

Cancer Therapy: Preclinical

Lapatinib, a Dual EGFR and HER2 Kinase Inhibitor, Selectively Inhibits HER2-Amplified Human Gastric Cancer Cells and is Synergistic with Trastuzumab *In vitro* and *In vivo*

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Abstract

Purpose: HER2 amplification occurs in 18% to 27% of gastric and gastroesophageal junction cancers. Lapatinib, a potent ATP-competitive inhibitor simultaneously inhibits both EGFR and HER2. To explore the role of HER family biology in upper gastrointestinal cancers, we evaluated the effect of lapatinib, erlotinib, and trastuzumab in a panel of molecularly characterized human upper gastrointestinal cancer cell lines and xenografts.

Experimental Design: EGFR and HER2 protein expression were determined in a panel of 14 human upper gastrointestinal cancer cell lines and HER2 status was assessed by fluorescent *in situ* hybridization. Dose-response curves were generated to determine sensitivity to lapatinib, erlotinib, and trastuzumab. In HER2-amplified cells, the combination of trastuzumab and lapatinib was evaluated using the median effects principal. The efficacy of lapatinib, trastuzumab, or the combination was examined in HER2-amplified xenograft models.

Results: Lapatinib had concentration-dependent antiproliferative activity across the panel with the greatest effects in HER2-amplified cells. There was no association between EGFR protein expression and sensitivity to any of the HER-targeted agents. Cell cycle analysis revealed that lapatinib induced G₁ arrest in sensitive lines and phosphorylated AKT and phosphorylated ERK were decreased in response to lapatinib as well. The combination of lapatinib and trastuzumab was highly synergistic in inhibiting cell growth with a combination index of <1. The combination also induced greater decreases in AKT and ERK activation, G₀-G₁ cell cycle arrest, and increased rates of apoptosis. *In vivo* studies showed that the combination of lapatinib and trastuzumab had greater antitumor efficacy than either drug alone.

Conclusion: Together, these data suggest that lapatinib has activity in HER2-amplified upper gastrointestinal cancer and supports the ongoing clinical investigation of lapatinib in patients with HER2-amplified disease. *Clin Cancer Res*; 16(5): 1509-19. ©2010 AACR.

Adenocarcinoma of the stomach is the leading cause of gastrointestinal cancer in the world and is the second leading cause of cancer death worldwide (1). Despite recent advances in the molecular understanding of gastric cancer, there is a noticeable lack of targeted therapies in clinical development for this malignancy. The human epidermal growth factor receptor (HER) family consists of four homologous receptors: epidermal growth factor receptor (EGFR/HER1), HER2, HER3, and HER4. All are transmembrane glycoproteins, but HER2 has no known ligand and HER3 has a nonfunctioning kinase (2). HER2 overexpres-

sion and/or amplification has been detected in 20% to 27% of invasive breast cancers and correlates with poorer clinical outcomes (3, 4). Although HER2 amplification was first described in breast cancer, the prognostic value of HER2 alteration has since been described for other neoplasms, most notably gastric cancer (5). In both gastric and breast cancers, HER2 gene amplification is accompanied by increased expression of the gene product in the cell membrane resulting in growth and transformation (6). Studies evaluating the incidence of the alteration in gastric cancer by immunohistochemistry and fluorescence *in situ* hybridization (FISH) have reported rates between 10% and 28% in patients with gastric cancer (5, 7-10). Specifically, amplification of HER2 has been associated with the intestinal pathologic subtype of gastric cancer as well as with tumors arising from the gastroesophageal junction (7, 11). The largest analysis to date of the incidence of HER2 amplification in gastric cancer was from the recently reported phase III clinical trial evaluating the combination of trastuzumab with chemotherapy in

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Translational Relevance

Over the last several years, we have seen numerous targeted therapies enter advanced stages of clinical development in gastric and esophageal cancer. Based on recent results, it is anticipated that the anti-HER2 monoclonal antibody trastuzumab will become the first targeted therapy approved for patients with metastatic gastric cancer. In this work, we evaluated the activity of lapatinib, a small molecule tyrosine kinase inhibitor in a large panel of gastric and esophageal cancer cell lines and we show its selectivity for the HER2-amplified subgroup. We provide a comparison between isolated EGFR inhibition with erlotinib and HER2 inhibition by both lapatinib and trastuzumab. We show for the first time that lapatinib and trastuzumab interact synergistically to inhibit human HER2-amplified gastric and esophageal cancer cells and that this combination acts by increasing apoptosis and by blocking downstream activation through the AKT and mitogen-activated protein kinase pathways. Clinical studies built on the described laboratory studies are currently ongoing.

patients with metastatic gastric cancer. In this study, the overall rate of HER2 amplification was reported to be 22%, with a higher percentage (34%) in patients with gastroesophageal junction tumors (12). Currently, several ongoing clinical trials are exploring the addition of anti-HER2 agents with chemotherapy in HER2-positive gastric or gastroesophageal adenocarcinomas (5).

In gastric cancer models, there has been limited examination of HER2-targeted agents; although one study, examining the combination of trastuzumab and docetaxel, showed impressive antitumor activity in a gastric cancer xenograft model (13). An alternative anti-HER2 strategy has been the development of small-molecule tyrosine kinase inhibitors that target not only HER2 but other HER family proteins. The simultaneous inhibition of multiple receptors may be an attractive strategy, as interactions between HER2 and EGFR provide a mechanism for signal diversification and augmentation (14). Lapatinib (Tykerb; GlaxoSmithKline) is a potent ATP-competitive inhibitor that simultaneously inhibits both EGFR and HER2. In cell-free biochemical kinase assays, lapatinib inhibits the recombinant EGFR and HER2 tyrosine kinases by 50% (IC_{50}) at concentrations of 10.8 and 9.3 nmol/L, respectively. In cell-based assays, lapatinib inhibits the growth of HER2-overexpressing BT474 breast cancer cells at comparably low concentrations (IC_{50} , 100 nmol/L; ref. 15).

Our group has previously shown that lapatinib has antiproliferative activity in human HER2-amplified breast cancer cell lines and in trastuzumab-conditioned breast cancer models (15). This *in vitro* observation has been validated clinically in breast cancer patients who

have progressed on trastuzumab and have improvements in clinical outcomes when placed on a lapatinib-containing regimen (16). In preclinical models, the combination of lapatinib and trastuzumab has shown synergistic behavior in breast cancer cell lines and recent clinical data suggests that targeting the HER2 axis with two distinct inhibitors may improve clinical outcomes (15, 17, 18, 26). These data have generated our hypothesis that HER2-directed therapy may be clinically active in HER2-amplified gastric cancer. To explore this, we did a preclinical evaluation comparing the biological effects of lapatinib and the isolated EGFR tyrosine kinase inhibitor erlotinib on a large panel of human gastric and gastroesophageal junction cell lines with and without HER2 amplification. We analyzed the growth-inhibitory effects of these agents with their effects on expression and activation of EGFR, HER2, and the downstream signaling molecules AKT and ERK. We used cell cycle analysis and apoptosis assays to further characterize the biological effects of lapatinib. Finally, we analyzed the combination of trastuzumab and lapatinib in HER2-amplified cell lines and xenografts and showed that they behave synergistically.

In summary, the current studies were intended to provide a rationale to test lapatinib either as a single agent or in combination with trastuzumab in patients with metastatic gastric cancer and support the ongoing investigation of the role of HER2 as a therapeutic target in upper gastrointestinal cancer.

Materials and Methods

Cell lines, cell culture, and reagents. The effects of lapatinib, erlotinib, and trastuzumab on malignant cell growth were studied in a panel of 14 established human gastric and esophageal cancer cell lines. The human gastric cancer cell lines AGS, NCI-N87, KATO III, SNU-1, SNU-5, and SNU-16 were obtained from the American Type Culture Collection, as were the human cell lines SKBR3 and A431. The human gastric cancer cell lines NUGC4, NUGC3, FU97, IM95, IM95m, MKN74, and MKN1 were obtained from the Japanese Health Science Research Resources Bank (Osaka, Japan). The cell line OE19 was obtained from the European Collection of Animal Cell Cultures (Sigma-Aldrich). SNU-1, SNU-5, AGS, N87, Kato III, SNU-16, MKN74, MKN1, NUGC-3, NUGC-4, and OE19 were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and PSF (Irvine Scientific). AGS, FU-97, IM95, and IM95m were cultured in MEM Eagle's medium (Irvine Scientific) supplemented with 10% fetal bovine serum and 10 mg/mL of insulin (Sigma Aldrich). Lapatinib was generously provided by Tona Gilmer of GlaxoSmithKline. It was prepared as a 10 mmol/L stock solution in DMSO (Fisher Scientific). Erlotinib was provided by OSI Pharmaceuticals and was prepared as a 10 mmol/L stock in DMSO (Fisher Scientific). Trastuzumab was obtained from UCLA Pharmaceutical Services and was prepared from a stock concentration of 21 mg/mL.

K-Ras mutation assays. Frozen pellets were obtained from culture and washed twice in PBS, pelleted, and digested using a Proteinase K buffer. DNA was purified from the collected cells using the MagneSil Genomic Fixed Tissue System DNA Isolation Kit (Promega). A NanoDrop 1000 was used to evaluate the concentration of the samples.

KRAS testing was done using the DxS KRAS Mutation Kit. Sample DNA was added to eight separate reactions. The reaction mixes contain a single primer set specific for either the wild-type sequence or one of seven mutations in codons 12 and 13. Mutation identification was based on allele-specific real-time PCR using Scorpions probes (provided by Clariant, Inc.).

Proliferation assays. Cells were plated into 24-well plates at a density of 2×10^4 to 6×10^4 and grown in cell line-specific medium in decreasing concentrations of both lapatinib (ranging between 10 and $0.3125 \mu\text{mol/L}$) and erlotinib (ranging between 10 and $0.3125 \mu\text{mol/L}$). The same cell lines were treated with trastuzumab at a fixed dose of $10 \mu\text{g/mL}$. Cells were harvested by trypsinization on day 6 and counted using a particle counter (Z1; Beckman Coulter, Inc.). Growth inhibition was calculated as a percentage of the untreated controls. Experiments were done twice and in duplicate for each cell line (Microsoft Excel).

Western blots and immunoprecipitation. Cells in log phase growth were washed in PBS and lysed at 4°C in lysis buffer. Insoluble material was cleared by centrifugation at $10,000 \times g$ for 10 min. Protein was quantitated using bi-

cinchoninic acid (Pierce Biochemicals), resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Invitrogen). Total HER2 and EGFR expression were detected, respectively, by monoclonal anti-HER2 (Calbiochem), anti-EGFR antibodies (Santa Cruz Biotechnology). For the evaluation of concentration-dependent activity of erlotinib and lapatinib on HER2, EGFR, AKT, and ERK phosphorylation, cells in log phase growth were treated with varying doses of lapatinib for 1 h prior to cell lysis. Tyrosine phosphorylation of HER2 and EGFR was analyzed as follows: immunoprecipitations were done by allowing $250 \mu\text{g}$ protein lysate to incubate with $3 \mu\text{g}$ of monoclonal anti-HER2 (Calbiochem) or anti-EGFR antibody (Santa Cruz Biotechnology), and protein A/G-agarose (Santa Cruz Biotechnology) at 4°C overnight with gentle agitation. The immunoprecipitates were washed thrice in lysis buffer and then denatured in Lemmli's buffer prior to SDS-PAGE. Immunoblotting was done using a monoclonal antiphosphotyrosine antibody (Upstate). Detection was done using ECL Plus chemifluorescent reagent (Amersham Biosciences) and imaging of the resulting Western blots was done using the chemifluorescence method by FluorChem Q MultiImage III (Alpha Innotech). Total AKT, total ERK, phosphorylated AKT, and phosphorylated ERK were detected by polyclonal anti-pAKT (Ser473) and anti-pERK (Thr²⁰²/Thr²⁰⁴) antibodies (Cell Signaling).

FISH. HER2 gene copy number was analyzed using FISH. Fourteen established upper gastrointestinal cancer cell lines were treated with Colcemid (0.05 g/mL) for

Table 1. Lapatinib and erlotinib concentrations that achieve IC_{50} and the corresponding k-Ras, and HER2 molecular status in gastric and esophageal cancer cells

| Cell line | Lapatinib IC_{50} (mean \pm SE, $\mu\text{mol/L}$) | Erlotinib IC_{50} growth (mean \pm SE, $\mu\text{mol/L}$) | Trastuzumab, growth inhibition (%) | HER2 amplification status | K-Ras mutation |
|-----------|---|--|---------------------------------------|------------------------------|-------------------|
| N87 | 0.01 ± 0.04 | 3.32 ± 0.37 | 15.50 ± 6.08 | Amplified | WT |
| OE19 | 0.09 ± 0.02 | 2.31 ± 0.50 | 34.53 ± 5.20 | Amplified | WT |
| NUGC4 | 0.35 ± 0.03 | 0.24 ± 0.04 | 0 | Not amplified | WT |
| NUGC3 | 2.24 ± 0.55 | 0.70 ± 0.01 | 0 | Not amplified | WT |
| FU97 | 4.86 ± 0.34 | 4.76 ± 0.96 | 0 | Not amplified | WT |
| SNU16 | 8.58 ± 0.69 | 6.50 ± 1.31 | 0 | Not amplified | WT |
| IM95 | >10 | >10 | 2.80 | Not amplified | WT |
| IM95m | >10 | >10 | 7.85 | Not amplified | WT |
| MKN74 | >10 | >10 | 10.60 | Not amplified | WT |
| MKN1 | >10 | 0.96 ± 0.48 | 5.83 | Not amplified | WT |
| KATOIII | >10 | 5.98 ± 0.98 | 4.18 | Not amplified | WT |
| AGS | >10 | >10 | 5.01 | Not amplified | G12D (exon 1) |
| SNU1 | >10 | >10 | 0 | Not amplified | G12D (exon 1) |
| SNU5 | >10 | >10 | 0 | Not amplified | WT |

NOTE: Fourteen gastric and esophageal cancer cell lines were treated with lapatinib, erlotinib, and trastuzumab as described. Lapatinib and erlotinib reported as concentrations that achieve IC_{50} whereas the effects of trastuzumab are reported as a percentage of growth inhibited. HER2 amplification status and k-Ras mutation status of the cell lines as measured by FISH and PCR, respectively.

24 h to obtain metaphase preparations. All samples were fixed in methanol/acetic acid (3:1). Specimen preparation, hybridization, and microscopy were done as previously described (19). A multicolor HER2 spectrum orange, and CEP17 spectrum green probe was used (Vysis, Inc.). Samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and scored as positive for amplification if they had either (a) greater than four copies of HER2/neu per chromosome 17 centromere in at least 20 cells, or had (b) uncountable clusters of signals suggestive of an amplification in 20 or more cells.

Cell cycle analysis and apoptosis studies. The effects of lapatinib on the cell cycle were evaluated using Nim-DAPI staining. Cells were plated evenly in control and experimental wells and allowed to grow to log phase. Cells were then treated with varying doses of lapatinib (100 nmol/L, OE19; 20 nmol/L, N87), 10 µg/mL of trastuzumab, or the combination of these two drugs. At the time of cell cycle analysis, supernatant was collected, cells were washed with PBS, and trypsin was applied to release cells, which were then centrifuged at 3,000 rpm for 5 min. Supernatant was aspirated and cells were then resuspended in 100 µL of Nim-DAPI (NPE Systems) and gently vortexed. Cells were analyzed with UV using a Cell Lab Quanta SC flow cytometer (Beckman Coulter). For apoptosis assays, the supernatant was aspirated and cells were then resuspended in 150 µL of binding buffer, and stained with 5 µL of Annexin V-FITC and 5 µL of propidium iodide at room temperature for 5 min (Medical & Biological Laboratories, Co.; refs. 20, 21). After incubation, cells were processed as directed in the kit and analyzed using a FITC signal detector and propidium iodide detector using a Cell Lab Quanta SC flow cytometer.

Multiple drug effect analysis. Aliquots of 20×10^3 N87 and OE19 cells were plated in 24-well microdilution plates. Following cell adherence (24 h), experimental culture fluids containing either control medium, lapatinib, trastuzumab, or the combination (lapatinib plus trastuzumab) were added to appropriate wells in duplicate, and serial 2-fold dilutions were done to span clinically relevant concentration ranges for the dose-effect analyses for lapatinib and drug combinations. Following incubation for 5 d, cells were harvested by trypsinization on day 6 and counted using a particle counter (Z1; Beckman Coulter). Growth inhibition was calculated as a percentage of the untreated controls. Experiments were done in duplicate for each cell line. For each assay, the log of the fractional growth inhibition was plotted against the log of the drug concentration and the linear regression curve fit correlation coefficient (*r* value) was calculated. Multiple drug effect analysis was done using computer software (Biosoft) as described in detail (22). In this analysis, synergy is defined as combination index (CI) values of <1.0, antagonism as CI values >1.0, and additivity as CI values equal to 1.0.

Tumor xenografts. Six-week-old CD-1 athymic nude mice were purchased from Charles River Laboratories. A total of 5×10^6 cells in 50% Matrigel were injected into

the right flanks of 44 mice (11 for each group). Tumor xenografts were measured with calipers thrice a week, and tumor volume in mm³ was determined by multiplying height × width × length. When tumors reached an average size of 150 to 200 mm³ (4 d after injection), mice were randomized and treatment was begun. Trastuzumab (10 mg/kg in sterile PBS) or sterile PBS (control) was given i.p. twice weekly. Lapatinib (100 mg/kg) was administered daily (Monday to Friday) by oral gavage in 0.5% hydroxypropyl methylcellulose and 0.1% Tween 80 (Sigma). After 23 d of treatment, the animals were euthanized. Results are presented as mean volumes for each group. Error bars represent the SD of the mean. Comparisons between all groups were made using a two-tailed Student's *t* test. Differences between groups were considered statistically significant at *P* < 0.05.

Statistical methods. Associations between the expression levels of biomarkers and the IC₅₀ values were analyzed using Spearman's ρ correlation, and differences in the IC₅₀ values between subgroups compared using the Mann-Whitney *U* test. All statistical tests were two-sided.

Results

Lapatinib selectively inhibits the growth of HER2-amplified esophageal and gastric cancer cell lines. To evaluate the effects of lapatinib on human gastric cancer cells, we used a panel of 14 established human gastric and esophageal cancer cell lines that express variable levels of EGFR and HER2. These lines were selected because they represent a spectrum of gastric and esophageal adenocarcinomas covering both intestinal and diffuse pathologic subtypes. In addition, some of these lines were derived from the gastroesophageal junction, a malignancy in increasing incidence in the western world (1). Table 1 shows the calculated IC₅₀ for each cell line, K-Ras, and HER2 amplification status. Lapatinib inhibited the proliferation of the upper gastrointestinal cancer cell lines in a concentration-dependent manner and the IC₅₀ values varied significantly among the individual cell lines (Fig. 1A). Using FISH, 2 of the 14 cell lines (NCI N87 and OE19) showed levels of HER2 amplification similar to that seen in the HER2-amplified breast cancer cell line SK-BR-3 (data not shown), a breast cancer cell line widely used as a standard for high-level HER2 amplification (Fig. 1B). These were also the two most sensitive cell lines to lapatinib and trastuzumab.

Effect of lapatinib on EGFR, HER2, AKT, and ERK signaling in gastric and esophageal cancer cells. EGFR and HER2 can signal through the AKT and mitogen-activated protein kinase pathways (6). Having previously shown that the effect of lapatinib in breast cancer cells is mediated through these pathways, we sought to examine the effect of lapatinib on EGFR, HER2, AKT, and mitogen-activated protein kinase phosphorylation in gastric and esophageal cancer cells (15). Fourteen human upper gastrointestinal cancer lines with differential EGFR and HER2 expression

were exposed to lapatinib at a concentration of 1 $\mu\text{mol/L}$. In the HER2-amplified cell lines (N87 and OE19), lapatinib decreased both EGFR and HER2 phosphorylation when compared with untreated controls (Fig. 2A and B).

In addition, there was a significant decrease in both AKT and ERK phosphorylation, suggesting that lapatinib may be blocking HER-driven signaling through mitogen-activated protein kinase and AKT.

In the most sensitive cell lines, there was a significant decrease in the candidate markers after exposure to 1 $\mu\text{mol/L}$ of lapatinib. In the more resistant lines, there were no significant changes in these markers with the exception of MKN1 and SNU1, which had a decrease in ERK. In addition, there was no correlation between pTEN status and response to lapatinib (data not shown). These studies show that lapatinib can suppress the phosphorylation of key pathways that are downstream of EGFR and HER2 at clinically achievable concentrations.

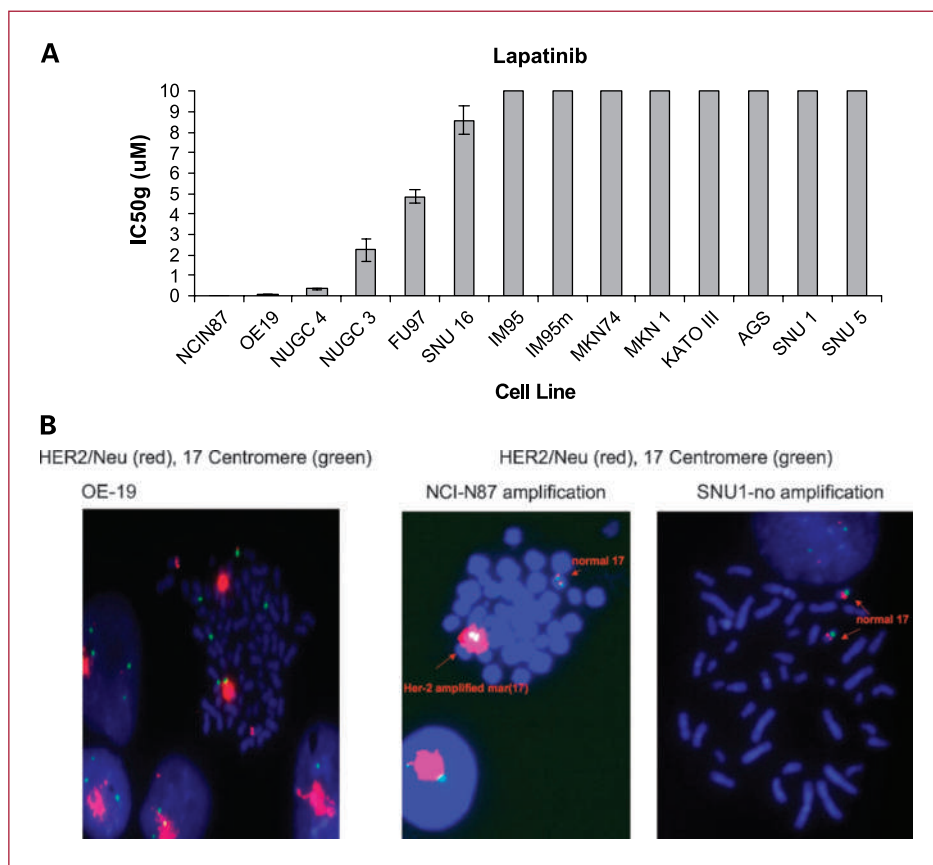
Lapatinib induces a G_0 - G_1 arrest and apoptosis in HER2-amplified cell lines. Having determined that lapatinib had very selective growth inhibition on the two HER2-amplified gastric cancer cell lines, its effects on the cell cycle were analyzed. The two most sensitive cell lines (N87 and OE19) and the two least sensitive cell lines (SNU1 and SNU5) were incubated for either 48 or 120 hours with 1 $\mu\text{mol/L}$ of lapatinib, and cells were analyzed by flow-

cytometry using Nim-DAPI staining. G_0 - G_1 arrest was seen in the two sensitive cell lines (N87 and OE19; Fig. 3A and B) but not in the resistant lines (SNU1 and SNU5). In addition, when compared with untreated controls, lapatinib induced apoptosis in both N87 and OE19 at both early (48 hours) and late (120 hours) time points, suggesting a continued effect on apoptotic cell death. Together, these data support the proposed mechanism of action of this agent in HER2-amplified upper gastrointestinal cancer cells.

The effects of lapatinib are distinct from the effects of anti-EGFR blockade by erlotinib or anti-HER2 blockade by trastuzumab. To understand the relative contributions of EGFR and HER2 inhibition, the growth-inhibitory effects of lapatinib were compared with those of erlotinib, an isolated EGFR tyrosine kinase inhibitor (Table 1). Interestingly, erlotinib had its greatest antiproliferative effect on the two cell lines (NUGC4 and NUGC3) that were also relatively sensitive to lapatinib.

No EGFR or k-Ras mutations were noted in these two cell lines. As in the case with lapatinib, no correlation was noted between the IC_{50} concentrations of erlotinib and EGFR expression, further supporting the lack of predictive value of EGFR expression with regard to the effects of EGFR inhibition. We also compared the effects of erlotinib and lapatinib on downstream signaling and

Fig. 1. A, growth-inhibitory effects of lapatinib in gastric and esophageal cancer cell lines. Cells were treated with lapatinib at ranges from 0.001 to 10 $\mu\text{mol/L}$. N87 and OE19 (two HER2-amplified cell lines) were the most sensitive cell lines to lapatinib. This data is the result of experiments repeated in triplicate. **B,** HER2 gene copy number was analyzed using FISH. Samples were counterstained with DAPI and scored as positive for amplification if they had either (A) greater than four copies of HER2/neu per chromosome 17 centromere in at least 20 cells, or had (B) uncountable clusters of signals suggestive of an amplicon in 20 or more cells. HER2/Neu amplification was seen in two cell lines: OE19 and N87. SNU1 is representative of nonamplification.



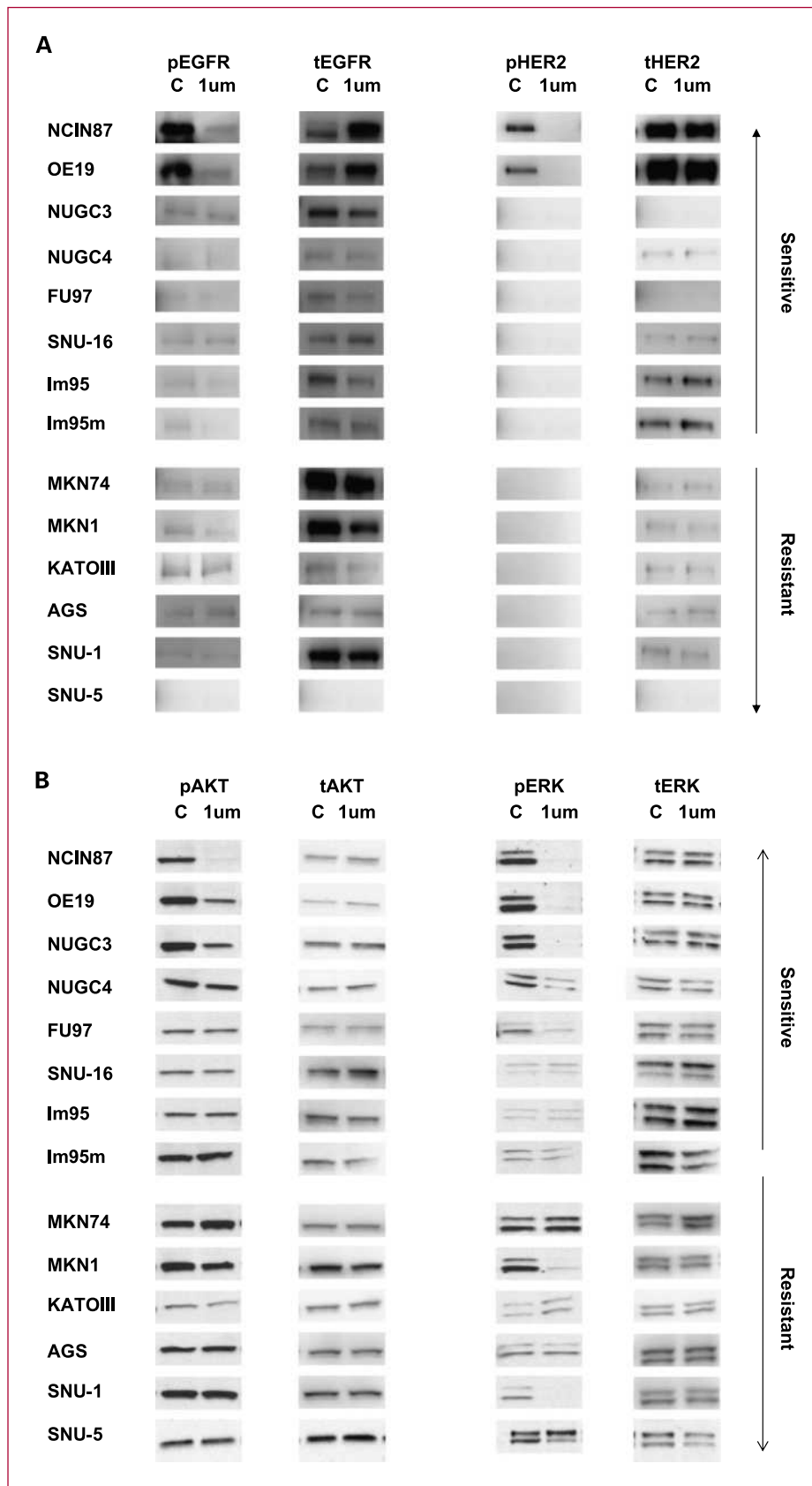


Fig. 2. Cell signaling after treatment with lapatinib and erlotinib in gastric cancer cell lines: all cell lines were treated with 1 $\mu\text{mol/L}$ of lapatinib. A, no appreciable differences in EGFR expression were seen after treatment with lapatinib but pEGFR was decreased in the two most sensitive cell lines. No differences in total HER2 expression were seen after treatment with lapatinib but pHER2 was also decreased in the two most sensitive cell lines. B, downregulation of pAKT was seen in the most lapatinib-sensitive cell lines. Similarly, downregulation of pERK was seen in the most lapatinib-sensitive cell lines.

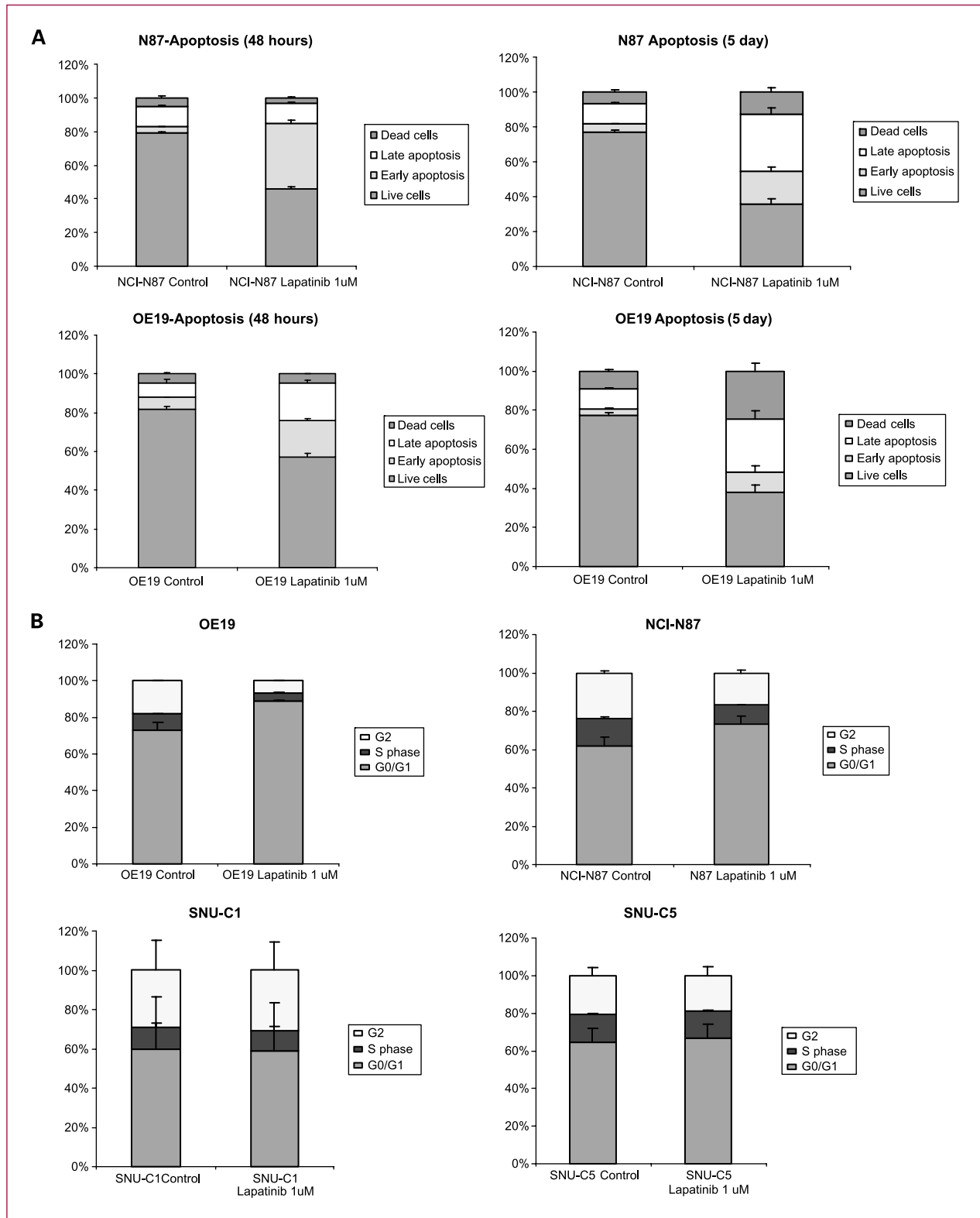


Fig. 3. Lapatinib induces G_0 - G_1 cell cycle arrest and apoptosis in HER2-amplified gastric cancer. A, N87 and OE19 cells were treated with 1 μ mol/L of lapatinib and cells were analyzed for DNA content by flow cytometry. The proportion of cells that undergo apoptosis is increased and is maintained both at 48 h and 5 d after treatment. B, N87 and OE19 both show an increase of the G_0 - G_1 fraction after lapatinib treatment. No appreciable differences in the cell cycle are seen in SNU1 and SNU5 after treatment with lapatinib.

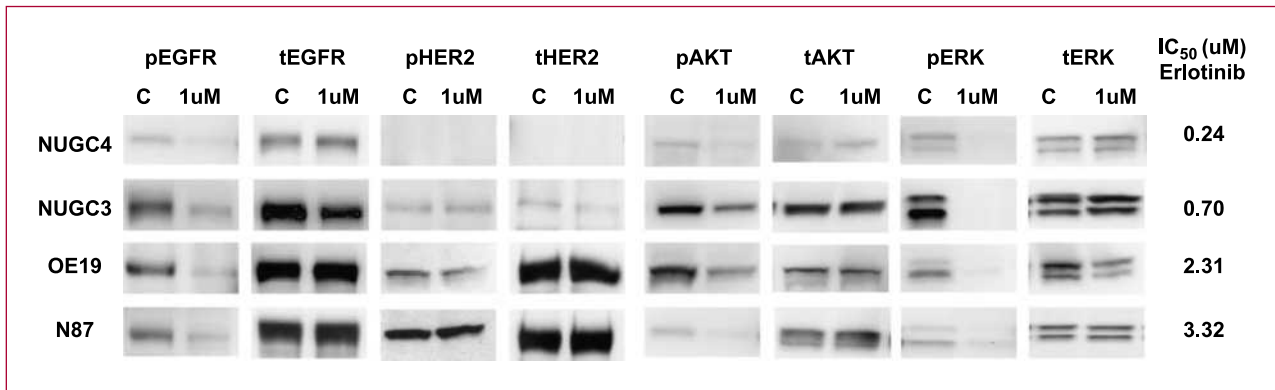


Fig. 4. The effects of lapatinib are distinct from the effects of anti-EGFR blockade by erlotinib or anti-HER2 blockade by trastuzumab. Cell signaling after treatment with erlotinib in gastric cancer cell lines: all cell lines were treated with 1 μ mol/L of erlotinib. Some downregulation of pEGFR was seen in the most erlotinib-sensitive cell lines and no effect was seen on total EGFR expression. As in lapatinib-sensitive cells, downregulation of pERK was seen in the most erlotinib-sensitive cell lines. No effects in pHER2 or total HER2 expression were seen after treatment with erlotinib.

found that in the most erlotinib-sensitive cells (NUGC3 and NUGC4) there was a decrease in pEGFR and a significant downregulation of pERK, suggesting that the effect of erlotinib is mediated through mitogen-activated protein kinase inhibition. Not surprisingly, there were no effects on either pHER2 or total HER2 expression in any of the cell lines treated with erlotinib (Fig. 4).

Finally, we also performed growth assays on all gastric and esophageal cancer cell lines with trastuzumab. *In vitro*, the two HER2-amplified cell lines that responded to lapatinib were also the most sensitive cells to inhibition with trastuzumab (Table 1).

The combination of lapatinib and trastuzumab is synergistic in HER2-amplified upper gastrointestinal cancer in vitro and in vivo. We have previously shown that the combination of trastuzumab and lapatinib is synergistic in HER2-amplified human breast cancer cells (15). To evaluate this combination in gastric cancer, we treated two HER2-amplified cell lines (N87 and OE19) with various clinically relevant concentration ranges of lapatinib and trastuzumab. The lapatinib concentrations used for these experiments ranged from 3.125 to 100 nmol/L for OE19 and 0.625 to 20 nmol/L for N87. For trastuzumab, doses ranged from 0.3125 to 10 μ g/mL for both cell lines.

Multiple drug effect analysis was done to determine the nature of the interaction (synergy, additive, or antagonism). When these two agents were combined, significant synergy in both HER2-amplified cell lines was seen (Fig. 5A and B). Mean CI for the dose-response curves were obtained: 0.228 ± 0.096 ($P < 0.0001$) in OE19 to 0.226 ± 0.042 ($P < 0.0005$) in N87 (Fig. 5B). The combination also induced a statistically significant increase in the proportion of cells that were undergoing G₀-G₁ cell cycle arrest (Fig. 5C). In addition, an increase in apoptosis was seen at both early (24 hours) and late (120 hours) time points. We measured the phosphorylation of several downstream markers that were treated

with this combination and found that it caused a greater decrease in pAKT and pERK when compared with either trastuzumab or lapatinib alone (Fig. 5D). This effect was most pronounced in N87, the most sensitive cell line to lapatinib and the one in which the greatest synergy was observed. There were no appreciable increases in the ability of the combination to block either pEGFR or pHER2 when cells were treated with the combination of lapatinib and trastuzumab as compared with lapatinib alone. In OE19, lapatinib also had maximal blockade of pEGFR and HER2 as a single agent and in combination with trastuzumab. However, in N87, the combination did not block pEGFR or HER2 as much as lapatinib alone.

To expand on our *in vitro* observations, we treated HER2-amplified N87 xenografts with either trastuzumab alone, lapatinib alone, the combination, or excipient control. Treatments were started on day 4 post-injection when tumors were well established. As seen in the *in vitro* studies, both single-agent lapatinib and trastuzumab caused tumor regression in N87 xenografts. In addition, the combination showed near complete tumor resolution by day 23 (Fig. 6). These observations were statistically significant, confirming the added benefit of total HER2 blockade.

Discussion

Lapatinib has recently been approved for the treatment of patients with HER2-amplified metastatic breast cancer whose disease has progressed on trastuzumab-based therapy (16). Encouraged by these data and the increasing interest of the role of HER2 in gastric and esophageal cancer, we sought to evaluate the therapeutic potential of this dual kinase inhibitor for the treatment of upper gastrointestinal cancer. We also sought to compare the effects of lapatinib with both erlotinib and trastuzumab and understand the mechanism of action of these agents.

Of 14 gastric and esophageal cancer cell lines evaluated, HER2 amplification and phosphorylated-protein overexpression were detected in two of them; NCI-N87 and OE19. Here we report that, as in breast cancer, HER2 amplification is the best predictive marker for the antiproliferative effects of lapatinib. In addition, as previously shown for endometrial and breast cancer, no correlation was shown between the activity of lapatinib and EGFR expression at the protein level (15, 23, 24).

A recent report by Kim et al. evaluated the role of lapatinib in gastric cancer cell lines (25). Our study adds additional data to the role of the HER family in upper gastrointestinal cancers in several respects. First, as our panel of cell lines was mostly derived from North American tissue banks, there were only four cell lines in common with that report. This is important, as distinct differences have long been noted between tumors of the upper gastrointestinal tract from Asian versus Western

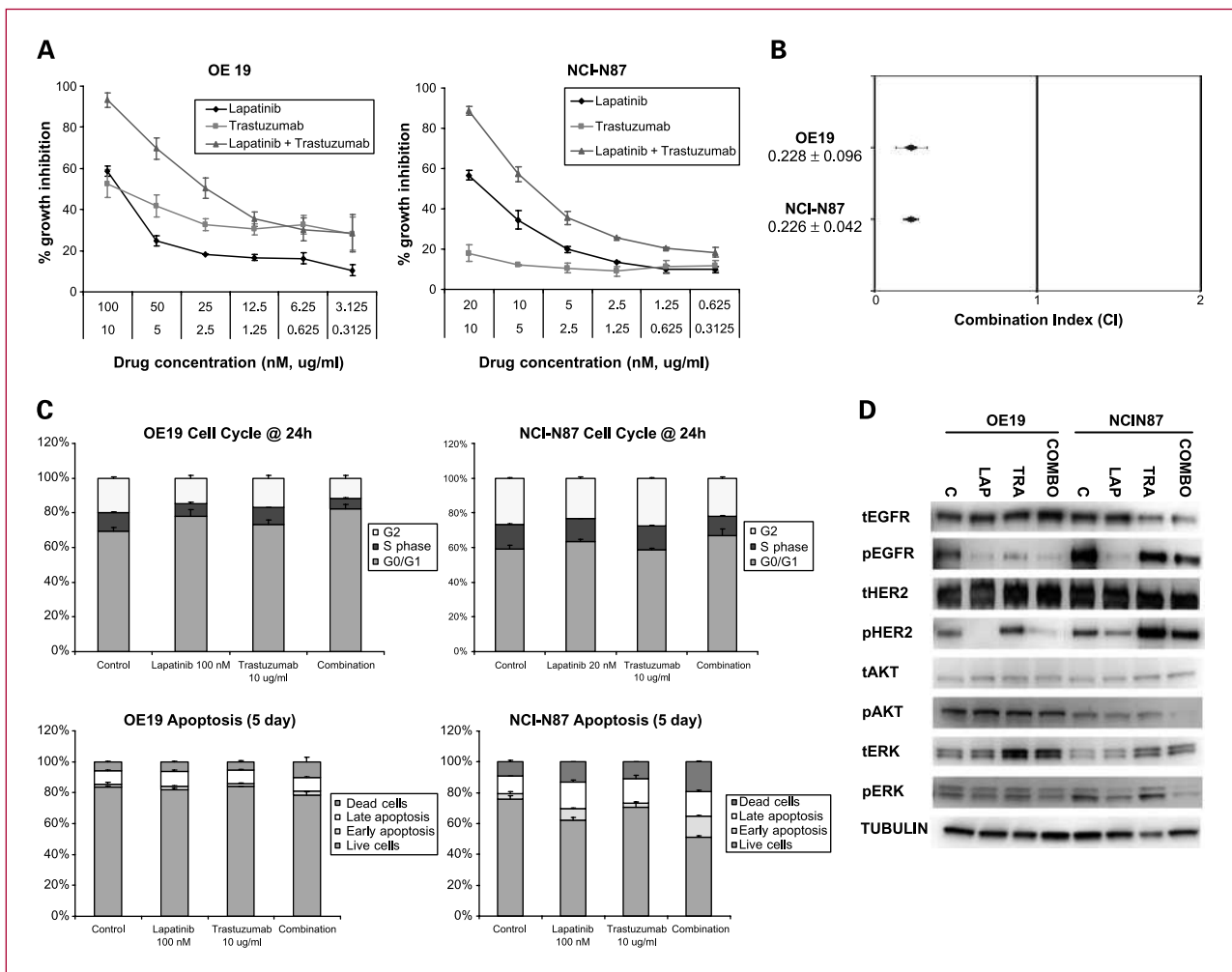


Fig. 5. The combination of lapatinib and trastuzumab is synergistic in HER2-amplified gastric cancer. **A**, in this experiment, the lapatinib concentrations ranged from 3.125 to 100 nmol/L for OE19 and 0.625 to 20 nmol/L for N87. For trastuzumab, doses ranged from 0.3125 to 10 μ g/mL for both cell lines. Growth inhibition was calculated as a percentage of the untreated controls. Experiments were done in duplicate for each cell line. **B**, mean CI values for lapatinib-trastuzumab combinations in two HER2-amplified gastric cancer cell lines. Error bars indicate the 95% CI of the mean value. CI values were derived from variables of the median effects plots, and statistical tests were used to determine significance. In this analysis, synergy is defined as CI values significantly lower than 1.0, antagonism as CI values significantly higher than 1.0, and additivity as CI values equal to 1.0. Synergistic interactions were observed in both cell lines ranging from 0.228 ($P < 0.0001$) in OE19 to 0.226 ($P < 0.0005$) in N87. **C**, cells were treated with lapatinib at 100 nmol/L (OE19) or 20 nm (N87), trastuzumab (10 μ g/mL) or the combination. Cells were analyzed for DNA content by flow cytometry. Both N87 and OE19 show a statistically significant increase in apoptosis with combination therapy than with either lapatinib or trastuzumab alone. There is also an increase in G_0 - G_1 cell cycle arrest in the combination than with either lapatinib or trastuzumab alone. **D**, comparisons of signaling and downstream markers with lapatinib alone, trastuzumab alone or the combination. In this experiment, lapatinib treatment was at 100 nmol/L (OE19) or 20 nm (N87) combined with trastuzumab at 10 μ g/mL. Minimal downregulation of pAKT, pERK are seen with lapatinib alone in both cell lines. Trastuzumab alone also has no effect on any of these markers. The combination of the two drugs causes a significant downregulation of pAKT and pERK in both HER2-amplified cell lines but is most pronounced in N87, the cell line with the highest level of amplification. No significant changes were seen with the combination in either pEGFR or pHER2 downregulation.

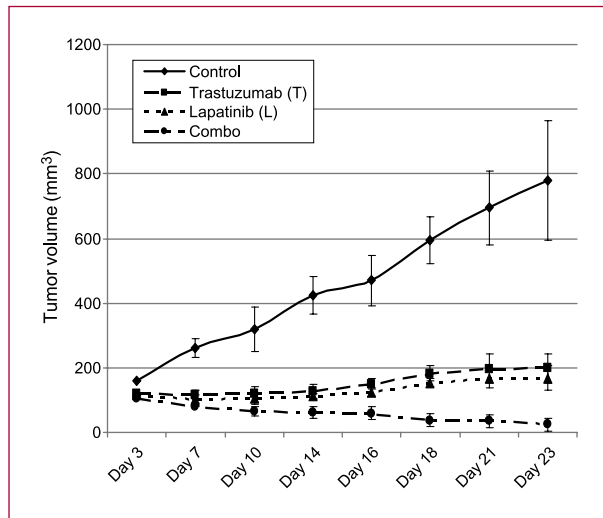


Fig. 6. Antitumor activity of lapatinib and trastuzumab on N87-bearing xenografts. N87 cells (5×10^6 cells with 50% Matrigel) were injected s.c. into nude mice and mice were randomized into one of four groups ($n = 11/\text{group}$). Treatment started at day 4 after injection with either lapatinib (100 mg/kg daily for 3 wk), trastuzumab (10 mg/kg i.p. twice weekly) or sterile PBS (i.p. twice weekly) or the combination of lapatinib and trastuzumab at the doses above. Tumors were measured biweekly. Student's *t* test was used to compare tumor sizes between the groups and results are presented as mean. Error bars represent SD of the mean. Student's *t* test was done at the end of the experiment. All comparisons were statistically significant between the following groups: combo versus L or T alone, $P < 0.05$; combo versus control, $P < 0.001$; L or T versus control, $P < 0.001$.

patients. In addition, several of our cell lines were derived from tumors of the distal esophagus; which has clinical implications as it reflects the increasing incidence of gastroesophageal junction cancer in the Western hemisphere (1). Our data support those of Kim et al. by demonstrating that the most significant effects of lapatinib are in the HER2-amplified lines. Furthermore, we found that its activity is modulated by blocking the phosphorylation of AKT and ERK. However, we also show distinct differences between lapatinib, trastuzumab, and erlotinib, three agents each with a distinct mechanism of targeting the HER pathway. Although the two HER2-amplified cell lines had minimal responses to erlotinib, the most sensitive cell lines to erlotinib were only moderately sensitive to lapatinib. In these cells, treatment with either lapatinib or erlotinib resulted in the downregulation of pEGFR and pERK, suggesting that lapatinib may have some anti-EGFR activity, albeit at higher concentrations. A comparison of the three drugs shows a significant correlation only between trastuzumab and lapatinib activity and HER2 amplification.

Lapatinib showed significant synergy when combined with trastuzumab in both of the HER2-amplified cell lines. Furthermore, in the N87 xenograft, the combination of lapatinib and trastuzumab induced a near com-

plete tumor regression in all the mice that were treated. These effects were much more pronounced than either lapatinib or trastuzumab alone. Similar observations have been made by our group and others in HER2-amplified breast cancer (15, 18). In this study, we show that the synergy seen with this combination is likely a consequence of both effects on apoptosis and cell signaling. Inhibition of pAKT and pERK was greater with the combination of lapatinib and trastuzumab than with either drug alone. The synergy described with this combination might have clinical implications, as recently shown in breast cancer patients that had progressed on trastuzumab seemed to have higher clinical responses when treated with the combination than with lapatinib alone (26). Our future work is aimed at demonstrating the effects of this drug in trastuzumab-conditioned N87 and OE19 cells in an effort to determine if clinical observations in breast cancer could also be extended to gastric cancer.

Our data suggest that inhibition of the HER2 pathway with the combination of a monoclonal antibody and tyrosine kinase inhibitor may augment the effects on downstream signaling pathways. Ongoing work in our laboratory is aimed at addressing the mechanism of these agents in combination in gastric cancer *in vivo* models. Recent work by Scaltriti et al. in breast cancer models showed that the accumulation of inactive HER2 receptor (induced by lapatinib) leads to enhanced trastuzumab activity through antibody-dependent cellular cytotoxicity (27). Although our results are preliminary, they support the ongoing investigation of lapatinib in gastric cancer as well as its possible combination with trastuzumab in HER2-amplified disease. The recently reported randomized phase III clinical trial, trastuzumab in gastric cancer (ToGA) met its prespecified clinical end points for overall survival (28). This suggests that, in certain settings, the addition of anti-HER2 therapy to standard chemotherapy could have direct clinical benefit and makes the investigation of additional anti-HER2 therapies in upper gastrointestinal cancers especially timely.

In this study, we focused on the effects of lapatinib in upper gastrointestinal cancers and comparisons between this agent and other HER-targeted molecules. These data do not rule out the possibility that the ability of lapatinib to block EGFR may be relevant in other malignancies, although data for its HER2 specificity *in vitro* for breast and endometrial cancers is well established. Ongoing studies are evaluating the role of lapatinib in other solid tumors not known to have significant HER2 amplification, i.e., lung cancer and colorectal cancer. Several clinical trials have suggested that the activity of anti-EGFR agents seems to be limited to tumors of the gastroesophageal junction, with responses to both erlotinib and gefitinib reported as ~10% (29–31). This differs from tumors derived from the body of the stomach in which no clinical responses were seen. In addition, in a clinical study of lapatinib in upper gastrointestinal cancers, disease control (prolonged stable disease) was reported only

in patients noted to have HER2-amplified disease (32). Our *in vitro* and *in vivo* observations support these clinical findings and the ongoing development of lapatinib in patients with HER2-amplified tumors.

Disclosure of Potential Conflicts of Interest

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Clinical Cancer Research

Lapatinib, a Dual EGFR and HER2 Kinase Inhibitor, Selectively Inhibits HER2-Amplified Human Gastric Cancer Cells and is Synergistic with Trastuzumab *In vitro* and *In vivo*

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