博 士 学 位 論 文

T790M 陽性 EGFR-TKI 耐性肺癌細胞株における第三世代 EGFR-TKI と Src 阻害薬ダサチニブの併用効果の検討

近 畿 大 学 大 学 院
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Doctoral Dissertation

T790M-selective EGFR-TKI combined with dasatinib as an optimal strategy for overcoming EGFR-TKI resistance in T790M-positive non-small cell lung cancer

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論文題目

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T790M-selective EGFR-TKI combined with dasatinib as an optimal strategy for overcoming EGFR-TKI resistance in T790M-positive non-small cell lung cancer

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Abstract

T790M mutation-selective epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have demonstrated clinical benefits in non-small cell lung cancer (NSCLC) patients harboring T790M mutation, which is the major cause of resistance to EGFR-TKI. However, their efficacy is limited, possibly due to the emergence of apoptosis resistance in T790M-positive NSCLC. We previously identified Src family kinases as co-oncogenic drivers along with T790M and found that the Src inhibitor dasatinib combined with an irreversible or a preclinical T790M-selective EGFR-TKI enhanced anti-tumor activity in T790M-positive cells. In the current study, we evaluated the efficacy of dasatinib combined with the clinically relevant T790M-selective EGFR-TKI or osimertinib in *EGFR* mutation-positive NSCLC with or without T790M mutation. A cell viability assay revealed that dasatinib had synergistic effects with these TKIs in T790M-positive cells, and simultaneously inhibited Src, Akt, and Erk, which remained activated upon single-agent treatment. Dasatinib also increased the rate of apoptosis in T790M-positive cells induced by T790M-selective EGFR-TKIs, as determined by the annexin-V binding assay; this was associated with downregulation of the anti-apoptotic Bcl-2 family member Bcl-xL, a finding that was confirmed in mice bearing T790M-positive xenografts. Our results suggest that Bcl-xL plays a key role in the apoptosis resistance of T790M-positive NSCLC, and that dasatinib combined with clinically relevant T790M-selective EGFR-TKIs is potentially effective in overcoming resistance to first-generation EGFR-TKIs in NSCLC patients with acquired T790M.

Key words : dasatinib, osimertinib, ASP8273, epidermal growth factor receptor (EGFR), non－small cell lung cancer (NSCLC)

Introduction

Treatment for advanced non-small cell lung cancer (NSCLC) depends on the molecular characteristics of the tumor [1]. Mutations in the gene encoding epidermal growth factor receptor (*EGFR*) are present in ～32% of Asians and ～7% of individuals of other ethnicities with NSCLC, with deletions in exon 19 and an L858R point mutation in exon 21 accounting for ～90% of genetic alterations detected at diagnosis [2]. NSCLC tumors harboring *EGFR* mutations including those mentioned above are oncogene addicted and therefore sensitive to treatment with EGFR-tyrosine kinase inhibitors (TKIs).

Despite initially responding to EGFR-TKIs, most patients acquire resistance to these agents within 1–2 years [3-5], which is associated with secondary mutations in *EGFR*; the most common of these is the substitution of methionine for threonine at position 790 (T790M), which is detected in approximately 50% of patients with acquired resistance to EGFR-TKIs [6-9]. T790M-selective or third-generation EGFR-TKIs −including osimertinib (AZD9291) and ASP8273 [10]−have been developed to overcome T790M-related resistance. Osimertinib was approved by the U.S. Food and Drug Administration for use in NSCLC patients harboring a T790M mutation whose disease progressed during treatment with other EGFR inhibitors, whereas ASP8273 is currently in clinical trials to evaluate the efficacy in patients with T790M-positive *EGFR*-mutated NSCLC.

Despite the improvement in progression-free survival (PFS) demonstrated by osimertinib compared to a combination of pemetrexed and platinum-based chemotherapy in patients with *EGFR* T790M, not all patients benefit from this treatment, with most developing resistance within approximately 10 months [11]. Further study is needed to optimize the treatment for T790M-positive NSCLC and improve patient survival.

We previously reported that Src family kinases (SFKs) act as co-drivers of resistance along with T790M and that the Src inhibitor dasatinib enhances the anti-tumor activity of the pan-EGFR-TKI afatinib or the T790M-selective inhibitor WZ4002 [12]. Given that WZ4002 is an agent only used in preclinical models, in the present study we investigated the efficacy of clinically relevant T790M-selective EGFR-TKIs in combination with dasatinib in T790M-positive *EGFR*-mutated NSCLC.

Materials and Methods

Cells and reagents

The human PC9 NSCLC cell line was provided from the Tokyo Medical University (Tokyo, Japan) in 1997. The PC9GR cell line was previously generated in our institution [12]. The human H1975 NSCLC cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) in 2009. Cells were maintained under a humidified atmosphere of 5% CO_2/95% air at 37℃ in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum (FBS). The cells were routinely tested for mycoplasma using MycoAlert (LT07; Lonza, Basel, Switzerland) and were negative. Erlotinib, dasatinib, and osimertinib were purchased from Chemietek (Indianapolis, IN, USA). Bosutinib (SKI-606) and navitoclax (ABT-263) [13] were purchased

from Selleck Chemicals (Houston, TX, USA). ASP8273 was provided by Astellas Pharma (Tokyo, Japan) thorough a material transfer agreement.

Cell viability assay

Cells were transferred to 96-well flat-bottomed plates and cultured overnight before exposure to various concentrations of erlotinib, ASP8273, osimertinib, and dasatinib in medium containing 5% FBS for 72 h. Cell Counting Kit-8 solution (CK04; Dojindo, Kumamoto, Japan) was added to each well, and cells were incubated for 3 h at 37℃ before measuring absorbance with a Multiskan Spectrum instrument (Thermo Fisher Scientific, Waltham, MA, USA) ; values are expressed as a percentage of the absorbance of untreated cells. The combination index (CI) was calculated using CalcuSyn v.2.1 software (Biosoft, Cambridge, UK) ; values < 1, $= 1$, and > 1 indicated synergism, additive effect, and antagonism, respectively.

Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with 1× Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) composed of 20 mmol/l of Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA (disodium salt), 1 mmol/l EGTA, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 μg/ml leupepsin, and 1 mmol/l phenylmethylsulfonyl fluoride. The protein concentration of lysates was determined with a bicinchoninic acid assay kit (Thermo Fisher Scientific), and equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel for analysis of intracellular signaling, or on a 12% gel for analysis of cell apoptosis (Bio-Rad, Hercules, CA, USA). Separated proteins were transferred to a nitrocellulose membrane that was incubated with Blocking One or (for phosphorylated proteins) Blocking One-P solution (both from Nakalai Tesque, Kyoto, Japan) for 20 min at room temperature before overnight incubation at 4℃ with primary antibodies against EGFR (#4405), phosphorylated EGFR [phospho (p-) Tyr1068; #2234], Src (#2108), p-Src (Tyr416; #2101), Akt (#9272), p-Akt (Ser473; #9271), Erk (#9102), cleaved poly (ADP-ribose) polymerase (PARP; #5625), Bcl-xL (#2762), and β-tubulin (#2128), which were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody against p-Erk (Thr202/Tyr204; sc-16982) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the antibody against β-actin (#10021) was from Sigma-Aldrich (St. Louis, MO, USA). The membrane was washed with PBS containing 0.05% Tween 20 before incubation for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (NA934; GE Healthcare, Indianapolis, IL, USA). Immune complexes were detected with enhanced chemiluminescence reagent (RPN3244; GE Healthcare).

Annexin-V binding assay

The binding of annexin-V to cells was measured using the Annexin-V-FLUOS Staining kit (#11858777001;

Roche, Basel, Switzerland). Cells were exposed to Accumax (#AM-105; Innovative Cell Technologies, San Diego, CA, USA), washed with PBS, and harvested by centrifugation at 200 × g for 5 min. Cell pellets were resuspended in 100 μl Annexin-V-FLUOS labeling solution, incubated for 10－15 min at 15℃－25℃, and then analyzed for fluorescence using a BD FACSCanto II system and BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA).

Mouse xenograft studies

Animal procedures were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations of Laboratory Animal Experiments, Kindai University. The study protocol was reviewed and approved by the Animal Ethics Committee of Kindai University. H1975 cells (5×10^6 per mouse) were subcutaneously injected into the flank of 7-week-old female athymic nude mice (BALB/cAJcl-nu/nu) obtained from CLEA Japan (Tokyo, Japan). The mice were divided into four treatment groups; treatments were initiated when tumors in each group achieved an average volume of 100–200 mm^3. Mice were treated over 3 weeks by daily oral gavage of vehicle, osimertinib (1 mg/kg), dasatinib (50 mg/kg), or osimertinib (1 mg/kg) + dasatinib (50 mg/kg); a 0.5% (wt/vol) aqueous solution of hydroxypropyl methyl cellulose and 50% propylene glycol were used as vehicles for osimertinib and dasatinib, respectively; the vehicle in the control group consisted of 50 μl each of 0.5% hydroxypropyl methylcellulose and 50% propylene glycol. The control group consisted for four mice and treatment groups of six mice each. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula $LW^2/2$. Tumor size and body weight were measured twice weekly. Mice were sacrificed at the end of the treatment period, and tumor tissue was flash frozen at －80℃ for immunoblot analysis.

Statistical analysis

Quantitative data are presented as mean ± SE unless otherwise indicated. The significance of differences in the annexin-V binding assay was evaluated with the Wilcoxon rank-sum test using GraphPad Prism v.7 software (GraphPad Inc., La Jolla, CA, USA). The repeated-measures model generated with STATA14 (StataCorp, College Station, TX, USA) was used to evaluate differences between groups in the *in vivo* study. A P value < 0.05 was considered statistically significant.

Results

Dasatinib enhances the decrease in cell viability induced by T790M-selective EGFR-TKIs in T790M-positive NSCLC cells

PC9 human NSCLC cells harbor an *EGFR* exon 19 deletion, which is sensitive to the first-generation EGFR-TKIs gefitinib and erlotinib. PC9GR cell line generated from PC9 cells harbors the T790M mutation and is

resistant to gefitinib and erlotinib [12]. H1975 is a *de novo* erlotinib-resistant cell line with L858R and T790M mutations. Consistent with previous reports, we confirmed that erlotinib decreased the viability of PC9 cells (Fig. 1A), whereas PC9GR and H1975 were resistant to erlotinib treatment (Fig. 1B, C). As expected, the T790M-selective EGFR-TKIs ASP8273 and osimertinib reduced PC9GR and H1975 cell viability (Fig. 1B, C, E, F). However, half-maximal inhibitory concentrations (IC50) for ASP8273 and osimertinib in these cells were approximately 2-fold higher than those in PC9 cells, suggesting that there is room for improvement in the treatment of T790M-positive NSCLC. We previously showed that dasatinib increased anti-tumor efficacy in combination with a pan-EGFR-TKI afatinib, or the T790M-selective EGFR-TKI WZ4002, which has only been used in preclinical models. Given these findings, we examined the efficacy of combined dasatinib and the novel T790M-selective EGFR-TKI ASP8273 or osimertinib in T790M-positive PC9GR or H1975 cells (Fig. 1B, C, E, F) [12]. In agreement with our previous study, we observed increased sensitivity to the combination of dasatinib and ASP8273 or osimertinib as compared to ASP8273 or osimertinib as single agents. In contrast, in T790M-negative PC9 cells, the anti-tumor effects of dasatinib were unaltered when administered in combination with ASP8273 or osimertinib.

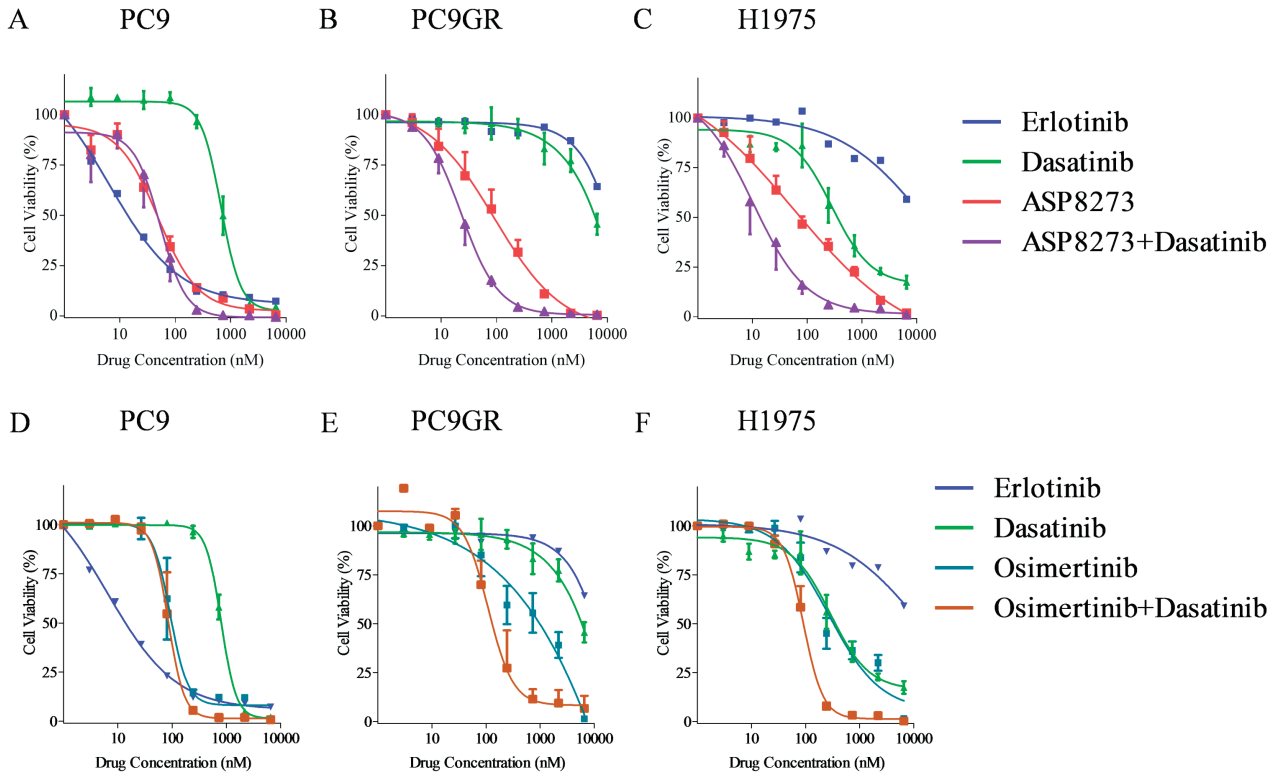


Figure 1. Evaluation of the efficacy of dasatinib combined with T790M-selective EGFR-TKIs in T790M-negative or -positive cells. (A, D) PC9 cells (ex19del), (B, E) PC9GR cells (ex19del+T790M), and (C, F) H1975 cells (L858R+T790M) were incubated for 72 h with increasing concentrations of erlotinib, ASP8273, osimertinib, dasatinib, and a combination of dasatinib and ASP8273 or osimertinib; cell viability was then assessed with Cell Counting Kit-8. Each point represents mean ± standard error of three independent experiments.

To evaluate the combined effects of dasatinib and osimertinib, CI values were calculated from the results of the cell viability assay (Table 1). All CI values in both PC9GR and H1975 cells were < 1, suggesting a synergistic effect between dasatinib and T790M-selective EGFR-TKIs. Since the 1:1 ratio of osimertinib and dasatinib showed the lowest CI value among the various ratios tested, this was used in subsequent experiments.

Table 1. Combination indices for each combination ratio*

	PC9GR			H1975		
Combination ratio of	Combination index at					
osimertinib (μM): dasatinib (μM)	ED50	ED75	ED90	ED50	ED75	ED90
1:5	0.49	0.48	0.48	0.45	0.49	0.54
1:1	0.32	0.36	0.41	0.44	0.46	0.47
5:1	0.43	0.52	0.64	0.88	0.76	0.66

*Values were calculated for each combination ratio; those < 1, = 1, and > 1 indicate synergism, additive effects, and antagonism, respectively.
ED = effective dose.

Dasatinib combined with T790M-selective EGFR-TKIs inhibits Src, Akt, and Erk activation

To clarify the anti-tumor mechanism of dasatinib in combination with ASP8273 or osimertinib, we examined the effects of these combinations on signaling pathways in PC9GR and H1975 cells (Fig. 2A–D). In both cell lines, erlotinib had a partial effect on the activation of Akt and Erk, which act downstream of EGFR, whereas ASP8273 or osimertinib caused an apparent reduction in p-Akt and p-Erk levels. Src activity was inhibited by dasatinib alone but not by either ASP8273 or osimertinib alone. Simultaneous inhibition of p-Src, p-Akt, and p-Erk was only achieved using a combination of dasatinib and ASP8273 or osimertinib.

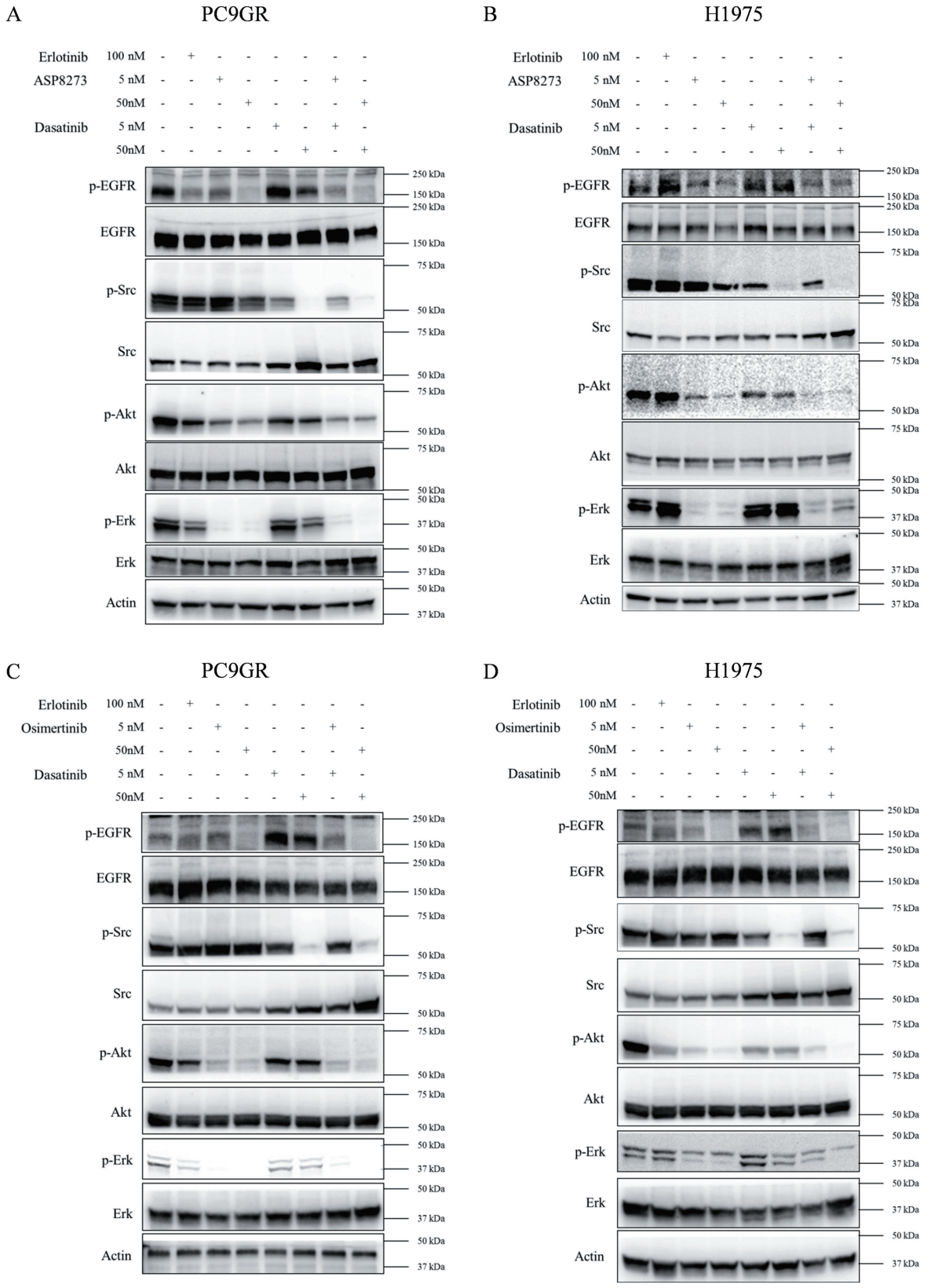


Figure 2. Effects of dasatinib and T790M-selective EGFR-TKIs on intracellular signaling in T790M-positive NSCLC cell lines. PC9GR and H1975 cells were incubated for 6 h in the absence or presence of (A, B) erlotinib (100 nM), ASP8273 (5 nM, or 50 nM), and dasatinib (5 nM, or 50 nM) ; or (C, D) erlotinib (100 nM), osimertinib (5 nM, or 50 nM), and dasatinib (5 nM, or 50 nM). Cell lysates were analyzed for expression of p-EGFR, EGFR, p-Src, Src, p-Akt, Akt, p-Erk, and Erk, with β-actin serving as a loading control.

Dasatinib enhances apoptosis induced by T790M-selective EGFR-TKIs in T790M-positive NSCLC cells

We investigated whether the combination of dasatinib and T790M-selective EGFR-TKIs could induce apoptosis in PC9GR and H1975 cells (Fig. 3A–D). The results of the annexin-V binding assay revealed that the rate of apoptosis was lower in cells treated with erlotinib or dasatinib as compared to ASP8273 or osimertinib alone. Although the number of annexin-V-positive apoptotic cells was higher upon combined treatment with dasatinib and erlotinib as compared to either agent alone, it was nonetheless lower than the rate of apoptosis of cells treated with ASP8273 or osimertinib alone. Dasatinib in combination with ASP8273 or osimertinib further increased the number of annexin-V-positive apoptotic cells as compared to either one of the T790M-selective EGFR-TKIs ($P < 0.05$), indicating that Src antagonism in T790M-positive NSCLC cells treated with ASP8273 or osimertinib potently induces apoptosis. Since dasatinib is a multi-kinase inhibitor that also inhibits Abl and c-Kit, we examined the effects of the Src inhibitor bosutinib in combination with osimertinib on apoptosis with the annexin-V binding assay (Fig. 3E, F). As expected, bosutinib in combination with osimertinib increased the number of annexin-V-positive apoptotic cells in PC9GR and H1975 cells, indicating that Src inhibition increases the antitumor activity of T790M-selective EGFR-TKI in T790M-positive cells.

Effects of combined dasatinib and T790M-selective EGFR-TKI treatment on apoptosis-related proteins in T790M-positive NSCLC cells

Given that dasatinib combined with ASP8273 or osimertinib induced apoptosis of T790M-positive cells, we examined the expression of apoptosis-related proteins by immunoblotting (Fig. 4A, B). The level of cleaved PARP, a marker of apoptosis, was increased in PC9GR and H1975 cells treated with dasatinib combined with osimertinib. Single-agent treatment induced Bcl-xL degradation, whereas Bcl-xL inhibition was enhanced by combining the two agents. To investigate if enhanced apoptosis was induced by inhibition of Bcl-xL, we evaluated the combination of navitoclax (ABT-263), an inhibitor of Bcl-xL and Bcl-2, and osimertinib in PC9GR and H1975 cells (Fig. 4C, D). The number of annexin-V-positive apoptotic cells was exacerbated by the combination of osimertinib and navitoclax in T790M-positive cells. This result supports the idea that inhibition of Bcl-xL is the cause of enhancement of apoptosis in T790M-positive cells.

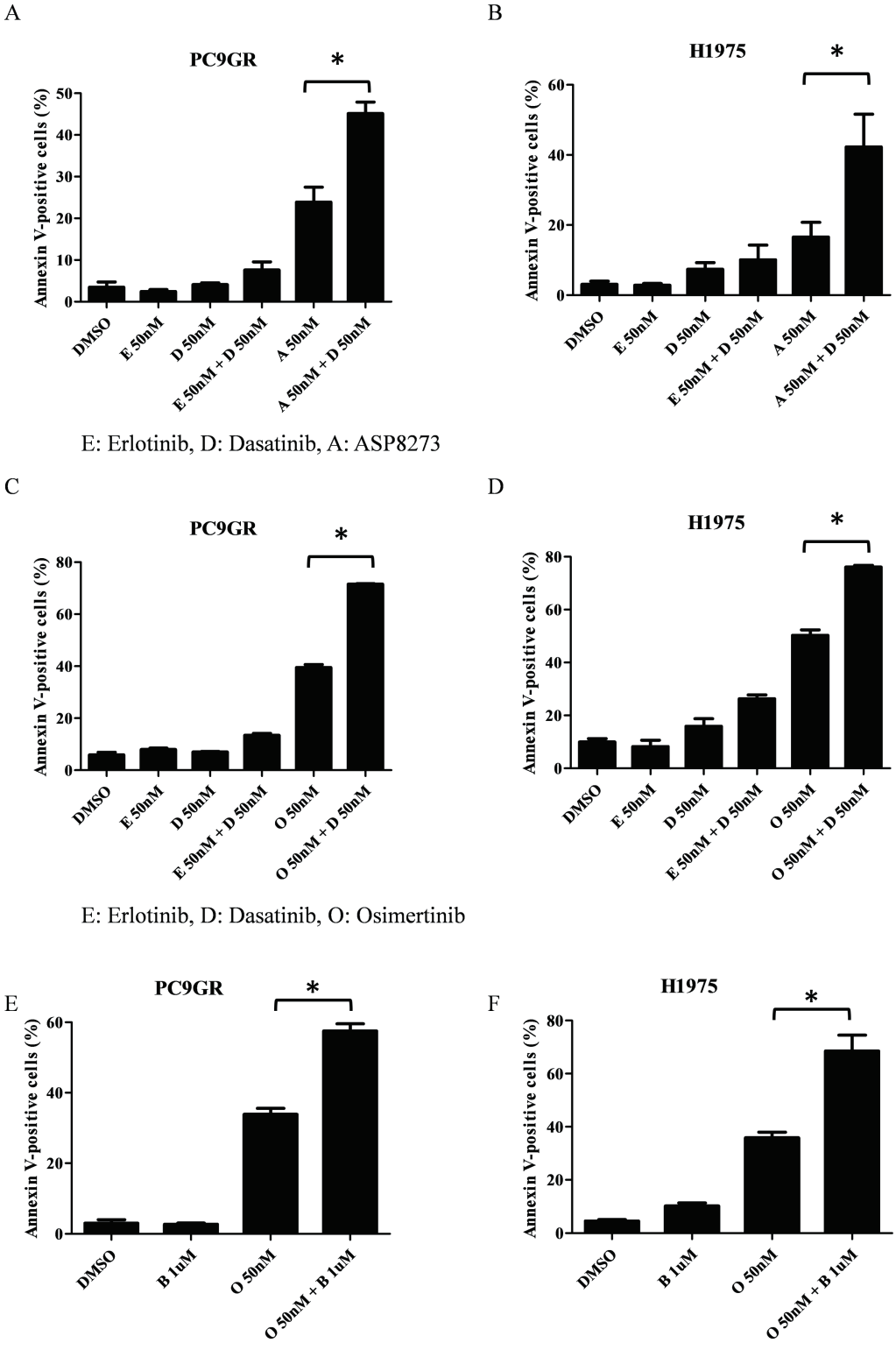


Figure 3. Effects of dasatinib or bosutinib combined with T790M-selective EGFR-TKIs on apoptosis in T790M-positive NSCLC cell lines. (A, C, E) PC9GR and (B, D, F) H1975 cells were cultured overnight in medium containing 5% serum and then incubated for 48 h in the absence or presence of (A–D) erlotinib alone (50 nM), dasatinib alone (50 nM), ASP8273 alone (50 nM), osimertinib alone (50 nM), or dasatinib combined with other agents, or (E, F) bosutinib alone (1 μM), osimertinib alone (50 nM), or bosutinib combined with osimertinib, after which apoptotic cells were detected by staining with propidium iodide and fluorescein isothiocyanate-labeled annexin-V followed by flow cytometry. Each point represents mean ± standard error of three independent experiments. *$P < 0.05$ (Wilcoxon rank-sum test).

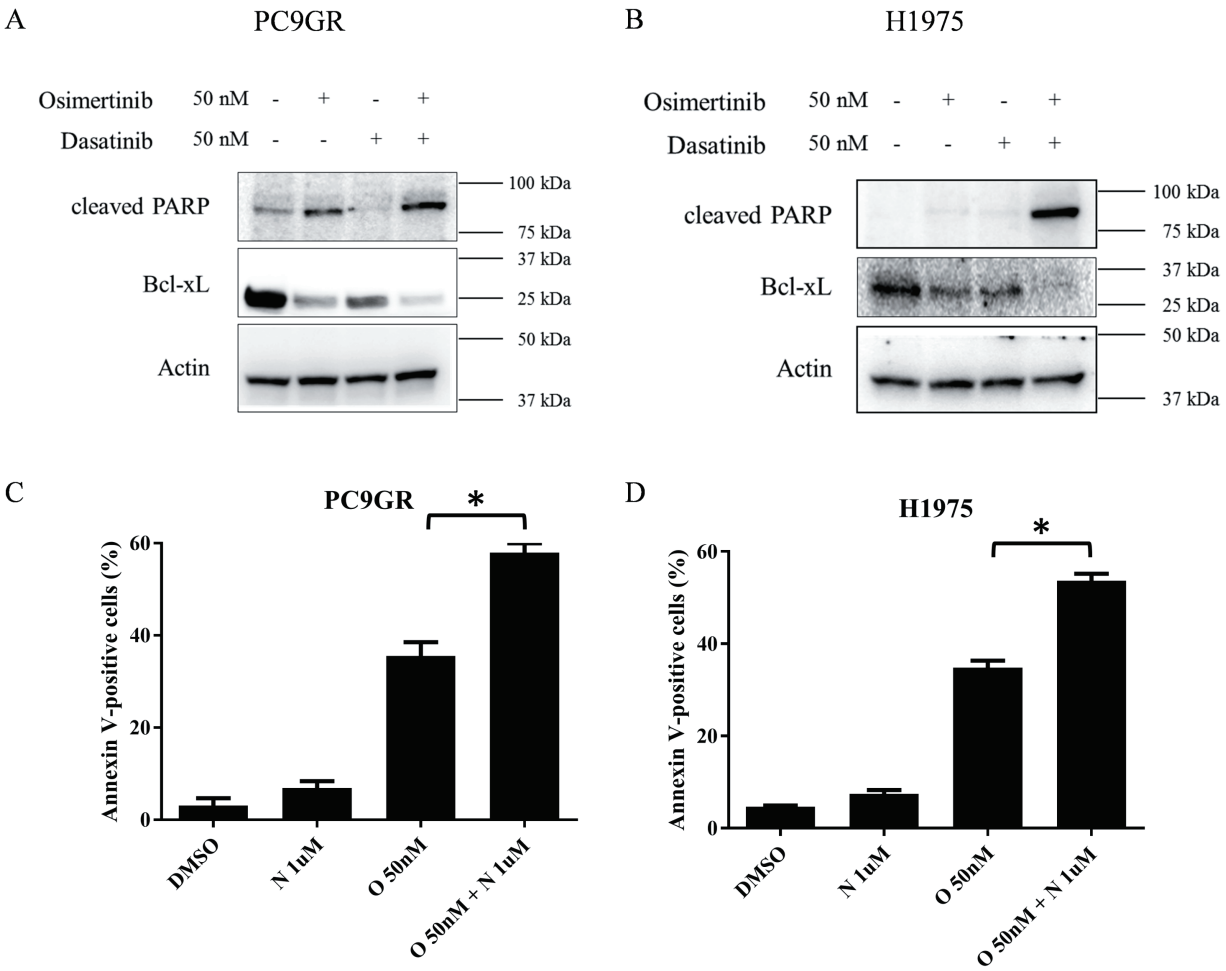
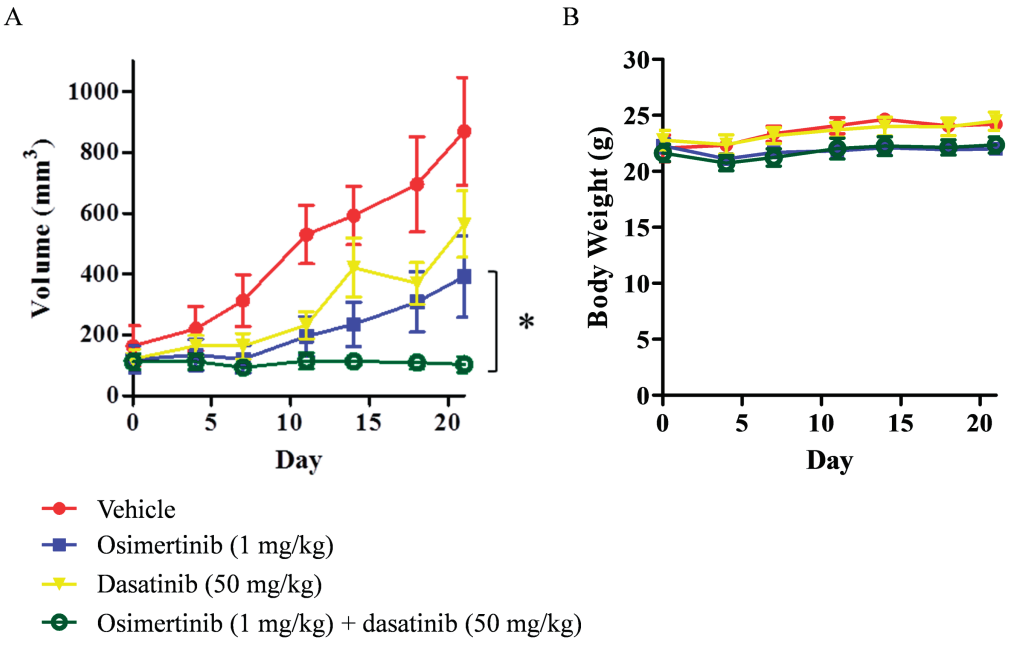


Figure 4. (A, B) Evaluation of apoptosis in T790M-positive NSCLC cell lines by immunoblotting. (A) PC9GR and (B) H1975 cells were incubated for 48 h in the absence or presence of osimertinib (50 nM) and dasatinib (50 nM) Cell lysates were analyzed for expression of cleaved PARP and Bcl-xL, with β-actin serving as a loading control. (C, D) Effects of navitoclax combined with osimertinib on apoptosis in T790M-positive NSCLC cell lines. (C) PC9GR and (D) H1975 cells were cultured overnight in medium containing 5% serum and then incubated for 48 h in the absence or presence of navitoclax alone (1 μM), osimertinib alone (50 nM), or navitoclax combined with osimertinib, after which apoptotic cells were detected by staining with propidium iodide and fluorescein isothiocyanate-labeled annexin-V followed by flow cytometry analysis. Each point represents mean ± standard error of three independent experiments. *P < 0.05 (Wilcoxon rank-sum test).

In vivo anti-tumor activity of combined dasatinib and osimertinib therapy

The combined effect of dasatinib and osimertinib in T790M-positive cells was evaluated *in vivo* using a H1975 xenograft model. Tumors treated with dasatinib (50 mg/kg) or osimertinib (1 mg/kg) alone inhibited tumor progression relative to the control group, whereas treatment with a combination of the two agents suppressed tumor growth (Fig. 5A). The combination therapy was well tolerated, as evidenced by the negligible reduction in body weight (< 5% of the starting weight) (Fig. 5B). We compared Bcl-xL and cleaved PARP levels in post-treatment tissue samples from xenograft-bearing mice by immunoblotting

(Fig. 5C) and found that co-administration of dasatinib and osimertinib increased PARP cleavage by decreasing Bcl-xL expression, consistent with our *in vitro* findings. This suggests that dasatinib enhances osimertinib-induced growth inhibition and apoptosis *in vivo*.



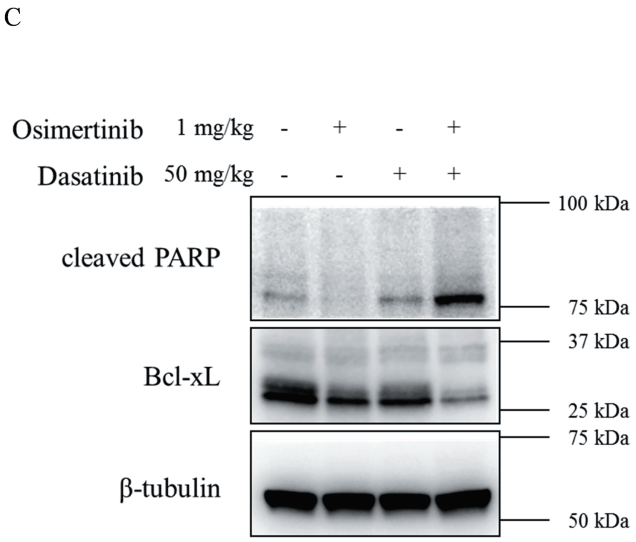


Figure 5. Efficacy of dasatinib combined with T790M-selective EGFR-TKIs in a H1975 tumor xenograft model. Vehicle (100 μl), osimertinib (1 mg/kg), dasatinib (50 mg/kg), and osimertinib (1 mg/kg) + dasatinib (50 mg/kg) were administered by oral gavage once daily to mice bearing H1975 cell xenografts. (A) Tumor volume and (B) body weight were measured twice a week. Data represent mean ± standard error. *P < 0.05 (repeated measures model). (C) Post-treatment expression of apoptotic proteins in tumors from xenograft-bearing mice, as determined by immunoblotting. Cell lysates were analyzed for cleaved PARP and Bcl-xL expression, with β-tubulin serving as a loading control.

Discussion

The results of this study demonstrate that the anti-tumor activity of dasatinib in T790M-positive NSCLC cells is enhanced *in vitro* and *in vivo* when administered in combination with clinically relevant T790M-selective EGFR-TKIs. Dasatinib combined with T790M-selective EGFR-TKIs synergistically inhibited the growth of T790M-positive cells, with simultaneous inhibition of Src, Akt, and Erk; it also increased apoptosis, as determined by the annexin-V binding assay. Dasatinib is a multi-kinase inhibitor; however, bosutinib－another Src inhibitor－also increased apoptosis in combination with osimertinib in T790M-positive cells. In addition, in our previous study, lentiviral transfection of PC9GR and H1975 cells with SFKs (SRC, FYN) harboring gatekeeper mutations caused resistance to dasatinib combined with afatinib, indicating that dasatinib acts as a Src inhibitor in T790M-positive cells [11]. Thus, concurrent inhibition of Src and EGFR signaling is an effective strategy for overcoming T790M-associated EGFR-TKI resistance. Targeting Src to overcome drug resistance has been investigated as a potential treatment approach in several cancers [14, 15]. Increased Src activation has been observed in trastuzumab-resistant breast cancer cells, and the Src inhibitor saracatinib combined with trastuzumab reduced trastuzumab resistance [14]. Dasatinib was also shown to sensitize *KRAS*-mutant colorectal cancer to cetuximab via Src inhibition [15]. Although the specific mechanism underlying Src inhibition is not fully understood, it is possible that Src has a universal role in mediating multiple resistance pathways, since SFKs are transducers of mitogenic signals originating from a number of receptor tyrosine kinases such as EGFR, human epidermal growth factor receptor 2, fibroblast growth factor receptor, platelet-derived growth factor receptor, colony-stimulating factor 1 receptor, and hepatocyte growth factor receptor (c-Met) [16－21]. Src is an upstream activator and downstream effector of EGFR, and is phosphorylated in about one-third of lung tumors [22, 23]. In our previous study, we profiled PC9 and PC9GR cell lines by immunoaffinity purification of tyrosine-phosphorylated peptides and mass spectrometry-based identification/quantification, and found that SFKs act as co-drivers of resistance along with T790M [12]. Simultaneously targeting both SFKs and *EGFR* T790M mutation with dasatinib combined with afatinib or WZ4002 had synergistic anti-tumor effects in T790M-positive cells. The synergism between dasatinib and ASP8273 or osimertinib observed in the current study is consistent with these previous findings. However, the mechanistic link between Src activity and T790M remains unclear and warrants further study, as there was no obvious difference in Src phosphorylation between T790M-positive and -negative cells [12].

T790M-positive *EGFR* mutants are resistant to the pro-apoptotic effects of T790M-selective EGFR-TKIs; this can reportedly be overcome by navitoclax [24]. Bcl-xL is an anti-apoptotic Bcl-2 family member that is a major determinant of the apoptotic response to phosphatidylinositol 3-kinase and mitogen-activated protein kinase kinase blockade [25, 26]. In the present study, we showed that the combination of dasatinib and T790M-selective EGFR-TKI induced apoptosis and inhibited Bcl-xL expression in T790M-positive cells, which was confirmed in a xenograft model. This finding suggests that existence of Bcl-xL is associated with intrinsic apoptosis resistance in T790M-positive cells, which can be overcome by dasatinib in conjunction with T790M-

selective EGFR-TKIs, which may function via Src inhibition [27–29]. Taken together, our results suggest that inhibition of Akt and Erk signaling by T790M-selective EGFR-TKIs as well as Src signaling by dasatinib leads to the suppression of Bcl-xL and induction of apoptosis. The effect of the dasatinib/osimertinib combination has been reported by another study in which Cripto-1 overexpression was found to contribute to intrinsic resistance to EGFR-TKIs via Src activation [30]. However, in this earlier report, H1975 cells harboring T790M showed low Cripto-1 expression. In the current study, we demonstrated for the first time the efficacy of dasatinib combined with osimertinib in T790M-positive NSCLC, which is the main cause (> 50% of cases) of EGFR-TKI resistance in clinical practice; additionally, we found that Bcl-xL downregulation is a mechanism underlying the antitumor effects of this drug combination, which has not been previously reported.

Treatment of *EGFR*-mutated NSCLC patients with dasatinib has not thus far been successful. In a phase II study, single-agent dasatinib showed limited anti-tumor effects in advanced NSCLC patients with *EGFR* mutation who had developed resistance to gefitinib or erlotinib [31]. Although dasatinib combined with the first-generation EGFR-TKI erlotinib showed tolerable response in 59% of patients in a phase I/II study [32], there have been no published clinical trials for dasatinib combined with T790M-selective EGFR-TKI. The current treatment recommended for T790M-positive NSCLC patients is T790M-selective EGFR-TKI [33]. Given the limited PFS of single-agent T790M-selective EGFR-TKI in T790M-positive NSCLC patients, the synergistic effects of dasatinib combined with T790M-selective EGFR-TKI reported here suggest a promising and novel therapeutic strategy. A clinical trial is currently underway (NCT02954523) to evaluate the combination of osimertinib and dasatinib in patients with *EGFR* mutation-positive NSCLC, including those with tumors harboring the T790M mutation.

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