Inhibition of the MAP kinase activity suppresses estrogen-induced breast tumor growth both in vitro and in vivo

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Abstract. Elevated expression of mitogen-activated protein kinase (Erk/MAPK) has been noted in a significant percentage of primary human breast cancers. To directly assess the importance of Erk/MAPK activation in estrogen (E$_2$)-induced tumor progression, we blocked E$_2$-signaling with MEK-inhibitor CI-1040 and/or tamoxifen (TAM). Our data show that both MEK-inhibitor CI-1040 and TAM blocked E$_2$-induced MAPK phosphorylation and cell proliferation in MCF-7 breast cancer cells in vitro. However, in vivo studies show that anti-tumor efficacy of combining the CI-1040 and TAM was similar to single agent(s). Furthermore, sequential treatment with TAM followed by CI-1040 or CI-1040 followed by TAM did not significantly reduce E$_2$-induced tumor growth. This suggests that the combination of CI-1040 and TAM may not be synergistic in inhibiting E$_2$-induced tumor growth. However, these findings also indicate that MAPK plays a critical role in E$_2$-induced tumor growth, and that this could be a potential therapeutic target to combat hormonally regulated growth in ER-positive tumors.

Introduction

Multiple lines of evidence have established that the steroid hormone estrogen (E$_2$) is essential not only for normal breast development but also plays a major role in the development and growth of breast cancer. Approximately three-quarters of all invasive breast tumors are estrogen and/or progesterone receptor-positive (1,2). Endocrine therapy remains the most important systemic treatment in this population of women with hormone receptor-positive breast cancer. It was shown that in estrogen receptor-positive disease, about five years of adjuvant tamoxifen reduces the annual breast cancer death rate by 31% (3). This benefit is achieved irrespective of the use of chemotherapy, age, progesterone receptor status, or other tumor characteristics. Most endocrine agents act by either blocking the production of estrogen (ovarian ablation and aromatase inhibitors) or the by competing for ER binding (tamoxifen and ICI). The choice of endocrine agent depends on the menopausal status of the patient, because this factor determines the source of estrogen: ovarian or adrenal.

There is an increasing body of evidence, however, that indicates such a therapeutic approach may be somewhat simplistic. Control of breast cancer growth is now perceived to be multifaceted, comprising of an elaborate network of interacting steroid hormone- and growth factor-driven pathways that reinforce individual pathway effects on gene expression (4,5). It has thus been postulated that aberrations in growth factor signal transduction would significantly influence estrogen action (4,6,7). Indeed, it is envisaged that such events may entirely circumvent the cellular requirement for this steroid hormone, supporting tumor growth that is resistant to anti-hormonal therapies despite retention of functional ER. Of particular interest is the mitogen-activated protein kinase (MAPK), also known as extracellular signal-regulated kinases (Erk1 and Erk2). Experimental studies have demonstrated that MAPKs are pivotal components of intracellular phosphorylation cascades both in cytoplasm and the nucleus (8,9). Interestingly, activated Erk/MAPK can drive ligand independent activation of ER (6,10). ER-regulated gene expression is subsequently initiated, as has been demonstrated using estrogen response element-containing reporter gene constructs transiently transfected in vitro (4,11). In addition, elevated Erk/MAPK has been demonstrated to occur within some breast tumors (12-14). Finally, changes in several key regulatory pathways such as ErbB, insulin-like growth factor receptor, and protein kinase C leading to Erk/MAPK activation are frequently elevated in clinical breast tumors, and these are associated with anti-hormonal resistance (15-18). In this study we investigated whether mitogen-activated extracellular signal regulated kinase kinase (MEK) inhibitor, CI-1040 used as a monotherapy or in combination with anti-estrogen TAM, is more efficient in inhibiting breast tumor growth both in vitro and in vivo.

Materials and methods

Materials. Human breast cancer MCF-7 cells were purchased from American Type Culture Collection. The cells were grown in DMEM supplemented with 10 ng/ml insulin, 5% fetal calf serum, and antibiotics (penicillin/streptomycin). DMEM,

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implanted s.c. at the time of cell inoculation. Treatment (CI-stradiol - 0.72 mg/biodegradable carrier-binder pellet) was purchased from Taconic Farms, Germantown, NY. The estrogen pellets (17ß-estradiol - 0.72 mg/biodegradable carrier-binder pellet) were purchased from Innovative Research of America, Inc. (Sarasota, FL). Phospho-MAPK antibody was purchased from New England Biolabs, MA. Anti-MAPK antibody was purchased from Zymed Laboratories, CA. ER antibody was purchased from Dako (Carpinteria, CA) and NeoMarkers.

Western immunoblot analysis. Western blotting was performed as described previously using a standard protocol (19). Crude protein extracts were obtained by lysing 5x10^6 cells in a buffer [50 mM Tris-HCl (pH 7.6), 1% NP-40, 2 mM EDTA, 0.5% Na deoxycholate, 150 mM NaCl, 1 mM Na orthovanadate, 2 mM EGTA, 4 mM Na p-Nitro phenyl phosphate, 100 mM Na fluoride] supplemented with protease inhibitors [leupeptin (0.5%), aprotonin (0.5%), and PMSF (0.02%)]. Samples containing 50 μg of total protein were electrophoresed on 7.5% (for EGFR) and 10% (for MAPK) SDS-polyacrylamide gels and transferred to nitrocellulose membrane by electroblotting. Membranes were probed with antibodies, followed by HRP-conjugated mouse or rabbit secondary antibodies (Amersham) and enhanced chemiluminescence detection (Amersham). For quantification of activity, band intensities of the phospho- and total protein(s) were quantified using Biorad ‘Quantity one’ software.

Monolayer growth assay. Cells were plated in 6-well tissue culture plates, at a density of 1x10^5 cells/well, in DMEM containing 5% FCS. After 24 h, the seeding medium was removed, cells were washed twice with PBS, and the medium was replaced with phenol red-free and serum-free DMEM with E2, Tam and with or without CI-1040. The medium was replaced with fresh medium, containing the same supplements, on day 3. The cells were counted in triplicate after being suspended in PBS with 1 mM EDTA, on day 5.

Animal efficacy studies. MCF-7 cells (1x10^6 cells/mouse) were suspended in matrigel and injected subcutaneous (s.c.) in ovarectomized female SCID-beige mice 5-6 weeks old (Taconic Farms, Germantown, NY). The estrogen pellets (17ß-estradiol - 0.72 mg/biodegradable carrier-binder pellet) were implanted s.c. at the time of cell inoculation. Treatment (CI-1040) was administered by gavage and/or tamoxifen (5 mg/biodegradable carrier-binder pellet) was implanted s.c. one day after tumor cell implantation. The E2 and TAM slow release pellets were obtained from Innovative Research of America. The CI-1040 (PD 184352) was prepared in a vehicle of 10% Cremophore EL (Sigma), 10% ethanol and 80% water. Ten animals were included in each group. Tumor size was evaluated periodically by caliper measurements, generally twice per week. Tumor size measurement of the length (a) and width (b) of tumor and volumes calculated as (a x b^2)/2.

Results

MEK-inhibitor CI-1040 blocks E2-induced MAPK phosphorylation and cell proliferation in vitro. CI-1040 is an oral inhibitor of mitogen-activated extracellular signal regulated kinase (MEK), a key enzyme in the Ras-Raf-MEK-MAPK (Erk) pathway known to be involved in key cellular activities including proliferation, differentiation, and apoptosis (20-22). To confirm that these compounds can block MAP kinase phosphorylation in breast cancer cells, we examined the effect of CI-1040 on MCF-7 breast cancer cells. After treating the cells with estrogen (E2) and/or TAM in the presence or absence of CI-1040, Western blot analysis using phospho-MAPK antibodies was performed. The data show that E2 (10^-8 M) significantly increases phosphorylated MAPK levels compared to control cells. E2-induced phosphorylated MAPK levels were inhibited by TAM and CI-1040. However, maximum inhibition of phosphorylated Erk/MAPK was seen in the presence of MEK-inhibitor CI-1040 (Fig. 1).

To investigate the effect of CI-1040 on E2-stimulated cell proliferation, we examined its effects on MCF-7 cell. In MCF-7 cells E2-induced ~2-fold increase in cell proliferation, and E2-induced cell proliferation was significantly inhibited by TAM (10^-6 M) and CI-1040 (10 μM) (Fig. 2). At concentrations of 20 μM, CI-1040 inhibited cell growth significantly below controls (data not shown), suggesting that, at this concentration, CI-1040 might inhibit other growth-regulatory processes, in addition to its effect on MEK mediated growth. Cells growing under these conditions in the presence of CI-1040 (10 μM) appeared to remain viable, as indicated by their morphological appearance, by the lack of floating cells, and by the ability of the cells to exclude trypan blue (>90% viable). These data suggested that the inhibitory effect of the CI-1040 was cyto-static rather than cytotoxic at this concentration.

Inhibition of MAPK signaling by CI-1040 inhibits estrogen-induced tumor growth in vivo. Exposure to CI-1040 and/or TAM inhibited E2-induced cell proliferation in vitro, we tested the ability of CI-1040 to inhibit E2-mediated tumor growth. CI-1040 significantly inhibited tumor growth compared to control cells (Fig. 3). The results suggest that CI-1040 inhibits tumor growth in vivo.
growth in vivo, either with or without TAM. MCF-7 cells were injected (s.c.) into 5-6 weeks old female ovariectomized SCID mice, which were implanted with E2-pellet (17ß-estradiol - 0.72 mg/biodegradable carrier-binder pellet, 90-day slow release pellet). Ten animals per treatment group were used. One day after the injection of the cells the mice were given CI-1040 (154 mg/kg) by oral gavage and/or TAM for 21 days. The dosage of CI-1040, estrogen and TAM was determined from previous studies (4,22). Our data show that mice receiving estrogen plus CI-1040 or estrogen plus TAM had significantly smaller tumors compared to estrogen-treated mice (p<0.05) (Fig. 3). However, concurrent administration of E2, CI-1040, and TAM did not show additive effect on inhibition of tumor growth. Phospho-MAPK levels in TAM and/or CI-1040 were significantly lower compared to E2-treated tumors (Fig. 4). However, differences with in the tumors either treated with TAM or CI-1040 or a combination of TAM and CI-1040 are not significantly different.

To determine if sequential treatment, with TAM followed by CI-1040 or reverse sequence will enhance the inhibitory effects of the drug compared to concurrent administration of CI-1040 and TAM. We conducted in vivo experiments were ten mice were first treated with CI-1040 for 21 days and then...
they were split into two groups (average tumor size in both the groups is roughly the same): one group of five mice continued to get CI-1040 and the other group of five mice received TAM (Fig. 5A). In the second treatment group ten mice were first treated with TAM for 21 days and then they were split into two groups (average tumor size in both the groups is roughly the same): one group of five mice continued to get TAM and the other group of five mice received CI-1040 (Fig. 5B). Our data do not show any significant advantages in sequential treatment compared to individual treatment with CI-1040 or TAM alone in vivo.

Discussion

In breast cancer, the steroid hormone estrogen is one of the most potent mitogens. Although it is generally believed that MAPK are activated in response to signals generated by cell surface receptors, it was recently reported that estrogen and progesterin activate MAPK signaling in human breast cancer cell lines (24). However, this activation is most likely not mediated by nuclear steroid receptors, but by cell surface forms of estrogen receptors. Furthermore, secretion of a growth factor upon estrogen stimulation of human breast cancer cells can also lead to autocrine/paracrine activation of MAPK (19,25,26). The Erk/MAPK signaling cascade is intimately involved in mediating proliferative signals for receptors whose overexpression and/or constitutive activation has been shown to play an important role in the pathogenesis and progression of breast cancer. Given the complexity of this signaling network and proliferative effect, therapeutic blocking of MEK, as key mediator of E2-mediated cell proliferation, could have significant clinical benefit in the treatment of breast cancer. To test this hypothesis we determined the role of Erk/MAPK in E2-induced cell proliferation both in vitro and in vivo models. We found that TAM partially blocked E2-induced MAPK activity and cell proliferation in vitro. However, addition of MEK-inhibitor CI-1040, inhibited most of the estrogen-induced MAPK activity. However, both TAM and CI-1040 inhibited E2-induced cell proliferation in MCF-7 human breast cancer cells. These experiments demonstrated an essential role of MEK/MAPK signaling for the maintenance of E2-induced cell proliferation in vitro.

Our data show that in vivo tumor models both TAM and CI-1040 as single agents, showed statistically significant reduction in E2-induced tumor volume. The anti-tumor efficacy of combined TAM and MEK-inhibitor CI-1040 was similar to single agents. We further tested to see if sequential treatment, TAM followed by CI-1040 or reverse sequence CI-1040 followed by TAM would have additive effect on reducing the tumor volume. However, sequential treatment did not significantly reduced the tumor volume compared to single agents. This suggests that either combination or sequential treatment with TAM and CI-1040 are ineffective in increasing efficacy, relative to either single agent that was associated with suppressed proliferation. One of the reasons may be that TAM competes with E2 to bind to ERα and decrease the synthesis and secretion of growth factors such as TGFα, HRG, IGF-II and this in turn reduce MAPK activity by autocrine and/or paracrine mechanism (9,26). CI-1040 also blocks MAPK activity; therefore combining TAM and CI-1040 may not be effective because both TAM and CI-1040 may be blocking E2-induced Erk/MAPK activity.

A majority of primary human breast cancers are considered estrogen receptor (ERα)-positive, and ER expression in breast tumors provides prognostic information, in addition to treatment response information (27). Targeting the ER using the selective ER modulator, tamoxifen, is efficacious in both the treatment and prevention of human breast cancer (28,29). However, despite tamoxifen therapy success, some primary ERα-positive tumors do not respond to TAM (30). In these cases inhibition of MEK may be used to prevent E2-induced tumor growth. However, phase II results using CI-1040, in breast, colon, and non-small cell lung cancer demonstrated insufficient CI-1040 anti-tumor activity (31). However, in these studies baseline tumor biopsies were not obtained for testing of phospho-MAPK expression in this study. Instead, archived tumor specimens available from an original diagnostic or a more recent biopsy were assessed. Therefore, it is possible that tumor specimens may underreport the true levels of constitutive expression present at the time of the original biopsy. Recent clinical data highlight the difficulty in identifying the patients who are most likely to respond to treatment with molecularly targeted agents (32). With the central role of MEK in mediating multiple oncogenic signaling pathways, inhibitors of this molecular target have the potential to have a broad spectrum of anti-tumor activity.

Although activation of the ER by Erk/MAPK has been implicated as a mechanism of resistance to anti-estrogen therapies (33,34). Recent data from our group showed that Erk/MAPK induced ERα activation lowers E2 requirement for optimal ER-signaling and accelerates E2-independent tumor growth without diminishing sensitivity to the inhibitory effects of anti-estrogens (4). Consistent with our observation Murphy et al have shown that Erk/MAPK-induced ER phosphorylation is associated with better disease outcome in women treated with TAM (35,36). These data suggest that Erk/MAPK phosphorylation plays a major role in breast cancer progression. Other studies have reported that receptor blocking antibodies and an inhibitor of Erk/MAPK can also inhibit growth of HER-2 overexpressing breast tumor cells when given together with TAM (33,34). However, our data show that anti-tumor efficacy of combined TAM and MEK-inhibitor CI-1040 was similar to single agents in vivo. Given the complexity of this signaling network and important proliferative effect of co-activation of multiple growth promoting pathways, the therapeutic blockade of MEK, could have significant clinical benefit in the treatment of E2-induced breast cancer.

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References