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SHORT COMMUNICATION

EGFR inhibitor and chemotherapy combinations for acquired TKI resistance in *EGFR*-mutant NSCLC models

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Abstract Acquired resistance to EGFR TKIs is the most important limiting factor for treatment efficiency in *EGFR*-mutant NSCLC. Although the continuation of EGFR TKI beyond disease progression in combination with chemotherapy is often suggested as a strategy for treating acquired resistance, the optimal treatment sequence for EGFR TKI and chemotherapy is unknown. In the current work, NSCLC cell lines PC9ER, H1975 and HCC827GR, representing the acquired TKI resistance genotypes (T790M, cMET), were exposed to a chemotherapeutic agent, cisplatin or paclitaxel, in combination with EGFR TKIs (erlotinib, WZ4002) in vitro and analysed for cytotoxicity and apoptotic response. The result showed that all the combinations of EGFR TKIs with a chemotherapeutic agent tested had a synergistic effect on cytotoxicity and increased the apoptotic response. The sequences involving a chemotherapeutic agent concurrently with an EGFR TKI or preceding it were the most efficient strategies. Our in vitro models suggest that the combination of an EGFR TKI and chemotherapy is beneficial in cases of acquired EGFR TKI resistance. Furthermore, the sequence of chemotherapy followed by EGFR TKI is significantly more powerful than the reversed order, so that an intercalated approach is likely to be the most active strategy in clinical use and ought to be tested in a randomized clinical trial.

Keywords NSCLC · *EGFR* mutant · Tyrosine kinase inhibitor · Acquired resistance · Chemotherapy

Introduction

Metastatic *EGFR*-mutant non-small cell lung cancer characterizes a disease subset in which tumours are highly responsive to EGFR TKIs. First-line EGFR TKI treatment is considered to be the 'standard of care' in these cases due to the marked improvement in progression-free survival (PFS) achieved and the better tolerability relative to a platinum doublet even though no benefit has been shown in terms of overall survival [1–6]. After a favourable response to EGFR TKI, acquired TKI resistance develops on average 9–13 mo after initiation of the therapy. Multiple mechanisms have been described for EGFR TKI acquired resistance, including T790M secondary mutation and activation of alternative pathways such as cMET or PI3KCA, and histological conversion to small cell lung cancer or a cancer stem-like phenotype. T790M is the most common mechanism behind acquired TKI resistance, occurring in half of all patients [7]. Furthermore, multiple concurrent resistance mechanisms have been identified in one patient [8].

There is currently no 'standard-of-care' approach to the treatment of acquired EGFR TKI resistance. If a progressive patient has been treated with first-line EGFR TKI, chemotherapy is often offered as a second-line treatment. Furthermore, the continuation of EGFR TKI beyond RECIST-defined progression alone or in combination with other treatment modalities could be considered, since disease flare has been described as occurring in about a quarter of all patients after TKI stoppage [9]. The continuation of EGFR TKI after local therapy directed at progressive lesions

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has been shown to result in impressive PFS figures [10, 11]. Furthermore, combining erlotinib with chemotherapy has been shown in a retrospective analysis to increase response rates as compared with chemotherapy alone [12]. However, a recently presented abstract of a clinical trial (IMPRESS) investigating concurrent use of chemotherapy and daily gefitinib to acquired EGFR TKI resistance in *EGFR*-mutant patient did not improve patient outcomes compared to chemotherapy alone. New pharmacological agents have been developed for acquired resistance, predominantly targeting T790M, the most common resistance mechanism. Irreversible inhibitors such as afatinib or dacomitinib proved effective in preclinical models but lacked activity in clinical trials, whereas newer, EGFR mutation-specific agents such as WZ4002, CO-1686, AZD9291, PKC412 and Gö6976 have shown encouraging activity in preclinical models [13–16], and the two currently being tested in clinical trials, CO-1686 and AZD9291, have yielded very promising early results.

In the present work, we characterize EGFR inhibitor and chemotherapy combinations using alternative dosing schedules in acquired EGFR TKI resistance models. The results suggest that the continuation of an EGFR inhibitor in combination with chemotherapy can be beneficial even in the presence of acquired resistance. Furthermore, the dosing schedule seems to be critical for the efficiency of therapy.

Materials and methods

Cell lines and reagents

EGFR-mutant NSCLC lines PC9 (ex19del), H1975 (L858R + T790M), HCC827 (ex19del) and HCC827GR (ex19del + cMET amplification) were kind gifts from Dr. Pasi Jänne (Dana–Farber Cancer Institute, Boston, USA), and PC9ER (ex19del + T790M) had previously been generated in the laboratory [16].

Erlotinib, WZ4002, cisplatin, and paclitaxel were purchased from LC Labs (Danvers, MA) and dissolved in DMSO or water (cisplatin) and stored in aliquots at −20 °C.

MTS cytotoxicity assay

In the MTS assay, 3000–5000 cells were plated onto 96-well plates and treated with drugs for 72 h. Three to six parallel wells for each treatment were used, and untreated cells were used as controls. After the drug treatments, the cells were incubated in an MTS reagent mix (Promega; Madison, WI) supplemented with phenazine methosulphate (Sigma-Aldrich; St. Louis, MO) in the medium. The absorbances of the plates at 490 nm were recorded on a plate reader. The results were displayed graphically using the GraphPad Prism software (GraphPad Software; La Jolla, CA), and the curves were fitted using a nonlinear regression model with a sigmoidal dose response. Decreases in cell survival are shown as percentage changes relative to the untreated cells.

Colony formation assay

A total of 300–1300 cells were plated onto 24-well plates and treated for the times indicated. At least two parallel wells were used for each treatment, and the experiments were repeated three times. The drugs were withdrawn after 7 days, and the cells were allowed to proliferate. The cell culture medium was changed when necessary. After differences in the growth of colonies had appeared, the cells were washed with PBS, fixed with methanol and dyed with 0.005 % crystal violet (Merck; Darmstadt, Germany), a chromatin-binding stain.

Western blot analysis

The cells were plated onto 6-well plates, allowed to attach for 1–2 days and then treated with the drugs. After the desired drug treatments, the cells were washed with phosphate-buffered saline (PBS) and lysed with NP-40 lysis buffer (1 % Igepal CA-630, 20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10 % glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The protein concentrations of the cell lysates were measured using the Bio-Rad Protein Assay (Bio-Rad; Hercules, CA), and the absorbances were read at a wavelength of 595 nm. After equalizing the protein concentrations of the samples with distilled water, 3× Laemmli buffer was added and the samples were boiled for 5 min and stored at −80 °C.

Equal amounts of protein samples were separated on SDS-PAGE, and the proteins were then transferred electrophoretically to a PVDF membrane. The membranes were blocked against unspecific binding of the antibodies with 5 % BSA (in PBS with 0.1 % Tween-20 and 0.0025 % sodium azide) and then incubated in the primary antibodies overnight at 4 °C. The next day, they were washed with PBS-T incubated in the horseradish peroxidase (HRP)-linked secondary antibody and washed again with PBS-T. The membranes were developed using an Immobilon Western Chemiluminescent HRP Substrate kit (Millipore; Billerica, USA), and the signal was detected on radiographic films. All the Western blot experiments were performed in duplicate.

The following antibodies were used: cleaved PARP, ERK1/2 and anti-rabbit IgG HRP-linked antibody (Cell

Signaling Technologies, Danvers, MA). The primary antibodies were diluted in 5 % BSA.

Results

EGFR inhibitors and chemotherapy in EGFR-mutant lines

To establish the cytotoxic concentration frame for the drugs, we first treated the *EGFR*-mutant cell lines with cisplatin or paclitaxel for 72 h and analysed them with a MTS cytotoxicity assay. With cisplatin treatment, cytotoxicity was seen in the PC9 and PC9ER lines at concentrations above 1 μM, while in the other lines tested, only minor toxicity was seen at the maximal concentration of 3.3 μM (Fig. 1a). When the cell lines were treated with paclitaxel, cytotoxicity was seen at a concentration of 3.3 nM, the PC9 lines being most sensitive to the drug (Fig. 1b). We also tested longer cisplatin, paclitaxel and EGFR TKI exposures in 7-day colony formation assays. With cisplatin, we observed that the drug caused some cytotoxicity in tested concentrations (1 μM for PC9 s and 3.3 μM for others; Fig. 1c). As expected, colony formation assay with paclitaxel showed similar cytotoxicity pattern as in MTS assay with some cytotoxicity evident in tested lines, H1975 being the most sensitive line. Colony formation with EGFR inhibitors followed the cytotoxicity pattern of previous studies showing resistant lines (PC9ER, HCC827GR and H1975) being highly resistant to erlotinib while PC9ER being sensitive to WZ4002 (Fig. 1c). In general, there were few differences in sensitivity to the chemotherapeutic agents between the EGFR TKI-sensitive and EGFR TKI-resistant PC9 and HCC827 lines, suggesting a difference in the mechanisms for sensitivity and resistance between TKI and chemotherapy (Fig. 1a, b). For the subsequent experiments, we selected cisplatin at 1 μM for the PC9 lines and at 3.3 μM for the other lines tested and 3.3 nM paclitaxel for all the lines since these concentrations were cytotoxic but did not result in complete cell killing. We drew concentrations for EGFR inhibitors (1 μM for both erlotinib and WZ4002) from previous works of others and us since these have been shown to result in maximal difference in cytotoxicity between sensitive and resistance lines [13, 16].

EGFR inhibitor and chemotherapy combinations in EGFR-mutant lines

We then set out to analyse whether combining EGFR TKIs with chemotherapeutic agents would be beneficial. We treated the cells with single agents, combinations or sequentially for 6 days and analysed them with a colony formation assay. HCC827GR and H1975 were more sensitive to both cisplatin and paclitaxel treatments than PC9 or PC9ER lines (Fig. 2a, b), while combinations of erlotinib or WZ4002 with cisplatin or paclitaxel increased cytotoxicity by comparison with single agents, especially in the PC9 and PC9ER lines (Fig. 2a, b). When EGFR TKIs and chemotherapy were given sequentially, treatments in which

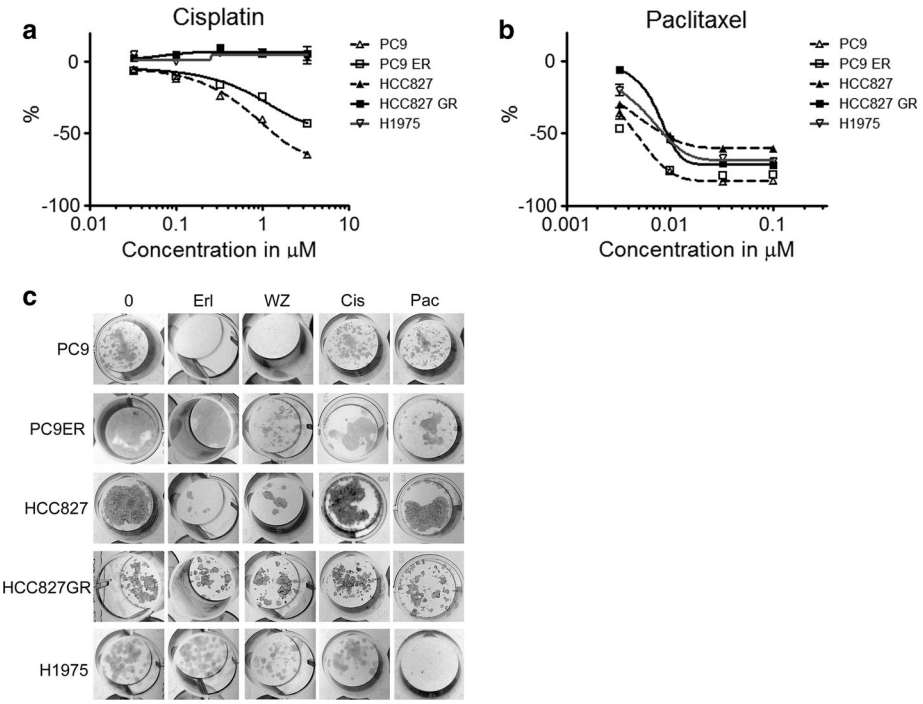

Fig. 1 Cytotoxicity assays in *EGFR*-mutant NSCLC lines. **a** MTS cytotoxicity assay for 72 h, Cisplatin treatments. **b** Paclitaxel treatment. The *x*-axis indicates the percentage down-regulation of viability and the *y*-axis the concentration in μM. (Cc, Colony formation assay in same lines treated with 1 μM erlotinib (Erl), 1 μM WZ4002 (WZ), 1/3.3 μM cisplatin (Cis) and 3 nM paclitaxel (Pac) for 7 days

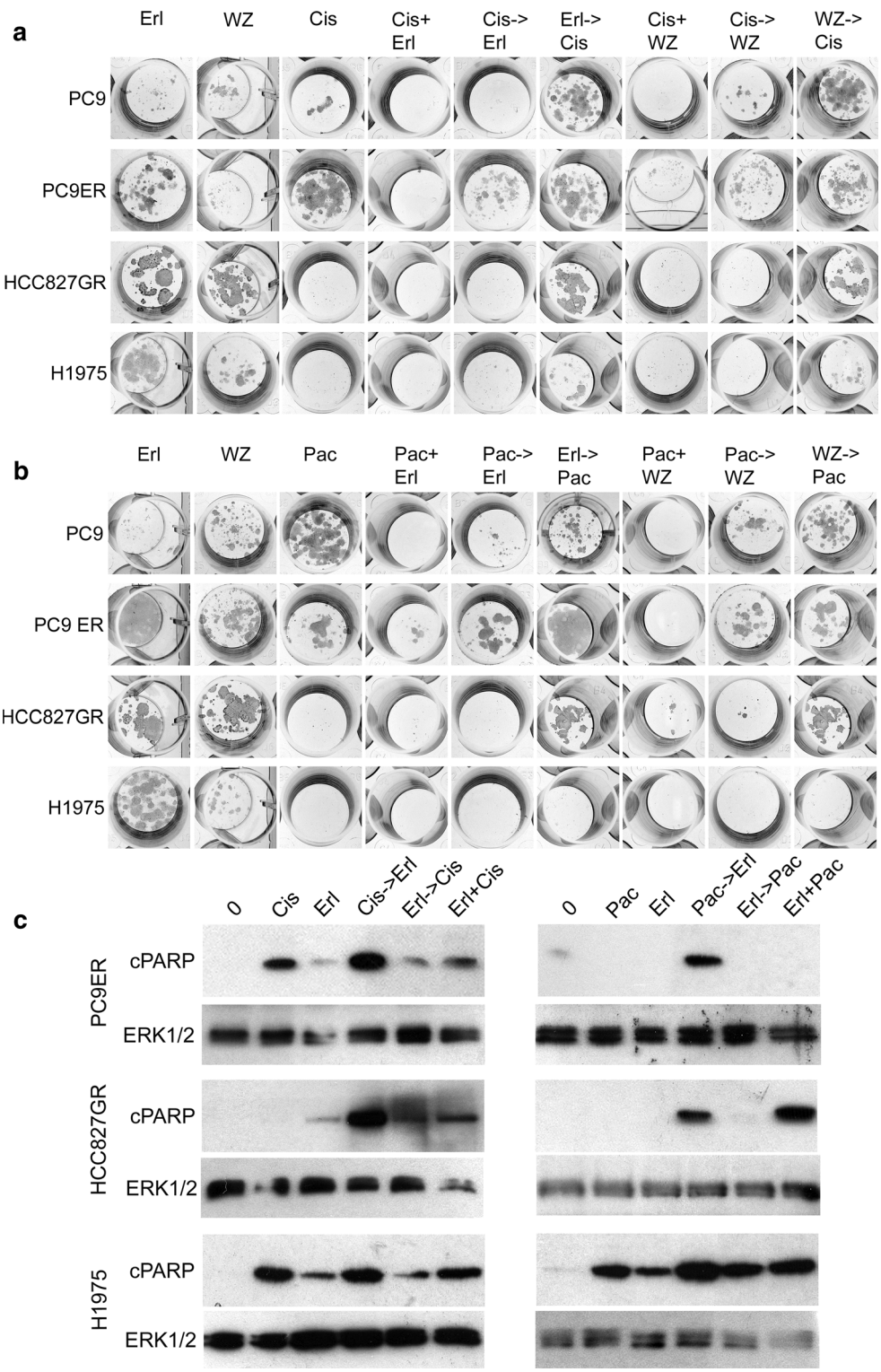

Fig. 2 Colony formation assay and apoptosis of *EGFR*-mutant NSCLC lines treated with an EGFR inhibitor and chemotherapy. PC9, PC9ER, HCC827GR and H1975 cells were treated with 1 μM erlotinib (Erl), 0.3 μM WZ4002 (WZ), 1 μM cisplatin (Cis) or 3 nM paclitaxel (Pac) for 3 or 6 days, after which they were switched to a regular medium, fixed and stained when growing colonies were present (colony formation) or lysed (Western blots). Lanes marked with an arrow were first treated with drug 1 for 3 days, after which they were switched to drug 2. **a** Treatment of cells with cisplatin and its combinations. **b** Treatment of cells with paclitaxel and its combinations. **c** Western blot assay for cleaved PARP (cPARP) and ERK1/2

the chemotherapy was given before TKI proved more cytotoxic than those in which the sequence was reversed. In fact, TKI given before chemotherapy totally blocked the cytotoxicity of the latter in most instances (Fig. 2a, b). Surprisingly, similar results were also seen with the lines that were resistant to EGFR TKIs and with the mutation-specific inhibitor WZ4002 (Fig. 2a, b). H1975 treated with cisplatin followed the same pattern, but we cannot make any conclusions concerning the paclitaxel combinations since this cell line was highly sensitive to the drug irrespective of

the treatment sequence (Fig. 2b). In preliminary experiments, we also tested the drug combinations in PC9ER line using different drug exposure schedules (4 days or 2 + 2 days) and the results did not differ from 6 days or 3 + 3 days exposures (not shown). We excluded the HCC827 line from the remaining experiments since it was so highly sensitive to erlotinib treatment; the efficacy of combinations proved difficult to assess.

Apoptosis in response to chemotherapy and erlotinib treatment in erlotinib-resistant models

Apoptotic responses to the various erlotinib and chemotherapy sequences were studied in the erlotinib-resistant models by treating the lines with erlotinib and chemotherapeutic agents concurrently or sequentially for 6 days or 3 + 3 days and performing Western blot analysis for cleaved PARP. Erlotinib itself induced only a minimal amount of apoptosis in the PC9ER and HCC827GR lines, but some apoptosis was seen in the H1975 line. Cisplatin induced some apoptosis in the PC9ER and H1975 lines and paclitaxel in the H1975 line. When the cells were exposed concurrently to chemotherapy and erlotinib, an increase in apoptosis compared with the single-agent treatments was seen only in the HCC827GR paclitaxel–erlotinib treatment. In some instances, even less apoptosis was seen with the concurrent treatment regimens. The most prominent apoptotic response was always seen when the cells were first treated with chemotherapy and then with erlotinib. The PC9ER lines treated with a paclitaxel–erlotinib sequence and HCC827GR treated with a cisplatin–erlotinib sequence were essentially the only experiments in which apoptosis was induced, while the other treatment schedules induced only a trace of apoptosis or none at all (Fig. 2c). In preliminary experiments, we also tested the drug combinations in PC9ER line using different drug exposure schedules (4 days or 2 + 2 days) and the results did not differ from 6 days or 3 + 3 days exposures (not shown). ERK1/2 was used as a control to verify uniform loading of proteins (Fig. 2c).

Discussion

Acquired resistance to EGFR TKIs is the major limiting factor for therapeutic efficiency in *EGFR*-mutant NSCLC. Although some therapeutic strategies have been suggested for treating acquired resistance, no standard-of-care approach exists. Chemotherapy, radiotherapy, continuation of TKI beyond progression and newer agents are among the strategies proposed, but there is limited evidence for the existence of any predictive factors that could be of help in selecting between them.

We set out here to investigate whether combining EGFR TKI with chemotherapy might be beneficial in acquired EGFR TKI resistance cell line models. The results show that the combining of TKI with either cisplatin or paclitaxel is beneficial when assessed in terms of colony formation or apoptotic response. We selected to use only one chemotherapy concentration, because of the narrow window of cytotoxicity with the drugs and 6-day drug exposures since preliminary experiments showed no difference between 4- or 6-days treatments. Surprisingly, TKI treatment is able to increase the cytotoxicity of chemotherapy, since the model cell lines tested show high-level resistance to the TKIs. Furthermore, we did not see any significant difference in the response between TKI resistance mediated by the most common mechanism, the T790M secondary mutation, and that mediated by cMET amplification. Likewise, our experiments yielded similar results in terms of TKI–chemotherapy synergy with both the first-generation TKI erlotinib, which has affinity for both wild-type and mutant *EGFR,* and the third-generation mutant EGFR-specific drug WZ4002, suggesting a significance for the mutant *EGFR* inhibition lying behind the observed synergy.

Some preclinical models have led to suggestions of synergy in the combination of EGFR TKIs with chemotherapy in *EGFR*-mutant disease [17]. Even though EGFR TKIs have led to impressive improvements in PFS in metastatic, *EGFR*-mutant NSCLC as compared head-to-head with first-line platinum doublet chemotherapy, none of the randomized studies have been able to show any benefit in overall survival (OS), a fact which has been thought to be a consequence of crossover between the treatment arms, since retrospective analysis has suggested that the OS of *EGFR*-mutant patients improved for >1 year after TKIs came into use [18]. The only investigators who have been able to point to any OS benefit with first-line EGFR TKI treatment in *EGFR* mutants combined a platinum doublet with erlotinib [19]. Continuation of TKI beyond progression is often suggested as a therapeutic option in the case of acquired resistance, and retrospective analyses have shown that TKI beyond progression can provide impressive PFS2 figures when combined with radiotherapeutic or surgical treatment of progressive lesions [10, 11] or higher response rates when combined with chemotherapy than with chemotherapy alone [12]. Contradictorily, a recently presented abstract (IMPRESS study) showed that the patient outcomes did not improve when daily gefitinib was added to chemotherapy in patients progressing on gefitinib.

The results of this work suggest that if EGFR TKI and chemotherapy are combined, the sequence of the drugs is crucial. If TKI is given before chemotherapy, no synergy is seen, but when TKI and chemotherapy are given

concurrently or TKI after chemotherapy, marked synergy is evident. Our work was carried out with TKI-resistant models, but similar results have previously been seen in TKI-sensitive NSCLC models [17]. Previous works have suggested that EGFR TKI-induced G1 arrest may be responsible for the antagonism with chemotherapy. Our work suggests a more complex background for the antagonism, since we used models that were resistant to TKI-induced G1 arrest and apoptosis. In clinics, NSCLC chemotherapy is given in cycles, and continuous concurrent therapy with TKI is likely to be synergistic only during the first cycle of chemotherapy if not proceeded by TKI therapy. In the light of our results, an intercalated approach to TKI and chemotherapy is likely to be the most efficient way of combining these for treating acquired EGFR TKI resistance. Interestingly, the only study showing OS benefit in the first-line treatment of *EGFR*-mutant disease not only used combined chemotherapy and TKI but also did so in an intercalated fashion [19]. One could speculate that intercalated chemotherapy and EGFR TKI could forestall the development of acquired resistance. It has been shown that multiple mechanisms of resistance can occur concurrently in patients, and it is likely that a combination of two treatment modalities is able to block more of the occurring resistance mechanisms. We have recently initiated a clinical investigation into intercalated EGFR TKI in combination with chemotherapy versus chemotherapy alone in the presence of acquired resistance to EGFR TKIs (ETAP, NCT02064491).

In conclusion, the results of the current investigation into the use of EGFR TKI in combination with chemotherapy based on acquired resistance models of *EGFR*-mutant NSCLC suggest that combining EGFR TKI with chemotherapy can create a synergistic effect. Furthermore, the drug sequence is crucial for this effect, in that an intercalated approach is likely to be clinically most potent.

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Conflict of interest Authors' declare no conflict of interest.

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