ARTICLE
Journal of Cellular Biochemistry 116:2778–2785 (2015)

Journal of Cellular Biochemistry

EGFR and MEK Blockade in Triple Negative Breast Cancer Cells

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Although evidence suggests that the RAF/MEK/ERK pathway plays an important role in triple negative breast cancer (TNBC), resistance to MEK inhibitors has been observed in TNBC cells. Different mechanisms have been hypothesized to be involved in this phenomenon, including receptor tyrosine kinase-dependent activation of the PI3K/AKT pathway. In this study, we analyzed the effects of the MEK1/2 inhibitor selumetinib in combination with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib in a panel of TNBC cell lines that showed different levels of sensitivity to single-agent selumetinib: SUM-149 and MDA-MB-231 cells resulted to be sensitive, whereas SUM-159, MDA-MB-468 and HCC70 cells were relatively resistant to the drug. Treatment of TNBC cells with selumetinib produced an increase of the phosphorylation of the EGFR both in selumetinib-sensitive SUM-149, MDA-MB-231 and in selumetinib-resistant MDA-MB-468 TNBC cells. The combination of selumetinib and gefitinib resulted in a synergistic growth inhibitory effect in all the TNBC cell lines, although the IC_{50} was not reached in SUM-159 and MDA-MB-468 cells. This effect was associated with an almost complete suppression of ERK1/2 activation and a reduction of selumetinib-induced AKT phosphorylation. In addition, in selumetinib-sensitive TNBC cells the combination of selumetinib and gefitinib induced a significant G0/G1 cell cycle arrest and apoptosis. Taken together, our data demonstrated that blockade of the EGFR might efficiently increase the antitumor activity of selumetinib in a subgroup of TNBC and that this phenomenon might be related to the effects of such combination on both ERK1/2 and AKT activation. J. Cell. Biochem. 116: 2778–2785, 2015.

KEY WORDS: SELUMETINIB; GEFITINIB; TRIPLE NEGATIVE BREAST CANCER

Triple negative breast cancer (TNBC) is a subtype of breast cancer characterized by a lack of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (ErbB-2) expression. Patients with triple-negative tumors have a poor outcome and do not respond to endocrine or anti-ErbB-2 therapies [Foulkes et al., 2010]. Currently, the mainstay of treatment for TNBC, in both the curative and metastatic settings, is conventional cytotoxic chemotherapy, because effective targeted therapies are not available [Crown et al., 2012].

The RAF/MEK/ERK signaling pathway plays an important role in breast cancer, regulating the growth and survival of breast cancer cells [De Luca et al., 2012]. Recently, analysis of TNBC tumor specimens showed evidence of high levels of activation of the RAF/MEK/ERK pathway, supporting MEK as suitable target for therapeutic intervention in TNBC [Craig et al., 2013]. The epidermal growth factor receptor (EGFR) is also overexpressed in at least 50% of TNBC and it is a negative prognostic factor in this tumor subset [Masuda et al., 2012].

Selumetinib (AZD6244), is a potent and selective adenosine triphosphate-uncompetitive inhibitor of MEK1/2 with anticancer activity both in vitro and in vivo [Yeh et al., 2007]. The drug is currently in phase III clinical development. However, selumetinib, as single agent, failed to demonstrate clinical activity in different tumour types [Zhao and Adjei, 2014]

Acquired or selected mutations can decrease the affinity of kinase inhibitors for their targets, but alternative routes of pathway activation may also reduce the efficacy of such drugs. In this regard, in basal-type breast cancer cells the antitumor activity of the MEK inhibitor CI1040 was found to be limited by the compensatory activation of the PI3K/AKT pathway [Mirzoeva et al., 2009]. Importantly, feedback activation of PI3K induced by MEK blockade was found to be mediated by the EGFR, although this phenomenon was formally proven in a single TNBC cell line (MDA-MB-231) [Mirzoeva et al., 2009]. In agreement with this hypothesis, selumetinib was found to induce EGFR activation in colorectal carcinoma cells bearing a mutant BRAF [Prahallad et al., 2012].

Grant sponsor: Associazione Italiana per la Ricerca sul Cancro (AIRC).

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Manuscript Received: 24 April 2015; Manuscript Accepted: 5 May 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 8 May 2015
DOI 10.1002/jcb.25220 • © 2015 Wiley Periodicals, Inc.

However, a more recent study suggested that selumetinib causes a marked loss of ERK activity in TNBC resulting in a rapid c-MYC degradation, with induction of the expression and activation of different receptor tyrosine kinases not including the EGFR [Duncan et al., 2012].

Although these differences might be due to the experimental approach used, these data raise the question of which receptor tyrosine kinase should be targeted to interrupt the feedback loop leading to PI3K activation following MEK inhibition. Actually, previous studies have suggested that combined treatment of TNBC cells with both MEK and PI3K inhibitors can overcome the EGFR activation induced by MEK blockade [Mirzoeva et al., 2009]. However, receptor tyrosine kinases can activate several different intracellular signaling pathways other than the RAS/MEK/ERK and the PI3K/AKT pathways. Therefore, identification of the target receptor involved in this phenomenon is mandatory to develop more adequate therapeutic strategies.

We previously demonstrated a synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib and the MEK1 inhibitor PD98059 in the MDA-MB-468 TNBC cell line [Normanno et al., 2006]. Therefore, we hypothesized that the simultaneous blockade of the RAS/MEK/ERK pathway and the EGFR may affect the proliferation and survival of TNBC cells in a more efficient manner. In this study, we explored the effects of selumetinib in combination with gefitinib in a panel of TNBC cells, in order to evaluate whether the simultaneous blockade of the EGFR and the RAS/MEK/ERK pathway might increase the antitumor activity of selumetinib in TNBC.

MATERIALS AND METHODS

MATERIALS

Gefitinib and selumetinib were kindly provided by Astra Zeneca (Macclesfield, UK).

CELL LINES

The human breast cancer cell lines MDA-MB-231, MDA-MB-468, HCC70 and AU565 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely grown in RPMI 1640 medium with GlutaMAX supplemented with 10% fetal bovine serum (FBS) (for AU565 cells) or 5% FBS (for MDA-MB-231, HCC70 cells) (all from LifeTechnologies/Thermo Fisher Scientific Milan, Italy). MDA-MB-468 cells were cultured as previously described [Normanno et al., 2006]. SUM-159 and SUM-149 human breast carcinoma cell lines were purchased from Asterand (Detroit, MI) and maintained in Ham's F12 medium with GlutaMAX (LifeTechnologies) supplemented with 5% FBS and 5 μg/ml insulin.

CELL PROLIFERATION ASSAYS

Cells were seeded into 96-wells plates (6×10^3 MDA-MB-468 cells; 5×10^3 SUM-159 cells; 1×10^4 SUM-149 cells and 3×10^3 AU565 cells) in serum-containing medium and allowed to attach for 24 h. Then, the medium was replaced and cells were treated for 72 h with different concentrations of gefitinib and selumetinib alone or in combination. Cell proliferation was evaluated using the tetrazolium-based (MTT) colorimetric assay as previously described [Normanno et al., 2006].

MDA-MB-231 and HCC70 cells (1×10^4 and 7.5×10^3 cells/well) were seeded in 48-wells plates. After 72 h of treatment with gefitinib and/or selumetinib, cells were trypsinized and counted with an automated cell counter (Coulter Model Z1; Instrumentation Lab., Milan, Italy).

ANALYSIS OF COMBINATION EFFECT

Combination analysis of treatment with gefitinib and selumetinib was performed using the Calcusyn software program (Biosoft, Cambridge, UK) that calculates a combination index (CI) according with Chou and Talalay-derived equations [Chou and Talalay, 1984]. CIs <1, $=1$, and >1 represent synergistic, additive and antagonist effects, respectively. The CI value for a fraction where 50% of the cells were affected (CI fa50) was used for our analysis of the experimental data.

WESTERN BLOT ANALYSIS

Whole protein extracts were prepared and analyzed by western blotting, according to a standard procedure. The following antibodies were used: anti-phospho p42/p44 MAPK (ERK1/2) (Thr202/Tyr204), anti-phospho AKT (Ser473); anti-phospho EGFR (Tyr 1068) (Cell Signaling Technology, Beverly, MA); anti-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA); anti-α-tubulin clone DM1A (Sigma-Aldrich, Milan, Italy).

CELL CYCLE ANALYSIS

MDA-MB-468 and SUM-149 cells were seeded in 60 mm dishes in serum-containing medium. After 24 h, the medium was replaced and cells were treated for 24 h with 1 μM gefitinib and/or selumetinib at the concentration of 0.2 μM (for SUM-149 cells) or 2.5 μM (for MDA-MB-468 cells). Cells were trypsinized, washed with PBS, and fixed in 95% ethanol. Then, cells were incubated in propidium iodide staining solution (50 μg/ml propidium iodide, 0.5 mg/ml RNase, 0.2% NP-40). After 30 min at room temperature in the dark, the DNA content was analyzed using a BD FACSAria II flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle data analysis was performed using the CellFIT program (Becton Dickinson).

APOPTOSIS ASSAY

SUM-149 were seeded in 60 mm dishes in serum-containing medium. Then, the medium was replaced and cells were treated for 24 h with gefitinib (1 μM) and/or selumetinib (0.2 μM). Apoptosis was measured using Annexin V-FITC kit (Alexis Biochemicals, San Diego, CA) according to the manufacturer's protocol. Cells were analyzed using the BD FACSAria II flow cytometer.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. Significance was determined using two-tailed Student t test. P values<0.05 were considered statistically significant.

RESULTS

EFFECTS OF SELUMETINIB ON TNBC CELL PROLIFERATION

We preliminarily assessed the effects of selumetinib on cell proliferation in a panel of TNBC cell lines (HCC70, MDA-MB-231, MDA-MB-468, SUM-149 and SUM-159) and in a luminal breast

cancer cell line (AU565). We found that TNBC cells had different sensitivity to selumetinib. Accordingly with a previous study [Dry et al., 2010] that classified as sensitive to selumetinib cell lines with $IC_{50} < 1$ μM and resistant cell lines with $IC_{50} > 10$ μM, SUM-149 and MDA-MB-231 cells resulted to be sensitive, whereas SUM-159, MDA-MB-468 and HCC70 cells were resistant to the drug. In agreement with previous findings indicating that luminal cell lines are less sensitive to MEK inhibitors than basal-like breast cancer cells, the luminal-type AU565 cell line was resistant to the drug ($IC_{50} > 10$ μM) (Table I) [Hoeflich et al., 2009; Mirzoeva et al., 2009].

EFFECTS OF THE BLOCKADE OF THE MEK/ERK PATHWAY ON EGFR ACTIVATION

We investigated in the panel of TNBC cell lines whether MEK inhibition with selumetinib was able to induce EGFR activation as previously hypothesized [Mirzoeva et al., 2009]. Treatment with selumetinib led to a marked and persistent increase in the phosphorylation of the EGFR in SUM-149, MDA-MB-231, and AU565 cells (Fig. 1). A slight and temporary increase of EGFR phosphorylation was observed in MDA-MB-468 cells, whereas in the HCC70 and SUM-159 cell lines no significant change in the levels of phosphorylated EGFR was observed. Taken together, our data confirmed that the EGFR might be activated in TNBC cell lines following treatment with selumetinib, although this phenomenon was not consistently found in all cell lines.

EFFECTS OF SELUMETINIB ALONE OR IN COMBINATION WITH GEFITINIB ON THE PROLIFERATION OF TNBC CELLS

Because selumetinib produced in a subset of TNBC cells a marked increase in the phosphorylation of the EGFR, we explored the effects of the drug in combination with gefitinib on the proliferation of TNBC cells. In agreement with a previous study [Corkery et al., 2009], we found that TNBC cell lines are generally less sensitive to gefitinib ($IC_{50} > 10$ μM), than the luminal AU565 cell line (IC_{50} 0.5 μM), with the only exception of SUM-149 cells that had an intermediate sensitivity to the drug (IC_{50} 5.0 μM) (Fig. 2).

Treatment with the combination of selumetinib and gefitinib produced a more significant growth inhibition as compared with the single agent in MDA-MB-231, SUM-149, and HCC70 cells. Although similar results were observed in SUM-159 and MDA-MB-468 cells, it must be emphasized that the combination of the two drugs did not achieve the IC_{50} value. In the luminal cell line the combined treatment produced a growth inhibitory effect similar to that induced by gefitinib alone (Fig. 2).

Combination analysis using the Chou and Talalay equation revealed that treatment with gefitinib and selumetinib was very strong synergistic in SUM-149 (CI = 0.094), strong synergistic in HCC70 (CI = 0.120), SUM-159 (CI = 0.136) and MDA-MB-468 cells (CI = 0.111); synergistic in the MDA-MB-231 cell line (CI = 0.508); nearly additive in AU565 cells (CI = 0.909) (Fig. 3). Collectively, our data suggested that the combination of gefitinib and selumetinib produced a synergistic growth inhibitory effect in TNBC cell lines.

TABLE I. IC_{50} of TNBC Cell Lines for the MEK Inhibitor Selumetinib

Cell Line	Subtype	Selumetinib IC_{50} (μM) ± SEM
AU565	Luminal	>10
HCC70	Basal A	9.90 ± 0.12
MDA-MB-468	Basal A	>10
MDA-MB-231	Basal B	0.68 ± 0.14
SUM-149	Basal B	0.51 ± 0.07
SUM-159	Basal B	>10

EFFECT OF TREATMENT WITH GEFITINIB AND/OR SELUMETINIB ON THE ACTIVATION OF SIGNALING PROTEINS IN TNBC CELL LINES

To identify the biochemical mechanisms involved in the synergism of selumetinib and gefitinib, we assessed the phosphorylation status of ERK1/2 and AKT after 24 h of treatment with selumetinib and/or gefitinib alone or in combination (Fig. 4).

Treatment with 0.5 μM selumetinib significantly reduced ERK1/2 phosphorylation in all cell lines. In agreement with previous findings, an increase in the phosphorylation of AKT was also observed in selumetinib-treated cells [Mirzoeva et al., 2009](Fig. 4). Following treatment with 1 μM gefitinib, a significant reduction in the levels of ERK1/2 phosphorylation in AU565, HCC70, and SUM-149 cells and a slight reduction in MDA-MB-468 were observed. The drug did not affect the activation of ERK1/2 in SUM-159 and MDA-MB-231 cells. No significant effects on AKT phosphorylation were observed in breast cancer cells following treatment with gefitinib. Combined treatment with gefitinib and selumetinib produced an almost complete suppression of ERK1/2 activation and a reduction of selumetinib-induced AKT phosphorylation (Fig. 4).

EFFECT OF GEFITINIB AND SELUMETINIB ON CELL CYCLE DISTRIBUTION IN TNBC CELLS

We next analyzed the alterations in cell cycle distribution produced by the combination of gefitinib and selumetinib in TNBC cell lines. For this purpose, we treated MDA-MB-468 and SUM-149 cells, that show different levels of sensitivity to selumetinib, with the combination of gefitinib and selumetinib for 24 hours (Fig. 5).

Treatment with selumetinib has been found to produce a pronounced G0/G1 arrest in sensitive breast cancer cell lines but not in resistant cells [Garon et al., 2010]. In addition, response to EGFR inhibition was associated with the induction of G1 cell cycle arrest and with the activation of an apoptotic cascade only in a subset of breast cancer cell lines [Campiglio et al., 2004; Corkery et al., 2009]. In agreement with these finding, in MDA-MB-468 cells that were resistant to both drugs, treatment with selumetinib or gefitinib alone did not induce an arrest in G0/G1. The combination produced a slight accumulation of cells in G0/G1 cell cycle phase and in G2/M and a mild reduction of cells in the S-phase, compared to untreated cells or to cells treated with the single agent (Fig. 5). In contrast, in SUM-149 cells, a significant accumulation of cells in G0/G1 and a reduction of cells in phase S were observed following treatment with either gefitinib, selumetinib or the combination. However, the effects of the combination on cell cycle progression were more pronounced as compared with single agent treatment. In agreement with these findings, in SUM-149 cells a sub-G1 peak, suggestive of apoptosis, was also more evident in cells treated with the combination than in cells treated with the single agent (Fig. 5). Using an Annexin V-FITC apoptosis assay, we confirmed that the combination of selumetinib and gefitinib induced apoptosis in SUM-

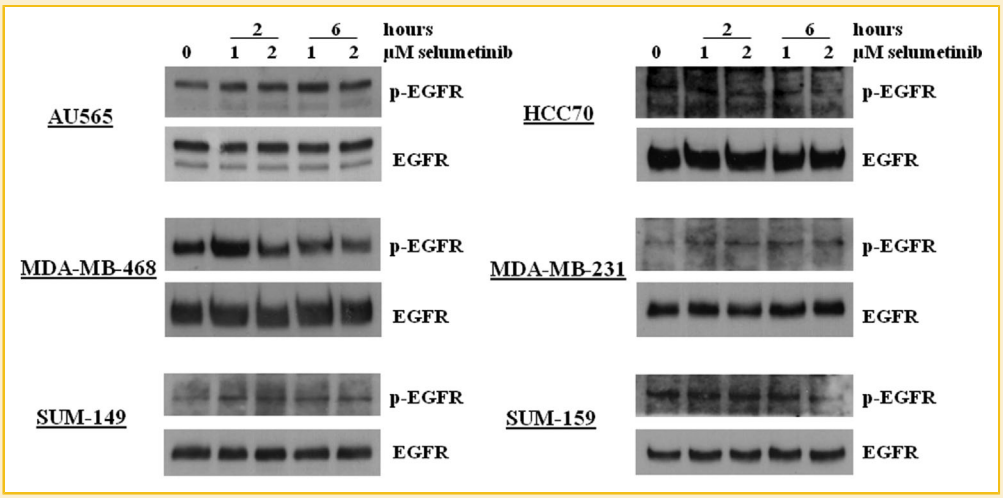


Fig. 1. Analysis of the phosphorylation of the EGFR in TNBC cells. Breast cancer cells were treated with different concentrations of selumetinib (1 or 2 µM) for 2 or 6 h. Western blot analysis for phosphorylated (p-EGFR) or total EGFR expression was performed with specific antibodies.

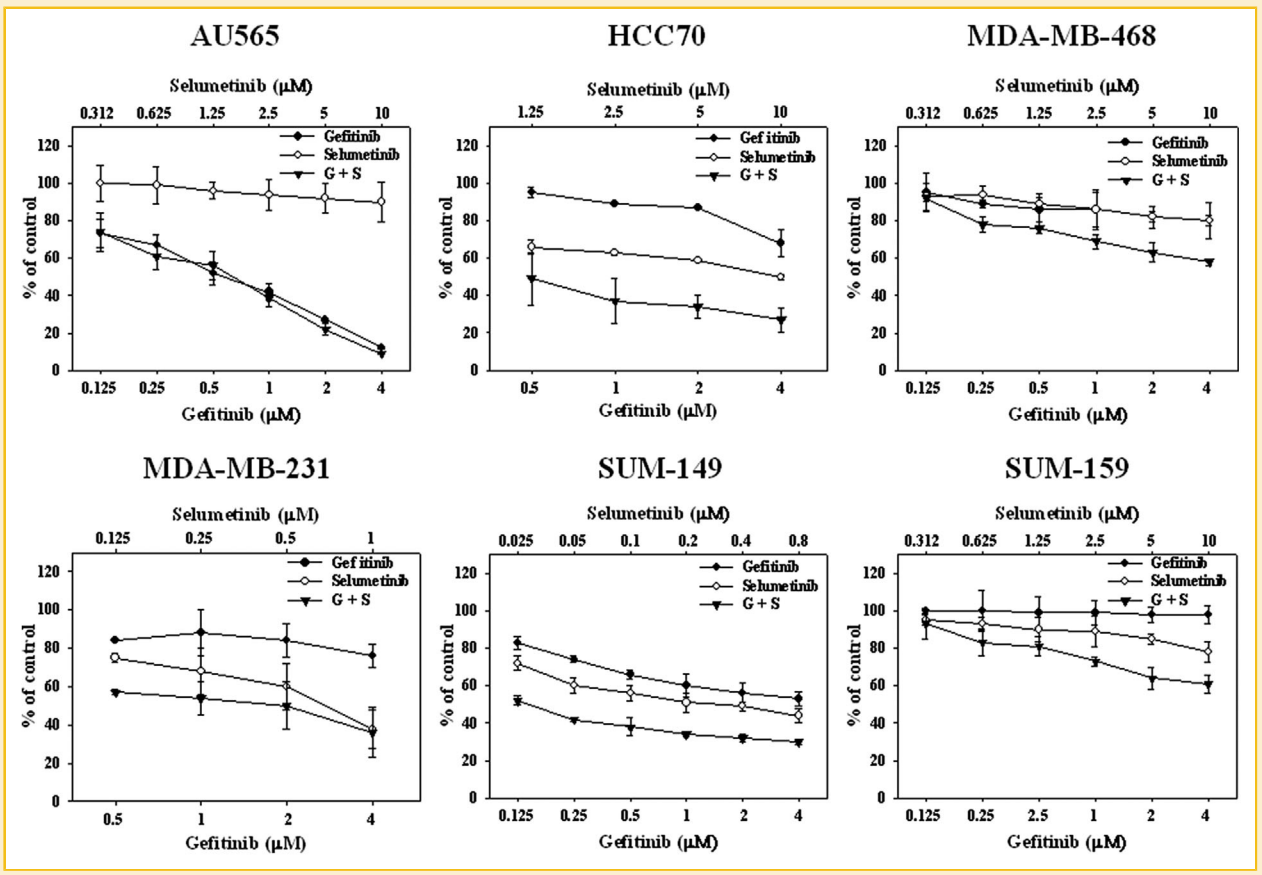


Fig. 2. Effects of treatment with selumetinib and gefitinib, alone or in combination, on the proliferation of TNBC cell lines. Cells were treated for 72 h with the indicated concentrations of the drugs. Cell proliferation was determined using an MTT assay or counting cells with an automated cell counter.

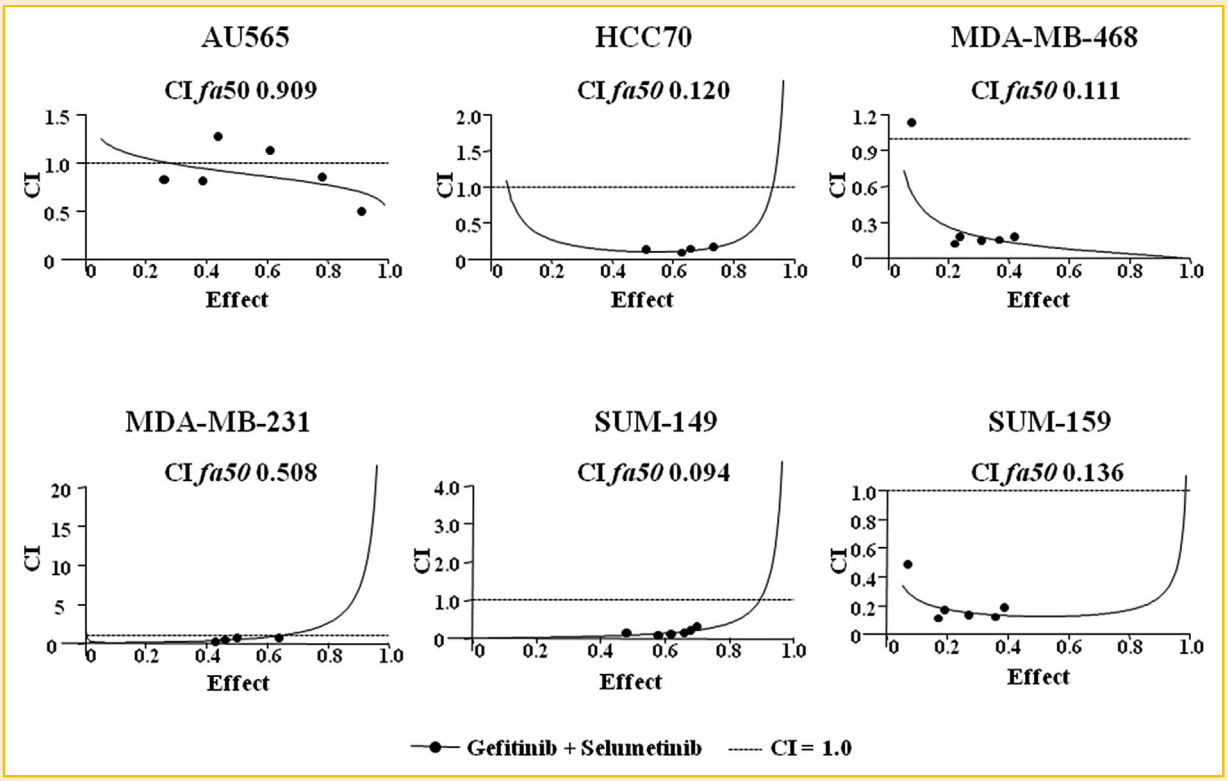


Fig. 3. Dose-effect analysis of the combination of selumetinib and gefitinib in TNBC cells. Combination analysis was performed using the method described by Chou and Talalay. CI fa50 = combination index at a fraction that affects 50%.

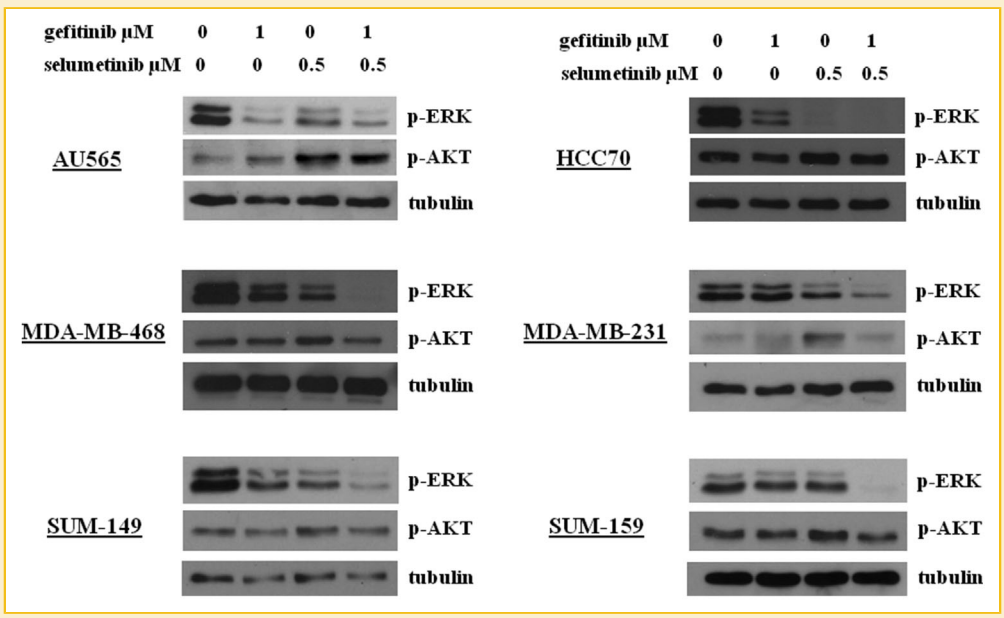
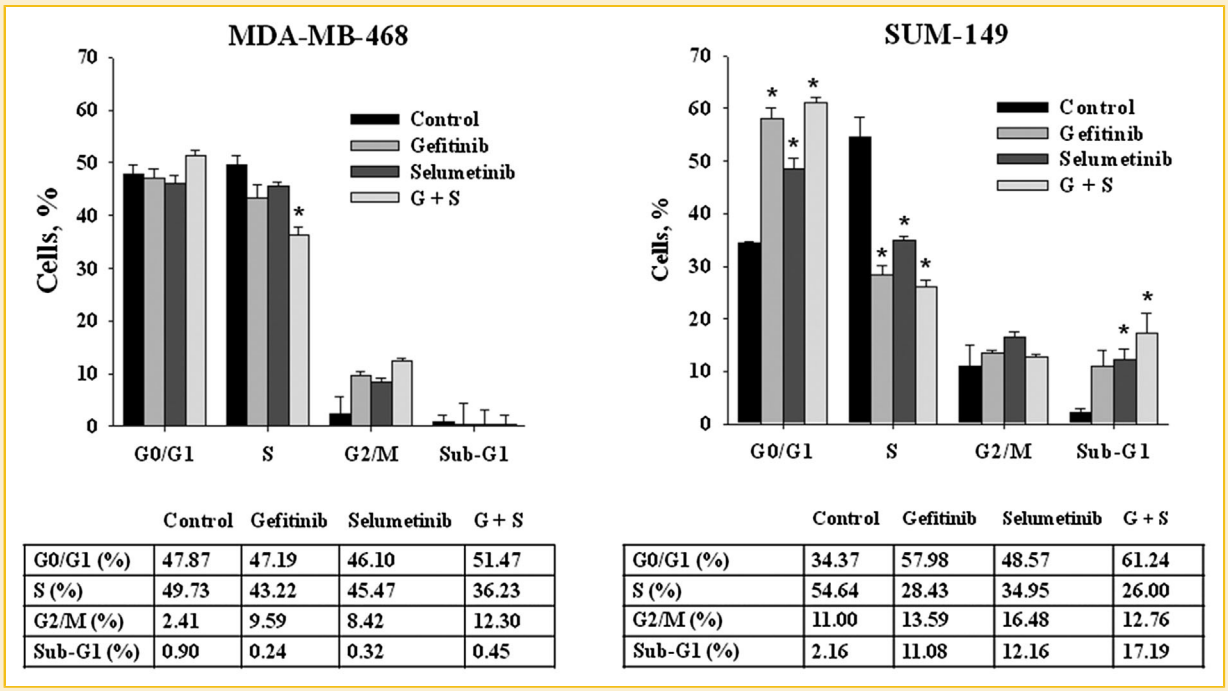


Fig. 4. Analysis of ERK and AKT activation in TNBC cells. Western blot analysis for the expression of the phosphorylated forms of ERK1/2 (p-ERK) and AKT (p-AKT) was performed in TNBC cells following treatment with 0.5 μM selumetinib and 1 μM gefitinib alone or in combination for 24 h. The blots were normalized to α-tubulin.



	Control	Gefitinib	Selumetinib	G + S
G0/G1 (%)	47.87	47.19	46.10	51.47
S (%)	49.73	43.22	45.47	36.23
G2/M (%)	2.41	9.59	8.42	12.30
Sub-G1 (%)	0.90	0.24	0.32	0.45

	Control	Gefitinib	Selumetinib	G + S
G0/G1 (%)	34.37	57.98	48.57	61.24
S (%)	54.64	28.43	34.95	26.00
G2/M (%)	11.00	13.59	16.48	12.76
Sub-G1 (%)	2.16	11.08	12.16	17.19

Fig. 5. Effect of gefitinib and selumetinib alone or in combination on cell cycle distribution in TNBC cells. Cell cycle distribution analysis was performed in untreated or treated MDA-MB-468 and SUM-149 cells with selumetinib 2.5 μM for MDA-MB-468 cells or 0.2 μM for SUM-149 cells alone or in combination with 1 μM gefitinib for 24 h. *$P < 0.05$ (Student *t*-test).

149 cells (Fig. 6). These results suggested that gefitinib and selumetinib in combination may have a cytotoxic effect in selected TNBC cell lines sensitive to selumetinib.

DISCUSSION

Identification of molecular mechanisms regulating the sensitivity/resistance of cancer cells to targeted agents is mandatory in order to improve our ability to select patients that might benefit from these agents. In this respect, the RAS/MEK/ERK signaling pathway has been identified as a key player in promoting TNBC cell proliferation, and different studies demonstrated that basal-like breast cancer cells are highly sensitive to MEK inhibition [Hoeflich et al., 2009; Mirzoeva et al., 2009; Garon et al., 2010]. Nevertheless, some TNBC cell lines have shown resistance to MEK inhibitors in pre-clinical studies and early clinical trials have not shown activity of these agents in different tumor types, thus underlining the need to better understand the mechanisms regulating resistance to these drugs [Zhao and Adjei, 2014].

Cancer cells can escape the blockade of a pathway by activating compensatory mechanisms that promote proliferation and survival [De Luca et al., 2012]. Our finding confirmed that feedback activation of AKT signaling occurs in breast cancer cells following treatment with MEK inhibitors [Mirzoeva et al., 2009]. However, this phenomenon was observed in all cell lines, irrespectively of their level of sensitivity to selumetinib thus implying that different mechanisms might be involved in the resistance of TNBC cells to MEK inhibition.

Different studies demonstrated that selective inhibition of specific kinases led to the up-regulation of tyrosine kinase receptors, which in turn can activate alternative intracellular signaling pathways to overcome the blockade [Chandarlapaty et al., 2011; Serra et al., 2011]. In this respect, contrasting results have been previously reported on the mechanisms involved in resistance to MEK inhibitors in TNBC. Mirzoeva et al. [2009] identified in the EGFR the main pathways leading to AKT activation in MEK inhibitor-treated breast cancer cells, whereas a more recent study [Duncan et al., 2012] suggested that other receptor tyrosine kinases, such as PDGFRβ, AXL, VEGFR2, RET, and ErbB-3 might be involved in this phenomenon [Mirzoeva et al., 2009; Duncan et al., 2012]. In this respect, we found that activation of the EGFR was not consistently observed in cell lines treated with selumetinib. However, it is important to underline that we found a persistent increase in phosphorylated EGFR in selumetinib-sensitive TNBC cell lines, whereas no significant increase in EGFR phosphorylation was observed in cell lines that are resistant to the drug. Interestingly, we observed the increased phosphorylation of the EGFR in the presence of serum, whereas the increase in the phosphorylation of the EGFR following treatment with the MEK inhibitor CI1040 in MDA-MB-231 cells was previously observed in low serum conditions and in presence of exogenous EGF [Mirzoeva et al., 2009]. This observation might suggest that selumetinib is more potent in inducing the phosphorylation of the EGFR, compared to other MEK inhibitors.

Although EGFR activation was not induced in 2/5 TNBC cell lines, we found that simultaneous blockade of MEK and EGFR produced a

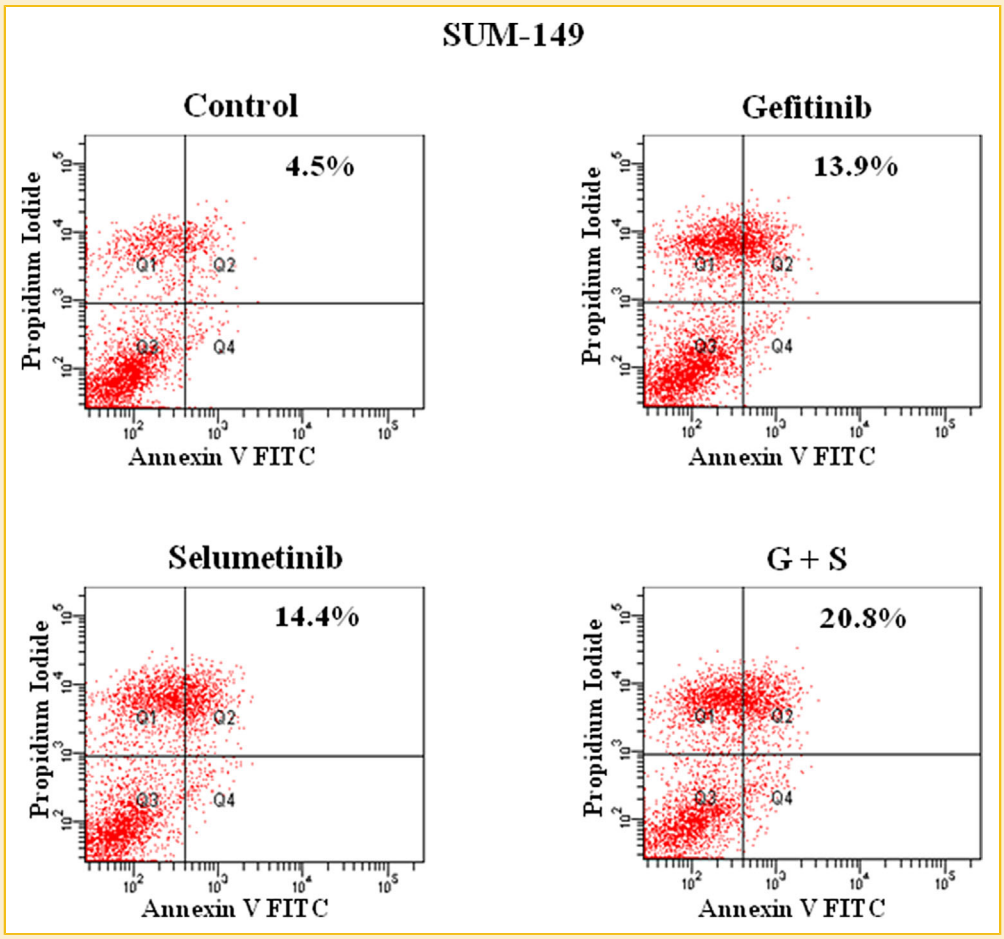


Fig. 6. Induction of apoptosis in SUM-149 cell line. SUM-149 cells were treated for 24 h with 0.2 μM selumetinib and/or 1 μM gefitinib. Annexin V/propidium iodide positive cells were evaluated by flow cytometry.

synergistic growth inhibitory effect in all the TNBC cell lines tested. These findings suggested that the EGFR is likely to be involved in either primary and/or acquired resistance to selumetinib in TNBC, although other mechanisms probably co-exist in these cells. Indeed, it must be emphasized that in the SUM-159 and MDA-MB-468 cell lines, despite the strong pharmacological synergism of gefitinib and selumetinib, the IC_{50} was not reached. Finally, these findings are apparently in contrast with Duncan et al. [2012] that reported involvement of other receptor tyrosine kinases in the resistance to selumetinib. However, the EGFR has interactions with several different membrane receptors that might be disrupted by EGFR inhibitors.

The above mentioned findings suggest that the combination of gefitinib and selumetinib might exert a significant clinical effect only in selected TNBC. In this respect, identification of the mechanism underlying the synergism of these two drugs in TNBC becomes mandatory to identify potential biomarkers of activity. We observed in all cell lines treated with the combination of the two agents a reduction in the levels of MEK inhibitor-induced AKT activation, as previously reported [Mirzoeva et al., 2009]. This phenomenon was observed in all cell lines, including AU565 cells in which no synergism occurred. In addition, the combination at best reduced the levels of AKT to those observed in untreated cells. In contrast, the combination of gefitinib and selumetinib produced a more significant inhibition of ERK activation as compared with single agent at 24 h of treatment in all cell lines with the exception of AU565 cells in which no synergism was observed. In this respect, Duncan et al. [2012] showed that treatment with selumetinib for 24 h resulted in reactivation of ERK following initial down-regulation, demonstrating that breast cancer cells are able to overcome the initial MEK inhibition [Duncan et al., 2012]. Our data suggest that the EGFR is a key player in reactivation of ERK signaling in MEK inhibitor-treated cells and highlight the potential of combinations of drugs targeting these kinases in the treatment of TNBC. Indeed, in SUM-149 cells that are highly sensitive to the combination, the simultaneous blockade of EGFR and MEK produced a marked induction of G1 arrest and a significant increase in the apoptosis. This observation is in agreement with a previous report showing that combined treatment with gefitinib and selumetinib synergistically induced apoptosis in gastric cancer cells [Yoon et al., 2009]. In addition, several preclinical studies suggested that MEK inhibitors in combination with other signaling inhibitors blocked more efficiently

the proliferation and survival of TNBC cell lines, compared with the single agent. Treatment of MDA-MB-231 and SUM-149 cell lines with a combination of a MEK and a PI3K inhibitor resulted in a synergistic inhibitory effect on cell viability [Mirzoeva et al., 2009]. A synergistic cell growth inhibitory effect was also observed when SUM-159 cells were treated with selumetinib in combination with the multikinase inhibitors sorafenib or foretinib [Duncan et al., 2012]. Generally speaking, the use of receptor kinase inhibitors in combination with selumetinib has the advantage to block different potential escape pathways, including a possible cross-talk between membrane receptors.

In conclusion, our data demonstrated that blockade of the EGFR with gefitinib might increase the antitumor activity of selumetinib in a subgroup of TNBC cell lines. These findings provide the rationale for testing selumetinib in combination with gefitinib in clinical trials in TNBC, although biomarkers to identify patients that might benefit of this approach are definitely needed.

ACKNOWLEDGMENT

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC) to N. Normanno.

REFERENCES

Campiglio M, Locatelli A, Olgiati C, Normanno N, Somenzi G, Vigano L, Fumagalli M, Menard S, Gianni L. 2004. Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level. J Cell Physiol 198:259–268.

Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, Majumder PK, Baselga J, Rosen N. 2011. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell 19:58–71.

Chou TC, Talalay P. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55.

Corkery B, Crown J, Clynes M, O'Donovan N. 2009. Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. Ann Oncol 20:862–867.

Craig DW, O'Shaughnessy JA, Kiefer JA, Aldrich J, Sinari S, Moses TM, Wong S, Dinh J, Christoforides A, Blum JL, Aitelli CL, Osborne CR, Izatt T, Kurdoglu A, Baker A, Koeman J, Barbacioru C, Sakarya O, De La Vega FM, Siddiqui A, Hoang L, Billings PR, Salhia B, Tolcher AW, Trent JM, Mousses S, Von Hoff D, Carpten JD. 2013. Genome and transcriptome sequencing in prospective metastatic triple-negative breast cancer uncovers therapeutic vulnerabilities. Mol Cancer Ther 12:104–116.

Crown J, O'Shaughnessy J, Gullo G. 2012. Emerging targeted therapies in triple-negative breast cancer. Ann Oncol 23(Suppl6):56–65.

De Luca A, Maiello MR, D'Alessio A, Pergameno M, Normanno N. 2012. The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches. Expert Opin Ther Targets 16(Suppl2):S17–S27.

Dry JR, Pavey S, Pratilas CA, Harbron C, Runswick S, Hodgson D, Chresta C, McCormack R, Byrne N, Cockerill M, Graham A, Beran G, Cassidy A, Haggerty C, Brown H, Ellison G, Dering J, Taylor BS, Stark M, Bonazzi V, Ravishankar S, Packer L, Xing F, Solit DB, Finn RS, Rosen N, Hayward NK, French T, Smith PD. 2010. Transcriptional pathway signatures predict MEK addiction and response to selumetinib (AZD6244). Cancer Res 70:2264–2273.

Duncan JS, Whittle MC, Nakamura K, Abell AN, Midland AA, Zawistowski JS, Johnson NL, Granger DA, Jordan NV, Darr DB, Usary J, Kuan PF, Smalley DM, Major B, He X, Hoadley KA, Zhou B, Sharpless NE, Perou CM, Kim WY, Gomez SM, Chen X, Jin J, Frye SV, Earp HS, Graves LM, Johnson GL. 2012. Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. Cell 149:307–321.

Foulkes WD, Smith IE, Reis-Filho JS. 2010. Triple-negative breast cancer. N Engl J Med 363:1938–1948.

Garon EB, Finn RS, Hosmer W, Dering J, Ginther C, Adhami S, Kamranpour N, Pitts S, Desai A, Elashoff D, French T, Smith P, Slamon DJ. 2010. Identification of common predictive markers of in vitro response to the Mek inhibitor selumetinib (AZD6244; ARRY-142886) in human breast cancer and non-small cell lung cancer cell lines. Mol Cancer Ther 9:1985–1994.

Hoeflich KP, O'Brien C, Boyd Z, Cavet G, Guerrero S, Jung K, Januario T, Savage H, Punnoose E, Truong T, Zhou W, Berry L, Murray L, Amler L, Belvin M, Friedman LS, Lackner MR. 2009. In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. Clin Cancer Res 15:4649–4664.

Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. 2012. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat 136:331–345.

Mirzoeva OK, Das D, Heiser LM, Bhattacharya S, Siwak D, Gendelman R, Bayani N, Wang NJ, Neve RM, Guan Y, Hu Z, Knight Z, Feiler HS, Gascard P, Parvin B, Spellman PT, Shokat KM, Wyrobek AJ, Bissell MJ, McCormick F, Kuo WL, Mills GB, Gray JW, Korn WM. 2009. Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition. Cancer Res 69:565–572.

Normanno N, De Luca A, Maiello MR, Campiglio M, Napolitano M, Mancino M, Carotenuto A, Viglietto G, Menard S. 2006. The MEK/MAPK pathway is involved in the resistance of breast cancer cells to the EGFR tyrosine kinase inhibitor gefitinib. J Cell Physiol 207:420–427.

Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, Beijersbergen RL, Bardelli A, Bernards R. 2012. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 483:100–103.

Serra V, Scaltriti M, Prudkin L, Eichhorn PJ, Ibrahim YH, Chandarlapaty S, Markman B, Rodriguez O, Guzman M, Rodriguez S, Gili M, Russillo M, Parra JL, Singh S, Arribas J, Rosen N, Baselga J. 2011. PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. Oncogene 30:2547–2557.

Yeh TC, Marsh V, Bernat BA, Ballard J, Colwell H, Evans RJ, Parry J, Smith D, Brandhuber BJ, Gross S, Marlow A, Hurley B, Lyssikatos J, Lee PA, Winkler JD, Koch K, Wallace E. 2007. Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. Clin Cancer Res 13:1576–1583.

Yoon YK, Kim HP, Han SW, Hur HS, Oh do Y, Im SA, Bang YJ, Kim TY. 2009. Combination of EGFR and MEK1/2 inhibitor shows synergistic effects by suppressing EGFR/HER3-dependent AKT activation in human gastric cancer cells. Mol Cancer Ther 8:2526–2536.

Zhao Y, Adjei AA. 2014. The clinical development of MEK inhibitors. Nat Rev Clin Oncol 11:385–400.