Suppression of Pro-metastasis Phenotypes Expression in Malignant Pleural Mesothelioma by the PI3K Inhibitor LY294002 or the MEK Inhibitor UO126

GEORGE W. COLE JR.*, ANNETTE M. ALLEVA*, JING T. ZUO*, SHAILEN S. SEHGAL**, WEN-SHUZ YEOW, DAVID S. SCHRUMP and DAO M. NGUYEN

Section of Thoracic Oncology, Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

Abstract. *Background: This study aimed to evaluate the impact of selective abrogation of either the MEK/ERK1/2 (UO126 or PD98059) or the PI3K/AKT (LY294002) signaling cascade on cell proliferation, motility and invasion and production of VEGF (collectively termed pro-metastasis phenotypes) in cultured malignant pleural mesothelioma (MPM) cells. Materials and Methods: Treatment-induced cytotoxicity was evaluated by MTT or Annexin V assays. Cell motility was assessed by wound healing and Matrigel invasion assays. VEGF in conditioned media of cancer cells was measured by ELISA. Results: LY294002 and UO126 significantly inhibited cell proliferation and clonogenicity of MPM cells in vitro. A substantial reduction of cell motility, Matrigel invasion as well as inhibition of basal or EGF-induced VEGF production were observed in drug-treated cells. Conclusion: The selective MEK or PI3K kinase inhibitors are equally effective in down-regulating the expression of pro-metastasis phenotypes, suggesting that MEK or PI3K are appropriate targets for the development of molecular therapeutics for malignant pleural mesothelioma.*

Malignant pleural mesothelioma (MPM) is a rare but deadly disease etiologically linked to asbestos exposure and possibly SV40 infection (1-3). The annual incidence of MPM in the USA (2,000 to 2,500 new cases/year) is expected to decrease in the next quarter of the century (4, 5) yet its incidence in other parts of the world, particularly in Europe, is projected to remain high in the same period of time (6, 7). MPM is characterized by unrelenting locoregional invasion causing death from encasement of contiguous intrathoracic organs. Current aggressive multimodality therapy for MPM, consisting of surgical resection, cytotoxic chemotherapy and radiation, offers survival benefit to only a small subset of patients with early stages of the disease (8). The major mode of treatment failure following ablative therapy is local/regional recurrence and/or contiguous metastasis to the peritoneal cavity. In conjunction with ongoing clinical efforts to design more effective therapy for MPM, considerable attention has been focused on the development of molecular therapy for this malignant disease by targeting the signal transduction pathways that regulate the process of tumorigenesis and metastasis formation.

The development of a malignant tumor from its microscopic focus, similar to the formation of metastasis, is a multistep process. This involves acquisition of multiple cellular characteristics such as enhanced proliferation, motility and invasion through extracellular ground substance matrix, resistance to apoptosis, as well as the ability to stimulate angiogenesis (collectively termed as pro-metastasis phenotypes) (9). Substantial evidence has indicated that extracellular signals transmitted into the cells *via* membrane receptors with intrinsic tyrosine kinase activity, such as those

*The authors contributed equally to this work.
**National Institutes of Health – Clinical Research Training Program, Bethesda, MD, U.S.A.

Abbreviations: EGFR, epidermal growth factor receptor; MPM, malignant pleural mesothelioma; MTT, 4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositide 3-kinase; PDK, 3-phosphoinositide-dependent kinase; VEGF, vascular endothelial cell growth factor; JAK/STAT, Janus kinase/signal transducers and activators of transcription; PLC, phospholipase C; PDGFR, platelet-derived growth factor; HGF, hepatocyte growth factor; IGFR, insulin growth factor receptor.

Correspondence to: Dao M. Nguyen, MD, Clinical Research Center, Room 4W-4-3940, 10 Center Drive, Bethesda, MD 20892, U.S.A. Tel: 301-496-2127, Fax: 301-451-6934, e-mail: Dao_Nguyen@nih.gov

Key words: EGFR, MAPK, MEK, ERK1/2, PI3K inhibitor LY294002, MEK inhibitor UO126, apoptosis, angiogenesis, prometastasis phenotypes, motility.

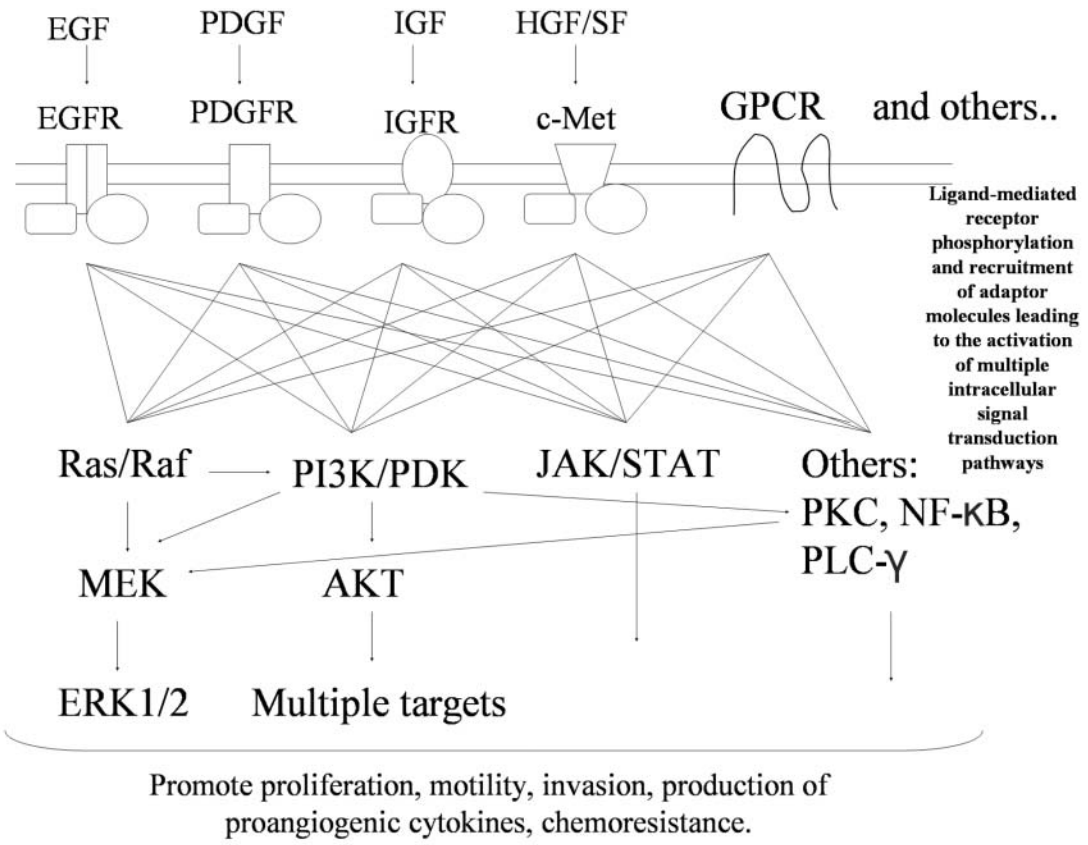

Figure 1. *Multiple membrane receptor tyrosine kinases (EGFR, IGFR, PDGFR, c-Met and others) converge on distinct intracellular parallel signaling pathways (PI3K/AKT, Ras/Raf/MEK, PLC-γ/PKC, JAK/STAT) that regulate cell proliferation, motility, invasion, angiogenesis and cellular responses to cytotoxic stresses. Direct inhibition of a membrane receptor tyrosine kinase (RTK) may not result in efficient suppression of downstream intracellular signaling pathways emanating from this particular receptor and biological responses due to multiplicity of other RTK's. Direct inhibition of each of the intracellular pathways at the converging point may circumvent this limitation.*

of the EGFR superfamily, PDGFR, IGFR and c-MET (HGF/SF receptor), regulate the expression of pro-metastasis phenotypes (10-17). Binding of ligands to their cognate receptors, regardless of receptor specificity, results in activation of the intrinsic tyrosine kinase activity of the membrane receptors, leading to autophosphorylation of tyrosine residues in their intracellular domains. Phosphorylated tyrosines within their unique amino acid motifs provide docking sites for a variety of adaptor molecules (for example, SOS, Grb2), as well as functional proteins (for instance PI3K p110 subunit) that lead to the activation of parallel yet interconnecting cascades of downstream intracellular signaling pathways such as the Ras/Raf/MEK/ERK, the PLC-γ, the PI3K/PDK/AKT and the JAK/STAT pathways. Activated receptors can also directly phosphorylate functional proteins (such as STAT or PLC-γ) (17). The biological responses from such diverse intracellular signaling include cell proliferation, survival, motility, invasion and angiogenesis. Blocking of upstream ligand/receptors will provide a global suppression of multiple downstream signals, but redundancy of the receptor tyrosine kinase repertoire may make selective targeting strategy less effective unless the targeted receptors exert a dominant signal transduction effect *via* receptor over-expression or hyper-functioning (Figure 1).

We have previously demonstrated that MPM cells express EGFR and yet the EGFR-selective tyrosine kinase inhibitor PD153035 mediated significant suppression of cell proliferation and of vascular endothelial growth factor (VEGF) production in a cell line-dependent fashion, particularly in those MPM cells expressing very high levels of EGFR (18). We postulated that PD153035-mediated inhibition of EGFR signaling may be inefficient in cell lines expressing normal levels of EGFR due to the presence of other growth factor receptors that, in parallel with EGFR, transduce their mitogenic signals to a similar set of intracellular pathways. A potentially more efficient strategy is to selectively target the intracellular signal transduction

pathways downstream of the membrane growth factor receptor repertoire. The theoretical advantage of targeting the intracellular pathways, such as PI3K/PDK/AKT or Ras/Raf/MEK, is the ability to collectively block inputs from multiple upstream receptors which converge on that particular pathway, while the potential disadvantage of this approach is that this would only alter the biological responses mediated by that single pathway alone. As there are extensive "cross-talks" between pathways that are somewhat unpredictable and cell-specific, selective down-regulation of a particular signal transduction pathway using pharmacological inhibitors or genetic manipulations to express dominant negative functional proteins may affect more than one intracellular signaling cascade. The roles of PI3K-mediated or MEK-mediated signal transduction on the cellular expression of pro-metastasis phenotypes have been well described (10, 19-25). Pharmacological inhibitors of these kinases have been extensively evaluated in preclinical models and, particularly in the case of the MEK inhibitor, in early phase clinical trials (26). The primary objective of this study, therefore, was to evaluate the effect of selectively blocking either the RTK's/PI3K/PDK/AKT pathway using the PI3K pharmacological inhibitor LY294002, or the RTK's/Ras/Raf/MEK/ERK1,2 pathway by the MEK inhibitors UO126 or PD98059, on the expression of prometastasis phenotypes in a panel of cultured MPM cells *in vitro*.

Materials and Methods

Cells and reagents. Cultured MPM cells (H513, H211, M28, H2373, H2595, H2052) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), glutamine (2 mmol/L) and antibiotics streptomycin (100 μg/mL) and penicillin (100 U/mL). The PI3K inhibitor LY294002 and the MEK inhibitors UO126 or PD98059, all purchased from Alexis (San Diego, CA, USA), were dissolved in DMSO as 100 mM stocks, aliquoted and stored at –20˚C.

Cell proliferation, clonogenic and apoptosis assays. Cultured MPM cells were seeded in 96-well plates (3,000 cells/well) and subsequently treated with LY294002 (5 to 80 μM) or UO126 (5 to 100 μM) for 96 hours with replacement of fresh media (with or without PD) at 48 hours. The cell viability of the control and drug-treated cells was quantified by MTT (Sigma-Aldrich, St. Louis, MO, USA). The IC_{50} values of LY294002 and of UO126 (concentrations of drugs that mediated 50% inhibition of cell proliferation) were extrapolated from the respective dose-response curves. Cell cycle analysis was performed in control (grown in 10% RPMI) and drug-treated cells (LY294002 at 20 and 40 μM or UO126 at 10 or 20 μM for 24 hours) using propidium iodine (PI) staining and flow cytometry. As UO126 creates autofluorescence of the treated cell (27), which interferes with fluorescence-based apoptosis assays, the MEK inhibitor PD98059 (28) was used in apoptosis experiments at drug concentrations and treatment conditions that mediated comparable degrees of inhibition of MEK kinase activity (reduction of phosphorylated ERK1/2 and inhibition of clonogenicity *in vitro*) as UO126. LY294002- or PD98059-induced apoptosis was determined by PE-conjugated Annexin V/7AAD staining and flow cytometry (Pharmigen-BD). Cells were seeded in 6-well plates (200,000 cells/well) and, after an overnight incubation, they were continuously treated with either LY294002 (40 μM) or PD98059 (50 μM) for 72 hours. The controls were cells grown in 10% RPMI. The cells were harvested and assayed for apoptosis as per protocol. A clonogenic assay was used to globally assess the growth inhibitory effect (cell cycle arrest and cell death) of these selective kinase inhibitors. Cells were plated in 12-well plates (500 cells/well) and, after an overnight incubation to allow complete attachment to the plastic surface, 2 ml of media without or with the respective kinase inhibitors (LY294002 at 10 or 40 μM, UO126 at 10 or 40 μM , PD98059 at 25 or 50 μM) were added and the cells were incubated for 10 days. As PD98059 is not sufficiently potent or soluble in culture media to sustain the inhibition of MEK activation (29), PD98059-containing media were replaced daily for 3 days and the cells were further incubated until clonogenicity was evaluated 7 days later. The magnitude of colony formation of the control and of UO126- or LY294002-treated cells was quantified by either digital photography of colonies stained with 0.1% crystal violet or by MTT assay to quantify cell viability which was then expressed as percentages of untreated control cells.

Cell motility assay. Cell motility was evaluated by the *in vitro* wound-healing assay as previously described (30). MPM cells were seeded in 6-well plates to obtain 90% confluence. After an overnight incubation, "wounds" were made by scratching the cell monolayer with sterile plastic pipette tips. The cells were then treated with LY294002 (40 μM) or UO126 (40 μM) for 24 to 48 hours. Movement of cells from the wound edges into the "wounds" was indicative of cell motility. The cells were fixed with 1% paraformaldehyde, stained and the magnitude of "wound-healing" was recorded by digital microphotography.

Matrigel invasion assay. Cell migration and invasion through the Matrigel membrane was quantitated using the commercially available cell invasion kit (Chemicon International, Temecula, CA, USA). MPM cells were treated with LY294002 (40 μM) or UO126 (40 μM) for 4 hours in FCS-free media prior to being seeded in the invasion chambers (in the presence of drug) and stimulated to migrate and invade the Matrigel-containing filter using serum-containing media in the lower wells. Filters containing cells that successfully traversed the Matrigel layer were stained with the staining solution provided in the kit and photographed with a digital camera.

VEGF assay. VEGF in conditioned media of cultured MPM cells (baseline control or following LY294002 (40 μM) or UO126 (20 μM) for 24 hours) was measured by ELISA (R&D Biosystems, Minneapolis, MN, USA). Cells were seeded in 12-well plates at 1.0 or $1.5x10^5$ cells/well then, after an overnight incubation, the cells were either incubated in 2 ml of 10% RPMI (baseline control) or in 10% RPMI with LY294002 or UO126 for 24 hours. The VEGF levels in the conditioned media were normalized for total cellular protein and expressed as pg VEGF/mg of cellular protein/24 h. To evaluate the ability of either LY294002 or UO126 to inhibit EGF-mediated up-regulation of VEGF production, the cells were serum-starved for 8 hours and then pre-treated with LY294002 (40 μM) or

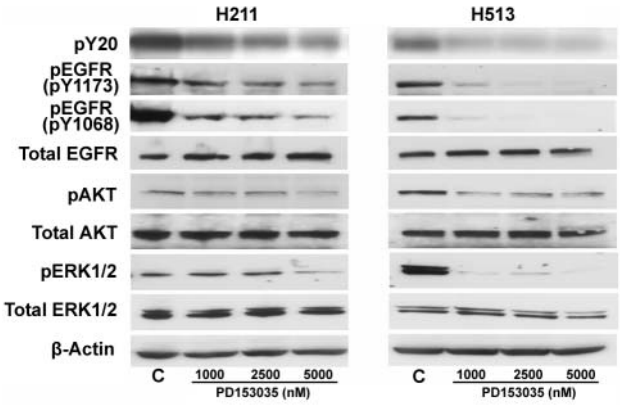

Figure 2. *The EGFR RTK inhibitor PD153035 mediated the dose-dependent suppression of tyrosine phosphorylation of EGFR (reduction of signals detected by pY1068, pY1173 and pY20 (at 180 kDa) antibodies) in both H513 and H211 cells, but profound suppression of pAKT and pERK1/2 was only observed in H513 cells. Cells were treated with PD153035 at 1000, 2500 or 5000 nM for 6 hours and harvested for Western blot analysis of pEGFR, pAKT and pERK1/2. β-actin was blotted to indicate equal loading of the proteins.*

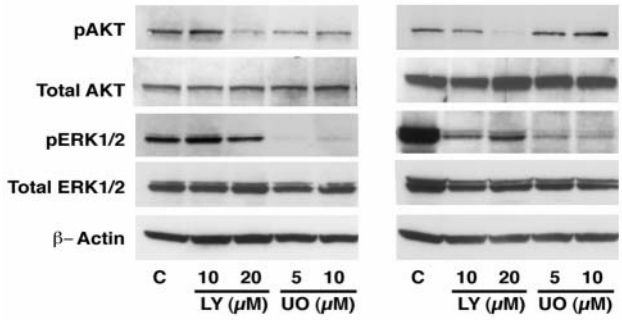

Figure 3. *Effect of UO126 or LY294002 on the levels of pERK1/2 or pAKT in H211 and H513 cells. Cells were treated with UO126 (5 or 10 μM) or LY294002 (10 or 20 μM) for 6 hours prior to harvesting for determination of pAKT and pERK1/2 expressions by Western blots using phospho-specific antibodies. Selective kinase inhibitors did not affect the total levels of AKT or ERK1/2. In addition to suppressing AKT activation (reduction of pAKT), LY294002 also down-regulated ERK1/2 activation (reduction of pERK1/2) in H513 cells. On the other hand, UO126 suppressed the activation of both AKT and ERK1/2 in H211 cells.*

UO126 (20 μM) for 4 hours prior to EGF (20 ng/ml) exposure while being continuously treated with the kinase inhibitors. The controls were cells incubated in serum-free media with or without LY294002 or UO126.

Western blots. The levels of total and phosphorylated EGFR, AKT and ERK1/2 in H211 and H513 cells treated with either the selective EGFR tyrosine kinase inhibitor PD153035 or LY294002 or UO126 were quantified by Western blot analysis using the following monoclonal antibodies: total EGFR (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500 dilution), pY1173 (Santa Cruz, 1:500 dilution), pY1068 for phosphorylated EGFR (Cell Signaling Technology, Beverly, MA, USA, 1:500 dilution), total phosphorylated tyrosine pY20 (BD Transduction Laboratories, San Jose, CA, USA, 1:2,500 dilution), phosphorylated AKT (ser473) and total AKT (Cell Signaling Biotechnology, 1:1,000 dilution), phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2 (Cell Signaling Biotechnology, 1:1,000 dilution) and β-actin (Oncogene Research Products, Cambridge, MA, USA, 1:10,000 dilution). Cells were plated in 10% FCS RPMI at a density of $5x10^6$ per plate in 10-cm^2 plates. The following day, the cells were treated with either PD153035 or LY294002 or UO126 (at concentrations indicated in the figure legends) for 6 hours. Cell pellets were lysed by Laemmli buffer, incubated at room temperature for 15 min and heated at 95˚C for 5 min to enhance protein extraction. The supernatants of the cell lysates were collected by centrifugation at 14,000 rpm for 5 min and their protein concentrations were measured by the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Proteins in the pre-cleared cell lysates were resolved by electrophoresis through 4% to 20% SDS-polyacrylamide gels, transferred to nitrocellulose membrane and immunoblotted with specific antibodies. The blots were also probed with β-actin to confirm equal loading of the proteins. In order to obtain an optimal signal, the amount of loading proteins was adjusted according to the sensitivity and specificity of the antibodies used in the study.

Data analysis. The data are expressed mean±SEM of 3 or 4 independent experiments that yielded similar results. Analysis of variance and the Student's *t*-test were used for statistical analysis and $p<0.05$ was considered statistically significant.

Results

Inhibition of downstream AKT and ERK1/2 activation by EGFR tyrosine kinase inhibitor PD153035. We have previously demonstrated that cultured MPM cells express EGFR and EGFR-mediated signaling contributes to cellular expression of pro-metastasis phenotypes in a cell line-dependent fashion (13). The selective EGFR TKI PD153035 (PD) was very efficient in suppressing multiple pro-metastasis cellular phenotypes in H513 cells but not in H211 cells, even though PD could significantly inhibit EGFR phosphorylation of both the cell lines. Indeed, treating these cells with PD while growing them in 10% RPMI resulted in a pronounced dose-dependent reduction of the levels of phosphorylated EGFR (using specific monoclonal antibodies to detect phosphorylated tyrosine residues pY1068, pY1173 or the general anti-phosphotyrosine antibody pY20 in the 170 kDa to 180 kDa range). Reduction of EGFR phosphorylation clearly correlated with the inhibition of AKT and ERK1/2 phosphorylation in H513 but not in H211 cells. Exposure of these cells to PD at the indicated concentrations did not affect the total levels of EGFR, AKT or ERK1/2 (Figure 2). The lack of efficient inhibition of AKT or ERK1/2 activation by a selective EGFR antagonist in H211 cells may be due to the presence of other upstream receptor tyrosine kinases besides EGFR that converge on these intracellular cascades, or the presence of Ras or Raf mutations that constitutively

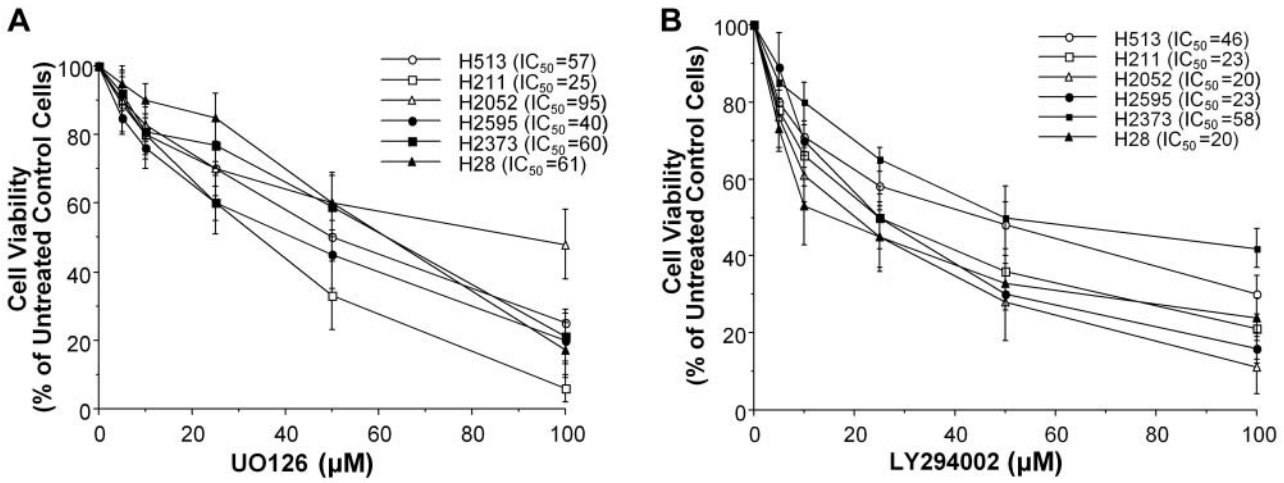

Figure 4. *Dose-dependent inhibition of cell viability by the MEK inhibitor UO126 (20 μM)) or the PI3K inhibitor LY294002 (40 μM) in MPM cells. Data are presented as means±SEM of 4 independent experiments.*

activate downstream signaling cascades. Direct inhibition of a downstream intracellular pathway in such an instance may be a better approach than selective inhibition of a single membrane receptor. This provides the impetus for further investigation into the direct inhibition of downstream pathways using currently available kinase inhibitors such as the PI3K inhibitor LY294002 or the MEK inhibitor UO126.

Inhibition of AKT activation by LY294002 and ERK1/2 activation by UO126. Treating H513 and H211 cells with UO126 or LY294002, as expected, resulted in profound reduction of the levels of pERK1/2 (Thr202/Thr204) and pAKT (ser473), respectively, in a dose-dependent fashion (Figure 3). Near complete inhibition of ERK1/2 phosphorylation in H153 and H211 was observed following 6-hour exposure to 10 μM of UO126. Similarly, LY294002 at 20 μM for 6 hours almost completely abrogated AKT phosphorylation in both cell lines. It is interesting to note that LY294002 also mediated the reduction of pERK1/2 in the H513 cells, probably *via* interference with the PI3K/PDK/MEK axis (31). This was not observed in H211 cells. Conversely, UO126, in addition to inhibiting the activation of ERK1/2, also caused a clear reduction of pAKT levels in H211 cells, but this was not observed in H513 cells. Such observations indicate the presence of significant "cross-talks" between parallel signaling pathways and selective pharmacological inhibitors may not be that specific in blocking a single pathway in living cells.

Growth inhibitory effect of LY294002 and UO126 on MPM cells. Continuous exposure of cultured MPM cells to either LY294002 or UO126 resulted in a dose-dependent and cell line-dependent inhibition of cell proliferation (Figures 4A and B). The IC_{50} values of LY294002 in MPM cells ranged from 15 to 47 μM. The IC_{50} values of UO126 ranged from 25 to 95 μM. Combining LY294002 (40 μM) with UO126 at 20 μM to achieve dual inhibition of both the PI3K/AKT and MEK/ERK1/2 pathways resulted in an additive growth inhibitory effect (data not shown). The underlying mechanisms of LY294002- or UO126-mediated growth inhibition in MPM cells were examined by evaluating the effect of these kinases on cell cycle dynamics and on induction of apoptosis. Cell cycle analysis using PI staining and flow cytometry indicated that 24-hour exposure of MPM cells to either LY294002 or UO126 caused cell cycle arrest at the G1/S checkpoint, as indicated by the significant accumulation of cells in the G0/G1-phases of the cell cycle together with corresponding reduction of cells in the S- and G2/M-phases (Figure 5A). An alternative MEK inhibitor, PD98059, had to be used in lieu of UO126 for apoptosis experiments as the strong autofluorescence observed in UO126-treated cells interfered with the apoptosis assays utilized for this study. PD98059, at the maximal recommended concentration of 50 μM, was slightly inferior to UO126 (20 μM) in inhibiting MEK activity (reduction of phosphorylated ERK1/2 at 6 or 24 hours of drug treatment as evaluated by ELISA) (Assay Designs, Ann Arbor, MI, USA) (data not shown). Nevertheless, PD98059 (50 μM) was as efficient as LY294002 (40 μM) in mediating mild to moderate apoptosis in 4 representative MPM cells (Figure 5B). The combination of cell cycle arrest and induction of apoptosis translated into a very strong inhibition of the clonogenicity of MPM cells treated with either LY294002 or with UO126 or PD98059 (Figure 5C).

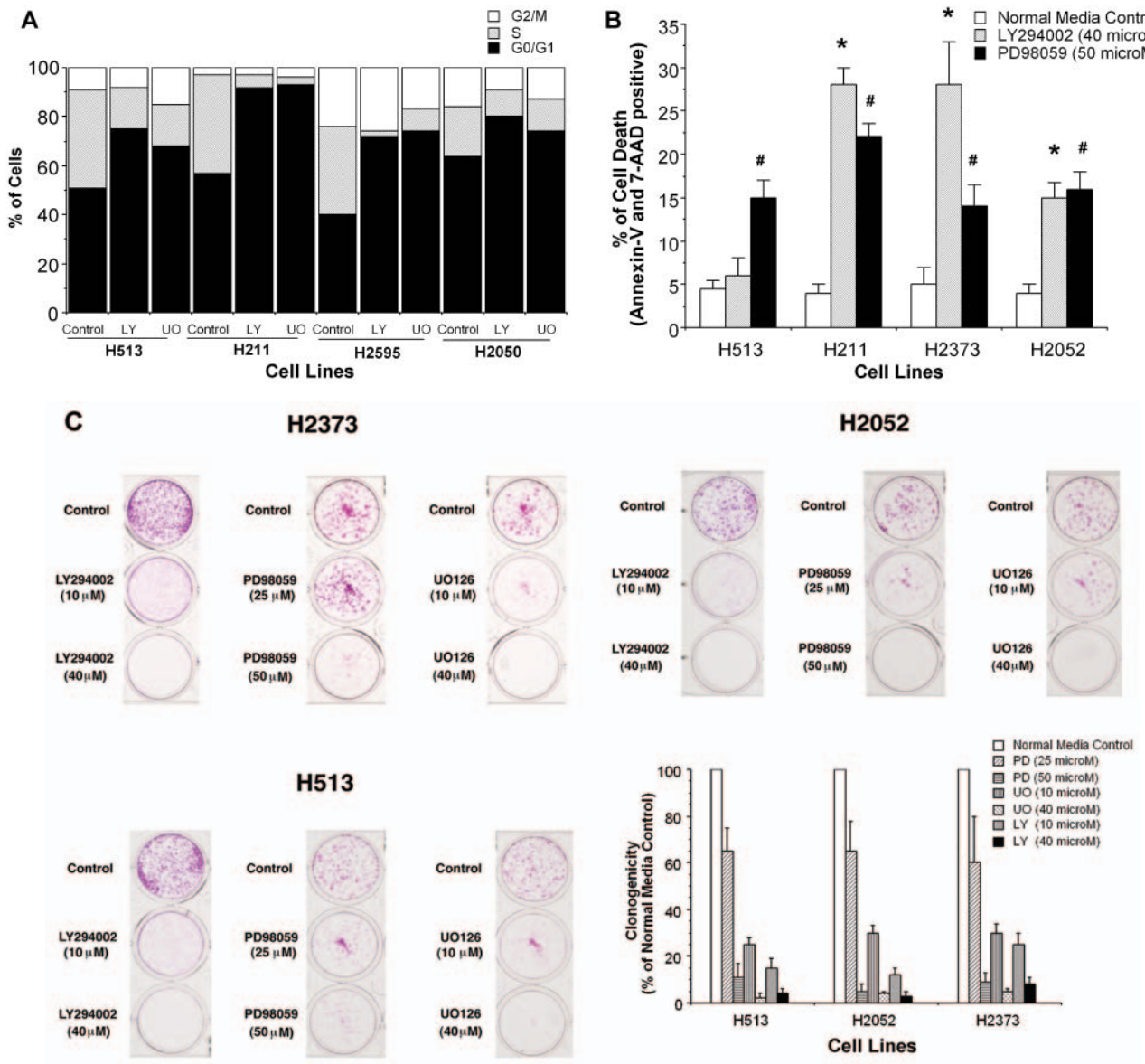

Figure 5. *A. Treating representative MPM cells H513, H211, H2595 and H2052 with either UO126 or LY294002 resulted in cell cycle arrest at the G1/S checkpoint with significant accumulation of cells in G1-phase and reduction of cells in S- and G2/M-phases. Representative data of 3 independent experiments that yielded similar results are shown here. B. Significant induction of apoptosis in MPM cells following exposure to PD98059 or LY294002. The cells were treated with either the MEK inhibitor (50 μM) or the PI3K inhibitor (40 μM) for 72 hours and apoptosis was quantified using Annexin V staining. Data are presented as means±SEM of 3 independent experiments, *,# $p<0.05$ versus normal media controls. C. Suppression of clonogenicity of MPM cells H2052, H513, H2595 and H2373 by UO126, PD98059 and LY294002. Digital photographs of representative experiments and quantitative analysis of inhibition of clonogenicity using MTT staining of viable clones are shown in the histogram (means±SEM of 3 independent experiments).*

LY294002- or UO126-mediated inhibition of cell motility and invasion through Matrigel membrane. The effect of inhibiting MEK or PI3K signaling on cell motility was assessed by the wound-healing assay. At the concentrations of drug that mediated >90% inhibition of AKT or ERK1/2 phosphorylation, there was significant retardation of cell movement from the wound edge into the denuded plastic surface created by "wounding" the cell monolayer when compared to the reference "fresh wound' and the control "healing wound" of cells incubated in drug-free 10% RPMI culture medium (Figure 6). Similarly, LY294002 or UO126 also strongly inhibited MPM cells to invade/migrate through the Matrigel extracellular matrix membrane (Figure 7) as determined

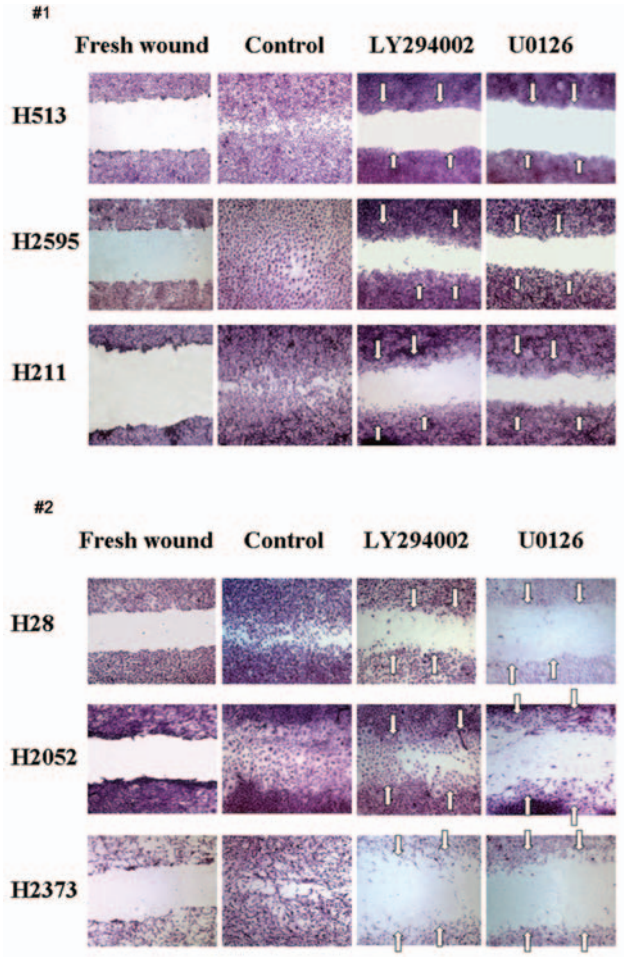

Figure 6. *UO126- and LY294002-mediated inhibition of motility of MPM cells. Little cell movement into the created wound was observed in cells treated with LY294002 or UO126 as compared to near complete filling of the wound by control cells grown in 10% RPMI culture medium. Fresh wounds (immediately fixed and stained after creation of wounds) are shown as baseline references. Representative pictures from 3 independent experiments with similar results are shown here.*

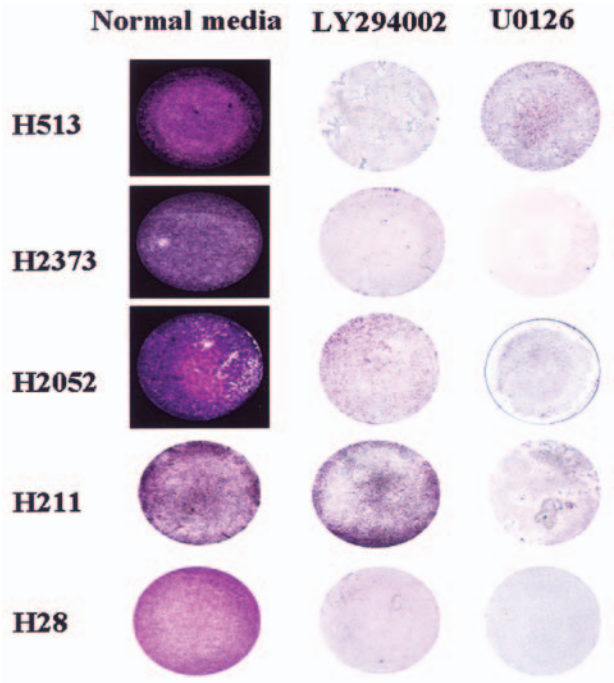

Figure 7. *Significant inhibition of cell movement through the extracellular matrix Matrigel by LY294002 or UO126 as evaluated by the cell invasion assay using the modified Boyden chamber technique. Representative pictures from 3 independent experiments with similar results are shown here.*

by the modified Boyden chamber method using 10% FCS as the chemo-attractant. Movement of cells through Matrigel requires a combination of cell motility and the production of matrix proteases to breakdown extracellular matrix ground substances. This *in vitro* assay has been shown to be predictive of the malignant behavior of cancer cells *in vivo* (32).

Inhibition of pro-angiogenic cytokine production by LY294002 or UO126. Tumor angiogenesis is a multifaceted process that involves a complex interaction of the cancer cells with the host stromal cells. Malignant cells initiate the angiogenesis process by secreting cytokines including VEGF and IL-8 to stimulate endothelial cell proliferation, vessel tube formation and in-growth of new blood vessels into the tumor masses to create tumor neovasculature (33). Either LY294002 (40 μM) or UO126 (20 μM) was equally effective in mediating a 30% to 55% ($p<0.05$ to $p<0.01$ *versus* untreated control) reduction of the levels of VEGF in the conditioned media of the 4 cultured MPM cells grown in 10% RPMI. To determine the relative role of either the PI3K- or MEK-mediated signaling cascades in mitogen-induced up-regulation of VEGF production, the cells were serum starved for 8 hours prior to EGF (20 ng/ml) stimulation, with or without pretreatment with LY294002 or UO126, respectively, and the culture supernatant collected 24 hours after EGF stimulation for VEGF measurement. There was a mild reduction of basal VEGF production in serum-free conditions (for instance, H211: 1,500±200 pg/mg cellular protein/24 h in 10% FCS RPMI *versus* 900±120 pg/mg cellular protein/24 h in serum-free media; H2595: 5,500±250 pg/mg cellular protein/24 h in 10% FCS RPMI *versus* 3,800±220 pg/mg cellular protein/24 h in serum-free media) (Figures 8A and B) and EGF mediated a 2- to 4-fold increase of VEGF concentrations in the conditioned media. EGF-mediated stimulation of VEGF production in H513 and H2595 cells was equally and significantly suppressed

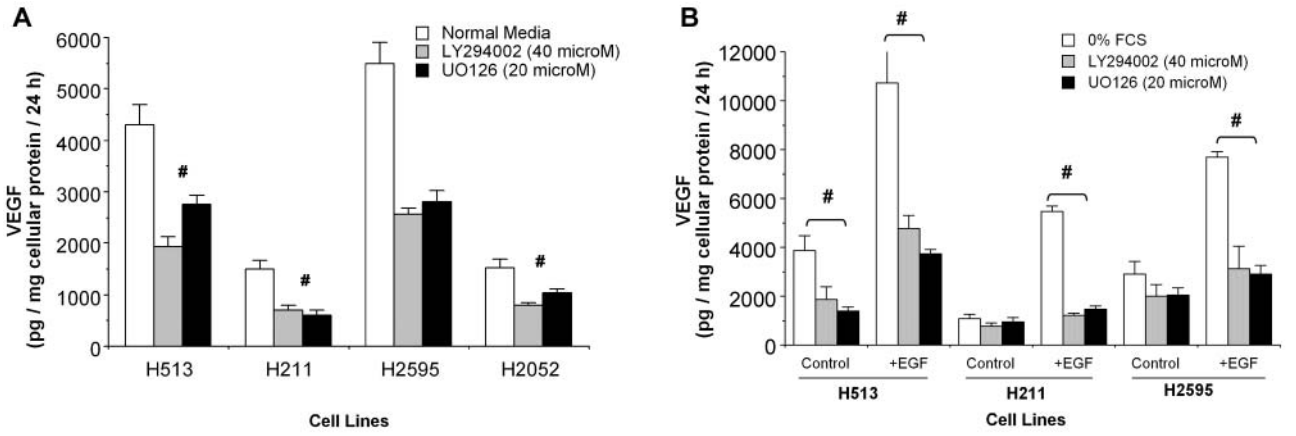

Figure 8. *Suppression of VEGF production and secretion into the conditioned media of MPM cells by LY294002 or by UO126, both at baseline condition (cell grown in 10% FCS RPMI culture media) (A) and following EGF stimulation (cells grown in serum-free media and stimulated with EGF at 20 ng/ml) (B). Data are presented as means±SEM of 4 independent experiments, # $p<0.01$ versus baseline control.*

either by the MEK or the PI3K inhibitor. Concurrent exposure of MPM cells H513 or H211 to LY294002 and UO126 caused further suppression of VEGF production in an additive manner, both under 10% RPMI and EGF-stimulated experimental conditions (data not shown), suggesting that these two pathways independently regulate the VEGF expression in cultured MPM cells.

Discussion

Constitutive activation of the Ras/Raf/MEK/ERK and the PI3K/AKT signal transduction cascades has been observed in many human cancers including those of the breast, colon, lung, pancreas as well as malignant melanoma, making them attractive targets for the development of novel molecular therapeutics (34). The purpose of our study was to functionally modulate intracellular molecular targets downstream from the membrane-bound receptor tyrosine kinases at the PI3K or the MEK levels to circumvent the apparent lack of antitumor efficacy of the anti-EGFR strategy in MPM. Little data exists regarding the baseline activity of these kinase pathways or the antitumor efficacy of pharmacological inhibitors of either PI3K or MEK in MPM cells. It was demonstrated in this study that either LY294002 or UO126 significantly down-regulated the expression of multiple malignant phenotypes in a panel of cultured MPM. It was interesting to note that LY294002, in addition to blocking PI3K activity, functionally inhibited the phosphorylative activation of the MEK substrate ERK1/2 in H513 cells. The PI3K inhibitor LY294002 has been shown to inhibit integrin-mediated Raf/MEK/ERK activation (35), Ras and ERK activation by lysophosphatidic acid in COS-7 cells (36), as well as basal MEK/ERK phosphorylation in leukemia cells (37). PI3K may directly influence MEK signaling *via* PKC or PDK1, both of which act as upstream regulators of MEK/ERK1/2 (38, 39). Moreover, while LY294002 selectively inhibited AKT phosphorylation in H211 cells, the MEK inhibitor MEK abrogated both phosphorylated ERK1/2 and AKT, in a similar way to previously reported studies (40, 41). These observations underscore the complexity of the "cross-talks" between intracellular signal transduction pathways and, thus, the unpredictable biological effects of "selective" kinase inhibitors *in vivo*. Inhibition of cell proliferation (cell cycle arrest at the G1/S checkpoint), cell motility, Matrigel invasion and VEGF production were observed following either LY294002 or UO126 treatments, in complete agreement with other studies (20-23, 42-52), implying that both PI3K- and the MEK-mediated signal transduction cascades regulate the expression of these cellular phenotypes. More interestingly, selective blocking of either the MEK or PI3K pathways in MPM cells resulted in a noticeable induction of apoptosis. This finding is in direct contrast to the previous study that reported the lack of MEK inhibitor-induced apoptosis in NSCLC (53), but in complete agreement with the study by Eisenmann and colleagues that described significant apoptosis of melanoma cells following 72-hour exposure to the MEK inhibitors PD184352 or PD98059 (54). PI3K-mediated signal transduction has been extensively characterized as anti-apoptotic, pro-survival pathways. PI3K transmits its anti-apoptosis signals *via* modulation of many downstream targets including activation of AKT, which in turn phosphorylates and thereby modulates the function of numerous target proteins, including IκK and the NF-κB p65/RelA subunit (to activate the NF-κB pathway), (55-59) caspase 9 and Bad (to inhibit

pro-apoptotic activity), (60, 61) and p21 or p27 (to suppress cell cycle regulation activity) (62, 63). The anti-apoptotic function of Ras/Raf/MEK/ERK1/2 has recently been shown to involve up-regulation of Bcl2, BclXL and MCL-1, phosphorylation of Bcl2 to prevent their degradation by proteosome (64 and refs therein), as well as phosphorylation of Bad (*via* a p90RSK-mediated mechanism) (54) or BIM (65) to inhibit their respective pro-apoptotic functions. The cumulative effect of cell cycle arrest and apoptosis, mediated by either MEK or PI3K inhibitors, was evident in the clonogeneic assay in which profound suppression of clonogenicity of the selected MPM cell lines was observed in LY294002-, UO126- or PD98059-treated cells (Figure 6A and B). The current focus of our laboratory effort is to further elucidate the molecular mechanism of MEK inhibitor-induced apoptosis in a larger panel of MPM cells and also in other cultured thoracic cancer cells in order to perform comparative analysis of the apoptosis-inducing effect of the MEK inhibitor in different kinds of thoracic malignancies.

In contrast to other tumor histology such as pancreatic, colon and lung cancers or malignant melanoma (66-68), mutations of K-ras or B-Raf were rare in MPM (69, 70). It is conceivable that, without upstream constitutively active K-ras that can activate other pathways in addition to MEK/ERK1/2, targeting MEK in this setting may be more efficacious in mediating meaningful cytotoxicity. Indeed, it has been shown by Wang and colleagues that K-ras mutation reduces the therapeutic efficacy of the MEK inhibitor CI-1040 in colon cancer cell lines *in vitro* (71). Growth factor-dependent signal transduction pathways employ extensive networks of intermediaries and posttranslational modifications of these proteins to transmit the signals to their intended targets. There is also ample redundancy of the receptor repertoire as well as the intracellular connections with positive and negative regulatory controls to ensure appropriate and diverse responses to vast arrays of environmental signals. This elaborate system of circuitry design of the signal transduction pathways poses opportunities as well as challenges for the development of targeted therapy. Opportunities are the multiple signaling proteins serving as potential targets. Targeting upstream proteins (for instance, EGFR or PDGFR) will provide a global alteration of downstream signaling, but the challenges arise since the multiplicity of upstream growth factor receptors may make selective targeting strategy less effective. Targeting overexpressed or overactive growth factor-mediated pathways, that are partially or totally responsible for the malignat behaviors of transformed cells, may circumvent the above-mentioned problem. One prime example is the clinical effectiveness of EGFR tyrosine kinase inhibitors such as Gefitinib or Erlotinib in mediating tumor regression of tumors harboring EGFR mutations, that make them particularly sensitive to this drug (72-74) or in patients whose tumors overexpress EGFR (75-77). Another is the efficacy of Trastuzumab (Herceptin®) on mediating the antitumor effect on breast cancers with erbB2 gene amplification and extreme overexpression of the erbB2 oncoprotein (78, 79). Similar to other carcinomas, the expressions of IGFR, PDGFR as well as members of the EGFR superfamily have been reported in cultured MPM cell lines (14, 15, 18, 80). In many instances, multiple receptor tyrosine kinases, some of which were overexpressed, were found on the same MPM cell lines (14, 18). Selective targeting of a single receptor tyrosine kinase may not be therapeutically effective, as we have observed when trying to modulate the expression of pro-metastasis phenotypes in MPM cells using the EGFR tyrosine kinase inhibitor PD153035 *in vitro* (18). Targeting intermediate intracellular signaling proteins such as PI3K, AKT, Ras or Raf offers the advantage of influencing upstream signals emanating from many distinct receptor/ligand interactions at their intracellular common convergent points which channel signals to distinct transduction cascades. We noticed that significant PD153035-mediated inhibition of cell growth or production of VEGF was only apparent in cell lines that express high levels of EGFR such as H513 or H2595, but not in other MPM cells such as H211, H2052, H2373 that have slightly elevated or normal levels of EGFR (18). The lack of biological effects of EGFR TKI PD153035 in MPM H211 cells was not due to its inability to suppress growth factor-mediated phosphorylation of EGFR, as indicated by the almost complete reduction of phosphorylated EGFR, but due to its inability to suppress the phosphorylative activation of AKT and ERK1/2 (Figure 2). On the other hand, PD153035 was very effective in down-regulating the pro-metastasis phenotype expression in H513 since it was able to inhibit the activation of EGFR, AKT and ERK1/2 (18).

Our study specifically focused on evaluating the effect of PI3K or MEK inhibitors on other cellular phenotypes besides cell proliferation as cellular processes, such as cell invasion, motility and the production of pro-angiogenesis cytokines which play crucial and equally important roles in the development of clinically relevant tumors from microscopic deposits. In this context, these kinase inhibitors may be ideal chemopreventive or even chemotherapeutic agents for appropriate tumors in the setting of minimal residual disease (post-resection adjuvant therapy). Even if these inhibitors mediate a cytostatic effect by arresting cancer cells from progressing through the cell cycle, or mild induction of apoptosis sufficient to counterbalance the fractions of cells undergoing division and contributing to the increase of biomasses, they, together with their ability to inhibit the production of VEGF to suppress tumor-derived

angiogenesis, may prevent established tumors from progressing further to become a health burden. In this aspect, these kinase inhibitors can not cause regression of tumor masses, but may keep them under control by maintenance therapy as in the cases of other tyrosine kinases like Gefitinib (81) or Gleevec (82) which can be safely administered over a long period of time. Of the two classes of kinase inhibitors, the MEK inhibitor (CI-1040) has entered phase I and II clinical trials as a single agent for solid tumors. CI-1040, however, was shown to be ineffective in patients with advanced non-small cell lung, breast, colon and pancreatic cancers (26). Even though continuous administration of CI-1040 (800 mg twice /day) resulted in plasma drug concentrations similar to those reported in the earlier phase I study, there were no complete or partial responses and stable disease was observed in only 12% of cases. Incomplete suppression of pERK1/2 expression, as determined by quantitative immuno-histochemical staining, was noted in the majority of cases with constitutive pERK1/2 expression at baseline, thus raising the possibility that the degree of MEK inhibition by CI-1040 may be insufficient to mediate a meaningful tumor response (26). There appeared to be a positive correlation between constitutive baseline pERK1/2 expression and stable disease with CI-1040 treatment. The clinical trial finding was not that surprising given the fact that Brognard and Dennis had demonstrated somewhat unpredictable responses of cultured non-small cell lung cancer cells lines, well characterized for their K-ras and p53 status, to MEK inhibition using either pharmacological inhibitors like UO126 or PD98059 or dominant negative MEK (53). Pharmacological inhibitors induced cell cycle arrest but no apoptosis and little sensitization of cancer cells to paclitaxel, in stark contrast to dominant negative MEK which efficiently induced apoptosis and chemosensitization of cultured lung cancer cells to cytotoxic chemotherapeutics. A more recent study, however, indicated that the clinically relevant MEK inhibitor CI-1040 synergistically interacted with paclitaxel to mediate potent antitumor effect on human heterotransplants in nude mice (83). Moreover, CI-1040, alone or in combination with paclitaxel, also significantly inhibited VEGF production and FGF-induced angiogenesis *in vivo* (83), compatible with our contention that the antitumor effect of the MEK inhibitor is more than just growth arrest or chemosensitization but also depends on its ability to modulate other cellular phenotypes such as the pro-angiogenic activities that are necessary for tumor development.

In summary, targeting either PI3K or MEK of cultured MPM cells by the pharmacological kinase inhibitors resulted in the deactivation of intended targets as well as modulation of other parallel pathways, which is most probable due to the "cross-talks" that exist between intracellular signaling cascades. In addition to mediating profound cytotoxicity *via* cell cycle arrest and induction of apoptosis, these kinase inhibitors also down-modulated the *in vitro* expression of multiple malignant phenotypes of transformed cells, further enhancing their anticancer properties that can only be evaluated in animal models or in human clinical trials. There has been a recent renewal of interest in targeting the Ras/Raf/MEK/ERK1/2 signal transduction pathway for the development of novel anticancer therapeutics based on exciting discoveries of the molecular mechanisms of pathway-mediated induction of apoptosis. This forms the impetus for our current work of evaluating MEK inhibitors as the molecularly-targeted drugs for MPM.

Acknowledgements

This research was supported by the Intramural Research Program of the National Cancer Institute, NIH, U.S.A.

References

1 Carbone M, Kratzke RA and Testa JR: The pathogenesis of mesothelioma. Semin Oncol *29*: 2-17, 2002.
2 Mossman BT and Churg A: Mechanisms in the pathogenesis of asbestosis and silicosis. Am J Respir Crit Care Med *157*: 1666-1680, 1998.
3 Schrump DS and Waheed I: Strategies to circumvent SV40 oncoprotein expression in malignant pleural mesotheliomas. Semin Cancer Biol *11*: 73-80, 2001.
4 Price B and Ware A: Mesothelioma trends in the United States: an update based on Surveillance, Epidemiology, and End Results Program data for 1973 through 2003. Am J Epidemiol *159*: 107-112, 2004.
5 Weill H, Hughes JM and Churg AM: Changing trends in US mesothelioma incidence. Occup Environ Med *61*: 438-441, 2004.
6 Hodgson JT, McElvenny DM, Darnton AJ, Price MJ and Peto J: The expected burden of mesothelioma mortality in Great Britain from 2002 to 2050. Br J Cancer *92*: 587-593, 2005.
7 Peto J, Decarli A, La Vecchia C, Levi F and Negri E: The European mesothelioma epidemic. Br J Cancer *79*: 666-672, 1999.
8 Zellos L and Sugarbaker DJ: Current surgical management of malignant pleural mesothelioma. Curr Oncol Rep *4*: 354-360, 2002.
9 Fidler IJ: The pathogenesis of cancer metastasis: the “seed and soil” hypothesis revisited. Nat Rev Cancer *3*: 453-458, 2003.
10 Kruger JS and Reddy KB: Distinct mechanisms mediate the initial and sustained phases of cell migration in epidermal growth factor receptor-overexpressing cells. Mol Cancer Res *1*: 801-809, 2003.
11 Aaronson SA: Growth factors and cancer. Science *254*: 1146-1153, 1991.
12 Kondapaka SB, Fridman R and Reddy KB: Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells. Int J Cancer *70*: 722-726, 1997.
13 Chakrabarty S, Rajagopal S and Huang S: Expression of antisense epidermal growth factor receptor RNA downmodulates the malignant behavior of human colon cancer cells. Clin Exp Metastasis *13*: 191-195, 1995.

14 Hoang CD, Zhang X, Scott PD, Guillaume TJ, Maddaus MA, Yee D and Kratzke RA: Selective activation of insulin receptor substrate-1 and -2 in pleural mesothelioma cells: association with distinct malignant phenotypes. Cancer Res *64*: 7479-7485, 2004.
15 Langerak AW, De Laat PA, Van Der Linden-Van Beurden CA, Delahaye M, Van Der Kwast TH, Hoogsteden HC, Benner R and Versnel MA: Expression of platelet-derived growth factor (PDGF) and PDGF receptors in human malignant mesothelioma *in vitro* and *in vivo*. J Pathol *178*: 151-160, 1996.
16 Huang SM, Li J and Harari PM: Molecular inhibition of angiogenesis and metastatic potential in human squamous cell carcinomas after epidermal growth factor receptor blockade. Mol Cancer Ther *1*: 507-514, 2002.
17 Nguyen DM and Schrump DS: Growth factor receptors as targets for lung cancer therapy. Semin Thorac Cardiovasc Surg 16: 3-12, 2004.
18 Cole GW, Alleva AM, Reddy RM, Maxhimer JB, Zuo J, Schrump DS and Nguyen DM: The selective EGFR tyrosine kinase inhibitor PD153035 suppresses expression of prometastasis phenotypes in malignant pleural mesothelioma cells *in vitro*. J Thorac Cardiovasc Surg. Ref Type: in press, 2005.
19 Takino T, Miyamori H, Watanabe Y, Yoshioka K, Seiki M and Sato H: Membrane type 1 matrix metalloproteinase regulates collagen-dependent mitogen-activated protein/extracellular signal-related kinase activation and cell migration. Cancer Res *64*: 1044-1049, 2004.
20 Okudela K, Hayashi H, Ito T, Yazawa T, Suzuki T, Nakane Y, Sato H, Ishi H, KeQin X, Masuda A, Takahashi T and Kitamura H: K-ras gene mutation enhances motility of immortalized airway cells and lung adenocarcinoma cells *via* Akt activation: possible contribution to non-invasive expansion of lung adenocarcinoma. Am J Pathol *164*: 91-100, 2004.
21 Nakamura T, Kanda S, Yamamoto K, Kohno T, Maeda K, Matsuyama T and Kanetake H: Increase in hepatocyte growth factor receptor tyrosine kinase activity in renal carcinoma cells is associated with increased motility partly through phosphoinositide 3-kinase activation. Oncogene *20*: 7610-7623, 2001.
22 Kermorgant S, Aparicio T, Dessirier V, Lewin MJ and Lehy T: Hepatocyte growth factor induces colonic cancer cell invasiveness *via* enhanced motility and protease overproduction. Evidence for PI3 kinase and PKC involvement. Carcinogenesis *22*: 1035-1042, 2001.
23 Menu E, Kooijman R, Van Valckenborgh E, Asosingh K, Bakkus M, Van Camp B and Vanderkerken K: Specific roles for the PI3K and the MEK-ERK pathway in IGF-1-stimulated chemotaxis, VEGF secretion and proliferation of multiple myeloma cells: study in the 5T33MM model. Br J Cancer *90*: 1076-1083, 2004.
24 Veit C, Genze F, Menke A, Hoeffert S, Gress TM, Gierschik P and Giehl K: Activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase is required for glial cell line-derived neurotrophic factor-induced migration and invasion of pancreatic carcinoma cells. Cancer Res *64*: 5291-5300, 2004.
25 Ge X, Fu YM and Meadows GG: U0126, a mitogen-activated protein kinase kinase inhibitor, inhibits the invasion of human A375 melanoma cells. Cancer Lett *179*: 133-140, 2002.
26 Rinehart J, Adjei AA, LoRusso PM, Waterhouse D, Hecht JR, Natale RB, Hamid O, Varterasian M, Asbury P, Kaldjian EP, Gulyas S, Mitchell DY, Herrera R, Sebolt-Leopold JS and Meyer MB: Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. J Clin Oncol *22*: 4456-4462, 2004.
27 Blank N, Burger R, Duerr B, Bakker F, Wohlfarth A, Dumitriu I, Kalden JR and Herrmann M: MEK inhibitor U0126 interferes with immunofluorescence analysis of apoptotic cell death. Cytometry *48*: 179-184, 2002.
28 Dudley DT, Pang L, Decker SJ, Bridges AJ and Saltiel AR: A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA *92*: 7686-7689, 1995.
29 Cohen P: The development and therapeutic potential of protein kinase inhibitors. Curr Opin Chem Biol *3*: 459-465, 1999.
30 Alper O, Bergmann-Leitner ES, Bennett TA, Hacker NF, Stromberg K and Stetler-Stevenson WG: Epidermal growth factor receptor signaling and the invasive phenotype of ovarian carcinoma cells. J Natl Cancer Inst *93*: 1375-1384, 2001.
31 Sato S, Fujita N and Tsuruo T: Involvement of 3-phosphoinositide-dependent protein kinase-1 in the MEK/MAPK signal transduction pathway. J Biol Chem *279*: 33759-33767, 2004.
32 Luo J, Lubaroff DM and Hendrix MJ: Suppression of prostate cancer invasive potential and matrix metalloproteinase activity by E-cadherin transfection. Cancer Res *59*: 3552-3556, 1999.
33 Hicklin DJ and Ellis LM: Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol *23*: 1011-1027, 2005.
34 Sliva D: Signaling pathways responsible for cancer cell invasion as targets for cancer therapy. Curr Cancer Drug Targets *4*: 327-336, 2004.
35 Hawes BE, Luttrell LM, van Biesen T and Lefkowitz RJ: Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. J Biol Chem *271*: 12133-12136, 1996.
36 King WG, Mattaliano MD, Chan TO, Tsichlis PN and Brugge JS: Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol Cell Biol *17*: 4406-4418, 1997.
37 Rahmani M, Yu C, Reese E, Ahmed W, Hirsch K, Dent P and Grant S: Inhibition of PI-3 kinase sensitizes human leukemic cells to histone deacetylase inhibitor-mediated apoptosis through p44/42 MAP kinase inactivation and abrogation of p21(CIP1/WAF1) induction rather than AKT inhibition. Oncogene *22*: 6231-6242, 2003.
38 Sato S, Fujita N and Tsuruo T: Interference with PDK1-Akt survival signaling pathway by UCN-01 (7-hydroxystaurosporine). Oncogene *21*: 1727-1738, 2002.
39 Goekjian PG and Jirousek MR: Protein kinase C inhibitors as novel anticancer drugs. Expert Opin Investig Drugs *10*: 2117-2140, 2001.
40 Yu CF, Roshan B, Liu ZX and Cantley LG: ERK regulates the hepatocyte growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. J Biol Chem *276*: 32552-32558, 2001.
41 Yu CF, Liu ZX and Cantley LG: ERK negatively regulates the epidermal growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. J Biol Chem *277*: 19382-19388, 2002.
42 Kawada M, Yamagoe S, Murakami Y, Suzuki K, Mizuno S and Uehara Y: Induction of $p27^{Kip1}$ degradation and anchorage independence by Ras through the MAP kinase signaling pathway. Oncogene *15*: 629-637, 1997.

43 Hoshino R, Tanimura S, Watanabe K, Kataoka T and Kohno M: Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of p27(Kip1). J Biol Chem *276*: 2686-2692, 2001.

44 Qi HL, Zhang Y, Ma J, Guo P, Zhang XY and Chen HL: Insulin/protein kinase B signalling pathway upregulates metastasis-related phenotypes and molecules in H7721 human hepatocarcinoma cell line. Eur J Biochem *270*: 3795-3805, 2003.

45 Caron RW, Yacoub A, Li M, Zhu X, Mitchell C, Hong Y, Hawkins W, Sasazuki T, Shirasawa S, Kozikowski AP, Dennis PA, Hagan MP, Grant S and Dent P: Activated forms of H-RAS and K-RAS differentially regulate membrane association of PI3K, PDK-1, and AKT and the effect of therapeutic kinase inhibitors on cell survival. Mol Cancer Ther *4*: 257-270, 2005.

46 Kobayashi H, Suzuki M, Kanayama N and Terao T: Genetic down-regulation of phosphoinositide 3-kinase by bikunin correlates with suppression of invasion and metastasis in human ovarian cancer HRA cells. J Biol Chem *279*: 6371-6379, 2004.

47 Brahmbhatt AA and Klemke RL: ERK and RhoA differentially regulate pseudopodia growth and retraction during chemotaxis. J Biol Chem *278*: 13016-13025, 2003.

48 Sliva D, Rizzo MT and English D: Phosphatidylinositol 3-kinase and NF-kappaB regulate motility of invasive MDA-MB-231 human breast cancer cells by the secretion of urokinase-type plasminogen activator. J Biol Chem *277*: 3150-3157, 2002.

49 Krystal GW, Sulanke G and Litz J: Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. Mol Cancer Ther *1*: 913-922, 2002.

50 Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ and Thun MJ: Cancer statistics, 2005. CA Cancer J Clin *55*: 10-30, 2005.

51 Skinner HD, Zheng JZ, Fang J, Agani F and Jiang BH: Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1alpha, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. J Biol Chem *279*: 45643-45651, 2004.

52 Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P and Cheresh DA: Regulation of cell motility by mitogen-activated protein kinase. J Cell Biol *137*: 481-492, 1997.

53 Brognard J and Dennis PA: Variable apoptotic response of NSCLC cells to inhibition of the MEK/ERK pathway by small molecules or dominant negative mutants. Cell Death Differ *9*: 893-904, 2002.

54 Eisenmann KM, VanBrocklin MW, Staffend NA, Kitchen SM and Koo HM: Mitogen-activated protein kinase pathway-dependent tumor-specific survival signaling in melanoma cells through inactivation of the proapoptotic protein bad. Cancer Res *63*: 8330-8337, 2003.

55 Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr and Mayo MW: Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol Cell Biol *20*: 1626-1638, 2000.

56 Sizemore N, Leung S and Stark GR: Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol *19*: 4798-4805, 1999.

57 Sizemore N, Lerner N, Dombrowski N, Sakurai H and Stark GR: Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. J Biol Chem *277*: 3863-3869, 2002.

58 Romashkova JA and Makarov SS: NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature *401*: 86-90, 1999.

59 Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM and Donner DB: NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature *401*: 82-85, 1999.

60 Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*: 231-241, 1997.

61 Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S and Reed JC: Regulation of cell death protease caspase-9 by phosphorylation. Science *282*: 1318-1321, 1998.

62 Li Y, Dowbenko D and Lasky LA: AKT/PKB phosphorylation of $p21^{Cip/WAF1}$ enhances protein stability of $p21^{Cip/WAF1}$ and promotes cell survival. J Biol Chem *277*: 11352-11361, 2002.

63 Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J and Arteaga CL: PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med *8*: 1145-1152, 2002.

64 Tan TT, Degenhardt K, Nelson DA, Beaudoin B, Nieves-Neira W, Bouillet P, Villunger A, Adams JM and White E: Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. Cancer Cell *7*: 227-238, 2005.

65 Harada H, Quearry B, Ruiz-Vela A and Korsmeyer SJ: Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. Proc Natl Acad Sci USA *101*: 15313-15317, 2004.

66 Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, Einhorn E, Herlyn M, Minna J, Nicholson A, Roth JA, Albelda SM, Davies H, Cox C, Brignell G, Stephens P, Futreal PA, Wooster R, Stratton MR and Weber BL: BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res *62*: 6997-7000, 2002.

67 Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR and Futreal PA: Mutations of the BRAF gene in human cancer. Nature *417*: 949-954, 2002.

68 Barbacid M: Ras oncogenes: their role in neoplasia. Eur J Clin Invest *20*: 225-235, 1990.

69 Metcalf RA, Welsh JA, Bennett WP, Seddon MB, Lehman TA, Pelin K, Linnainmaa K, Tammilehto L, Mattson K and Gerwin BI: p53 and Kirsten-ras mutations in human mesothelioma cell lines. Cancer Res *52*: 2610-2615, 1992.

70 Dote H, Tsukuda K, Toyooka S, Yano M, Pass HI and Shimizu N: Mutation analysis of the BRAF codon 599 in malignant pleural mesothelioma by enriched PCR-RFLP. Oncol Rep *11*: 361-363, 2004.

71 Wang Y, Van Becelaere K, Jiang P, Przybranowski S, Omer C and Sebolt-Leopold J: A role for K-ras in conferring resistance to the MEK inhibitor, CI-1040. Neoplasia *7*: 336-347, 2005.
72 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med *350*: 2129-2139, 2004.
73 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304*: 1497-1500, 2004.
74 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M and Varmus H: EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA *101*: 13306-13311, 2004.
75 Tsao MS, Sakurada A, Cutz JC, Zhu CQ, Kamel-Reid S, Squire J, Lorimer I, Zhang T, Liu N, Daneshmand M, Marrano P, da Cunha SG, Lagarde A, Richardson F, Seymour L, Whitehead M, Ding K, Pater J and Shepherd FA: Erlotinib in lung cancer – molecular and clinical predictors of outcome. N Engl J Med *353*: 133-144, 2005.
76 Taron M, Ichinose Y, Rosell R, Mok T, Massuti B, Zamora L, Mate JL, Manegold C, Ono M, Queralt C, Jahan T, Sanchez JJ, Sanchez-Ronco M, Hsue V, Jablons D, Sanchez JM and Moran T: Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. Clin Cancer Res *11*: 5878-5885, 2005.
77 Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn PA Jr and Varella-Garcia M: Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst *97*: 643-655, 2005.
78 Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ and Press M: Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol *20*: 719-726, 2002.
79 Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J and Norton L: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med *344*: 783-792, 2001.
80 Janne PA, Taffaro ML, Salgia R and Johnson BE: Inhibition of epidermal growth factor receptor signaling in malignant pleural mesothelioma. Cancer Res *62*: 5242-5247, 2002.
81 Birnbaum A and Ready N: Gefitinib therapy for non-small cell lung cancer. Curr Treat Options Oncol *6*: 75-81, 2005.
82 Cohen MH, Johnson JR and Pazdur R: U.S. Food and Drug Administration Drug Approval Summary: conversion of imatinib mesylate (STI571; Gleevec) tablets from accelerated approval to full approval. Clin Cancer Res *11*: 12-19, 2005.
83 McDaid HM, Lopez-Barcons L, Grossman A, Lia M, Keller S, Perez-Soler R and Band HS: Enhancement of the therapeutic efficacy of Taxol by the mitogen-activated protein kinase inhibitor CI-1040 in nude mice bearing human heterotransplants. Cancer Res *65*: 2854-2860, 2005

Received January 12, 2006
Accepted January 24, 2006