Video Article

Establishing Dual Resistance to EGFR-TKI and MET-TKI in Lung Adenocarcinoma Cells *In Vitro* with a 2-step Dose-escalation Procedure

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Abstract

Drug resistance is a major challenge in cancer therapy. The generation of resistant sublines *in vitro* is necessary for discovering novel mechanisms to overcome this challenge. Here, a 2-step dose-escalation method for establishing dual-resistance to an epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI), gefitinib, and a MET-TKI, PHA665752, is described. This method is based on simple stepwise dose-escalation of inhibitors for inducing acquired resistance in cell lines. The alternate method for generating resistant sublines involves exposing the cells to high concentrations of the inhibitor in one step. The stepwise dose-escalation method has a higher possibility of successfully inducing acquired resistance than this method. Activating EGFR mutations are biomarkers of a response to treatment with EGFR-TKI, which is an applied first-line treatment for non-small cell lung cancers (NSCLC) that harbor these mutations. However, despite reports of effective responses, the use of EGFR-TKI is limited because tumors inevitably acquire resistance. The major mechanisms behind EGFR-TKI resistance include a secondary mutation at the gatekeeper site, T790M in exon 20 of EGFR, and a bypass signal of MET. Thus, a potential solution for this issue would be a combination of EGFR-TKI and MET-TKI. This combined treatment has been shown to be effective in an *in vitro* study model. Acquired gefitinib-resistance was established through MET-amplification by stepwise dose-escalation of gefitinib for 12 months, and a cell line named PC-9MET1000 was generated in a previous study. To further investigate the mechanisms of acquired MET-TKI and EGFR-TKI resistance, a MET-TKI, PHA665752, was administered to these cells with stepwise dose-escalation in the presence of gefitinib for 12 months. This protocol has also been successfully applied for a number of combination therapies to establish acquired resistance to other inhibitor molecules.

Video Link

The video component of this article can be found at https://www.jove.com/video/55967/

Introduction

Oncogenic mutations in the epidermal growth factor receptor (EGFR) are found in a subset of patients with non-small cell lung cancer (NSCLC) and are important predictive biomarkers of this disease[1,2]. These activating EGFR mutations most commonly occur as either an in-frame deletion in exon 19 (delE746-A750) or a point mutation replacing leucine with arginine at codon 858 of exon 21 (L858R) in the EGFR tyrosine kinase domain[3,4]. Treatment with EGFR-tyrosine kinase inhibitors (TKIs) is a clinically effective therapy for patients with NSCLC harboring EGFR mutations. However, this therapy is limited by the inevitable acquisition of resistance to EGFR-TKIs such as gefitinib and erlotinib. The most common acquired resistance occurs through a secondary T790M mutation in exon 20 of EGFR, which was detected in approximately 60% of patients who acquired EGFR-TKI (gefitinib or erlotinib) resistance. Other molecular mechanisms associated with acquired resistance to EGFR-TKIs are activation of bypass signaling caused by MET amplification, transformation of small-cell lung cancers, and epithelial-to-mesenchymal transition[5,6]. The receptor for the hepatocyte growth factor, encoded by the MET gene, which is dysregulatedin many tumors, has been reported to be an important candidate for targeted therapies[7,8].

Strategies for overcoming the acquired resistance to EGFR-TKIs are now undergoing clinical evaluation. Preclinical and clinical studies have shown that third-generation EGFR inhibitors such as osimertinib are effective for patients with the T790M mutation[9]. In addition, the growth of EGFR-mutant cancers with MET amplification can be inhibited by combined treatment with EGFR- and MET-TKIs[6,10]. Clinical trials assessing a combination of MET and EGFR inhibitors in patients with acquired resistance to gefitinib and erlotinib are underway now[11].

To study the molecular mechanism of acquired resistance to chemotherapeutic agents, cell lines that initially respond to inhibitors are treated continuously with these inhibitors. Two methods are available to establish acquired resistance in cell lines. One is stepwise dose-escalation, and the other is high-concentration exposure. The stepwise escalation method has been more commonly used, because it has a higher success rate. The cells are first exposed to low concentrations of the inhibitors, nearly a tenth of the half-maximal inhibitory concentration (IC_{50}) value, and the concentration is then gradually increased by 10-30% until the target concentration is achieved. The high-concentration exposure method

involves exposing the cells to the final dose of inhibitor in the culture medium, which is more than the IC_{50} value, so the parental cells are almost completely killed. This high-concentration exposure method has much a lower success rate than the stepwise escalation method.

In a previous study, combined treatment with EGFR-TKI and MET-TKI was shown to be effective in an *in vitro* system. Acquired resistance to an EFGR-TKI, gefitinib, was established through stepwise escalation for 12 months, along with MET-amplification, and thus, a gefitinib-resistant cell line called PC-9MET1000 was generated[12].

The protocol presented here is a two-step dose-escalation procedure for obtaining EGFR-mutated NSCLC PC-9 cells with dual resistance to the EGFR-TKI, gefitinib, and a MET-TKI, PHA665752 (**Figure 1**). This method for establishing acquired resistant in cell lines is relatively easy to perform, with a high success rate. The described protocol can be modified for application with inhibitors of other molecular target drugs and cytotoxic agents as well.

Protocol

1. Establish Gefitinib-resistant Cells

1. **Determine the initial gefitinib concentration by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay.**
    1. Culture PC-9 cells in growth medium (RPMI-1640 medium with 10% FBS and 1% antibiotics (penicillin: 100 U/mL and streptomycin: 100 µg/mL)), in a 5% CO_2 incubator at 37 °C.
    2. Adjust the cells to 1.0 × 10^5 cells/mL using an automated cell counter (see **Table of materials**) and seed 50 µL of this in each well of a 96-well plate using the growth medium; the final concentration of cells should be 5.0 × 10^3 cells/well.
    3. Incubate the plate overnight at 37 °C.
    4. Add 50 µL of gefitinib (at 2X final concentration) at different concentrations: 0, 0.002, 0.006, 0.02, 0.06, 0.2, 0.6, 2, 6, and 20 µM to these wells such that the final volume in each well is 100 µL.
    5. Incubate the plate for 72 h at 37 °C.
    6. Add 15 µL of the dye solution (see **Table of Materials**) to each well (use final 13% concentration of the dye solution), and incubate for 4 h at 37 °C.
    7. Add 100 µL of the solubilization/stop solution (see **Table of Materials**) for solubilizing the formazan produced by the dye solution (see **Table of Materials**) in each well, and incubate it overnight at 37 °C.
    8. Measure the optical density at 570 nm (OD_{570}) using a microplate reader for obtaining the IC_{50} value by generating a cell-proliferation curve.
    9. Use a statistical software (see **Table of Materials**) to graphically display the data, and calculate the IC_{50} value (**Figure 2**).
    10. Prepare 6-12 replicates and repeat the experiments at least three times.

2. **Continuous stepwise dose-escalation of gefitinib for treatment of PC-9 cells.**
    1. Culture PC-9 cells (1 mL in sub-confluent condition) in p100 dishes with 10 mL of growth medium in a 5% CO_2 incubator at 37 °C.
    2. Add one-tenth of the IC_{50} value of gefitinib into the p100 dish.
        1. When the cells in the culture medium become sub-confluent, add 1-2 mL of cells to 10 mL of fresh growth medium using a 1-mL pipet with 10-30% higher concentration of gefitinib in the same culture conditions.
    3. Increase the gefitinib concentration progressively by 10-30% with each split until it reaches 1 µM; this might take about 6-12 months. NOTE: When the concentration of gefitinib reaches the IC_{50} value, the cell proliferation becomes considerably slower, and so, 2-4 mL of cells may be added to 10 mL of the fresh growth medium with higher concentration of gefitinib; reducing the increase in the concentration of gefitinib might solve the problem. Moreover, it would be ideal to store the remaining cells at -80 °C, and reuse them at the previous concentration. As PC-9 cells are in suspension, it is unnecessary to use trypsin for passaging; however, during the process of increasing gefitinib concentration, the cells may attach to the bottom of culture dish. In such a situation, a cell-scraper can be used for detaching the adherent cells.
    4. Culture the gefitinib-resistant PC-9 cells in the growth medium with 1 µM of gefitinib.
    5. Perform the MTT assay to confirm the gefitinib-resistance of the cells[13]; these gefitinib-resistant cells were named PC-9MET1000 (designated as MET1000 cells), because they exhibited increased protein and RNA levels of MET (**Figure 3A**, **Figure 4A**, **B**).

2. Establish Dual-resistance to Gefitinib and PHA665752

1. **Determine the initial concentration of PHA665752 by MTT assay.**
    1. Culture the MET1000 cells in p100 dishes with 10 mL of growth medium and 1 µM gefitinib in a 5% CO_2 incubator at 37 °C.
    2. Seed the cells into a 96-well plate at 5.0 × 10^3 cells/well with 50 µL of the growth medium in each well. Adjust the final concentration of the cells to 1.0 × 10^5 cells/mL using an automated cell counter (see **Table of Materials**).
    3. Incubate the plate overnight at 37 °C.
    4. Add 50 µL of PHA665752, a MET-TKI (at 2X final concentration), to the cells in different concentrations: 0, 0.002, 0.006, 0.02, 0.06, 0.2, 0.6, 2, 6, and 20 µM including 2 µM gefitinib (at 2X final concentration); the final volume in each well should be 100 µL.
    5. Incubate the plate for 72 h at 37 °C.
    6. Add 15 µL of the dye solution (see **Table of Materials**) to each well, and incubate for 4 h at 37 °C.
    7. Add 100 µL of the solubilization/stop Solution for solubilizing the formazan produced by the dye solution (see **Table of Materials**) to each well, and again incubate overnight at 37 °C.
    8. Measure the optical density at 570 nm (OD_{570}) using a microplate reader for obtaining the IC_{50} value by generating a cell-proliferation curve.

9. Use a statistical software (see **Table of Materials**) to graphically display the data, and calculate the IC_{50} value of PHA665752 in the presence of gefitinib (**Figure 3B**).
10. Prepare 6-12 replicates and repeat the experiments at least three times.

2. **Establish the dual-resistance in MET1000 cells to gefitinib and PHA665752.**
    1. Culture the MET1000 cells in p100 dishes with 10 mL of growth medium, and 1 µM gefitinib in a 5% CO_2 incubator at 37 °C.
    2. Add one-tenth of the IC_{50} value of PHA665752 into the growth medium in the presence of 1 µM of gefitinib with the same culture conditions.
    3. Increase the PHA665752 concentration progressively by 10-30% with each split until it reaches 1 µM. This might take about 6-12 months.
       NOTE: When the concentration of PHA665752 reaches the IC_{50} value, the cell proliferation becomes considerably slower, and so, 2-4 ml of cells may be added using a 1-mL pipet to 10 mL of the fresh growth medium with higher concentration of PHA665752; reducing the increase in the concentration of PHA665752 might solve the problem. Moreover, it would be ideal to store the remaining cells at -80 °C, and reuse them at the previous concentration. It is unnecessary to use trypsin for passaging; however, during the increase of PHA665752 concentration, the cells may attach to the bottom of the culture dish. In such situations, a cell-scraper can be used to detach the cells.
    4. Finally, culture the PC-9 cells resistant to both gefitinib and PHA665752 in the growth medium with 1 µM each of gefitinib and PHA665752 in a 5% CO_2 incubator at 37 °C.
    5. Perform the MTT assay to confirm that the cells are dual-resistant to gefitinib and PHA665752[13], and that they are not sensitive to PHA665752 in the presence of gefitinib; these dual-resistance cells were named PC-9DR (designated as DR cells) (**Figure 5**).

Representative Results

The overview of the protocol presented in this manuscript is shown in **Figure 1**, including the induction of acquired dual-resistance to gefitinib and PHA665752 in PC-9 cells by a 2-step dose-escalation procedure. **Figure 2** shows a decrease in the proliferation of parental PC-9 cells as the concentration of gefitinib increases. This indicates that the PC-9 cells became sensitive to gefitinib after the dose-escalation procedure was performed. **Figure 3** shows that PC-9MET1000 cells exhibit resistance to gefitinib, but are sensitive to a MET-TKI, PHA665752, in the presence of gefitinib. The PC-9 cell proliferation was suppressed at higher concentrations of gefitinib, indicating that they were sensitive to gefitinib. However, PC-9MET1000 cells did not show decreased proliferation even at high concentrations of gefitinib, indicating that they had acquired resistance to gefitinib. When PC-9MET100 cells were treated with increasing concentrations of PHA665752 in the presence or absence of gefitinib for 72 h, its proliferation, as measured by an MTT assay, was significantly decreased. There was also a modest decrease in the proliferation of PC-9MET1000 cells in the absence of gefitinib. Thus, a combination of gefitinib and PHA665752 would suppress cell proliferation more effectively than treatment with PHA665752 alone.

Figure 4 shows amplified levels of MET (both protein and RNA) in PC-9MET1000 and PC-9DR cells; no acquired mutations are present in EGFR and MET. Therefore, the emergence of bypass signaling is highly suggested as a cause of the acquired resistance to EGFR-TKI and/or MET-TKI. **Figure 5** shows the resistance of PC-9DR cells to PHA665752 in the presence of 1 µM of gefitinib, as measured by a MTT assay. Although the proliferation of PC-9MET1000 cells were inhibited by treatment with gefitinib and PHA665752, PC-9DR cells survived the treatment, indicating that they were dual-resistant to gefitinib and PHA665752. Thus, through a 2-step dose-escalation procedure, PC-9 cells acquired resistance to gefitinib and PC-9MET1000 cells acquired resistance to PHA665752 in the presence of 1 µM of gefitinib. **Figure 6** shows the anchorage-independent proliferation of MET1000 and DR cells in soft agar in the presence or absence of PHA665752 and gefitinib. The growth of both PC-9MET1000 and PC-9DR cells were observed on soft agar, indicating that these sublines could grow in both 2D and 3D systems. Thus, the resistant sublines had overcome the limitation of not being able to grow in 2D systems. Furthermore, PC-9DR cells, but not PC-9MET1000 cells, could grow in an agar with 1 µM of gefitinib and PHA665752. This result indicates that PC-9DR cells were resistant to both gefitinib and PHA665752, and could grow in a 3D culture system as well. The tumorigenic property of the established resistant sublines should be checked by a colony formation assay or by *in vivo* transplantation[14].

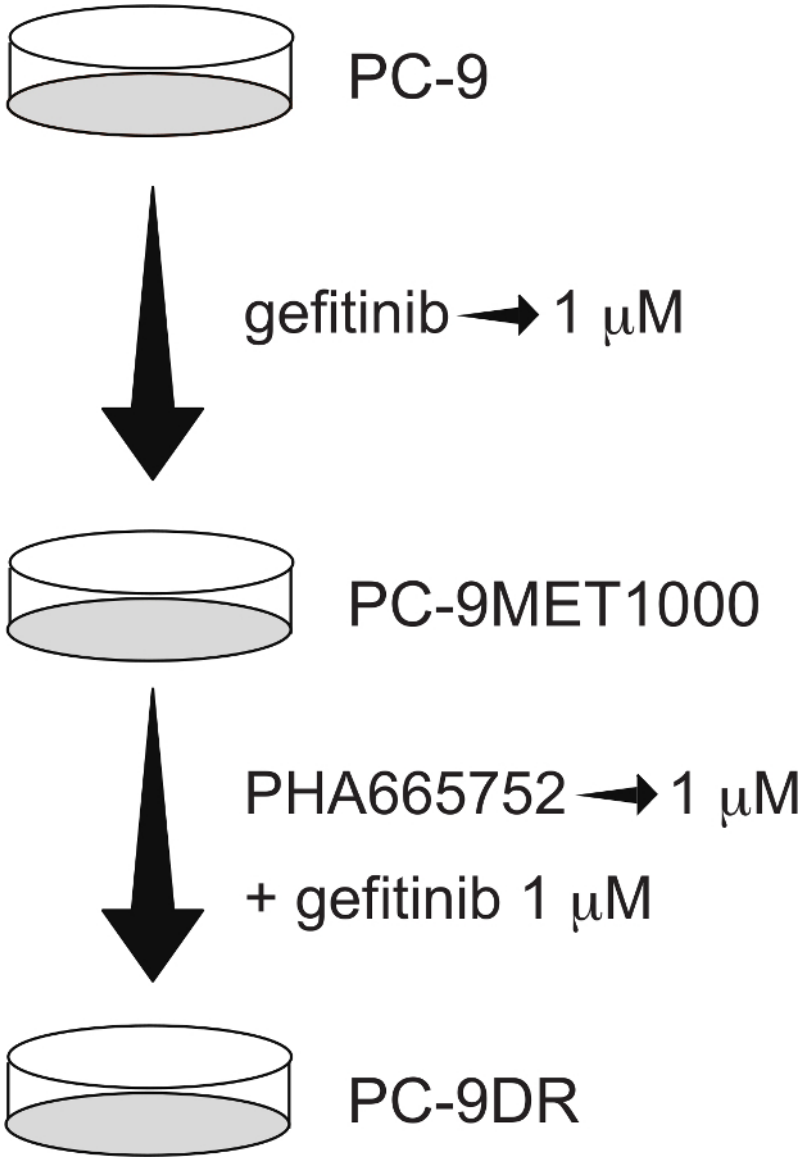


Figure 1: Schema of generating dual-resistance: PC-9DR cells from PC-9 cells.
Diagram for generating dual-resistant clones from PC-9 cells by a 2-step dose-escalation procedure. Please click here to view a larger version of this figure.

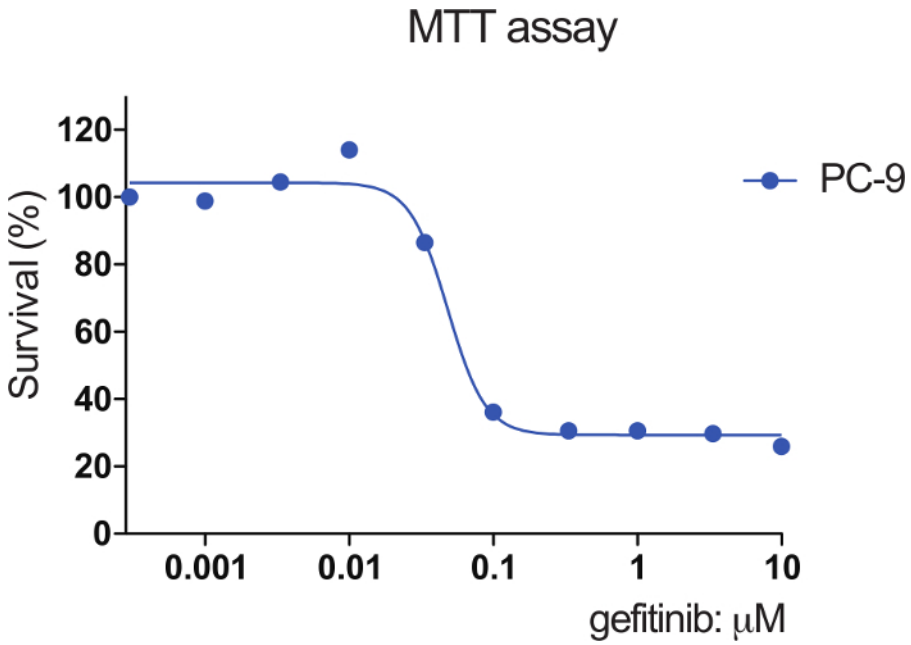


Figure 2: EGFR-TKI gefitinib inhibited the proliferation of PC-9 cells.
PC-9 cells were seeded into a 96-well plate at 5 x 10^3 cells/well with 50 µL of growth medium in each well, pre-incubated overnight, and then treated with gefitinib at the indicated concentrations for 72 h. An MTT assay was performed and the OD_{570} was measured by a microplate reader. The IC_{50} value was 0.048 ± 0.01 µM. Data are shown as the mean ± SEM for 6 wells. Please click here to view a larger version of this figure.

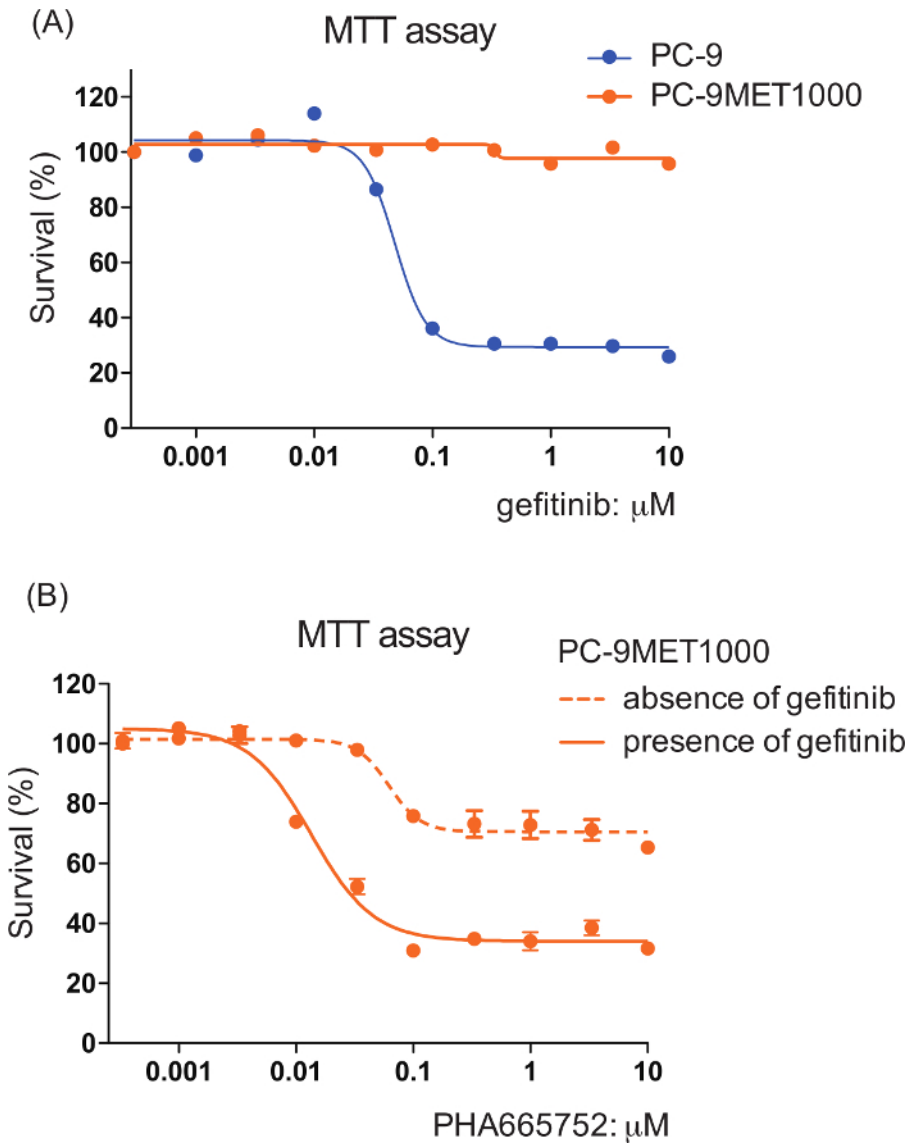
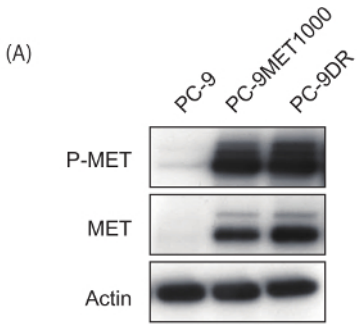
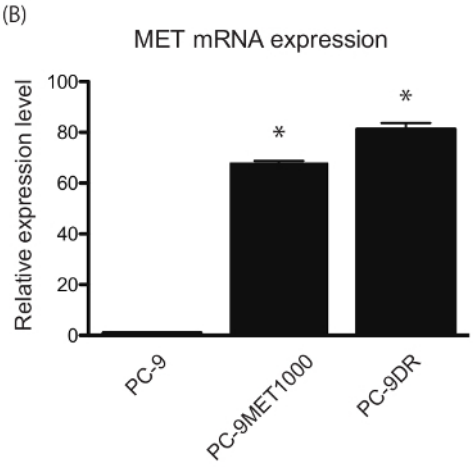


Figure 3: PC-9MET1000 cells exhibited resistance to gefitinib, but treatment with PHA665752 in the presence of 1 μM of gefitinib suppressed its proliferation.
(**A**) PC-9 and MET1000 cells were seeded into 96-well plates at 5 x 10^3 cells/well with 50 μL of growth medium in each well, pre-incubated overnight, and then treated with gefitinib at the indicated concentrations for 72 h. (**B**) MET1000 cells were seeded into a 96-well plate at 5 x 10^3 cells/well with 50 μL of the growth medium in each well, pre-incubated overnight, and then treated with PHA665752 at the indicated concentrations in the presence or absence of 1 μM of gefitinib for 72 h. An MTT assay was performed and the OD_{570} was measured by a microplate reader. The IC_{50} value in the presence of gefitinib was 0.014 ± 0.01 μM, while that in the absence of gefitinib was not determined. Data are shown as the mean ± SEM for 6 wells. Please click here to view a larger version of this figure.





(C)

	PC-9	MET1000	DR
EGFR exon 19	15bp del	15bp del	15bp del
EGFR exon 20	wt	wt	wt
EGFR exon 21	wt	wt	wt
MET exon 14 - 21	wt	wt	wt

Figure 4: MET amplification was observed in PC-9MET1000 and PC-9DR cells with no acquired mutations in EGFR and MET. (**A**) Expression levels of phosphor-MET and total MET were determined by Western blot analysis. β-actin was used as a loading control. (**B**) mRNA transcripts from PC-9, PC-9MET1000, and PC-9DR cells were quantified using real-time RT-PCR and normalized with that of GAPDH. Data are presented relative to PC-9 values, as mean ± SEM (n = 8). Statistical significance was estimated using the two-tailed Student *t*-test, and a $p < 0.05$ was considered statistically significant. *, $p < 0.05$, compared with the PC-9 value. (**C**) Exon 19-21 of the EGFR gene and exon 14-21 of the Met gene were amplified from the genomic DNA by PCR. PCR products were then purified and analyzed. Please click here to view a larger version of this figure.

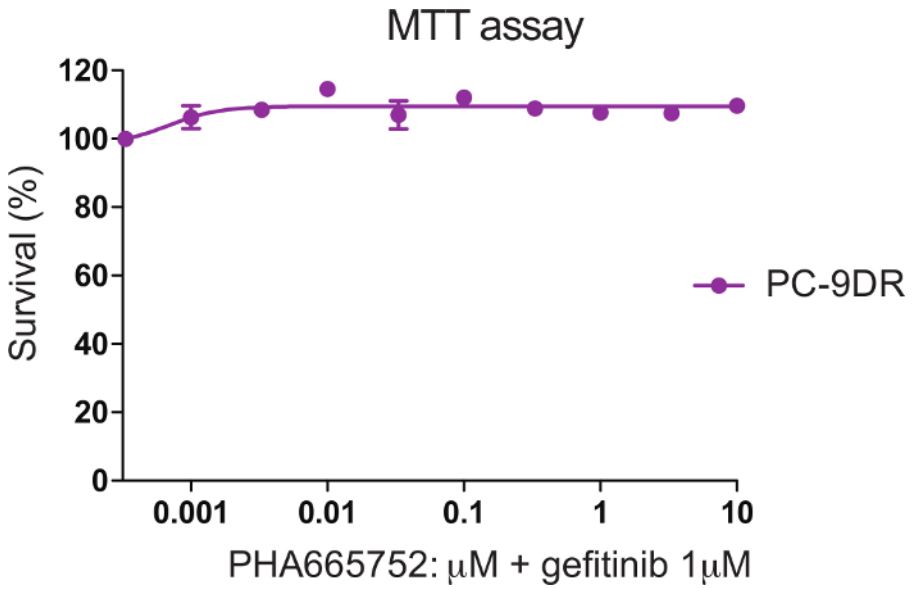


Figure 5: PC-9DR cells are resistant to PHA665752 in the presence of 1 μM of gefitinib.
DR cells were seeded into a 96-well plate at 5 x 10^3 cells/well with 50 μL of growth medium in each well, pre-incubated overnight, and then treated with PHA665752 at the indicated concentrations in the presence of 1 μM of gefitinib for 72 h. An MTT assay was performed and the OD_{570} was measured by a microplate reader. Data are shown as the mean ± SEM for 6 wells. Please click here to view a larger version of this figure.

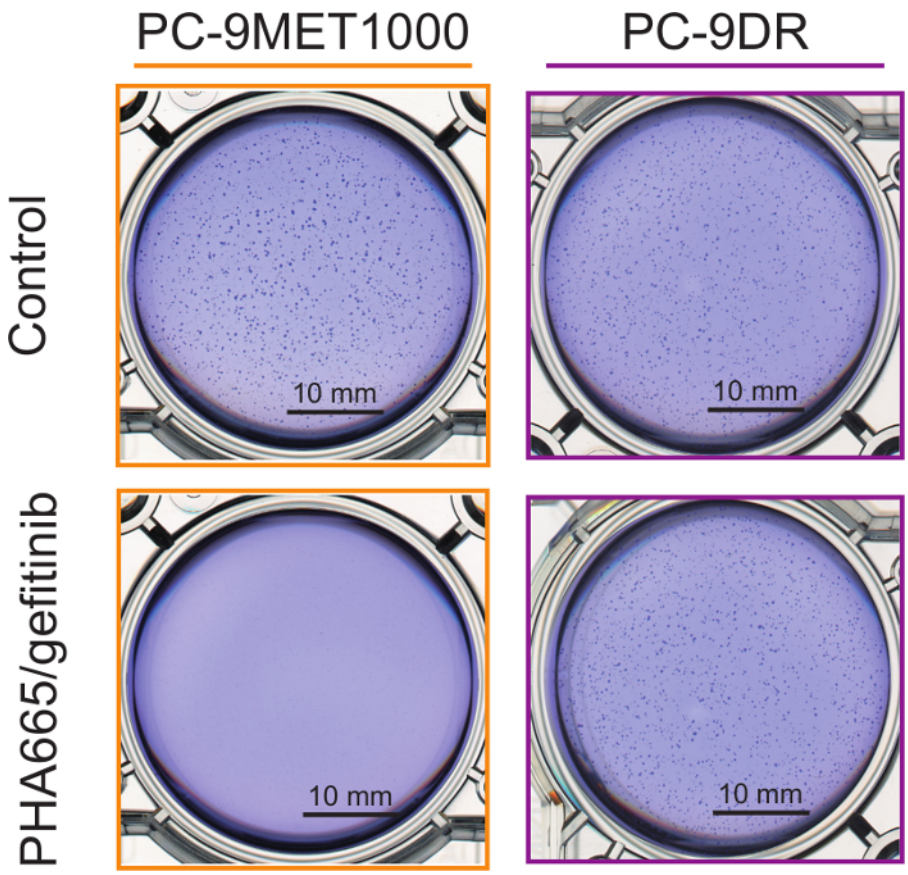


Figure 6: **Anchorage-independent proliferation of PC-9MET1000 and PC-9DR cells on soft agar in the presence or absence of PHA665752 and gefitinib.**
Cells (1 × 10^4) re-suspended in 0.3% agar with 10% FBS were seeded into 6-well plates pre-coated with 0.6% soft agar. The following day, cells were treated with gefitinib (1 μM) and PHA665752 (1 μM) and then incubated for 14-21 days. Colonies were stained with 0.005% crystal violet for 1 h. Please click here to view a larger version of this figure.

Discussion

The protocol described here can be used for the establishment of acquired dual-resistance in cancer cells using a 2-step dose-escalation method *in vitro*. Many research papers have reported that PC-9 cells developed EGFR-TKI (gefitinib or erlotinib) resistance through T790M EGFR mutations[15,16]. However, we did not detect T790M mutations in exon 20 of our PC-9MET1000 cells by direct sequencing (**Figure 4C**). Moreover, MET amplification was observed to act as a bypass signaling pathway. Thus, PC-9MET1000 cells are extremely rare. We previously reported that lung adenocarcinoma PC-9 cells harboring a 15-bp deletion in exon 19 of EGFR developed gefitinib-resistance with MET amplification after continuous stepwise dose-escalation was performed for 12 months until the dose reached 1 μM (named PC-9MET1000 cells)[17]. This cell line was sensitive to a MET-TKI in the presence of gefitinib. In order to determine the mechanism behind resistance to EGFR- and MET-TKIs, a continuous stepwise dose-escalation method was developed to induce dual-resistance to MET-TKI, PHA665752, and EGFR-TKI, gefitinib, in PC-9MET1000 cells[12]. This method relied on the results of a clinical trial to study the progression of the disease beyond gefitinib treatment. In the case of MET-amplified EGFR-TKI-resistance, a single MET inhibitor would not significantly inhibit the cell proliferation. Because the signal transduction between the receptor and the downstream molecules are separated (EGFR-ERK1/2 and MET-AKT), the combined suppression of EGFR and MET would be required. In another lung adenocarcinoma cell line, HCC827, which also harbors an EGFR mutation, EGFR-TKI resistance was developed through MET amplification. Suda *et al.* reported that HCC827 cells exposed to increasing concentrations of erlotinib in the presence of MET-TKI PHA665752 developed resistance to MET-TKI and EGFR-TKI[18]. Furthermore, clinical trials are being conducted to study cases of MET-amplification with EGFR-TKI-resistance. Thus, to investigate the mechanism of acquired MET-TKI resistance after treatment with EGFR-TKI, it is necessary to induce dual-resistance to MET inhibitors and EGFR inhibitors.

The protocol described here involved stepwise dose-escalation of a MET-TKI, PHA665752, in the presence of 1 μM of gefitinib. The stepwise dose-escalation method is the most reliable and the most commonly used method[18,19]. The most critical step in this protocol is obtaining the IC_{50} value of the inhibitor. If this value is not accurately determined, the cells will fail to grow at all in the culture dish. Therefore, if the cell growth begins to slow, reducing the increase in the concentration of the inhibitor might solve the problem. Moreover, if the cells begin dying, it would be ideal to store the remaining cells at -80 °C, to reuse them at the previous concentration.

An alternate method for establishing resistance in cell lines is the high-concentration exposure method, in which the cells are exposed to the target concentration of the inhibitor directly, and the cells are cultured until the surviving resistant cells start proliferating. Although this method is more clinically relevant, it has a lower success rate because the target concentration of the inhibitor, which is administrated initially, might kill all the parental cells immediately. Interestingly, it has been reported that resistant sublines obtained through these two methods exhibit different cellular properties[19,20].

Another method for establishing resistance is the *in vivo* resistance method, in which a cell suspension (1.0×10^6-2.0×10^7) is injected into the flanks of mice, and when the tumor reaches a volume of 200 mm^3, the mice are subjected to continuous treatment with inhibitors. Tumors that respond completely and then recur during maintained therapy are harvested, minced, digested with trypsin, and then filtered and seeded onto culture dishes. This method, although somewhat complicated, can be used effectively in cases of antibody-resistance. However, when the tumors that show resistance in the *in vivo* system are transferred to an *in vitro* system, the cells sometimes fail to grow 2D culture systems. Therefore, 3D culture systems such as a basement membrane matrix should be used[21].

Although all three of these methods require a relatively long time to perform, the stepwise dose-escalation method is the most economical one. It is also the most suitable method for clearly understanding and studying the process of acquiring inhibitor-resistance. In addition, the success rate of the other two methods is significantly lower than that of the stepwise dose-escalation method.

Once the resistance is established, it is necessary to test the tumorigenic potency of these cell lines. Colony formation tests or *in vivo* transplantations are frequently employed for this purpose (**Figure 6**). These methods might overcome the disadvantage of using 2D culture systems.

A major limitation of these methods is that cells with acquired resistance often do not accurately reflect the resistance in human cancer cells. Therefore, the mechanisms discovered through studies in *in vitro* models should be confirmed in human tissue samples through repeated tests in biopsy samples. In addition, although these protocols could be useful for examining the mechanism behind acquired resistance during the development of tumor cells, it is possible that the acquired resistance was induced by alterations of the tumor microenvironment. This is another serious limitation of investigating the efficacy of these protocols in only cancer cells. It would be better to use tissues from human tissue banks, which are available for many researchers.

The protocol presented here can be modified for establishing resistance to other molecular inhibitors as well. Therefore, this method is potentially useful for developing therapeutic tools to control cancer in the future.

Disclosures

The authors have no conflicts of interest to disclose.

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