MEK1/2 Inhibitor Selumetinib (AZD6244) Inhibits Growth of Ovarian Clear Cell Carcinoma in a PEA-15–Dependent Manner in a Mouse Xenograft Model

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Abstract

Clear cell carcinoma (CCC) of the ovary tends to show resistance to standard chemotherapy, which results in poor survival for patients with CCC. Developing a novel therapeutic strategy is imperative to improve patient prognosis. Epidermal growth factor receptor (EGFR) is frequently expressed in epithelial ovarian cancer. One of the major downstream targets of the EGFR signaling cascade is extracellular signal–related kinase (ERK). PEA-15, a 15-kDa phosphoprotein, can sequester ERK in the cytoplasm. MEK1/2 plays a central role in integrating mitogenic signals into the ERK pathway. We tested the hypothesis that inhibition of the EGFR–ERK pathway suppresses tumorigenicity in CCC, and we investigated the role of PEA-15 in ERK-targeted therapy in CCC. We screened a panel of 4 CCC cell lines (RMG-I, SMOV-2, OVTOKO, and KOC-7c) and observed that the EGFR tyrosine kinase inhibitor erlotinib inhibited cell proliferation of EGFR-overexpressing CCC cell lines through partial dependence on the MEK/ERK pathway. Furthermore, erlotinib-sensitive cell lines were also sensitive to the MEK inhibitor selumetinib (AZD6244), which is under clinical development. Knockdown of PEA-15 expression resulted in reversal of selumetinib-sensitive cells to resistant cells, implying that PEA-15 contributes to selumetinib sensitivity. Both selumetinib and erlotinib significantly suppressed tumor growth (P < 0.0001) in a CCC xenograft model. However, selumetinib was better tolerated; erlotinib-treated mice exhibited significant toxic effects (marked weight loss and severe skin peeling) at high doses. Our findings indicate that the MEK–ERK pathway is a potential target for EGFR-overexpressing CCC and indicate that selumetinib and erlotinib are worth exploring as therapeutic agents for CCC.
Erlotinib inhibits the activation of the ERK pathway in several human non–small cell lung cancer cell lines (7). Inhibition of ERK by EGFR-TKIs intercepts ERK’s upstream aberrant mitogenic signals. To date, no therapeutically useful direct ERK inhibitors has been adopted in clinical practice. MEK1/2 plays a central role in integration of mitogenic signals into the ERK pathway, as MEK has no downstream substrates except for ERK1/2. Thus, MEK is an excellent alternative therapeutic target with which to modulate ERK activity. The MEK inhibitor PD-0325901 selectively binds to and inhibits MEK, which results in inhibition of ERK phosphorylation and inhibition of tumor cell proliferation. However, PD-0325901 has been discontinued from Pfizer’s drug development pipeline because the phase II trial of PD-0325901 showed no objective tumor response (8, 9). However, selumetinib (AZD6244), a potent, selective, ATP-competitive inhibitor of MEK1/2 kinases (10, 11), is currently in phase II clinical trial development for melanoma and non–small cell lung cancer.

PEA-15 is a phosphoprotein that slows cell proliferation by binding to and sequestering ERK in the cytoplasm (12). The structure of PEA-15 indicates that phosphorylation could regulate PEA-15 binding to ERK (13). We previously showed that transfection of low PEA-15–expressing ovarian cancer cells with PEA-15 inhibited cell proliferation (14). Analysis of PEA-15 expression in a tissue microarray of epithelial ovarian cancer samples revealed that PEA-15 expression is associated with prolonged overall survival in patients with epithelial ovarian cancer (15).

We hypothesized that inhibition of the EGFR–ERK pathway suppresses tumorigenicity in ovarian CCC. To evaluate this hypothesis, we tested the impact of erlotinib and selumetinib on cell proliferation. We found that erlotinib suppressed cell proliferation in EGFR-overexpressing CCC cells, partly because of suppression of ERK. Furthermore, erlotinib-sensitive cells were also sensitive to selumetinib. This selumetinib sensitivity of CCC cells was partially dependent on the phosphorylation status of PEA-15. Erlotinib and selumetinib suppressed CCC tumor growth in a mouse intraperitoneal xenograft model. Our findings indicate that the MEK/ERK pathway may be a promising therapeutic target in EGFR-overexpressing CCC.

Materials and Methods

Cell lines and cell cultures

Four human ovarian CCC cell lines (RMG-I, KOC-7c, SMOV-2, and OVTOKO) were used. RMG-I was obtained from Professor Shiro Nozawa, Keio University, Tokyo, Japan; KOC-7c from Dr. Toru Sugiyama, Kurume University, Japan; SMOV-2 from Dr. Tomohiro Iida, St. Mariana University, Kawasaki, Japan; and OVTOKO from Dr. Hiroshi Minaguchi, Yokohama City University, Japan. RMG-I cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 medium (GIBCO). KOC-7c, SMOV-2, and OVTOKO cells were maintained in RPMI-1640 medium (GIBCO).

Plasmid constructs

The plasmid construct PEA-15-AA, the nonphosphorylated variant of PEA-15 (S104A/S116A), was a gift from Dr. Mark Ginsberg (University of California, San Diego, CA; ref. 13). To construct PEA-15-AD, we exchanged serine 104 (Ser-104) for Ala. pcDNA3-PEA-15-S116D served as a template using the primer 5’-CAAGCTT-AACCCGTATCCCCGCCAAGAAGTACAAAGATTTG-3’ and its reverse complement GTCTTTGTACTTTGTGGCAGCGGGATACGGGTAGCTTG. The structure of PEA-15 indicates that phosphorylation by binding to and sequestering ERK in the cytoplasm could regulate PEA-15 binding to ERK (13). To construct PEA-15-AA, the nonphosphorylated variant of PEA-15 (S104A/S116A), was a gift from Dr. Mark Ginsberg (University of California, San Diego, CA; ref. 13). To construct PEA-15-AD, we exchanged serine 104 (Ser-104) for Ala. plasmid (12). The plasmid construct PEA-15-AA, the nonphosphorylated variant of PEA-15 (S104A/S116A), was a gift from Dr. Mark Ginsberg (University of California, San Diego, CA; ref. 13). To construct PEA-15-AD, we exchanged serine 104 (Ser-104) for Ala. pcDNA3-PEA-15-S116D served as a template using the primer 5’-CAAGCTT-AACCCGTATCCCCGCCAAGAAGTACAAAGATTTG-3’ and its reverse complement GTCTTTGTACTTTGTGGCAGCGGGATACGGGTAGCTTG.

Drugs

Erlotinib was purchased from ChemieTek. Selumetinib was provided by AstraZeneca under the auspices of the National Cancer Institute’s Cancer Therapy Evaluation Program.

Western blot analysis

Cell pellets were lysed as described previously (16). Primary antibodies were rabbit anti-EGFR antibody (diluted 1:500; Santa Cruz Biotechnology), rabbit anti-phospho-EGFR (Tyr1173; 1:200; Santa Cruz Biotechnology), rabbit anti-phospho-p42/44 MAP kinase (Thr202/Tyr204; 1:500; Cell Signalling), rabbit anti-PEA-15 polyclonal antibody (1:1000; SynPep), rabbit anti-pPEA-15 (5104; 1:500; Cell Signalling), rabbit anti-pPEA-15 (S116; 1:500; Invitrogen), mouse anti-α-tubulin (1:5,000; Sigma-Aldrich), and mouse anti-β-actin (1:2,000; Sigma-Aldrich). Signals were detected using an Odyssey IR imaging system (LI-COR Biosciences).

WST-1 assay

Cell viability was assayed using cell proliferation reagent WST-1 (Roche Applied Science) as described previously (17). Ovarian CCC cells (RMG-I, 4 × 10⁴/90 μL; SMOV-2, 3 × 10⁴/90 μL; OVTOKO, 2 × 10⁴/90 μL; or KOC-7c, 2 × 10⁴/90 μL) were seeded into each well of a 96-well plate and treated the next day with erlotinib or selumetinib at a final concentration of 0.001, 0.01, 0.1, 1, or 10 μmol/L for 72 hours.

Short interfering RNA against EGFR

Cells were seeded in 6-well culture plates at 3.0 × 10⁵ cells/well (30%–50% confluence). The next day, cells were transfected with ON-TARGET SMART pools against EGFR or a scrambled control (Dharmacon) at a final concentration of 100 nmol/L using DharmaFECT 1 (Dharmacon) following the manufacturer’s protocol.

Cell-cycle distribution analysis

Briefly, cells were plated in a 6-well plate, cultured overnight, and then treated or untreated with selumetinib for 48 hours (final concentration 0.1 or 1 μmol/L for RMG-I; 1 or 5 μmol/L for SMOV-2) or selumetinib for 24 hours (final concentration 0.01 or 0.1 μmol/L for RMG-I).
Cell-cycle distribution was analyzed by flow cytometry as described previously (16).

**Mutation screening**

Mutation screening was carried out as described previously (18, 19). Genomic DNA was purified from all 4 CCC cell lines using a Gentra Puregene Cell Kit (Qiagen). PCR primers were designed to amplify the sequence of interest, using the same as previously reported (18, 19). Annealing temperatures were 59°C for EGFR exons 19 and 21 and KRAS exons 2 and 3. PCR primers used to amplify the sequence of interest were those of parental RMG-I cells. RMG-I/luc cells have a morphology and proliferation rate similar to those of parental RMG-I cells. RMG-I/luc cells in log-phase growth were trypsinized, washed twice with PBS, and the medium was replaced. The next day, selumetinib (2 µmol/L) was added to the culture medium. Forty-eight hours later, cells were stained with 0.2% trypan blue, and living cells and dead cells were counted under a microscope.

**Constitutive active MEK1 transfection**

Constitutive active MEK1 (MEK1^{C/A})-expressing plasmid was a gift from Dr. M.C. Hung (MD Anderson Cancer Center, Houston, TX; ref. 20). For MEK1^{C/A} transfection, briefly, cells were suspended in electroporation buffer. The MEK1^{C/A} or control vector pcDNA3 plasmids were transfected by electroporation using Nucleofector (Amaxa Biosystems). Stable transfectants were established by G418 selection. pERK protein expression was used for evaluating efficacy of the gene transduction. The stable transfectant RMG-1-vector and RMG-1-MEK cells were plated on 6-well plates (2 × 10^4 cells per well), and beginning the next day, cells were treated with erlotinib (2 or 5 µmol/L) for 48 or 72 hours and a trypan blue assay was carried out. In addition, RMG-1-vector and RMG-1-MEK cells were plated on 96-well plates (5 × 10^3 cells per well), and beginning the next day, cells were treated with erlotinib (1, 2, 5, or 10 µmol/L) for 72 hours and then subjected to WST-1 assay.

**Nuclear/cytoplasmic fractionation**

Cells were plated at 2 × 10^5 cells per well in 6-well plates and pelleted, after which the nuclear and cytoplasmic fractions were separated using a fractionation kit according to the manufacturer’s protocol (Chemicon International).

**PEA-15 transfection**

OVTOKO cells (1 × 10^5 cells per well) were plated on 35-mm plates. The next day, cells were transfected using FuGENE HD transfection reagent (Roche Applied Sciences) with PEA-15-AD (S104A, phosphoinhibitory at S104, S116D, phosphomimetic at S116), PEA-15-AD (S104A, phosphoinhibitory at S104, S116A, phosphoinhibitory at S116), or control vector pcDNA3 in serum-free medium. Twenty-four hours later, cells were washed with PBS, and selumetinib 10 µmol/L was added to the culture medium. Cells were stained with 0.2% trypan blue 44 hours after addition of drug, and living cells and dead cells were counted under a microscope.

**siRNA against PEA-15**

RMG-I cells were seeded in 6-well culture plates at 6.0 × 10^4 per well (70%–80% confluence) in DMEM/F12 medium supplemented with 10% FBS. The next day, cells were transfected with ON-TARGET plus siRNA SMART pools against PEA-15 or a scrambled control (Dharmacon) at a final siRNA concentration of 100 nmol/L using DharmaFECT 2 (Dharmacon) according to the manufacturer’s instructions. Six hours later, cells were washed with PBS, and the medium was replaced. The next day, selumetinib (2 µmol/L) was added to the culture medium.

**ERK-targeted therapy in an ovarian cancer xenograft model**

For xenograft experiments, luciferase-expressing RMG-I cells (RMG-I/luc) were established using firefly luciferase + gene pGL3 lentivirus vector (Lentigen). These cells have a morphology and proliferation rate similar to those of parental RMG-I cells. RMG-1/luc cells in log-phase growth were trypsinized, washed twice with PBS, and centrifuged at 250 × g. Viable cells were counted, and 4 × 10^7 viable cells (in 0.5 mL of PBS) were injected under aseptic conditions into the peritoneal cavities of female athymic mice. Mice were subjected to whole-body luciferase imaging using an IVIS 100 Imaging System (Xenogen) 4 days after inoculation. Before imaging, mice were injected intraperitoneally with luciferin (Caliper Life Sciences) at 150 mg/kg body weight with a 25-gauge syringe. Then mice were kept under anesthesia with isoflurane. Starting 5.5 minutes after the luciferin injection, images were collected for 30 seconds each in the ventral and dorsal positions. Images and amounts of bioluminescent signals were analyzed using Living Image Software (Xenogen).

We carried out 3 sets of animal experiments. In the first set, mice were randomly divided into 3 groups (7 mice per group), and treatment was started 5 days after tumor cell inoculation and continued for 3 weeks. Group 1 was given vehicle (0.5% hydroxypropyl methyl cellulose and 0.1% Tween-80, 10 mL/kg/d); group 2 received selumetinib 100 mg/kg/d; and group 3 was administered selumetinib 25 mg/kg/d. In the second set of experiments, mice were randomly divided into 4 groups (10 mice per group), and treatment was started 5 days after tumor cell inoculation and continued for 5 weeks. Group 1 was given vehicle as in the first set of experiments; group 2, selumetinib 50 mg/kg/d; group 3, selumetinib 50 mg/kg/d; and group 4, selumetinib 100 mg/kg/d. All agents were administered by oral gavage. Bioluminescent imaging was done weekly to assess intraperitoneal tumor growth.

In the third set of experiments, mice were randomly divided into 3 groups (4 mice per group), and treatment was started 5 days after tumor cell inoculation and continued for 2 weeks. Groups 1 to 3 received the same daily treatments as groups 1 to 3 in the second set of
experiments, by oral gavage. At the end of the 2 weeks, tissue samples were collected, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Paraffin blocks were sliced in 4-μm sections and deparaffinized. Expression of ERK, pERK, PEA-15, pPEA-15 (S116), and Ki-67 in tumor tissue sections was detected with mouse monoclonal anti-p42/44 MAP kinase (3A7; Cell Signalling), rabbit anti-phospho-p42/44 MAP kinase (Thr202/Tyr204), rabbit anti-PEA-15 polyclonal antibody, rabbit anti-pPEA-15 (S116), and rabbit monoclonal Ki-67 (SP6) antibody.

Statistical analysis
Statistical analyses were carried out with Prism version 5 (GraphPad Software Inc). Data are presented as means ±1 SE. Means for all data were compared by one-way ANOVA with post hoc testing or by unpaired t test. P values of <0.05 were considered statistically significant.

Results
Expression of EGFR and ERK in CCC cell lines
We hypothesized that suppression of the EGFR–ERK pathway inhibits cell proliferation in human ovarian CCC cells. We first screened a panel of 4 human CCC cell lines, RMG-I, SMOV-2, OVTOKO, and KOC-7c, for EGFR expression. RMG-I and SMOV-2 cells expressed high levels of EGFR, and OVTOKO and KOC-7c cells expressed low levels of EGFR. In RMG-I and SMOV-2 cells, stimulation with EGF increased expression of pEGFR and pERK, which indicated that the EGFR-downstream pathway was activated in these 2 cell lines (Fig. 1A). In contrast, in OVTOKO and KOC-7c cells, stimulation with EGF increased expression of pERK but not pEGFR.

Next, we investigated whether proliferation of RMG-1 cells with a functional EGFR pathway is EGFR driven. Transfection of RMG-1 cells with siRNA specific for EGFR reduced EGFR protein levels to 25% of the levels in siRNA-control-treated cells (Fig. 1B) and reduced the number of cells to 60% of the number of siRNA-control-treated cells (P < 0.05; Fig. 1C). These results indicate that RMG-I cell proliferation is dependent on the EGFR pathway. We observed similar results in SMOV-2 cells (data not shown).

Erlotinib suppresses proliferation of CCC cells via pERK inhibition and induction of G1 arrest
To determine whether erlotinib could suppress proliferation of CCC cells, we treated cells with different concentrations of erlotinib and assessed cell viability by WST-1 assay 72 hours later. RMG-I and SMOV-2 cells were sensitive to erlotinib [50% inhibitory concentration (IC50): 0.1 and 1 μmol/L, respectively], whereas OVTOKO and KOC-7c cells were resistant (IC50: 6 and 9 μmol/L, respectively; Fig. 2A).

When RMG-I cells were treated with erlotinib, EGF-induced pEGFR and pERK expression were incompletely suppressed in a dose-dependent manner (Fig. 2B). In addition, erlotinib treatment suppressed pAkt expression in RMG-I cells, but the baseline expression of pAkt and pERK did not correlate with sensitivity to erlotinib in CCC cell lines (data not shown). Similar results were observed
in SMOV-2 cells (data not shown). These cell lines had no EGFR mutations at exon 19 or exon 21, which are known to correlate with response to EGFR-TKI in non–small cell lung cancer (18).

We then examined the effect of erlotinib on cell-cycle distribution in erlotinib-sensitive CCC cell lines. RMG-I cells exhibited G1 arrest, but did not exhibit apoptosis (denoted by an increased proportion of cells in sub-G1) after 72 hours of treatment with 0.1 and 1 μmol/L erlotinib (Fig. 2C). We observed similar G1 arrest in sensitive to erlotinib), G1 arrest was associated with a significant increase in p27 expression after 72 hours of erlotinib treatment, whereas in SMOV-2 cells (moderately sensitive to erlotinib), there was only a modest increase in p27 expression after 72 hours of erlotinib treatment (data not shown).

After erlotinib treatment, p27 was located in both the cytoplasm and the nucleus of untreated cells but was sequestered in the nucleus in treated cells (data not shown).

**MEK1CA transfection renders RMG-I cells resistant to erlotinib**

Because activated ERK is suppressed by erlotinib in a dose-dependent manner in erlotinib-sensitive cells, we hypothesized that overexpression of activated ERK reduces the sensitivity of these cells to erlotinib. Given that MEK has no downstream substrates except for ERK, we used MEK1CA to increase the expression of ERK. We examined cell viability after erlotinib treatment with or without MEK1CA. MEK1CA significantly reduced sensitivity to erlotinib in RMG-I cells (WST-1 assay, \( P < 0.001 \); trypan blue assay, \( P < 0.005 \); Fig. 2D), indicating that erlotinib sensitivity is partially dependent on ERK.

**Selumetinib suppresses proliferation in erlotinib-sensitive CCC cell lines**

Given that MEK1CA reduced the sensitivity of RMG-I cells to erlotinib, we hypothesized that suppression of ERK inhibits proliferation of erlotinib-sensitive CCC cells. To date, no therapeutically nontoxic ERK inhibitors have been identified for ovarian cancer. Selumetinib is a highly selective MEK inhibitor that is in clinical trials and inhibits ERK activation, which is downstream of MEK. Consequently, we examined the sensitivity of erlotinib-sensitive and erlotinib-insensitive CCC cell lines to selumetinib. Similar to what we saw with erlotinib, RMG-I and SMOV-2 cells were sensitive to selumetinib (IC50 = 0.076 and 0.85 μmol/L, respectively), whereas OVTOKO and KOC-7c cells were resistant (IC50 > 10 μmol/L for both; Fig. 3A). These cell lines had no mutations at exon 2 or 3 of KRAS or
exon 15 of BRAF, which are known to correlate with sensitivity to selumetinib in human cancer cells (21).

**Selumetinib suppresses proliferation of CCC cells via pERK inhibition and induction of G1 arrest**

Treatment of RMG-I cells with selumetinib for 24 hours reduced the expression of pERK and increased the expression of p27 (Fig. 3B) concomitantly with the observed G1 arrest (G1 fraction; 56.9% for control, 85.4% for 24-hour treatment with 0.01 μmol/L selumetinib, and 88.2% for 24-hour treatment with 0.1 μmol/L selumetinib; Fig. 3C). Similar results were observed in SMOV-2 cells (data not shown). In contrast, OVTOKO and KOC-7c cells did not show inhibition of ERK phosphorylation even after exposure to high selumetinib concentrations (data not shown). We did not observe apoptosis in these cell lines with selumetinib treatment.

Translocation of ERK from the cytoplasm to the nucleus is important for ERK-dependent transcription, which regulates cell proliferation by phosphorylating ERK's nuclear substrates. We, thus, examined the nuclear and cytoplasmic expression level of ERK after selumetinib treatment in CCC cell lines. In RMG-I cells (selumetinib sensitive), the levels of nuclear and cytoplasmic pERK were suppressed after 72-hour exposure to 0.1 μmol/L selumetinib. In contrast, in OVTOKO cells (selumetinib resistant), there was a less significant suppression of nuclear pERK expression even though the cells were treated with a drug concentration 10 times as great (1 μmol/L; Fig. 3D).

**PEA-15 knockdown renders CCC cells resistant to selumetinib**

Overall, ERK activity in a cell could be defined not only by duration and magnitude of enzymatic activity but also by its location (cytoplasm vs. nucleus; ref. 22). Therefore, we investigated the possible role of PEA-15 in selumetinib sensitivity. PEA-15 is known to sequester ERK in the cytoplasm by binding to ERK but does not directly change ERK kinase activity (12). After selumetinib treatment, pPEA-15 (S116) and total PEA-15 expression increased in RMG-I cells (selumetinib sensitive; 2.8 and 2.5 times of control, respectively) but did not change in OVTOKO cells (selumetinib resistant; Fig. 4A).

To determine whether upregulation of PEA-15 by selumetinib contributes to the antiproliferative action of selumetinib, we knocked down PEA-15 by siRNA-PEA-15 in RMG-I cells and then examined their sensitivity to selumetinib. PEA-15 knockdown completely cancelled the growth-inhibitory effect of selumetinib (P < 0.05; Fig. 4B). To determine the role of PEA-15 phosphorylation at S116 in selumetinib sensitivity, we transfected OVTOKO cells (selumetinib resistant) with mutant PEA-15-AD (phosphoinhibitory at S104 and phosphomimetic at S116). OVTOKO cells transfected with PEA-15-AD were significantly more sensitive to selumetinib than OVTOKO cells transfected with PEA-15-AA (nonphosphorylated...
Erlotinib and selumetinib suppress tumor growth in a
CCC xenograft model

All mice injected with RMG-I/luc cells (luciferase expressing RMG-I cells) developed intraperitoneal
tumors by the fifth day after inoculation.

In our first set of xenograft experiments, all of the mice treated with a high dose of erlotinib (100 mg/kg/d) exhibited severe skin peeling with yellow discoloration on treatment day 6 (11 days after inoculation) and marked weight loss on treatment day 9 (14 days after inoculation; Fig. 5A). The mice continued to lose weight even after treatment was stopped for 2 days (P = 0.0015). Consequently, we reduced the dose of erlotinib to 50 mg/kg per day starting on day 11 (16 days after inoculation). In the mice treated with selumetinib 25 mg/kg/d, there were no signs of overt toxicity (skin toxicity, weight loss, etc.). Bioluminescent signals (to assess intraperitoneal tumor growth) of the erlotinib group (100 mg/kg/d and then 50 mg/kg/d) were significantly lower than those of the vehicle control group on days 18 and 25 after inoculation (P = 0.0072 and P = 0.0004, respectively; data not shown). The bioluminescent signals of the selumetinib group (25 mg/kg/d; data not shown) were lower (but not significantly lower) than those of the vehicle control group on days 11 and 18 after inoculation and increased to the same level as the vehicle control group on day 25. After treatment cessation, tumor regrowth was apparent within 6 days in the mice treated with selumetinib 25 mg/kg/d (data not shown). This dose was chosen because it effectively inhibited tumor growth in multiple MEK inhibitor-sensitive models (21).

To evaluate whether higher doses of selumetinib could be more effective, we conducted a second RMG-I xenograft study to evaluate sensitivity to selumetinib 50 and 100 mg/kg/d. Concurrently, we examined the effect of erlotinib 50 mg/kg/d (this time, we used a fixed dose of 50 mg/kg/d). In our second set of xenograft experiments, no obvious toxicity was observed with higher doses of selumetinib (50 and 100 mg/kg/d) or the fixed dose of erlotinib (50 mg/kg/d). Bioluminescent signals of the erlotinib group and of both selumetinib groups were significantly lower than those of the vehicle control group on days 21, 28, and 35 after inoculation, indicating significant inhibition of CCC tumor growth (P = 0.0001; Fig. 5B). Bioluminescent signals for the 2 selumetinib dose groups were similar (selumetinib 50 mg/kg vs. selumetinib 100 mg/kg; P = 0.67, P = 0.12, and P = 0.1434, respectively, on days 21, 28, and 35), and the signals for the 2 selumetinib dose groups were similar to the signals for the erlotinib group (selumetinib 50 mg/kg vs. erlotinib 50 mg/kg; P = 0.177, P = 0.4220, and P = 0.6241, respectively, on days 21, 28, and 35; selumetinib 100 mg/kg vs. erlotinib 50 mg/kg; P = 0.2242, P = 0.355, and P = 0.3827, respectively, on days 21, 28, and 35).

In the third set of xenograft experiments, tumor tissues were analyzed for ERK1/2, pERK1/2, PEA-15, and pPEA-15 (S116) expression and proliferation (Ki-67) in mice treated with erlotinib 50 mg/kg/d for 2 weeks and mice treated with selumetinib 50 mg/kg/d for 2 weeks. As expected, levels of pERK1/2 and Ki-67 expression were reduced in the erlotinib-treated mice and markedly reduced in the selumetinib-treated mice (Fig. 5C). As in our in vitro experiments, PEA-15 and pPEA-15 (S116) expression were increased in selumetinib-treated groups in vivo.
Discussion

Our results show that both selumetinib and erlotinib effectively suppress pERK expression in sensitive cell lines and suppress tumor growth in a CCC human xenograft model. However, mice treated with selumetinib 50 or 100 mg/kg/d showed significant tumor growth inhibition with no major toxicity, whereas mice treated with erlotinib 100 mg/kg/d showed skin peeling and marked weight loss. We speculate that the antitumorigenic effects of selumetinib and, to a lesser extent, erlotinib are attributable to targeting of ERK, which is downstream of EGFR. In addition, we showed that in the selumetinib-sensitive cell lines, PEA-15, a protein that binds ERK and sequesters it in the cytoplasm, contributes to selumetinib’s effects. Through these findings, we have shown that suppression of ERK pathway signaling by selumetinib can be effective in preclinical models of ovarian CCC.

In previous studies, it was shown that gefitinib, another EGFR-TKI, decreased the proliferation and invasion of CCC cells and inhibited tumor growth in a xenograft model in a dose-dependent manner (23). In the present study, we confirmed that overexpression of MEK1CA rendered erlotinib-sensitive CCC cells resistant to erlotinib. These findings indicate that sensitivity of ovarian CCC to erlotinib is partially dependent on ERK suppression, implying that ERK itself may be a potential therapeutic target in ovarian CCC.

In addition, erlotinib-insensitive cell lines were insensitive to the MEK inhibitor selumetinib. The low expression of EGFR and pEGFR and the lack of BRAF or KRAS mutation in these insensitive cell lines indicate possible involvement of other known pathways, such as the insulin-like growth factor (IGF)–IR and phosphoinositide 3-kinase (PI3K)–Akt pathways (24). Recently, it has been shown that patients with CCC have a high frequency of activating PIK3CA mutations (25). In our present study, the baseline expression of pAkt and pERK did not correlate with sensitivity or resistance of ovarian CCC to selumetinib.

Patients with ovarian cancer with high PEA-15–expressing tumors have been shown to survive longer than those with low PEA-15–expressing tumors (15). In contrast,
Stassi and colleagues reported that exogenous expression of a dominant-negative AKT cDNA or a PEA-15 antisense cDNA in breast cancer cells induced a significant downregulation of PEA-15 and sensitized cells to chemotherapy-induced cell death (26). However, little is known about the relationship between PEA-15 and sensitivity to molecular targeted therapies related to the EGFR-ERK pathway. In the present study, we showed that expression of PEA-15 and pPEA-15 (S116) increased after selumetinib treatment only in the sensitive cell lines. Furthermore, we showed that siRNA knockdown of PEA-15 reduced sensitivity to selumetinib in selumetinib-sensitive CCC cells and that this transfection with mutant PEA-15, which mimics phosphorylation at S116, significantly increased the sensitivity to selumetinib in selumetinib-insensitive CCC cells. Although further studies are needed to elucidate the role of pPEA-15 (S116) and the mechanism of its involvement in the antiproliferative action of selumetinib, our findings indicate that phosphorylated PEA-15 contributes to sensitivity to selumetinib and might be a useful tumor marker to predict sensitivity to ERK-targeted therapy in CCC. Further studies are warranted to define the role of PEA-15 phosphorylation in selumetinib sensitivity.

Other investigators have shown that pPEA-15 (S116) binds to Fas-associated protein with death domain (FADD) and inhibits apoptosis, whereas unphosphorylated PEA-15 binds to ERK and sequesters it in the cytoplasm (12, 13, 27). In addition, PEA-15 binds to RSK2, which is a substrate of ERK, implying that PEA-15 acts as a scaffold for ERK and RSK2 (28). In this context, at low levels, PEA-15 expression enhances ERK binding to RSK2, whereas at very high levels, PEA-15 expression inhibits ERK binding to RSK2 (29, 30). Although whether pPEA-15 (S116) can bind to RSK2 is unknown, overexpression of S116D may cause a similar inhibitory effect.

In summary, we have shown that the ERK signaling pathway is a potential therapeutic target for CCC and that both selumetinib, which was less toxic than erlotinib in vivo, and erlotinib are worth exploring as therapeutic agents for patients with CCC. Further studies are warranted to determine whether PEA-15 and/or phosphorylated PEA-15 may be useful to predict sensitivity to ERK pathway-targeted therapy in CCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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MEK1/2 Inhibitor Selumetinib (AZD6244) Inhibits Growth of Ovarian Clear Cell Carcinoma in a PEA-15 –Dependent Manner in a Mouse Xenograft Model

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