**ZD1839 induces p15INK4b and causes G₁ arrest by inhibiting the mitogen-activated protein kinase/extracellular signal–regulated kinase pathway**

Makoto Koyama, Youichirou Matsuzaki, Shingo Yogosawa, Toshiaki Hitomi, Mayumi Kawanaka, and Toshiyuki Sakai

Department of Molecular-Targeting Cancer Prevention, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyō-ku, Kyoto, Japan

**Abstract**

Inactivation of the retinoblastoma protein pathway is the most common abnormality in malignant tumors. We therefore tried to detect agents that induce the cyclin-dependent kinase inhibitor p15INK4b and found that ZD1839 (gefitinib, Iressa) could up-regulate p15INK4b expression. ZD1839 has been shown to inhibit cell cycle progression through inhibition of signaling pathways such as phosphatidylinositol 3'-kinase-Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) cascades. However, the mechanism responsible for the differential sensitivity of the signaling pathways to ZD1839 remains unclear. We here showed that ZD1839 up-regulated p15INK4b, resulting in retinoblastoma hypophosphorylation and G₁ arrest in human immortalized keratinocyte HaCaT cells. p15INK4b induction was caused by MAPK/ERK kinase inhibitor (PD98059), but not by Akt inhibitor (SH-6, Akt-III). Moreover, mouse embryo fibroblasts lacking p15INK4b were resistant to the growth inhibitory effects of ZD1839 compared with wild-type mouse embryo fibroblasts. Additionally, the status of ERK phosphorylation was related to the antiproliferative activity of ZD1839 in human colon cancer HT-29 and Colo320DM cell lines. Our results suggest that induction of p15INK4b by inhibition of the MAPK/ERK pathway is associated with the antiproliferative effects of ZD1839.

**Introduction**

Cell cycle regulation is important in growth control, and therefore the deregulation of cell cycle machinery has been implicated in carcinogenesis (1). Cyclins and cyclin-dependent kinases (CDK), in association with each other, play key roles in promoting the G₁-to-S phase transition of the cell cycle by phosphorylating the retinoblastoma (RB) protein (2, 3). Activation of cyclin-CDK complexes is counterbalanced by CDK inhibitors including the INK4 family. The INK4 family consists of p15INK4b, p16INK4a, p18INK4c, and p19INK4d, and their members, with similar affinity, are specific inhibitors of cyclin D-CDK4/6 complexes (4, 5).

A great number of cancer studies have shown that most of the abnormalities leading to malignancies involve inactivation of tumor suppressor molecules, such as the INK4 family, associated with the RB pathway (6, 7). Consistently, for example, mice deficient in p16INK4a or p18INK4c are highly susceptible to various types of malignant tumors (8–10). In many malignant tumors, p16INK4a is inactivated by homozygous deletions, gene methylation, or mutations (11, 12). In contrast to p16INK4a, alterations of p15INK4b, p18INK4c, and p19INK4d genes are rare events in malignant tumors, although these genes have functions similar to that of p16INK4a (6). Regarding the association between p15INK4b and carcinogenesis, p15INK4b is an essential mediator in cell cycle arrest in response to mitotic signals (13). The p15INK4b gene is one of the major target genes in an oxidative stress–induced rat renal carcinogenesis model (14). There is also a significant relationship between p15INK4b methylation and overall survival of patients with non–small-cell lung cancer (15). We therefore have focused on p15INK4b as a member of the INK4 family and have found that the histone deacetylase inhibitor trichostatin A causes G₁ arrest by inducing p19INK4d (16) and p18INK4c (17), and that trichostatin A (18) and a naturally occurring compound, indole-3-carbinol (19), up-regulate p15INK4b, resulting in hypophosphorylation of the RB protein. Moreover, we previously established the screening system to detect p15INK4b-inducing agents using a branched-DNA assay. As a result, we found several p15INK4b inducers, including an agent whose structural formula is similar to that of ZD1839, and a pyrido-pyrimidine derivative as a novel mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-1/2 inhibitor.1 We subsequently confirmed that ZD1839 also induces p15INK4b expression. ZD1839 has been shown to block epidermal growth factor receptor

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Requests for reprints: Toshiyuki Sakai, Department of Molecular-Targeting Cancer Prevention, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyō-ku, Kyoto 602-8566, Japan. Phone: 81-75-251-5339; Fax: 81-75-241-0792. E-mail: tsakai@koto.kpu-m.ac.jp

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1 Yamaguchi et al., submitted for publication.
(EGFR) phosphorylation and its downstream pathways such as phosphatidylinositol 3’-kinase (PI3K)-Akt and MAPK/ERK cascades, resulting in inhibition of proliferation in tumor cell lines (20–22). EGFR is expressed in most human tissues and is often highly expressed in human solid tumors (23, 24). However, the level of EGFR expression alone is not sufficient to predict the response of tumor cells to ZD1839 (25, 26). Several groups have shown that ZD1839 effectively induces a marked response in patients with non–small-cell lung cancer who have somatic mutations in exons 18 to 21 in the ATP-binding region of the EGFR kinase domain (27–29). These findings indicate that EGFR mutations might be a determinant of ZD1839 sensitivity in non–small-cell lung cancer. On the other hand, activating EGFR mutations are rare in colorectal cancer, and patients whose colorectal cancer possessed no EGFR mutations experienced disease regression following treatment with ZD1839 and chemotherapy (30). Therefore, other downstream events may influence responsiveness to EGFR inhibition.

We here show that ZD1839 induces p15INK4b by inhibiting ERK phosphorylation, and that p15INK4b status is at least partially associated with ZD1839 sensitivity. We suggest for the first time that the ability of ZD1839 to induce p15INK4b by inhibiting signals through the MAPK/ERK pathway could be one of the mechanisms of the antiproliferative activity of this agent.

Materials and Methods

Cell Culture and Reagents

Human immortalized keratinocyte HaCaT cells (a kind gift from Dr. N. E. Fusenig, German Cancer Research Center, Heidelberg, Germany) and human colon cancer cell lines (HT-29 and Colo320DM) were maintained in DMEM containing 10% fetal bovine serum, 2 mmol/L glutamine, and antibiotics (penicillin/streptomycin), and incubated at 37°C in a humidified atmosphere with 5% CO2. Wild-type and p15 INK4b-deficient [p15(−/−)] mouse embryonic fibroblasts (MEF; ref. 9; a kind gift from Dr. Paul Krimpenfort, Division of Molecular Genetics and Centre of Biomedical Genetics, Netherlands Cancer Institute, Amsterdam, the Netherlands) were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 0.1 mmol/L MEM nonessential amino acids, 55 mmol/L 2-mercaptoethanol, and antibiotics (penicillin/streptomycin), and incubated at 37°C in a humidified atmosphere of 9% CO2. ZD1839 (Gefitinib, Iressa, Zymed Laboratories), mouse anti-human p18INK4c monoclonal antibody (mAb; G175-1239, PharMingen), rabbit anti-human p27KIP1 polyclonal antibody (C-19, Santa Cruz Biotechnology), mouse anti-human CDK6 mAb, mouse anti-human CDK4 mAb (H-22, Santa Cruz Biotechnology), mouse c-myc antibody (C-19, Santa Cruz Biotechnology), mouse anti-human p21WAF1 mAb (2D2G4, Zymed Laboratories), mouse anti-human p21WAF1 mAb (6B6, PharMingen), rabbit anti-human p27KIP1 polyclonal antibody (C-19, Santa Cruz Biotechnology), mouse anti-human p15INK4b polyclonal antibody (C-20, Santa Cruz Biotechnology, CA), mouse anti-human p16INK4a polyclonal antibody (G175-1239, PharMingen), rabbit anti-human p19INK4d, p21WAF1, p27KIP1, c-myc, cyclin D1, CDK4/6, and α-tubulin detection) or a 10% [for phospho-Akt, Akt, phospho-p42/44 MAPK (p-ERK1/2), p42/44 MAPK (ERK1/2), and phospho-GSK3β detection] or a 7% (for RB detection) polyacrylamide gel, subjected to electrophoresis, and transferred to a nitrocellulose membrane. The following antibodies were used as the primary antibody: rabbit anti-human p15INK4b polyclonal antibody (C-20, Santa Cruz Biotechnology, CA), mouse anti-human p16INK4a monoclonal antibody (mAb; G175-1239, PharMingen), rabbit anti-human p18INK4c monoclonal antibody (N-20, Santa Cruz Biotechnology), mouse anti-human p19INK4d mAb (2D2G4, Zymed Laboratories), mouse anti-human p21WAF1 mAb (6B6, PharMingen), rabbit anti-human p27KIP1 polyclonal antibody (C-19, Santa Cruz Biotechnology), mouse c-myc mAb (9E10, Santa Cruz Biotechnology), CDK4 mAb (H-22, Santa Cruz Biotechnology), mouse anti-human CDK6 mAb (D-83, MBL), mouse anti-human pRB mAb (G3-245, PharMingen), rabbit anti-human Akt, phospho-Akt (Ser473), phospho-p42/44 MAPK, p42/44 MAPK, phospho-GSK3β (Ser9) antibody (phospho-Akt Pathway Sampler Kit, Cell Signaling Technology, Inc.), and α-tubulin antibody (Oncogene Research Products). The signal was then developed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK Ltd.).

Cell Viability Assay

Cells were seeded at 4,000 per well in 96-well plates. The number of viable cells was determined by Cell Counting Kit-8 assay according to the manufacturer’s instructions (Dojindo, Kumamoto, Japan). After incubation with the indicated concentrations of ZD1839 or PD98059, kit reagent WST-8 was added to the medium and incubated for a further 1 h. The absorbance of samples (450 nm) was tested. For flow cytometry analysis, unsynchronized HaCaT cells were exposed to the agents for the indicated times. The cells were then treated with Triton X-100 and RNase A, and their nuclei were stained with propidium iodide before DNA content was measured using a Becton Dickinson FACSCalibur. At least 10,000 cells were counted and the ModFit LD V2.0 software package (Becton Dickinson) was used to analyze the data.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated using a Sepasol RNA isolation kit (Nacalai Tesque, Inc.) and poly(A)+ mRNA was separated from 200 μg of total RNA using an Oligotex-dT30 (Super) mRNA purification kit (Takara Bio, Inc.). Poly(A)+ mRNA was fractionated on 1% agarose gels, transferred to nylon filters, and probed according to standard procedures. Exon 1 of p15 cDNA was used as a probe. Northern blot analysis was done using standard methods (31). For mRNA half-life studies, 5 μg/mL actinomycin D (Sigma) was added directly to the medium. The relative band intensity was assessed by densitometric analysis of digitalized photographic images using Scion Image software (Scion Corp.).

Protein Isolation and Western Blot Analysis

Cells were lysed in lysis buffer [50 mmol/L Tris- HCl (pH 7.5), 1% SDS]. The protein extract was then boiled for 5 min and loaded onto a 12% [for p15INK4b, p16INK4a, p18INK4c, p19INK4d, p21WAF1, p27KIP1, c-myc, cyclin D1, CDK4/6, and α-tubulin detection] or a 10% [for phospho-Akt, Akt, phospho-p42/44 MAPK (p-ERK1/2), p42/44 MAPK (ERK1/2), and phospho-GSK3β detection] or a 7% (for RB detection) polyacrylamide gel, subjected to electrophoresis, and transferred to a nitrocellulose membrane. The following antibodies were used as the primary antibody: rabbit anti-human p15INK4b polyclonal antibody (C-20, Santa Cruz Biotechnology, CA), mouse anti-human p16INK4a monoclonal antibody (mAb; G175-1239, PharMingen), rabbit anti-human p18INK4c monoclonal antibody (N-20, Santa Cruz Biotechnology), mouse anti-human p19INK4d mAb (2D2G4, Zymed Laboratories), mouse anti-human p21WAF1 mAb (6B6, PharMingen), rabbit anti-human p27KIP1 polyclonal antibody (C-19, Santa Cruz Biotechnology), mouse c-myc mAb (9E10, Santa Cruz Biotechnology), CDK4 mAb (H-22, Santa Cruz Biotechnology), mouse anti-human CDK6 mAb (D-83, MBL), mouse anti-human pRB mAb (G3-245, PharMingen), rabbit anti-human Akt, phospho-Akt (Ser473), phospho-p42/44 MAPK, p42/44 MAPK, phospho-GSK3β (Ser9) antibody (phospho-Akt Pathway Sampler Kit, Cell Signaling Technology, Inc.), and α-tubulin antibody (Oncogene Research Products). The signal was then developed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK Ltd.).

Growth Inhibition Assay and Cell Cycle Analysis

One day after inoculation of cells, various concentrations of ZD1839, PD98059, or Act-II were added to the culture medium. The number of viable cells was counted 24 to 48 h after drug addition with the trypan blue dye exclusion test. For flow cytometry analysis, unsynchronized HaCaT cells were exposed to the agents for the indicated times. The cells were then treated with Triton X-100 and RNase A, and their nuclei were stained with propidium iodide before DNA content was measured using a Becton Dickinson FACSCalibur. At least 10,000 cells were counted and the ModFit LD V2.0 software package (Becton Dickinson) was used to analyze the data.
determined using a scanning multiwell spectrophotometer that serves as an ELISA reader.

Statistical Analysis
Statistical evaluation of the data was done using the Student’s t test for simple comparison between groups and treatments. \( P < 0.05 \) was considered statistically significant.

Results

Cell Growth Inhibition and G1 Arrest by ZD1839 in HaCaT Cells
We first investigated the antiproliferative effects of ZD1839 in human immortalized keratinocyte HaCaT cells. We observed that ZD1839 inhibits the growth of HaCaT cells in a dose-dependent manner and 600 nmol/L ZD1839 had a cytostatic effect (Fig. 1A). To examine the effects of ZD1839 on cell cycle progression, the DNA content of the cell nuclei was measured by flow cytometry analysis. The treatment with 600 nmol/L ZD1839 increased the percentage at the G1 phase and decreased that at the S phase in a time-dependent manner (Fig. 1B). These data show that ZD1839 arrests the cell cycle of HaCaT cells at the G1 phase.

\( p15^{INK4b} \) Induction and RB Hypophosphorylation by ZD1839 in HaCaT Cells
We tried to elucidate whether the expression of \( p15^{INK4b} \) protein could be influenced by treatment with ZD1839 in HaCaT cells. The time course study showed that \( p15^{INK4b} \) was markedly increased at 24 h or more after treatment with ZD1839 in a time-dependent manner, compared with the treatment with solvent DMSO alone (Fig. 1C). These results suggest that \( p15^{INK4b} \) is an important molecular target of ZD1839 among the INK4 family, whose members are associated with G1 cell cycle arrest. There were no obvious changes in the abundance of other INK4 family members such as \( p16^{INK4a}, p18^{INK4c}, \) and \( p19^{INK4d} \); however, the expression of \( p21^{WAF1} \) and \( p27^{KIP1} \) was slightly up-regulated after treatment with ZD1839 as previously reported (32, 33). The protein \( p15^{INK4b} \) is a specific inhibitor of cyclin D–dependent kinases and the subsequent dephosphorylation of the RB protein causes G1 cell cycle arrest. We therefore examined whether ZD1839 could alter the phosphorylation status of the RB protein in HaCaT cells. A hyperphosphorylated form of the RB protein (ppRB) was converted into a hypophosphorylated form (pRB) 24 to 48 h after the treatment (Fig. 1C). Taken together, these results indicate that ZD1839 up-regulates \( p15^{INK4b} \) protein levels and subsequently converts a hyperphosphorylated form of the RB protein into a hypophosphorylated form in HaCaT cells.

\( p15^{INK4b} \) mRNA Up-regulation by ZD1839 in HaCaT Cells
We next investigated in a time course study using Northern blot analysis whether ZD1839 could also affect \( p15^{INK4b} \) mRNA in HaCaT cells. The result indicated that \( p15^{INK4b} \) mRNA was up-regulated at 12 h or more after treatment with ZD1839 in a time-dependent manner (Fig. 2A). The longer transcript of \( p15^{INK4b} \) mRNA is \( ~3.2 \) kb and the shorter transcript is \( ~2.2 \) kb. These two transcripts were detected in a previous report (13). Because we found that \( p15^{INK4b} \) mRNA is induced by ZD1839, we evaluated the effect of ZD1839 on the promoter activity in a \( p15^{INK4b} \) promoter-luciferase fusion plasmid by transient assay (18). However, we did not detect significant up-regulation of the \( p15^{INK4b} \) promoter by ZD1839.

Figure 1. Effects of ZD1839 on cell growth and CDK inhibitors expression in HaCaT cells. A, one day after inoculation of HaCaT cells, ZD1839 at 50 (●), 100 (○), 200 (△), 400 (□), or 600 (●) nmol/L was added, and cell growth was compared with control treated with equivalent DMSO (□). The number of viable cells was counted by trypan blue dye exclusion test. Points, mean of duplicate experiments. B, unsynchronized HaCaT cells were incubated in the presence of either DMSO or 600 nmol/L ZD1839 for 24 h (top) and 48 h (bottom), and the DNA content of the cells was determined by flow cytometry. Data represent means of duplicate experiments. C, HaCaT cells were exposed either to DMSO alone (−) or to 600 nmol/L ZD1839 (+) for the indicated times. The expressions of INK4 family proteins (\( p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, \) and \( p19^{INK4d} \)), CIP/KIP family proteins (\( p21^{WAF1} \) and \( p27^{KIP1} \)), and RB protein were analyzed by Western blotting. \( \alpha \)-Tubulin was chosen as the loading control in all blots.
To investigate whether a posttranscriptional regulation mechanism could be involved in p15INK4b expression, we next did a series of mRNA half-life studies in HaCaT cells. Actinomycin D was added to HaCaT cells to prevent mRNA synthesis followed by a 36-h incubation period in the presence or absence of 600 nmol/L ZD1839. At the indicated times after actinomycin D treatment, total RNA was isolated and p15INK4b mRNA levels were examined by Northern blot analysis. The half-life of p15INK4b mRNA was calculated to be 1.6 h in control cells. On the other hand, in cells treated with ZD1839, the half-life of p15INK4b mRNA increased ~2-fold (3.2 h; Fig. 2B). These results suggest that an increase of p15INK4b mRNA stability could at least partially contribute to the up-regulation of p15INK4b by ZD1839.

**Effects of ZD1839 on the Expression of Cell Cycle Regulatory Molecules in HaCaT Cells**

We examined the effects of ZD1839 on the expression of cell cycle regulatory molecules in HaCaT cells. The time course study indicated that cyclin D1 and its transcription factor c-myc were down-regulated 12 h after treatment with ZD1839; however, no apparent change in phosphorylation of Akt and its downstream molecule GSK3β was observed (Fig. 3). These results suggest that the inhibition of ERK phosphorylation is involved in ZD1839-mediated cell cycle arrest of HaCaT cells.

**Effects of Inhibiting Downstream Signals of EGFR**

The MAPK/ERK and PI3K-Akt pathways are two main signaling pathways downstream of EGFR. We examined the effects of MEK inhibitor PD98059 and Akt inhibitor Akt-III on the cell cycle regulation and p15INK4b induction in HaCaT cells. Both agents inhibited the cell growth in a dose-dependent manner (Fig. 4A) and increased the percentage at the G1 phase and decreased that at the S phase, respectively (Fig. 4B). We then did Western blot analysis 48 h after treatment with 40 μmol/L PD98059 or 20 μmol/L Akt-III. PD98059 induced p15INK4b and p27KIP1 and down-regulated c-myc. This pattern of the expression profile is similar to that observed with ZD1839 (Figs. 1C, 3, and 4C). Akt-III increased p21WAF1 and p27KIP1 and decreased cyclin D1 expression; however, unlike PD98059, Akt-III did not induce p15INK4b (Fig. 4C). These results suggest that p15INK4b induction is associated with the inhibition of the MAPK/ERK pathway in HaCaT cells.

**Association between p15INK4b or ERK Phosphorylation Status and the Antiproliferative Effect of ZD1839**

To reveal whether p15INK4b contributes to the antiproliferative activity of MEK inhibitor as a result of the phosphorylation of ERK1/2 (Fig. 2B). These results suggest that an increase of p15INK4b mRNA stability could at least partially contribute to the up-regulation of p15INK4b by ZD1839.
inhibition of ERK phosphorylation, we did the Cell Counting Kit-8 assay to assess the viability of wild-type and p15INK4b-deficient [p15(-/-)] MEFs treated with PD98059. The p15(-/-) MEFs exhibited decreased PD98059 sensitivity relative to wild-type MEFs (Fig. 5A). To analyze the physiologic relevance of the induction of p15INK4b in ZD1839-induced growth inhibition, we next examined the effects of ZD1839 on the cell growth of wild-type and p15(-/-) MEFs. As expected, the p15(-/-) MEFs grew more rapidly than wild-type MEFs in the absence of ZD1839. The p15(-/-) MEFs were also more resistant to the growth inhibitory effects of ZD1839 than were wild-type MEFs, and 2 μmol/L ZD1839 had a cytostatic effect in wild-type MEFs (Fig. 5B and C). Furthermore, we found that ERK phosphorylation was inhibited in both types of MEFs; however, p15INK4b protein induction by treatment with ZD1839 was detected only in wild-type MEFs (Fig. 5D).

To assess the influence of the activation status of ERK on ZD1839-mediated cell growth inhibition, we evaluated the antiproliferative effects of ZD1839 in two human colon cancer cell lines, HT-29 and Colo320DM (Fig. 6A). The graph shows that the growth of HT-29 cells was inhibited in a dose-dependent manner; however, that of Colo320DM cells was not inhibited by ZD1839. To investigate whether the difference in growth inhibition by ZD1839 between the two cell lines depends on ERK phosphorylation and p15INK4b induction, we did Western}

**Figure 4.** Effects of the inhibition of the PI3K-Akt and MAPK/ERK pathways in HaCaT cells. A, one day after inoculation of HaCaT cells, PD98059 (left) at 10 (■), 20 (○), or 40 (▲) μmol/L or Akt-III (right) at 5 (□), 10 (●), or 20 (▲) μmol/L was added, and cell growth was compared with control treated with equivalent DMSO (●). The number of viable cells was counted by trypan blue dye exclusion test. Points, mean of duplicate experiments. B, unsynchronized HaCaT cells were incubated in the presence of DMSO (left), 40 μmol/L PD98059 (middle), or 20 μmol/L Akt-III (right) for 48 h, and the DNA content of the cells was determined by flow cytometry. Data represent means of duplicate experiments. C, HaCaT cells were exposed to DMSO alone (●) or to 40 μmol/L PD98059 (+) or to 20 μmol/L Akt-III (+) for 48 h. The expressions of p15INK4b, p21WAF1, p27KIP1, CDK4/6, cyclin D1, and c-myc proteins were examined by Western blotting. α-Tubulin was chosen as the loading control in all blots.
blot analysis. The level of ERK in Colo320DM cells was higher than that in HT-29 cells; however, constitutive phosphorylation of ERK was not observed in Colo320DM cells (Fig. 6B). Moreover, p15INK4b induction and the inhibition of ERK phosphorylation by ZD1839 were detected only in HT-29 cells. Taken together, these results suggest that loss of p15INK4b influences the cellular response to the antiproliferative effects of ZD1839 and that, in addition to induction of p15INK4b, status of ERK phosphorylation and inactivation of the MAPK/ERK pathway play important roles in the cell growth inhibition induced by ZD1839.

**Discussion**

A great number of cancer studies have shown that the RB pathway is the most frequently inactivated in human malignant tumors (6, 7). If the RB pathway is inactivated, activation of the INK4 family leads to functional restoration of this pathway. Interestingly, it has been reported that ZD1839 induces p21WAF1 and p27KIP1 (32, 33) and imatinib mesylate (STI571, Gleevec) up-regulates p18INK4c (34), resulting in hypophosphorylation of the RB protein.

In the present study, we disclosed that ZD1839 up-regulates p15INK4b protein and subsequently converts a
physiologic relevance of the induction of p15 INK4b in wild-type MEFs (Fig. 5A). Moreover, to emphasize the MEFs exhibited decreased PD98059 sensitivity relative to withMEFsinhibitorPD98059. We revealed that the p15/C0 assess the viability of wild-type and p15/C0 proliferative activity of ZD1839, wedidcellviabilityassaysto inhibition of ERK phosphorylation contributes to the anti- results suggest that p15 INK4b induction through inhibition of MAPK pathways, such as the c-jun NH2-terminal kinase, p38, and ERK cascade, contribute to the stabilization and destabilizing activities of ARE-binding factors in the regulation of half-lives of ARE-containing mRNAs (40, 41). Taken together, it could suggest that ZD1839 inhibition of the MAPK/ERK pathway may affect p15 INK4b mRNA stability through ARE-like sequences in the 3’-untranslated region.

Akt and ERK are important molecules of EGFR downstream signals because activation of the PI3K-Akt and MAPK/ERK pathways depends on the phosphorylation status of such signaling components in response to EGFR activation. Cappuzzo et al. (42) suggested that ZD1839 may

difference may depend on particular properties of different cell lines.

Because we found that p15 INK4b mRNA is induced by ZD1839, we next investigated the molecular mechanism of p15 INK4b activation by ZD1839. To clarify whether the activation of the p15 INK4b promoter was responsible for the up-regulation of its mRNA, we examined the effects of ZD1839 on the promoter activity in a p15 INK4b promoter-luciferase fusion plasmid by transient assay. It was previously reported that transforming growth factor-β signaling induces p15 INK4b promoter activation by a combination of two coupled events: transactivation and relief of repression by c-myc (38). As a positive control, we showed that the p15 INK4b promoter was activated ∼7.5-fold with 5.0 ng/μL transforming growth factor-β; however, we did not detect significant activation of the p15 INK4b promoter by ZD1839 (data not shown). Therefore, posttranscriptional mechanisms may be responsible for the induction of p15 INK4b by ZD1839.

We next revealed that stabilization of p15 INK4b mRNA could be associated with the up-regulation of p15 INK4b by ZD1839 (Fig. 2B). The regulation of mRNA stability is a central mechanism in the control of gene expression. It has been shown that adenylate/uridylate (AU)-rich elements (ARE), often containing one to several copies of the consensus sequence AUUUA, are critical cis-acting regulatory motifs located in the 3’-untranslated region of many mRNAs of cellular growth regulators, and that AREs are targets for trans-acting proteins to modulate mRNA stability (39). We found that the human p15 mRNA contains six separate AUUUA repeats in the 3’-untranslated region (Table 1). It has been shown that MAPK pathways, such as the c-jun NH2-terminal kinase, p38, and ERK cascade, contribute to the stabilizing and destabilizing activities of ARE-binding factors in the regulation of half-lives of ARE-containing mRNAs (40, 41). Taken together, it could suggest that ZD1839 inhibition of the MAPK/ERK pathway may affect p15 INK4b mRNA stability through ARE-like sequences in the 3’-untranslated region.

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Table 1. RNA sequence of the human p15 mRNA 3’-untranslated region position sequence

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-221 to 225</td>
<td>...UAUUUAUUAAUUUA</td>
</tr>
<tr>
<td>-1,053 to 1,057</td>
<td>...UAUUUAAG</td>
</tr>
<tr>
<td>-1,376 to 1,380</td>
<td>...ACAUUAUUU</td>
</tr>
<tr>
<td>-1,409 to -1,413</td>
<td>...AAUUUAUUAAUUUA</td>
</tr>
</tbody>
</table>

NOTE: The ARE sequences are shown with AUUUA motifs underlined. The position numbers are derived from the human p15 INK4b cDNA sequence (GenBank).
be most effective against tumors with activated Akt; however, they found no correlation between activated ERK status and effectiveness of ZD1839. On the other hand, Ono et al. (43) indicated that high sensitivity to ZD1839 in cancer cell lines is closely correlated with dependence not only on Akt but also on ERK activation for cellular survival and proliferation. In our study, we showed that ZD1839 inhibited ERK phosphorylation but did not block Akt phosphorylation in HaCaT cells (Fig. 3). These results suggest that inhibition of ERK phosphorylation plays an important role in ZD1839-mediated cell cycle arrest. Furthermore, we found that the status of ERK phosphorylation and p15INK4b induction through inhibition of ERK phosphorylation are involved in the antiproliferative activity of ZD1839 (Fig. 6A and B). Ogiño et al. (30) reported that EGFRI mutations are uncommon in colorectal cancer, and therefore other downstream molecules may influence responsiveness to EGFRI inhibition. Taken together, our results may suggest that p15INK4b induction through inhibition of ERK phosphorylation is associated with the antiproliferative effects of ZD1839.

Our results have suggested that ZD1839 induces p15INK4b through inhibition of ERK phosphorylation, resulting in hypophosphorylation of the RB protein. Therefore, anticancer agents activating RB function, such as ZD1839, may contribute to new strategies for the therapy of malignancies, which we have termed "gene-regulating chemotherapy" (44, 45). A common strategy to detect a novel molecular-targeting anticancer agent has been based on finding direct inhibitors of various malignancy-related molecules in in vitro conditions. On the other hand, using the screening system we established, we were able to identify several p15INK4b-inducing agents containing ZD1839. This suggests that INK4 family-inducing agents, toward activation of RB function, will be useful as new molecular-targeting anticancer drugs against malignant tumors.

Acknowledgments

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References

29. Pao W, Miller V, Zakowski M, et al. EGFR receptor gene mutations are common in lung cancers from "never smokers" and are associated with


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ZD1839 induces p15\(^\text{INK4b}\) and causes G\(_1\) arrest by inhibiting the mitogen-activated protein kinase/extracellular signal–regulated kinase pathway

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