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HSP90 inhibitor, AUY922, debilitates intrinsic and acquired lapatinib-resistant HER2-positive gastric cancer cells

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Running Title: AUY922 overcomes lapatinib resistance in Gastric Cancer

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ABSTRACT

Human epidermal growth factor receptor 2 (HER2) inhibitors such as trastuzumab and lapatinib are used for breast cancer or gastric cancer (GC) that tests HER2 positive. However, as with other targeted therapies, the occurrence of intrinsic or acquired resistance to HER2 inhibitors such as trastuzumab and lapatinib is an unresolved therapeutic problem for HER2-positive gastric cancer. The present study describes how the heat shock protein 90 (HSP90) inhibitor AUY922 could be an alternative treatment for primary lapatinib-resistant (ESO26 and OE33) and lapatinib-sensitive gastric cancer cells (OE19, N87, and SNU-216) harboring HER2 amplification. In order to investigate whether AUY922 could overcome intrinsic and acquired resistance to HER2 inhibitors in HER2-positive gastric cancer, we generated lapatinib-resistant gastric cancer cell lines (OE19/LR and N87/LR) by continuous exposure to lapatinib *in vitro*. We found that activation of HER2 and protein kinase B (AKT) was a key factor in inducing intrinsic and acquired lapatinib-resistant gastric cancer cell lines, and AUY922 effectively suppressed activation of both HER2 and AKT in acquired lapatinib-resistant gastric cancer cell lines. In conclusion, AUY922 shows a synergistic anti-cancer effect with lapatinib and could sensitize gastric cancer cells that have intrinsic resistance to lapatinib. In addition, this dual inhibition of the HSP90 and HER2 signaling pathways could represent a potent therapeutic strategy to treat HER2-positive gastric cancer with intrinsic and acquired resistance to lapatinib.

Keywords: Gastric cancer, HER2, Lapatinib, AUY922, Drug Resistance

INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer in 2018 and is the third most common cause of cancer-related death in the world (1). South Korea had the highest rate of gastric cancer worldwide in 2018 (1). While surgery remains a major curative treatment modality for localized gastric cancer, palliative chemotherapy is recommended in patients with unresectable gastric cancer to prolong survival and improve quality of life (2). However, the median overall survival in metastatic gastric cancer patients is less than 12 months with standard combination chemotherapy of fluoropyrimidine and platinum, indicating the need for more effective therapy (3, 4). Human epidermal growth factor receptor 2 (HER2) inhibitors, such as trastuzumab and lapatinib, have been developed for gastric or breast cancers harboring HER2 amplification. Following a success in breast cancer, trastuzumab, a humanized monoclonal antibody targeting HER2 was also approved for unresectable or metastatic HER2-positive gastric cancer based on the survival benefit of combining trastuzumab with chemotherapy in a randomized phase III study (ToGA)(5). In contrast, lapatinib, a dual HER2 and human epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor did not demonstrate the overall survival benefit despite improvement in objective response rate or progression-free survival in two phase III studies for HER2-positive gastric cancer in the first-line (LOGiC) and second-line (TyTAN) settings (6). These unsatisfactory efficacy outcomes suggest the presence of drug resistance mechanisms or alternative pathways of escape from lapatinib in gastric cancer. Although the molecules that render intrinsic or acquired resistance to lapatinib are not well known, suggested lapatinib-resistance mechanism are FOXO1 suppression (7) and activation of alternative signaling pathways, such as MET (8), Testican-1 (9), AXL (10), HER2 (11-13) and AKT (14, 15) which induce bypass of HER2 inhibition by trastuzumab or lapatinib.

AUY922, luminespib, is a representative heat shock protein 90 (HSP90) inhibitor and shows

anti-cancer effects via binding to the ATPase domain of HSP90 to cause loss of chaperone functions. Many misfolded client proteins, including HER2, EGFR, IGF1R, AKT, RAF-1, IKK, c-Kit, v-SRC, NPM-ALK, BCR-ABL, p53, STAT3, HIF1, and CDK4/6, by a HSP90 inhibitor are degraded by proteasome (16, 17). This induces anti-apoptosis and anti-proliferation effect in various tumors (18-22). While AUY922 monotherapy showed the promising activity in non-small cell lung cancers harboring EGFR exon 20 insertions (23), various stages of preclinical and clinical development as a component for combination therapies with AUY922 are still ongoing (18, 24).

AUY922 inhibited growth and proliferation via significant decrease of AKT and ERK, as well as HER2 levels in HER2-positive breast cancer and gastric cancer cell lines (15, 20, 22). Furthermore, combining AUY922 (15) or another HSP90 inhibitor 17-AAG (25) with trastuzumab showed promising antitumor activity in HER2-positive trastuzumab-resistant breast and gastric cancer preclinical models or metastatic breast cancer patients. These results suggest HSP90 inhibition could be a treatment strategy to overcome resistance to HER2-targeted therapies. However, the effect of AUY922 on HER2-positive and lapatinib-resistant gastric cancer remains to be investigated.

In this study, we first describe the dramatically synergistic anti-cancer effect of an HSP90 inhibitor AUY922 with lapatinib in intrinsic and acquired lapatinib-resistant gastric cancer harboring HER2 amplification.

RESULTS

AKT activation bypasses HER2 inhibition in HER2-positive gastric cancer cells with intrinsic resistance to lapatinib.

Western blotting was performed to determine the expression of HER2 and assess signaling

pathway in five gastric cancer cell lines. Interestingly, although HER2 was highly expressed and lapatinib inhibited the cell growth in a concentration-dependent manner in all these cell lines (Figure 1A), the sensitivity to lapatinib was larger in OE19, N87 and SNU-216 cells than ESO26 and OE33. lapatinib significantly inhibited the growth of OE19, N87 and SNU-216 cells but not ESO26 and OE33 cell lines in a concentration-dependent manner (Figure 1B). Whereas lapatinib remarkably inhibited pAKT in lapatinib-sensitive OE19 cell line, the AKT activation persisted even after lapatinib treatment in intrinsic lapatinib-resistant ESO26 cell line (Figure 1C and 1D).

Both intrinsic lapatinib-resistant and lapatinib-sensitive gastric cancer cells are all sensitive to AUY922 via suppression of HER2 and AKT activation.

To determine IC50 of AUY922 in HER2-positive gastric cancer cells, five gastric cancer cells, OE19, ESO26, N87, OE33 and SNU-216, were exposed to AUY922 (From 1 nM to 1 μM). AUY922 showed the potent anti-cancer effect with low IC50 values in both intrinsic lapatinib-resistant and lapatinib-sensitive gastric cancer cells, even though AUY922 could not kill these cancer cells completely even at 1 μM (Figure 2A).

To assess if AUY922 could block HER2 and AKT activation, western blot analyses were conducted to measure pHER, HER2, pAKT, and AKT in OE19 and ESO26 cells upon exposure to AUY922 (50 nM and 100 nM) for 24 hrs. AUY922 effectively suppressed pHER2 as well as pAKT in both lapatinib-sensitive OE19 (Figure 2B) and intrinsic lapatinib-resistant ESO26 (Figure 2C), equally.

Activation of HER2 and AKT bypass HER2 inhibition in HER2-positive gastric cancer cells with acquired resistance to lapatinib.

To establish lapatinib-resistant gastric cancer cells, we exposed OE19 and N87 cells to

increasing concentrations of lapatinib in culture over 3-month period. We then treated these resistant cells and parental cells with lapatinib (from 1 nM to 1 μM). Lapatinib-resistant cells, OE19/LR and N87/LR, exhibited higher IC50 than their parental cells, OE19 and N87, in response to lapatinib, respectively (Figure 3A). AKT and HER2 phosphorylation was not inhibited by lapatinib treatment in lapatinib-resistant OE19/LR and N87/LR cells, in contrast to lapatinib-sensitive OE19 and N87 cells, implying that activation of AKT and HER2 signaling may play a role in lapatinib resistance (Figure 3B and 3C). Caspase 3/7 assays revealed that these two resistant cells attenuated lapatinib–induced apoptosis (Figure 3D and 3E).

AUY922 sensitizes HER2-positive gastric cancer cells with acquired resistance to lapatinib

We assessed the effect of the combination of AUY922 and lapatinib for 72 hrs in OE19/LR and N87/LR cells. Western blot analysis revealed that both pHER2 and pAKT was more downregulated in OE19/LR and N87/LR cells exposed to the drug combination than in cells exposed to AUY922 or lapatinib alone (Figure 4A). The levels of the apoptosis marker cleaved PARP were also more elevated in the combination treatment when compared with either drug alone (Figure 1A). To determine if AUY922 can overcome acquired resistance to lapatinib and synergize with lapatinib in OE19/LR and N87/LR cells, we exposed two cell lines to different concentrations of AUY922 alone, lapatinib alone, and their combinations. In cell viability studies, the combination of AUY922 with lapatinib of 1 M or 500 nM showed significant synergy in OE19/LR (Figure 4B) and N87/LR (Figure 4C), respectively, compared with either agent alone. In addition, we confirmed these synergistic effects using CalcuSyn program (BIOsoft, UK) (Figure 4D and 4E). These observed synergy effects occurred via increased caspase-3/7 activity (Figure 4F and 4G). Taken together, these results indicate that

dual targeting of HER2 and HSP90 overcomes acquired resistance to HER2 inhibition, lapatinib, *in vitro*.

DISCUSSION

Although the prognosis for HER2-positive advanced gastric cancer patients has been significantly improved because of the development of anti-HER2 targeted therapy such as trastuzumab (26), appearance of primary or acquired resistance to anti-HER2 therapy remains a major therapeutic challenge. While trastuzumab was established as standard therapy for both HER2-positive breast cancer and gastric cancer (5), other anti-HER2 targeting agents such as lapatinib, trastuzumab emtansine, and pertuzumab showed different efficacy outcomes in clinical trials between both cancers, resulting in approval only in breast cancer. Among them, lapatinib enhanced antitumor efficacy in terms of overall response rate or progression-free survival when combined with cytotoxic chemotherapy in HER2-positive metastatic gastric cancer, but this did not translate to the overall survival benefit (6, 27). This failure of clinical trials for lapatinib in gastric cancer might have been caused by inadequate patient selection, HER2 heterogeneity, or statistically under-powered study design. However, probably, one of the most important reasons for that would be intrinsic or acquired resistance to lapatinib in gastric cancer. Considering multiple escape mechanisms to circumvent inhibition of the HER2 signaling pathways including compensatory activation of the HER network or activation of other redundant survival pathways (7-15), the combination strategy to block these escape mechanisms could be pursued to overcome resistance to lapatinib in HER2-positive gastric cancer.

In this study, we focused on a HSP90 inhibitor as a counterpart for combination treatment with lapatinib in acquired lapatinib-resistant HER2-positive gastric cancer cells. HSP90 is highly expressed in various tumors including HER2-positive gastric cancers, and has

significant association with poor prognosis (28, 29). HSP90 is a molecular chaperone to stabilize client proteins, many of which are oncoproteins including HER2 (18, 19, 30). HSP90 is a good therapeutic target to treat HER2-positive cancer becuase it is in charge of protein stabilization of multiple proteins involved in tumor progression as well as HER2. AUY922, an HSP90 inhibitor induces degradation of HER2 via ubiquitinylation and lysosomal pathways in proteasomes (31). Preclinical data suggested that AUY922 could play a role in overcoming resistance to trastuzumab in HER2-positive breast and gastric cancer cells (15, 32). Besides clinical antitumor activity in non-small cell lung cancer where AUY922 showed the promising activity in cases harboring EGFR exon 20 insertions (23) or ALK rearrangements (33), AUY922 showed encouraging antitumor efficacy when combined with trastuzumab in metastatic HER2-positive breast cancer patients who have progressed on trastuzumab-based therapy (34). However, it has not been studied yet whether an HSP90 inhibitor AUY922 in the combination with lapatinib could overcome lapatinib-resistant HER2-positive gastric cancer cells.

In this research, we first suggested that AUY922 has potent anti-proliferative effects in two intrinsic lapatinib-resistant cells (OE33 and ESO26) as well as lapatinib-sensitive HER2-positive gastric cancer cell lines (OE19, N87 and SNU-216). In addition, AUY922 markedly decreased the levels of HER2 and AKT in both lapatinib-sensitive and lapatinib-resistant cell lines. These results suggest that AUY922 could be useful to treat intrinsic lapatinib-resistant HER2-positive gastric cancer.

We also investigated if AUY922 show effective activity in two acquired lapatinib-resistant HER2-positive gastric cancer cell lines OE19/LR and N87/LR. The acquired lapatinib-resistant cell lines were also highly sensitive to AUY922 even though this drug could not kill completely even at the high concentration (1 M). In our study, lapatinib-resistant cells were still dependent on the HER2 signaling pathway, including AKT pathway. Therefore, the

combination of AUY922 with lapatinib showed dramatic synergistic effect in OE19/LR and N87/LR cell lines.

In summary, our results support the clinical development of AUY922 as a treatment strategy for overcoming intrinsic or acquired resistance to lapatinib in HER2-positive gastric cancer.

MATERIALS AND METHODS

Cell culture and reagents

The human gastric cell lines OE19, OE33, N87, ESO26, and SNU-216 were purchased from the American Type Culture Collection (Manassas, VA, USA) and Korea cell line bank (Seoul, Korea). These cell lines were grown at 37 °C in 5% CO_2 in RPMI-1640 and DEME containing 10% fetal bovine serum from GIBCO (Waltham, MA, USA). NVP-AUY922 and lapatinib were purchased from Selleck Chemicals (Houston, TX, USA), dissolved in DMSO to a final concentration of 10 mmol/L, and stored at −20 °C.

Cell viability assay

Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Approximately 3×10^3 cells were transferred to white 96 well plates. The next day, the culture medium was removed, and the desired concentrations of AUY922 or/and lapatinib (CP358774) were added to a volume of 100 μL. After 72 h, 100 μL of CellTiter-Glo reagent was added, and the plates were incubated for 10 min at room temperature. The luminescence was measured using a Wallac 1420 apparatus (PerkinElmer, Boston, MA, USA).

Western blot analysis

Cells were suspended in modified RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris–HCl [pH 7.4]) with protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (1 mM sodium fluoride and 2 mM sodium orthovanadate) on ice for 30 min and centrifuged at 15,000 × g for 30 min to collect whole cell lysate. The proteins (10–20 μg) were separated on an 8%–12% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). Western blotting was done with specific primary antibodies and peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. Proteins were visualized with ECL Plus enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA). The commercial antibodies used in this study included HER2, pHER2, HSP90, AKT, pAKT, pERK, cPARP, BIM, and β-actin (Cell Signaling Technology, Danvers, MA, USA).

Caspase 3/7 assay

Caspase 3 and 7 activation was measured by using the Caspase-Glo 3/7 Luminescence Assay (Promega Corp., Madison, WI, USA) following the manufacturer's protocol. Protein samples from cells were prepared using RIPA buffer in the same manner as western blot sample preparation. Ten micrograms of protein samples in 100 μL total volume were transferred to white 96 wells, and 100 μL of equilibrated Caspase-Glo 3/7 reagent was added to protein samples and incubated for 1 h at room temperature. Luminescence was measured by using the Wallac 1420 apparatus (PerkinElmer, Waltham, MA, USA).

Calculation of the combination index

Activity of the drugs, used singly or in combination treatment, was estimated using CalcuSyn software (Biosoft, Ferguson, MO, USA). Briefly, this program calculated and determined the combination index, a quantitative measure of the degree of drug interaction. combination

index (CI) < 1, CI = 1, and CI > 1 indicated synergistic, additive, and antagonistic effects, respectively.

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CONFLICTS OF INTEREST

Dea Ho Lee declares honoraria from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, CJ HealthCare, Eli Lilly and Company, Janssen Pharmaceutica, Merck & Co., MSD, Mundipharma, Novartis, Ono Pharmaceutical Co., Ltd., Pfizer, Roche, Samyang Biopharmaceuticals, and ST Cube. The other authors declare that they have no conflict of interest.

FIGURE LEGENDS

Figure 1. *In vitro* response to lapatinib in HER2-positive gastric cancer cells.

(A) Baseline expression of HER2, HSP90, and β-Actin measured by western blotting in five parental HER2-positive gastric cancer cells. (B) The anti-cancer effects of lapatinib alone in five HER2-positive gastric cancer cell lines. Cell proliferation was measured by the CellTiter-Glo luminescent cell viability assay. The average results (±SD) of three independent experiments are shown. (C and D) Effects on the downstream pathway by lapatinib alone. Western blotting of pHER2, pAKT, and pERK levels following treatment with lapatinib in lapatinib-sensitive HER2-positive gastric cancer cells (OE19) and intrinsic lapatinib-resistant

HER2-positive gastric cancer cells (ESO26). β-Actin was included as a loading control.

Figure 2. Both intrinsic lapatinib-resistant and lapatinib-sensitive gastric cancer cells are sensitive to AUY922 via suppression of HER2 and AKT activation.

(A) The anti-cancer effects of AUY922 alone in five HER2-positive gastric cancer cell lines. Cell proliferation was measured by the CellTiter-Glo luminescent cell viability assay. The average results (±SD) of three independent experiments are shown. (B and C) The effects on the downstream pathway by lapatinib alone. Western blotting of pHER2, HER2, pAKT, and AKT levels following treatment with AUY922 in lapatinib-sensitive HER2-positive gastric cancer cells (OE19) and intrinsic lapatinib-resistant HER2-positive gastric cancer cells (ESO26). β-Actin was included as a loading control.

Figure 3. Activation of HER2 and AKT bypass HER2 inhibition in HER2-positive gastric cancer cells with acquired resistance to lapatinib.

(A) The anti-cancer effect of lapatinib in two parental HER2-positive gastric cancer cells, OE19 and N87, and acquired lapatinib-resistant HER2-positive gastric cancer cells, OE19/LR and N87/LR. Cell proliferation was measured by the CellTiter-Glo luminescent cell viability assay. The average results (±SD) of three independent experiments are shown. (B and C) Expression of pHER2, pAKT, cPARP, and BIM was determined following 72 h incubation with increasing doses of lapatinib in OE19, OE19/LR, N87, and N87/LR cell lines. β-Actin was included as a loading control. (D and E) Caspase 3/7 activity of parental cells (OE19 and N87) and acquired lapatinib-resistant cells (OE19/LR and N87/LR) after treatment with 100 nM lapatinib.

Figure 4. AUY922 sensitizes HER2-positive gastric cancer cells with acquired resistance

to lapatinib.

(A) Effect of the combination of lapatinib and AUY922 on downstream signaling. Western blotting of HER2, pHER2, pAKT, and cPARP following treatment with lapatinib and AUY922 for 72 h in OE19/LR and N87/LR cell lines. (B and C) Synergistic effect of the combination of lapatinib and AUY922. Proliferation assays were performed in OE19/LR and N87/LR cells treated with 1 μM or 500 nM of lapatinib plus increasing concentrations of AUY922 (from 1 nM to 1 μM) for 3 d. (D and E) CI, combination index, and CI table regarding B and C. (F and G) Caspase 3/7 activity of acquired lapatinib-resistant cells (OE19/LR and N87/LR) after single-agent (AUY922 or lapatinib) or combination (AUY922 plus lapatinib) treatment.

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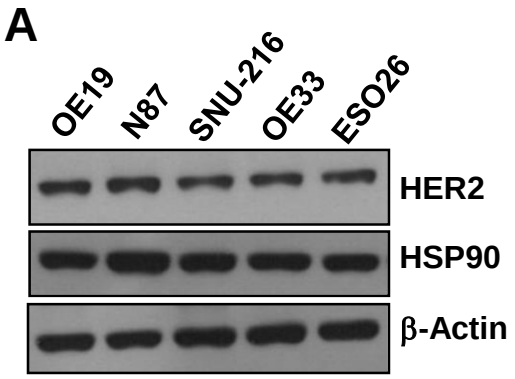
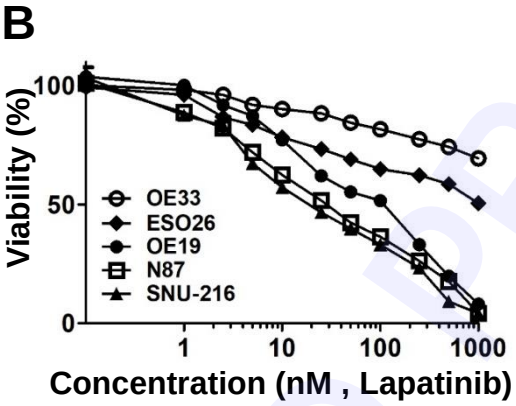
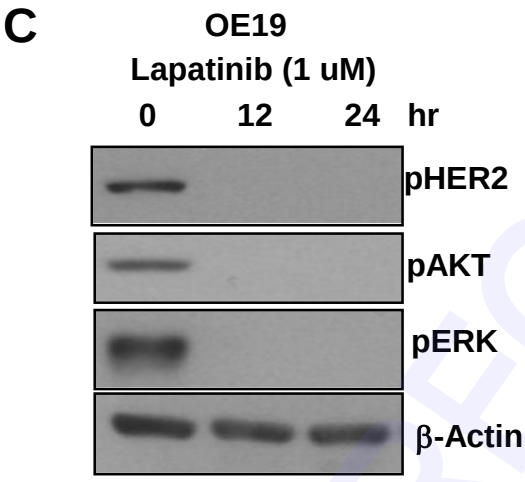
Figure 1. In vitro response to lapatinib of HER2-positive gastric cancer cells.





Cell lines	Lapatinib (IC50)
OE19	100 nM
ESO26	1 μM
N87	30 nM
OE33	> 1 μM
SNU-216	20 nM



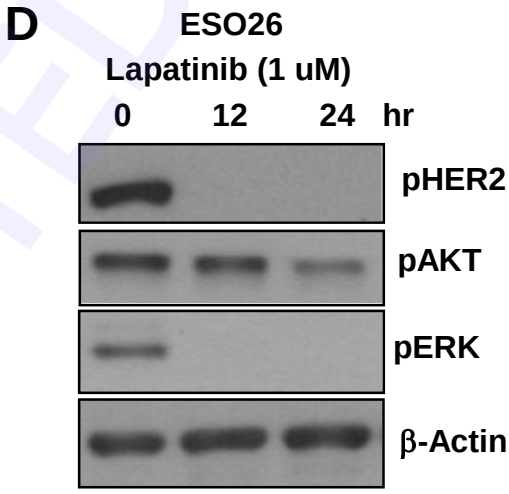
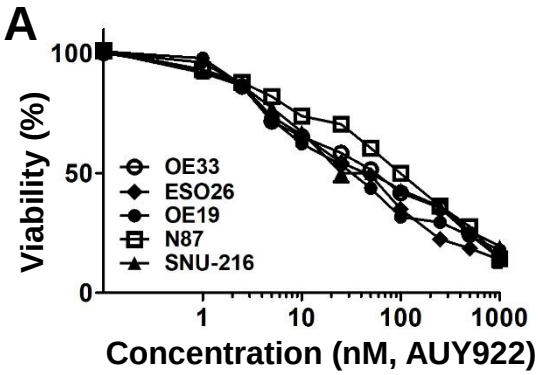


Figure 2. Both intrinsic lapatinib-resistant and lapatinib-sensitive gastric cancer cells are all sensitive to AUY922 via suppression of HER2 and AKT activation.



Cell lines	AUY922 (IC50)
OE19	20 nM
ESO26	50 μM
N87	80 nM
OE33	40 nM
SNU-216	50 nM

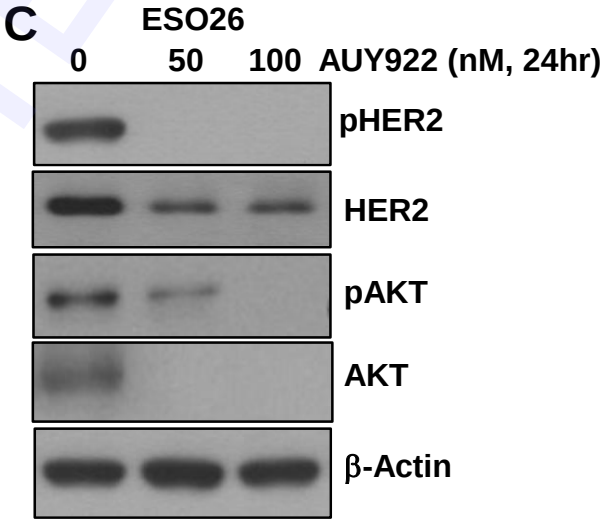
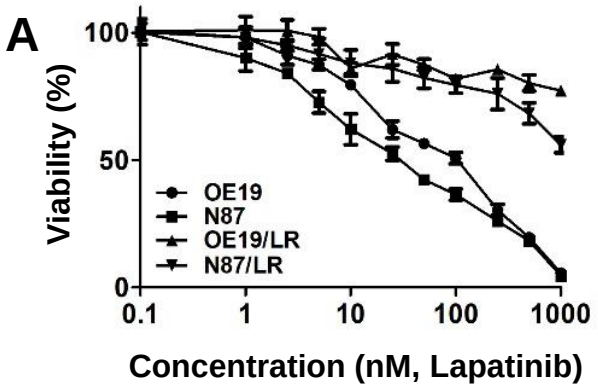
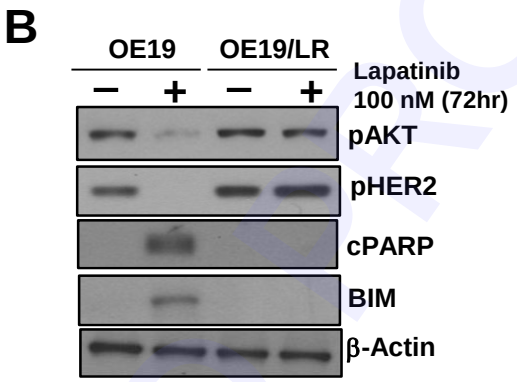
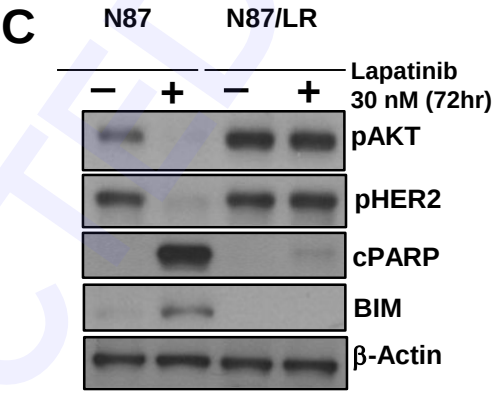


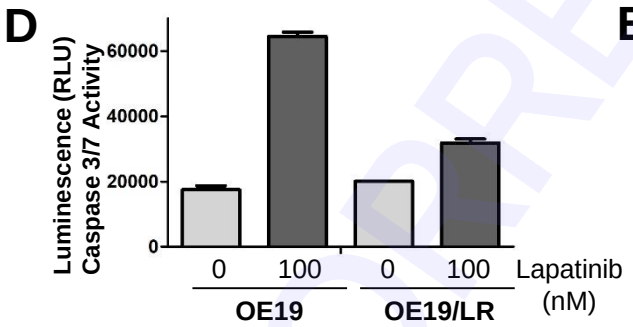
Figure 3. Activation of HER2 and AKT bypass HER2 inhibition in HER2-positive gastric cancer cells with acquired resistance to lapatinib.



Cell lines	Lapatinib (IC50)
OE19	100 nM
OE19/LR	> 1 μM
N87	30 nM
N87/LR	> 1 μM







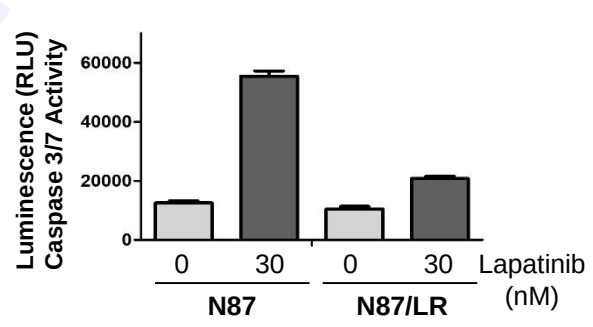
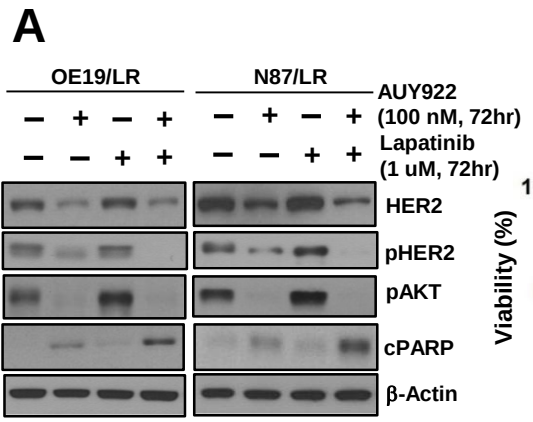
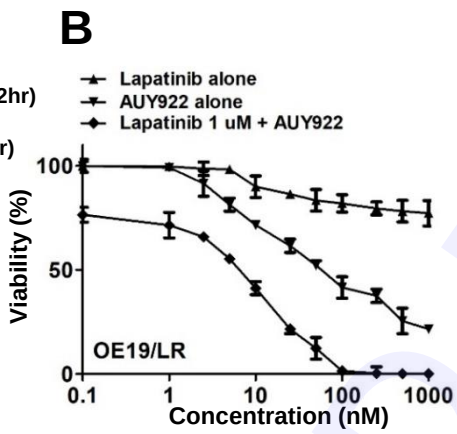
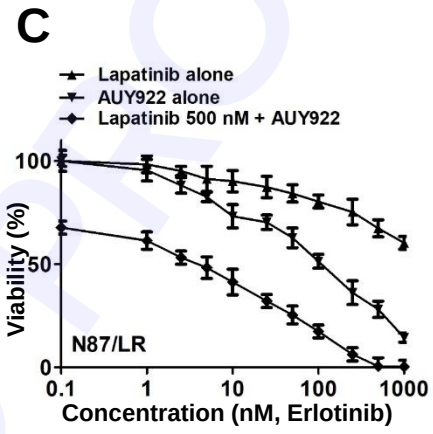
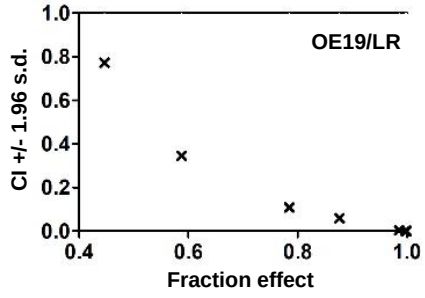


Fig 4. AUY922 sensitizes HER2-positive gastric cancer cells with acquired resistance to lapatinib

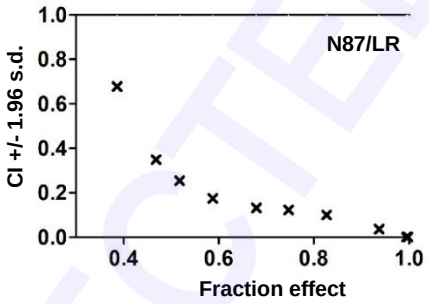








No.	Lapatinib (nM)	AUY922 (nM)	Fa	CI
1	1000	5	0.446635	0.773
2	1000	10	0.587546	0.346
3	1000	25	0.784575	0.109
4	1000	50	0.876664	0.06
5	1000	100	0.985463	0.004
6	1000	250	0.997245	0.001
7	1000	500	0.998755	0.001
8	1000	1000	0.998536	0.002



Lapatinib (nM)	AUY922 (nM)	Fa	CI
500	1	0.386266	0.678
500	2.5	0.468265	0.349
500	5	0.517518	0.255
500	10	0.587266	0.175
500	25	0.678826	0.132
500	50	0.746516	0.123
500	100	0.826628	0.1
500	250	0.937255	0.037
500	500	0.994853	0.001
500	1000	0.997623	0.001