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Authors: Takamasa Koga, Yoshihisa Kobayashi, Kenji Tomizawa, Kenichi Suda, Takayuki Kosaka, Yuichi Sesumi, Toshio Fujino, Masaya Nishino, Shuta Ohara, Masato Chiba, Masaki Shimoji, Toshiki Takemoto, Makoto Suzuki, Pasi A. Jänne, Tetsuya Mitsudomi

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Activity of a novel HER2 inhibitor, poziotinib, for HER2 exon 20 mutations in lung cancer and mechanism of acquired resistance: an *in vitro* study

Takamasa Koga, MD, [a, b] Yoshihisa Kobayashi, MD, PhD, [a, c] Kenji Tomizawa, MD, PhD, [a, d] Kenichi Suda, MD, PhD, [a] Takayuki Kosaka, MD, PhD, [c, e, f] Yuichi Sesumi, MD, PhD, [a] Toshio Fujino, MD, [a] Masaya Nishino, MD, [a] Shuta Ohara, MD, [a] Masato Chiba, MD, PhD, [a] Masaki Shimoji, MD, PhD, [a] Toshiki Takemoto, MD, PhD, [a] Makoto Suzuki, MD, PhD, [b] Pasi A. Jänne, MD, PhD, [c, f, g] Tetsuya Mitsudomi, MD, PhD. [a]*

[a]Department of Thoracic Surgery, Kindai University Faculty of Medicine, Osaka-Sayama, Japan.

[b]Department of Thoracic Surgery, Graduate School of Medical Science, Kumamoto University, Kumamoto, Japan.

[c]Department of Medical Oncology, Harvard Medical School, Boston, Massachusetts.

[d]Department of Thoracic Surgery, Izumi City Hospital, Izumi, Japan.

[e]Department of General Surgical Science, Gunma University Graduate School of Medicine, Maebashi, Japan.

[f]Lowe Center for Thoracic Oncology, Harvard Medical School, Boston, Massachusetts.

[g]Belfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, Massachusetts.

***Corresponding author.**

Tetsuya Mitsudomi, MD, PhD, at Department of Thoracic Surgery, Kindai University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, 589-8511, Japan. Tel.: +81 72 366 0221, Fax: +81 72 365 7161, E-mail: mitsudom@med.kindai.ac.jp.

Highlights:

- The treatment strategy for NSCLC with HER2 exon 20 insertion remains unclear.
- Poziotinib showed potent activity against HER2 exon 20 insertions *in vitro* model.
- C805S was identified as a mechanism underlying acquired resistance to poziotinib.
- HSP90 inhibitors were able to overcome this resistant clone.

ABSTRACT

Objectives

Oncogenic HER2 mutations are present in 2-4% of lung adenocarcinomas, but the relevant clinical trials are unsatisfactory. The novel HER2 inhibitor poziotinib was recently developed and clinical trials are ongoing. We compared poziotinib with nine tyrosine kinase inhibitors (TKIs), and derived poziotinib-resistant clones to investigate the resistant mechanism.

Materials and Methods

We introduced three common HER2 mutations A775_G776insYVMA (YVMA), G776delinsVC (VC) and P780_Y781insGSP (GSP), which account for 94% of HER2 exon 20 insertions in the literature, into Ba/F3 cells. We then compared the activity of poziotinib with that of nine TKIs (erlotinib, afatinib, dacomitinib, neratinib, osimertinib, AZ5104, pyrotinib, lapatinib, and irbinitinib), determined the 90% inhibitory concentration (IC_{90}) through a growth inhibition assay, and defined a sensitivity index (SI) as IC_{90} divided by the trough concentration at the recommended dose as a surrogate for drug activity in humans. We also generated resistant clones by exposure to poziotinib in the presence of N-ethyl-N-nitrosourea, and HER2 secondary mutations that might serve as a resistance mechanism were searched.

Results

YVMA showed resistance to all tested drugs except neratinib, poziotinib and pyrotinib.

Poziotinib was the only drug with an SI less than 10 for YVMA, the most common HER2 exon 20 insertion. We established 62 poziotinib-resistant clones, and among these, only C805S of HER2, which is homologous to C797S of the EGFR, was identified as a secondary mutation in 19 clones. We also revealed that heat shock protein (HSP) 90 inhibitors show potent anti-growth activity to the C805S secondary mutant clone.

Conclusions

Poziotinib showed the most potent activity against HER2 exon 20 mutations. We identified the secondary C805S at the covalent binding site of HER2 to poziotinib as a potential mechanism of acquired resistance. HSP90 inhibitors might be a therapeutic strategy for the C805S secondary mutation.

Keywords:

Lung cancer; HER2 mutation; Exon 20; Poziotinib; Acquired resistance; C805S

1. INTRODUCTION

Targeted therapies against lung cancer harboring EGFR mutations, ALK or ROS1 fusions and BRAF mutation using respective tyrosine kinase inhibitors (TKI) comprise the standard of care [1]. The list of these driver genes of NSCLC is continuously expanding, and

the human epidermal growth factor receptor 2 (ERBB2/HER2) mutation is one of these emerging driver mutations that were originally shown in 2004 to be present in NSCLC patients [2].

According to the literature, HER2 mutations are present in 2-4% of lung adenocarcinoma (ADC) patients. Most HER2 mutations are in-frame insertional mutations occurring at exon 20 in the protein kinase domain [2-5]. Although several small molecules and HER2 antibodies have been tested for treatment of these tumors, the results were generally unsatisfactory. For example, a recently published large retrospective study found that the overall response rate (ORR) for HER2-targeted TKIs, including neratinib, lapatinib and afatinib, is only 7.4% [6].

A novel pan-HER TKI, poziotinib, was recently developed for lung cancer with EGFR and HER2 exon 20 mutations. Early clinical trials for NSCLC with EGFR and HER2 exon 20 mutation are ongoing, and the ORR in the EGFR exon 20 cohort was found to equal 73% in a phase II study [7]. In addition, another novel pan-HER TKI, pyrotinib, achieved an ORR of 55% in a phase II study for HER2 mutant-pozitive advanced NSCLC [8]. The development of these de novo reagents inspired us to comprehensively evaluate the antitumor activities of poziotinib and pyrotinib for common HER2 exon 20 insertional mutations and

compare them with those of eight preexisting TKIs using a mouse Ba/F3 system. Furthermore, we derived resistant clones against effective TKI through chronic exposure to the drug and searched for the resistant mechanism.

2. MATERIAL AND METHODS

2.1 Cell lines

The immortalized murine pro-B cell line Ba/F3 was obtained from the RIKEN Bio Resource Center (Tsukuba, Japan). NCI-H1781 cells, which belong to a lung adenocarcinoma cell line harboring the HER2 exon 20 G776delinsVC (VC) mutation [4], were purchased from the American Type Culture Collection (Virginia, USA). All the cells were cultured in RPMI1640 (Wako, Osaka, Japan) containing 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) with or without murine IL-3 at 37°C in a humidified atmosphere with 5% CO_2. All cells were routinely checked and verified to be free from mycoplasma contamination using the TaKaRa PCR Mycoplasma Detection Set (Takara, Kusatsu, Japan).

2.2 Establishment of Ba/F3 cells with HER2 mutations

We reviewed the literature and summarized the frequency of HER2 exon 20 mutations (Supplementary Fig. 1, Supplementary Table 1), and the results revealed that 94% of the HER2 exon 20 mutations were A775_G776insYVMA (YVMA) (73%), VC (13%), and GSP (8%). Therefore, we decided to generate Ba/F3 cells harboring these three mutations. YVMA and VC were introduced into Ba/F3 cells using a retrovirus system, as previously described [9]. In brief, a pBABE retrovirus vector was subcloned with the full-length cDNA fragment encoding the human wild-type (WT) ERBB2 gene, and transduced YVMA was purchased from Addgene (Cambridge, MA, USA). VC was introduced into the pBABE of HER2-WT using a Prime STAR Mutagenesis Basal Kit (Takara). To generate viral particles, the pBABE constructs were transfected with a pVSV-G vector (Clontech, Fremont, CA, USA) into gp-IRES 293 cells using the FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Forty-eight hours are transfection, the culture medium was collected, and the viral particles were concentrated using a Retrovirus Concentration kit (Clontech) for 24 hours at 4°C. After centrifugation at 1,500× g for 40 min., the viral pellet was diluted in RPMI 1640 and stored at -80°C. We seeded 2 x 10^3 / well Ba/F3 cells onto 24-well plates. Twenty four hours seeding, diluted viral suspension was added to the cells, and this step was repeated 24 hours later. The infected Ba/F3 cells were incubated for 3-5 days, and then selected using

puromycin (1.5 μg/mL) in the presence of interleukin-3 (IL-3). Puromycin-selected cells were incubated without IL-3 for approximately one week. Ba/F3 cells harboring GSP were generated as previously described [10].

2.3 Reagents

We used erlotinib, which is a first-generation (1G) EGFR TKI, afatinib, dacomitinib, neratinib, which are second-generation (2G) EGFR TKIs, osimertinib and its metabolite AZ5104, which are third-generation (3G) EGFR TKIs, as well as in poziotinib, pyrotinib, lapatinib and irbinitinib which are HER2 targeting TKIs. We also used heat shock protein (HSP) 90 inhibitors, ganetespib and luminespib. All the drugs except pyrotinib were purchased from Selleck Chemicals (Houston, TX, USA), and pyrotinib was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Each drug was dissolved to a concentration of 10 mmol/L in dimethyl sulfoxide (Sigma-Aldrich) and stored at -80°C until use.

2.4 Cell proliferation assay

Overall, 3×10^4 transfected Ba/F3 cells were seeded onto six-well plates. Ba/F3 cells

expressing HER2 WT, YVMA, VC and GSP were cultured without interleukin-3 (IL-3), and HER2 WT cells were cultured with 5 ng/ml murine IL-3 (Cell Signaling Technology, Danvers, MA, USA). Ba/F3 cells were also cultured in the presence and absence of IL-3 as a control. The total numbers of cells in each well were counted in triplicate every 24 hours for a total experimental period of 72 hours using OneCell Counter (Biomedical Medical Science, Tokyo, Japan).

2.5 Growth inhibition assay

Cells were seeded at a density of 2-4 x 10^3/well onto 96-well plates. After 24 hours of incubation, the cells were exposed to each HER2 inhibitor at the indicated drug concentrations for 72 hours. Then, 10 μL of tetrazolium salt WST-8 was added to the cells, and the plates were incubated for 2-4 hours using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). To measure the formazan dye generated by the reduction of tetrazolium by NADH, which reflects cell viability, the absorbance at 450 nm was read using a multiplate reader (Tecan, Mannedorf, Switzerland).

2.6 Generation of resistant clones through N-ethyl-N-nitrosourea (ENU) mutagenesis

and chronic exposure to poziotinib.

We established poziotinib-resistant clones using ENU (Sigma-Aldrich), as previously reported [11]. Ba/F3 cells harboring YVMA, VC or GSP were exposed to 100 μg/ml ENU for 24 hours, washed and cultured in RPMI1640 supplemented with 10% FBS for 24 hours. Then, 5-20 × 10^4 cells were seeded onto 96-well plates in the presence of various concentrations of poziotinib (10 nM, 100 nM and 200 nM). These plates were incubated for 2 weeks, the medium was changed twice weekly and surviving clones were isolated.

In the chronic exposure assay, parental YVMA, VC and GSP Ba/F3 cells were cultured with an increasing concentration of poziotinib in the absence of ENU. The final target concentration was 200 nM because the maximum concentration of poziotinib in the phase I study was 167 nM [12].

2.7 HER2 mutation analyses

The total RNA from the cells was extracted using a mirVana miRNA Isolation Kit (Qiagen, Hilden, Germany). The RNA was transcribed to cDNA using ReverTra Ace (TOYOBO, Osaka, Japan). Using the obtained cDNA as the template, the tyrosine kinase domains (TKD) of HER2 exon 18 to 24 were amplified using designed primers. We

performed agarose gel electrophoresis to confirm the PCR-amplified products. Sanger sequencing was performed using a Genetic Analyzer 3130 or 3500XL (Applied Biosystems, Waltham, MA).

3. RESULTS

3.1 Establishment of transfected Ba/F3 cells harboring HER2 exon 20 insertions and HER2 WT

The Ba/F3 cells transfected with YVMA, VC or GSP proliferated well in the absence of IL-3, which confirmed that these three mutations were oncogenic drivers (Fig. 1). The Ba/F3 cells transfected with WT HER2 grew without IL-3 but more slowly than the Ba/F3 with mutated HER2 (Fig. 1). Chromatograms of each mutant sequences are shown in Supplementary Fig. 2.

3.2 Drug sensitivity spectrum of HER2 exon 20 insertions

Growth inhibition curves of the Ba/F3 cells with HER2 mutations and WT HER2 are shown in Fig. 2A. The specific IC_{90} values are summarized in Supplementary Table 2. Since Ba/F3 cells are of mouse origin, there is concern that results obtained using the Ba/F3 system

might not be applicable to humans. Therefore, we compared the sensitivity patterns against 10 TKIs of Ba/F3 cells transduced with VC and H1781 human lung cancer cell lines with VC. As shown in Fig. 2B, these two cells showed similar patterns, suggesting the validity of our experimental system.

We originally planned to compare the IC_{90} of mutant HER2 with that of WT HER2 (with/without IL-3). However, the cells with WT HER2 tended to be more sensitive to HER2-targeting TKIs in the absence of IL-3 than many of those with HER2 mutants, as previously reported [13]. In contrast, the cells with WT HER2 were completely resistant to TKIs in the presence of 5 ng/ml IL-3. To distinctly compare the cell growth inhibition activities, we used a sensitivity index (SI), which was defined as the IC_{90} divided by the trough concentration of a given drug ($IC_{90}/C_{trough} \times 100$) at the recommended dose for humans described in the literature, as a surrogate for drug activity in humans [12,14-21]. The C_{trough} of pyrotinib was unavailable.

In general, poziotinib and 2G EGFR TKIs (afatinib, dacomitinib and neratinib) showed lower SI values for HER2 mutants than the 3G EGFR TKIs (osimertinib and AZ5104) and reversible TKIs (erlotinib, lapatinib and irbinitinib). All the drugs except lapatinib and irbinitinib showed their highest activity against VC. The most common

mutation, YVMA, was less sensitive than other mutations to all the drugs except poziotinib, neratinib and pyrotinib. For YVMA, poziotinib was the only drug that had a SI of less than 10. In fact, the SI of poziotinib for YVMA was 2-10-fold lower than those of other irreversible TKIs (2G and 3G EGFR TKIs) and 5-100-fold lower than those of reversible TKIs. Furthermore, poziotinib was the most potent of the tested drugs for VC and GSP, with the exception for dacomitinib for GSP (Fig. 2C, 2D).

3.3 Identification of the secondary mutation that causes poziotinib resistance through ENU mutagenesis

To examine the mechanisms of acquired resistance, poziotinib-resistant clones of Ba/F3 cells harboring HER2 exon 20 insertions were generated by ENU mutagenesis. We derived 62 poziotinib-resistant clones that grew vigorously in the presence of 10 nM (n=39), 100 nM (n=16) and 200 nM (n=7) poziotinib (Fig. 3A). We sequenced all the clones to identify secondary HER2 mutations and found that 19/62 (31%) had a secondary C805S mutation homologous to C797S in the EGFR gene (Fig. 3B). Most C805S clones were derived from parental cells with YVMA or VC treated with a high concentration of poziotinib (100 nM or 200 nM). However, the remaining 43 clones including all those obtained from

parental cells with GSP, had no secondary mutation in the tyrosine kinase domain.

We also attempted to induce resistance by chronic exposure to increasing concentration of poziotinib. Although we established resistant clones with VC and YVMA that were able to grow in the presence of 200 nM and 50 nM of poziotinib, respectively, these did not harbor any secondary HER2 mutation.

3.4 In vitro sensitivities of poziotinib-resistant Ba/F3 cells to HER2 TKIs

To confirm the drug resistance conferred by the C805S secondary mutation, we examined the IC_{90}s of poziotinib-resistant cells harboring YVMA+C805S or VC+C805S to various TKIs. Cell growth inhibition assays revealed that these cells with the C805S secondary mutant showed approximately 100-fold higher resistance to poziotinib than the parental cells (Fig. 3C). However, the IC_{90}s of reversible TKIs (erlotinib, lapatinib and irbinitinib) were not affected by the addition of C805S secondary mutation and remained at 200 nM or more (Fig. 3D, Supple. Table 3). Therefore, the proliferation of these poziotinib-resistant cells were not inhibited by any TKIs used in this study.

3.5 HSP90 inhibitor showed potent anti-growth activity against poziotinib-resistant cells

Previous studies demonstrated that heat shock protein (HSP) inhibitors exhibit inhibitory activity in cell models of acquired resistance to EGFR- or ALK-TKI [22,23]. To overcome acquired resistance due to the C805S secondary mutation, we tested the activity of the HSP 90 inhibitors, ganetespib and luminespib. HSP90 inhibitors impede the chaperone function of HSP90 in the maturation and stability of oncogenic client proteins such as HER2 and EGFR [24]. As shown in Fig. 4A and 4B, both HSP inhibitors exhibited strong activity against Ba/F3 cells with HER2 mutations, regardless of the presence of C805S. The SI values of luminespib were approximately 870-fold lower for YVMA+C805S and 25-fold lower for VC+C805S compared with the values of poziotinib (Fig. 4C).

4. DISCUSSION

Here, we showed that among 10 relevant TKIs, poziotinib exihibited the most potent activity against three common HER2 exon 20 insertions, with the exception that GSP was confirmed to be sensitive to dacomitinib as reported previously [13,25]. The fact that poziotinib showed superior activity for H1781 harboring VC compared with afatinib was consistent with the results of a previous preclinical study [26].

To our knowledge, this study provides the first evidence showing that the HER2

C805S secondary mutation is a potential mechanism of acquired resistance against poziotinib. Because C805 forms a covalent bond with irreversible TKI, similarly to C797 of the EGFR, C805 is likely to interfere with binding to poziotinib. This result is concordant with the findings report previously by Kosaka *et al.* who found that YVMA with secondary C805S was resistant to neratinib or dacomitinib. [10] However, there have been no clinical reports on secondary HER2 mutations in NSCLC harboring HER2 exon 20 insertions.

Strategies to overcome C805S should be developed before this acquired resistance becomes a matter of clinical concern. The C797S secondary mutation does not affect ATP competitive inhibition in EGFR-mutated cells. Hence, EGFR-mutated cells with C797S remain sensitive to reversible EGFR TKI [27]. Similarly, C805S did not affect the sensitivity to reversible HER2 TKI. However, this strategy is not applicable to HER2-mutated lung cancer because HER2 exon 20 insertions are inherently resistant to reversible TKIs (Fig. 3D).

Alternatively, HSP90 inhibition can be considered as another strategy to cope with HER2 C805S because various RTK products, including HER2/EGFR/ALK, are client proteins of HSP90. Indeed, a HSP90 inhibitor, 17-AAG, exhibits inhibitory activity against the V1180L secondary mutation in the ALK gene [22]. In a previous study, ganetespib showed anti-tumor activity against a mouse model with YVMA, and luminespib

downregulated HER2 expression in human tumor xenografts [28,29]. As expected, C805S did not alter sensitivity to the HSP90 inhibitors, ganetespib and luminespib. Therefore, HSP90 inhibitors may be an option for addressing the HER2 C805S secondary mutation, even though disappointing results were obtained in a phase III study of ganetespib for EGFR/ALK-negative pretreated lung adenocarcinoma [30].

According to the literature, the ORRs of NSCLC with HER2 exon 20 insertions treated with EGFR TKIs have been unsatisfactory, except for the treatment of the GSP mutation with dacomitinib (Table 1). [25,31-41]. During the preparation of this manuscript, Robichaux *et al*. found that poziotinib exhibits potent activity against HER2 exon 20 insertions by comparing the absolute IC_{50} values of various TKIs [42]. Moreover, these researchers reported a successful treatment of a rare HER2 A771insAYVM with poziotinib. More recently, Oh *et al*. reported two responders, one with three YVMA and one with two VC, although the number of patients was limited [41]. The results of currently ongoing two phase II trials are eagerly awaited (NCT03066206, NCT033189399).

In conclusion, compared with other TKIs, poziotinib showed the most potent activity for HER2 exon 20 insertions. We provide the first identification of the C805S secondary mutation as the potential mechanism responsible for the acquisition of poziotinib resistance.

Furthermore, HSP90 inhibitors are expected to overcome the HER2 C805S secondary mutation.

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Conflict of interest:

Dr. Kobayashi has received a lecture fee from Boehringer Ingelheim and research funding from Novartis during the study. Professor Pasi A. Jänne has received research funding from Astellas Pharmaceuticals, AstraZenenca, Daiichi Sankyo, and PUMA; has been a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Roche, Pfizer, Ariad/Takeda and Chugai Pharmaceuticals; and has received post marketing royalties from DFCI owned IP on EGFR mutations licensed to Lab Corp outside the submitted work. Dr. Mitsudomi has received lecture fees from AstraZeneca, Boehringer Ingelheim, Chugai, and Pfizer and research funding from Astra Zeneca, Boehringer Ingelheim, and Chugai during the

study; has been on the advisory board of Novartis; has received lecture fees from Bristol-Myers Squibb, Eli Lilly, Merck Sharp and Dohme; and has received research funding from Daiichi Sankyo, Taiho, and Ono Pharmaceutical for studies not described in this paper.

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FIGURE LEGENDS

Figure 1. Cell growth and proliferation of transfected Ba/F3 cells. Thirty thousand transfected and non-transfected Ba/F3 cells were seeded onto six-well plates. Ba/F3 cells expressing A775_G776insYVMA (YVMA), G776delinsVC (VC) and P780_Y781insGSP (GSP) were cultured without interleukin-3 (IL-3). Ba/F3 cells with wild type (WT) HER2 and non-transfected Ba/F3 cells were also cultured in the presence and absence of 5 ng/ml IL-3 as a control. We counted these cells every 24 hours for a total experimental period of 72 hours.

Figure 2. Drug sensitivities of transfected Ba/F3 cells harboring each HER2 exon 20 insertion against the indicated TKIs. **A.** Cell growth inhibition curves of infected Ba/F3 cells with the three HER2 mutations and wild-type HER2 that were treated with ten TKIs. **B.** Comparison of the sensitivity of Ba/F3 cells transfected with VC and H1781 cells, which harbors VC, to treatment with various TKIs. **C.** The measured IC_{90} values are plotted as a bar graph. The growth inhibition assay was performed in triplicate and the mean value with the standard deviations was calculated. Trough concentrations (C_{trough}) were obtained from the

literature at the recommended doses. The values with asterisks were extracted from the plasma drug concentration curves of original figures in the literature. **D.** The inhibition activities of each TKI for HER2 exon 20 insertions were compared according to the selectivity index (SI, IC_{90} / C_{trough} ×100). The measured SI values were color coded as follows: ≤10, blue: 11-30, light orange; and >30, red.

Figure 3. Secondary HER2 mutation of the poziotinib-resistant clones established by ENU mutagenesis. Sequencing of the resistant clones revealed that all the acquired secondary mutations were C805S. **A.** Number of poziotinib-resistant clones established through ENU mutagenesis. The purple bar indicates YVMA + C805S or VC + C805S clones, and the gray bar indicates the clones harboring no secondary mutation. **B.** Chromatogram of a wild-type C805 and the secondary C805S mutation gained by Ba/F3 cells expressing YVMA before and after the acquisition of poziotinib resistance through ENU mutagenesis. **C.** Growth inhibition curves of Ba/F3 cells harboring YVMA, VC, YVMA + C805S and VC + C805S that were treated with poziotinib. Ba/F3 cells expressing YVMA + C805S and VC + C805S both acquired resistance to poziotinib. **D**. Growth inhibition curves of Ba/F3 cells harboring YVMA, VC, YVMA + C805S and VC + C805S treated with each TKI.

Figure 4. Growth inhibition curves of cells with YVMA, VC, YVMA+C805S and VC+C805S after treatment with ganetespib (**A**) and luminespib (**B**). The growth inhibition curves of cells with YVMA+C805S and VC+C805S that were treated with poziotinib are included as a control. **C.** HSP90 inhibitors (ganetespib and luminespib) showed significant inhibition activity against these secondary C805S clones. The sensitivity index (SI) of luminespib for the YVMA+C805S and VC+C805S clones was 870- and 25-fold lower than that of poziotinib, respectively. The trough concentration of ganetespib at the recommended dose was not available. Abbreviations: Gane, ganetespib; Lumi, luminespib.

Figure 1.

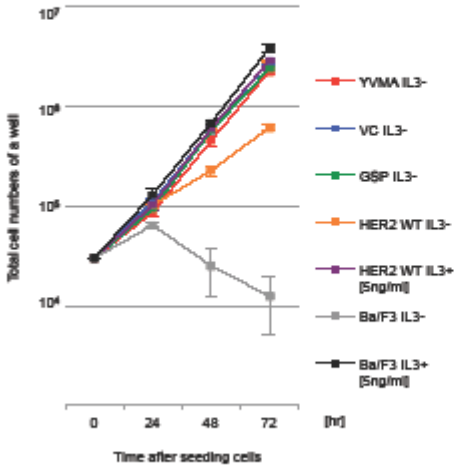


Figure 2.

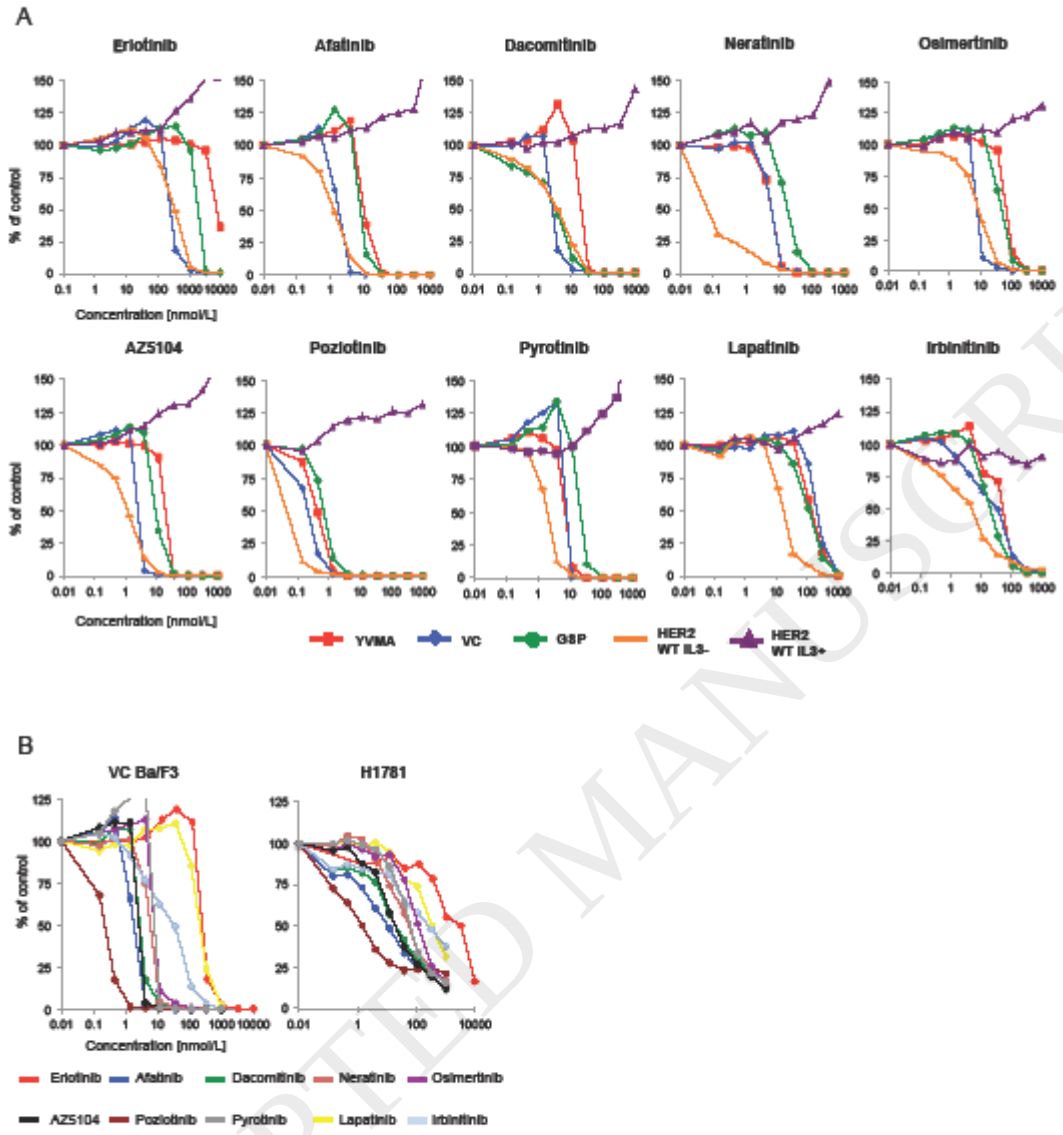


Figure 3.

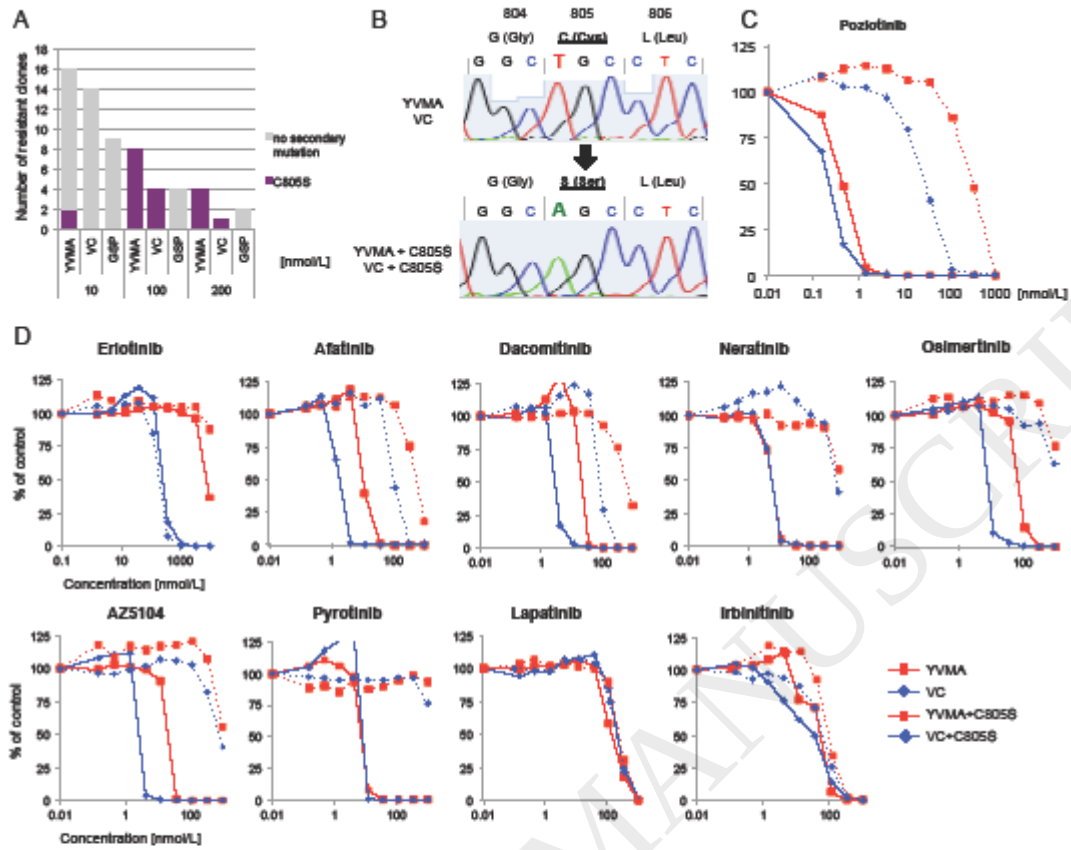
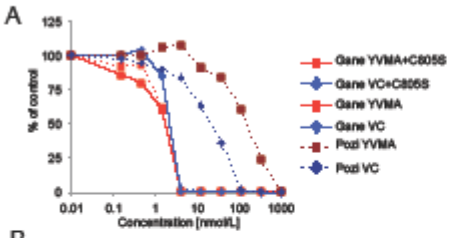
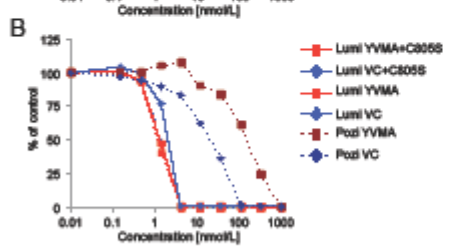


Figure 4.





C

	YVMA+C805S	VC+C805S
Pozlotinib	3215	100
Luminespib	3,7	4,0

TABLES

Table 1. Summary of response rate to HER2 targeting mono-therapies in reported cases harboring HER2 exon 20 insertion mutations investigated in this study.

			Response to HER2 inhibitors				
Exon 20 insertion	Drug	N	CR/PR	SD/PD	NE	ORR(%)	Reference
YVMA	Afatinib	31	5	24	2	16	(31-32, 34-38)
	Dacomitinib	13	0	13		0	(25)
	Erlotinib	1	0	1		0	(36)
	Neratinib	9	0	9		0	(33)
	Poziotinib	3	1	2		33	(41)
VC	Afatinib	3	0	3[a]		0	(31-32, 40)
	Dacomitinib	2	0	2		0	(25)
	Erlotinib	1	0	1		0	(34)
	Neratinib	1	0	1		0	(33)
	Lapatinib	1	0	1[a]		0	(40)
	Poziotinib	2	1	1		50	(41)
GSP	Afatinib	4	1	3		25	(31, 36-37, 39)
	Dacomitinib	2	2	0		100	(25)
	Neratinib	1	0	1		0	(33)

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; ORR, overall response rate; YVMA, A775_G776insYVMA; VC, G776delinsVC; GSP, P780_Y781insGSP.

[a]One patient was treated with lapatinib as the first-line treatment and with afatinib as the

third-line treatment.