ONO-7475, a novel AXL inhibitor, suppresses the adaptive resistance to initial EGFR-TKI treatment in EGFR-mutated non-small lung cancer

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Translational relevance

A recently identified small subpopulation of reversibly “drug-tolerant” cells resulting from anexelekto (AXL) activation was reported to maintain cell viability in the EGFR mutant non-small cell lung cancer (NSCLC). We investigated the best therapeutic strategy to combat AXL-induced tolerance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) using the novel AXL inhibitor ONO-7475 in AXL-expressing EGFR mutated NSCLC cells treated with the new generation EGFR-TKIs osimertinib and dacomitinib. Our findings demonstrated that the pivotal role of AXL inhibition in the intrinsic resistance, but not acquired resistance, of EGFR mutated lung cancer and the emergence of drug tolerant cells. These results showed the clinical importance of combined initial treatment with osimertinib and ONO-7475 to suppress the development of intrinsic resistance and the emergence of drug tolerant cells, and led to the prevention of tumor heterogeneity in EGFR mutated lung cancer.
Abstract

Purpose

Currently, an optimal therapeutic strategy comprising molecularly targeted agents for treating EGFR-mutated non-small cell lung cancer (NSCLC) patients with acquired resistance to osimertinib is not available. Therefore, the initial therapeutic intervention is crucial for the prolonged survival of these patients. The activation of anexelekto (AXL) signaling is known to be associated with intrinsic and acquired resistance to EGFR-TKIs. In this study, we investigated the best therapeutic strategy to combat AXL-induced tolerance to EGFR-TKIs using the novel AXL inhibitor ONO-7475.

Experimental design

We examined the efficacy of ONO-7475 in combination with EGFR-TKIs in EGFR mutated NSCLC cells using in vitro and in vivo experiments. We investigated the correlation between AXL expression in tumors and clinical outcomes with osimertinib for EGFR mutated NSCLC patients with acquired resistance to initial EGFR-TKIs.

Results

ONO-7475 sensitized AXL-overexpressing EGFR-mutant NSCLC cells to the EGFR-TKIs osimertinib and dacomitinib. In addition, ONO-7475 suppressed the emergence and maintenance of EGFR-TKI tolerant cells. In the cell line-derived xenograft models of AXL-overexpressing EGFR mutated lung cancer treated with osimertinib, initial combination therapy of ONO-7475 and osimertinib markedly regressed tumors and delayed tumor re-growth compared to osimertinib alone or the combination after acquired resistance to osimertinib. AXL expression in EGFR-TKI refractory tumors did not correlate with the sensitivity of osimertinib.

Conclusion

These results demonstrate that ONO-7475 suppresses the emergence and maintenance of tolerant cells to the initial EGFR-TKIs, osimertinib or dacomitinib, in AXL-overexpressing EGFR-mutated NSCLC cells, suggesting that ONO-7475 and osimertinib is a highly potent combination for initial treatment.
Introduction

EGFR tyrosine kinase inhibitors (EGFR-TKIs) are effective for the treatment of non-small cell lung cancer (NSCLC) harboring EGFR mutations, such as the exon 19 deletion and the L858R mutation in \textit{EGFR}. Of them, the 1st generation EGFR-TKIs gefitinib and erlotinib, and the 2nd generation afatinib and dacomitinib have been approved for untreated \textit{EGFR} mutated advanced NSCLC patients in countries such as the U.S. and Japan (1) (2) (3) (4). However, almost all EGFR-positive NSCLC patients ultimately acquired resistance to EGFR-TKIs after approximately one year. The most commonly acquired resistance mechanism, known as the secondary resistant mutation, exon20 T790M mutation in \textit{EGFR}, is detected in half of the EGFR positive patients (5). The 3rd generation EGFR-TKI osimertinib showed promising efficacy for overcoming EGFR T790M mutation positive NSCLC in the AURA2 and AURA3 studies (6,7). However, an optimal therapeutic strategy using molecularly targeted agents for treating NSCLC patients without the EGFR T790M mutation after acquired resistance to initial EGFR-TKIs is not available. Current Phase 3 clinical trials demonstrated that the treatment with osimertinib or the novel 2nd generation EGFR-TKI dacomitinib showed better outcomes than that of 1st generation EGFR-TKIs at the first-line setting for advanced \textit{EGFR} mutated NSCLC patients (8,9). However, the intervention with EGFR-TKI treatment for NSCLC cells with EGFR activating mutations has facilitated tumor evolution and leads to acquired resistance to such EGFR-TKIs, resulting in the lack of an optimal therapeutic strategy with molecularly targeted therapy after the acquired resistance to osimertinib. In addition, approximately 20% of \textit{EGFR} mutated NSCLC patients show intrinsic resistance to the new generation EGFR-TKIs, dacomitinib and
osimertinib. Therefore, the initial therapeutic intervention plays a crucial role in the survival of NSCLC patients with \textit{EGFR} mutations.

Anexelekto (AXL) is a tyrosine kinase receptor belonging to the TAM family of proteins. AXL is usually expressed in epithelial and mesenchymal cells, as well as in breast, pancreatic, lung, and bone marrow cancers (10) (11) (12) (13) (14). AXL in malignant tumors plays a pivotal role in contributing to proliferation, migration, survival, and the epithelial-to-mesenchymal transition (EMT); its overexpression is consequently correlated with poor prognosis in several cancers (15) (16) (17) (18) (19). The activation of AXL signaling in tumors is associated with acquired resistance to several targeted molecular therapy drugs and chemotherapeutic agents (15) (20) (21) (22). Pre-clinical studies showed that the Gas6-AXL axis induced acquired resistance to the EGFR-TKI erlotinib or osimertinib in \textit{EGFR} mutated NSCLC cells, and that the addition of the AXL inhibitor in combination could overcome this resistance (20) (21) (22). In melanoma harboring NRAS and BRAF mutations, AXL inhibition has a pivotal role in overcoming intrinsic or acquired resistance to BRAF/MEK inhibitors (23). We previously revealed the role of the AXL pathway in the intrinsic resistance to osimertinib and the emergence of osimertinib-tolerant cells in \textit{EGFR} mutated NSCLC cells (24). Hence, the regulation of the AXL pathway is expected to be a promising therapeutic strategy against malignant progress in several cancers.

Several AXL inhibitors are being developed in clinical trials. Of them, multi-targeted kinase inhibitors, such as cabozantinib, crizotinib, and sunitinib are approved for advanced cancers, while bosutinib is applied for refractory tumors that are resistant to other RTK inhibitors. Recent clinical studies show that AXL inhibitors are promising combination partners (25). Of them, ONO-7475 is a novel inhibitor of TAM
receptor tyrosine kinase family that has been shown to inhibit the phosphorylation of AXL and Mer, and to suppress the growth of acute myeloid leukemia with FLT3 mutations (26,27) and solid tumors (28) (29). A phase I clinical trial of ONO-7475 is currently underway for acute leukemia and advanced or metastatic solid tumors, although the results remain unpublished (ClinicalTrials.gov identifier NCT03176277 and NCT03730337).

In this study, we investigated the best therapeutic strategy to combat AXL-induced tolerance to EGFR-TKIs using the novel AXL inhibitor ONO-7475 in combination with osimertinib or dacomitinib in AXL-overexpressing EGFR mutated NSCLC cells.

Materials and Methods

Cell cultures and reagents

Ten human NSCLC cell lines with mutations in EGFR were utilized. As previously described (24), HCC4011 and H3255 were generously provided by Dr. David P. Carbone (Ohio State University Comprehensive Cancer Center, Columbus, OH) and Dr. John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX), respectively. The H1975 human lung adenocarcinoma cell line with the EGFR-L858R/T790M double mutation was kindly provided by Dr. Yoshitaka Sekido (Aichi Cancer Center Research Institute, Japan) and Dr. John D. Minna. The human cell lines HCC827 and HCC4006 were purchased from the American Type Culture Collection (Manassas, VA), and the PC-9 cell line was obtained from RIKEN Cell Bank (Ibaraki, Japan). The PC-9KGR cells, which contain deletions in EGFR exon 19 and the T790M mutation, were developed from PC-9 cells by stepwise exposure to gefitinib.
with limiting dilution analysis (30). The PC-9GXR cells, which contain deletions in \( EGFR \) exon 19 and the T790M mutation, were established at Kanazawa University (Kanazawa, Japan) from PC-9 cell xenograft tumors in nude mice that had acquired resistance to gefitinib. The PC-9AR1 cells and HCC827 OR1 cells, which both contain deletions in \( EGFR \) exon 19, were developed from PC-9 cells or HCC827 cells, respectively, by stepwise exposure to afatinib or osimertinib, respectively, using the limiting dilution method. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 g/mL) in a humidified CO\(_2\) incubator at 37 °C. All cells were passaged for less than three months before being renewed with frozen, early-passage stocks. Cells were regularly screened for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza). Cell lines were authenticated by DNA fingerprinting.

Patient-derived xenograft (PDX) from a 44-year-old woman with \( EGFR \) L858R-positive lung cancer (TM0199) was purchased from The Jackson Laboratory (Bar Harbor, ME). Female NOD.Cg-Prkdc \(^{scid}\)/Il2rg \(^{tm1Wjl}\)/SzJ (NSG) mice aged 6–8 weeks were implanted with tumor fragments at passage 7 at the Jackson Laboratory. The mice were transferred to the animal facility at Kanazawa University and the tumors were resected. The study protocol was approved by the Ethics Committee on the Use of Laboratory Animals and the Advanced Science Research Center, Kanazawa University, Kanazawa, Japan. Osimertinib and dacomitinib were obtained from Selleckchem (Houston, TX). ONO-7475 was supplied by Ono Pharmaceutical Co., Ltd.

**Kinase inhibitor assays**
The IC50 value of ONO-7475 against recombinant human AXL was determined using the Off-chip Mobility Shift Assay (MSA) (Carna Biosciences, Inc., Kobe, Japan), according to the manufacturer’s instructions. The ATP concentration in the assay was set at the Km value of AXL for ATP (32 μM). The IC50 value was calculated from the concentration vs. % inhibition curve by fitting a four-parameter logistic curve to the data.

For cell-based AXL inhibition, IC50 value of ONO-7475 was determined using the ACD cell-based tyrosine kinase assay (Advanced Cellular Dynamics, Inc., San Diego, US). Recombinant human AXL-expressing mouse Ba/F3 cells, which depend on AXL kinase activity for survival and proliferation, were exposed to ONO-7475. After 48 h, viable cells were analyzed for ATP concentration using CellTiter-Glo® Luminescent Cell Viability Assay. IC50 value was determined using non-linear regression analysis.

**Kinase selectivity profiling of ONO-7475 in cell-based kinase assay**

Inhibitory activity of 100 nmol/L ONO-7475 against 60 tyrosine kinases was assessed using the ACD cell-based tyrosine kinase assay (Advanced Cellular Dynamics, Inc., San Diego, US). For the 52 kinases showing < 50% inhibition, the ratio of IC50 for each kinase to IC50 for AXL was considered to be >100. For the remaining 8 kinases (Mer, TYRO3, TrkB, EphB2, PDGFRA, TRKa, TRKc, and FLT3), further studies were conducted at various concentrations of ONO-7475, and IC50 values were determined. The ratio of IC50 for each kinase to IC50 for AXL (0.7 nmol/L) was calculated.

**Antibodies and western blotting**

Protein aliquots of 25 μg each were resolved by SDS polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA), as previously described (24) (31).
Electrophoresed protein samples were transferred to polyvinylidene difluoride membranes (Bio-Rad). After washing three times, the membranes were incubated with blotting-grade blocker (Bio-Rad) for 1 h at room temperature and overnight at 4 °C with primary antibodies to p-AXL (Tyr702), t-AXL, p-EGFR (Tyr1068), p-AKT (Ser473), t-AKT, p-p70S6K (Thr389), t-p70S6K, cleaved PARP, β-actin (13E5) (1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA), p-Erk1/2 (Thr202/Tyr204), t-Erk1/2, and t-EGFR (1:1,000 dilution, R&D systems).

After washing three times, the membranes were incubated for 1 h at room temperature with HRP-conjugated species-specific secondary antibody. Immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology). Each experiment was independently performed at least three times.

**Cell viability assay**

Cell viability was determined using the MTT dye reduction method, as previously described (24) (31). Briefly, tumor cells (2–3 × 10^3 cells/100 μL/well) in RPMI 1640 medium supplemented with 10% FBS were plated in 96-well plates and cultured with the indicated compound for 72 h. After culturing, 50 μg of MTT solution (2 mg/mL, Sigma, St. Louis, MO) was added to each well. Plates were incubated for 2 h, the medium was removed, and the dark blue crystals in each well were dissolved in 100 μL of dimethyl sulfoxide (DMSO). Absorbance was measured with a microplate reader at a test wavelength of 550 nm and a reference wavelength of 630 nm. The percentage of growth was determined relative to untreated controls. Experiments were repeated at least three times with triplicate samples. As another cell viability assay, cells were treated with DMSO, EGFR-TKI osimertinib or dacomitinib, ONO-7475, or a
combination for 7 and 15 days where the drugs were replenished every 72 h. The plates were stained with crystal violet and visually examined. A plate representative of three independent experiments is shown.

**Wound healing assay**

For the wound healing assay, the PC-9 and HCC4011 cells (1×10^5 cells) were seeded and incubated for 24 hours at 37 °C. After achieving confluence, the cellular layer in each plate was scratched using a plastic pipette tip. The migration of the cells at the edge of the scratch was analyzed at 0 and 24 hours after treatment with the EGFR-TKIs osimertinib or dacomitinib, ONO-7475, or a combination, when microscopic images of the cells were captured.

**Transfection of siRNAs**

Duplexed Silencer® Select siRNAs against AXL (s1845 and s1846; Invitrogen, Carlsbad, CA) were transfected into cells using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. In all experiments, Silencer® Select Negative Control no.1 siRNA (Invitrogen) was used as the scrambled control, as previously described (24). Knockdown of AXL was confirmed by western blotting. Each sample was tested in triplicate in three independent assays.

**Plasmid construction**

pWPXL plasmids expressing empty vector (Vector) and human AXL (AXL-WT) were purchased from Addgene, Inc. (32). X-tremeGene HP DNA Transfection Reagent (Roche) was used to transfect HCC827 cells with Vector or
AXL-WT plasmid, following the manufacturer's instructions. Whole-cell lysates (25 µg) were analyzed by western blot.

**Cytokine production**

Cells (2 × 10⁵) were cultured in RPMI-1640 medium with 10% FBS for 24 h, washed with PBS, and incubated for 48 h in 2 mL of the same medium. The culture medium was harvested and centrifuged, and the supernatant stored at −70°C until analysis, as previously described (24) (31). Level of Gas6 was determined with Human Gas6 DuoSet ELISA kit (R&D Systems), according to the manufacturer's protocols. All culture supernatants were tested twice. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Concentrations of growth factors were determined from standard curves.

**Cell-line-derived xenograft models**

Suspensions of 5×10⁶ cells were injected subcutaneously into the flanks of five-week-old male mice with severe combined immunodeficiency (SCID) obtained from Clea Japan (Tokyo, Japan), as previously described (24). Once the mean tumor volume reached approximately 100–200 mm³, five or six mice each were injected with the PC-9 and PC-9KGR cell-line-derived xenografts (CDX) at the time of initial treatment or at the time of sequential therapy. Drugs were administered seven days a week by oral gavage and the body weight and general condition of the mice were monitored daily. Tumors were measured twice weekly using calipers and their volumes were calculated as width² × length/2. Approval was obtained from the institutional review board at University Hospital, Kyoto Prefectural University of Medicine for a study using mice (approval no. M29-529). According to institutional guidelines, surgery was performed after the animals
were anesthetized with sodium pentobarbital and efforts were made to minimize animal suffering.

**DNA extraction**

Genomic DNA was extracted from frozen samples using a nucleospin tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Concentrations of the extracted DNAs were measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNAs were stored at -20 °C until use.

**PNA-LNA PCR clamp**

The PNA-LNA PCR clamp reaction was carried out using the LightCycler 480 II (Roche, Pleasanton, CA, USA) as previously reported (33). Quantitative PCR was then carried out with a 30 second hold at 95 °C followed by 45 cycles at 95 °C for 3 seconds, and 62 °C for 30 seconds.

**Patients**

Specimens of *EGFR*-positive tumors were obtained from 7 lung adenocarcinoma patients hospitalized at University Hospital, Kyoto Prefectural University of Medicine (Kyoto, Japan) prior to osimertinib treatment. Re-biopsy specimens of tumors containing *EGFR*-activating mutations with the T790M mutation, after acquired resistance to initial treatments with the EGFR-TKIs gefitinib, erlotinib, afatinib and prior to treatment with osimertinib, were obtained from 28 lung adenocarcinoma patients hospitalized at the Kanazawa University Hospital (Kanazawa, Japan), Japanese Red Cross Kyoto Daiichi Hospital (Kyoto, Japan), Niigata University Hospital (Niigata, Japan), Nagasaki University Hospital (Nagasaki, Japan), or Japanese Red Cross Nagasaki Genbaku Hospital (Nagasaki, Japan). The study was conducted according to the Declaration of
Helsinki. All patient participated in the Institutional Review Board of University Hospital, Kyoto Prefectural University of Medicine (approval no. ERB-C-812 and ERB-C-1349-2) and each hospital-approved study, and provided written informed consent.

**Histological analyses of tumors**

Formalin-fixed, paraffin-embedded tissue sections (4 μm thick) were deparaffinized. Antigen was retrieved by microwaving the tissue sections in 10 mM citrate buffer (pH 6.0). Proliferating cells were detected by incubating the tissue sections with Ki-67 antibody (Clone MIB-1; DAKO Corp, Glostrup, Denmark), as previously described (24). Cell apoptosis was quantitated using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method, according to the manufacturer’s instructions. Based on expression patterns, tumor cells in tissue specimens were separately evaluated for expression of AXL using an anti-AXL antibody (1:200; goat polyclonal, R&D SYSTEMS). Since immunohistochemical studies have shown that AXL is present primarily in the cytoplasm of cells and that its staining varies in intensity, we quantified its expression as negative (0), weak (1+), moderate (2+), and strong (3+) compared with vascular endothelial cells as an internal control (34). After incubation of the specimens with the secondary antibody and treatment using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA), peroxidase activity was visualized using 3,3’-diaminobenzidine (DAB) as a chromogen. The sections were counterstained with hematoxylin.

**Quantification of immunohistochemistry results**
The five areas containing the highest numbers of positively stained cells within each section were selected for histological quantitation using light microscopy at a 400-fold magnification, as previously described (24) (31).

**Statistical analysis**

Data from the MTT assays and tumor progression of xenografts were expressed as means ± standard deviation (SD) and as means ± standard error (SE), respectively. The statistical significance of differences was analyzed using one-way ANOVA and Spearman rank correlations. Progression-free survival (PFS) and 95% confidence intervals (CIs) were determined using the Kaplan-Meier method and compared using the log-rank test. Hazard ratios (HRs) of clinical variables for PFS were determined using a univariate Cox proportional hazards model. All statistical analyses were performed using GraphPad Prism Ver. 7.0 (GraphPad Software, Inc., San Diego, CA, USA), with a two-sided \( P \)-value less than 0.05 being considered statistically significant.

**Results**

**The AXL inhibitor ONO-7475 sensitized AXL-overexpressing EGFR-mutant NSCLC cells to EGFR-TKIs**

We first tested ten \( \text{EGFR} \)-mutated NSCLC cell lines and one PDX tumor, which were divided into those with high levels of AXL expression and those with low levels of AXL expression. Phosphorylated EGFR tended to be higher in cells with lower relative AXL expression levels, as we previously described (24) (**Figure 1A**). The chemical structure of ONO-7475 is shown in **Figure 1B**. IC\(_{50}\) values of ONO-7475 against recombinant human AXL were 0.414 and 0.7 nmol/L using Off-chip MSA and ACD cell-based tyrosine kinase assay, respectively (**supplementary Figure 1A**). Ratio of
IC50 for each kinase to that for AXL (0.7 nmol/L) was calculated (supplementary Figure 1B).

We examined the potency of the AXL inhibitor ONO-7475 on the sensitivity of EGFR-mutated NSCLC cells to the EGFR-TKIs osimertinib or dacomitinib. Two different doses with ONO-7475 for 72 h increased the sensitivity to osimertinib and dacomitinib and reduced viability of high AXL-expressing PC-9 and HCC4011 cells, but not of low-AXL-expressing HCC827 cells (Figure 1C, supplementary Figure 2). In addition, ONO-7475 enhanced osimertinib efficacy on the viability of cell lines PC-9, PC-9KGR, and HCC4011, and H1975, all of which express high levels of AXL, but had only a marginal effect on the viability of cell lines HCC827, HCC4006, and H3255, which express low levels of AXL. Similar results were observed in the treatment with dacomitinib (Figure 1D). In addition, the continuous co-treatment of EGFR-TKI osimertinib or dacomitinib and ONO-7475 in PC-9 and HCC4011 cells reduced the cell viability for 15 days of incubation, but did not affect viability of low-AXL-expressing HCC827 cells (Figure 1E). To prove further evidence that AXL is an important mediator of the therapeutic action of osimertinib on EGFR-mutated NSCLC cells, we surmised that enforced expression of AXL in low-AXL-expressing cells might suppress their sensitivity to osimertinib. To test this hypothesis, we transfected HCC827 cells with Vector or AXL-WT. Western blot showed an increase in total AXL protein in the AXL-WT-expressing cells (supplementary Figure 3A), which reduced their sensitivity to osimertinib, as expected. Moreover, ONO-7475 in combination with osimertinib restored viability of AXL-WT-transfected cells (supplementary Figure 3B).

To elucidate the underlying mechanisms of the combined therapy of ONO-7475 and the EGFR-TKIs osimertinib or dacomitinib, we investigated protein expression by
western blot. The combination of osimertinib and ONO-7475 for 4 h markedly inhibited the phosphorylation of AXL, AKT, and p70S6K compared with treatment of the high-AXL-expressing cell lines treated with osimertinib alone (Figure 1F). The combined use of ONO-7475 with osimertinib for 48 h increased cleaved PARP in PC-9 and HCC4011 cells compared with the treatment with osimertinib alone. These findings were also observed in the dacomitinib treatment. Moreover, the co-treatment of ONO-7475 with osimertinib on PC-9 and HCC4011 cells inhibited cell migration for 24 h incubation, compared to osimertinib alone (Supplementary Figure 4).

These results showed that cell sensitivity to the EGFR-TKIs osimertinib or dacomitinib is enhanced by the combined treatment with an AXL inhibitor ONO-7475, resulting in reduced viability and migration, and induced apoptosis of high-AXL-expressing EGFR-mutated NSCLC cell lines, but not with cells expressing low levels of AXL.

The AXL inhibitor ONO-7475 suppressed the emergence and maintenance of EGFR-TKI tolerant cells

Drug-tolerant (DT) cells are reported as a small subpopulation of cells with remarkably reduced sensitivity to targeted drugs. They are generated within several days to several weeks of exposure to target drugs (35). Thus, we isolated DT cells from PC-9, HCC4011, PC-9KGR, and H1975 cells after nine days of exposure with high doses of osimertinib (3 µM) or dacomitinib (1 µM). As expected, these DT cells were resistant to the EGFR-TKIs osimertinib or dacomitinib, compared with their parental cells (Figure 2A, Supplementary Figure 5A). We next examined the expression level of AXL, and its ligand Gas6, in both parental cells and DT cells. The DT cells from osimertinib and
dacomitinib expressed high level of total AXL compared with parental cells in high-AXL-expressing cells, but not low-AXL-expressing cells (Figure 2B, Supplementary Figure 6). Production of Gas6 could not be detected in culture medium of either parental cells or osimertinib-DT cells isolated from PC-9 and HCC4011 cells, indicating that AXL activation in DT cells is independent of ligand stimulation (Supplementary Table 1). ONO-7475 treatment decreased the viability of DT cells, but not that of the parental PC-9, HCC4011, PC-9KGR, or H1975 cells (Figure 2C, Supplementary Figure 5B, 7A). In addition, knockdown of AXL decreased the viability of DT cells compared to scrambled control (Supplementary Figure 7B, 7C).

Western blot analysis showed that while osimertinib and dacomitinib slightly inhibited the phosphorylation of EGFR in the DT cells, ONO-7475 suppressed the phosphorylation of AKT, p70S6K, ERK, as well as EGFR (Figure 2D). Moreover, the continuous treatment of PC-9DT and HCC4011DT cells with ONO-7475 inhibited the viability of these DT cells for 7- and 15-days of incubation (Figure 2E, 2F).

These results indicated that activated AXL played a critical role in the emergence and the maintenance of DT cells by treatment with the EGFR-TKIs osimertinib or dacomitinib, and that ONO-7475 could suppress the formation of these DT cells though AKT and ERK signals.

Initial combination therapy of ONO-7475 and osimertinib delayed re-growth of cell-line derived xenograft tumors

We evaluated the effect of ONO-7475 and osimertinib in a CDX model using high-AXL-expressing PC-9KGR cells, which has an exon 19 deletion and the exon21-T790M mutation in EGFR. Mice were continuously administered osimertinib
alone, ONO-7475 alone, or a combination of the two drugs seven days a week by oral gavage until day 29. Treatment with osimertinib alone caused tumor regression within one week, but the tumors reappeared within three weeks, indicating that recurrence was induced by acquired resistance. In contrast, treatment with ONO-7475 alone had little effect on the tumor growth. The combined initial treatment with osimertinib and ONO-7475 caused tumor regression compared to osimertinib alone, and the size of tumors was maintained for 4 weeks (Figure 3A). No apparent adverse events, including weight loss, were observed during these treatments (Supplementary Figure 8A).

To further elucidate the best therapeutic strategy of ONO-7475 plus osimertinib, we next evaluated the effect of the combination in a CDX model of PC-9 cells, using two different treatment schedules, 1) during the initial phase, and 2) during the acquired resistance phase with osimertinib. For the initial phase studies, mice were treated as above with PC-9KGR cells (Figure 3B). The results were similar, except that the combined initial treatment with osimertinib and ONO-7475 caused tumor regression within one week and the size of the regressed tumors was maintained for 7 weeks. The number of Ki-67 positive proliferating tumor cells was significantly lower in the combination-treated tumors than in osimertinib-treated tumors derived from PC-9 cells, whereas the number of TUNEL-positive tumor cells was not significantly different between the two (Figure 3C, 3D, supplementary Figure 9A, 9B). In the PC-9 cell-derived tumors, expressions of phosphorylated AKT and p70S6K were inhibited by a combination of ONO-7475 with osimertinib, compared to osimertinib alone (Figure 3E).

These results indicated that combined treatment with osimertinib and ONO-7475 during the initial phase prevented the growth of high-AXL-expressing EGFR-mutated NSCLC cells with or without the EGFR-T790M mutation in vivo. For the
acquired resistance phase studies, the PC-9 tumor-bearing mice were initially treated with osimertinib alone. The size of tumors gradually increased, despite the continued osimertinib treatment, indicating that the tumor cells had acquired resistance to osimertinib. On day 29, the mice were randomly divided into two groups. One group was continuously treated with osimertinib alone and the other was additionally treated by a combination with ONO-7475. The tumors treated with osimertinib alone grew persistently until day 57. Tumor growth when treated with the combination of osimertinib with ONO-7475 from the acquired resistance phase was slightly slower than those treated with osimertinib alone until day 47. From day 47 to day 57, tumor growth was similar to osimertinib alone (Figure 3B). No apparent adverse events, including weight loss, were observed during these treatments (Supplementary Figure 8B). Overall, the results from the rapid recurrence model using PC-9 cells indicated that the therapeutic intervention of combined treatment with osimertinib and ONO-7475 prevents more tumor re-growth at the initial phase than at the osimertinib-resistant phase, indicating the crucial role of initial therapeutic intervention for high-AXL-expressing EGFR-mutated NSCLC cells.

PC-9OR1 cells established from acquired in vivo resistance to osimertinib are insensitive to combined therapy with osimertinib and ONO-7475

We harvested subcutaneous tumors from mice on day 29 after osimertinib treatment and cultured them in vitro. The expanded tumor cells were named PC-9OR1 (Figure 4A). We first examined the EGFR-mutation status of both PC-9 and PC-9OR1 cells. Both the PC-9 and PC-9OR1 cells possessed deletions in the EGFR exon 19 gene, however the C797S EGFR-mutation reported as the osimertinib resistance-inducing mutation, was not detected in both cell lines (Supplemental Table 2). While the growth
of PC-9OR1 cells was slightly faster than that of PC-9 cells, both cell lines grew at a constant rate in vitro (Figure 4B). To evaluate the role of AXL activation between the initial and acquired resistance phases of osimertinib treatment, we assessed PC-9OR1 cells compared to PC-9 cells in subsequent experiments. The expression of AXL and phosphorylation of AKT, but not phosphorylation of EGFR and ERK, increased in PC-9OR1 cells compared to PC-9 cells by Western blot (Figure 4C). Next, we examined the drug sensitivity of PC-9OR1 cells in vitro. As expected, PC-9OR1 cells were resistant to osimertinib compared with PC-9 cells (Figure 4D; IC50 PC-9OR1, 1424 nmol/L; IC50 PC-9, 2 nmol/L). Importantly, consistent with in vivo experiments, PC-9OR1 cells were also less sensitive to osimertinib in combination with ONO-7475 than PC-9 cells, even though PC-9OR1 cells have higher AXL expression than PC-9 cells (Figure 4E). Western blot analysis showed that osimertinib inhibited phosphorylation of EGFR and ERK in both cell lines, whereas the EGFR downstream molecule AKT was activated in PC-9OR1 cells, but not in PC-9 cells. These findings suggest that osimertinib failed to inhibit AKT phosphorylation in PC-9OR1 cells, resulting in acquired osimertinib resistance. Moreover, the combination of ONO-7475 and osimertinib did not completely inhibit AKT phosphorylation in PC-9OR1 cells compared with PC-9 cells (Figure 4F).

To characterize DT cells from the acquired resistance phase, we tested the growth of PC-9OR1 DT cells selected by osimertinib treatment. PC-9OR1 DT cells were less sensitive to ONO-7475 compared with the PC-9 DT cells, suggesting that DT cells from the osimertinib-resistant phase reduced AXL-dependent cell viability (Figure 4G).

These results showed that the survival of osimertinib-acquired resistant cells was independent of the AXL pathway. Cells could not recover sensitivity to osimertinib by
treatment with a combination of an AXL inhibitor, suggesting the progression of drug tolerance with clonal evolution during osimertinib treatment.

**AXL expression in tumors correlated with sensitivity to osimertinib in the pre-treatment EGFR-mutant NSCLC patients but not those with the T790M mutation diagnosed by re-biopsy**

To assess the role of AXL expression in osimertinib-treated tumors, we performed a retrospective study in patients with TKI-naive EGFR-mutant NSCLC (N=7). Pre-treatment expression of AXL in the cytoplasm of tumor cells was evaluated using immunohistochemistry (IHC) staining, and scored as high (3+), intermediate (2+), low (1+), or negative (0). High AXL protein expression was detected in 1 (14.3%), intermediate in 0 (0%), low in 3 (42.9%), and negative in 3 (42.9%) of the 7 tumors. We next assessed whether AXL expression in tumors could serve as a negative predictor for treatment with osimertinib. The response to osimertinib was high (83.3%) and low (0%) in patients with AXL expression scores of 0 to 1+ and 3+, respectively. Additionally, the average tumor shrinkage rate relative to baseline was high (53.3%) and low (-19%) in osimertinib-treated patients with AXL expression scores of 0 to 1+ and 3+, respectively (Figure 5A, 5B).

To assess the role of AXL expression in tumors after acquired resistance to EGFR-TKIs with osimertinib treatment, we next performed a retrospective study in patients with EGFR mutant tumors containing the T790M mutation (N=28). Time to progression with osimertinib ranged from 0.3 to 28 months. Among 28 tumors obtained from 28 patients with the EGFR-T790M mutation, high AXL protein expression was detected in 2 (7.1%), intermediate in 7 (25.0%), low in 19 (67.9%), and negative
expression in 0 (0%). The response rate to osimertinib for the patients with AXL expression scores of 0 to 1+ was low (62.3%), while for those patients with AXL expression scores of 2+ to 3+, the response rate to osimertinib was relatively higher (77.8%) \( (p = 0.67) \). Progression-free survival with osimertinib treatment was not significantly altered in patients with low and high AXL expression \( (p = 0.111) \) (Supplemental Figure 10A, 10B).

These findings show that AXL expression in tumors may be a good predictor of osimertinib sensitivity, although the sample size was small. In contrast, after acquired resistance to initial EGFR-TKIs, AXL expression in tumors may not be a good predictor for the acquired resistance to osimertinib. It appears to be difficult to select promising populations for combined therapy with osimertinib and an AXL inhibitor in \( EGFR \) mutated tumors obtained from the acquired resistance phase.

**Discussion**

Acquired resistance to 1st and 2nd generation EGFR-TKIs is caused by various mechanisms, such as gatekeeper mutations like the EGFR-T790M second site mutation, activation of an alternate pathway, activation of EGFR downstream signals, transformation to small cell lung cancer, and the EMT (36). Several clinical trials targeting the bypass signals related to drug resistance have been conducted in \( EGFR \) mutated NSCLC patients with acquired resistance to initial EGFR-TKIs. Of these, several clinical trials with an AXL inhibitor have been ongoing for \( EGFR \)-mutated NSCLC patients at the acquired resistance setting (37) (38). However, previous clinical studies demonstrated that the outcomes are limited for NSCLC patients with acquired resistance to EGFR-TKIs (39) (40) (41). These observations suggest that maintenance of residual
tumor cells during EGFR-TKI treatment may be accelerated by various molecular mechanisms, such as minor subpopulations with a resistance mechanism, the tumor microenvironment, and the reversible drug-tolerant state (42), which ultimately leads to tumor heterogeneity and drug resistance.

Currently, DT cells are thought to play a pivotal role in the progression of tumor heterogeneity because these cells are considered to eventually enhance tumor recurrence (43). However, it is not completely understood how to prevent the development of drug tolerant cells and tumor heterogeneity, which is related to epigenetic changes in treated cells (44). Previous studies demonstrated that a small subpopulation of reversibly DT cells maintain viability through the activation of the IGF-1 receptor, aurora kinase A, and GPX4 (35) (45) (46). However, a clinical study using the combination of an IGF1-receptor inhibitor plus the EGFR-TKI erlotinib in unselected patients with lung cancer failed to demonstrate clinical benefits (47), although pre-clinical studies showed that the combination reduced these DT cells in EGFR mutated NSCLC (35). These pre-clinical and clinical observations suggest that a predictive biomarker for the emergence of DT cells needs to be developed for the clinical setting.

We previously reported that addition of an AXL inhibitor NPS1034 during the initial phase of osimertinib treatment reduced tumor growth in AXL-expressing EGFR-mutated NSCLC cells. In addition, in analysis of clinical specimens, high AXL expression in the pre-treatment of EGFR tumors correlates with a poor response to initial EGFR-TKIs, including osimertinib, suggesting the high AXL expression in tumors may be a promising negative biomarker to detect the response to EGFR-TKIs (24). However, in cases of refractory EGFR-mutated tumors to initial EGFR-TKIs, a significant
correlation was not observed between those with AXL expression and clinical outcomes with osimertinib. Additionally, CDX mice models and cell line-based analysis showed that the combination of ONO-7475 and osimertinib is more effective at intervention of the initial phase than at the osimertinib-acquired resistance phase in high-AXL-expressing EGFR mutated NSCLC cells. These results indicate that in high AXL-expressing tumors, intervention with initial EGFR-TKIs may not be sufficient to combat the resistance to initial EGFR-TKIs by inhibition of the AXL-dependent pathway. Interestingly, PC-9OR1 expressed more AXL proteins compared to the pre-treatment PC-9 and their DT cells, whereas the DT cells from PC-9OR1 showed less sensitivity to ONO-7475 than that of PC-9 DT cells, indicating that the intervention of EGFR-TKIs expanded the diversity of drug tolerant mechanisms in addition to AXL activation and led to insensitivity to an AXL inhibitor. In contrast, our results showed that EGFR mutated NSCLC cells harboring the T790M mutation are also sensitive to combined therapy of ONO-7475 and osimertinib at the initial therapeutic intervention, suggesting that the combination of ONO-7475 and osimertinib is effective for EGFR-T790M mutated NSCLC cells, without the progression of tumor heterogeneity. Collectively, our findings support the idea that therapeutic intervention with initial EGFR-TKI leads to clonal evolution and facilitates tumor heterogeneity in EGFR mutated NSCLC cells. Therefore, an AXL-targeted therapy to conquer drug tolerant mechanisms at the initial phase might be promising to reduce the emergence of tumor heterogeneity compared with those at the resistance phase of initial treatment in AXL-overexpressing EGFR mutated NSCLC cells.

In summary, a novel AXL inhibitor ONO-7475 suppresses the emergence and maintenance of cells tolerant to the initial EGFR-TKI osimertinib or dacomitinib in
AXL-overexpressing EGFR-mutated NSCLC cells, suggesting that the combination of ONO-7475 and osimertinib is highly potent for the initial treatment phase. Further clinical investigations are warranted for the development of novel strategies using an AXL inhibitor at the initial therapeutic setting to overcome the tolerance to the EGFR-TKIs osimertinib and dacomitinib in AXL-overexpressing EGFR mutated NSCLC patients.

Acknowledgments

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Reference


Figure legends

Figure 1. The AXL inhibitor ONO-7475 sensitized AXL-expressing EGFR-mutant NSCLC cells to the EGFR-TKIs osimertinib and dacomitinib.

(A) The EGFR-mutated NSCLC cell lines PC-9, PC-9KGR, PC-9GXR, PC-9AR1, HCC4011, H1975, HCC827, HCC827OR1, HCC4006, H3255, and PDX tumor cells (TM0199), were lysed and the indicated proteins were detected by western blot. (B)
Structure of ONO-7475. (C) PC-9 and HCC4011 cells were incubated with osimertinib or dacomitinib in the presence or absence of the AXL inhibitor ONO-7475 (0.1, 1 μmol/L) for 72 h and then cell viability was determined using MTT assays. Data are representative of three independent experiments that produced similar results. (D) Quantitative determination of the inhibition of cell viability of high-AXL-expressing and low-AXL-expressing EGFR-mutant cells treated with the EGFR-TKIs osimertinib or dacomitinib in the presence or absence of ONO-7475. Paired Student’s t tests were used for comparisons. (E) Cells were treated with DMSO, 300 nmol/L osimertinib or 10 nmol/L dacomitinib, 1 μmol/L ONO-7475, or a combination of 300 nmol/L osimertinib or 10 nmol/L dacomitinib, and 1 μmol/L ONO7475 for 15 days where the drugs were replenished every 72 h. The plates were stained with crystal violet and visually examined. A plate representative of three independent experiments is shown. (F) The indicated cells were incubated with osimertinib (100 nmol/L) or dacomitinib (100 nmol/L) with or without ONO-7475 (1 μmol/L) for 4 h or 48 h. The cells were lysed and the indicated proteins were detected by western blot.

Figure 2. The AXL inhibitor ONO-7475 suppressed the emergence and maintenance of tolerant cells resulting from treatment of EGFR-TKIs.
(A) Drug-tolerant (DT) cells previously treated with 3 μmol/L osimertinib or 1 μmol/L dacomitinib for 9 days were treated with the indicated concentrations of osimertinib or dacomitinib for 72 h and their viability was assessed using MTT assays. (B) PC-9 parental cells, and DT cells (generated by treatment of parental cells with 3 μmol/L osimertinib or 1 μmol/L dacomitinib for 9 days) were lysed and the indicated proteins were detected by western blotting. (C) PC-9, HCC4011 parental cells, and each DT cells generated by treatment with 3 μmol/L osimertinib (top panel) or 1 μmol/L dacomitinib (bottom panel) were treated with the EGFR-TKI osimertinib or dacomitinib, 1 μmol/L of ONO-7475, or a combination of these agents for 72 h and the cell viability was assessed using MTT assays. (D) The indicated DT cells from PC-9 were incubated with osimertinib (100 nmol/L) or dacomitinib (100 nmol/L) with or without ONO-7475 (1 μmol/L) for 4 h. The cells were lysed and the indicated proteins were detected by western blot. (E) PC-9 DT cells were generated for 9 days of treatment with 3 μmol/L of osimertinib (top panel) or 1 μmol/L of dacomitinib (bottom panel) exposed to either EGFR TKI alone (osimertinib 300 nmol/L or dacomitinib 10 nmol/L) or with the addition of ONO-7475 (1 μmol/L) for the indicated times. Paired Student’s t tests were used for comparisons. (F) PC-9 DT and HCC4011 DT cells were treated with DMSO, 300 nmol/L osimertinib or 10 nmol/L dacomitinib, 1 μmol/L ONO-7475, or a combination of 300
nmol/L osimertinib or 10 nmol/L dacomitinib and 1 μmol/L ONO-7475 for 15 days
where the drugs were replenished every 72 h. The plates were stained with crystal violet
and visually examined. A plate representative of three independent experiments is shown.

Figure 3. Initial combination therapy of ONO-7475 and osimertinib delayed
re-growth of cell-line derived xenograft tumors.

(A) PC-9KGR cell-line-derived xenograft (CDX) tumors were treated with vehicle
(control), ONO-7475 10 mg/kg, osimertinib 5mg/kg, or ONO-7475 10 mg/kg plus
osimertinib 5mg/kg (n = 6 per group). Tumor volumes were measured over time from the
start of treatment and the results are shown (mean ± SEM). *, p < 0.05. (B) PC-9 CDX
tumors were treated with vehicle (control), ONO-7475 10 mg/kg, osimertinib 5mg/kg, or
ONO-7475 10 mg/kg plus osimertinib 5mg/kg (n = 6 per group). In the osimertinib
treatment group, tumors on day 29 were randomized, and treated with continuous
administration osimertinib 5mg/kg or ONO-7475 10 mg/kg plus osimertinib 5mg/kg until
day 57 (n = 5 per group). The results of tumor volume are plotted (mean ± SEM). *, p <
0.001. (C) Quantification of proliferating cells, as determined by their Ki-67-positive
proliferation index (percentage of Ki-67-positive cells) as described in the Methods.
Columns, mean of five evaluated areas; bars, SD. Comparisons by paired Student’s t
tests. *, \( p < 0.05 \). (D) Representative images of PC-9 xenografts containing the following immunohistochemical staining with antibodies specific for human Ki-67. Bar, 100 μm.

**Figure 4. Osimertinib acquired resistant cells are insensitive to combined therapy with osimertinib and ONO-7475.**

(A) PC-9 parental and the osimertinib acquired resistant PC-9OR1 cells were evaluated using a light microscope. Bar, 200 μm. (B) Cells were incubated for the indicated times and their viability assessed using MTT assays. (C) Protein expression was analyzed by Western blot with the indicated antibodies. (D) Growth inhibition was assessed by MTT assay of PC-9 and PC-9OR1 cells treated with the indicated concentrations of osimertinib for 72h. (E) Growth inhibition assessed by MTT assay of PC-9 and PC-9OR1 cells treated with 300 nmol/L osimertinib or 1 μmol/L of ONO-7475, or a combination of these agents for 72 h. *, \( p < 0.01 \). (F) The indicated cells were incubated with osimertinib (100 nmol/L) or dacomitinib (100 nmol/L) with or without ONO-7475 (1 μmol/L) for 4 h. The cells were lysed and the indicated proteins were detected by western blot. (G) The indicated cells were treated with DMSO, 300 nmol/L osimertinib, 1 μmol/L ONO-7475, or a combination of 300 nmol/L osimertinib and 1 μmol/L ONO-7475 for 15 days where the drugs were replenished every 72 h. The plates were stained with crystal violet and visually examined. A plate representative of three independent experiments is shown.
Figure 5. The correlation between osimertinib sensitivity and AXL expression in pre-treatment tumors derived from EGFR-mutated NSCLC patients.

(A) Waterfall plot of EGFR-mutated NSCLC patients treated with osimertinib. (B) Correlation between cytoplasmic AXL protein expression levels (determined immunohistochemically) and the response to osimertinib treatment, including the average tumor shrinkage rate relative to baseline in seven patients with EGFR-mutated NSCLC treated with osimertinib.
Fig. 2

A. Osimertinib or Dacomitinib

Parent  ➔ Drug-tolerant (DT) cells

Fig. 2B

Control  Osimertinib-DT  Dacomitinib-DT

Fig. 2C

PC-9  HCC4011

Control  Osimertinib  ONO  COMB

Fig. 2D

ONO7475

EGFR-TKI

pEGFR  EGFR  pERK  ERK  pAKT  AKT  pp70S6K  p70S6K  β-actin

Fig. 2E

PC-9  HCC4011

Medium  ONO7475

Osimertinib  Osimertinib + ONO7475

Medium  ONO7475

Dacomitinib  Dacomitinib + ONO7475

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Fig. 4

A. PC-9 and PC-9OR1 images showing cell morphology.

B. Graph showing % cell viability over 5 days with PC-9 and PC-9OR1.

C. Western blot analysis comparing PC-9 and PC-9OR1 for AXL, pEGFR, EGFR, pERK, ERK, pAKT, AKT, and β-actin.

D. Graph depicting % cell viability in response to varying concentrations of Osimertinib (μM) for PC-9 and PC-9OR1.

E. Bar graph illustrating % cell viability for Control, Osimertinib, ONO, and COMB.

F. Table summarizing treatment conditions for PC-9 and PC-9OR1.

G. Petri dish images showing cell growth under different conditions: Medium, Osimertinib, ONO7475, and combination treatment.
The average tumor shrinkage rate relative to baseline (Range)

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ONO-7475, a novel AXL inhibitor, suppresses the adaptive resistance to initial EGFR-TKI treatment in EGFR-mutated non-small lung cancer


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