

# Inhibition of Epidermal Growth Factor Receptor Signaling in Malignant Pleural Mesothelioma

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## ABSTRACT

Malignant pleural mesothelioma (MPM) is a rare malignancy with no known curative modality. Approximately 70% of MPMs have high levels of expression of the epidermal growth factor receptor (EGFR), and a subset of cell lines derived from MPM patients express both EGFR and transforming growth factor  $\alpha$ , suggesting an autocrine role for EGFR in MPM. We have determined the effects of EGFR inhibition in MPM cell lines *in vitro*, using four MPM cell lines derived from previously untreated patients with epithelial (H2461 and H2591), sarcomatoid (H2373), and biphasic (MSTO-211H) MPM. All four cell lines expressed EGFR at levels comparable with the non-small cell lung carcinoma (NSCLC) cell line A549, as shown by Western blot analysis. ZD1839 significantly inhibited epidermal growth factor-dependent cell signaling including phosphorylation of AKT and extracellular signal-regulated kinases 1 and 2 in all MPM cell lines. Furthermore, treatment with ZD1839 led to a significant dose-dependent reduction of colony formation (41–89% at 10  $\mu$ M) when MPM cells were grown in soft agarose. MSTO-211H, H2461, and H2373 were more sensitive to the growth-inhibitory effects of ZD1839 than was the NSCLC cell line A549, whereas H2591 had similar sensitivity to A549. This variability in growth-inhibitory effects is not related to the amount of EGFR present on MPM cells or to the degree of inhibition of EGFR phosphorylation by ZD1839. We show that H2373 MPM cells, which show 89% growth inhibition at 10  $\mu$ M ZD1839, undergo a dose-dependent arrest at the G<sub>1</sub>-S phase of the cell cycle and a corresponding increase in p27 levels. However, H2591 cell lines, which show 41% growth inhibition at 10  $\mu$ M ZD1839, undergo no significant cell cycle changes or changes in p27 levels. Our findings demonstrate that *in vitro*, ZD1839 is as effective or more effective against MPM cell lines as it is against the NSCLC cell line A549 and suggest that ZD1839 may be an effective therapeutic option for patients with MPM.

## INTRODUCTION

MPM<sup>2</sup> is a rare malignancy with an estimated annual incidence of 3000 cases in the United States. MPM is comprised of three major histological subtypes: epithelial, sarcomatoid, and biphasic or mixed (containing both epithelial and sarcomatoid elements) types. There is no known curative modality for MPM, and the median survival ranges from 6–18 months despite treatment (1, 2). There is no standard of care for MPM, and current treatment approaches range from aggressive surgical treatments to chemotherapy to only palliative care (3–5). Despite surgery, MPM will recur in the majority of patients. MPM is primarily a regionally recurrent disease, and with improvements in regional therapies such as surgery, systemic metastases are likely to become more evident. These patients are candidates for systemic therapies, but most chemotherapy agents are not very effective against MPM, with typical single-agent response rates of <25% (4, 6–9). Thus, more effective systemic treatments are needed.

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<sup>2</sup> The abbreviations used are: MPM, malignant pleural mesothelioma; EGFR, epidermal growth factor receptor; TGF- $\alpha$ , transforming growth factor  $\alpha$ ; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; NSCLC, non-small cell lung carcinoma; FBS, fetal bovine serum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; FACS, fluorescence-activated cell-sorting.

The EGFR, a receptor tyrosine kinase, is overexpressed in a wide variety of epithelial malignancies including NSCLCs, head and neck cancers, colon cancers, and breast cancers (10–13). EGFR signaling leads to increases in cellular proliferation, increases in cell motility, angiogenesis, inhibition of apoptosis, and expression of extracellular matrix proteins (14–17). High levels of EGFR expression are associated with a poor prognosis in some but not all studies of NSCLC and breast cancer (10, 13, 18, 19). Several inhibitors of EGFR have been developed including monoclonal antibodies (C225; Imclone) and small molecule inhibitors [such as ZD1839 (Iressa) and OSI-774 (Tarceva)], which have been shown to be effective in preclinical studies and animal models and are in late stages of clinical trials (20–24). EGFR inhibition, either by tyrosinase inhibitor AG 1478, a small molecule inhibitor, or by C225, leads to inhibition of mitogen-activated protein kinase and phosphatidylinositol 3'-kinase signaling pathways and induction of cell cycle arrest at the G<sub>1</sub>-S boundary mediated by an increase in p27 levels (25–27). EGFR is also expressed by immunohistochemistry in 68% of paraffin-embedded MPM specimens (28). Upon immunohistochemical examination, EGFR expression was observed in  $\geq$ 20% of cells in 11 of 16 (69%) epithelial MPM specimens, but in only 4 of 9 (44%) sarcomatoid specimens and 2 of 9 (22%) mixed MPM specimens, respectively. However, EGFR overexpression does not appear to be an independent prognostic factor for survival in MPM (28). *In vitro* studies suggest that EGFR may function in an autocrine fashion in MPM because coexpression of EGFR and one of its ligands, TGF- $\alpha$ , has been detected in MPM cells, and TGF- $\alpha$  has been detected in the supernatant of both epithelial and sarcomatoid MPM cell lines (29, 30). Asbestos (a known carcinogen for MPM; Ref 31)-transformed rat mesothelial cells also express EGFR and TGF- $\alpha$ , and the growth of these cells can be inhibited by neutralizing antibodies to TGF- $\alpha$  (32). Asbestos fibers also induce the phosphorylation of EGFR and downstream signals including mitogen-activated protein kinases and ERK1/2 in rat pleural mesothelial cells (33). In rat pleural mesothelial cells, the phosphorylation of EGFR appears to correlate with the carcinogenicity of the asbestos fibers, with a greater degree of phosphorylation observed after treatment with fibrous preparations (34). Exposure of pleural mesothelial cells to crocidolite, a fibrous asbestos preparation, also increases activator protein-1 DNA binding activity approximately 2-fold, which, along with EGFR phosphorylation, can be significantly reduced in the presence of tyrosinase inhibitor AG 1478, a specific inhibitor of EGFR (33, 34). These studies suggest that EGFR may play an important role in the pathogenesis of MPM and that its inhibition provides a rational therapeutic target.

In the present study we examine the role of inhibition of EGFR signaling in cell lines derived from patients with previously untreated epithelial, sarcomatoid, and mixed histology MPM. We demonstrate that inhibition of EGFR signaling by ZD1839 leads to marked antiproliferative effects of all MPM cell types examined *in vitro*. We also characterize the biochemical and cell cycle effects of EGFR inhibition in MPM cell lines.

## MATERIALS AND METHODS

**Cell Culture.** A549 NSCLC cells and MSTO-211H biphasic MPM cells (35) were purchased from American Type Culture Collection and maintained in RPMI 1640 (Cellgro; Mediatech, Inc., Herndon, CA) supplemented with

10% FBS (Gemini-Bio-Products, Inc., Woodland, CA), 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine. H2461 (epithelial), H2591 (epithelial) and H2373 (sarcomatoid) MPM cell lines were kindly provided by the National Cancer Institute and have been characterized previously (30). All mesothelioma cell lines were derived from previously untreated patients. MSTO-211H, H2461, and H2591 cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine, whereas H2373 cells were maintained in ACL-4 media (Life Technologies, Inc., Rockville, MD) supplemented with 5% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine.

**Drugs.** Tyrphostin AG 1478 was purchased from Sigma (St. Louis, MO). ZD1839 (Iressa) was a kind gift of AstraZeneca. Stock solutions were prepared in DMSO and stored at  $-20^{\circ}\text{C}$ . The drugs were diluted in fresh media before each experiment. In all experiments, the final DMSO concentration was  $<0.1\%$ . EGF was purchased from Biosource International Inc. (Camarillo, CA), dissolved in distilled  $\text{H}_2\text{O}$ , and stored at  $-70^{\circ}\text{C}$  before use.

**In Vitro Cytotoxicity Assays.** The *in vitro* drug sensitivity was assessed by MTS assay using the manufacturer's instructions (Promega, Madison, WI). The MTS assay is a colorimetric method in which the intensity of the dye is proportional to the number of the cells. It is used to determine the number of viable cells in chemosensitivity assays. Cells were seeded at 2,500–20,000 cells/well in 96-well flat-bottomed plate (Corning Inc., Corning, NY) to allow exponential growth for the 4 days of the assay to give an absorbance of 1.0–2.2. The optimum number of cells required to reach an absorbance between 1.0 and 2.2 was determined for each cell line (data not shown). In a typical experiment, cells were trypsinized, seeded in 96-well plates, and allowed to recover for 24 h before the addition of ZD1839 or tyrphostin AG 1478. Drug concentrations ranged from 33 nM to 10  $\mu\text{M}$ . All experimental points were set up in 8 replicate wells, performed in duplicate, and all experiments were repeated at least three times. The MTS assay was developed after a 72-h incubation. One hundred  $\mu\text{l}$  of media [20  $\mu\text{l}$  of MTS and phenazine methosulfate (Sigma) solution at a (v/v) ratio of 19:1] were added to each well 3 h before termination of incubation. The absorbance was then measured by a spectrophotometer at 490 nm. The *in vitro* cytotoxicity assays were repeated in media containing full (10% or 5%) or reduced FBS (0.1%). EGF-dependent growth was assayed in a similar manner. Exponentially growing cells were trypsinized, washed twice, resuspended in RPMI 1640 containing 0.1% BSA (Sigma), and then seeded into 96-well plates. Twenty-four h later, ZD1839 was added as described above, and after 1 h of incubation, EGF (final concentration, 10 ng/ml) was added to all wells. Cell viability was assayed 72 h later using the MTS assay.

**Antibodies and Western Blotting.** Cells were lysed in lysis buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP40, and 0.42% NaF] containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM  $\text{Na}_3\text{VO}_4$ , 2  $\mu\text{g}/\text{ml}$  aprotinin, and 5  $\mu\text{g}/\text{ml}$  leupeptin). Proteins were separated by gel electrophoresis on 8% polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), and detected by immunoblotting using an enhanced chemiluminescence system (New England Nuclear Life Science Products Inc., Boston, MA). The EGFR (SC-03) and AKT antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific EGFR (pY1068) and ERK antibodies (pT185/pY187) were purchased from Biosource International Inc. Phospho-AKT (pS473) was purchased from Cell Signaling Technology (Beverly, MA). The p27 antibody (K25020) was purchased from Transduction Laboratories (Lexington, KY). The  $\beta$ -actin antibody (AC-15) was purchased from Sigma. All antibodies were used according to the manufacturer's instructions.

**Soft Agarose Assays.** Cells (between  $4 \times 10^3$  and  $1 \times 10^4$ , depending on the cell line) were resuspended in 1.5 ml of 0.3% ultrapure agarose (Life Technologies, Inc.) in complete culture media containing different concentrations of ZD1839. This suspension was layered over 3 ml of 0.6% agarose in complete culture media in 6-well plates (Corning Inc.). Every 4–7 days, 250  $\mu\text{l}$  of fresh complete culture media, alone or containing different concentrations of ZD1839, were added to each of the wells. The cells were incubated for 3 weeks (except for MSTO-211H cells, which were incubated for 2 weeks), and all visible colonies were counted manually with the aid of the ChemiImager 4400 image system (Alpha Innotech Corp., San Leandro, CA). Images obtained using the ChemiImager 4400 image system were verified to correspond to colonies on the plates. All experiments were performed in duplicate and repeated three times. Colony numbers are represented as a percentage of control colonies. Colonies were photographed using an Olympus IX70 microscope (Melville, NY) with a

DVC1310 digital video camera and a QED camera with Standalone 145 software (QED Imaging Inc., Pittsburgh, PA).

**In Vitro Wound Healing Assays.** Cell wounding studies were performed using methods similar to previously published methods (36). Briefly, cells were seeded onto 6-cm plates in full culture media for 24 h, and then 1  $\mu\text{M}$  ZD1839 was added for the subsequent 24 h. Using the tip of a p-20 pipette-man, a line was drawn through the middle of the plates. The plates were photographed at 0, 24, and 48 h using an Olympus IX70 microscope with a DVC1310 digital video camera and a QED camera with Standalone 145 software (QED Imaging Inc.). The experiments were repeated three times.

**FACS Analysis.** The FACS analysis was performed using methods similar to those described previously (37). Briefly,  $1\text{--}1.5 \times 10^6$  cells were seeded into 10-cm<sup>2</sup> plates, and increasing concentrations of ZD1839 were added 24 h later. The cells were then trypsinized and fixed overnight in ethanol at  $4^{\circ}\text{C}$ . Fixed cells were then resuspended in 500  $\mu\text{g}/\text{ml}$  RNase A (Sigma), centrifuged, resuspended in 69  $\mu\text{M}$  propidium iodide (Sigma) in 38 mM sodium citrate, incubated at room temperature for 30 min, and analyzed by FACScan using Cell Quest software (Becton Dickinson Labware, Franklin Lakes, NJ). All experiments were repeated three times. The absolute change in the percentage of  $\text{G}_0\text{--}\text{G}_1$ ,  $\text{G}_2\text{--}\text{M}$ , and S fractions is shown with respect to ZD1839 concentration in Fig. 6. Each data point represents the mean and SD (error bars) of three experiments.

## RESULTS

**EGFR Is Expressed in Mesothelioma Cell Lines.** The relative expression of EGFR in MPM cell lines was determined using Western blotting. To control for protein loading, the blot was stripped and rehybridized with the  $\beta$ -actin antibody. Fig. 1 demonstrates that all four of the MPM cell lines examined [two epithelial MPM cell lines (H2591 and H2461), one sarcomatoid MPM cell line (H2373), and one biphasic MPM cell line (MSTO-211H)] express EGFR at a level comparable with that of the NSCLC cell line A549 (positive control). Lane 2 (H2461) contains significantly more protein than the other lanes, but the relative expression of EGFR, in the context of  $\beta$ -actin expression, is similar to that of the other cell lines.

**ZD1839 Blocks Basal and EGF-induced Phosphorylation of EGFR in Mesothelioma Cell Lines.** We sought to examine the effects of ZD1839 on EGF-induced and basal phosphorylation of EGFR. Because all of the MPM cell lines expressed EGFR, we focused our studies on an epithelial cell line (H2461 or H2591) and a sarcomatoid cell line (H2373). H2461 and H2373 cells were grown in RPMI 1640 or ACL-4 media containing 0.1% serum overnight and then subsequently treated with 1  $\mu\text{M}$  ZD1839 for 30 min. The concentration of ZD1839 was based on previously published reports (38). EGF (final concentration, 10 ng/ml) was then added for various time points, cell lysates were prepared, and the proteins were separated by PAGE and analyzed by Western blotting. Fig. 2 demonstrates the time-dependent EGF phosphorylation of EGFR in H2461 cells. As can be seen in Fig. 2, the degree of phosphorylation reaches a maximum after 5 min of treatment. This is significantly inhibited by pretreatment with ZD1839 without changing the relative amount of EGFR. We also examined the effects of ZD1839 treatment on the

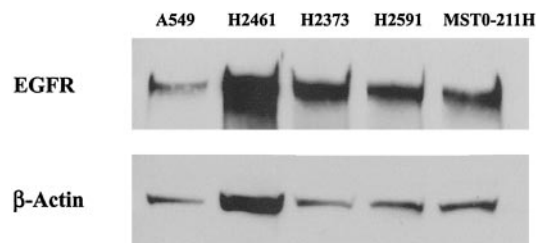


Fig. 1. Western analysis of EGFR expression (top panels) in A549 NSCLC cells (positive control) and epithelial (H2461 and H2591), mixed histology (MSTO-211H), and sarcomatoid (H2373) MPM cell lines. The blot was stripped and rehybridized with a  $\beta$ -actin antibody (bottom panels).



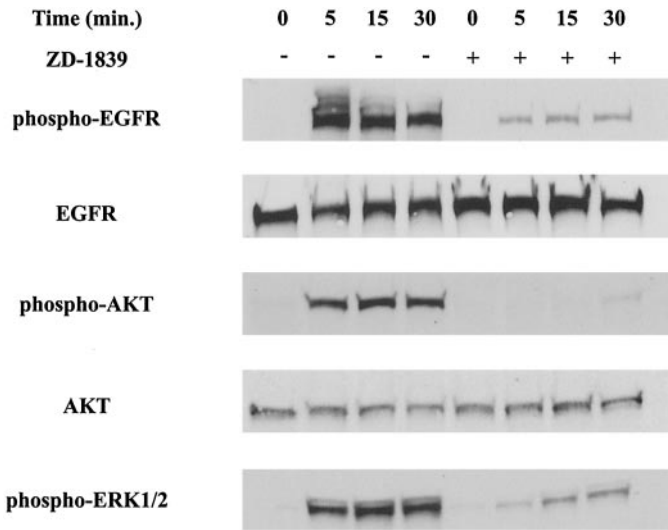


Fig. 2. ZD1839 inhibits EGF-induced phosphorylation of EGFR and downstream intermediates in H2461 MPM cells. EGFR becomes phosphorylated within 5 min after treatment with EGF. AKT and ERK1/2 also become phosphorylated with similar kinetics. The phosphorylation of EGFR, AKT, and ERK1/2 is significantly reduced by pretreatment with 1  $\mu\text{M}$  ZD1839 for 30 min. The levels of EGFR and AKT do not change significantly with treatment. -, no pretreatment with ZD1839; +, pretreatment with ZD1839.

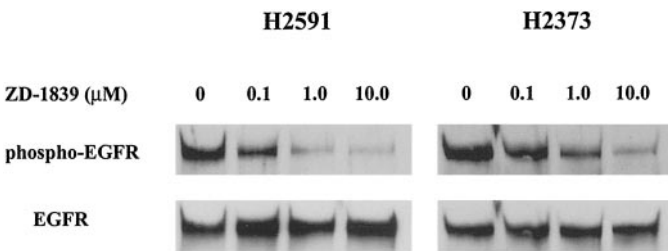


Fig. 3. ZD1839 inhibits constitutive EGFR phosphorylation in a dose-dependent manner in H2591 and H2373 cells. H2591 and H2373 cells grown in full media were treated with increasing concentrations of ZD1839 for 24 h. The phosphorylation of EGFR decreased in a dose-dependent manner without a change in the amount of EGFR (bottom panels).

phosphorylation of known proteins downstream of EGFR. Fig. 2 demonstrates that the phosphorylation of AKT was also significantly inhibited by pretreatment with ZD1839, without affecting the amount of AKT. Similarly, the phosphorylation of ERK1/2 was significantly reduced by pretreatment with ZD1839. Similar findings were obtained with a 30-min pretreatment with 250 nM tyrphostin AG 1478 and using H2373 cells (data not shown). We also examined the effects of EGFR phosphorylation in H2373 and H2591 cells grown in complete A4 or RPMI 1640 media supplemented with 5% or 10% FBS. We compared these cell lines to elucidate their significant growth differences in soft agarose (Fig. 4A). Subconfluent cells were treated for 24 h with 0–10  $\mu\text{M}$  ZD1839 and analyzed as described above. Fig. 3 shows that the phosphorylation of EGFR is decreased in a dose-dependent manner in both cell lines without changing the amount of the EGFR itself.

**In Vitro Cytotoxicity Assays.** We examined the effects of ZD1839 treatment on MPM cell lines grown *in vitro* using a MTS assay. MPM cells were treated with increasing concentrations (33 nM to 10  $\mu\text{M}$ ) of ZD1839 for a duration of 72 h. As measured by MTS, no changes in absorbance were observed between untreated and treated cells (data not shown). The experiments were repeated using cells grown in 0.1% serum with identical findings (data not shown). We subsequently examined the effects of ZD1839 inhibition on EGF-dependent growth. Serum-starved cells grown in 0.1% BSA were treated with increasing concentrations of ZD1839 (33 nM to 10  $\mu\text{M}$ ) and subsequently stimulated with EGF (10

ng/ml). The mean  $\text{IC}_{50}$  for growth inhibition was 6.6  $\mu\text{M}$  for A549 NSCLC cells and 6.2  $\mu\text{M}$  for H2461 MPM cells. These studies could not be repeated for H2591, H2373, and MSTO-211H cells because they could not be maintained under serum-starved conditions.

**ZD1839 Inhibits Growth of MPM Cells in Soft Agarose.** To further examine the growth-inhibitory effects of ZD1839, we examined cell growth in soft agarose. Cells were grown in the presence of increasing concentrations of ZD1839. Fig. 4A shows the growth curves. As can be seen, ZD1839 inhibits colony formation in a dose-dependent manner but has differential inhibitory effects on various MPM cell lines. The NSCLC cell line A549 and H2591 are the least sensitive to the inhibitory effects of ZD1839, MSTO-211H cells have an intermediate sensitivity, and H2461 and H2373 colony for-

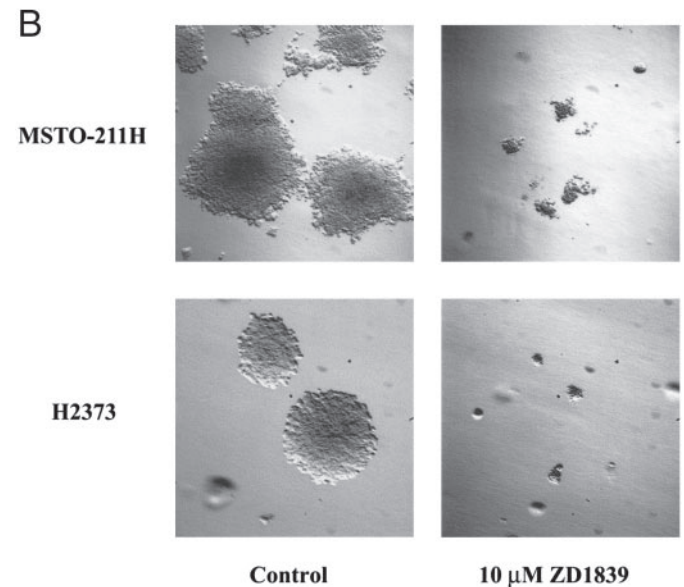
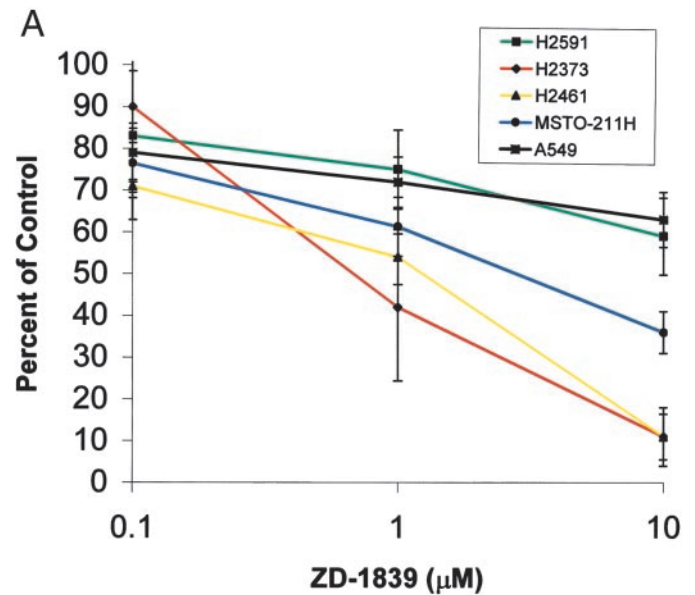


Fig. 4. A, ZD1839 inhibits growth of A549 NSCLC and mesothelioma cells grown in soft agarose. MPM cells were grown in soft agarose in the presence of increasing concentrations of ZD1839 (see "Materials and Methods"). The number of colonies is shown as a percentage of control on the Y axis. The X axis shows the concentration of ZD1839 and is plotted in a logarithmic scale. Error bars represent 1 SD. B, the size and morphology of MSTO-211H and H2373 cells grown in soft agarose in either the absence (left panels) or presence of 10  $\mu\text{M}$  ZD1839 (right panels). The size and morphology of the colonies are substantially different after treatment with ZD1839. Magnification:  $\times 4$  (MSTO-211H) and  $\times 7$  (H2373).

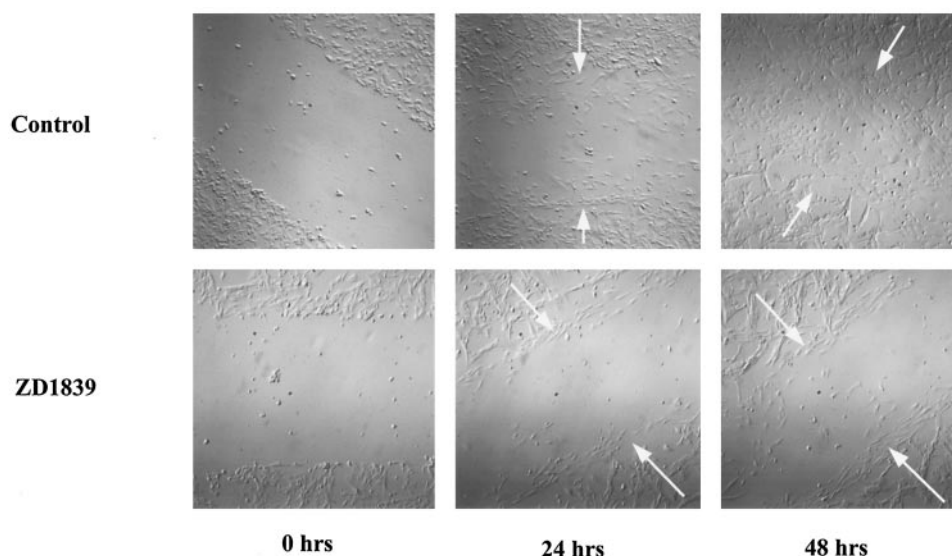


Fig. 5. *In vitro* wounding assays of H2373 cells. H2373 cells migrate into an *in vitro* wound created onto a subconfluent plate. Cells were seen to be migrating into the wound over the subsequent 48 h, and migration was significantly inhibited in the presence of 1  $\mu\text{M}$  ZD1839. The edge of the wound is shown by the white arrows.

mation is most inhibited by ZD1839 treatment. There are also qualitative differences in the colonies that grow in the presence of ZD1839. Fig. 4B demonstrates H2373 and MSTO-211H colonies grown in the absence and presence of 10  $\mu\text{M}$  ZD1839. As can be seen, the control colonies are about four to seven times larger in size and have a more uniform appearance to them than those grown in the presence of ZD1839. The colonies in Fig. 4B would have been counted in the total number of colonies because they were visible under our detection system (see “Materials and Methods”).

**Treatment with ZD1839 and Wound Healing.** MPM is a locally advancing disease with frequent extension and growth into adjacent vital structures such as the chest wall, heart, and esophagus. In an effort to study this process *in vitro*, we performed wound healing assays using previously described techniques (36). When a wound was introduced into subconfluent H2373 cells, over the course of the next 24–48 h cells would progressively migrate into the area of the wound (Fig. 5). However, when cells were pretreated with 1  $\mu\text{M}$  ZD1839 for 24 h, and the wound was introduced, this migration was virtually completely prevented (Fig. 5). H2461, H2591, and MSTO-211H cells did not demonstrate this migratory activity even when allowed to grow for 120 h after the formation of the wound (data not shown).

**Cell Cycle Distribution.** To determine the cellular effects of ZD1839 treatment of MPM cells, we performed cell cycle analyses. Subconfluent H2373 cells were treated with 0–10  $\mu\text{M}$  ZD1839 for 24 h, and cell cycle distribution was examined by FACS analysis. Treatment of H2373 cells led to a dose-dependent cell cycle arrest at the G<sub>1</sub>-S boundary (Fig. 6A). This is seen by an increase in the G<sub>0</sub>-G<sub>1</sub> fraction and a decrease in the G<sub>2</sub>-M and S fractions. Treatment with 10  $\mu\text{M}$  ZD1839 did not lead to a substantial increase in the G<sub>1</sub>-S fraction compared with 1  $\mu\text{M}$  ZD1839. However, treatment of H2591 cells led to no significant changes in cell cycle parameters (Fig. 6B). No sub-G<sub>1</sub> fraction indicative of apoptosis was observed with ZD1839 treatment in either cell line. We subsequently examined the effects on p27 levels (Fig. 7). Treatment of H2373 cells led to a slight increase in p27 levels, especially with 1 and 10  $\mu\text{M}$  ZD1839. There was no substantial difference in the p27 levels in cells treated with 1 or 10  $\mu\text{M}$  ZD1839 (Fig. 7). In contrast, p27 was also present in H2591 cells, but its levels did not change appreciably after treatment with ZD1839.

## DISCUSSION

MPM is a rare and aggressive malignancy with no known curative modality. Current chemotherapeutic options for patients with MPM

are limited. Over the last decade, a variety of receptor tyrosine kinases have been identified to play a central role in various aspects of tumorigenesis. One of these, EGFR, is overexpressed in the majority of MPMs and appears to play an important role in the pathogenesis of the disease (28). In the present study, we examined *in vitro* the effects of the p.o. active small molecule inhibitor of EGFR, ZD1839, against MPM cell lines derived from untreated patients with MPM of the three major histological subtypes. We demonstrate that the EGFR signaling axis is active in MPM cell lines and that its inhibition with ZD1839 leads to decreases in proliferation.

The findings from these studies suggest that ZD1839 treatment leads to primarily cytostatic rather than cytotoxic effects in MPM cell lines. This is supported by the lack of cytotoxicity observed in our MTS assays, the inhibition of EGF-dependent growth in H2461 cells, and the induction of a G<sub>1</sub>-S arrest without an increased apoptotic fraction in the cell cycle analyses. Similar findings have been observed in prior *in vitro* studies using ovarian, breast, and colon cancer cell lines (38). When grown in soft agarose, ZD1839 produced a dose-dependent inhibition of colony growth in all four MPM cell lines examined (Fig. 4A). The degree of inhibition was variable, with H2373 and H2461 cells being the most sensitive, H2591 cells being the least sensitive, and MSTO-211H cells having an intermediate sensitivity. Sirotnak *et al.* (39) have examined the effects of ZD1839 inhibition in human tumor xenografts. They demonstrate that the antitumor activity of ZD1839 against vulvar (A431), lung (A549, SK-LC-16, and LX-1), and prostate (PC-3 and TSU-PR1) cancers was variable and correlated with the amount of EGFR present in the cell lines as determined by reverse transcription-PCR and immunohistochemistry. In contrast, Ciardiello *et al.* (38) demonstrate that growth inhibition of GEO colon cancer cells, ZR-75-1 and MCF-10A breast cancer cells, and OVCAR-3 ovarian cancer cells grown in soft agarose by ZD1839 was dose dependent but did not differ significantly among the cell lines, even though the amount of EGFR varies considerably among these cell lines [from 20,000 binding sites/cell (ZR-75-1) to 250,000 binding sites/cell (MCF-10A)]. As analyzed by Western blotting, there appears to be no significant difference in the amount of EGFR present in H2373 and H2591 cells, although the former are much more sensitive to the effects of ZD1839 (Fig. 4A). Furthermore, in both cell lines when grown in full media, ZD1839 treatment decreased the phosphorylation of EGFR in a dose-dependent manner (Fig. 3). Thus, in MPM cell lines, sensitivity to ZD1839 inhibition is not clearly related to the amount or degree of inhibition of EGFR. Additional studies in xenograft models and in patients with mesothelioma will be needed to

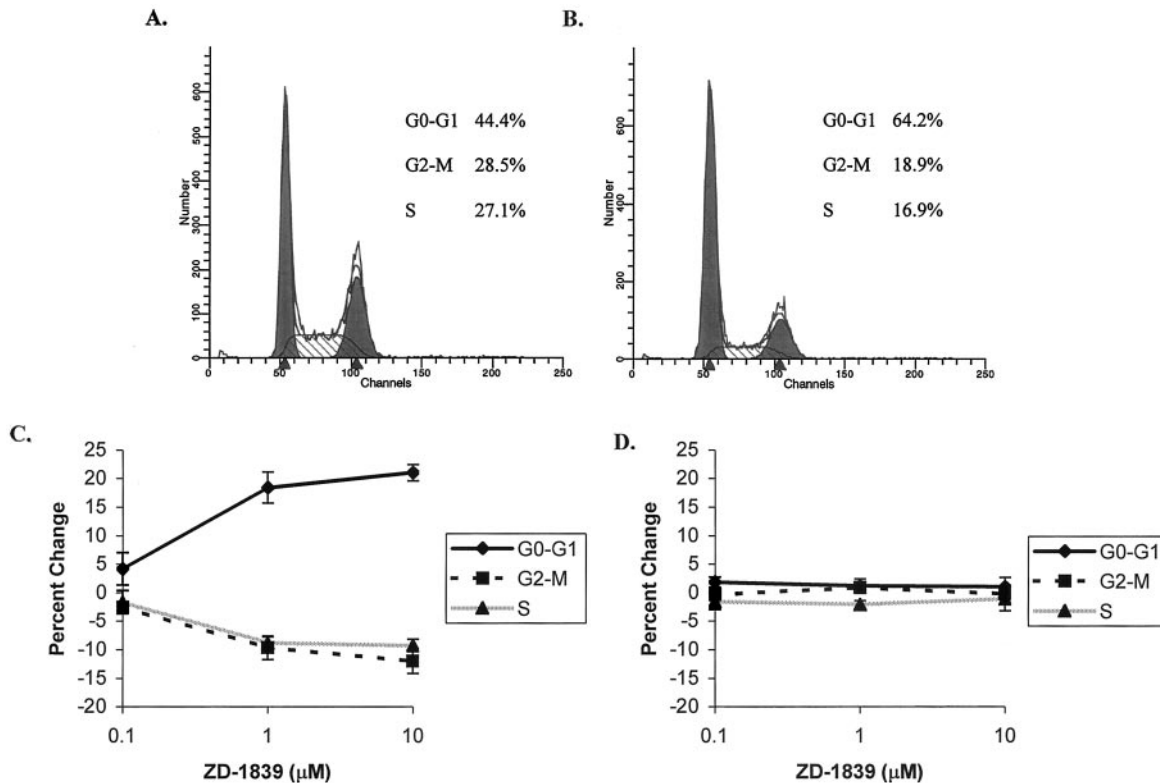


Fig. 6. Change in cell cycle parameters in H2373 and H2591 cells as a result of ZD1839 treatment. An example of FACS analysis of H2373 cells treated with 0 (A) and 10  $\mu\text{M}$  (B) ZD1839 for 24 h is shown. The proportion of cells in the G<sub>0</sub>-G<sub>1</sub>, G<sub>2</sub>-M, and S phases of the cell cycle is shown at the top right corner. Cell cycle analysis was performed on H2373 (C) and H2591 (D) cells after treatment with increasing amounts of ZD1839 for 24 h. The results are depicted as absolute changes in the percentage of cells in each of the cell cycle phases (Y axis) plotted against the concentration of ZD1839 (X axis).

further characterize the relationship between the EGFR and sensitivity to ZD1839.

Previous studies have examined the cellular effects of EGFR inhibition (25–27). In A431 squamous cell carcinoma cells, treatment with tyrphostin AG 1478 led to a dose-dependent increase in p27 levels associated with recruitment of cells into the G<sub>1</sub> phase of the cell cycle (25). When p27 expression was blocked by pretreatment using an antisense oligonucleotide, tyrphostin AG 1478 treatment did not lead to a significant increase in p27, and only a minimal change in the proportion of cells in G<sub>1</sub> phase was observed (25). Similar findings have been observed using C225 treatment of DiFi human colon cancer cell lines (26). In the present study, treatment with ZD1839 led to a dose-dependent increase in G<sub>1</sub> arrest in H2373 cells, but not in H2591 cells. In fact, there is virtually no cell cycle inhibition of H2591 cells by ZD1839. This variable difference in cell cycle arrest likely accounts for the differences observed in growth inhibition between these two cell lines in soft agarose. Consistent with previous studies, p27 levels also increase in H2373 cells, but not in H2591 cells, after inhibition of EGFR with ZD1839 treatment (Fig. 7; Refs. 25 and 26). Thus, at least in H2591 cells, receptor inhibition and the presence of p27 are not sufficient to lead to a significant cell cycle arrest. However, given the dose-dependent, albeit modest, growth inhibition of H2591 in soft agarose, ZD1839 must exert some growth-inhibitory effects. The mechanisms behind the differences in H2373 and H2591 have not been elucidated but are the focus of ongoing investigations.

These studies with ZD1839 in MPM have several clinical implications. MPM is a relatively chemotherapy-resistant disease. Most chemotherapy agents have a response rate of <25% and are associated with significant toxicities (4). ZD1839 is a p.o. active agent that has been well tolerated in previous clinical studies. ZD1839 is undergoing extensive clinical studies in patients with advanced NSCLC and has demonstrated antitumor activity (40). Our findings suggest that

ZD1839 treatment leads to greater growth-inhibitory effects in three of the four MPM cell lines examined (MSTO-211H, H2461, and H2373) compared with the NSCLC cell line A549. Based on the *in vitro* inhibitory data, ZD1839 may also be an active antitumor agent for patients with MPM. The Cancer and Leukemia Group B is presently conducting a clinical trial of ZD1839 in patients with mesothelioma. The sarcomatoid and mixed histology variants of MPM are associated with poor survival and a worse response rate to chemotherapy but not to ZD1839 in our *in vitro* studies (5, 41). The migratory capability of sarcomatoid mesothelioma cells may also contribute to their aggressive behavior, which is also inhibited *in vitro* by ZD1839. This finding is consistent with those of Alper *et al.* (15), who studied NIH: OVCAR-8 EGFR-expressing ovarian cancer cells. Because mesothelioma can recur at sites of previous surgical biopsies, postbiopsy or postsurgical treatment with ZD1839 of patients with sarcomatoid MPM may provide an effective means of decreasing the chance of tumor infiltration into the incisions (42, 43).

Many studies have also demonstrated a potentiation of the antitumor effects of cytotoxic chemotherapy agents by ZD1839 in soft agarose and in xenograft models and radiosensitization by an anti-

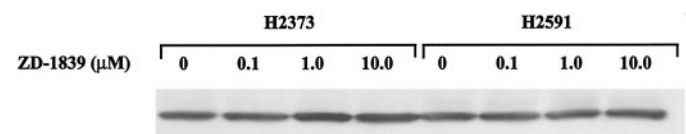


Fig. 7. Western analysis of p27 levels in H2373 and H2591 cells as a result of ZD1839 treatment. H2373 and H2591 cells were treated with increasing concentrations of ZD1839 for 24 h and then subjected to Western analysis of p27 protein. There was a slight increase in the amount of p27 in the 1.0 and 10.0  $\mu\text{M}$  lanes in H2373 cells but no significant change in the H2591 cells.



sense EGFR construct or by C225 *in vitro* and *in vivo* (24, 39, 44, 45). Both chemotherapy and radiation are used in the treatment of MPM, and ZD1839 treatment may enhance the effects of these modalities. *In vitro* studies in mesothelioma combining radiation and chemotherapy with ZD1839 are presently under way.

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