1R pathway, and IGF-1R inhibitor enhanced the antitumor effect of trastuzumab in HER2-amplified breast cancer (49). The MEK inhibitor has been found to exert efficacy against KRAS-mutant cancer cell lines that are resistant to gefitinib. A combination of MEK and EGFR inhibitors showed synergistic effects via inhibition of the EGFR/HER3/AKT pathway activated in MEK inhibitor resistant cells (34). Moreover, the combination of RAD001 plus trastuzumab was more effective than either drug alone, suggesting inhibition of PI3K and mTOR is required for the growth-inhibitory effect of HER2 antagonists (50). Therefore, we evaluated the combined effects of PF00299804 with molecular targeted agents in gastric cancer cell lines. PF00299804 exerted synergistic effects when combined with targeted agents (trastuzumab, CP751871, PD0325901, or PF04691502) in cell lines with various types of genetic status including KRAS mutation, FGFR2 amplification, MET amplification, or MYC amplification (Figure 1B and Table 2). These evaluations provide a rationale for the combination of PF00299804 with molecular targeted agents according to each genetic status.

Overall, our findings suggest that PF00299804 is an effective agent in gastric cancers with
HER2 amplification through inhibition of EGFR/HER2, HER2/HER3, and HER3/HER4 heterodimer formation as well as HER3 with p85. In addition, the results of the combination study indicate that coadministration of PF00299804 with cytotoxic chemotherapeutic agents exerts synergistic effects in vitro and in vivo. Furthermore, PF00299804 produced synergistic effects with trastuzumab, IGF1R inhibitors, ERK1/2 inhibitors, and PI3K/mTOR inhibitors. These findings provide rationale for future clinical development of PF00299804 alone or in combination with chemotherapeutic and/or molecular targeted agents for the treatment of gastric cancer.

Acknowledgement

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References


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NOTE: Shown are the IC\textsubscript{50} values of each drug using MTT assay, as described in the Methods on gastric cancer cell lines.

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<tr>
<th>Cell Line</th>
<th>Genetic Status</th>
<th>PF00299804 IC\textsubscript{50} (µmol/L)</th>
<th>Gefitinib IC\textsubscript{50} (µmol/L)</th>
<th>Lapatinib IC\textsubscript{50} (µmol/L)</th>
<th>BBW-2992 IC\textsubscript{50} (µmol/L)</th>
<th>CI-1033 IC\textsubscript{50} (µmol/L)</th>
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<td>0.02±0.003</td>
<td>0.02±0.001</td>
<td>0.15±0.008</td>
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<td>HER2 amp</td>
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<td>0.01±0.001</td>
<td>0.02±0.001</td>
<td>0.13±0.002</td>
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<td>KRAS mt</td>
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<td>3.8±0.52</td>
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<td>1.61±0.03</td>
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Table 2. Combined effects of PF00299804 with molecular targeted agents

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NOTE: Shown are combination index for the combination of PF00299804 with molecular targeted agents (Trastuzumab, CP751871, PD0325901 or PF04691502) at the 50% fraction affected in 18 gastric cancer cell lines (CI > 1: antagonistic effect, CI = 1: additive effect, CI <1: synergistic effect).
**Figure Legends**

Figure 1. HER2 amplification is potent predictive marker for response to PF00299804. A, N87 cells were treated with the indicated agents for 72hr, after which the cell growth inhibition was analyzed by an MTT assay. B, Mutation data was obtained from the COSMIC database, CNV data was obtained from the CONAN dataset, and additional SNP6.0 arrays were profiled in-house. The sensitivity to PF00299804 was determined by a cell viability assay in 18 gastric cancer cell lines. C, In the left panel, HuPrime® gastric cancer xenograft were treated with vehicle or PF00299804 (7.5 mg/kg, PO, QD x 21). Then, tumor growth inhibition values (T/C value) were calculated from the median tumor size of the treatment group divided by that of the control group on day 27. In the right panel, each data point represents a given SNP of a tissue sample. Norm Theta represents genotype. Norm R represents the intensity value of the SNP signal. The data points in red (AA), blue (BB), and purple (AB) represent SNPs of normal human tissues, displaying normal genotypes. The data points in other colors are SNPs of tumor tissues from a panel of HuPrime® gastric cancer models. D, Shown are Tumor growth inhibitory effects in HuPrime® gastric cancer xenograft model, GA#110. Significant tumor growth inhibition was observed in the PF00299804-treated group from Day 6 through the end of the study (p=0.001), achieving complete regression (CR). Data are shown as mean ± SE.

Figure 2. PF00299804 induces apoptosis and G1 arrest in HER2 amplified gastric cancer cells. A, SNU216, N87 and SNU668 cells were treated with the indicated concentrations of PF00299804 for 48h. The cell cycle distribution was then analyzed by flow cytometric analysis. The percentage of cells in the subG1, G1, S and G2/M phase of the cell cycle are shown. Columns, mean of three independent experiments; bars, ±SE. B and C, SNU216, N87 and SNU668 cells were exposed to the increasing doses of PF00299804 (0.001, 0.01, 0.1 μmol/L) for 48h. Cells were subjected to immunoblotted with the indicated antibodies.
Figure 3. PF00299804 decreases the activity of the HER family and downstream signaling molecules and it inhibits the formation of EGFR/HER2, HER3/HER2, HER3/HER4 heterodimers and the association of HER3 with p85α in HER2 amplified cell lines. A, SNU216, N87 and SNU668 cells were treated with increasing doses of PF00299804 (0.001, 0.01, 0.1 μmol/L) for 48h, after which the extracts were immunoblotted with the indicated antibodies. 

B, SNU 216 or SNU668 cells were serum starved for 24 h, then grown for 3h in the increasing doses of PF00299804 (0.1, 1 μmol/L) for 3h, followed by 30 minutes of EGF (100ng/mL) stimulation. After cell lysis, EGFR was immunoprecipitated with specific antibodies. The immunoprecipitates were then subjected to immunoblot analysis using the indicated antibodies against EGFR and HER2, respectively. C, SNU216 or SNU668 cells were serum starved for 24h, then grown for 3h in the increasing concentrations of PF00299804 (0.1, 1 μmol/L) for 3h, followed by 30 minutes of HRG (100ng/mL) stimulation. After cell lysis, HER3 was immunoprecipitated with specific antibodies. The immunoprecipitates were subjected to immunoblot analysis using the indicated antibodies against HER3, HER2, HER4 or p85α, respectively.

Figure 4. PF00299804 enhances antitumor efficacy of chemotherapeutic agents in vitro and in vivo. A, Cells were treated with increasing concentrations of PF00299804, chemotherapeutic agents (5-FU(left), cisplatin(right)) or a combination of the two at a fixed ratio. The antiproliferative effects were then assessed and a median effect analysis was conducted. The combination index values at the 50% fraction affected are shown. B and C, Mice bearing subcutaneous N87 tumors of approximately 200 mm³ were orally administered PF00299804 daily or in combination with 5-FU (B) or cisplatin (C) interperitoneally weekly. Tumor volume was measured twice weekly until group tumor volume reached the predetermined endpoint of 800 mm³. *; significant when compared with either single agent (TGI t-test & OS Logrank P<0.01).
Figure 1.

A. N87 (HER2+)

% of viable cells

Concentration (μmol/L)

B. Mutations and amplifications of different cell lines.

C. EGFR amplification in #110 (2 SNPs)

D. Tumor Size (mm³) vs. Days of Treatment

- Vehicle
- PF00299804 7.5 mg/kg
Figure 2.

A

SNU 216 (HER2-amp)

N87 (HER2-amp)

SNU668 (HER2 non-amp)

% cell cycle phases

0 20 40 60 80 100

Sub G1 G1 S G2/M

% cell cycle phases

0 20 40 60 80 100

Sub G1 G1 S G2/M

% cell cycle phases

0 20 40 60 80 100

Sub G1 G1 S G2/M

B

SNU 216

N 87

SNU 668

PARP

Cleaved caspase-3

Cleaved Caspase-7

MCL-1

Bcl-2

Bim

Actin

C

SNU 216

N 87

SNU 668

Cyclin D

Cyclin E

CDK2

p 27kip1

Actin
Figure 3.

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Evaluation of the antitumor effects and mechanisms of PF00299804, a pan-HER inhibitor, alone or in combination with chemotherapy or targeted agents in gastric cancer


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