Published in final edited form as:

Lung Cancer. 2014 January ; 83(1): 37–43. doi:10.1016/j.lungcan.2013.09.019.

Dual ALK and EGFR inhibition targets a mechanism of acquired resistance to the tyrosine kinase inhibitor crizotinib in *ALK* rearranged lung cancer

Norihiro Yamaguchi, MD, MPH^{1,*}, Antonio R. Lucena-Araujo, PhD^{1,*}, Sohei Nakayama, MD¹, Lorena L. de Figueiredo-Pontes, MD, PhD¹, David A. Gonzalez, MSc¹, Hiroyuki Yasuda, MD¹, Susumu Kobayashi, MD, PhD¹, and Daniel B. Costa, MD, PhD^{1,*}

¹Department of Medicine, Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Abstract

Introduction—The multitargeted tyrosine kinase inhibitor (TKI) crizotinib is active against *ALK* translocated non-small-cell lung cancer (NSCLC); however acquired resistance invariably develops over time. *ALK* mutations have previously been implicated in only a third of resistant tumors. We sought to evaluate alternative mechanisms of resistance and preclinical strategies to overcome these in a cell line driven by *EML4-ALK*.

Methods—We selected the NSCLC cell line NCI-H3122 (H3122: *EML4-ALK* E13;A20) and derived resistant variants that were able to grow in the presence of 1μM crizotinib. These were analyzed for *ALK* mutations, sensitivity to crizotinib in combination to other TKIs, and for activation of alternative tyrosine kinases.

Results—All H3122 crizotinib resistant (CR) clones lacked amplification or mutations in the kinase domain of *ALK*. To evaluate if possible alternative kinases functioned as “bypass” tracks for downstream signaling activation in these resistance cells, we performed of phospho-receptor tyrosine kinase array that demonstrated that CR clones had higher phospho-EGFR signals than H3122 cells before and after exposure to crizotinib. A functional approach of dual ALK TKI (with crizotinib) with combinatory TKI inhibition was used as a secondary screen for possible targets. Crizotinib + erlotinib (reversible EGFR TKI) and crizotinib + afatinib (irreversible EGFR/ERBB2 TKI) were able to inhibit the growth of H3122 CR clones, confirming EGFR activation as a

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*Correspondence to: Daniel B. Costa, MD, PhD - Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, 330 Brookline Av., Boston, MA 02215, Phone: 617-667-9236, Fax: 617-975-5665, dbcosta@bidmc.harvard.edu.

*these authors contributed equally

Preliminary results of this work were presented at the 2013 Annual Meeting of the American Association for Cancer Research (AACR) in Washington, DC, United States

CONFLICT OF INTEREST STATEMENT

Daniel B. Costa has received consulting fees from Pfizer, Roche and AstraZeneca. Norihiro Yamaguchi, Antonio R. Lucena-Araujo, Sohei Nakayama, Lorena L. de Figueiredo-Pontes, David A. Gonzalez, Hiroyuki Yasuda and Susumu Kobayashi have no conflicts to disclose. No other conflict of interest is stated.

Contributors: SSK and DBC were involved in the conception of this study; NY, SN, DG, HY, LLFP, SK and DBC were involved in data acquisition, analysis and interpretation; SK and DBC provided administrative and funding support; NY, SN, HY, LLFP, SK and DBC were involved in writing the report; all authors approved the final version.

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mechanism of resistance. The removal of crizotinib from the culture media re-sensitized CR cells to crizotinib.

Conclusions—We identified activation of EGFR as a mechanism of resistance to crizotinib in preclinical models of *ALK* translocated NSCLC. If EGFR activation is confirmed as a predominant mechanism of ALK TKI-induced resistance in patient-derived tumors, the use of ALK plus EGFR TKIs could be explored for this important cohort of NSCLCs.

Keywords

lung cancer; non-small-cell lung cancer; tyrosine kinase; kinase inhibitor; epidermal growth factor receptor; EGFR; EGF; anaplastic lymphoma kinase; ALK; crizotinib; erlotinib; afatinib

INTRODUCTION

Lung cancer - with its most prevalent form non-small-cell lung cancer (NSCLC) - has been for the last decade the leading cause of cancer-related mortality for both men and women worldwide (1)(2). NSCLCs are heterogeneous diseases characterized by driver oncogenic alterations that can be targeted with precision tyrosine kinase inhibitors (TKIs). The most prevalent mutated or rearranged oncogenes identified in NSCLCs are *KRAS*, epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), *ROS1*, among others (3). *ALK* rearrangements in NSCLC brought forth new treatment options for advanced NSCLC with the use of ALK TKIs (4). Crizotinib, a multitargeted TKI (5) with activity against MET, ALK and ROS1 was approved in 2011 by the Food and Drug Administration for metastatic NSCLC that is positive for *ALK* rearrangements (6–8). Crizotinib has a reported response rate of over 60%, a median progression-free survival (PFS) that exceeds 9 months, and an overall survival of near 75% at one year in *ALK* rearranged NSCLC (9). Therefore, from identification to inhibitor approval, the story of *ALK* in NSCLC stands as a testament of the promises of molecular targeted medicine (10).

However, pharmacokinetic (11) and systemic acquired resistance (10) to crizotinib in patients with *ALK* rearranged NSCLC remains the main limitation of the prolonged palliative benefit of this compound. Acquired resistance to TKI therapy is a common thread of many oncogene addicted NSCLCs and much has been learned from the evolving story of EGFR TKIs for *EGFR* mutated NSCLC (12;13). In the latter cancer, second site mutations (i.e., EGFR-T790M) that disrupt kinase-drug binding interactions and activation of downstream shared signaling pathways via other aberrant oncogenes (i.e., “bypass tracks” or “oncogene kinase co-dependence states”) are the predominant models for acquired resistance under pressure of a TKI (12–15). In the case of *ALK* rearranged NSCLC exposed to crizotinib, it seems that the aforementioned mechanisms of acquired resistance (mutations and activation of bypass tracks) can also be observed (16–21); however a detailed understanding of the most prevalent mechanism(s) of resistance and of strategies to overcome the most common forms of acquired resistance to ALK TKIs is lacking.

We sought to use a robust preclinical model of *EML4-ALK* driven NSCLC to model bypass track-mediated acquired resistance to crizotinib with an attempt to establish the preclinical rationale for treatment of *ALK* rearranged crizotinib-resistant disease.

MATERIALS AND METHODS

Reagents

Crizotinib, sorafenib, imatinib, erlotinib and afatinib were purchased from LC Laboratories (Woburn, MA). All reagents were dissolved in dimethyl sulfoxide (DMSO) and stored at

–80°C. Cetuximab was obtained from the clinical research pharmacy at Beth Israel Deaconess Medical Center and stored at 4°C.

Cell culture

NCI-H3122 (H3122) cells, which harbor *EML4-ALK* E13;A20 and are dependent on ALK signaling (i.e., oncogene addicted), were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere with 5% CO₂.

H31222 crizotinib resistant cell (CR) derivation, sequencing of *ALK* and evaluation of copy number changes

H3122 were made resistant to crizotinib by incremental and continuous exposure to a formulation of crizotinib provide by its manufacturer (Pfizer Inc., La Jolla, CA). Initially H3122 cells were treated with 0.01μM of crizotinib and surviving cells were grown in subsequent passages through the ensuing 12 weeks with incremental increases of 5-fold drug levels every 3 weeks. At the end of 12 weeks, a bulk population of H3122 CR cells to 1μM of crizotinib was subcloned using limited dilution into 3 separate CR clones in a process that took 10 weeks. The resulting CR bulk clones were names H3122 CR_A, CR_B and CR_C; and all were able to grow in the presence of 1μM of crizotinib. DNA and RNA were isolated from H3122 CR cells, and the kinase of *ALK* sequenced using previously described methods (22). DNA copy number alterations were analyzed using methods described previously (23;24), with DNA probed onto Affymetrix SNP6.0 arrays (Affymetrix Inc., Santa Clara, CA).

Cell line proliferation assays

Cells were plated in 96-well plates, allowed to attach and then treated with or without tyrosine kinase inhibitors for 72 hours. Cell viability was determined by CellTiter 96 Aqueous One solution proliferation kit (Promega, Madison, WI) according to the manufacture's protocol. All experiments were performed in triplicate.

Western blotting and antibodies

Cells were lysed and lysates prepared using previously described methods (5) Total EGFR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Total extracellular signal regulating kinase 1/2 (ERK 1/2) antibody was purchased from BD Transduction Laboratories (Lexington, KY). Phospho-EGFR (pT1068) antibody was purchased from Invitrogen (Carlsbad, CA). Protein kinase B (AKT), phospho-AKT (pS473), phospho-ERK 1/2 (pT202/pY204), phospho-ALK (pT1604) and ALK were purchased from Cell Signaling Technology (Beverly, MA). All primary antibodies were diluted 1:1000, while their recommended secondary antibodies were diluted 1:10000.

Phospho receptor tyrosine kinase (RTK) array

The phospho-RTK array was performed according to manufacturer's instructions (R&D Systems, Minneapolis, MN). 300 μg of total protein was used for each membrane. Signals were semi-quantified with ImageJ software (<http://rsb.info.nih.gov/ij/>).

Epidermal growth factor (EGF) measurement

Levels of EGF in media were measured using an enzyme-linked immunosorbent assay (ELISA) based on the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

The paired Student's t-test was used to determine the difference in cell viability between control and inhibitor treated cells. All tests were performed with STATA version 12 (STATA Corp, TX).

RESULTS

***EML4-ALK* E13;A20 driven cell lines (H3122) and acquired resistance to crizotinib**

To simulate acquired resistance to crizotinib, we used H3122 cell lines harboring *EML4-ALK* E13;A20. In H3122 cells, crizotinib completely inhibited ALK phosphorylation and this was accompanied by inhibition of AKT and ERK phosphorylation (Figure 1A); thus indicating that the AKT/phosphatidylinositol-3-kinases (PI3K) and ERK/mitogen-activated protein kinase (MAPK) are linked to ALK signaling in these cells.

In a cell viability assay, H3122 cells were intrinsically sensitive to crizotinib with a calculated 50% inhibitory concentration (IC₅₀) of 0.21 μM and significant cell proliferation inhibition with 1 μM of drug (Figure 1B). All H3122 CR cells, which were derived to be resistant to crizotinib (Methods), displayed higher proliferative ability in the presence of 1 μM crizotinib and had IC₅₀s that exceed 7 μM (Figure 1B). Therefore, these resistant cells had IC₅₀s that were more than 10 times higher than the reported median trough plasma total concentration of crizotinib at 250 mg twice daily (its recommended dosing scheme in NSCLC) of 0.57 μM (i.e, 256 ng/mL) (11;25).

All H3122CR cells did not harbor mutations in the tyrosine kinase domain of *ALK*, spanning exons 20 to 28 of *ALK* (data not shown) or *ALK* amplification (data not shown). Therefore, we suspected other mechanisms played a role in the observed resistance to crizotinib. To investigate the biological response to crizotinib of H3122 CR cells, we treated them with 1 μM crizotinib and monitored ALK and its downstream targets (Figure 1C). Interestingly, ALK phosphorylation was completely inhibited in H3122 CR cells (Figure 1C) in a pattern indistinguishable from the ALK TKI-sensitive H3122 cells (Figure 1A). However, different than H3122 cells, all H3122 CR cells did not display inhibition of AKT or ERK phosphorylation (Figure 1C). The latter findings were indicative of a possible bypass track mediated by an alternative oncogene/tyrosine kinase that continued to stimulate the AKT/PI3K and ERK/MAPK pathways – and hence cell survival – despite ALK inhibition.

Activation of EGFR as a mechanism of acquired resistance to the ALK TKI crizotinib in H3122 cells

To screen for other activated RTKs in both H3122 and H3122 CR cells, we utilized a human phosphor-RTK array that was able to simultaneously interrogate close to fifty different RTK antibodies. H3122 CR cells were observed to have at least 4 times higher EGFR phosphorylation signals than H3122 cells (Figure 2A), and the signal intensity was boosted by treatment with crizotinib (Figure 2A). We were not able to identify another RTK with the same degree of activation when comparing H3122 with H3122 CR cells.

To confirm the results of the RTK array, we also obtained protein extracts from H3122 CR cells and demonstrated that both phosphorylated and total EGFR signals in H3122 CR cells were more intense than those of H3122 cells (Figure 2B). In all H3122 CR cells, amplification of *EGFR* was not observed (data not shown). These findings led us to believe that EGFR signaling was activated in H3122 CR cells by a non-genomic event. The precise mechanism of interaction between ALK, crizotinib and EGFR signaling remains elusive but may involve an intertwined network already present (but not completely activated) in H3122 cells prior to crizotinib therapy.

Proliferation of H3122 crizotinib sensitive and resistant cell lines upon exposure to multitargeted kinase inhibitors in combination with crizotinib

Our aforementioned findings from H3122 CR cells indicated that the EGFR signaling pathway was the potential bypass track that led to evasion of inhibition of ALK phosphorylation by crizotinib in these cells. To confirm the functional significance of these observations, we performed a functional screening using combinations of crizotinib plus other TKIs as a dual multitargeted TKI strategy to identify/confirm possible targets (outside EGFR) and establish potential therapeutic strategies in H3122 CR cells (Figure 3).

Using a limited panel, we were able to confirm that two multitargeted TKIs without anti-EGFR activity were ineffective in H3122 CR cells when combined with crizotinib. The combination of sorafenib (potentially targets BRAF, RET, PDGFR, and VEGFR pathways) and crizotinib did not reveal a statistically significant difference compared with crizotinib monotherapy alone in these crizotinib-resistant cells (Figure 1A). In addition, the combination of imatinib and crizotinib failed to show a statistically significant difference from crizotinib monotherapy. (Figure 3B); highlighting that ABL, KIT, and PDGFR (imatinib targets) may also not play an important role in the predominant bypass mechanism of H3122 CR cells.

On the other hand, erlotinib (a reversible EGFR TKI) and afatinib (an irreversible EGFR TKI) combined with crizotinib demonstrated a more robust, and statistically significant, inhibitory pattern than crizotinib monotherapy alone in all H3122 CR cells (Figure 3C and 3D). We also attempted - using our *in vitro* models - the combination of cetuximab (up to 100µg/mL) and crizotinib in H3122 CR cells, however this was ineffective (data not shown) likely due to the known limitations of using EGFR monoclonal antibodies using cell lines in culture (19).

The enhanced response of the combination of ALK and EGFR TKIs in H3122 CR cells confirmed our prior results implicating EGFR signaling as the potential bypass track in this model of acquired resistance to crizotinib. It also provided a functional demonstration that the combination of crizotinib and erlotinib or crizotinib and afatinib can inhibit H3122 CR cells that are driven by EML4-ALK and EGFR activation.

The combination of crizotinib plus an EGFR TKI inhibits both the PI3K and MAPK downstream pathways in H3122 crizotinib resistant cell lines

To determine if the combination of ALK and EGFR TKIs can abrogate common downstream signaling pathways in H3122 CR cells, we treated H3122 CR_A cells with the combinatory crizotinib and afatinib. Different than each drug alone, which was only able to inhibit its target without significant affecting the AKT/PI3K and ERK/MAPK pathways, the combination of crizotinib and afatinib was able to inhibit the phosphorylation of ALK, EGFR, AKT and ERK (Figure 4). This result supports the observed cell viability inhibition by crizotinib and EGFR TKIs (Figure 3C and 3D) in combination. It also establishes that dual inhibition of ALK and a crizotinib-resistant oncogene-mediated bypass track can lead to downregulation of both the PI3K and MAPK downstream pathways.

Continuous pressure from crizotinib exposure is necessary for maintained EGFR signaling and crizotinib resistance in H3122 CR cells

We hypothesized that continued exposure of H3122 CR cells to crizotinib was important in maintaining the EGFR mediated bypass track and the ensuing resistance to ALK TKIs. Therefore, we removed crizotinib from the growth media of H3122 CR_A cells over the course of 4 weeks. The aforementioned cells were then treated with crizotinib and shown to be partially re-sensitized to the inhibitory effects of crizotinib when compared to H3122

CR_A cells that were maintained on media with 1 μ M crizotinib (Figure 5A). In addition, the H3122 CR_A that had been removed from continuous exposure to crizotinib showed lower levels of EGFR and activated EGFR (Figure 5B). These results indicate that continuous exposure to crizotinib is important in mediating the induction of EGFR signaling in H3122 CR cells.

EGFR ligands can also induce resistance to crizotinib in H3122 cells

We were also interested in determining if EGFR ligands alone, even in the absence of prior crizotinib exposure, could also shift the sensitivity pattern of the hypersensitive H3122 cells. A few investigators had previously reported that the EGFR ligands amphiregulin, epidermal growth factor (EGF) and others were able to potentiate resistance to crizotinib in EML4-ALK driven models (17;19). To determine if EGF was secreted in higher levels from H3122 CR_A than H3122 cells, we measured media-derived EGF in both cells and were unable to detect measurable EGF (< 0 μ g/mL, data not shown). We tried to induce temporary resistance to crizotinib in H3122 cells with EGF. H3122 cells were incubated with 100ng/ml EGF for three days and were subsequently treated with crizotinib. The cell viability of H3122 cells co-incubated with EGF was significantly higher than that of H3122 cells even after exposure to 1 μ M crizotinib (Figure 5C). Therefore, it seems that the axis of EGFR ligand-EGFR is able to mediate resistance to crizotinib in this model of EML4-ALK driven NSCLC.

DISCUSSION

ALK rearranged NSCLCs are intrinsically sensitive to inhibition by the multitargeted ALK TKI crizotinib (5;6;9;10); however despite the drastic clinical responses initially, the median PFS does not exceed 10 months (9). The latter observation highlights that pharmacokinetic (11) – in the case of isolated central nervous system progression – and systemic acquired resistance to this precision TKI are the main limitations of the clinical benefit of use of this class of drug for ALK rearranged NSCLC. Similar to other oncogene addicted NSCLCs treated with matched TKIs, tyrosine kinase mutations and oncogene activation of bypass tracks seem to be the major determinants of biological acquired resistance to crizotinib in ALK-positive NSCLC (10;16–21).

Interestingly, point mutations in the kinase domain of ALK have only been observed in a third of the tumors from re-biopsy specimens of patients with ALK rearranged NSCLC and in a few cell line models with acquired resistance to crizotinib (10;18). Of the ALK mutations identified in patient tumors - including L1196M (this represents the gatekeeper position of ALK), L1151Tins, L1152R, C1156Y, F1174L, G1202R, S1206Y and G1269A –all have increased IC₅₀ when compared to EML4-ALK without an ALK kinase domain mutation (10;16–19;21;26). The identification of ALK mutations in the minority of cases with acquired resistance to crizotinib has spawned the development of novel ALK TKIs – such as LDK378, AP26113, AF802 or ASP3026 - that are in general more potent than crizotinib against EML4-ALK (10). However, the potency of these next generation ALK TKIs seems to be different depending on the type of ALK mutation tested; and none of the ALK TKIs in development is significantly more effective than crizotinib for all ALK kinase domains mutations identified (10;17;18). The later observations could hypothetically limit the development of these compounds in clinical trials aimed at patients with ALK rearranged NSCLC with acquired resistance to crizotinib; however initial results of these second generation inhibitors have shown exceedingly high response rates in crizotinib-resistant tumors (10) that may indicate that ALK rearranged tumors remains ALK-dependent irrespective of the presence or absence of resistant mutations.

The fact that over two thirds of cases of *ALK* rearranged NSCLC with acquired resistance to crizotinib lack *ALK* kinase mutations is potentially indicative that other mechanisms are more prevalent and therefore clinically pertinent for all types of *ALK* TKIs. The activation of downstream shared signaling pathways via the activation of bypass tracks (i.e., activation of other oncogenes in an oncogene kinase co-dependence state) has been recently scrutinized in preclinical models and clinical samples of TKI-resistant cases (10;17–20). Multiple investigators have previously identified activation of the EGFR axis as a common potential mechanism of bypassing the inhibition of *ALK* phosphorylation conferred by crizotinib and more potent *ALK* TKIs (with close homology to LDK378) and maintaining the downstream AKT/PI3K and ERK/MAPK signals in *EML4-ALK* driven cell lines made resistant to *ALK* TKIs (17;19;20;26). The precise means by which the EGFR signaling axis is activated in *ALK* rearranged cells with acquired resistance to *ALK* TKIs is uncertain, although the upregulation of EGFR ligands and EGFR itself have been observed in the aforementioned studies (10;17;19;20). In our own preclinical work, we were able to determine that all our *EML4-ALK* rearranged cells (H3122 CR_A, B and C) with acquired resistance to crizotinib had upregulation of EGFR and activation of the EGFR signaling cascade as the major mechanism of resistance to crizotinib. In addition, we also confirmed that EGF ligands alone can lead to resistance to crizotinib and showed that the activation of EGFR signaling (and hence the resistance to crizotinib) requires continued exposure to crizotinib in resistant cells. Investigating how EGFR and *ALK* interact with each other will likely shed light in why EGFR is the main bypass track in these cells. It is unclear how often EGFR as a bypass track is present in clinical specimens from patients with *ALK* rearranged NSCLC with acquired resistance to crizotinib. Only one report has evaluated this issue and observed that in close to a third of the eighteen crizotinib resistant samples described there was an increase in the levels of EGFR activation (as measured by an immunohistochemical stain against phosphorylated EGFR) in the resistant tumor when compared to the crizotinib-naïve sample (10;17). The lack of a standardized method to identify EGFR activation (in the absence of a genomic – amplification or mutation – event) has hampered the ability to reproduce the aforementioned data in other cohorts.

In case the activation of EGFR is confirmed as the most prevalent mechanism of resistance to crizotinib and other *ALK* TKIs in *ALK* rearranged NSCLC, it will be important to translate preclinical strategies of treatment quickly into the clinical realm. Our observations lend robust evidence that the dual inhibition of *ALK* and EGFR (as a crizotinib-resistant oncogene-mediated bypass track) in preclinical models of acquired resistance to crizotinib is effective, and that both reversible (erlotinib) and irreversible (afatinib) EGFR TKIs can reverse part of the resistant phenotype of *EML4-ALK* rearranged cells with EGFR-mediated resistance. Our data not only confirms observations made by other groups using other EGFR TKIs, such as gefitinib and dacomitinib (17;19), but also highlights that combinatory treatment strategies are putative approaches that should be tested in clinical trials for *ALK* rearranged NSCLC with acquired resistance to TKIs mediated by bypass tracks. Indeed, ongoing clinical trials of crizotinib plus erlotinib and crizotinib plus dacomitinib have been initiated with the intent of identifying the doses, toxicities and potential efficacies of these approaches in NSCLC (10). Results, and analyses of biopsy specimens for EGFR activation, are eagerly anticipated from these clinical trials.

In summary, we identified activation of EGFR as the predominant mechanism of acquired resistance to crizotinib in a preclinical model of *ALK* rearranged NSCLC and establish that the dual inhibition of *ALK* and EGFR in this model was efficacious. If EGFR activation is confirmed as a predominant mechanism of TKI-induced resistance in patient-derived tumors, the use of *ALK* plus EGFR TKIs may be beneficial and should merit clinical investigation for this important cohort of NSCLCs.

Acknowledgments

We would like to thank Drs. Keith Wilner and James Christensen from Pfizer, Inc (La Jolla, CA) for providing the initial formulation of crizotinib used to derive H3122 resistant cells, and Megan Hanna and Matthew Meyerson at the Center for Cancer Genome Discovery (Dana-Farber Cancer Institute, Boston, MA) for help with experimental data.

This work was funded in part through fellowships from the American Society of Clinical Oncology Conquer Cancer Foundation (DBC), an American Cancer Society grant RSG 11-186 (DBC), and National Institutes of Health grants CA090578 (DBC, SK) and CA126026 (SK).

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FIGURE 1.

H3122 crizotinib resistant (CR) cells. A. H3122 cells are sensitive to crizotinib (1 μM) and western blot analysis demonstrates inactivation of ALK, AKT and ERK 1/2 phosphorylation after 6 hours of exposure to crizotinib. B. Proliferation assay evaluating the inhibitory effect of 1 μM crizotinib in H3122 cells and three H3122 CR cells: CR_A, CR_B and CR_C. The 50% inhibitory concentration (IC₅₀) was calculated using dose-response curves (data not shown) and indicated in parenthesis. C. Western blot analysis of protein extracts from H3122 CR cells demonstrates inactivation of ALK phosphorylation but persistence of AKT and ERK 1/2 phosphorylation after 6 hours of exposure to crizotinib. Crizotinib was removed from the growth media of H3122 CR cells two days prior to commencing the experiment.

FIGURE 2.

Activation of EGFR in H3122 CR cells. A. Phospho-receptor tyrosine kinase (RTK) array of H3122 and H3122 CR cells exposed to 6 hours of control conditions (DMSO) or 1 μ M crizotinib. In all H3122 CR cells, EGFR was the main signal observed (black arrows). B. Western blot analysis of protein extracts from H3122 and H3122 CR cells demonstrates increased levels of phosphorylated EGFR and upregulation of EGFR in all H3122 CR cells after 6 hours of exposure to control conditions (DMSO, indicated as minus sign) or crizotinib (1 μ M, indicated as positive sign). Crizotinib was removed from the growth media of H3122 CR cells two days prior to commencing the experiment.

FIGURE 3.

Proliferation assay evaluating multitargeted tyrosine kinase inhibitors (TKIs) in combination with crizotinib in H3122 NSCLC cell lines. Cells were plated at a density of 3000–5000 cells/well for H3122 and CR clones. All experiments were performed in triplicate. A. Inhibitory profile of imatinib (1 μ M) in combination with crizotinib (1 μ M). B. Inhibitory profile of sorafenib (1 μ M) in combination with crizotinib (1 μ M). C. Inhibitory profile of erlotinib (1 μ M) in combination with crizotinib (1 μ M). D. Inhibitory profile of afatinib (1 μ M) in combination with crizotinib (1 μ M). Results are displayed in bar columns with standard deviation in relation to cell viability. Treatment with DMSO (indicated as a concentration of 0) was used as the standard for 100% cell viability in each cell line. * indicates a p-value < 0.05 (see text for exact p-values) for treated cells.

FIGURE 4.

Combination of EGFR and ALK TKIs in H3122 CR_A cells inhibit downstream targets. Western blot analysis of protein extracts from H3122 CR_A cells after 6 hours of exposure to control conditions (DMSO), crizotinib (1 μM), afatinib (1 μM) or the combination of 1 μM crizotinib with 1 μM afatinib. The extracts obtained in the combination experiment demonstrate inactivation of ALK, EGFR, AKT and ERK 1/2 phosphorylation; while the treatment with single agents was unable to inhibit all these signals simultaneously. Crizotinib was removed from the growth media of DMSO and afatinib alone conditions a day prior to commencing the experiment.

FIGURE 5.

Continuous exposure to crizotinib is important to maintain acquired resistance to crizotinib in H3122 CR_A cells. A. Proliferation assay evaluating the inhibitory effect of 1μM crizotinib in H3122 CR_A cells that were maintained in growth media with 1μM crizotinib or H3122 CR_A cells that were grown in growth media without crizotinib for 4 weeks (indicated in parenthesis). Treatment with DMSO (indicated as a minus sign) was used as the standard for 100% cell viability in each cell line. B. Western blot analysis of protein extracts from H3122 CR_A cells demonstrates decreased levels of phosphorylated EGFR and downregulation of EGFR in H3122 CR_A cells that were grown in the absence of crizotinib in the growth media when compared to H3122 CR_A cells grown in the presence of crizotinib, after re-exposure to crizotinib (1μM) for 6 hours. C. Proliferation assay evaluating the inhibitory effect of 1μM crizotinib in the presence or absence of EGF (at 100 ng/mL). Treatment with DMSO (indicated as a minus sign) was used as the standard for 100% cell viability in each cell line.