ABSTRACT

Increased growth factor receptor signaling is implicated in antiestrogen-resistant breast tumors suggesting that abrogation of such signaling could restore or prolong sensitivity to antihormonal agents. Activation of the mitogen-activated protein/extracellular regulated kinase cascade (MEK)-extracellular regulated kinase (ERK)1/2 cascade is a common component of such pathways. We investigated the ability of the MEK activation inhibitor U0126 to block the increased growth of estrogen receptor-positive MCF-7 breast cancer cells caused by fibroblast growth factor 1 (FGF-1), heregulin β1 (HRGβ1), and epidermal growth factor (EGF) in the presence of the pure antiestrogen ICI 182780 (Faslodex; fulvestrant). We found that either FGF-1 or HRGβ1 but not EGF substantially reduced the inhibitory effects of U0126 on growth and ERK1/2 activation, including the combined inhibitory effects of U0126 and ICI 182780. FGF-1 and HRGβ1 also reduced the inhibition of ERK1/2 phosphorylation by the MEK inhibitors PD98059 and PD184161. Interestingly, a transiently transfected dominant-negative MEK1 completely abrogated activation of a coexpressed green fluorescent protein-ERK2 reporter by all three of the factors. Despite a short-lived activation of Ras and Raf-1 by all three of the growth factors, both FGF-1 and HRGβ1, unlike EGF, induced a prolonged activation of MEK and ERK1/2 in these cells. Thus, activation of FGF-1- and HRGβ1-specific signaling causes MEK-dependent prolonged activation of ERK1/2, which is incompletely susceptible to known MEK inhibitors. We also demonstrate that the cytosolic phospholipase A2 inhibitor arachidonyl trifluoro methyl ketone and the pan PKC inhibitor bisindolylmaleimide abrogated U0126-resistant phosphorylation of ERK1/2 induced by HRGβ1 but not by FGF-1. Phosphorylation of ERK5 by all three of the factors was also resistant to U0126 suggesting that its activation is not sufficient to override inhibition due to diminished ERK1/2 activation. Therefore, therapy combining antiestrogens and MEK inhibitors may be ineffective in some antiestrogen-resistant estrogen receptor-positive breast cancers.

INTRODUCTION

Estrogen receptor (ER)-positive breast cancers that initially respond to antiestrogens like tamoxifen frequently become resistant to it and to subsequent hormonal therapies. Understanding the mechanisms of development of antiestrogen resistance could lead to therapeutic strategies that might overcome such resistance in patients. Animal models of acquired tamoxifen resistance have shown that development of antiestrogen-resistant growth in MCF-7 cells (3, 6). In addition, epidermal growth factor (EGF) and transforming growth factor α, known ligands of EGF receptor/HER1 have also been shown to induce either estrogen-independent or antiestrogen-resistant growth in MCF-7 cells (6, 7). Thus, these studies suggest that overexpression of the ligands themselves or aberrant expression or activity of other erbB receptor family members could also participate in the development of antiestrogen resistance.

A number of investigators have shown that different growth factors induce antiestrogen-resistant growth to varying degrees (8–13). Our laboratory has shown that signaling mediated by fibroblast growth factor (FGF)-4 and FGF-1 can also provide an alternative growth stimulus to that provided by ER activation in MCF-7 breast adenocarcinoma cells, which results in antiestrogen resistant growth (8, 14–16). Cross-talk between growth factor signaling and ER can also involve ER activation by phosphorylation independent of estrogen or phosphorylation of coactivators (17–19).

Distinct growth factors including FGFs, EGF, and HRGβ1 are thought to regulate proliferation by activating tyrosine kinase receptors that result in the activation of a common signaling pathway involving mitogen-activated protein kinases (MAPK) extracellular regulated kinase (ERK)1 and ERK2 (20–24). Moreover, constitutively active Raf-1 and constitutively active MEK1 have also been shown to mediate antiestrogen-resistant growth in MCF-7 cells (25, 26). Therefore, it was reasonable to speculate that the MEK-ERK1/2 cascade represented a point of convergence of many signaling pathways that when blocked could abrogate multiple antiestrogen resistance mechanisms. U0126, a small-molecule inhibitor of the MAPK signal transduction pathway, used in nanomolar to low micromolar doses, has been shown to abrogate growth stimulation in response to a number of growth factors including factors that activate receptor tyrosine kinases (27, 28). U0126 has been shown to specifically inhibit the MAPK kinases mitogen-activated protein/ERK kinase (MEK)1 and MEK2 both in vitro and in vivo (29). Blockade of the MAPK pathway using the MEK inhibitor PD 184352 (a structural analog of PD 184161), has also been shown to suppress growth of colon tumors and leukemic cell lines, and tumor growth reduction has been correlated with a dose-dependent reduction in levels of activated tyrosine kinase receptor (also called HER2/new; Ref. 1). Whereas it was shown recently that long-term estrogen deprivation of ER-positive MCF-7 breast carcinoma cells can result in enhanced erbB2 expression (2), others have found that long-term treatment of these cells with antiestrogen did not result in altered levels of any of the erbB family members (3). In addition, studies with patient samples suggest that tumors that are initially negative for expression of HER2 do not acquire expression during tamoxifen treatment and subsequent relapse, suggesting that additional mechanisms might also account for such resistance (4).

Heregulin β1 (HRGβ1), which binds to HER2/HER3 and HER2/HER4 heterodimers (5), has also been shown to induce antiestrogen-resistant growth in MCF-7 cells (3, 6). In addition, epidermal growth factor (EGF) and transforming growth factor α, known ligands of EGF receptor/HER1 have also been shown to induce either estrogen-independent or antiestrogen-resistant growth in MCF-7 cells (6, 7). Thus, these studies suggest that overexpression of the ligands themselves or aberrant expression or activity of other erbB receptor family members could also participate in the development of antiestrogen resistance.

Received 8/24/03; revised 4/28/04; accepted 5/4/04.

Grant support: Susan G. Komen Breast Cancer Foundation Grant BCTR00-000456 (J. Thottassery), National Cancer Institute ROI CA50376-12 (F. Kern), University of Alabama at Birmingham Comprehensive Cancer Center Grant P30 CA13148, and Adolph Weil Endowed Chair in Cancer Biology at Southern Research Institute (F. Kern).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Francis G. Kern, Oncology Department, Lexicon Genetics Inc., 8800 Technology Forest Place, The Woodlands, TX 77381. Phone: (281) 863-3780; Fax: (281) 863-8094; E-mail: fker@lexgen.com.
MAPKs (30, 31). Therefore, combining MEK blockers with drugs like tamoxifen (or the pure ER antagonist ICI 182780) might improve therapy by blocking nonestriol (E2)-dependent MAPK signaling that could potentially cause antiestrogen resistance in ER-positive breast cancers. Indeed, in two separate studies U0126 has been demonstrated to enhance the effects of tamoxifen and ICI 182780, respectively, against HER2-overexpressing ER-positive breast cancer cells in vitro (26, 32).

To assess whether MEK inhibitors might be useful in combination with antiestrogens we investigated the role of the MEK-ERK1/2 cascade in antiestrogen-resistant growth of MCF-7 cells in response to EGF, FGF-1, and HRGβ1. Transient cotransfection of a green fluorescent protein (GFP)-ERK2 fusion construct with a dominant-negative MEK1 indicated that the ERK1/2 activation induced by all of these factors was MEK1/2-dependent. However, contrary to our expectation, we found that the MEK inhibitor U0126 was not able to completely block ERK1/2 activation and antiestrogen-resistant growth induced by either FGF-1 or HRGβ1. We also found that the MEK inhibitors PD98059 and PD184161 were not able to completely block ERK1/2 activation induced by these factors. This appeared to be related to the prolonged activation of ERK1/2 that occurs after addition of either factor. However, the underlying mechanism for the prolonged activation of the MEK-ERK1/2 cascade by these factors appears to be different. ERK1/2 phosphorylation induced by HRGβ1 but not by FGF-1 was abrogated by cotreatment with U0126 plus either the cytosolic phospholipase A2 (cPLA2) inhibitor arachidonyl trifluoro methyl ketone (AACOCF3) or the pan-conventional protein kinase C (PKC) inhibitor bisindolylmaleimide. Thus, FGF-1 and HRGβ1-induced ERK 1/2 activation is prolonged and U0126-resistant but not MEK-independent. Instead, we suggest that factor-specific MEK1/2 activation mechanisms, which, in the case of HRGβ1, is PKC- and PLA2-dependent, leads to incomplete susceptibility to U0126-mediated inhibition. Therefore, MEK inhibitors may not be very effective in reducing antiestrogen-resistant growth phenotypes induced by FGF-1- and HRGβ1-mediated pathways in ER-positive breast cancer.

MATERIALS AND METHODS

Chemicals, Growth Factors, and Antibodies. U0126 was obtained from Promega (Madison, WI), PD98059 was from Calbiochem (La Jolla, CA), and PD184161 was a gift from Pfizer Inc. (Groton, CT). Recombinant human FGF-1 and EGF were obtained from Promega, and recombinant human HRGβ1 (residues 176–246 corresponding to the EGF-like domain) was from R&D Systems (Minneapolis, MN). ICI 182780 (Faslodex) was a gift from AstraZeneca (Macclesfield, Cheshire, United Kingdom) and was dissolved in ethanol before use. Bisindolylmaleimide and AACOCF3 were obtained from Calbiochem. Phenol red-free and phenol red containing Improved Modified Eagle’s Medium (IMEM) were obtained from Invitrogen (Carlsbad, CA). In some experiments charcoal-stripped calf serum was used to strip cells of endogenous estrogens. Calf serum was treated with dextran-coated activated charcoal as described before (8, 33) to obtain charcoal-stripped calf serum. Anti-ERK1/2 bands for every time point. An average of all of the normalized values was performed on lysates prepared from ML20 cells treated with the appropriate conditions. Forty μg of cell lysate was run on Criterion prestained 10% SDS-PAGE gels (Bio-Rad, Hercules, CA). Proteins were transferred onto Protran filters (Bio-Rad) by electroblotting. The blots were then probed with phosphospecific antibodies against the following: phospho-STAT1, phospho-ERK1/2, phospho-ERK5, and phospho-p90 Rsk-1 (Cell Signaling Technology), or phospho-ERK5 antibodies (BioSource International). After probing with phospho-STAT1, phospho-ERK1/2, phospho-p90 Rsk-1, and phospho-ERK5 antibodies, blots were stripped with Restore stripping solution (Pierce, Rockford, IL) and reprobed with antibodies against total ERK1/2. Blots that were first probed for phospho-ERK5, phospho-p90 Rsk-1, or phospho-ERK1/2 were subsequently reprobed for total MEK, total p90 Rsk-1, and total ERK5, respectively, after stripping. Enhanced chemiluminescence with the Supersignal kit (Pierce) was used to detect bands. Densitometric analysis of the Western blots that examined the time course of activation of ERK1/2 and MEK was performed by using the NIH Image J Program. The sum of the intensities of the phospho-ERK1/2 bands was normalized to that of the total ERK1/2 bands for every time point. An average of all of the normalized values for the zero time point was then obtained. The normalized values for each time point in each of the treated conditions was then expressed as fold change over the averaged normalized intensity for the zero time point. Means ± SE of the fold changes were subjected to the Student’s t test and were represented graphically (SigmaPlot v8.0, SPSS Inc.) as a function of time post-treatment.

| Table 1 Description of estradiol-, ICI 182780-, and growth factor-supplemented medium conditions used |
| Nomenclature | Components in the medium |
| F | 5% FBS* |
| FI | 5% FBS, ICI 182780 (10−7 M) and EGF 10 ng/ml |
| FIE | 5% FBS, ICI 182780 (10−7 M) and EGF 10 ng/ml |
| FIF | 5% FBS, ICI 182780 (10−7 M) and FGF-1 20 ng/ml |
| FIFH | 5% FBS, ICI 182780 (10−7 M) and HRGβ1 25 ng/ml |
| FIE2 | 5% FBS, ICI 182780 (10−7 M) and ZHβ- Estradiol (10−7 M) |
| FIE3 | 5% FBS and EGF 10 ng/ml |
| FF | 5% FBS and EGF 10 ng/ml |
| FH | 5% FBS and HRGβ1 25 ng/ml |

* FBS, fetal bovine serum; EGF, epidermal growth factor; HRGβ1, heregulin β1; FGF-1, fibroblast growth factor 1.
ERK 1/2 ACTIVATION IN BREAST CANCER AND MEK INHIBITORS

The Western blots examining MEK activation were subjected similarly to densitometric analysis.

**Ras Activation Assays.** Ras activation was assessed by using a Raf-1 ras binding domain affinity binding assay. Cells were lysed in 1× lysis buffer (Cell Signaling Technology) supplemented with 1 mM of phenylmethylsulfonyl fluoride, and 500 μg of cellular lysate was incubated with 10 μl Raf-1 ras binding domain agarose from the Ras activation kit (Upstate Biotechnology, Lake Placid, NY) at 4°C for 30 min. The agarose pull-downs were washed three times with lysis buffer and boiled with 2× Laemmli sample buffer [10% glycerol, 150 mM β-mercaptoethanol, 3% SDS, and 150 mM Tris-HCl (pH 6.8)]. Proteins were fractionated on 12% SDS-PAGE gels and transferred to Protran nitrocellulose membranes (Bio-Rad), followed by immunoblotting with anti-Ras antibodies (Upstate Biotechnology).

**Raf-1 Activation Assays.** Raf-1 was immunoprecipitated using an antibody directed against the COOH terminus of Raf-1 (sc-133; Santa Cruz Biotechnology, Santa Cruz, CA) coupled to protein G-Sepharose. Immunoprecipitates were washed twice with lysis buffer and then incubated with 30 μg protein G-Sepharose coupled to Raf-1 antibody from the Ras activation kit (Upstate Biotechnology, Santa Cruz, CA) for 30 min before addition of conditions. As shown in Fig. 1A, C and H, Raf-1 activation was decreased by treatment with U0126 for the entire duration of the experiment (5 days) with a quantitative decrease noted at all U0126 doses (Fig. 1A, C, and H). Importantly, we demonstrate that even a 3 μM dose of U0126 for 5 days, or in its absence (CT), was sufficient to decrease Raf-1 activation in estrogen-responsive breast cancer cells.

**RESULTS**

**FGF-1 and HRG β1 Induce Both Antiestrogen-Resistant and U0126-Resistant Growth in MCF-7 Cells.** To assess whether MEK blockade would restore or prolong sensitivity of ER-positive breast cancer cells to antioestrogens, we tested the ability of U0126 to block FGF-1-induced growth of ML20 cells in the presence and absence of ICI 182780 in an exponential growth assay. Estrogen-deprived ML20 cells were plated in a 24-well format and were then fed with 30 μl kinase assay buffer (25 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM MnCl₂, 1 mM DTT, 1 μM ATP, and 30 μCi [γ-32P]ATP) containing inactive MEK1 expressed in Escherichia coli as a fusion with glutathione S-transferase (US Biologicals, Swampscott, MA) as substrate. After incubating for 30 min at 30°C, reactions were spotted onto P81 phosphocellulose squares, which were subsequently washed 10 times with 150 mM phosphoric acid. Radioactivity on the P81 squares was counted using a Bioscan QC 4000 Benchtop Radioisotope Counter (Washington, DC). 

Unpublished data from our laboratory and reports from other investigators have shown that EGF and HRG can also induce antioestrogen-resistant growth in these cells (3, 6, 7). To test whether these factors would also induce U0126-insensitive growth, we performed a MTs growth assay with estrogen-deprived ML20 cells treated with all three of the growth factors in the presence and absence of ICI 182780 in 5% FBS containing medium. We found that similar to its modest effects in FGF-1 supplemented medium, U0126 only moderately
inhibited growth in HRGβ1-containing conditions (Fig. 1B, compare FH with F and FIH with FI in U0126-treated cells). However, U0126 inhibited growth in EGF supplemented medium, both in the presence and in the absence of ICI 182780 (Fig. 1B, FIE and FE conditions). Thus, like FGF-1 and HRGβ1, EGF was equally capable of inducing antiestro-
gen-resistant growth. However, unlike FGF-1 or HRGβ1, and like E2, the growth stimulatory effect of EGF was very sensitive to the effects of U0126.

**FGF-1 and HRGβ1 Induces Prolonged ERK1/2 and MEK1/2 Activation Even in the Presence of ICI 182780.**

**Fig. 2.** Fibroblast growth factor 1 and heregulin β1 induce prolonged extracellular regulated kinase (ERK)1/2 activation in the presence and absence of ICI 182780. A, ML20 cells were stripped of estrogens with four changes of 5% charcoal-treated calf serum-containing medium and then incubated overnight in serum-free medium. They were then treated with F, FI, FIF, FIH, FIE, FIE2, FF, FH, or FE medium (see Table 1 for a description of these media) for the indicated times. The cells were then lysed, and lysate aliquots (40 μg) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Blots shown are representative of at least 3 separate experiments. B, densitometric analysis of Western blots from three separate experiments. *, significantly different from the normalized phospho-ERK1/2 levels in FI condition (left) or F condition (right) at that time point; P < 0.05; bars, ±SD.

**Fig. 3.** Fibroblast growth factor 1 and heregulin β1 induce prolonged mitogen-activated protein/extracellular regulated kinase (MEK) activation in the presence and absence of ICI182780. A, ML20 cells (MCF-7 transfected with LacZ) were stripped of estrogens with four changes of 5% charcoal-stripped calf serum-containing medium and then incubated overnight in serum-free medium. They were then treated with F, FI, FIF, FIH, FIE, FIE2, FF, FH, or FE medium (see Table 1 for a description of these media) for the indicated times. The cells were then lysed, and lysate aliquots (40 μg) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total MEK. Blots shown are representative of at least three separate experiments. B, densitometric analysis of Western blots from 3 separate experiments. *, significantly different from the normalized phospho-MEK levels in FI condition (left) or F condition (right) at that time point; P < 0.05; bars, ±SD.
growth factor application (35–38). Therefore, we first established the magnitude and longevity of phosphorylation of ERK1/2 and its upstream activators MEK1/2 subsequent to growth factor or E2 exposure in the presence or absence of ICI 182780 in ML20 cells. Estrogen-deprived ML20 cells that had been serum-starved for 24 h were subjected to the various conditions listed in Table 1. Treatment with 5% FBS causes a rapid but modest activation of ERK1/2 that is sustained for at least 2 h (Fig. 2, A and B, F condition). ICI 182780 (1 × 10−7 m) does not affect this activation (Fig. 2, A and B, FI condition) nor does E2 alter it appreciably (Fig. 2, A and B, FIE2 condition). FGF-1 supplementation in the presence or absence of ICI 182780 causes a more robust and prolonged activation of ERK1/2 for at least 8 h (Fig. 2, A and B. FIF and FF conditions) as compared with 5% FBS alone. HRGβ1 supplementation in these conditions also caused a very robust and prolonged activation of ERK1/2 (Fig. 2, A and B, FIH and FH conditions). In contrast, EGF supplementation in 5% FBS-containing medium resulted in a relatively short-lived, albeit robust activation of ERK1/2, which dropped down to control levels within 30 min in the presence of ICI 182780 and lasted only 2 h in its absence (Fig. 2, A and B, FIE and FE conditions). In some experiments, a second bolus of EGF was added 5 min before harvesting to the 2-h time point and all of the subsequent samples. Results from these indicated no differences when compared with experiments where only a single bolus was administered. Therefore, the data suggest that the relatively rapid drop in phospho-ERK1/2 levels in EGF-supplemented conditions could not be attributed to its half-life.

We also assessed the time course of activation of MEK1/2, which are the direct upstream activators of ERK1/2. As shown in Fig. 3, A and B, treatment of serum- and estrogen-deprived ML20 cells with 5% FBS, either in the absence or presence of ICI 182780, led to modest increases in phospho-MEK1/2 levels that lasted 2 h. Similar results were obtained in the FIE2 condition (Fig. 3, A and B). However, in the presence of FGF-1-supplemented medium, phospho-MEK1/2 levels remained very high for at least 8 h in the presence of ICI 182780 and 24 h in its absence. HRGβ1 also maintained sustained activation of MEK1/2, which remained high for at least 4 h in the presence of ICI 182780 and at least 8 h in its absence. EGF supplementation in serum-containing medium did not cause such a prolonged activation of MEK1/2 (even when an additional bolus of EGF was added 5 min before harvesting the 2 h and all of the subsequent samples; data not shown), which correlated with the time course of its activation of ERK1/2. EGF-induced increases in phospho-MEK1/2 levels were, however, of similar magnitude with respect to FGF-1 and HRGβ1-induced increases at earlier time points.

**FGF-1 and HRGβ1-Induced Ras and c-Raf-1 Activation Is Transient Unlike ERK1/2 Activation.** To determine whether the differential duration of MEK1/2 and ERK1/2 activation by FGF-1 or HRGβ1 versus EGF correlated with the activation of the upstream components of this cascade, we investigated the time course of activation of Ras and Raf-1 in response to these factors in the presence of 5% FBS. The ras binding domain of Raf-1 was used to selectively capture active Ras, and anti-ras antibodies were used to detect it (Fig. 4A). The activation of Ras by 5% FBS was very minimal (Fig. 4A). On the other hand, the activation of Ras by all three of the growth factors was rapid, starting as early as 2 min in the case of HRGβ1 and EGF and peaking at 5 min. In the case of FGF-1, active Ras levels were modestly induced and peaked at 5 min (Fig. 4A). However, Ras activation in all three of the cases returned to basal levels rapidly, with FGF-1 and HRGβ1-induced Ras levels dropping to near basal levels by 30 min and within 2 h in the EGF-treated condition (Fig. 4A).

Active Raf-1 was determined by an *in vitro* kinase assay by using an inactive recombinant MEK1 fused to glutathione S-transferase (glutathione S-transferase-MEK), as a substrate. As shown in Fig. 4B, Raf-1 activation was maximal in the case of all three of the factors by 5 min and dropped rapidly by 30 min, consistent with Ras activation (Fig. 4A). The peak active Raf-1 levels induced by HRGβ1 were the highest and were almost as high as active Raf levels measured in a MCF-7 clone stably expressing a constitutively active Raf-1 NH2-terminal deletion mutant (Fig. 4B). Peak active Raf-1 levels induced by FGF-1 were lower than that induced by EGF or HRGβ1. Active Raf-1 induced by serum alone was minimal, again consistent with Ras activation levels.

**HRG β1 and FGF-1 but not Serum or EGF Can Stimulate MAPK and p90 Rsk-1 Activation in the Presence of U0126.** To determine whether the reduced growth-inhibitory effect of U0126 in FGF-1- or HRGβ1-containing medium correlated with an inability to effectively delay the proliferation MAPK activity, we examined phospho-ERK1/2 levels under these conditions (Fig. 5, A and B). We used serum and estrogen-deprived ML20 cells (Fig. 5, A and B) and treated them with 5% FBS supplemented with the three factors for 2 h, a time point at which substantial phospho-ERK1/2 levels are found under all of the conditions (Fig. 5, A and B, and Fig. 2). In parallel, dishes with the same conditions were also treated with 10 μM U0126 for 2 h (with a 30-min pretreatment before addition of conditions). To determine the concentration of U0126 to be used in these assays we used a clonally isolated MCF-7 line called Raf14c that expresses a stably transfected constitutively active c-Raf mutant (25). Serum-starved cells from this line (lane UT, Fig. 5A, bottom) exhibit a high level of
ERK 1/2 ACTIVATION IN BREAST CANCER AND MEK INHIBITORS

PD98059, the least potent MEK inhibitor (39), when used at 200 μM, a dose shown previously to block ERK1/2 phosphorylation in Raf14c cells (40), was nearly as effective as U0126 in reducing ERK1/2 phosphorylation in Raf14c line even in the presence of serum (Fig. 5, A and B). However, U0126 only modestly inhibited ERK1/2 activation by FGF-1 and HRGβ1 under these conditions (Fig. 5, A and B).

Data shown in Fig. 6, A and B, depict results from experiments where three structurally distinct MEK blockers (see Table 2) were used to assess whether growth factor-induced ERK1/2 activation seen in ICI 182780-treated conditions was susceptible to MEK inhibition. PD98059, the least potent MEK inhibitor (39), when used at 200 μM, a dose shown previously to block ERK1/2 phosphorylation in Raf14c cells (40), was nearly as effective as U0126 in reducing ERK1/2 activation in the F and FI conditions (Fig. 6, A and B). In these experiments we also used a new, highly potent MEK inhibitor PD184161 at 10 μM (41). PD184161 is a close structural analog of PD184352, which is currently in clinical trials and which directly inhibits MEK1 with an IC50 of 17 nM (30). All of the three inhibitors nearly completely abrogated phosphorylation of ERK1/2 when E2 was added to negate the effects of ICI 182780 (Fig. 6, A and B). We also found that all three of the MEK inhibitors do not effectively abrogate ERK1/2 activation induced by FGF-1- and HRGβ1-supplemented medium in the presence of antiestrogen (Fig. 6, A and B). Even 30 μM U0126 did not completely inhibit ERK1/2 activation by FGF-1 and HRGβ1 in the presence or absence of ICI 182780 (data not shown).

We also asked whether the observed U0126-resistant phosphorylation of ERK1/2 in FGF-1- and HRGβ1-containing medium results in activation of its downstream target p90 Rsk-1 (Fig. 7). The anti-phospho-p90 Rsk-1 antibody recognizes p90 Rsk-1 only when it is phosphorylated at Thr359/Ser363, both of which are ERK targets. As shown in Fig. 7, p90 Rsk-1 was substantially phosphorylated by FGF-1 and HRGβ1 signaling in the presence of U0126, consistent with phospho-ERK1/2 levels. However, U0126 treatment completely

Table 2 Inhibitors used in this study

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Enzyme(s) inhibited</th>
<th>Concentration(s) used</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>U0126</td>
<td>MEK1/2 and MEKS</td>
<td>10 and 30 μM</td>
<td>29, 39, 48, 49, 50</td>
</tr>
<tr>
<td>PD 98059</td>
<td>MEK1/2 and MEK5</td>
<td>200 μM</td>
<td>39, 49, 50</td>
</tr>
<tr>
<td>PD 184161a</td>
<td>MEK1/2 and MEK5 (?)</td>
<td>10 μM</td>
<td>41, 42</td>
</tr>
<tr>
<td>AACOCF3</td>
<td>ePLA2</td>
<td>10 μM</td>
<td>42</td>
</tr>
<tr>
<td>Bis-indolylmaleimide</td>
<td>Conventional PKCs</td>
<td>10 μM</td>
<td>42</td>
</tr>
</tbody>
</table>

a MEK, mitogen-activated protein/extracellular regulated kinase kinase; AACOCF3, arachidonoyl trifluoro methyl ketone; PKC, protein kinase C.  
a PD184161a is a close structural analog of PD184352, which has completed Phase I trials. Whereas the ability of PD184352 to inhibit activation of MEK5-extracellular regulated kinase 5 has been established (49, 65), similar data for PD184161 have not been reported.

Fig. 5. 1,4 Diamino-2,3-dicyano-1,4-bis [2-aminophenyl]thiobutadiene (U0126) does not effectively block fibroblast growth factor 1- and heregulin β1-stimulated extracellular regulated kinase (ERK1/2) phosphorylation in estrogen receptor-positive breast cancer cells. A, ML20 cells or Raf14c, a MCF-7 line that had been stably transfected with a constitutively active c-Raf (Δraf; Ref. 25), were stripped of estrogens as described in Fig. 1 legend and incubated overnight in serum-free medium. Where indicated, cells were then pretreated for 30 min with 10 μM U0126 and then exposed to the indicated conditions (see Table 1) for 2 h in the presence of the inhibitor. The cells were then lysed, and aliquots (40 μg) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Lane marked UT represents lysates from Raf14c cells that did not receive any conditions or U0126. Blots shown are representative of at least three separate experiments. B, densitometric analysis of Western blots from three separate experiments. *, significantly different from the normalized phospho-ERK1/2 levels in F and FE conditions within the U0126 treated group; P < 0.05.

Fig. 6. The mitogen-activated protein/extracellular regulated kinase (ERK) kinase inhibitors PD98059, PD184161, and 1,4 diamino-2,3-dicyano-1,4-bis [2-aminophenyl]thiobutadiene (U0126) do not effectively block fibroblast growth factor 1- and heregulin β1-stimulated ERK1/2 phosphorylation in estrogen receptor-positive breast cancer cells in the presence of the antiestrogen ICI 182780. A, ML20 cells were stripped of estrogens as described in Fig. 1 legend and incubated overnight in serum-free medium. Cells were then pretreated for 30 min with PD98059 (200 μM), PD184161 (10 μM), or U0126 (10 μM) and were then exposed to the indicated conditions (see Table 1) for 2 h in the presence of the inhibitor. The cells were then lysed, and aliquots (40 μg) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Blots shown are representative of at least three separate experiments. B, densitometric analysis of Western blots from three separate experiments. *, significantly different from the normalized phospho-ERK1/2 levels in F and FI conditions within the mitogen-activated protein/extracellular regulated kinase inhibitor-treated groups; P < 0.05.
abrogated p90 Rsk-1 phosphorylation in F, FE, FI, FIE, and FIE2 conditions.

MAPK Phosphorylation in HRGβ1- and FGF-1-Stimulated MCF-7 Cells Is MEK Dependent. The experiments with U0126 described above did not completely rule out a role for MEK1/2 in the prolonged ERK1/2 activation and growth observed in the presence of the compound in FGF-1 and HRGβ1 containing medium, because it could be argued that factor-specific MEK activation mechanisms that are insensitive to U0126 are involved. To additionally test the MEK dependence of the prolonged ERK1/2 activation induced by FGF-1 and HRGβ1 we did transient cotransfection assays using a GFP-ERK2 reporter and a dominant-negative MEK (S218A and S222A) construct.

The GFP-ERK2 fusion used in this assay has been shown to be a bona fide surrogate for the endogenous ERKs by others in similar experiments (34, 36). As shown in Fig. 8, 5% FBS either unsupplemented or with added EGF, FGF-1, or HRGβ1 caused robust phosphorylation of GFP-ERK2 when cotransfected with the pCMV vector.

![Fig. 9. 1,4 Diamino-2,3-dicyano-1,4-bis [2-amino phenyl] thiobutadiene (U0126)-resistant extracellular regulated kinase (ERK)1/2 phosphorylation induced by heregulin β1 is protein kinase C and cytosolic phospholipase A2-dependent.](image)

![Fig. 8. Extracellular regulated kinase (ERK)1/2 phosphorylation induced by heregulin β1 and fibroblast growth factor 1 is mitogen-activated protein/extracellular regulated kinase kinase (MEK)-dependent.](image)

The GFP-ERK2 fusion used in this assay has been shown to be a bona fide surrogate for the endogenous ERKs by others in similar experiments (34, 36). As shown in Fig. 8, 5% FBS either unsupplemented or with added EGF, FGF-1, or HRGβ1 caused robust phosphorylation of GFP-ERK2 when cotransfected with the pCMV vector.
shown in Fig. 10, HRGβ1 was the most potent in terms of induction of phospho-ERK5. However, U0126 did not decrease this (Fig. 10). Surprisingly EGF, which does not stimulate growth in U0126-treated conditions, was a potent inducer of phospho-ERK5, more so than FGF1, and even in the presence of 30 μM U0126 (Fig. 10). U0126 did inhibit the minimal ERK5 activation observed in response to 5% FBS treatment (Fig. 10). Thus, these results indicate that ERK5 activation alone, in the absence of active ERK1/2, is not sufficient for U0126-resistant growth.

**DISCUSSION**

Growth factor-induced ERK1/2 activation has been characterized as rapid and transient in some cases and prolonged in others. Prolonged ERK1/2 activation has been shown to be required for S-phase transit in some systems (35, 37, 38). We found that two factors capable of inducing antiestrogen-resistant growth, FGF-1 and HRGβ1, are both able to induce a prolonged activation of ERK1/2 in MCF-7 cells in either the presence or absence of MEK activation blockers. The MEK-ERK1/2 pathway has also been shown to be important in the estrogen-dependent growth of the MCF-7 breast carcinoma (6, 27). In our study E2 supplementation negated the effects of ICI 182780 on growth but did not increase the amplitude nor the duration of the ERK1/2 activation response induced by 5% FBS (Fig. 2, A and B). However, U0126 completely abrogated the growth effects of E2 and the ERK1/2 activation levels in E2-supplemented conditions (Fig. 1, A and B, and Fig. 6, A and B). This suggests that the inability of E2, like EGF and unlike FGF-1 and HRGβ1, to maintain a sustained ERK1/2 activation response in the presence of U0126 renders it unable to overcome the inhibitory effects of U0126.

Although the mechanisms regulating the differences in the magnitude and duration of MAPK signaling in MCF-7 cells subsequent to exposure with FGF-1 and HRGβ1 versus EGF are still not entirely clear, a proposed scheme is depicted in Fig. 11. Neve et al. (51) have suggested that the EGF receptor is less able to maintain sustained effector activity, because it is rapidly internalized, ubiquitinated, and degraded upon ligand stimulation, unlike the HRGβ1-activated ErbB2 receptor, which has a relatively slow rate of endocytosis. However, we have demonstrated that the signaling components Ras and Raf-1, which are proximal to the receptors and upstream of MEK, are activated only transiently when cells are stimulated by EGF, HRGβ1, or FGF-1 (Figs. 4 and 11). This suggests that continually active receptor tyrosine kinase may not entirely explain the prolonged MAPK signaling. It has also been suggested that regulation and activation of another Ras-like small G-protein, Rap1, can also govern the kinetics of ERK1/2 activation (Fig. 11). Prolonged Rap1 activation is induced when nerve growth factor stimulates TrkA (unlike EGF stimulation), which then forms a complex with phosphorylated SNT-1 and other downstream components resulting in prolonged ERK1/2 activation (52). Because SNT-1 is also phosphorylated and activated upon FGF-1 stimulation in our system3 it is possible that Rap1 could also mediate FGF-1-induced prolonged ERK1/2 activation (Fig. 11). Experiments exploring the role of Rap1 in U0126-resistant induction of ERK1/2 activity by FGF-1 are in progress. In a recent report examining computer models of the Ras-MAPK pathway, Yamada et al. (53) have also proposed a role for SNT-1 in the prolonged activation of MAPK by FGF versus EGF.

Positive feedback loops generated by ERK1/2 activation of cPLA2 and PKCs have also been implicated in sustained activation of this cascade (42, 43, 54). This is a possible explanation for the sustained HRGβ1-induced ERK1/2 activation, because treatment with bisindolylmaleimide or the cPLA2 inhibitor AACOCF3 plus U0126 results in a complete inhibition of ERK1/2 phosphorylation (Fig. 9). Although FGF-2 has also been shown to activate cPLA2 (45), it does not seem to play a role in FGF1-induced ERK1/2 activation in these cells (Figs. 9 and 11). Sustained ERK1/2 activation in other systems can be

---

3 Unpublished observations.
ERK 1/2 ACTIVATION IN BREAST CANCER AND MEK INHIBITORS

blocked by inhibitors of phosphatidylinositol 3'-kinase (55). However, studies in our laboratory demonstrate that neither combined treatment of U0126 plus the phosphatidylinositol 3'-kinase inhibitor LY294002 nor LY294002 alone altered phospho-ERK1/2 levels induced by FGF-1 in MCF-7 cells.3

Signal duration through the ERK1/2 cascade can also potentially reflect dephosphorylation reactions in addition to phosphorylations. Thus, it is possible that Ser-Thr phosphatases like PP2A, dual specificity phosphatases such as MAPK phosphatases, and tyrosine phosphatases such as PTP1B, can all play a role in the sustained activation of ERK1/2. Although it is widely acknowledged that activation of the MEK-ERK pathway results in increased expression, enhanced stabilization, and elevated catalytic activity of some MAPK phosphatases (56, 57), resulting in feedback inhibition of the ERKs themselves, a recent report also shows that ERK1/2 signaling can trigger MKP-1 degradation, thereby accomplishing sustained kinase activation (58).

Whether the activity of these phosphatases in our system is reduced by HRGβ1 or FGF-1 and whether it correlates with the duration of ERK1/2 activation is not known at present.

Sustained activation of MAPKs has been shown to be a requirement for S-phase entry in some systems, whereas it correlates with migration, metalloproteinase expression, invasive capacity, and various other specific biological outcomes in others (35, 59–61). In our system, the sustained and stronger nature of ERK1/2 activation induced by FGF-1 and HRGβ1 correlates with the U0126-insensitivity of this pathway. It is not clear why the amplitude and duration of MEK activation should determine the extent of inhibition by a MEK inhibitor administered for 30 min before addition of the growth factor and throughout the length of the experiment. U0126 has been used to test MEK 1/2 dependence of growth factor-elicted signaling pathways, because it can block the activity, both in vitro and in vivo, of native MEK 1/2 and also activated MEK 1/2. Interestingly, whereas only nanomolar to low micromolar concentrations of U0126 are required to inactivate MEK in stimulated cells, the IC50 for inhibiting MEK catalytic activity in vitro is 10 μM. In in vitro experiments U0126 has also been shown to block the activation of MEK by both Raf-1 and a catalytic fragment of MAPK kinase kinase at low concentrations (39). This has led to the suggestion that in cells U0126 predominantly blocks the activation of MEK rather than blocking its enzymatic activity.

Phosphorylation of MEK1 at serines 218 and 222 and the subsequent activation is mediated directly by not only Raf-1 (of which there are three isotypes) but also c-mos and MAPK kinase kinase 1. It is not clearly known whether MEK blockers are equally effective in inhibiting MEK activated by all of these pathways in vitro, thus perhaps explaining the differential susceptibility observed in these studies. It is also not known whether in addition to these phosphorylations, interactions with other proteins in scaffolding complexes or other signaling complexes play a role in MEK activation in vitro (62). Both MAPK kinase kinase 1 and Raf-1 are found in scaffolding complexes with MEK (63) along with other proteins that might serve to provide specificity in routing signals generated by different growth factors. Consequently, different conformations of MEK and protein-protein interactions in such scaffolding complexes in response to different stimuli could potentially result in differential susceptibility to MEK inhibitors. Because cells were pretreated with U0126 in these experiments for 30 min before addition of FGF-1 or HRGβ1 containing medium, this could suggest that U0126 prebound MEK might be in rapid equilibrium with free MEK. This equilibrium is likely shifted to the right with different MEK complexes being made upon treatment with these conditions that are no longer capable of binding U0126. On the other hand it could also suggest that specific active MEK-containing complexes that are made subsequent to HRGβ1 or FGF-1 treatment are still capable of activating ERK1/2 despite being pre-bound to U0126.

It is well accepted now that although U0126 inhibits Raf-1 and MAPK kinase kinase-mediated activation of MEK, it does not block the activating phosphorylations of MEK at Ser 218 and 222 (39). It has also been suggested that sustained activation of MEK1 requires not only its phosphorylation at Ser 218 and Ser222 but also dephosphorylation at Ser 212 (64). Thus, it is also possible that it is the increased dephosphorylation at Ser 212 that results in sustained ERK1/2 activation. It is also possible that U0126 functions by blocking this “activating” dephosphorylation at Ser 212, a residue that is normally phosphorylated in intact cells (64). Therefore, another possibility is that FGF-1- and HRGβ1-mediated pathways can induce Ser 212 dephosphorylation of U0126-prebound MEK resulting in a partially active MEK, whereas serum, EGF, and E2 are unable to do so. Thus, mechanistic details about U0126-mediated inhibition in a cellular context need to be further elucidated.

U0126 has also been reported to inhibit the MEK5-ERK5 kinase cascade, albeit in some cells at higher doses than that required for MEK1/2 inhibition (48, 49). The MEK5-ERK5 pathway has also been implicated in the proliferative responses to EGF and HRGβ1 in some cell types (46, 47, 65). However, it is not known whether this pathway plays a role in antiestrogen-resistant growth induced by either of these growth factors or by FGF-1 in MCF-7 cells. In our experiments we found that HRGβ1 was the most potent inducer of ERK5 activation and that U0126 at high doses did not decrease this (Fig. 10). Surprisingly, EGF, which does not stimulate growth in U0126-treated conditions, was also a potent inducer of phospho-ERK5, even more so than FGF-1 and even in the presence of 30 μM U0126 (Fig. 10). This lack of correlation between phospho-ERK5 levels and the growth phenotypes of these factors suggests that ERK5 activation cannot surmount the growth reduction resulting from diminished ERK1/2 activation. In fact, in certain conditions, ERK5 phosphorylation was enhanced in the presence of 10 μM U0126 (data not shown). This is consistent with two other reports that suggest that the ERK1/2 cascade negatively regulates the ERK5 pathway (49, 66). It is possible, however, that ERK5 activation can contribute to growth in EGF, FGF-1, and HRGβ1 conditions. However, consistent with another recent report, our data with EGF suggest that ERK5 activation is not sufficient if ERK1/2 is also not activated (66).

The implications of the studies described in this report could be important for future endocrine therapy strategies for breast cancer. Because different growth factors induce varying levels of antiestrogen resistance, it would be logical to target signaling components common to multiple pathways. Because activation of the Ras-Raf-MEK-ERK1/2 pathway represents a common paradigm in multiple proliferative pathways, inhibition of components of this cascade is now being investigated both in laboratory and clinical studies as a means of combating antiestrogen resistance (67). Increased dependence on MAPK signaling is now being recognized to play a major role in antiestrogen resistance and adaptive resistance to estrogen deprivation (2, 68). Preclinical experiments with hormone-resistant MCF-7 xenografts that use combined treatment of antiestrogens and small molecule inhibitors of components of the Ras/Raf/MEK/MAPK pathway have been performed and have demonstrated significant synergistic effects (68). Because MAPK signaling can be activated by both Ras-dependent and Ras-independent pathways, multiple approaches to blocking this cascade will need to be considered. Currently available MEK inhibitors function in vitro predominantly by blocking MEK activation. Our results raise the possibility that distinct factorspecific MEK activation mechanisms will likely determine the level of susceptibility to such inhibitors. Therefore, a lack of complete understanding of MEK activation mechanisms and alternate input
ERK 1/2 ACTIVATION IN BREAST CANCER AND MEK INHIBITORS

routes into the MAPK signaling cascade that are operative in patient tumors could possibly result in frequent failures of therapy regimens that use MEK inhibitors in combination with antihermangonal agents.

ACKNOWLEDGMENTS

We thank Dr. Kun Liang-Guan for the DNAE plasmid, Dr. Eisuke Nishida for the GFP-ERK2 plasmid, Dr. Bernard Vose (AstraZeneca, Cheshire, United Kingdom) for ICI182780, and Dr. Dorraya El-Ashey for review of the manuscript.

REFERENCES

55. Grammer TC, Blenis J. Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. Oncogene 1997;14:1635–42.
Prolonged Extracellular Signal-Regulated Kinase 1/2 Activation during Fibroblast Growth Factor 1- or Heregulin β1-Induced Antiestrogen-Resistant Growth of Breast Cancer Cells Is Resistant to Mitogen-Activated Protein/Extracellular Regulated Kinase Kinase Kinase Inhibitors

Jaideep V. Thottassery, Yanjie Sun, Louise Westbrook, et al.

Cancer Res 2004;64:4637-4647.

Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/13/4637

Cited articles  This article cites 64 articles, 37 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/13/4637.full.html#ref-list-1

Citing articles  This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/64/13/4637.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.