Anti-EGF nanobodies enhance the antitumoral effect of osimertinib and overcome resistance in non-small cell lung cancer (NSCLC) cellular models

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Abstract
Osimertinib is a third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) that is effective against the EGFR T790M mutation in patients with advanced non-small-cell lung cancer (NSCLC). However, acquired resistance appears invariably due to several mechanisms. The strategy of using EGF-targeted nanobodies (Nbs) to block the initial step of the EGFR pathway constitutes a new research area. Nbs offer several advantages compared to traditional mAbs, such as their reduced size, increased stability, and tissue penetration, which provide key advantages for targeting soluble tumoral growth factors. In this study we investigated the efficacy of anti-EGF Nbs to reduce Osimertinib resistance. Two anti-EGF Nbs, generated in our laboratory, were shown to inhibit cell viability and colony formation in PC9 and PC9-derived osimertinib-resistant cell lines. The combination of these Nbs with osimertinib improved the antitumor efficacy of this EGFR-TKI in cell viability and colony formation experiments. In a mechanistic study of the EGFR pathway, the combination treatment dampened the activation of downstream proteins such as Akt and Erk1/2 MAP kinases. In addition, it increased cellular apoptosis and decreased the expression of Hes1, a cancer stem cell marker involved in metastasis and osimertinib resistance. We conclude that the addition of anti-EGF nanobodies enhances the antitumor properties of osimertinib, thus representing a potentially effective strategy for NSCLC patients.

Keywords Nanobody · Osimertinib · EGF · Lung cancer · Resistance

Introduction
Lung cancer is one of the main causes of cancer deaths in the world. Specifically, advanced non-small-cell lung cancer (NSCLC), which represents nearly 85% of the cases of lung cancer [1], is characterized by a poor prognosis and few second- or third-line effective therapies. In the past decade, research on NSCLC therapy has been centered on the inhibition of EGFR mutations through EGFR-tyrosine kinase inhibitors (EGR-TKIs), such as, erlotinib, gefitinib, afatinib or osimertinib [2]. In spite of first-generation EGFR TKIs produced significant clinical benefit, acquired resistance often occurs, most commonly via T790M mutation in the kinase domain. Osimertinib is an EGFR-TKI that has been authorized by the Food Drug Administration (FDA) for treating patients with EGFR T790M mutant NSCLC with disease progression. Osimertinib is selective for both T790M and activating EGFR mutations over wild-type EGFR, but it is highly active when T790M is present, especially EGFR(L858R/T790M), and moderately active when T790M is absent [2, 3]. Although initial response occurs, inevitably resistance to osimertinib appears [4–6]. The mechanisms of resistance might be divided into two groups: EGFR-dependent and EGFR-independent. Between the first, C797S mutation in EGFR exon 20 is the main cause...
of resistance. T790 mutation reduction or disappearance, EGFR amplification, and high EGF levels, might also causes resistance to osimertinib. EGFR-independent mechanisms are induced by activation of compensatory pathways, such as HER2 or Met [7], PIK3CA, KRas, NRas, or BRAF mutations, PTEN loss, or activation of RAS-MAPK and IGF-1R pathways, and in addition the FGF-FGFR1 autocrine loop and epithelial-mesenchymal transition are involved in this resistance mechanisms [8].

An interesting strategy to avoid osimertinib resistance due to EGFR-dependent mechanisms, is the use of anti-EGFR monoclonal antibodies, such as cetuximab or panitumumab, in monotherapy, or in combination with other drugs, such as brigatinib [9]. In addition, another possible therapeutic strategy is directly to sequester ligands that activate EGFR signaling. One example is the drug named suramin, that targets several growth factors, such as EGF, hepatocyte growth factor, platelet-derived growth factor and vascular endothelial growth factor (VEGF). However, poor efficacy and safety problems have arrested the clinical use of this compound [10]. Recently, various vaccines have been developed to block EGFR family members, particularly, CIMA-vax-EGF, a vaccine that targets the ligand EGF. In a phase III clinical trial including 405 unselected NSCLC patients, median survival was 12.4 months versus 9.4 months for the control arm. Patients with high EGF concentration in serum baseline had superior benefit than patients with low EGF concentration [11, 12]. In this way, we showed in a previous study, that antibodies raised by the EGF vaccine enhanced the efficacy of EGFR-TKIs, such as gefitinib, and delayed the emergence of resistance to EGFR-TKIs in cellular models of NSCLC [13]. Furthermore, a recent phase Ib clinical trial has evaluated the combination of first-line afatinib with anti-EGF vaccination in EGFR-mutant metastatic NSCLC [14].

Besides to monoclonal antibodies (mAbs) and vaccines, the research in oncology has seen a growing interest in the use of single-domain antigen-binding antibodies, also named nanobodies (Nbs). Nbs are the smallest functional antibody-based biologics known to date, with a molecular weight (14 kDa) that is ten times smaller than a typical mAb. Their small size, combined with a compact structure rich in β-strands, endows them with higher biochemical stability and increased solid tumor penetration, compared to conventional IgGs. Furthermore, they display a convex paratope that is well-suited for molecular recognition of small and flexible epitopes, such as the one presented by EGF, which are typically not accessible to full-length IgGs [15]. These unique features have allowed Nbs to enter clinical development for several indications, including oncology [16].

We recently reported the discovery of the first Nbs that directly target EGF and block EGFR phosphorylation and pathway activation through a new mechanism of action [17]. The most potent inhibitors (Nb1 and Nb6) showed remarkably different target recognition mechanisms, which were characterized, at the biophysical level, by distinct kinetics and thermodynamics fingerprints [18]. Here, we studied the cellular and molecular effects of these anti-EGF Nbs, alone or in combination with osimertinib, a third-generation TKI, in NSCLC cellular models, including osimertinib-resistant clones. We show that combination treatment of osimertinib with anti-EGF Nbs effectively reversed the oncogenic effects of EGF ligand, potentiated the antitumor activity of osimertinib, and delayed the appearance of resistance to osimertinib.

Materials and methods

Cell lines

Human lung adenocarcinoma PC-9 cell line, that possesses EGFR exon 19 deletion (E746-A750), were provided by F. Hoffmann-La Roche Ltd. with the authorization of Dr. Mayumi Ono (Kyushu University, Fukuoka, Japan). Human lung adenocarcinoma PC-9-OR4 is an osimertinib-resistant cell line and was generated in our laboratory by exposing PC-9 cells to increasing concentrations of osimertinib [19]. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 1% penicillin/streptomycin/glutamine (Gibco) and 10% fetal bovine serum (FBS) (Gibco) in 5% CO2, 37 °C cell culture incubator and were routinely evaluated for mycoplasma contamination. Media and supplements were obtained from Life Technologies (Gaithersburg, MD, USA).

Chemical and reagents

Osimertinib (AZD9291) was purchased from Selleck Chemicals (Houston, TX, USA). Nbs against EGF (Nb1 and Nb6) were produced as previously reported [17]. The following antibodies used were from Cell Signaling Technology (Beverly, MA, USA): EGFR (Cat#4267); phospho-EGFR (Tyr 1068) (Cat#3777); AKT (Cat#9272); phospho-AKT (Ser473) (Cat#9271); ERK1/2 (Cat#9102); phospho-ERK 1/2 (Thr202/Tyr204) (Cat#9101); Bmi1 (Cat#6964); HES1 (Cat#11988); Axl (Cat#8661); phospho-Rb (ser 780) (Cat#3390); E2F-1 (Cat#3742); p21 (cat#2947) and Cyclin D2 (Cat#3741). PARP (Cat#11835238001) was purchased by Roche. α-Tubulin from Sigma-Aldrich (Cat#T9026).

Secondary antibodies horseradish linked peroxidase anti-mouse (Cat#NX934) were purchased from GE Healthcare.
Cell viability assay

The inhibitory properties of different compounds on the cell viability of PC-9 and PC-9-OR4 were checked by the use of the colorimetric M Thiazolyl Blue Tetrazolium Bromide (MTT) method. Two thousand cells were seeded in 100 µL 0.1% FBS media and incubated for 24 h before treatment; 100 µL of various 2 × compound concentrations (1 × final concentration) were added to the wells, and the cells were incubated for 72 h. Cell viability was assessed by the MTT assay (Sigma, St Louis, MO, USA). Cells from each cell line were seeded at 2000 cells per well in 96-well plates, allowed to attach for 24 h and treated for 72 h (for details of the combination experiments, see below). After treatment, cells were incubated with culture medium containing MTT (0.75 mg/mL in medium) for 1–2 h at 37 °C. Culture medium with MTT was removed and formazan crystals resuspended in 100 µL DMSO (Sigma). Cell numbers were estimated by measuring the absorbance at 495 nm, using a microplate reader (BioWhittaker, Walkersville, MD, USA). The data reported represent the mean ± SD of a minimum of 3 experiments of each drug concentration tested.

Western blotting

In Western blot assays, cells were cultured in cell culture flasks and left untreated or treated as indicated in each experiment. Cells were lysed in ice-cold RIPA buffer [20 mM Tris–HCl (pH:7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Na3VO4, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na2VO4, 1 µg/mL leupeptin, 1 mM PMSF], and then incubated for 20 min at 4 °C, and then centrifuged. The supernatant was collected and kept at −80 °C. Protein concentration was determined by bicinchoninic acid protein assay (BSA). Equal amounts of protein from each cell lysate (30 µg/lane) were subjected to SDS polyacrylamide gel electrophoresis (SDS/PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, New Bedford, MA, USA). The membranes were blocked in Tris-buffered saline containing 5% fat free dry milk and then probed with primary antibodies at 4 °C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer’s instructions (Pierce Biotechnology, Rockford, IL, USA) and analyzed by Chemidoc MP Imaging System (Bio Rad). β-actin was used as an internal control to confirm equal gel loading.

Colony formation assay

PC-9 and PC-9-OR4 cells were seeded at 200 cells per well in a 6-well plate and incubated overnight. The cells were treated with compounds, Nb1 or Nb6 in combination with osimertinib, or DMSO as vehicle control. Culture media (supplemented with 10 ng/mL, except in the control condition) was changed every three days. On day 12, cell culture medium was removed and each well was washed with phosphate buffered saline (PBS). Then the colonies were stained with 0.5% of violet crystal at room temperature for 15 min and distilled water was used to wash the plates several times until the dye stopped coming off and was allowed to dry overnight.

Emergence of osimertinib resistance

To study the acquisition of resistance to TKIs, we seeded 350 PC-9 cells per well in 96-well plates, using two plates per treatment (120 wells) [18]. Cells were allowed to attach and the treatments were started with Nb1 or Nb6 alone (at 20 µM) or in combination with osimertinib (at 1 µM) after 2 h in RPMI + 10% FBS. Media were changed every week, plates were inspected thrice a week under the microscope, and wells >50% confluent were scored as positive [20].

Results

Anti-EGF Nbs single agent and combination with osimertinib potentiates cell viability and colony formation inhibition

To determine the antitumor activity of anti-EGF Nbs (Nb1 and Nb6), we used human lung adenocarcinoma PC-9 cells, which harbor a Glu746-Ala750 deletion in exon 19, as well as a PC-9 osimertinib-resistant cell line (PC-9-OR4), which was generated by stepwise long-term exposure to the drug [21]. Both cell types were treated for 72 h with the anti-EGF Nbs, in a dose-dependent manner to assess their effect on cellular viability. As shown in Fig. 1, Nb1 and Nb6 cause a small decrease in the viability of PC-9 cells, while their effect on osimertinib-resistant cells is negligible. Next, we assessed whether our anti-EGF Nbs could overcome intrinsic resistance to osimertinib in PC-9 and PC-9-OR4 cells, by combining different doses of osimertinib combined with Nb1 or Nb6. In these assays, the combination treatment resulted in a reduced cellular viability, which was more pronounced for the PC-9 cell line. Among the two antibodies, Nb1 exerted a more potent effect than Nb6, in agreement with their relative binding affinity and especially with their on-target residence time, which is significantly longer for Nb1 [17]. At concentrations below the half-maximal
inhibitory concentration (IC50) of each drug (e.g., 10 nM of osimertinib plus 10 µM of Nb1), a significant effect on cell viability can be observed. In PC-9-OR4 cells, however, the EGF-withdrawal effect of the Nbs is not able to fully rescue the sensitivity of PC-9 cells to osimertinib.

To study if the effect on cell viability translates into a decreased colony formation capacity, we performed clonogenic assays with PC-9 and PC-9-OR4 cells. As shown in Fig. 2, the Nbs alone did not have a significant effect on colony formation, in agreement with the cell viability results. In contrast, a dose-dependent reduction in colony formation was observed for the combined treatment, showing a clear effect at the dose of 10 µM of Nb1/Nb6 Nbs. Thus, the combination treatment reduces the formation of new colonies to a greater extent than a single treatment strategy.

**EGFR signaling pathway is inhibited by the combination of anti-EGF Nbs and osimertinib**

To assess the effects of anti-EGF Nbs in expression and activation of proteins related to EGFR-dependent osimertinib resistance [22], Western blotting analyses were conducted. In PC-9 and PC-9-OR4 cells treated with anti-EGF Nbs, nanobodies, Nb1 (in blue) or Nb6 (in red, both tested at 0, 10, or 20 µM) combined with increasing concentrations of osimertinib (Os) (0–50 nM for PC-9, 0–2500 nM for PC-9-OR4). Cell viability was analyzed by MTT. Experiments were performed in triplicate.

a dose-dependent attenuation of EGFR phosphorylation was observed, as well as of downstream molecules Akt and Erk1/2 (Fig. 3). In agreement with the effects observed in the cell viability assay, the combination of osimertinib and Nbs produced a more pronounced effect that suppressed EGFR, Akt and Erk1/2 phosphorylation.

**Anti-EGF nanobody treatment modulate the regulation of the expression of proteins involved in osimertinib resistance**

Since the combination of anti-EGF Nbs and osimertinib blocks the phosphorylation of protein kinases downstream of EGFR, which are involved in osimertinib resistance mechanisms, we aimed at studying the capacity of these Nbs to change the expression of several proteins involved in cell cycle regulation, cancer stem cell (CSC) development and apoptosis, and compared by Western blot the effects of anti-EGF Nbs alone plus osimertinib combination.

To this aim, we selected Nb1, since it shows a more potent and lasting effect on the tumor cells. In PC-9 cells, we observed that Nb1 alone produced a concentration-dependent decrease in the expression of p21, similar to...
that observed upon osimertinib treatment. A similar reduction was observed for Hes1, a key signature protein that is involved in maintaining quiescent cells and CSCs in a nondividing state. In addition, Nb1 enhanced the expression of the cell-cycle-related protein E2F, which is essential for cell cycle progression. At the higher dose of Nb1, a subtle decrease of AXL was observed, while no effects were shown for the CSC marker Bmi1, nor for the cell cycle protein Rb (Fig. 4). These effects were similar, albeit milder, in PC-9-OR4 cells.

We further assessed that the combination of osimertinib and anti-EGF Nbs combination caused a stronger effect in the expression of several of these proteins. Osimertinib was shown to increase p21 expression, thus confirming its inhibitory effect on cell cycle progression mediated via the inhibition of p53-p21-Cyclin D1 pathway [23]. However, the addition of Nb1 significantly dampened this inhibitory effect. On the other hand, the combination treatment caused a major reduction in the expression levels of Hes1 (Fig. 4), while E2F levels were increased in PC-9 cells treated with osimertinib and anti-EGF Nbs combination.
the combination. Finally, a clear increment in the cleaved PARP form was observed, which might indicate that the addition of the anti-EGF Nb potentiates the apoptotic effect of osimertinib (Fig. 4).

**Anti-EGF Nbs delay the emergence of resistance to osimertinib**

Activation of the EGFR pathway is one of the mechanisms associated with intrinsic and acquired resistance to osimertinib. In consequence, we decided to investigate the effects of anti-EGF Nbs on the emergence of resistance to osimertinib in vitro. To this end, low-confluence PC-9 cells growing in 96-well plates were treated with either osimertinib, anti-EGF Nbs, or with the combination treatment. Wells were inspected twice per week and those reaching 50% confluence were scored as positive. We found that the presence of the anti-EGF Nbs significantly delayed the emergence of resistant colonies to osimertinib. In particular, > 60% of the wells with cells treated only with osimertinib reached 50% of confluence at week 16, while only < 40% of wells in the combination treatment group reached the 50% confluence threshold. As in the previous assays, there were no significant differences between Nb1 and Nb6 in the delayed emergence of TKI resistance (Fig. 5).

**Discussion**

The administration of EGFR-TKI inhibitors is one of the most relevant therapeutic strategies in NSCLC patients. Osimertinib is currently approved by the FDA and EMA for treating patients who are positive for the T790M mutation in EGFR. Despite producing an initial clinical response, acquired resistance to osimertinib occurs, largely due to EGFR-dependent mechanisms. One of the strategies to address this problem is the selective blockade of circulating EGFR ligands such as EGF. This approach has been
applied to block similar tyrosine-kinase receptors, such as VEGF with mAbs (bevacizumab); however, drugs able to specifically target EGF have been scarcely developed. Along this line, a method based on active immunotherapy (EGF vaccine) has been developed and is currently in clinical trials [14]. In vitro, sera from the vaccinated patients—but not from control patients—blocked the EGF-EGFR interaction as well as EGFR phosphorylation, thus confirming the mechanism of action of the vaccine. We previously developed drug-like peptides targeting EGF, which blocked the EGF-EGFR interaction [24, 25]. However, their relatively weak potency resulted in incomplete EGF inhibition; thus highlighting the need for antibody-like high-affinity binders.

To this end, we recently developed a family of camelid single-domain antibodies that bind with low nanomolar affinity to EGF and block EGFR activation [17]. Compared to conventional mAbs, Nbs have a smaller size that allows them to achieve increased penetration in tumor tissues. In addition, they can be produced in prokaryotic cells at considerably reduced time and costs. In recent years, several anti-EGFR nanobodies have been developed, showing that they can efficiently inhibit cancer cell growth in vitro and tumor proliferation in vivo [26]. Beyond targeted therapy against NSCLC, Nbs are also being used in several types of drug delivery modalities, such as nanoparticles [27], quantum dots [28], and chimeric antigen receptor T-cells (CAR-T) [29]. In addition, EGFR-targeted nanobodies can be conjugated to different photosensitizing agents to induce cell death through local light activation, thanks to their shorter in vivo half-life compared to mAbs, resulting in a potent

Fig. 4 Combination of Nb1 with osimertinib downregulates expression of proteins involved in osimertinib resistance. PC-9 and PC-9-OR4 cell lines were incubated with the indicated concentrations of Nb1, alone or in combination with osimertinib (20 and 500 nM, for PC-9 and PC-9-OR4 cells, respectively) for 24 h. Protein expression and activation were analyzed by Western blotting, α-tubulin was used as the housekeeping protein. Experiments were performed twice.

Fig. 5 Effects of anti-EGF nanobodies in the emergence of resistance to osimertinib in EGFR-mutated PC-9 cells. Cells were incubated with osimertinib alone or in combination with Nb1 or Nb6. 50% of confluence was marked as positive.
antitumor effect, high tissue selectivity, and low toxicity [30, 31].

Our results showed that EGF-blocking Nbs potentiate osimertinib’s effect on cell viability, as well as in reducing cell colony formation. These effects were observed in the EGFR-mutated cell line PC-9, as well as in a PC-9-osimertinib-resistant cell line (PC-9-OR4). The analysis of the molecular basis underlying the superior efficacy of the combinatorial strategy by Western blot revealed inhibition of proteins involved in EGFR signaling pathway, such as EGFR, Akt and Erk1/2, thus showing that anti-EGF Nbs and osimertinib have a convergent impact in the EGFR signaling pathway. We further observed that the combination reduced the expression of proteins associated with cancer progression and resistance to EGFR-TKIs, such as Hes1 and Axl, in both cell line models, and Bmi1, only in PC-9-OR4 cell line. Among them, Hes1 is known to participate in drug resistance, metastasis, and cancer cell stemness [32]. Activation of Axl causes resistance to EGFR-targeted therapy in lung cancer [33] and it is closely related to epithelial-mesenchymal transition [34]. Finally, Bmi1 overexpression is a robust prognostic marker in patients with NSCLC [35] and is related with initiation and progression of hepatocellular carcinoma [36].

In both cell models tested, the expression of the apoptosis marker PARP was decreased by anti-EGF Nb1, which has a more sustained inhibitory effect than Nb6. Notably, the detection of PARP cleaved was higher when in the combination treatment in the PC-9 cell line. E2F1 has been reported to play crucial roles in proliferation, chemoresistance, metastasis and apoptosis processes through the activation of different downstream effectors. Hypophosphoryylation of Rb is indispensable for the release of active E2F1, which is required to drive the cell cycle from G1 to S phase [37]. Anti-EGF Nb1 was able to inhibit Rb phosphorylation, while the expression of E2F was decreased. Finally, we observed a reduction of p21 expression, which inhibits cyclin-dependent kinase (CDK) activity [38] and interferes with the apoptotic machinery that is often induced by chemotherapy. p21 is involved in the expression of genes that regulate cell cycle progression, DNA repair, and apoptosis, being E2F1 one of these genes [39].

In summary, the data presented herein demonstrates that anti-EGF Nbs potentiate the antitumor effects of osimertinib, increasing the antiproliferative effects, inhibiting colony formation, delaying the emergence of resistance clones, and effectively blocking, in combination, the activation of downstream signaling. These effects are very similar to those elicited by an anti-EGF vaccination, a strategy that is currently being clinically evaluated in NSCLC patients. If this approach results successful, our EGFR-targeted Nbs could be employed to temporarily deprive EGFR-addicted cancer cells of their main growth stimulus, thus extending the cancer vaccine approach to immunosuppressed patients (i.e., most advanced cancer patients) who do not respond to active vaccination. We also envisage that this combination treatment could be extended to other TKIs, thus representing a valuable strategy to improve the efficacy and duration of anti-EGFR therapy in NSCLC patients.

Author contributions Conception and design: JC-S, SG, MS–N, EG, RR; Development of methodology: JC-S, SG, MS–N. Collection and assembly of data: JC-S, SG, MS–N, EG, RR; Data analysis and interpretation: JC-S, SG, MS–N, EG, RR; Manuscript writing: JC-S, SG; Final approval of the manuscript: JC-S, SG, MS–N, EG, RR.

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Declarations

Conflict of interest S.G., M.S.-N., E.G., J.C.-S., R.R. declare no conflict of interest.

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