A Combination Of Approved Antibodies Overcomes Resistance Of Lung Cancer To Osimertinib By Blocking Bypass Pathways

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Running title: Two antibodies overcome resistance to osimertinib

Keywords: antibody, EGFR, lung cancer, osimertinib, T790M

Additional information: Our laboratory has been supported by the European Research Council, the Israel Science Foundation, the Israel Cancer Research Fund and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation. Y.Y. is the incumbent of the Harold and Zelda Goldenberg Professorial Chair. Our studies were performed in the Marvin Tanner Laboratory for Research on Cancer.

Conflict of interests: Yeda, the technology transfer office of the Weizmann Institute, protected relevant intellectual property. All authors declare that they have no other conflicts of interests.

Manuscript information: The total number of words in the main text is 4,953. This manuscript contains 6 figures and 3 supplementary figures.

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TRANSLATIONAL RELEVANCE
Mutations in epidermal growth factor receptor (EGFR) drive 10-15% of non-small cell lung cancer. Although reversible EGFR tyrosine kinase inhibitors (TKIs) are superior to chemotherapy, tumors invariably develop drug resistance. Osimertinib, an
irreversible TKI, targets T790M-EGFR, the most common mode of resistance, but several compensatory mechanisms confer resistance to osimertinib. Currently, clinicians have no targeted treatment to offer their patients once they develop resistance to osimertinib. Herein we used an animal tumor model that initially responds to osimertinib but eventually relapses. The results show that a combination of intraperitoneal cetuximab and trastuzumab, along with low-dose oral osimertinib, can persistently prevent tumor relapses following either continuous or intermittent schedules. The observed long-term reversal of osimertinib resistance is attributable to simultaneous blockade of several compensatory mechanisms previously reported in patients. Hence, the ability to nullify resistance to osimertinib in a model system using commercially available drugs may inform clinical trial development.

**ABSTRACT**

**Purpose:** Because of emergence of resistance to osimertinib, a third-generation EGFR tyrosine kinase inhibitor (TKI), no targeted treatments are available for patients with lung cancer who lose sensitivity due to new mutations or bypass mechanisms. We examined in animals and in vitro an alternative therapeutic approach making use of antibodies.

**Experimental Design:** An osimertinib-sensitive animal model of lung cancer, which rapidly develops drug resistance, has been employed. To overcome compensatory hyper-activation of ERK, which we previously reported, an anti-EGFR antibody (cetuximab) was combined with other antibodies, as well as with a sub-therapeutic dose of osimertinib, and cancer cell apoptosis was assayed.

**Results:** Our animal studies identified a combination of three clinically approved drugs, cetuximab, trastuzumab (an anti-HER2 mAb) and osimertinib (low dose), as an effective and long-lasting treatment, able to prevent onset of resistance to osimertinib. A continuous schedule of concurrent treatment was sufficient for effective tumor inhibition and for prevention of relapses. Studies employing cultured cells and analyses of tumor extracts indicated that the combination of two mAbs and a sub-therapeutic TKI dose sorted EGFR and HER2 for degradation, cooperatively enhanced apoptosis, inhibited activation of ERK, and reduced abundance of several bypass proteins, namely MET, AXL and HER3.
Conclusions: Our in vitro assays and animal studies identified an effective combination of clinically approved drugs, which might overcome resistance to irreversible TKIs in clinical settings. The results we present attribute the long-lasting effect of the drug combination to simultaneous blockade of several well-characterized mechanisms of drug resistance.

INTRODUCTION
Defective vesicular trafficking and intracellular degradation of surface receptors, such as receptor tyrosine kinases (RTKs) or integrins, is emerging as a common feature of malignant cells (1). Accordingly, pharmacological interception of the propensity of tumor cells to derail their signaling or adhesion receptors may identify novel targets for cancer therapy. Herein we address the therapeutic potential of the ability of anti-EGFR monoclonal antibodies (mAbs) to sort mutant EGFRs for intracellular degradation. Somatic mutations in the EGFR gene characterize approximately 15% of all non-small cell lung cancers (NSCLC) from Caucasian patients. While treatment with reversible EGFR-specific tyrosine kinase inhibitors (TKIs) is associated with response rates that are superior to treatment with chemotherapy, almost all patients acquire resistance within 10-14 months. The most common mechanism (>50%) of acquired resistance involves a second site mutation, T790M (2-5). Unlike patients who develop non-T790M resistance, for whom no targeted treatment options exist, second- and third-generation irreversible TKIs may be offered to T790M carriers (6). The most recently approved irreversible TKI is osimertinib, which selectively inhibits T790M-EGFR (7). Osimertinib demonstrated >50% response rate among T790M mutant tumors (8), greater efficacy than chemotherapy (9), as well as durable responses (10). Unfortunately, patients treated with osimertinib acquire resistance due to aberrant expression of NRAS and KRAS (11), activation of HER2 and MET (12), or emergence of a third site EGFR mutation, C797S (11, 13).

Experimental strategies able to overcome resistance to osimertinib include an allosteric EGFR inhibitor (14) and an ALK inhibitor that cross-inhibits EGFR (15). Clinically approved anti-EGFR mAbs like cetuximab may offer an alternative to TKIs. Four clinical trials that investigated the addition of cetuximab to chemotherapy, observed small but consistent improvements in outcome in unselected patients with NSCLC (16). Similarly, a phase Ib study which combined an irreversible inhibitor, afatinib, and cetuximab found an overall objective response rate of 29% (17).
However, the rate was comparable in T790M-positive and T790M-negative tumors. We previously examined the ability of anti-EGFR mAbs to overcome resistance to TKIs and uncovered compensatory loops that up-regulated both HER2 and HER3, as well as hyper-stimulated ERK, in response to an anti-EGFR mAb (18). Preventing this by means of a triple combination of mAbs to EGFR, HER2 and HER3 (hereinafter, 3XmAbs) arrested cell growth, sorted the three receptors for degradation, and robustly inhibited tumor growth in animal models. More recently, we demonstrated that 3XmAbs not only nullified osimertinib resistance in animal models, but it also synergized with sub-therapeutic doses of this TKI (19).

Notably, T790M tumor-bearing mice were practically cured following a short (3 weeks) treatment with 3XmAbs plus osimertinib (19). Hence, we assumed that continuous or intermittent treatments with just two mAbs might retain efficacy. In line with this prediction the animal studies we present herein reveal that blocking EGFR+HER2 is as effective as blocking EGFR+HER3, but blocking HER2+HER3 is much less effective. Because no anti-HER3 drug has so far been approved, in the present study we supplemented osimertinib with cetuximab (anti-EGFR) and trastuzumab (anti-HER2). We report that no relapses occurred in animals continuously, or intermittently, treated with a combination of osimertinib and the two antibodies (2XmAbs). Furthermore, while all animals treated with osimertinib displayed relapses after a phase of tumor regression, treatment of the re-growing tumors with 2XmAbs+osimertinib resulted in rapid and persistent tumor inhibition. According to our studies, the underlying mechanism involves forced degradation of EGFR and HER2, inhibition of several bypass routes, along with enhanced cancer cell apoptosis.

**MATERIALS AND METHODS**

**Materials**

Cetuximab, trastuzumab and osimertinib were obtained from Merck, Roche and Selleckchem, respectively, and a mAb to HER3 was generated in our lab (20). Unless indicated, antibodies were purchased from Cell Signaling Technology. PC9ER cells were cultured as described (Mancini et al., 2015). All cell lines were obtained from the American Tissue Type Culture Collection less than 3 years before starting the experiments. Periodic tests for Mycoplasms were performed using a commercially available kit.
Cell viability assays
Viability of PC9ER cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, cells (6X10^4) were seeded in triplicates in 96-well plates and treated with the indicated drugs for 48 hours. Subsequently, MTT was added and following 3 hours at 37°C, we dissolved in DMSO the water-insoluble formazan crystals, which form in metabolically active cells. Optical density was measured at 570 nm.

Cell cycle analysis and apoptosis assays
Following 48 hours of treatment, PC9ER cells were incubated for 60 minutes with bromodeoxyuridine (BrdU; 10 μM) and then washed, harvested and fixed in ethanol (at 4°C). Thereafter, cells were incubated in a denaturation solution (2N HCl, 0.5% Triton-X100; 30 min), followed by a neutralization solution (0.1 M sodium borate, pH 8.5; 30 min). BrdU that incorporated into newly synthetized DNA was then assayed using an APC-conjugated anti-BrdU antibody. Total DNA content was determined by using a propidium iodide (PI) solution supplemented with RNase A. Cell cycle distribution was detected by flow cytometry. Apoptosis assays were performed using the FITC Annexin V Apoptosis Detection Kit with 7-AAD (from BioLegend) and analyzed by flow cytometry. Both assays were performed on a BD FACSaria Fusion instrument controlled by BD FACS Diva software v8.0.1 (BD Biosciences). Further analysis was performed using the FlowJo software v10.2 (Tree Star).

Immunofluorescence analyses
Surface expression levels of specific RTKs were detected in vitro by means of immunofluorescence. The following anti-receptor antibodies were used: anti-EGFR (Cell Signaling, #4267S), anti-HER2 (Cell Signaling, #4290S) and anti-HER3 (Cell Signaling #12708S). Cells were grown for 24 hours on sterile coverslips and treated as indicated. Following 24 hours of treatment, cells were washed in PBS containing 0.01% of Tween 20 (w/v). Thereafter, they were fixed in 4% formaldehyde in PBS (overnight at 4°C). After washing the cells on the next day, they were blocked with 2% of fetal bovine serum, for 30 min. Next, cells were incubated overnight with anti-receptor antibodies (1:50 dilution) at 4°C. Thereafter, cells were washed thrice, followed by the FITC-conjugated secondary antibody (for 45 min in dark),
counterstained with DAPI and mounted on slides for image capturing using a confocal microscope (40X magnification). Images were processed using the Zeiss ZEN2011 software.

**Flow cytometry analyses of surface receptors**

To evaluate surface receptor levels, cells were treated with trypsin and washed twice in saline containing albumin (1% w/v). Thereafter, cells were incubated for 30 min at 4°C using antibodies to EGFR (clone AY13), HER2 (clone 24D2) and HER3 (clone 1B4C3), which were conjugated to the following fluorophores: Alexa Fluor 488, allophycocyanin and phycoerythrin (BioLegend Inc). Fluorescence intensity was measured using BD FACSARia Fusion flow cytometer.

**Tumorigenic cell growth in mice**

All animal studies were pre-approved by the Weizmann Institute’s Review Board (IRB) and adhered to the NIH Guide for the Care and Use of Laboratory Animals. Mice were injected subcutaneously in the right flank with cancer cells (3-4X10^6 per mouse). Antibodies were injected intraperitoneally at 200 μg (total) per mouse per injection, twice weekly. Daily administration of osimertinib used oral gavage. Tumor width (W) and length (L) were measured twice a week using a caliper and tumor volume (V) was calculated according to the following formula: V = 3.14×(W^2 ×L)/6. Body weight was evaluated once per week. Mice were euthanized when tumor size reached 1,500 mm³. Investigators were not blinded to the group allocation during the experiment and when assessing outcome.

**Statistical data analyses**

All data were analyzed using the Prism GraphPad software and statistical analyses were performed using one or two-way ANOVA with the Dunnett’s or Tukey’s test (*, p≤0.05; **, p≤0.01; ***. p≤0.001; ****, p≤0.0001). Flow cytometry analysis was performed on a BD FACSARia Fusion Instrument. Further analysis was performed using the FlowJo software v10.2 (Tree Star). Staining intensity was determined using ImageJ.

**RESULTS**
Short treatments combining two or three anti-receptor antibodies with osimertinib variably inhibit an EGFR-driven NSCLC model

No targeted therapy is currently available for treatment of NSCLC patients after they develop resistance to osimertinib (21). We previously reported that a 3-week long treatment of an erlotinib-resistant animal model (del746/50 and T790M) using a mixture of three antibodies and a sub-therapeutic dose of osimertinib inhibited tumor relapses, for at least 4 months after termination of all treatments (19). The unusually high and prolonged efficacy we observed in tumor-bearing mice prompted us to test simpler mixtures of mAbs, which might associate with reduced toxicity. Accordingly, we implanted erlotinib-resistant PC9ER cells carrying two EGFR mutations in the flanks of athymic mice, and randomized the animals to several arms once tumors reached approximately 450 mm$^3$. To resolve small differences in efficacy, we limited all treatments to three weeks only: two antibody injections per week (intraperitoneal delivery) or a single daily treatment with osimertinib (oral delivery). As expected, a 3-week long treatment with osimertinib (monotherapy), either high dose (H; 5 mg/kg/day) or low dose (L; 1 mg/kg/day) resulted in complete or partial tumor shrinkage, respectively, but tumors re-appeared immediately after we stopped oral administrations (Figs. 1A and 1C). In contrast, treatment with 3XmAbs (cetuximab, trastuzumab and mAb33, a murine antibody specific to HER3) induced slower shrinkage of pre-established tumors, and subsequent relapses were delayed for 7-8 weeks in this arm.

As expected, the combination of low-dose osimertinib and 3XmAbs induced the strongest therapeutic effect: a 3-week treatment completely prevented any subsequent relapses (Figs. 1A and 1C). Notably, when tested alone or pairwise, none of the three antibodies caused tumor shrinkage comparable to the rapid and strong effect of high dose osimertinib. In contrast, although low-dose osimertinib (1 mg/kg/day) caused only limited tumor shrinkage, when it was combined with antibodies we observed clear cooperative effects: three pairwise combinations of mAbs (topped with low dose osimertinib) were tested, and while none prevented relapses, two combinations: cetuximab + trastuzumab + osimertinib and cetuximab + mAb33 + osimertinib, were comparably effective and clearly superior to the effect of trastuzumab + mAb33 + osimertinib (Figs. 1A and 1B). Notably, a recent study combined cetuximab and the therapeutic dose of osimertinib (25 mg/kg/day), and
reported that nine out of ten PC9-T790M xenografts remained progression-free at 52 weeks (Della Corte et al., 2018). Yet another study reported that one tenth of this dose (2.5 mg/kg) was sufficient for blocking both PC-9 (ex19del) and H1975 (L858R/T790M) tumor xenografts (Cross et al., 2014). We conclude that antibody-mediated targeting of the mutant form of EGFR, along with either co-receptor (i.e., HER2 or HER3) can achieve comparable delays of relapses, provided that a relatively low dose of osimertinib is simultaneously delivered.

**In vitro, osimertinib enhances the ability of a combination of cetuximab and trastuzumab to inhibit both viability and migration of NSCLC cells**

In comparison to 3XmAbs+osimertinib, the results presented in Figure 1 identified two simpler and relatively effective combinations: cetuximab+trastuzumab or cetuximab+mAb 33 (anti-HER3). Although several anti-HER3 antibodies have reached clinical trials, so far none has been approved for medical applications (reviewed in (22)). Because the profile of toxicity of trastuzumab is well characterized, but toxicity of anti-HER3 antibodies is only partly known, we focused all our subsequent animal studies on the combination of osimertinib (low dose) and the two clinically approved mAbs, namely cetuximab and trastuzumab (hereinafter, 2XmAbs).

In vitro assays that examined cell viability following 48 hours of drug treatment detected no marked effects of either antibody, or a combination of the two mAbs (Fig. 2A). In contrast, all treatments that used osimertinib, which we tested either at high (H; 500 nM) or low (L, 100 nM) concentrations, strongly inhibited cell viability. Notably, this in vitro assay detected no benefit of concurrent application of the TKI and the two antibodies. Hence, we employed two additional assays with the aim of understanding the cooperative in vivo effect of the drug combination. The first assay examined cell cycle distributions of PC9ER cultures following 48 hours of treatment with osimertinib, either alone or combined with antibodies (Fig. 2B).

Unlike the viability assay, the cell cycle test detected a clear effect of 2XmAbs when applied alone: both the S-phase fraction and the G2/M fraction were reduced in response to the treatment, whereas the G0/G1 phase increased. As expected, osimertinib strongly reduced the S-phase fraction, but we observed a further diminution when the TKI was co-administered with 2XmAbs. Conceivably, 2XmAbs
arrests erlotinib-resistant PC9ER cells at the G0/G1 phase by activating a senescence-like process (23-25), which we previously observed following treatment of NSCLC cells with 3XmAbs (19). Consistent with this interpretation, prolonged treatment of PC9ER cells with 2XmAbs strongly induced senescence-associated β-galactosidase (SA-β-Gal; Supplementary Fig. S1), a hallmark of cellular senescence (26). We noted that both cetuximab and trastuzumab induced partial effects on senescence, but prolonged treatments (>12 days) that included osimertinib promoted extensive cell death.

Similar to the additive effects on cell growth, the other test we employed, a cell migration assay, detected a moderate cooperative action of the TKI and two mAbs (Fig. 2C). The ability of PC9ER cells, which were pre-plated on a porous filter, to migrate for 24 hours through the pores was strongly or weakly inhibited by osimertinib or 2XmAbs, respectively. A further, rather moderate but reproducible decrease in the ability of cells to migrate was observed when co-treated with a combination of osimertinib and the two antibodies (see data quantification in the lower part of Fig. 2C). In conclusion, the in vitro tests we employed detected cooperative inhibitory effects of the TKI+2XmAbs combination on both cell migration and G0/G1 cell cycle arrest. It is worth noting that the assays we used may be considered as relatively short snapshots, such that the observed, rather moderate cooperative effects, are likely amplified by the longer-term scale of the animal tests (weeks/months).

Osimertinib and anti-receptor antibodies differently affect surface abundance of EGFR, HER2 and HER3

Previous studies linked the anti-tumorigenic effects of anti-RTK antibodies to their ability to accelerate receptor endocytosis and degradation (27-29). Hence, we tested the ability of osimertinib, 2XmAbs and their combination to regulate the abundance of HER/ERBB family members. The first assay employed confocal microscopy, which was used to determine cell surface abundance of EGFR, HER2 and HER3 following a 24-hour long treatment with the drugs (Fig. 3A). This assay revealed that exposure to osimertinib strongly up-regulated surface HER3, in line with a previous report, which inhibited AKT signaling and observed elevated expression of HER3 (30). Unlike osimertinib, the mixture of two mAbs reduced staining with antibodies to EGFR and HER2, but HER3 displayed only moderate alterations. Congruent with
these observations, the combined treatment with 2XmAbs+osimertinib induced up-regulation of HER3, along with downregulation of EGFR and HER2. To further characterize effects on HER3 and EGFR we used immunoblotting, which detects both surface and intracellular receptor levels (Figs. 3B). As expected, treatment with osimertinib alone blocked phosphorylation of EGFR, ERK and AKT, as well as increased HER3. In addition, 2XmAbs downregulated EGFR and HER2 and increased HER3 and pHER3. Notably, cells treated with the osimertinib+2XmAbs displayed very low levels of active EGFR, HER2, HER3, ERK and AKT. To quantify these effects and also focus on receptor molecules localized to the cell surface, we employed flow cytometry (Fig. 3C). As expected, the mixture of two antibodies, when tested alone, downregulated surface levels of both EGFR and HER2. In addition, we observed similarly strong (4-fold) increases in surface HER3 after treatment of cells with either osimertinib or the combination of osimertinib and 2XmAbs. In conclusion, the three complementary assays we employed raised the possibility that the therapeutic effects observed when combining in animals osimertinib and 2XmAbs relate to the ability of this drug combination to downregulate signaling downstream to EGFR and the co-receptor, HER2. Nevertheless, the combination of mAbs and TKI was unable to abrogate (within 24 hours) the ability of osimertinib to up-regulate surface HER3, a receptor involved in acquired resistance to TKIs (31).

The combination of osimertinib and 2XmAbs increases apoptosis of erlotinib-resistant NSCLC cells

Previous studies reported that osimertinib induces apoptosis in a process involving up-regulation of PUMA (32) and BIM, along with downregulation of MCL1 (33). To examine the effect of 2XmAbs in combination with osimertinib, we probed PC9ER cells for annexin V and 7-amino-actinomycin D (7-AAD), which is excluded from early apoptotic cells, while late stage apoptotic cells stain positively due to passage of the dye into the nucleus. Due to long-term incubation times and cooperative effects of 2XmAbs and sub-maximal osimertinib, our apoptosis tests made use of a relatively low concentration of osimertinib (40 nM). These tests detected strong apoptosis of PC9ER cells that were pre-treated for 72 hours with osimertinib, but 2XmAbs induced very weak apoptosis (Fig. 4A). Interestingly, however, treatment with a
combination of cetuximab, trastuzumab (each at 0.01 mg/ml) and osimertinib (40 nM), followed by quantification of the fractions of early and late apoptotic cells showed that, in comparison to osimertinib alone, the antibodies increased cell death, primarily by enhancing early apoptosis. Next, we analyzed BIM, a pro-apoptotic protein, the abundance of which predicts TKI-induced suppression of lung tumors (34). Using an antibody specific to the three forms of BIM, we confirmed that protein levels were rather low in untreated cells, but a 24- to 48-hour long treatment with either 2XmAbs or osimertinib elevated the large (BIM_{EL}), as well as the other forms of BIM (Figs. 4B and 4C). Interestingly, while osimertinib strongly induced cleavage of caspase 3, only weak cleavage was observed in 2XmAbs-treated cells. Nevertheless, co-treatments that used a combination of the TKI and the two mAbs more strongly activated caspase 3 and enhanced BIM’s abundance after 24 hours. In conclusion, the set of in vitro assays we performed confirmed the enhanced anti-tumorigenic potential of co-treatments employing a TKI and a mixture of two mAbs. Notably, while osimertinib strongly inhibits cell cycle progression and induces death of EGFR-mutant expressing cells, treatment with 2XmAbs emerges as an inducer of cell cycle arrest, which can collaborate with osimertinib.

Both continuous and intermittent treatments using a combination of two antibodies and a TKI robustly prevent resistance to osimertinib

The in vitro validated positive mAb-TKI interactions raised the possibility that animal treatments longer than the three weeks period we applied (see Figure 1) might delay onset of resistance. To test this prediction, we compared the drug holiday scenario with either a continuous administration protocol or with an intermittent drug delivery. The continuous protocol was first applied to mono-therapy osimertinib, either low (1 mg/kg) or high dose (5 mg/kg; Fig. 5A). For this, we implanted PC9ER cells in the flanks of 9 athymic mice and, once tumors became palpable, orally delivered osimertinib once daily. As expected, within the first 2-3 weeks of treatment all animals displayed rapid tumor shrinkage (note that each color represents one animal). However, following a variable length remission (50-110 days), eight of nine animals displayed relapses and their tumors were sampled for future transcriptomic/proteomic analyses.
Using a similar tumor implantation protocol, which was followed by a 3-week long treatment with 2XmAbs plus osimertinib (1 mg/kg), we confirmed the initial pattern of rapid shrinkage of pre-established tumors (Fig. 5B). However, approximately 60 days after termination of all drug deliveries, we observed the beginning of a variable phase of relapse. Altogether, relapses took place in 6 out of the 16 animals used in this arm. Two variants of the protocol of Figure 5B were used side-by-side: (i) a ‘No Holiday’ group, which was continuously treated with low-dose osimertinib and 2XmAbs (Fig. 5C), and (ii) an ‘Intermittent’ group, which was similarly treated for 21 days, but this was followed by 4 cycles of a holiday (14 days) and treatment (7 days; Fig. 5D). Note that for ethical reasons, the regimen applied to the ‘No Holiday’ group was changed from a daily oral osimertinib administration to once every other day, but the concomitant 2XmAbs treatment remained unaltered.

The results we observed in the ‘No Holiday’ group were quite encouraging: no relapses were observed, provided that treatment was carried out continuously. Similarly, although small and transient relapses were initially observed in the ‘Intermittent’ group, following 2-3 treatment-holiday cycles all tumors, with no exception, were completely eradicated. In conclusion, admixing two antibodies and a sub-therapeutic dose of the TKI completely and persistently inhibited resistance of T790M-expressing mouse models. However, the striking effects we observed required continuous or intermittent treatments using a combination of three drugs.

In response to the combined ‘antibodies plus TKI’ treatment, tumors shut down multiple bypass pathways and strongly activate apoptosis

Mechanisms of acquired resistance to EGFR-specific TKIs may be divided into EGFR-dependent (e.g., new mutations or \(\text{EGFR} \) amplification) and EGFR-independent modes (35). The latter mode of resistance often involves activation of a surrogate RTK. For example, activation of MET and HER2 was found in patients who acquired resistance to osimertinib (36). Another RTK, AXL, is an oncogenic receptor implicated in both inhibition of apoptosis and resistance to drugs (37-39). Notably, the most prevalent mechanism of resistance, namely amplification of \(\text{MET} \) (40, 41), promotes formation of MET-HER3 complexes, which restore an AKT-mediated anti-apoptosis pathway (42). Similarly, \(\text{HER2} \) is amplified in 12% of tumors with acquired resistance (43). To examine RTK alterations and apoptosis, we treated pre-established PC9ER xenografts with either osimertinib, a mixture of two antibodies (2XmAbs), or
a combination of all three drugs. Tumor extracts were prepared seven days after treatment initiation, and they were subjected to immunoblotting using anti-RTK antibodies. As shown in Figure 5E, a seven-day long treatment with 2XmAbs, either alone or in combination with osimertinib, effectively downregulated not only EGFR and HER2, but also HER3, MET and AXL. By contrast, animal treatment with osimertinib alone exerted much weaker effects on RTK levels and, in similarity to the in vitro assays shown in Figure 3, strongly elevated HER3 expression, a potential mechanism of therapeutic resistance (44). Notably, however, this putative bypass loop was nullified when osimertinib was combined with 2XmAbs (Fig. 5E). To corroborate the observed effects on RTK abundance, we analyzed tumors from additional animals. Altogether, six tumor-bearing mice were analyzed. This is exemplified by Supplementary Figure S2, which quantitatively analyzed two additional mice and confirmed simultaneous downregulation of five RTKs (EGFR, HER2, HER3, MET and AXL) by 2XmAbs+osimertinib, already after 3 days of treatment.

Because all three treatments we examined in animals inhibited, in part, the active forms of both ERK and AKT, a survival-supporting kinase, we probed for two markers of apoptosis, caspase 3 and BIM. Tumor extracts were prepared 3, 7 and 14 days following treatment initiation and probed with the respective antibodies. In line with the in vitro assays of apoptosis (see Fig. 4), we observed cooperative activation of caspase 3, along with a marked induction of all three forms of BIM, which is known to release cytochrome C from mitochondria and consequently activate caspase 3 (Fig. 5F). In conclusion, biochemical analyses of NSCLC xenografts attributed the anti-tumor efficacy of the combination of antibodies and osimertinib to an ability to degrade EGFR, as well as to block its intrinsic catalytic activity, and to a concomitant antibody-induced shutdown of several mechanisms of resistance involving surrogate RTKs, namely HER2, HER3, MET and AXL.

Combining osimertinib and two (or three) mAbs eradicates tumors after they acquired resistance to osimertinib

No upfront polytherapy is currently used to treat patients with the T790M mutation (21). Instead, once patients are diagnosed with a primary site EGFR mutation they receive treatment with a first-generation drug (e.g., elrotinib), and those who develop
resistance due to the T790M mutation receive osimertinib until resistance emerges. To simulate this scenario and test if a combination of osimertinib and mAbs can inhibit tumors after they acquired resistance to osimertinib, we adopted a second line protocol: erlotinib-resistant cells were implanted under the skin of mice and once tumors reached a volume of 400-600 mm$^3$, mice were orally treated with osimertinib (1 mg/kg), once per day. Invariably, all tumors regressed and after 3-4 weeks they started to relapse. Once they re-gained 400-600 mm$^3$, mice were randomized and received the following treatments: (i) 3XmAbs plus low-dose osimertinib (Fig. 6A), or (ii) 2XmAbs plus low-dose osimertinib (Fig. 6B). Remarkably, all animals treated with the combination of osimertinib and either two or three mAbs showed complete tumor elimination 2-6 weeks later, and no relapses were detected.

Next, we asked if osimertinib was necessary after commencement of antibody treatment. To this end, we used the protocol of Figure 6, but once regrowth was in full swing we switched to treatments containing mAbs only, either three antibodies (3XmAbs; Supplementary Figure S3A) or two antibodies (2XmAbs; Supplementary Figure S3B). Unlike the uniform eradication of tumors observed when we combined osimertinib and antibodies, the omission of osimertinib resulted in variable responses, ranging from rapid tumor eradication to tumor progression. Taken together, this and other observations we made underscore the power of concurrent, rather than sequential, treatments making use of antibodies and a TKI. Furthermore, our results attribute the cooperative TKI-mAb effects to shutdown of evasion mechanisms previously reported in osimertinib-treated patients. Thus, the animal studies and the results we obtained in vitro identify 2XmAbs plus osimertinib as a highly effective and commercially available combination, which might warrant trials in clinical settings.

DISCUSSION

Our motivation to examine the antibody alternative to lung cancer treatment has stemmed from the recurring relapses observed in almost all patients treated with first-, second- and third-generation TKIs. The cellular system we applied, namely PC9ER cells, reliably recapitulates the recurring cycles of drug sensitivity followed by tumor relapses. For example, the data shown in Figure 5A demonstrate rapid and general tumor relapses, which exhibited animal-specific variation in terms of onset and rate of re-growth. We previously reported that emergence of the C797S mutation and
overexpression of EGFR characterize some relapsing tumors in the PC9ER model (19). To overcome insurgence of resistance, our immunological strategy underwent a three-step evolutionary process, which led to the herein proposed combination of 2XmAbs (i.e., cetuximab and trastuzumab) and a sub-therapeutic dose of osimertinib. In the first step we examined a single, home-made anti-EGFR antibody (18). Although the antibody robustly downregulated EGFR, we observed marked activation of downstream signalling due to two bypass feedback loops, which elevated HER2 and HER3. Concurrent inhibition of EGFR, HER2 and HER3 prevented over-activation of ERK and effectively inhibited tumor growth. In the second step we contrasted the mechanisms underlying the anti-tumorigenic effects of osimertinib and 3XmAbs, a combination of cetuximab, trastuzumab and a home-made anti-HER3 antibody (19). Because osimertinib induced apoptosis of PC9ER cells but 3XmAbs arrested cell growth, while activating a senescence-like process, we combined the two treatments. Remarkably, tumors were persistently eradicated after just 3 weeks of treatment. To reduce potential skin and gastrointestinal toxicities, due to the inclusion of an anti-HER3 antibody, we tested in the present study a simpler combination, which included three clinically approved drugs, namely cetuximab, trastuzumab and osimertinib.

The major finding reported herein is the surprisingly strong and durable effects exerted in vivo by a combination of two antibodies and a sub-optimal dose of osimertinib. Only a fraction of this synergistic effect was captured by our in vitro assays of cell cycle progression and migration, but this might be due to the short-term nature of the in vitro treatments. Notably, it has previously been reported that a combination of a TKI (i.e., afatinib) and cetuximab might overcome T790M-mediated resistance in clinical settings (17). In our animal model a single mAb was insufficient; the combined effect of osimertinib and cetuximab was greatly enhanced by either trastuzumab or by an antibody to HER3 (Fig. 1). By contrast, a triplet containing trastuzumab, our home-made anti-HER3 antibody and osimertinib, was inferior in animals, in line with the driver function of mutant EGFRs.

In view of the added value of simultaneously blocking both the mutant form of EGFR and the wild type form of HER2, it is worthwhile reviewing the role played by HER2 in NSCLC (45). HER2 aberrations, including amplification and mutations, have been classified as oncogenic drivers involved in 2-6% of lung adenocarcinomas.
HER2 amplification serves also as an important mechanism of acquired resistance to EGFR kinase inhibitors, including osimertinib (46). FISH analysis revealed that HER2 was amplified in 12% of tumors with acquired resistance to EGFR inhibitors, versus only 1% of untreated lung adenocarcinomas (47). Moreover, HER2 amplification and presence of EGFR(T790M) were mutually exclusive. In addition, HER2 amplification, as well as amplification of MET, have been found in patients who developed resistance to osimertinib (12). Altogether, these lines of evidence and the herein reported cooperative effects of cetuximab and trastuzumab, portray HER2 as a major partner of mutant forms of EGFR in lung tumors.

Understanding how exactly do cetuximab and trastuzumab collaborate with osimertinib remains a matter for further investigation. Evidently, the two antibodies not only block the EGFR pathway, but also inhibit multiple surrogate routes, namely HER2, HER3, MET and AXL, which are often activated in drug-resistant tumors (48). In line with concurrent inactivation of several, partly redundant, survival pathways, we observed growth arrest both in vitro and in animals, along with sensitization of cells to the apoptosis-inducing action of osimertinib. Our working hypothesis assumes that cetuximab sorts EGFR to degradation in lysosomes, while osimertinib inhibits kinase activity of the remaining T790M-EGFR molecules, and the combination with trastuzumab blocks frequent routes of drug resistance. Unlike the well-understood inhibition of EGFR by both a TKI and a mAb, mechanisms enabling inhibition of several bypass receptors remain unknown. For example, this might be achieved by a RAB-family member, such as RAB25, which regulates recycling of both RTKs and integrins (49). Alternatively, catalytic activation of ADAM-family surface proteases by the combination of mAbs and osimertinib may underlay shedding of the ectodomain of several RTKs, thereby explains the observed disappearance of RTKs like MET and AXL (Fig. 5E).

In summary, our study reports for the first time a potential therapeutic intervention for TKI-resistant lung cancer, namely a combination of two mAbs and a sub-therapeutic dose of osimertinib. The proposed intervention is able to completely disarm mutant EGFRs and, at the same time, block several bypass routes. Hence, regardless of the underlying molecular mechanisms, the cocktail of three clinically approved drugs offers hope for patients who are currently developing resistance to osimertinib and remain with no targeted treatment options.
REFERENCES


ACKNOWLEDGMENTS
Our laboratory has been supported by the European Research Council, the Israel Science Foundation, the Israel Cancer Research Fund and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation. Y.Y. is the incumbent of the Harold and Zelda Goldenberg Professorial Chair. Our studies were performed in the Marvin Tanner Laboratory for Research on Cancer.

AUTHOR CONTRIBUTIONS

DR, LM, MM and YY designed the experiments.

DR, LM, MM, IM, AN, SS, SG, ML, TMS and RE performed the experiments.

DR, LM, MM and YY analyzed the data.

DR and YY wrote the manuscript.

All authors declare no competing interests.

COMPETING FINANCIAL INTERESTS

Yeda, the technology transfer office of the Weizmann Institute, protected relevant intellectual property. All authors declare that they have no other conflicts of interests.

FIGURES AND LEGENDS TO FIGURES

Fig. 1. Combining different anti-receptor antibodies with a kinase inhibitor variably inhibits an EGFR-driven lung cancer model. PC9ER cells (4X10^6 cells per animal) were subcutaneously implanted in CD1-nu/nu mice. When tumors became palpable, mice were randomized into groups of 7-9 animals that were later treated for 3 weeks (hatched area) with the indicated antibodies (total dose: 0.2 mg/mouse/injection) once every 3 days, or with osimertinib, either high dose (H; 5 mg/kg/day), or low dose (L; 1 mg/kg/day). In addition, we tested the indicated combinations of mAbs and the kinase inhibitor. Note that all treatments were stopped on Day 21. However, tumor growth was followed for approximately 100 additional days (A and B), and animal survival was followed for 180 days and presented as Kaplan-Meyers curves (C and D). Mice were euthanized when tumor size reached 1,500 mm^3. Data are means ± SEM from 7-9 mice of each group. Note that for technical reasons, we aborted the cetuximab + osimertinib (L) arm on day 61.
Fig. 2. Osimertinib treatment decreases cell viability and, when combined with 2XmAbs, it decreases cell migration and cell cycle progression. (A) PC9ER cells were cultured for 48 hours and later incubated with the following agents: osimertinib, either low dose (L; 0.1 μM) or high dose (H; 0.5 μM), cetuximab (0.02 mg/ml), trastuzumab (0.02 mg/ml) or a combination of the two antibodies. Cell viability was determined using the MTT assay. Data are means ± S.D. values from three experiments. (B) PC9ER cells (100,000 per sample) were treated for 48 hours with saline, 2XmAbs (cetuximab plus trastuzumab; 0.02 mg/ml), osimertinib (0.1 μM), or with the two treatments combined (2XmAbs and osimertinib). Following incubation with BrdU (60 min), cells were fixed and subjected to BrdU and propidium iodide (PI) detection. Shown are the means of cell cycle distributions observed in three experiments that used cytometry. (C) PC9ER cells (2X10^4) were seeded in duplicates on filters of transwell chambers under the indicated treatments (see B for the doses). The upper panel shows representative images of cells that migrated to the lower side of the filters (scale bars, 500 μm). The histogram shows quantification of migrated cells relative to control. *, p=0.0173; ns, not significant.

Fig. 3. The combination of osimertinib and two mAbs reduces EGFR and HER2 abundance. (A) PC9ER cells were seeded on cover slips and treated for 24 hours with saline (Control), 2XmAbs (cetuximab and trastuzumab; 20μg/ml total), osimertinib (100 nM), or with the combination (2XmAbs plus osimertinib). Cells were later fixed in formaldehyde (4%) and incubated with specific primary antibodies, followed by a secondary, FITC-conjugated antibody. DAPI staining (blue) indicates locations of nuclei. Images were captured using a confocal microscope (40X magnification). Bar (white, vertical), 50 μm. (B) PC9ER cells were treated for 24 hours with vehicle (DMSO), increasing concentrations (25, 50, 100 nM) of osimertinib (Os.), 2xmAbs (cetuximab and trastuzumab, 20 μg/mL total), or the combination (2xMabs plus Os.). Cell extracts were blotted and probed for the indicated proteins. GAPDH and tubulin served as loading control. (C) PC9ER cells were treated for 24 hours with saline (CTRL), 2XmAbs (20 μg/ml), osimertinib (100 nM), or with the combination (2XmAbs plus osimertinib). Thereafter, cells were analyzed, using flow cytometry, for surface expression levels of EGFR, HER2 and
HER3. Normalized surface expression levels (means of two independent experiments) are shown.

**Fig. 4.** The combination of osimertinib and 2XmAbs increases apoptosis of erlotinib-resistant NSCLC cells. (A) PC9ER cells were treated for 72 hours with the following agents: saline (PBS), 2XmAbs (cetuximab and trastuzumab, each at 0.01 mg/ml), osimertinib (40 nM), or the combination of 2XmAbs and osimertinib. Shown are results of an apoptosis assay performed using an annexinV/7-AAD kit (BioLegend, Inc.). Quantification of the fractions of early and late apoptotic cells is shown in histograms. The experiment was repeated three times. Note the isoforms of BIM. (B) PC9ER cells were treated for 24 or 48 hours with saline, osimertinib (40 nM), 2XmAbs (cetuximab and trastuzumab, each at 0.01 mg/ml), or with a combination of 2XmAbs and osimertinib. Shown is a representative immunoblot probed with antibodies to caspase 3 or to BIM. GAPDH levels were used to control gel loading. Control samples were treated with the solvent of osimertinib (DMSO) or with saline. (C) PC9ER cells were treated with osimertinib (as indicated) for 48 hours, and extracts were blotted using the indicated antibodies.

**Fig. 5.** Continuous treatments using a combination of two antibodies reverses resistance to osimertinib, downregulates several RTKs and cooperatively activates apoptosis. (A-D) PC9ER cells (2x10⁶) were subcutaneously engrafted in CD-1 nu/nu mice. Once tumors became palpable, mice were subdivided into several groups. Note that each colored line represents one animal. Mice of the OSIMERTINIB group (A) were treated daily, for 60 days, with oral osimertinib, either low dose (1 mg/kg; dotted lines) or high dose (5 mg/kg; solid lines). Thereafter, we switched to osimertinib treatment every other day (dotted underneath line). (B) Palpable tumors of the HOLIDAY group were treated twice a week with 2XmAbs (0.2 mg/mouse/injection) plus daily oral osimertinib administrations (1 mg/kg). All treatments were stopped on day 21 and tumor volumes were monitored in the next 4 months. (C) Tumors of the NO HOLIDAY group were treated as in B twice a week with a combination of cetuximab and trastuzumab, combined with daily administrations of osimertinib (1 mg/kg). This regimen was changed on day 60 to osimertinib treatment once every other day, but mAb treatment remained unaltered (underneath dotted line). (D) The INTERMITTENT group was treated for 21 days,
twice a week, with 2XmAbs plus daily osimertinib (1 mg/kg). This was followed by 4 cycles of a break (14 days) and treatment (7 days), as indicated by the underneath segmented line. \( (E-F) \) Tumor-bearing mice (see above) were randomized into groups of 3 animals. Osimertinib (1 mg/kg/dose) was daily administered using oral gavage, whereas the antibody combination (2XmAbs, cetuximab and trastuzumab; 0.2 mg/mouse/injection) was administered intraperitoneally once every three days. Shown are immunoblots of tumor extracts isolated one week after treatment initiation (E) or 3, 7 and 14 days after treatment onset (F). Note that the three forms of BIM are shown.

**Fig. 6. Combining a low dose of osimertinib with mixtures of either two or three monoclonal antibodies (2XmAbs or 3XmAbs) eradicates osimertinib-resistant tumors.** PC9ER cells \((2 \times 10^6)\) were subcutaneously injected in the flanks of CD-1 nu/nu mice. Once tumors reached a volume of 300-600 mm\(^3\), mice were orally treated with osimertinib (1 mg/kg), once per day. Invariably, tumors regressed and thereafter relapsed. Once relapsing tumors reached their original volume, mice were randomized and injected intraperitoneally twice a week with either 3XmAbs (A) or with 2XmAbs (B), while still receiving daily oral treatments with osimertinib. Note that each panel displays results obtained using one animal.
Figure 1

A

- Vehicle
- osimertinib (L)
- osimertinib (H)
- Cetuximab
- Cetuximab + osimertinib (L)
- Cetuximab + Trastuzumab
- Cetuximab + Trastuzumab + osimertinib (L)
- Cetuximab + mA33
- Cetuximab + mA33 + osimertinib (L)
- Trastuzumab + mA33
- Trastuzumab + mA33 + osimertinib (L)
- 3XmAbs
- 3XmAb + osimertinib (L)

B

C

D

ANIMAL SURVIVAL (%)

TUMOR VOLUME (mm³)

TIME (days)

TIME (days)
Figure 3

A

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B

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C

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Figure 4

A

Control

2XmAbs

Osimertinib

Osimertinib + 2XmAbs

7-AAD-A

Annexin FITC-A

Cell Count (%)

Control 2XmAbs Osimertinib Osimertinib + 2XmAbs

Late apoptosis

Early apoptosis

live

200

150

100

50

0

B

24h 48h

Osimertinib (40nM)

2XmAbs (20µg/mL)

kDa

35

19/17

23

15

12

37

Caspase 3

Cleaved-Caspase 3

BIMeli

BIMli

BIMsi

GAPDH

C

- - 20 20 40 40

Osimertinib (nM)

2XmAbs (20µg/mL)

kDa

35

19/17

23

15

12

37

Caspase 3

Cleaved-Caspase 3

BIMeli

BIMli

BIMsi

GAPDH
A Combination Of Approved Antibodies Overcomes Resistance Of Lung Cancer To Osimertinib By Blocking Bypass Pathways

Donatella Romaniello, Luigi Mazzeo, Maicol Mancini, et al.

Clin Cancer Res  Published OnlineFirst July 2, 2018.

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