의학박사 학위논문

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2015 년 7 월

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최세훈

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지도교수 전상훈

이 논문을 의학박사 학위논문으로 제출함

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최 세 훈

최세훈의 의학박사 학위논문을 인준함

2015 년 7 월

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Abstract

Efficacy of AUY922 in lung cancer cell lines with the acquired resistance to epidermal growth factor receptor-tyrosine kinase inhibitor

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The Graduate School

Seoul National University

The vast majority of non-small cell lung cancer patients have developed resistance towards epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), despite of the favorable responses during the initial periods. Resistance to EGFR-TKI therapy can occur via the introduction of a secondary T790M mutation, or the activation of bypass signals, such as MET or AXL, etc. Because heat shock protein 90 (HSP90) is essential for stability and maturation of many oncoproteins, HSP90 inhibitors have been recognized as an attractive therapeutic agent for the treatment of malignancies. The AUY922 is a newly developed HSP90 inhibitor with low toxicity and nano-molar potency. We report the efficacy of AUY922 to inhibit the EGFR-TKI resistance in non-small cell lung cancer cell lines that had acquired resistance through T790M mutations, MET- or AXL- bypass.

We established EGFR-TKI resistant PC-9 and HCC827 cell lines through continuous exposing to gefitinib or erlotinib more than 6 months, and named

PC-9/GR, PC-9/ER, HCC827/GR, and HCC827/ER, respectively. Using pyrosequencing and western blotting, we confirmed that PC-9 cells acquired resistance to EGFR-TKIs via T790M mutation, HCC827/GR cells via MET activation, and HCC827/ER cells via AXL bypass.

We confirmed that AUY922 is effective in inhibiting the resistant lung cancer cell lines in dose- and time- dependent manner. We have also confirmed that AUY922 can suppress not only major oncoproteins, such as EGFR, MET or AXL but also their downstream signaling proteins, such as Akt and Erk. AUY922 induced apoptosis and cell cycle arrest, and inhibited the cellular ability to migrate and invade as well. The antitumor effect of AUY922 was also verified in a xenograft mouse model using HCC827/GR and /ER cells.

Our study shows that AUY922 is a promising agent to overcome resistant non-small cell lung cancers via three major mechanisms of resistance, namely T790M mutation, MET- or AXL- bypass.

Keywords: non-small cell lung cancer, EGFR, resistance, heat shock protein 90, MET, AXL.

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List of Figures

List of abbreviations

EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
HSP	Heat shock protein
MAPK	Mitogen-activated protein kinase
NF	Nuclear factor
NSCLC	Non-small cell lung cancer
PARP	Poly ADP ribose polymerase
PI3K	Phosphoinositide 3-kinase
RTK	Receptor tyrosine kinase
STAT	Signal transducers and activators of transcription
TAM	Tyro-Axl-Mer
TKI	Tyrosine kinase inhibitor
TUNEL	Transferase-mediated dUTP nick end labeling

Contents

Introduction

Ever since epidermal growth factor receptor (EGFR) mutation was reported to predict sensitivity towards gefitinib (1, 2), EGFR-tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib have been one of the most successful targeted therapies for patients with non-small cell lung cancer. However, despite of the favorable responses during the initial stages, the vast majority of patients have developed resistance towards EGFR-TKIs (3-6). Resistance towards EGFR-TKI therapy can occur via the introduction of a secondary T790M mutation in the EGFR gene (encoding a p.Thr790Met mutation) (6, 7), increased expression of MET kinase (8, 9), activation of the nuclear factor (NF)-κB pathway (8, 10, 11), or the activation of bypass signals such as AXL (10, 12, 13), etc. EGFR mutation at T790M accounts for more than 50% of acquired TKI resistance through conformational changes in the ATP-binding pocket of EGFR, which leads to reduced affinity for small molecule tyrosine kinase inhibitors (5, 11, 13, 14). MET amplification contributes to the acquisition of resistance to EGFR-TKIs as this is a redundant pathway for the activation of phosphoinositide 3-kinase (PI3K)/Akt signaling which facilitates the survival of cancer cells, thus bypassing the inhibition of EGFR signaling upstream (8, 11, 13-15). AXL contributes to the survival

of EGFR-mutant lung cancer cells in a manner similar to that observed for MET in approximately 20% of the cases with acquired resistance towards TKIs (10, 16). AXL belongs to the Tyro-Axl-Mer (TAM) receptor tyrosine kinase (RTK) family and TAM signaling is related to cell survival, proliferation, migration, and phagocytosis, which may affect the downstream PI3K/Akt and Janus kinase-signal transducers and activators of transcription (STAT) signaling pathways (13, 14). AXL upregulation may activate AKT, mitogen-activated protein kinase (MAPK) or NF-κB signaling, promoting resistance to erlotinib treatment in EGFR-mutant NSCLCs (10).

Heat shock protein 90 (HSP90) is essential for protein maturation and stability, and for maintaining cellular homeostasis (17-21). Because HSP90 is essential for malignant transformation, progression, and the stability of various oncoproteins including kinases such as ErbB2, EGFR, Bcr-Abl tyrosine kinase, MET tyrosine kinase, PKB/Akt, b-Raf, hypoxia-inducible factor-1α and telomerase depend on HSP90. Hence HSP90 has been recognized as an attractive therapeutic target to overcome the acquired resistance to EGFR-TKIs (20-22). A major benefit of HSP90 inhibitors is the combined impact on multiple oncogenic pathways while antagonizing of all the hallmark traits of cancer, including proliferation, evasion of

apoptosis, immortalization, invasion, angiogenesis and metastasis, and such combined action should markedly reduce the opportunity for cancer cells to develop resistance to HSP90 inhibition (19, 21). AUY922 is one of these newly designed small-molecule HSP90 inhibitors which has a much higher affinity for HSP90 than previous geldanamycin analogues (23, 24). Preclinical data from various types of human cancer have shown an anti-proliferative effect of AUY922, with nano-molar potency both in vivo and in vitro, without any major adverse effects being observed in mice (23-25). However, the study was not sufficient on how can AUY922 suppress secondary T790M mutations, MET- or AXL- mediated resistance towards EGFR-TKI in lung cancer.

In this study, we report the efficacy of AUY922 to inhibit the acquired EGFR-TKI resistance in non-small cell lung cancer cell lines that had acquired resistance through T790M mutations, MET- or AXL bypass.

Material and Methods

Establishment of Gefitinib- and Erlotinib-resistant cell lines

To establish the gefitinib- and erlotinib- resistant variants of PC-9 and HCC827 cells, EGFR-TKI sensitive parental PC-9 and HCC827 human lung cancer cell lines with a deletion mutation at exon 19 (p.Glu746_Ala750del) exposed to stepwise increasing doses of gefitinib and erlotinib. Briefly, PC-9 and HCC827 cells were initially treated with 10 nmol/L gefitinib or 5 nmol/L erlotinib (the approximate IC_{50} dose) for 72 hours in RPMI 1650 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37° C in an atmosphere of 5% CO_2. Subsequently, cells were cultured in drug-free medium until 80% confluence, and then cells were repeatedly exposed to increasing dose of drug. PC-9 cells became able to grow in 1 μmol/L of gefitinib or erlotinib within 6 months after initial exposure (PC-9/GR(L) and PC-9/ER(L), respectively). We kept to expose the cell lines for more than 3 months in 1 μmol/L of EGFR-TKIs and named the cell lines as PC-9/GR(H) and PC-9/ER(H), each. It took more than 8 months for HCC827 cells to become able to grow up to 1 μmol/L drug concentration. The established resistant cell lines were maintained in medium containing 1 μmol/L gefitinib or erlotinib (HCC827/GR

and HCC827/ER, respectively). For all in vitro studies, resistant cells were maintained in gefitinib- or erlotinib-free medium for at least 2 weeks before experiments to eliminate the effects of the drugs.

Cell viability assays

Cell proliferation and viability was assessed by the MTT assay. Briefly, cells in the logarithmic growth phase were harvested, and seeded onto 96-well flat-bottomed tissue culture plates (Becton Dickinson, San Jose, CA) with complete culture medium and allowed to adhere to the plate for 24 hour. Then the cells were incubated in the presence of the drug of various doses (0, 0.01, 0.1, 1, and 5 or 10 μmol/L) or durations (0, 24, 48, 72 hours) of gefitinib, erlotinib, afatinib (a second generation EGFR-TKI), or AUY922 in medium containing 1% FBS. After 72 hours at 37◦C in a humidified atmosphere of 5% CO2 in air, the attached cells were stained with a 0.2% trypan blue solution containing 50% methanol. Cell viability was determined using an ADAM-MC automatic cell counter (NanoEnTek, Seoul, Korea) in accordance with the manufacturer's instructions.

Western blot analysis

Protein expression was analyzed by western blotting. Proteins were separated on SDS-polyacrylamide gels and electro-transferred to Immobilon-P membranes (Millipore, Bedford, MA). Antibodies specific for EGFR, AXL, MET, Akt, p-Erk, Erk, Myc, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies for p-Akt, Poly ADP ribose polymerase (PARP) and caspase-3 were obtained from Cell Signaling Technology (Beverly, MA). Proteins were detected using an enhanced chemiluminescence western blotting kit (Amersham Biosciences, NJ) in accordance with the manufacturer's instructions.

Pyrosequencing assay for T790M mutation

Pyrosequencing was done as follows. The PCR amplification primers used were exon 20 EGFR-forward, 5′-ctgggcatctgcct-cacct-3′, and EGFR-reverse biotinylated primer, 5′-tgtgttcccgga-catagtcca-3′. Each PCR mix contained forward and reverse primers (each 0.4 pmoL), 0.2 mmoL each of deoxynucleotide triphosphates, 1.5 mmol/L $MgCl_2$, 1× PCR buffer, 1.5 units of Immolase DNA polymerase (Bioline), and 5 μL of genomic DNA in a total volume of 50 μL. PCR conditions consisted of initial denaturing at 94° C for 1 min, 50 amplification cycles (95° C for 20 s, 58° C for 20 s, and 72° C for 40 s), and a final extension at 72° C for 1 min. The obtained PCR products were

electrophoresed in 2% agarose gel to confirm successful amplification of PCR products, 20 μL of which were sequenced using a Pyrosequencing PSQ96 HS System (Biotage) according to the manufacturer's instructions. The pyrosequencing primer sequence was 5′ -ccgtgcagctcatca-3′ .

Cell cycle analysis

Cells were seeded onto 6-well plates and treated for the indicated times with 1 μmol/L AUY922. Both adherent and floating cells were collected for analysis. Cells were fixed in 70% ice-cold ethanol for 1 hour and incubated in 50 μg/mL RNase A and 25 μg/mL propidium iodide (PI) for 30 minutes at 37° C. Quantitative analysis of the cell cycle distribution was performed using FASCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Migration and Invasion Assays

The cell migration and invasion assays were performed using Transwell (6.5 mm diameter, 8 μm pore polycarbonate membrane), which was obtained from Corning (Cambridge, MA). Cells ($1*10^5$ per chamber) in 200 μL medium were placed in the upper chamber, and the lower chamber was filled with 1 mL serum-free media supplemented with 0.1% bovine serum albumin. After incubation for 24 hours, non-migrating cells were removed using cotton swabs,

and the cells that invaded to the lower surface of the filter were stained using the Diff-Quick kit (Fisher Scientific, Pittsburgh, PA). Cells were counted using a microscope. The migration assay was performed using the same procedure with filters that had been coated with extracellular matrix on the upper surface (BD Biosciences, Bedford, MA). Results are representative of at least three independent experiments, and are expressed as means ± SDs.

In vivo study

Female severe combined immunodeficiency (SCID) mice (18-20 g; 5 weeks of age; 5 mice/group) were purchased from Charles River Laboratories. Tumors were grown by implanting cells (1×10^6 HCC827/GR cells or 5×10^6 HCC827/ER cells) in Matrigel (BD Biosciences) and subsequently into the mouse flanks. Treatment with the vehicle control commenced when the tumors reached a volume of 50-100 mm^3 (20 mg/kg AUY922 via intra-peritoneal injection; 5 days/week). Treatment was stopped on the indicated day, and mice received follow-up examinations to document tumor recurrence. To measure tumor size, the length (L) and width (W) of each tumor was measured using calipers, and tumor volume (TV) was calculated as $TV = (L \times W^2)/2$. To evaluate the proliferation and apoptosis of the xenografts, immunohistochemical staining was performed using a specific

primary antibody (Ki-67; DakoCytomation, Los Angeles, CA), the EnVision Plus staining kit (DakoCytomation), and the APO-Direct terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Millipore) as directed by the supplier's instructions. Quantitative analysis of each stained section was performed by counting all immunopositive cells in 5 arbitrarily selected fields (× 400 magnifications).

Results

1-1. The establishment of the resistant PC-9/GR(L), PC-9/ER(L), PC-9/GR(H), and PC-9/ER(H) cell lines via T790M mutation

Gefitinib- and erlotinib- resistant cells were established by long-term exposure of stepwise increasing concentrations of gefitinib or erlotinib (up to a final concentration of 1 μmol/L) over a period of 6 months (PC-9/GR(L), PC-9/ER(L), each), and more than 9 months (PC-9/GR(H), PC-9/ER(H), each) as described in Materials and Methods. The cell lines exposed to the longer time to EGFR-TKIs, i.e. PC-9/GR(H) and PC-9/ER(H) cells, showed higher IC_{50} values to gefitinib and erlotinib than PC-9/GR(L) and ER(L) sublines (Figure 1-A). To evaluate the resistant mechanism of these cell lines, we analyzed the sequence of the EGFR gene at exons 18 to 21. Semi-quantitative pyrosequencing revealed that the secondary T790M mutation was present in all the resistant PC-9 sublines whereas the mutation was absent in parental PC-9 cell line. Only a small amount of mutated EGFR (~13-14%) was in both PC-9/GR(L) and ER(L) cells as compared with higher proportion of mutation in PC-9/GR(H) or ER(H) cell lines (~50-53%) (Figure 1-B). Because the cell lines showed already the cross-resistance

to the first generation of EGFR-TKIs, i.e. gefitinib and erlotinib, we examined the effectiveness of afatinib, as a second generation EGFR-TKI, to control these resistant sublines. MTT assay and cell counting showed higher resistance to afatinib in PC-9/GR(H) and /ER(H) cell lines than PC-9/GR(L) and /ER(L) sublines (Figure 1-C).

1-2. The effectiveness of AUY922 to control T790M mutation mediated resistance of PC-9 cell lines.

AUY922 shows dose-dependent control in not only EGFR-TKI sensitive parental PC-9 cell lines but also EGFR-TKI resistant sublines in MTT assay (Figure 2-A). When we investigated EGFR activation and their downstream molecules using western blotting, the expressions of EGFR, phosphorylated EGFR (p-EGFR), p-Akt and p-Erk in all PC-9 sublines were significantly decreased by AUY922 while the expression of β-actin, the "house-keeping" protein, was consistent (Figure 2-B).

2-1. HCC827/GR cell lines acquired resistance via MET amplification, and HCC827/ER cells via AXL bypass

The parental HCC827 cells were sensitive to both gefitinib and erlotinib, however HCC827/GR cell line became resistant to gefitinib (Figure 3). When we analyzed the EGFR family proteins through

western blotting, MET protein was significantly amplified (Figure 3-A, asterisk). We confirmed that MET amplification is the key factor to acquire resistance to gefitinib using PHA665752, a known MET inhibitor. The growth of HCC827/GR cells was not suppressed by gefitinib or PHA665752 alone, but when PHA665752 was combined with gefitinib, HCC827/GR cells were controlled effectively, which means MET amplification is the key factor for the resistance of the HCC827/GR cell line. (Figure 3-B). Similarly, HCC827/ER cell line showed erlotinib-resistance in cell viability test, and the mechanism of resistance is confirmed as AXL bypass by western blotting (Figure 4) (10).

2-2. Effectiveness of AUY922 to control MET- and AXL-mediated resistance of HCC827 cell lines

To verify the effectiveness of AUY922 and compare the efficacy with other EGFR-TKI drugs, we treated HCC827/GR or ER cells with various dose concentration and exposure time of gefitinib, erlotinib, afatinib, and AUY922. Though both HCC827/GR and ER cell lines showed cross-resistance to EGFR-TKIs, the resistant cells as well as parental HCC827 cells were effectively controlled by AUY922 (Figure 5-A). AUY922 could inhibit the growth of resistant cell even when the drug concentration was very low (0.01 μM) (Figure 5-B). Because HSP90 plays a critical role in stability of

client proteins and progression of cancer as a chaperone molecule (18, 20, 26), we tried to reveal whether the stability of AXL and MET was broken by AUY922. The AUY22 induced dramatic degradation of EGFR, MET, AXL and their downstream proteins in dose- (Figure 5-B) and time-dependent manner (Figure 5-C).

2-3. Effect of AUY922 on cell cycle arrest, apoptosis, and cellular mobility in HCC827/GR or ER cell lines

It is fully suggested that HSP90 inhibitors could induce apoptosis and cell cycle arrest (27-31). We investigated the effects of AUY922 on cell cycle changes in PI-stained HCC827/GR or ER cells using FACS analysis. When the cells treated with AUY922, the number of cell in G2/M phase was increased at first, and after 48 hours the proportion of sub-G1 cells were increased which means cell death (Figure 6-A). Consistent with these findings, cleaved Poly ADP ribose polymerase (PARP) and cleaved caspase-3 which is developed in apoptotic cells were also increased (Figure 6-B). These data suggested that AUY922 showed antitumor effect through the induction of cell cycle arrest and apoptosis. It also reported that cellular mobility increased when the MET amplification or AXL bypass were induced in cancers (10, 12, 32-34). To verify the effects of AUY922 on cellular mobility in HCC827 cells, we checked the migration and invasion abilities in both resistant cell lines, and

compared those with parental cells. As expected, the migration and invasion in both resistant sublines were dramatically increased in comparison with parental cells (Figure 7), and those were significantly reduced when AUY922 was treated in the parental and both resistant cell lines.

2-4. Effect of AUY922 in a xenograft model

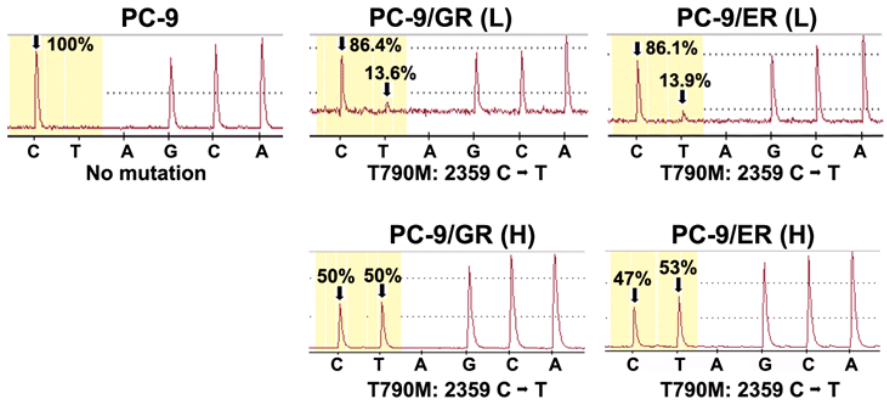
To evaluate the antitumor effect of AUY922 in xenograft model, HCC827/GR or ER tumor cells were injected in the flank of SCID mice. HCC827/ER cell showed lower growth rate than HCC827/GR. AUY922 was intraperitoneally injected 5 days per week for 25 days (Figure 8-A, arrow), and followed up for 3 weeks after the last injection (Figure 8-A, asterisk). AUY922 injection could effectively suppress the tumor growth in all the cell lines. When we followed up for 3 weeks after AUY922 treatment, the suppression of growth of HCC827/ER xenograft tumor was maintained, however HCC827/GR tumors grew slowly after cessation of AUY922 treatment. We also confirmed the inhibitory effect of AUY922 on cellular proliferation and the induction of apoptosis in both cell lines using immunohistochemical staining for Ki-67 and TUNEL (Figure 8-B).

Figure 1. Establishment of PC-9/GR(L), PC-9/GR(H), PC-9/ER(L), and PC-9/ER(H) cell lines. A. The IC_{50} values (μM) of parental and EGFR-TKI resistant PC-9 cell lines, B. Each cell lines were tested for EGFR exon 20 mutation using pyrosequencing. All the resistant cell lines showed C to T mutation at the 2359 position of EGFR sequence (arrows). In PC-9/GR(H) and ER(H) cell lines, the proportion of mutation was higher compared to PC-9/GR(L) or ER(L) sublines. C. In MTT assay and relative cell counting, PC-9/GR(H) and ER(H) cells show resistance to afatinib, the second generation EGFR-TKI.

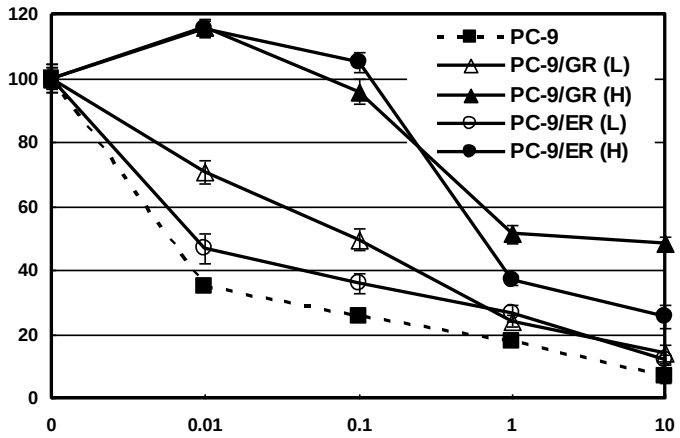
A.

Drug	IC_{50} values (μM)				
	PC-9	PC-9/GR (L)	PC-9/GR (H)	PC-9/ER (L)	PC-9/ER (H)
Gefitinib	< 0.01	4±1.2	9.6±2.5	5.5±2.1	8.2±1.1
Erlotinib	< 0.01	8±2.5	> 10	8.8±3.2	> 10

B.



C.


120
100
80
60
40
20
0
0
0.01
0.1
1
10
PC-9
PC-9/GR (L)
PC-9/GR (H)
PC-9/ER (L)
PC-9/ER (H)

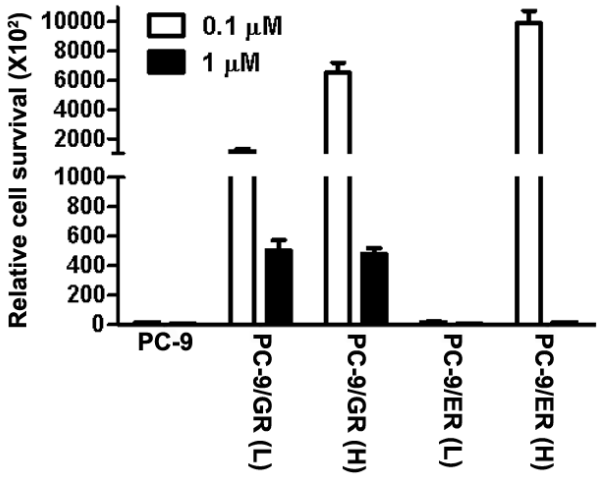
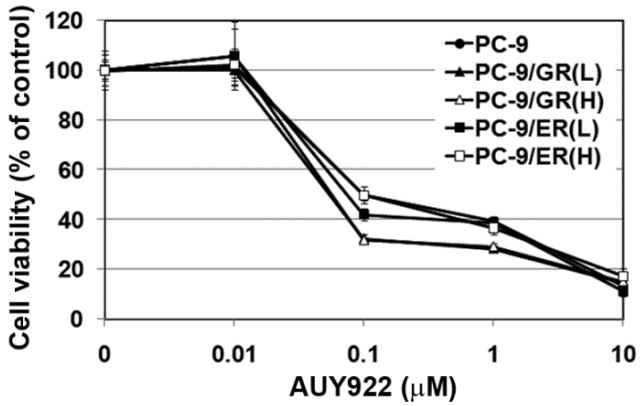

Relative cell survival (X10²)
10000
8000
6000
4000
2000
1000
800
600
400
200
0
0.1 μM
1 μM
PC-9
PC-9/GR (L)
PC-9/GR (H)
PC-9/ER (L)
PC-9/ER (H)

Figure 2. The effect of AUY922 on PC-9 cell lines. A. AUY922 shows dose-dependent control in both parental PC-9 cell line and its resistant sublines. B. AUY922 suppressed EGFR and phosphorylation of its downstream molecules, e.g. Akt and Erk, in all PC-9 sublines.

A.



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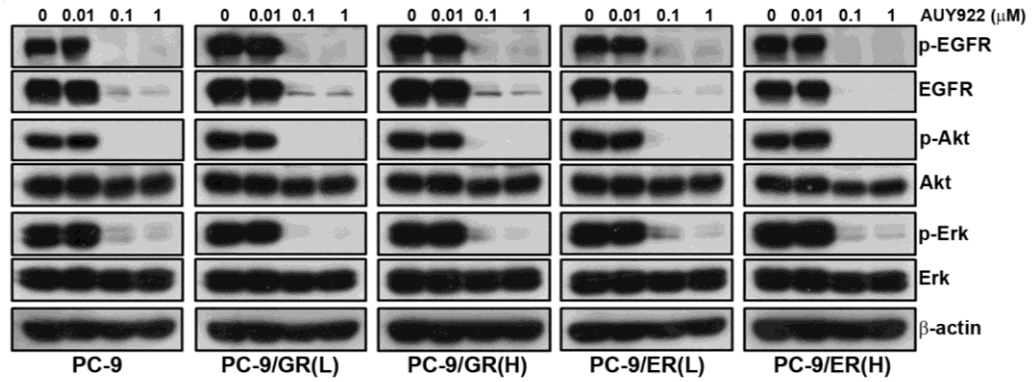
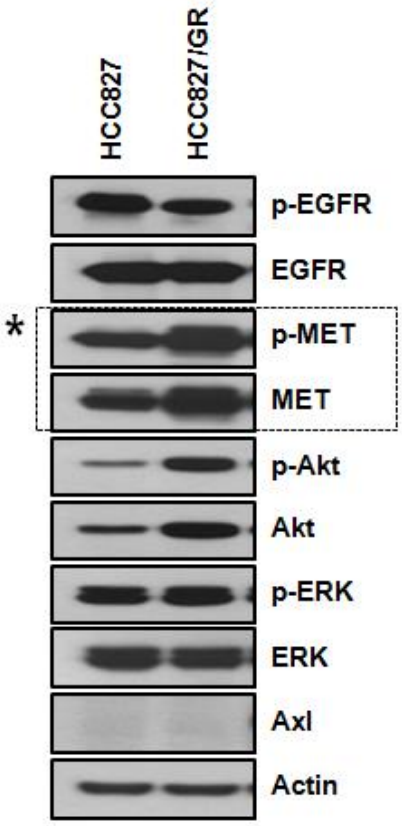


Figure 3. HCC827/GR cell line and MET amplification. A. Both MET and phosphorylated MET were amplified in HCC827/GR sublines compared with the parental HCC827 cell line. B. HCC827/GR cells showed definite resistance to gefitinib compared with its parental cells. By adding PHA665752, the MET inhibitor, to gefitinib, the resistance could be reversed, which means HCC827/GR cell lines acquired the resistance by MET amplification.

A.



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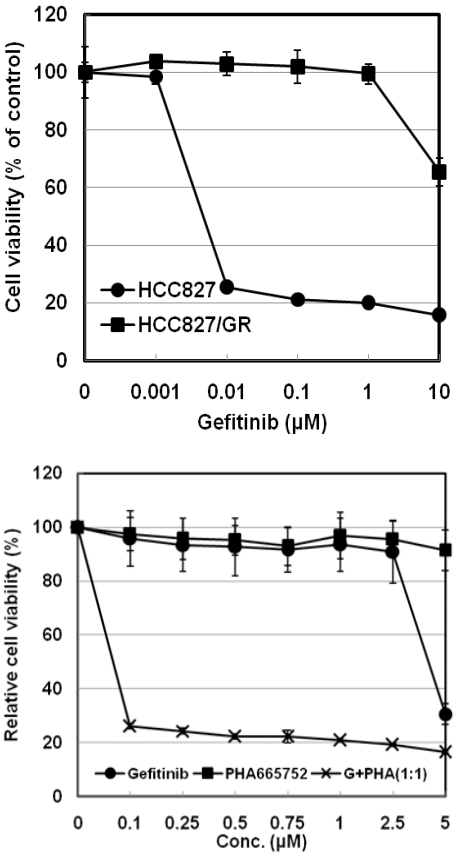


Figure 4. HCC827/ER cell line and AXL bypass. A. HCC827/ER subline was resistant to erlotinib compared with the parental HCC827 cell line. B. By western blotting, the AXL protein was overexpressed in the HCC827/ER subline, while EGFR, MET, Akt, or ERK did not show significant elevation (asterisk).

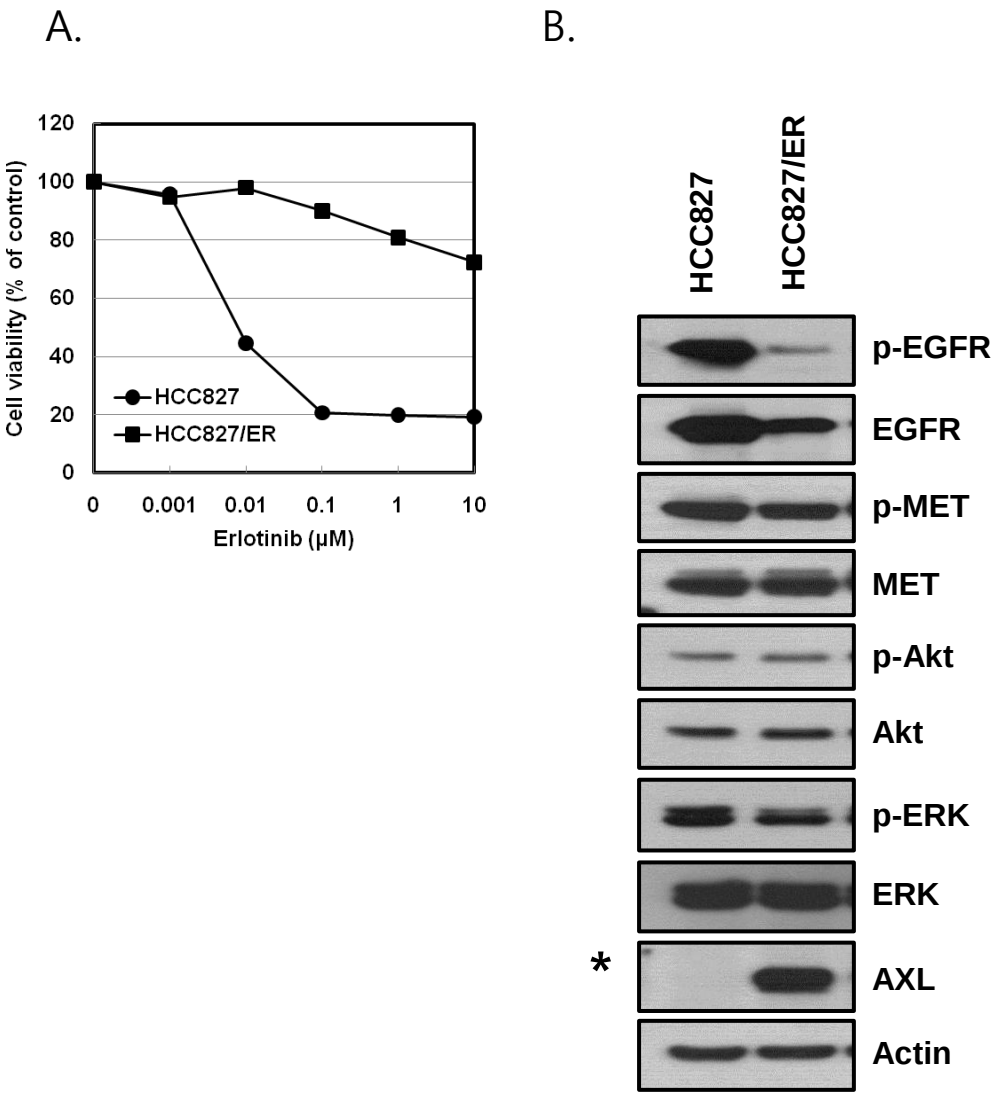
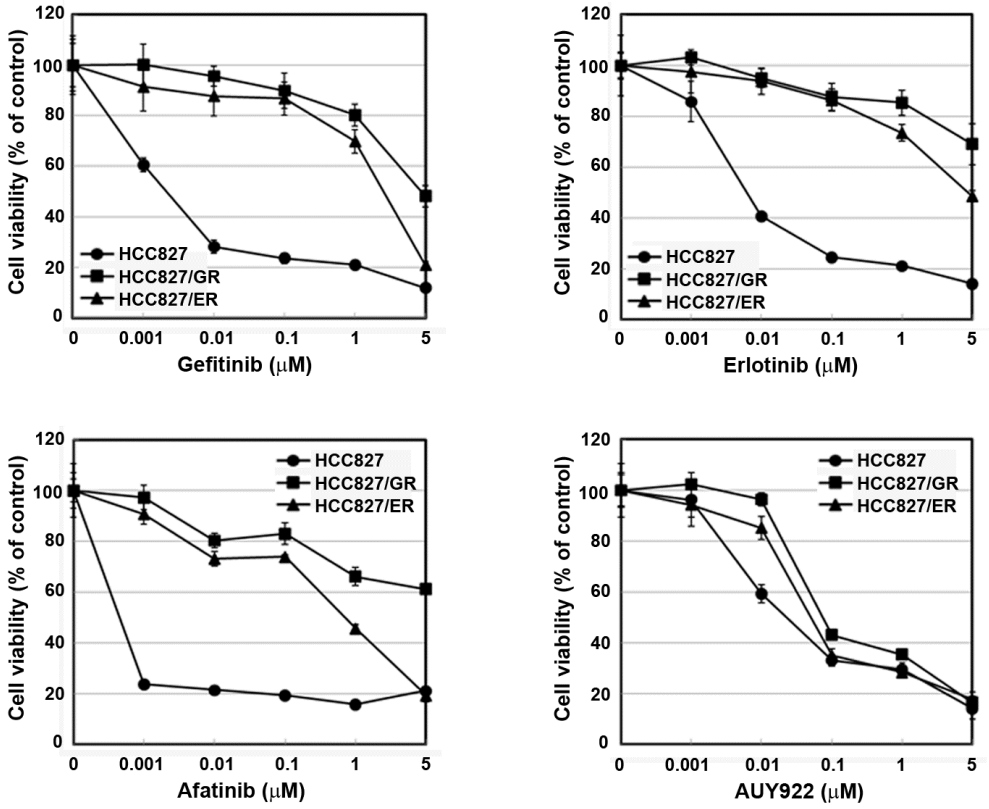


Figure 5. The effect of AUY922 on cell viability in the parental HCC827 cell line and its resistant sublines. A. When each cells cultured in the media of the indicated doses of gefitinib, erlotinib, afatinib, or AUY922 for 72 hours, AUY922 showed effective control in all the HCC827 sublines by MTT assay. B., C. The viable cells of each HCC827 sublines were significantly decreased by AUY922 in dose- and time- dependent manner. The significant degradation of EGFR, MET, AXL, Akt and ERK were observed also.

A.



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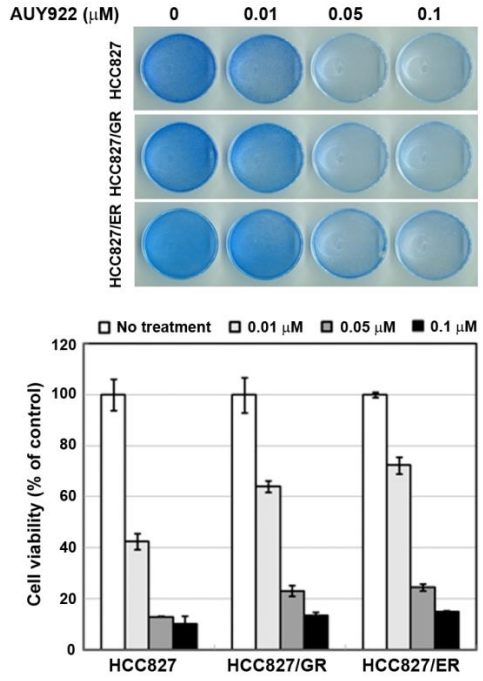
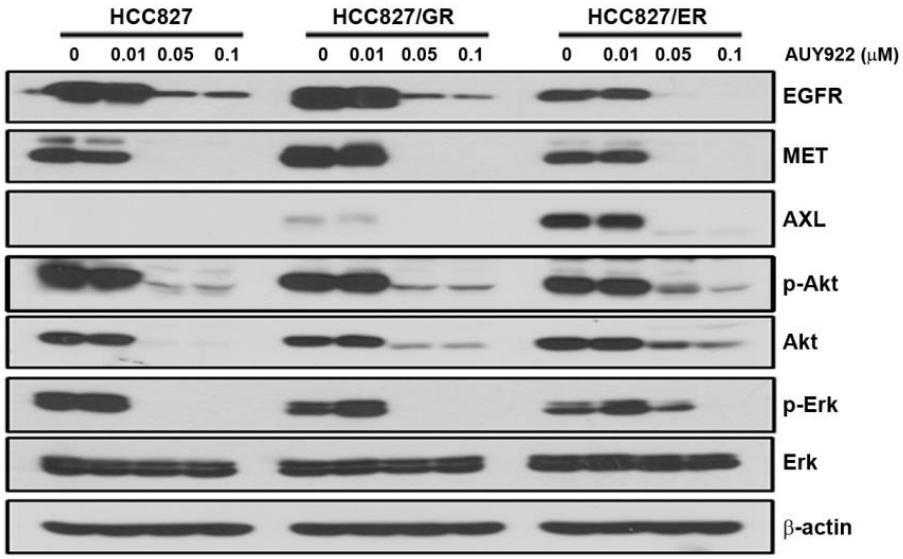

AUY922 (μM)
0
0.01
0.05
0.1
HCC827
HCC827/GR
HCC827/ER
No treatment
0.01 μM
0.05 μM
0.1 μM
Cell viability (% of control)
120
100
80
60
40
20
0
HCC827
HCC827/GR
HCC827/ER


HCC827
HCC827/GR
HCC827/ER
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AUY922 (μM)
EGFR
MET
AXL
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p-Erk
Erk
β-actin

C.

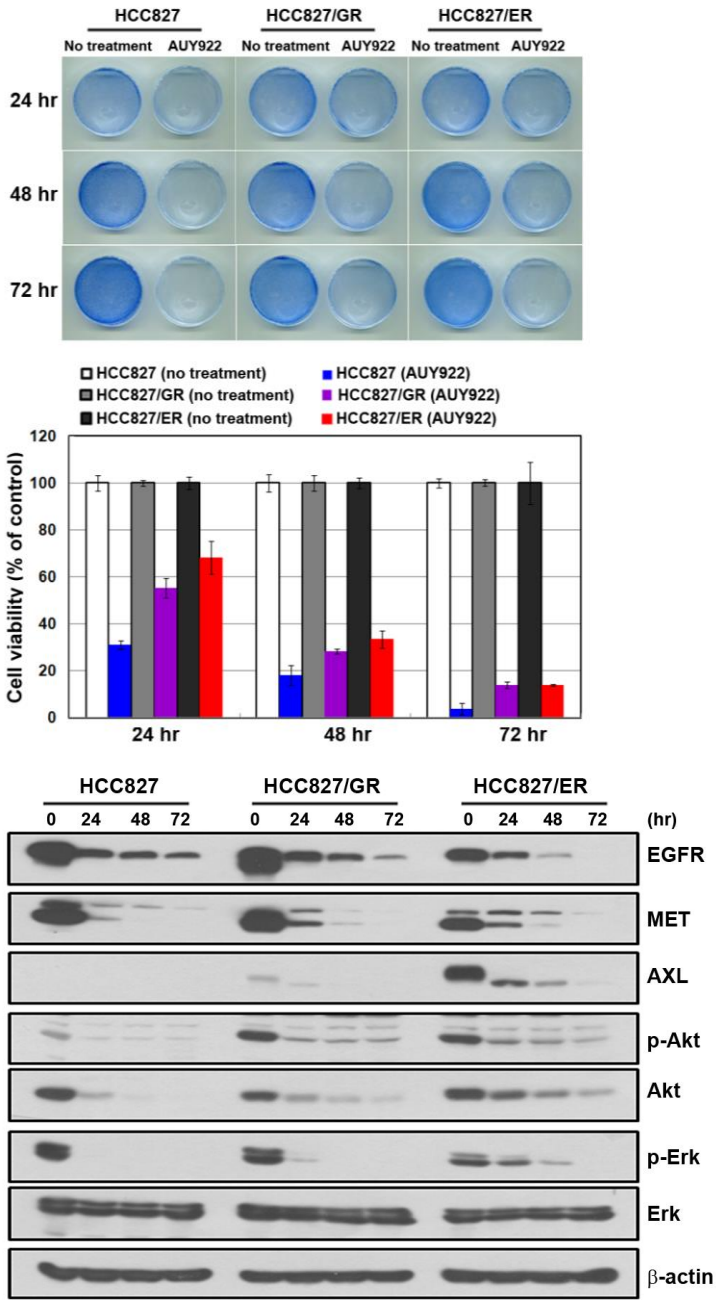
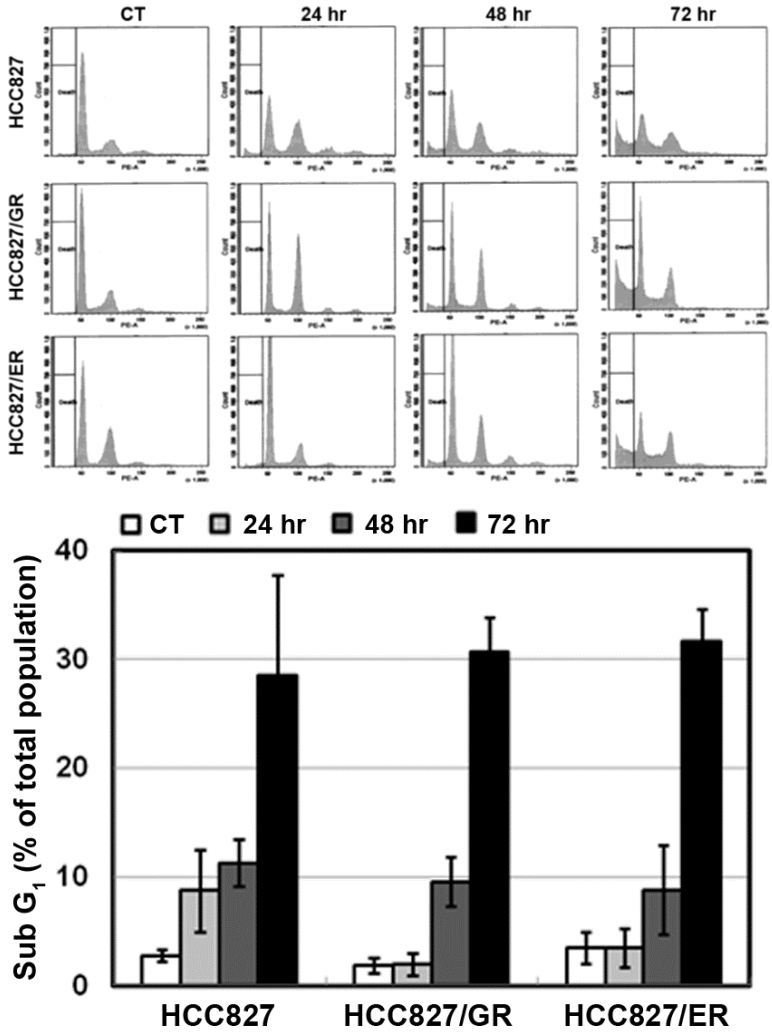

HCC827
HCC827/GR
HCC827/ER
No treatment
AUY922
24 hr
48 hr
72 hr
HCC827 (no treatment)
HCC827/GR (no treatment)
HCC827/ER (no treatment)
HCC827 (AUY922)
HCC827/GR (AUY922)
HCC827/ER (AUY922)
Cell viability (% of control)
120
100
80
60
40
20
0
24 hr
48 hr
72 hr
HCC827
HCC827/GR
HCC827/ER
0
24
48
72
(hr)
EGFR
MET
AXL
p-Akt
Akt
p-Erk
Erk
β-actin

Figure 6. The effect of AUY922 on cell cycle and apoptosis of the HCC827 sublines. A. Cells were treated with 1 μmol/L AUY922 and observed at 24, 48, and 72 hours using flow cytometry. B. Cleaved PARP and caspase-3 were increased in time dependent manner which means apoptosis progressed.

A.



B.

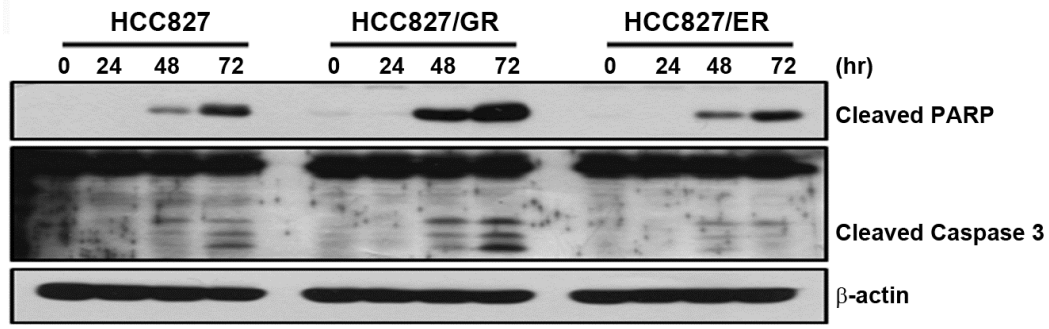

HCC827
HCC827/GR
HCC827/ER
0 24 48 72
0 24 48 72
0 24 48 72
(hr)
Cleaved PARP
Cleaved Caspase 3
β-actin

Figure 7. The effect of AUY922 on cellular migration and invasion in the HCC827 sublines. Cells were cultured in the indicated concentrations of AUY922 for 24 hours. The cells that invaded or migrated through the filter were stained and counted. Bars represent the mean ± SD of three wells. *p< 0.01 and **p< 0.001 in comparison with HCC827 cells.

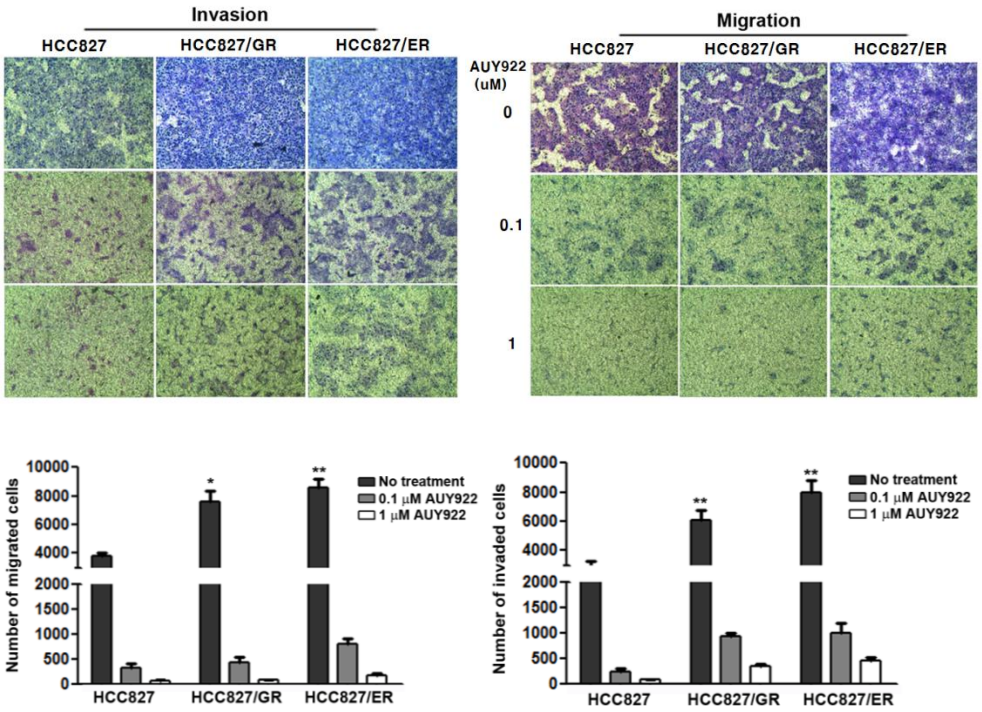
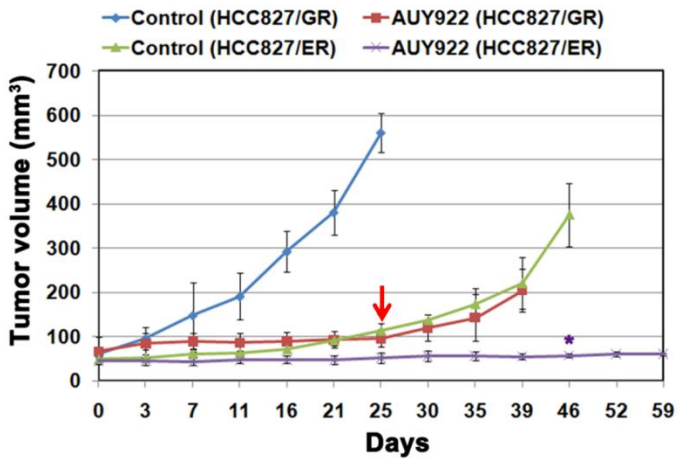
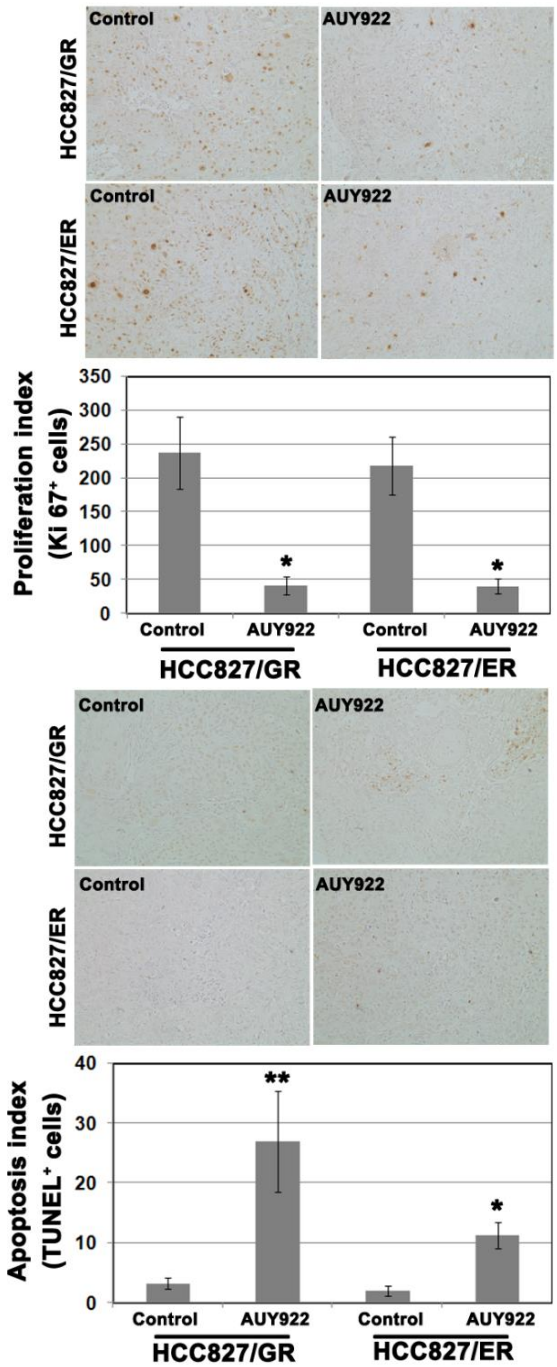


Figure 8. The Efficacy of AUY922 in a xenograft mouse model. A. SCID mice with established HCC827/GR and HCC827/ER tumor cell xenografts were treated with AUY922. AUY922 suppressed tumor growth significantly in both HCC827/GR and ER cells. HCC827/ER tumor showed no growth after cessation of AUY922 injection (arrow). B. Immunohistochemical staining for Ki-67^{+} cells and TUNEL^{+} cells. Ki-67 antibody is for indicating proliferation and TUNEL for apoptosis. $*p < 0.01$ and $**p < 0.001$ in comparison with the control.

A.



B.


Control
AUY922
HCC827/GR
Control
AUY922
HCC827/ER
Proliferation index
(Ki 67+ cells)
350
300
250
200
150
100
50
0
*
*
Control
AUY922
Control
AUY922
HCC827/GR
HCC827/ER
Control
AUY922
HCC827/GR
Control
AUY922
HCC827/ER
Apoptosis index
(TUNEL+ cells)
40
30
20
10
0
**
*
Control
AUY922
Control
AUY922
HCC827/GR
HCC827/ER

Discussion

In this study, we confirmed that AUY922 is effective in inhibiting the three major mechanisms of resistance towards EGFR-TKIs, i.e. T790M, MET and AXL, both in vitro and in xenograft models. We have also confirmed that AUY922 can suppress not only major oncoproteins such as EGFR, MET, or AXL but also their key-signaling proteins, such as Akt and Erk, in dose- and time-dependent manner. AUY922 induced cell cycle arrest as well as apoptosis and suppressed cellular motility. We believe that our study reflects the clinical setting more accurately as our cell lines were established through long-term exposure to EGFR-TKIs, compared with many reports which used gene transfection (18, 35-38). Moreover, there are few reports about resistance via AXL bypass as well as the mechanism how HSP90 may inhibit AXL-mediated resistance (10). Considering that the majority of cancers present with redundant and complex signaling pathways resulting in lack of success in treating lung cancer, the fact that AUY922 could control the activity of multiple aberrant signaling proteins simultaneously has strong implications (23, 24, 39).

HSP serves as a biochemical buffer for the genetic instability, and its expression enhances cell survival in tissues damaged by stressors, including hypoxia, or acidosis, which are common within

tumors (20, 21, 39). From this aspect HSP90 provides a potentially attractive target for the treatment of cancer, however HSP90 has only recently become accepted as a mainstream therapeutic target, due to concerns about the potential toxicity associated with the inhibition of such a ubiquitous chaperone, which involved in various biological pathways (19, 39). However, in early phase I and II studies HSP90 showed an acceptable safety profile based on its high selectivity for cancer cells over healthy cells (20, 25). HSP90 demonstrated high selectivity to the tumor for the following reasons: 1. Cancer cells are more dependent on oncoproteins, i.e. 'addicted' to oncoproteins. 2. In the tumor microenvironment consisting of hypoxia, nutrient deprivation, and acidosis, cancer cells become much more dependent on HSP90 to control cellular stress (19, 22). 3. The exposure of HSP90 inhibitors in tissue is greater than in plasma, with more tumor retention (24). The first generation of HSP90 inhibitors was natural products such as geldanamycin and its derivatives. 17-AAG (17-(Allylamino)-17-demethoxygeldanamycin), which is a semisynthetic analogue of geldanamycin, its more soluble forms such as 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin) and IPI-504 (17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride), and the hydroquinone form of 17-AAG have undergone clinical trials (19, 21, 28, 36). Some preclinical studies

have reported promising results (35, 36, 40), however the complexity of natural products and their potential for off-target effects brought a widespread search for synthetic, lower molecular mass inhibitors of Hsp90 (19).

AUY922 (5-(2,4-dihydroxy-5-isopropyl-phenyl)-N-ethyl-4-[4-(morpholinomethyl)phenyl]isoxazole-3-carboxamide) is a highly potent, newly designed non-geldanamycin small-molecule HSP90 inhibitor that inhibits the ATPase activity of HSP90 (24). AUY922 has a much higher affinity for HSP90 than previous analogues of geldanamycin, and has nano-molar efficacy (with IC_{50} of 13nM/21nM for HSP90α/β) against a wide range of human cancer cell models in vitro and in vivo with no major adverse effects (23-25, 41). The first in-human phase I dose-escalation study of the Hsp90 inhibitor AUY922 in patients with advanced solid tumors was reported in 2013 (25). AUY922 was administered as a 1-hour intravenous infusion once a week for 28 days, and when patients were treated at doses of 40 mg/m2 or higher, the trough concentrations were maintained above 20 ng/mL, which previously reported as the average efficacious plasma concentration (24, 25). AUY922 has shown an acceptable safety profile at doses of 70 mg/m^2, therefore a recommended dose was determined as 70 mg/m^2 intravenously as a once-weekly schedule. The most common

adverse effects (mostly grade 1–2) included diarrhea, nausea, fatigue, and visual disturbance which were considered manageable and reversible following interruption or discontinuation (25). At this time, several clinical trials on the effect of AUY922 have been completed (http://clinicaltrials.gov, http://www.cancer.gov/clinicaltrials/search), however few formal reports have been published to date. Another approach is combination treatment of AUY922 with conventional cytotoxic drugs or other molecular targeted drugs, such as crizotinib, to enhance anti-tumor effects while maintaining toxicity at tolerable levels. Further investigation is mandatory for the clinical use of AUY922 to treat NSCLC patients, especially those with various mutations or acquired resistance to EGFR-TKIs.

In conclusion, AUY922 is a promising agent to overcome resistant NSCLC via the three major mechanisms of resistance, namely T790M mutation, MET and AXL bypass.

Reference

1. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating Mutations in the Epidermal Growth Factor Receptor Underlying Responsiveness of Non-Small-Cell Lung Cancer to Gefitinib. *N Eng J Med.* 2004;350:2129-39.

2. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR Mutations in Lung Cancer: Correlation with Clinical Response to Gefitinib Therapy. *Science.* 2004;304:1497-500.

3. Paz-Ares L, Soulieres D, Melezinek I, Moecks J, Keil L, Mok T, et al. Clinical outcomes in non-small-cell lung cancer patients with EGFR mutations: pooled analysis. *J Cell Mol Med*. 2010;14:51-69.

4. Perez-Soler R, Chachoua A, Hammond LA, Rowinsky EK, Huberman M, Karp D, et al. Determinants of tumor response and survival with erlotinib in patients with non--small-cell lung cancer. *J Clin Oncol*. 2004;22:3238-47.

5. Su KY, Chen HY, Li KC, Kuo ML, Yang JC, Chan WK, et al. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. *J Clin Oncol*. 2012;30:433-40.

6. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Eng J Med.* 2005;352:786-92.

7. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib

or erlotinib is associated with a second mutation in the EGFR kinase domain. *Plos Med*. 2005;2:e73.

8. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science.* 2007;316:1039-43.

9. Rho JK, Choi YJ, Lee JK, Ryoo BY, Na, II, Yang SH, et al. The role of MET activation in determining the sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors. *Mol Cancer Res.* 2009;7:1736-43.

10. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, et al. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nat Genet.* 2012;44:852-60.

11. Bean J, Brennan C, Shih JY, Riely G, Viale A, Wang L, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *P Natl Acad Sci USA*. 2007;104:20932-7.

12. Zhang YX, Knyazev PG, Cheburkin YV, Sharma K, Knyazev YP, Orfi L, et al. AXL is a potential target for therapeutic intervention in breast cancer progression. *Cancer Res.* 2008;68:1905-15.

13. Lin L, Bivona TG. Mechanisms of resistance to epidermal growth factor receptor inhibitors and novel therapeutic strategies to overcome resistance in NSCLC Patients. *Chemother Res Pract.* Vol. 2012, DOI:10.1155/2012/817297

14. Rho JK, Choi YJ, Kim SY, Kim TW, Choi EK, Yoon SJ, et al. MET and AXL inhibitor NPS-1034 exerts efficacy against lung cancer cells resistant to EGFR kinase inhibitors because of MET or

AXL activation. *Cancer Res.* 2014;74:253-62.

15. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Bio*. 2003;4:915-25.

16. Postel-Vinay S, Ashworth A. AXL and acquired resistance to EGFR inhibitors. *Nat Genet*. 2012;44:835-6.

17. Wandinger SK, Richter K, Buchner J. The Hsp90 chaperone machinery. *J Biol Chem.* 2008;283:18473-7.

18. Whitesell L, Lin NU. HSP90 as a platform for the assembly of more effective cancer chemotherapy. *Biochim Biophys Acta*. 2012;1823:756-66.

19. Pearl LH, Prodromou C, Workman P. The Hsp90 molecular chaperone: an open and shut case for treatment. *Biochem J*. 2008;410:439-53.

20. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. *Nat Rev Cancer*. 2010;10:537-49.

21. Banerji U. Heat shock protein 90 as a drug target: some like it hot. *Clin Cancer Res.* 2009;15:9-14.

22. Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC, et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature*. 2003;425:407-10.

23. Ueno T, Tsukuda K, Toyooka S, Ando M, Takaoka M, Soh J, et al. Strong anti-tumor effect of NVP-AUY922, a novel Hsp90 inhibitor, on non-small cell lung cancer. *Lung cancer*. 2012;76:26-31.

24. Jensen M, Schoepfer J, Radimerski T, Massey A, Guy C, Brueggen J, et al. NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models. *Breast Cancer Res*. 2008;10:R33.

25. Sessa C, Shapiro GI, Bhalla KN, Britten C, Jacks KS, Mita M, et al. First-in-Human Phase I Dose-Escalation Study of the HSP90 Inhibitor AUY922 in Patients with Advanced Solid Tumors. *Clin Cancer Res*. 2013;19:3671-80.

26. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer*. 2005;5:761-72.

27. Georgakis GV, Li Y, Rassidakis GZ, Martinez-Valdez H, Medeiros LJ, Younes A. Inhibition of heat shock protein 90 function by 17-allylamino-17-demethoxy-geldanamycin in Hodgkin's lymphoma cells down-regulates Akt kinase, dephosphorylates extracellular signal-regulated kinase, and induces cell cycle arrest and cell death. *Clin Cancer Res*. 2006;12:584-90.

28. Georgakis GV, Li Y, Younes A. The heat shock protein 90 inhibitor 17-AAG induces cell cycle arrest and apoptosis in mantle cell lymphoma cell lines by depleting cyclin D1, Akt, Bid and activating caspase 9. *Brit J Haematol*. 2006;135:68-71.

29. Graham B, Curry J, Smyth T, Fazal L, Feltell R, Harada I, et al. The heat shock protein 90 inhibitor, AT13387, displays a long duration of action in vitro and in vivo in non-small cell lung cancer. *Cancer Sci*. 2012;103:522-7.

30. Lamottke B, Kaiser M, Mieth M, Heider U, Gao Z, Nikolova Z, et al. The novel, orally bioavailable HSP90 inhibitor NVP-HSP990

induces cell cycle arrest and apoptosis in multiple myeloma cells and acts synergistically with melphalan by increased cleavage of caspases. *Eur J Haematol*. 2012;88:406-15.

31. Okawa Y, Hideshima T, Steed P, Vallet S, Hall S, Huang K, et al. SNX-2112, a selective Hsp90 inhibitor, potently inhibits tumor cell growth, angiogenesis, and osteoclastogenesis in multiple myeloma and other hematologic tumors by abrogating signaling via Akt and ERK. *Blood*. 2009;113:846-55.

32. Li Y, Ye X, Tan C, Hongo JA, Zha J, Liu J, et al. Axl as a potential therapeutic target in cancer: role of Axl in tumor growth, metastasis and angiogenesis. *Oncogene.* 2009;28:3442-55.

33. Robinson KW, Sandler AB. The role of MET receptor tyrosine kinase in non-small cell lung cancer and clinical development of targeted anti-MET agents. O*ncologist*. 2013;18:115-22.

34. Webb CP, Taylor GA, Jeffers M, Fiscella M, Oskarsson M, Resau JH, et al. Evidence for a role of Met-HGF/SF during Ras-mediated tumorigenesis/metastasis. *Oncogene*. 1998;17:2019-25.

35. Shimamura T, Li D, Ji H, Haringsma HJ, Liniker E, Borgman CL, et al. Hsp90 inhibition suppresses mutant EGFR-T790M signaling and overcomes kinase inhibitor resistance. *Cancer Res.* 2008;68:5827-38.

36. Kobayashi N, Toyooka S, Soh J, Yamamoto H, Dote H, Kawasaki K, et al. The anti-proliferative effect of heat shock protein 90 inhibitor, 17-DMAG, on non-small-cell lung cancers being resistant to EGFR tyrosine kinase inhibitor. *Lung cancer*.

2012;75:161-6.

37. Koizumi H, Yamada T, Takeuchi S, Nakagawa T, Kita K, Nakamura T, et al. Hsp90 inhibition overcomes HGF-triggering resistance to EGFR-TKIs in EGFR-mutant lung cancer by decreasing client protein expression and angiogenesis. *J Thorac Oncol*. 2012;7:1078-85.

38. Xu L, Kikuchi E, Xu C, Ebi H, Ercan D, Cheng KA, et al. Combined EGFR/MET or EGFR/HSP90 inhibition is effective in the treatment of lung cancers codriven by mutant EGFR containing T790M and MET. *Cancer Res*. 2012;72:3302-11.

39. Bagatell R, Whitesell L. Altered Hsp90 function in cancer: A unique therapeutic opportunity. *Mol Cancer Ther*. 2004;3:1021-30.

40. Shimamura T, Lowell AM, Engelman JA, Shapiro GI. Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins. *Cancer Res*. 2005;65:6401-8.

41. Lee K-H, Lee J-H, Han S-W, Im S-A, Kim T-Y, Oh D-Y, et al. Antitumor activity of NVP-AUY922, a novel heat shock protein 90 inhibitor, in human gastric cancer cells is mediated through proteasomal degradation of client proteins. *Cancer Sci.* 2011;102:1388-95.

초록

서론: 상피세포 성장인자 수용체 (Epidermal growth factor receptor, EGFR) 변이 폐암의 경우 티로신 카이나제 억제제 (Tyrosine kinase inhibitor, TKI) 에 좋은 초기 반응을 보이더라도 결국 저항성을 획득하게 되는 바, 그 저항성을 보이는 원인으로, T790M 이차 변이나 MET 또는 AXL 등의 회피 신호의 발현이 규명되었다. 열충격단백질 90 (Heat Shock Protein 90, HSP90) 은 여러 종류의 종양발현단백질의 기능과 안정성에 중요한 역할을 하는 단백질로서, 본 연구에서는 T790M 이차변이, MET 와 AXL 회피 기전을 통하여 EGFR-TKI 에 저항성을 획득한 폐암세포주에서 HSP90 억제제의 일종인 non-geldanamycin 계열의 AUY922 약제의 종양억제 효과를 확인하고자 한다.

방법: 본 연구자들은 EGFR 유전체의 exon 19 에 deletion 변이가 있어 EGFR-TKI 에 민감한 PC-9 및 HCC827 폐암세포주에 gefitinib 과 erlotinib 의 농도를 올리며 장기간 노출시킴으로써, 각각의 약제에 저항성을 보이는 세포주를 확립하였으며, 이를 각각 PC-9/GR, ER 및 HCC827/GR, ER 로 명명하였다. 각 저항성 세포주를 대상으로 AUY922 약제의 농도와 시간을 달리하여 적용하였으며 그 반응을 확인하였다.

결과: PC-9 세포주에서는 T790M 이차변이를 통하여, HCC827/GR 에서는 MET 증폭, HCC827/ER 세포주는 AXL 회피경로를 통하여

EGFR-TKI 저항성을 획득함을 확인하였다. AUY922 는 EGFR, MET, AXL 의 종양발현단백질뿐 아니라 Akt, Erk 등 하위 단백질의 발현을 억제함을 확인할 수 있었다. 또한 모든 저항성 세포주에서 효과적으로 세포 성장을 억제하고 세포사를 유도할 뿐 아니라 침습능력과 이동능력을 억제하는 것을 확인하였다. 이종이식 생쥐 모델을 통하여 AUY922 가 EGFR-TKI 저항성을 보이는 종양의 성장을 in vivo 에서도 억제함을 확인하였다.

결론: EGFR-TKI 저항성을 보이는 비소세포폐암의 주요 원인인 T790M 이차변이, MET 및 AXL 매개 저항성 폐암세포주에서 AUY922 치료가 좋은 치료효과를 보임을 확인하였다.

주요어: 비소세포폐암, EGFR, 저항성, 열충격단백질90, MET, AXL

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