# 博士学位論文

## クロルプロマジンはEGFR変異肺癌細胞株に対してチロシンキナーゼ阻害薬によるアポトーシスを増強しT790Mを有する耐性株のゲフィチニブに対する感受性を回復させる

近畿大学大学院
医学研究科医学系専攻

**藤原　亮介**

Doctoral Dissertation

# Chlorpromazine cooperatively induces apoptosis with tyrosine kinase inhibitors in EGFR-mutated lung cancer cell lines and restores the sensitivity to gefitinib in T790M-harboring resistant cells

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Major in Medical Sciences
Kindai University Graduate School of Medical Sciences

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課博

# 同　意　書

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# Chlorpromazine cooperatively induces apoptosis with tyrosine kinase inhibitors in EGFR-mutated lung cancer cell lines and restores the sensitivity to gefitinib in T790M-harboring resistant cells

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ABSTRACT

We previously reported that the antipsychotic drug chlorpromazine (CPZ), which inhibits the formation of clathrin-coated vesicles (CCVs) essential for endocytosis and intracellular transport of receptor tyrosine kinase (RTK), inhibits the growth/survival of acute myeloid leukemia cells with mutated RTK (KIT D816V or FLT3-ITD) by perturbing the intracellular localization of these molecules. Here, we examined whether these findings are applicable to epidermal growth factor receptor (EGFR). CPZ dose-dependently inhibited the growth/survival of the non-small cell lung cancer (NSCLC) cell line, PC9 harboring an EGFR-activating (EGFR exon 19 deletion). In addition, CPZ not only suppressed the growth/survival of gefitinib (GEF)-resistant PC9ZD cells harboring T790 M, but also restored their sensitivities to GEF. Furthermore, CPZ overcame GEF resistance caused by Met amplification in HCC827GR cells. As for the mechanism of CPZ-induced growth suppression, we found that although CPZ hardly influenced the phosphorylation of EGFR, it effectively reduced the phosphorylation of ERK and AKT. When utilized in combination with trametinib (a MEK inhibitor), dabrafenib (an RAF inhibitor), and everolimus (an mTOR inhibitor), CPZ suppressed the growth of PC9ZD cells cooperatively with everolimus but not with trametinib or dabrafenib. Immunofluorescent staining revealed that EGFR shows a perinuclear pattern and was intensely colocalized with the late endosome marker, Rab11. However, after CPZ treatment, EGFR was unevenly distributed in the cells, and colocalization with the early endosome marker Rab5 and EEA1 became more apparent, indicating that CPZ disrupted the intracellular transport of EGFR. These results suggest that CPZ has therapeutic potential for NSCLC with mutated EGFR by a novel mechanism different from conventional TKIs alone or in combination with other agents.



## 1. Introduction

Tyrosine kinases (TKs) are classified into two families. One is receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR) (Type 1), insulin receptor (Type 2), c-KIT, and FMS-like tyrosine kinase 3 (FLT3) (Type 3), and so on [1,2]. The other is cytoplasmic tyrosine kinases including Src, Tec, and Jak families. Both families play essential roles in various cellular phenomena including cell development, growth, differentiation, mobility, and cell death[2]. In addition, constitutive activation of TK by somatic gene mutation or chromosomal translocation is critically involved in the pathogenesis and the progression of various types of malignancies such as lung cancer, breast cancer, and leukemia as driver mutations [3,4].

From the crucial roles of these mutations in the regulation of growth and survival of cancer cells, mutated TKs have been considered to be good therapeutic targets. Indeed, a number of tyrosine kinase inhibitors (TKIs) have been developed and are being widely utilized in clinical practice [2,4–6]. In the case of non-small cell lung cancer (NSCLC), malignant cells harboring constitutively

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active EGFR mutations (L858R, exon 9 deletion, etc.) are sensitive to EGFR TKIs such as 1st generation gefitinib (GEF) [7,8] and 2nd generation erlotinib [9]. Although most patients initially respond to those TKIs, many patients subsequently acquire resistance, which can occur via various mechanisms, such as MET amplification, overexpression of hepatocyte growth factor (HGF), and activating mutations in the downstream molecules such as BRAF and Ras [10–12]. However, the most common mechanism is the emergence of secondary EGFR mutations [13]. Especially, the T790 M mutation in EGFR, also known as the "gatekeeper mutation", is detected in about half of the patients with acquired resistance to EGFR TKIs [14,15]. To overcome T790 M mutation, 3rd generation TKIs, Osimertinib (OSIM) was developed and has shown to be effective for patients with T790 M [16,17]. However, most of the patients eventually became resistant to OSIM [18], requiring new therapeutic options.

Clathrin assembly lymphoid myeloid leukemia protein (CALM) is an essential component of clathrin-coated vesicles (CCVs) and regulates endocytosis of ligand-bound RTKs and subsequent intracellular transport from early to late endosomes as a cargo [19,20]. We previously reported that CALM knockdown severely impaired the growth of acute myeloid cells (AML) cells with activating mutation in FMS-like tyrosine kinase 3 (FLT3) (FLT3-internal tandem Duplication [ITD]) or KIT (KIT D816V), both of which are causative mutations and poor prognostic factors for AML [21]. Consistent with these findings, the antipsychotic drug chlorpromazine (CPZ), which inhibits CCV formation, severely suppressed the growth of AML cells with KIT D816V or FLT3-ITD, while its inhibitory effects on AML cells without these mutations were marginal. As for this mechanism, we found that CPZ reduced CALM protein expression and perturbed the intracellular localization of FLT3-ITD and KIT D816V, thereby blocking their compartment-dependent signals [21,22].

In this study, we examined whether our findings from FLT3-ITD and KIT D816V were applicable to another RTK, EGFR. As a result, we found that CPZ inhibited the growth/survival of NSCLC cells harboring activating mutations of EGFR. Furthermore, we found that CPZ not only suppressed the growth/survival of GEF-resistant PC9ZD with T790 M but also restored their sensitivity to GEF. These results suggest that CPZ may have therapeutic utility NSCLC, especially in patients harboring activating mutations of EGFR.

## 2. Materials and methods

### 2.1. Cells and reagents

The human lung adenocarcinoma cell lines PC9, HCC827, and EGFR TKI-resistant human lung adenocarcinoma cell lines PC9ZD and HCC827GR55 were established in 2005 [23] and 2007 [24], respectively and kindly provided by Prof. Okamoto I. (Kyushu University, Fukuoka, Japan). Cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% Fetal calf serum (FCS). Chlorpromazine (CPZ) was purchased from Sigma-Aldrich (St. Louis, MO). GEF, OSIM, trametinib, dabrafenib, and everolimus were purchased from Selleck Chemicals (Houston, TX).

### 2.2. Evaluation of cell growth

For proliferation assays, cells were seeded into RPMI 1640 medium with 10% FCS at an appropriate cell density (5–50/μl) and cultured with or without TKIs and CPZ at various concentrations at 37 °C for the indicated times. Cell viability was measured with the Cell Titer Glo Reagent (Promega, Madison, WI) according to the manufacturer's recommendation using an Envision plate reader (Wallac, 1420 ARVO MX-2, Turku, Finland).

### 2.3. Annexin V detection

Cells were cultured with or without TKIs and/or CPZ for 72 h. Then, cells were harvested, washed, and stained with a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Apoptosis cell death was analyzed by Flow cytometry using BD FACSCanto II (BD Biosciences).

### 2.4. Cell cycle analysis

To identify cell cycle status of cultured cells, cells ($1 \times 10^3$/μl) were fixation with 70% ice cold ethanol and stained with 100 ng/ml Propidium Iodide (BD Biosciences) with RNase (Merck, Darmstadt, Germany) for 15 min at room temperature. Cell cycle was analyzed by Flow cytometry using BD FACSCanto II (BD Biosciences).

### 2.5. Immunoblot analysis

Cells were cultured with CPZ for 36 h. After washing with PBS, cells were lysed with RIPA buffer (Nacalai Tesque, Kyoto, Japan) containing Phosphatase Inhibitor Cocktail (Nacalai Tesque) and insoluble materials were removed by centrifugation. Cell lysates (15 μg per lane) were subjected to SDS-PAGE with PAGEL (Atto, Tokyo, Japan) and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). After incubating with TBST blocking buffer (4% nonfat dry milk in Tris-buffered saline-Tween 20, 0.15 M NaCl, 0.01 M Tris-HCl pH 7.4, 0.05% Tween 20), immunoblotting was performed with the appropriate antibodies (Abs). Primary Abs against EGFR (#2232), phosphorylated EGFR [phosphor- (p-)Tyr1068; #3777], Erk (#9102), p-Erk (Tyr202/Tyr204; #4376), Akt (#4691), p-Akt (Ser473; #4058), and GAPDH (#5174) were purchased from Cell Signaling Technology (Danvers, MA). Secondary Ab, horseradish peroxidase-conjugated anti-Rabbit IgG was purchased Promega (W4011). The immune complex was visualized by an enhance chemiluminescent kit (LAS4010, GE healthcare, Cleveland, OH).

### 2.6. Immunofluorescence analysis

After culturing in Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific) for 24 h, cells were further incubated with or without 5.0 μM CPZ for 36 h. Then, cells were washed with PBS and fixed with 4% formalin for 15 min. After washing, cells were incubated with PBS containing 0.1% (v/v) Triton X-100 and 1% (v/v) bovine serum albumin and reacted with the primary Ab and appropriate secondary Ab (each for 45 min). Primary Abs against EGFR (#2232), EEA1(#3288), Rab5 (#3547), Rab7(#9367), and Rab11(#5589) were purchased from Cell Signaling Technology. Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) Fluoromount-G (Southern Biotech, Birmingham, USA). After washing with PBS, the coverslips were mounted on glass slides and observed under a BZ-X710 All-in-One fluorescence microscope (Keyence Corp, Osaka, Japan). Each image showed single sections with a 60 × oil immersion objective, adjusted to give the same x, y, and z position in all channels.

### 2.7. Statistical analyses

All statistical analyses were performed with the EZR software (Saitama Medical Center, Jichi Medical University) [25]. At least three independent cell samples were included for statistical analysis. Results were presented as means ± SEM. Data were analyzed

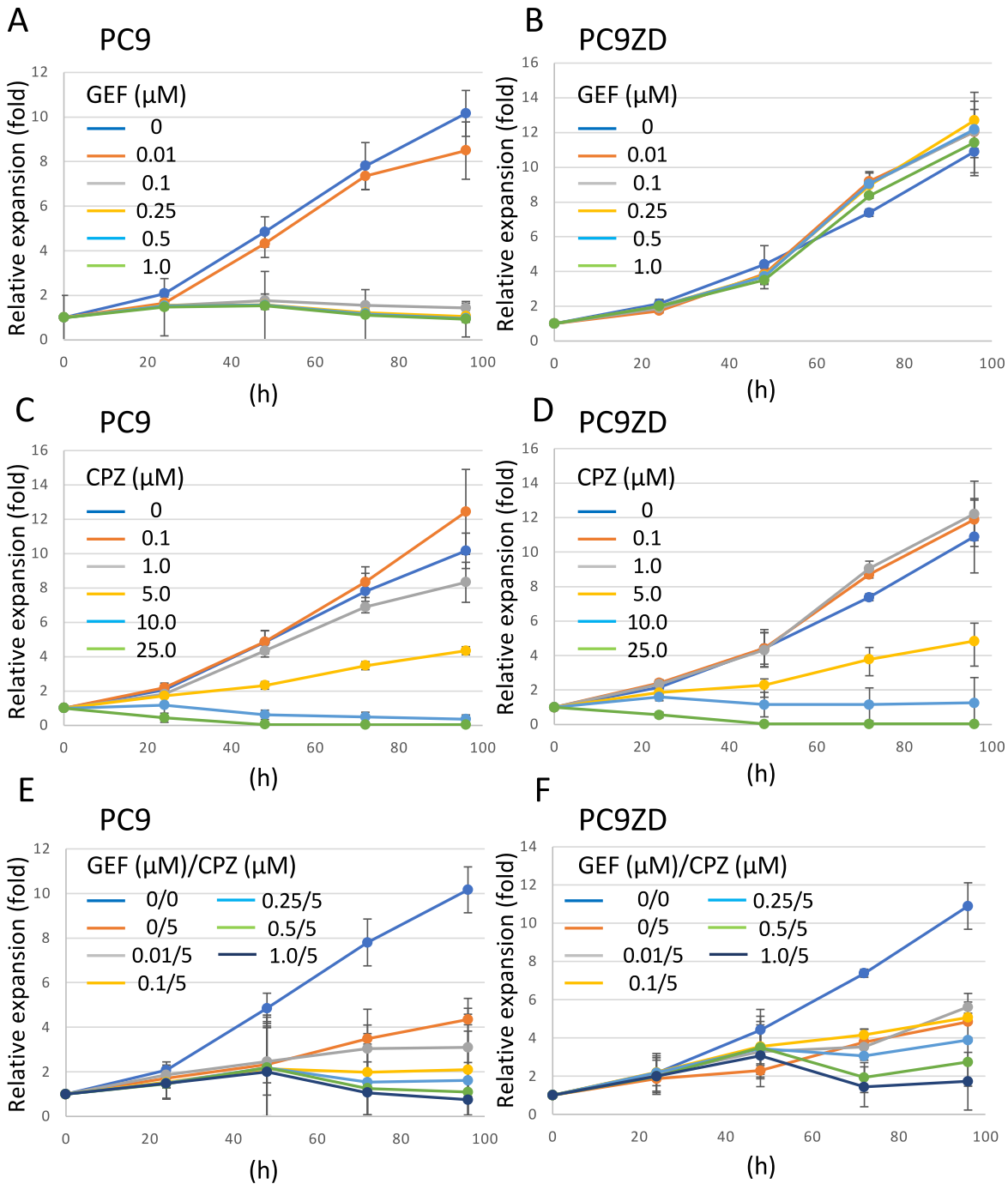


**Fig. 1.** CPZ inhibited the growth/survival of PC9 and PC9ZD cells and sensitized PC9ZD cells to GEF. **A, B.** PC9 (**A, C, E**) and PC9ZD (**B, D, F**) cells were cultured with GEF alone, CPZ alone, or their combination as indicated, and their growth was evaluated with an ATP assay. The relative proliferation at indicated points was shown as the value of day 0 as 1. The aata shown are the mean ± SEM from three independent experiments. **G-I.** PC9 and PC9ZD cells were cultured with GEF alone, CPZ alone, or their combination for 72 h. Then, apoptotic cells were detected as Annexin V-positive cells. Dot plots are representative of three independent experiments. The results obtained from the indicated cells are shown as the mean ± SEM from three independent experiments. Two-sided unpaired Student's t-test, *p < 0.05, **p < 0.01.

and compared with a One-way analysis of variance (ANOVA) with Dunnett's post-hoc test, repeated-measures ANOVA, and Welch's ANOVA. Statistical significance was accepted when the P value was <0.05. The FlowJo software package (version 10.7.1, Ashland, Oregon, USA) was used for flow cytometry analysis.

## 3. Results

### *3.1. CPZ alone inhibited the growth of PC9 and PC9ZD cells and restored the sensitivity to GEF in PC9ZD cells*

PC9 with EGFR exon 19 deletion is sensitive to GEF. PC9ZD was generated from PC9, which harbors EGFR T790 M gatekeeper

mutation and is resistant to GEF. At first, we examined the effects of GEF on the growth of both cell lines. Consistent with the previous reports [23], GEF suppressed the growth of PC9 at concentrations ≥0.1 μM (Fig. 1A), while PC9ZD was resistant to GEF at concentrations up to 1 μM (Fig. 1B). Next, we evaluated the effects of CPZ on these cell lines. As shown in "Figure" 1C and 1D, CPZ inhibited the growth of PC9 and PC9ZD in a dose-dependent manner from 0.1 to 25 μM, with no apparent difference in the inhibitory effects on both cell lines. Since the IC50 of CPZ at PC9 and PC9ZD was 5 μM, we next cultured PC9 and PC9ZD with 5 μM CPZ and various concentrations of GEF. The growth of PC9 was suppressed by 5 μM CPZ, and GEF dose-dependently augmented this growth inhibition (Fig. 1E, Suppl. "Figure" 1). Of note, while PC9ZD cells were resistant to GEF alone ("Fig. 1B), their growth was dose-dependently inhibited by GEF in the presence of 5 μM CPZ (Fig. 1F, Suppl. "Figure" 2).

### 3.2. CPZ induced apoptosis in PC9 and PC9ZD cells alone and in combination with GEF

Next, we evaluated whether CPZ induced apoptosis in PC9 and PC9ZD in combination with GEF. After 3-day cultures of PC9 cells, both 0.01 μM, 0. 1 μM GEF and 5 μM CPZ increased Annexin V-positive apoptotic cells to 12.8%, 52.4% and 34.1%, respectively, compared with the control (CTL) culture (2.1%) (Fig. 1G, 1I). In addition, the combination of 5 μM CPZ with 0.01 μM and 0. 1 μM GEF further increased the apoptotic fraction to 58.5% and 64.0%, respectively.

In PC9ZD cells, 0.5 μM GEF alone hardly increased the proportion of Annexin V-positive cells compared with the CTL culture (CTL *vs*. GEF: 12.2% vs 11.4%, Fig. 1H). On the other hand, 5 μM CPZ increased the apoptotic cells to 35.3% (Fig. 1H, 1I). Moreover, the addition of 0.5 μM GEF to cells treated with 5 μM CPZ further increased the apoptotic fraction to 69.1%. These results were almost

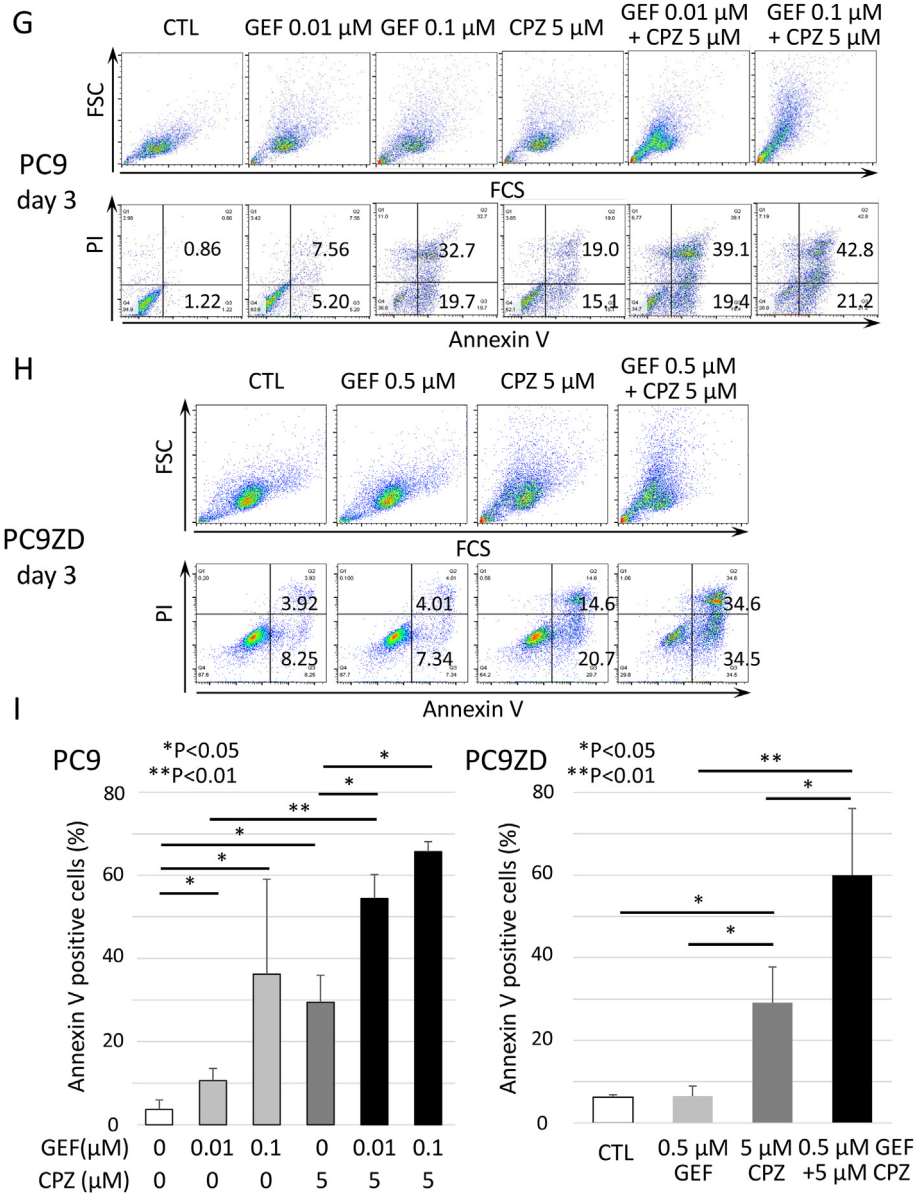


**Fig. 1.** (*continued*).

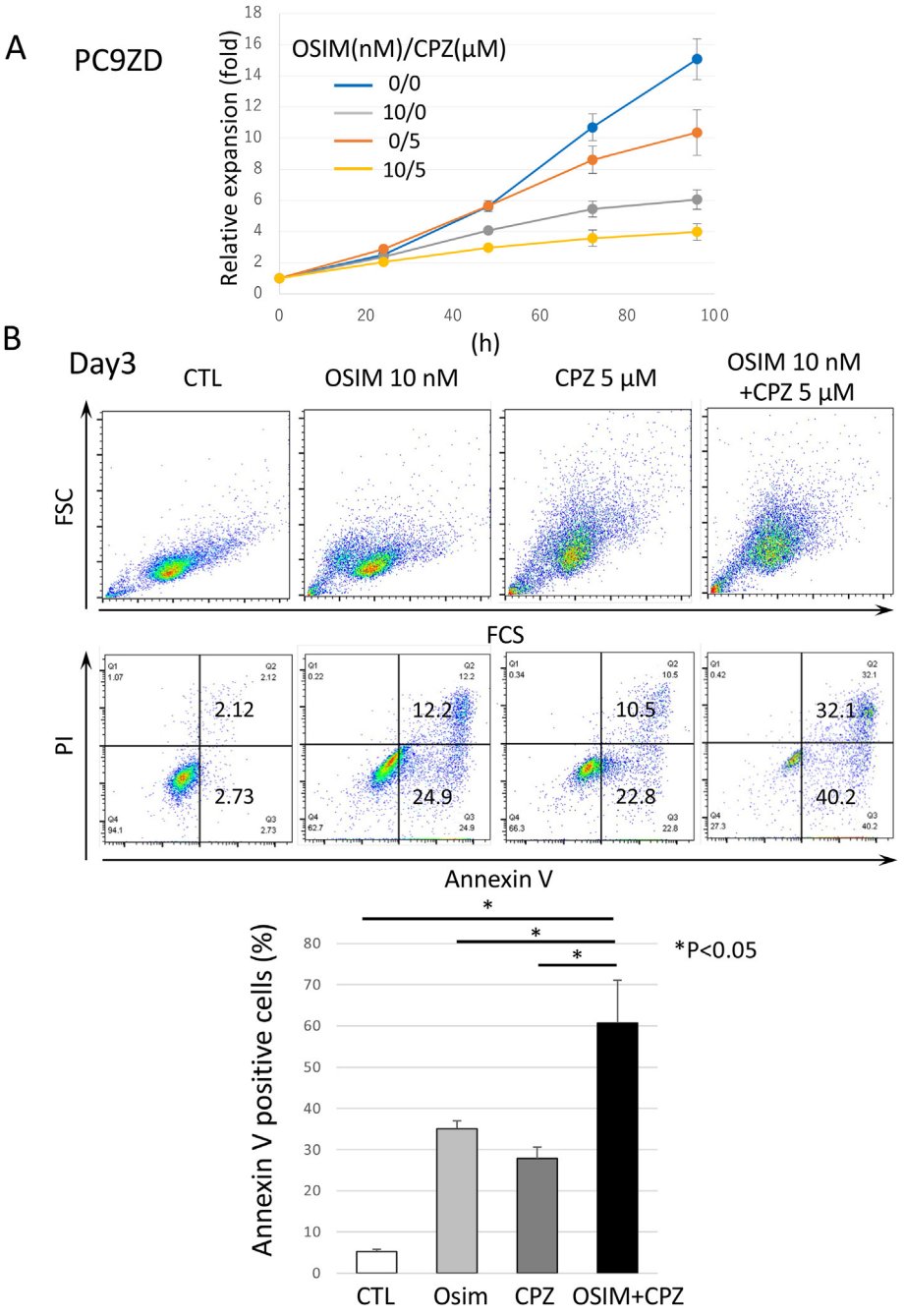


**Fig. 2.** CPZ and OSIM cooperatively induced apoptosis in PC9ZD cells and overcame GEF resistance caused by Met amplification in HCC827GR5 cells..
**A.** PC9ZD cells were cultured with 10 nM OSIM alone, 5 μM CPZ alone, or their combination as indicated, and their growth was evaluated with an ATP assay. The relative proliferation at indicated points was shown as the value of day 0 as 1. The data shown are the mean ± SEM from three independent experiments. **B.** After 72 h treatment with OSIM alone, CPZ alone, or their combination, apoptotic cells were detected as Annexin V-positive cells. Dot plots are representative of three independent experiments. The results obtained from the indicated cells are shown as the mean ± SEM from three independent experiments. Two-sided unpaired Student's t-test, *p < 0.05. **C, D.** Parental HCC827 cells harboring exon 9 deletion (**A**) and HCC827GR5 cells with Met amplification (**B**) were cultured with 0.5 μM GEF alone, 5 μM CPZ alone, and their combination as indicated, and their growth was evaluated with an ATP assay. The relative proliferation at indicated points was shown as the value of day 0 as 1. The data shown are the mean ± SEM from three independent experiments. Two-sided unpaired Student's t-test, *p < 0.05, **p < 0.01.

consistent with the results observed in Fig. 1.

Cell cycle analyses showed that, although GEF induced G1 arrest and yielded sub-G1 apoptotic fraction in PC9, it scarcely influenced the cell cycle status in PC9ZD. Meanwhile, CPZ alone and in combination with GEF induced apoptosis and increased the cell fraction in the G2/M phase (Suppl. "Figure" 3).

### *3.3. CPZ induced apoptosis of PC9ZD alone and in combination with OSIM*

OSIM is a third-generation EGFR TKI that can overcome the gate keeper mutation T790 M. We conducted similar experiments using CPZ and OSIM. In contrast to GEF, 10 nM OSIM effectively inhibited

the growth of PC9ZD, which was enhanced by the addition of 5 μM CPZ (Fig. 2A). After 3-day cultures, both OSIM and CPM induced apoptosis in about 30% of cells, which was further increased to about 60% by their combination, suggesting that CPZ may inhibit the growth of PC9ZD cells independently of OSIM (Fig. 2B).

### 3.4. CPZ overcame GEF-resistance caused by met amplification in HCC827GR5 cells

We next evaluated whether CPZ could overcome TKI-resistant mechanisms other than gatekeeper mutations, using HCC827GR5 with exon 9 deletion (del E746-A750) and Met amplification. As observed in PC9 cells, both GEF and CPZ efficiently suppressed the growth of parental HCC827 cells, and their combination was more effective that when using each drugs separately (Fig. 2C). In accord with a previous report [24], GEF scarcely inhibited the growth of HCC827GR5 cells, while CPZ inhibited their growth substantially (Fig. 2D). However, in contrast to the result observed in PC9ZD, the addition of GEF didn't enhance the inhibitory effects of CPZ in PC9ZD. Thus, it was supposed that GEF and CPZ would act differently depending on the resistant mechanism and/or cellular context.

### 3.5. CPZ inhibited phosphorylation of ERK and AKT without affecting EGFR phosphorylation

To clarify the mechanism underlying growth inhibitory effects of CPZ on PC9ZD, we examined whether EGFR signaling was affected by CPZ treatment with Western blotting analyses. We compared the phosphorylation status of ERK and PI3K/AKT, both of which were located downstream of EGFR and closely related to tumor growth and survival, before and after CPZ treatment. After 36-h treatment, CPZ didn't affect the amounts of EGFR, ERK, or AKT ("Figure" 3). In this culture condition, up to 10 μM of CPZ hardly influenced the phosphorylation status of EGFR. However, CPZ effectively reduced the phosphorylation status of ERK and AKT at concentrations ≥5 μM.

### 3.6. CPZ and mTOR inhibitor cooperatively suppressed the growth/survival of PC9ZD cells

Next, we explored the effects of trametinib (a MEK inhibitor), dabrafenib (an RAF inhibitor), and everolimus (an mTOR inhibitor) alone or in combination with CPZ on the growth of PC9ZD cells. As shown in Fig. 3B, 3C, and 3D, 10 nM trametinib, 100 nM dabrafenib, and 10 nM everolimus each suppressed the growth of PC9ZD cells by about 30–45%. Also, the addition of CPZ significantly augmented the effect of everolimus, while the additive effects on trametinib and dabrafenib were only marginal.

### 3.7. CPZ perturbed the intracellular localization of EGFR

Next, we analyzed the effect of CPZ on EGFR localization in PC9ZD by immunofluorescent staining. To analyze the cytoplasmic localization of EGFR, we used early endosome antigen-1 (EEA1) and Rab5 as markers for early endosomes, and Rab7, and Rab11 as markers for late, and recycling endosomes, respectively. Without CPZ treatment, EGFR revealed a perinuclear pattern and was colocalized with EEA1, Rab5 and Rab7, and more intensely with Rab11 (Fig. 4A). In contrast, after 36-h treatment with CPZ, the perinuclear pattern was disturbed and EGFR was unevenly distributed in the cells. Also, colocalization with EEA1, and Rab5 became more visually apparent ("Figure" 4B). These results indicated that CPZ treatment impaired EGFR transport from early endosome to other organelles.

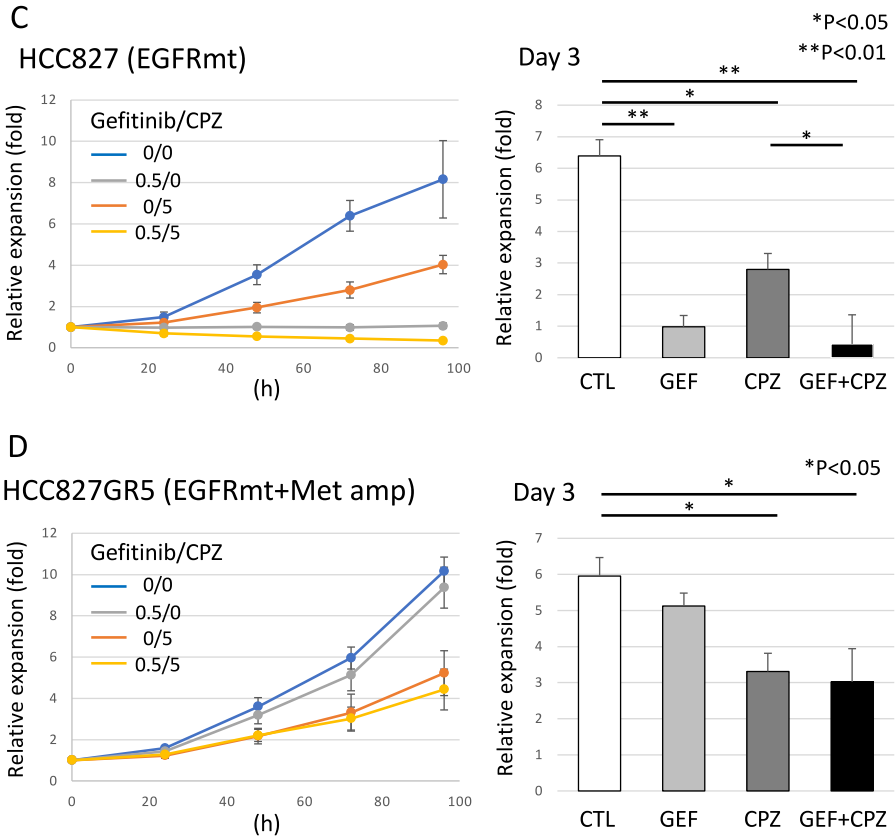


**Fig. 2.** (*continued*).

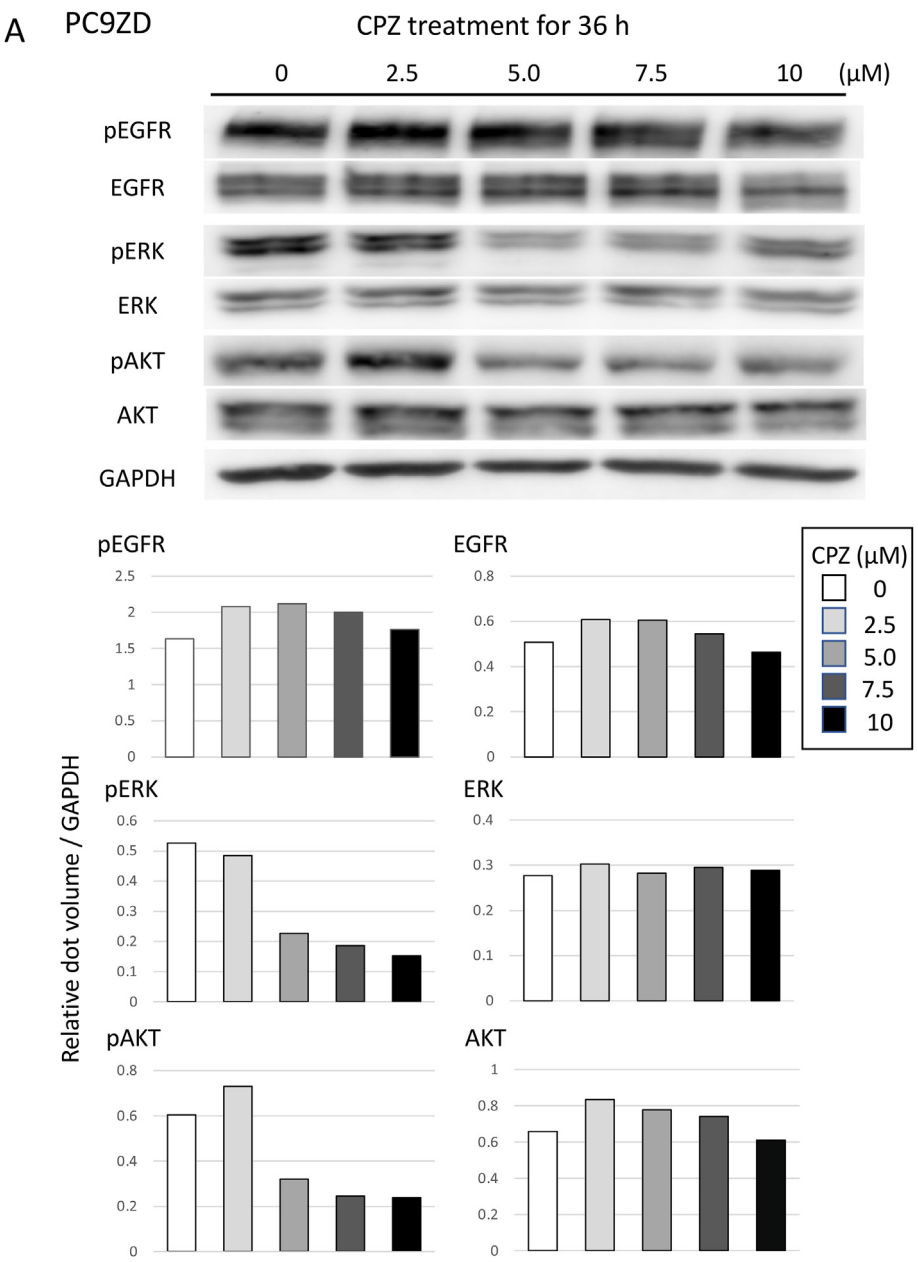


**Fig. 3.** CPZ inhibited phosphorylation of ERK and AKT without affecting EGFR phosphorylation in PC9ZD cells.
**A.** PC9ZD cells were cultured with various concentrations of CPZ for 36 h and cellular lysates were isolated. Whole-cell lysates were subjected to immunoblot analyses with the indicated Abs. Densitometry analyses were carried out using an Image Quant TL with data from two independent experiments. Each dot volume is shown in comparison to that of the endogenous control GAPDH. **B-D.** PC9ZD cells were cultured with 10 nM Trametinib (Tram, a MEK inhibitor) alone, 100 nM Dabrafenib (Dab, an RAF inhibitor) alone, 10 nM Everolimus (Ever, an mTOR inhibitor) alone, or in combination with 5 μM CPZ, and their growth was evaluated with an ATP assay. The relative proliferation at indicated points was shown as the value of day 0 as 1. The data shown are the mean ± SEM from three independent experiments. Two-sided unpaired Student's t-test, *$p < 0.05$.

## 4. Discussion

Using knockdown experiments, we previously showed that CALM plays a crucial role in the growth/survival of AML cells harboring mutant (MT) RTK (FLT3-ITD or KIT D816V) by regulating their intracellular trafficking [21,22,26]. Also, we found that CPZ severely inhibited the growth/survival of AML cells with FLT3-ITD or KIT D816V *in vitro* and *in vivo*, while it showed only marginal inhibitory effects on AML cells without these mutations. As for this mechanism, we found that CPZ reduced CALM protein at the post-transcriptional level and perturbed the intracellular localization of MT RTKs, thereby blocking their compartment-dependent signals to downstream molecules, STAT5 and Akt. These results raised a possibility that CPZ may be effective to induce cell death not only in leukemia cells but also in other malignant cells with MT RTKs through a novel mechanism different from conventional TKIs.

Upon ligand-binding, WT EGFR undergoes endocytosis via clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE), and are transported from early endosomes to either recycling endosomes or lysosomes for degradation [6,27]. Although EGFR signaling is supposed to occur mainly at the plasma membrane, activated MT EGFRs transmit oncogenic signals from

endosomes [28,29]. In the current study, we examined the effects of CPZ on MT EGFR with exon 19 deletion using several NSCLC cell lines. As a result, we found that CPZ inhibited the growth/survival of PC9 cells. Also, consistent with the observed in cells harboring FLT3-ITD and KIT D816V mutations [21], the intracellular localization of MT EGFR was perturbed by CPZ treatment. However, it should be noted that the mode of action of CPZ on EGFR would be rather different from that observed in FLT3-ITD or KIT D816V. That is, although CPZ treatment dose-dependently suppressed tyrosine phosphorylation of FLT3-ITD and KIT D816V in leukemia cell lines MV4-11 and HMC-1, respectively [21], tyrosine phosphorylation of EGFR was scarcely affected by CPZ treatment in PC9ZD. Nonetheless, CPZ suppressed the phosphorylation of downstream ERK and Akt in PC9ZD cells as observed in MV4-11 and HMC-1 cells. These results suggest that CPZ would act on MT RTKs differently according to the types of RTKs and/or cellular context.

As expected, CPZ suppressed the growth/survival of gefitinib-resistant PC9ZD harboring T790 M mutation and HCC827GR5 cells with Met amplification. In addition, we here found that CPZ restored the sensitivity to gefitinib in PC9ZD cells. This result is largely consistent with a recent paper reporting that responses to GEF were reversed by a CME inhibitor, phenylarsine oxide (PAO), in GEF-resistant H358 and Celu-3 cells [30]. Interestingly, tyrosine phosphorylation of EGFR was not affected by PAO in these cells, resembling CPZ-treated PC9ZD cells. Because CPZ also blocks CMEs, the inhibitory effects on CME may be involved in the anti-cancer effects of CPZ as well as the inhibition of intracellular trafficking.

Although we here analyzed the growth/survival inhibitory effects of CPZ from the aspect of the cellular transport of MT EGFR, CPZ has been reported to reveal cytotoxic activities through several mechanisms such as induction of autophagy [31–34], inhibition of sirtuin 1 [35], upregulation of PRB [36], and induction of S1PR2 [37] in various cancer cells [38]. So, these mechanisms might also participate in the growth inhibitory effects of CPZ observed in this study. So, to utilize CPZ as the anti-MT RTK drug optimally, further analyses are required to clarify the molecular effects of CPZ in future studies.

We here found that CPZ inhibited ERK and Akt activities in PC9ZD cells. To determine which drug would be the most appropriate partner of CPZ in treating NSCLC, we tried several agents

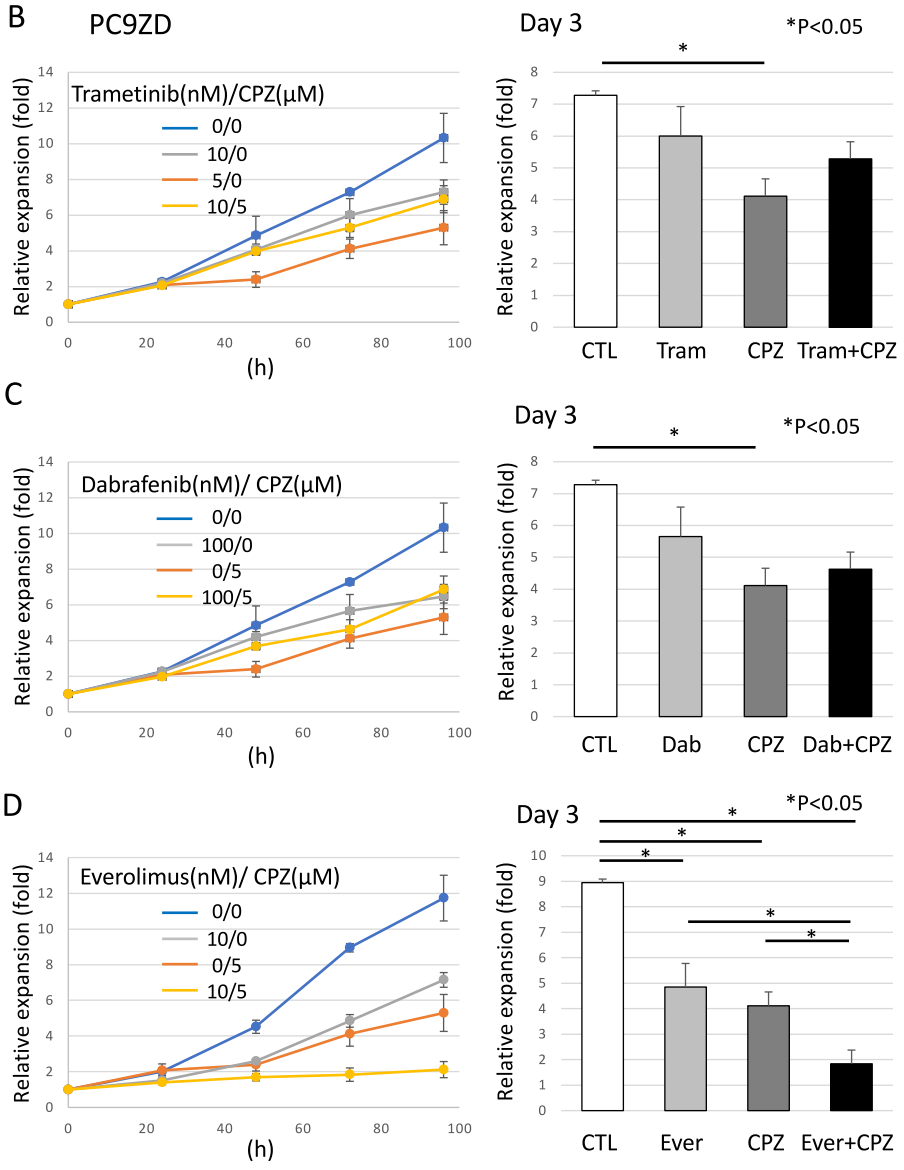


**Fig. 3.** (*continued*).

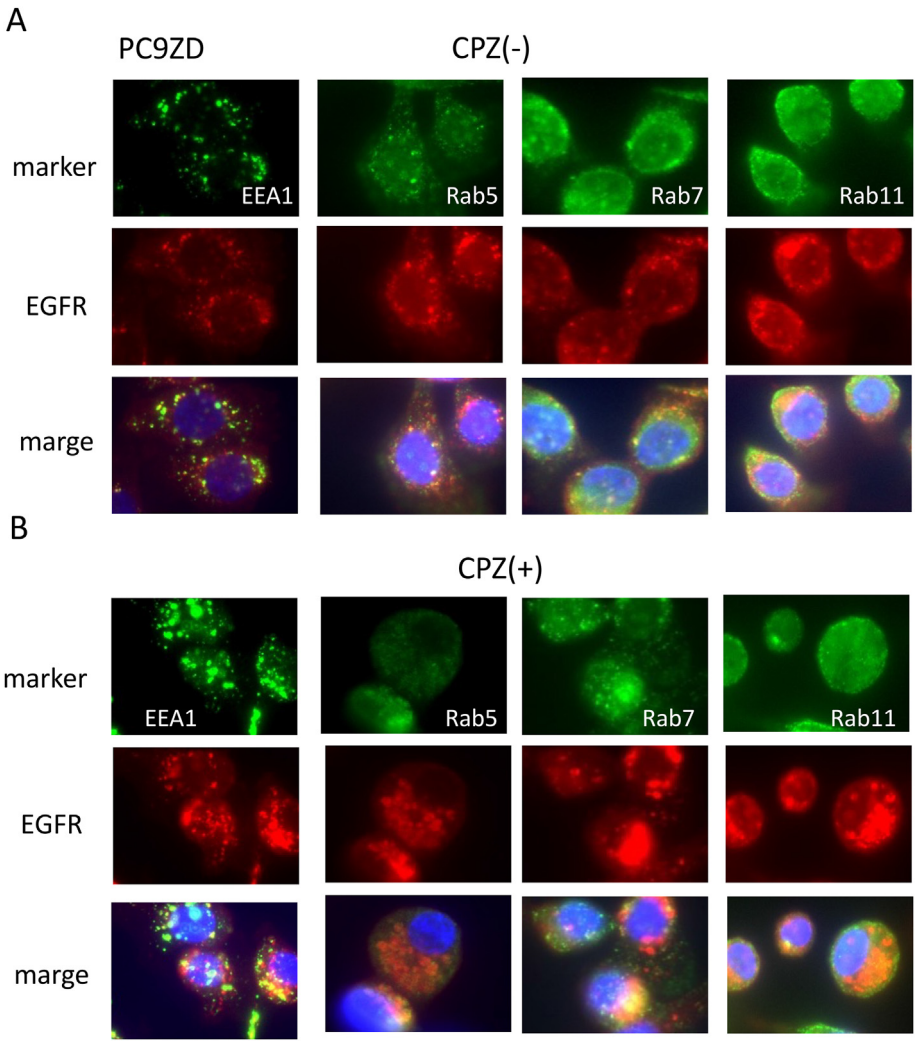


**Fig. 4.** CPZ impaired intracellular trafficking of EGFR from early endosome in PC9ZD cells.
**A. B.** Intracellular localization of EGFR in PC9ZD cells was examined by immunofluorescence analyses with the anti-EGFR Ab after 36-h culture with or without CPZ. EEA1 and Rab5 were used as markers of early endosomes. Rab7 and Rab11 were used as markers of late and recycling endosomes, respectively. Arrows indicate regions of colocalization (Inset shows the region of higher magnification).

alone or in combination with CPZ. As a result, a MEK inhibitor (trametinib), a Raf inhibitor (dabrafenib), and an Akt inhibitor (everolimus) each suppressed the growth of PC9ZD cells by about 30–45%. Also, CPZ significantly augmented the effect of everolimus, while the additive effects on trametinib and dabrafenib were only marginal. These results were consistent with the previous paper indicating the importance of the PI3K/Akt pathway in NSCLC cells with MT EGFR [39,40] and would be useful to explore the possibility of the combinational therapy using CPZ and everolimus.

## 5. Conclusions

In conclusion, we here found that CPZ was effective for NSCLC with MT EGFR (exon 9 deletion). CPZ not only suppressed the growth/survival of GEF-resistant PC9ZD cells with T390 M, but it also restored the sensitivity of these cells to GEF. Although some aspects to the molecular basis of how CPZ acts on NSCLC cells remains to be more precisely clarified, CPZ emerges as a promising agent to treat NSCLC patients with MT EGFR through a novel mechanism different from conventional TKIs.

## Authors contributions

H.T. and I.M. designed and supervised research.
F.R., Y.T., S.R, Y.I., A.F., K.F., T.K., K.S., and H.T. analyzed the data.
F.R., Y.T., and H.T. performed statistical analysis.
F.R., Y.T., R.S., L.E., and H.T. prepared Figures and tables.
F.R., Y.T., H.T., L.E., and I.M. wrote the manuscript.
Y.Y, and H.H. revised the manuscript.

## Ethics statement

- Approval of the research protocol by an Institutional Reviewer Board. N/A.
- Informed Consent. N/A.
- Registry and the Registration No. of the study/trial. N/A.
- Animal Studies. N/A.

## Declaration of competing interest

S.R. received personal fees from Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Bristol-Myers Squibb (Celgene), Janssen

Pharmaceutical K.K, Takeda Pharmaceutical Company Limited., and Amgen BioPharma K.K., and Mundipharma K.K.

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The remaining authors declare no competing financial interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.08.010.

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