Chemistry

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ZD1839 (Gefitinib, 'Iressa'), an epidermal growth factor receptor-tyrosine kinase inhibitor, enhances the anti-cancer effects of TRAIL in human esophageal squamous cell carcinoma

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Short Title: Antitumor effect of ZD1839 and TRAIL

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Footnotes:

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma; FACS, fluorescence-activated cell-sorting; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLCs, non-small cell lung cancers; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; TKI, tyrosine kinase inhibitor; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

Abstract

The EGF (epidermal growth factor) receptor-tyrosine kinase inhibitor ZD1839 (Gefitinib, 'Iressa') blocks the cell signaling pathways involved in cell proliferation, survival, and angiogenesis in various cancer cells. TRAIL (TNF-Related Death Apoptosis Inducing Ligand) acts as an anticancer agent. We investigated the antitumor effects of ZD1839 alone or in combination with TRAIL against human esophageal squamous cell cancer (ESCC) lines. Although all ESCC cells expressed EGF receptor at a protein level, the effect of ZD1839 on cell growth did not correlate with the level of EGFR expression and phosphorylation of EGF receptor protein in ESCC lines. ZD1839 caused a dose-dependent growth arrest at G_0-G_1 phase associated with increased p27 expression. As TE8 cells are resistant to TRAIL, we tested whether ZD1839 combined with TRAIL induced apoptosis of TE8 cells via the inhibition of EGF receptor signaling by ZD1839. ZD1839 inhibited the phosphorylation of Akt, and enhanced TRAIL-induced apoptosis via activation of caspase-3 and caspase-9, and inactivation of Bcl-xL. Our results indicated that ZD1839 has anti-cancer properties against human esophageal cancer cells. ZD1839 also augmented the anti-cancer activity of TRAIL, even in TRAIL-resistant tumors. These results suggest that treatment with ZD1839 and TRAIL may have potential in the treatment of ESCC patients.

1. Introduction

Esophageal squamous cell cancer (ESCC) is associated with a poor prognosis and high recurrence rate despite improvement in early diagnosis and multimodal treatments including operation, chemotherapy and irradiation. Searching for molecular targets of ESCC is critical to improve the effect of treatment [1-3]. According to recent reports, epidermal growth factor receptor (EGFR) is highly expressed in invasive ESCC, and patients with EGFR-overexpressing esophageal cancers have a poorer prognosis and more lymph node metastases than those with EGFR-negative tumors [4-6].

EGFR belongs to the type I receptor tyrosine kinase family, which includes ErbB2/ HER2, ErbB3, and ErbB4 and is implicated in the cell proliferation, angiogenesis, invasion and survival of neoplastic cells [7]. The regulation of these family members is complex, as the receptors can be transactivated by homodimeric interaction between two family members and thus uses multiple pathways to execute their biological functions [8]. The presence of deregulated EGFR-tyrosine kinase (TK) has been confirmed in many types of solid tumors, such as head and neck cancers, colon cancers, non-small cell lung cancers (NSCLC), and breast cancers. Considered together, growth factors and their receptors, therefore, play an essential role in cancer pathogenesis and progression, and they are considered important targets for anticancer therapy [9-11].

ZD1839 (Iressa®, AstraZeneca) is an orally administered, small-molecule

EGFR-TK inhibitor (EGFR-TKI) that blocks signal transduction pathways implicated in proliferation and survival of cancer cells, as well as other host-dependent processes that promote cancer growth. ZD1839 has broad antitumor effect against various types of solid tumors, including NSCLC, colorectal, breast, and head and neck cancers [12-14], and has been clinically approved for the treatment of advanced NSCLC. Moreover, various additive/cooperative effects have been reported for many kinds of solid tumors both *in vitro* and *in vivo* when ZD1839 is combined with other chemotherapeutic agents or radiotherapy [15-17]. Based on the above studies, ZD1839 is anticipated to exert antitumor effects on ESCC. When clinically assessed in a Phase II trial as second-line treatment for advanced esophageal cancer, in which 9 out of 36 enrolled patients had ESCC, a total of 3 patients (10%) had partial responses and seven patients (23%) had stable disease [18]. The results were less than ideal and multidisciplinary strategies are required.

Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) is a member of the TNF family and a potent proapoptotic ligand in broad variety of tumor cells but not in most normal cells [19]. Interestingly, several recent reports have shown that phosphorylated active Akt protected cancer cells against TRAIL-induced apoptosis [20, 21], and that EGFR-TKI can inactivate Akt [22]. In this regard, a recent study also reported that various ESCC cell lines were resistant to TRAIL including TE8 cells [23]. These findings led us to hypothesize that ZD1839 might potently enhance TRAIL-induced apoptosis of TRAIL-resistant cancer cells via inhibition of Akt phosphorylation secondary to EFGR TK inhibition. To test this hypothesis, we

designed the present study with the following aims. First, to characterize the antitumor properties of ZD1839 in ESCC cell lines. Second, to determine the intracellular mechanisms of action of ZD1839 alone and/or when combined with TRAIL by examining inhibition of EGFR TK by ZD1839 on the phosphorylation of Akt and TRAIL-mediated apoptosis of ESCC cells.

The results showed that inhibition of EGFR mediated Akt phosphorylation by ZD1839 led to Bcl-xL inactivation and caspase-9 activation in TE8 cells, thereby promoting apoptosis of cells through a mitochondrial-dependent apoptotic pathway. The findings suggest that treatment with ZD1839 alone or in combination with TRAIL is a promising strategy for ESCC.

2. Materials and methods

2.1 Cells and culture conditions

The human ESCC cell lines (TE1, TE6, TE8 and TTn) were propagated in monolayer cultures in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 units/ml penicillin, and 100 mg/ml streptomycin. ESCC cells were maintained under 5% CO_2 at 37°C. Cells were treated with the indicated concentration of ZD1839 (10-100 µM) or TRAIL (0.1-500 ng/ml) for the indicated interval. TE8 cells were treated with 1 µM ZD1839 combined with or without 100 ng/ml TRAIL for various time intervals (1, 3, 6, 12, and 24 h). Cells were harvested, and prepared for each experiment as described below.

2.2 Reagents

ZD1839 (Gefitinib, 'Iressa') was a kind gift from AstraZeneca (Macclesfield, United Kingdom), dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at –20 ^{o}C as a master stock solution. The drugs were diluted in fresh media before each experiment. EGF was purchased from Sigma Chemical Co. (St. Louis, MO), dissolved in DMSO at 100 µg/ml, and stored at –20°C. Human recombinant TRAIL was purchased from Calbiochem (San Diego, CA), dissolved in phosphate-buffered saline

(PBS) at 100 µg/ml, and stored at –80 ^{o}C. In all experiments, the final DMSO

concentration was < 0.1%.

2.3 Cell proliferation assay

The cell growth inhibitory effects of ZD1839 or/and TRAIL on ESCC cells were

determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) assay as described previously [24]. Cell viability was calculated: 100 x

$A_{treatment}/A_{control}$ (%). The IC_{50} value was the concentration of ZD1839 that resulted in

50% inhibition in absorbance according to the Curve Fit 1.3 program.

2.4 Flow cytometry analysis

Cells treated with various concentrations of ZD1839 were harvested in 0.125%

trypsin, washed twice in PBS with 2% FCS. After centrifugation, 1×10^{6} cells were

resuspended in PBS containing 0.1% Triton X-100 and 10 µg/ml RNase A (Sigma)

for 5 min at room temperature. The samples were then stained with 10 µg/ml PI

(Sigma) and incubated at room temperature for 30 min. Cell cycle analysis was

determined by the fluorescence-activated cell sorter (FACScan) flow cytometer with

the Cell Quest software program (Becton Dickinson Labware, Franklin Lakes, NJ).

All experiments were repeated at least twice.

2.5 Antibodies and Western blot analysis

The following antibodies were used: anti-EGFR, cyclinD1 and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-EGFR (BioSource International Inc., Camarillo, CA), anti-HER2 (Neomarkers, Fremont, CA), anti-phospho-tyrosine (Upstate Biotechnology, Lake Placid, NY), anti-phospho-Akt (Ser473, Upstate USA, Inc., Charlottesville, VA), Akt, Caspase-3, -7, -9, and PARP (Cell Signaling Technology, Beverly, MA), anti-p21, p27, cdk4 (Transduction Laboratories, Lexington, KY), and anti-β-actin (Sigma). Cells were washed twice in cold PBS and collected, then lysed in lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% glycerol, 0.5% NP40] containing proteinase inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.5mM Na_3VO_4). After 20 min on ice, the lysates were spun at 14,000 rpm in a microcentrifuge at 4^oC for 10 min. The supernatants were used as whole cell extracts. Protein concentration was determined using the Bio-Rad protein determination method (Bio-Rad, Richmond, CA). Equal amounts (50 µg) of proteins were boiled for 5 min and electrophoresed under reducing conditions on 6-12.5% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to a Hybond-polyvinylidene difluoride (PVDF) transfer membranes (Amersham Life Science, Buckinghamshire, UK), and incubated with the primary, followed by peroxidase-linked secondary antibody. An Amersham ECL chemiluminescent western system (Amersham) was used to detect secondary probes.

2.6 Caspase-3 activity assay

TE8 cells in 10-cm dishes were treated with ZD1839 in the presence or absence of TRAIL for 3 h and then harvested in cell lysis buffer. The cell lysates were collected by centrifugation at 12,000 rpm for 10 min, and their protein concentration was evaluated using a Protein Assay (Bio-Rad). Caspase-3 activity in cell lysates (25 µg) was determined by using caspase-3 Fluorometric Protease Assay Kit (Medical & Biological Laboratories, Nagoya, Japan) according to the instructions provided by the manufacturer. Fluorescence was detected using a fluorescence spectrometer at 400 nm excitation and 505 nm emission. The relative caspase activity was determined as the level of substrate-labeled AFC (7-amino-4-trifluoromethyl coumarin) cleavage in each treated cell compared with that in control cells.

2.7 Statistical analysis

Data are expressed as mean ± SD. Differences in the cell growth inhibition effect for the treatment groups were compared using the Student's *t*-test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1 Overexpression of EGFR and effects of ZD1839 on cell growth in human ESCC cells

ESCC cell lines expressed different levels of EGFR, HER2, phosphorylation of EGFR, and phosphorylation of tyrosine determined by Western blot analysis (Fig. 1A). EGF, the specific ligand of EGFR, increased the level of phosphorylation of EGFR and tyrosine, and was used as positive control in further experiments. TE8 cells were autophosphorylated to EGFR without EGF.

We next determined the effects of ZD1839, an EGFR-TKI, on the cell proliferation using the MTT assay. Cells were treated with various concentrations (0.01-100 μM) of ZD1839 for 3 days. ZD1839 induced differential cell growth inhibitory effects on four esophageal cancer cells (Fig. 1B). Treatment of TE1 cells with 1 μM of ZD1839 decreased cell survival rate by over 50%, suggesting that TE1 cells were the most sensitive to ZD1839 among the cell lines used. However, treatment with 1 μM of ZD1839 had little effect on the cell growth of TE8. These results indicated that the effect of ZD1839 did not correlate with the level of EGFR expression. The mean IC_{50} values for ZD1839 were TE1 0.13, TE6 18.73, TE8 16.29, and TTn 8.96 μM.

3.2 ZD1839 treatment induces G_1 phase arrest and up-regulation of p27

To elucidate the mechanisms of ZD1839 induced growth inhibition in ZD1839-sensitive TE1 cells, we first tested the effects of ZD1839 on the phosphorylation of EGFR. ZD1839 treatment for 24 h down-regulated phosphorylation of EGFR in TE1 cells, while basal EGFR protein level did not change over the same period (Fig. 2A). These data confirmed that ZD1839 effectively inhibited TK-related phosphorylation of EGFR. Moreover, treatment with ZD1839 resulted in inhibition of TE1 cell growth but not control cells, as confirmed by morphological examination (Fig. 2B). To determine the effects of ZD1839 on the cell-cycle profile, TE1 cells were treated with 0-10 μM ZD1839 for 24 h and then the cell cycle distribution was analyzed by FACS. The cell cycle was arrested at G_1 phase in a ZD1839 dose-dependent manner, but sub-G_1 fraction indicative of apoptosis was very small (Fig. 2C). Western blot analysis performed to evaluate the molecules related to the cell cycle regulation revealed that ZD1839 treatment of TE1 cells led to a slight increase in p21 protein level, and marked increase in p27 protein level in dose- and time-dependent manners, but had no effect on cyclin D1 and cdk4 protein levels (Fig. 2D, E). These results suggest that ZD1839 treatment induced cell cycle arrest at G_1 phase via induction of p21 and p27 rather than apoptosis.

3.3 ZD1839 inhibits phosphorylation of EGFR and PI3k-Akt signaling pathway

ESCC cells expressed variable levels of the Akt and phosphorylated Akt, and EGF activated phosphorylation of Akt (Fig. 3A). In TE6 and TE8 cells, Akt was constitutively phosphorylated and the level of phosphorylated Akt expression did not

change with EGF treatment. TE1 cells showed no phosphorylated Akt expression following EGF treatment, whereas phosphorylation of Akt was induced with EGF in TTn cells. We next examined whether ZD1839 treatment inhibits phosphorylation of Akt as a downstream molecule of EGFR in TE8 cells. TE8 cells were pretreated with 1 μM ZD1839 for 30 min, and then 10 ng/ml EGF was added for the indicated time intervals. As we expected, ZD1839 inhibited both the phosphorylation of EGFR and Akt in TE8 cells within 1 hour of ZD1839 treatment, while basal EGFR and Akt protein levels were not changed (Fig. 3B). These results indicated that ZD1839 does not only inhibit phosphorylation of EGFR expression, but also blocked the activation of phosphatidylinositol 3-kinase (PI3k)-Akt pathway, including phosphorylation of Akt. As 1 μM of ZD1839 was sufficient to inhibit phosphorylation of EGFR as well as Akt, this concentration was used for further combination experiments with TRAIL.

3.4 ZD1839-augmented TRAIL-induced apoptosis of TE8 cells involves activation of caspases and inactivation of Bcl-xL

Recently, one report demonstrated that various ESCC cell lines including TE8 cells were resistant to TRAIL [23]. Other studies showed that activation of the PI3k-Akt pathway rendered several types of cancer cells resistant to TRAIL-induced apoptosis [20, 21]. In the next step, we investigated whether ZD1839 could alter the TRAIL sensitivity of TE8 cells via Akt inactivation. In fact, TE8 cells overexpressing phosphorylated EGFR and Akt, and treatment with 500 ng/ml TRAIL did not significantly inhibit cell growth, suggesting that TE8 cells are resistant to TRAIL.

Moreover, TTn cells were also insensitive to 500 ng/ml TRAIL, and more resistant to

TRAIL treatement than TE8 cells (Fig. 4A). We further examined the effects of

TRAIL on the growth of TE8 and TTn cells *in vitro* in the presence or absence of

ZD1839. As shown in Fig. 4B, a synergistic increase in the growth inhibition rate was

observed in TE8 cells treated with 100 ng/ml of TRAIL combined with 1 μM of

ZD1839 for 24 h. TRAIL alone inhibited cell growth in approximately 17.5% of the

cells, while treatment with ZD1839 and TRAIL further inhibited cell growth in 46.1%

of the cells. ZD1839 alone had little effect on tumor cell viability (11%). We also

found that treatment of TTn cells with 2 μM ZD1839 and 500 ng/ml TRAIL resulted

in a marked inhibition of cell growth compared with TRAIL alone or ZD1839 alone;

the magnitude of the synergism in TTn cells, however, was less dramatic than that in

TE8 cells, presumably due to the increased resistance in TTn cells. Microscopic

examination showed numerous dead TE8 cells treated for 24 h with TRAIL plus

ZD1839, indicating apoptosis of these cells (Fig. 4C). Interestingly, ZD1839 or

TRAIL alone had little effect on caspase-3 activity, but the combination with ZD1839

and TRAIL resulted in a marked increase of caspase-3 activity 3 h after treatment (Fig.

4D). In addition, the result of Western blotting showed no effect for ZD1839 or little

effect for TRAIL on the processing of caspase-3 or PARP. In contrast, the

combination of ZD1839 and TRAIL resulted in cleavage of both of caspase-3 and

PARP (Fig. 5A). Taken together, these results indicate that ZD1839 augmentes

TRAIL-induced apoptosis of TRAIL-resistant TE8 cells.

We further examined the effects of ZD1839 and TRAIL on changes of Bcl-xL,

Bcl-xS and caspase-9 protein by western blotting. ZD1839 induced a slight induction of Bcl-xS protein. Moreover, ZD1839 plus TRAIL downregulate Bcl-xL, and activated caspase-9 (Fig. 5B). These findings suggest that the augmentation of TRAIL-induced apoptosis by ZD1839 is mediated by an increase in active caspase-9, which is considered to be mediated by down-regulation of Bcl-xL.

4. Discussion

ESCC is an aggressive human malignancy and prognosis is poor except for a few patients with early stage ESCC. Although the currently used chemotherapeutic agents have improved the outcome of patients with advanced ESCC, their efficacies are still limited and they may cause serious toxicity. Therefore, there is a general agreement for the identification of new therapeutic targets and development of new agents with high efficacy and tolerability [25, 26]. Recently, a wide variety of molecular targets involved in the initial development and progression of ESCC have been identified. Certainly, EGFR could potentially be one such factor as it is overexpressed in most ESCCs [4]. These findings led us to test the antitumor effect of ZD1839 against ESCC cells.

The results of cell proliferation assay showed that ZD1839 inhibited the cell growth of four ESCC cell lines in a dose-dependent manner. The cell growth inhibitory effect of ZD1839 varied, with TE1 cells being the most sensitive, TTn cells being moderate, and TE6 and TE8 cells being the least sensitive. Although TE8 cells overexpressed both non-phosphorylated and phosphorylated EGFR, which were much more than TE1 cells, the antitumor activity of ZD1839 on TE8 cells was less than that on TE1 cells. This means that the antitumor activity of ZD1839 against ESCC cells is not related to EGFR expression or phosphorylation of EGFR proteins. These results are consistent with those of previous study, which showed that the growth inhibition of several cancer cells by ZD1839 was dose-dependent but did not correlate with the

expression level of EGFR [27].

ZD1839 inhibited phosphorylation of EGFR at concentrations between 1.0 and 10 μM in TE1 cells. In addition, the results of flow cytometric analysis showed that ZD1839 resulted in a dose-dependent increase of cell cycle arrest at G_1 phase in TE1 cells, whereas these effects were not observed in other cell types (data not shown). This diversity in cell cycle arrest may account for the differences observed in growth inhibition between TE1 cells and other cells in MTT assay. Several reports have previously shown that EGFR inhibition and resultant increase in p27 expression correlated with accumulation of cells with cell cycle arrest at G_1 phase. When p27 expression was blocked by an antisense oligonucleotide, only a small proportion of cells in G1 phase was observed [28, 29]. Similar findings were been observed in human malignant pleural mesothelioma cell lines treated with ZD1839 [30]. Our results are consistent with these findings. We also demonstrated that ZD1839 induced a slight increase in p21 protein expression in TE1 cells. p21 is known as an inducer of cell cycle arrest at G_1 phase [24]. In this regard, it has been reported that AG1478, an ErbB kinase inhibitor, reduced cyclin D1 and cdk4 before it increased p27 expression in BT-474 breast cancer cells [31]. Cyclin D1 and cdk4 are well known as the main regulators of cell cycle progression through the G_1-S transition. However, our results showed that ZD1839 treatment had no effect on cyclin D1 and cdk4 in TE1 cells. The underlying mechanisms of the differences in TE1 and other ESCC cells remain to be clarified in future studies.

Akt/PI3K, the downstream signaling of ErbB, is a key molecule in the control

of neoplastic cell proliferation, apoptosis, and angiogenesis [32-34]. Inhibition of PI3K signaling pathway by various chemicals delays tumor development and enhances the activity of death-inducing ligands, such as TRAIL [35, 36]. In addition, previous reports showed that the PI3K signaling pathway plays an essential role in regulating cells to escape from TRAIL-induced apoptosis [20, 21, 37-40]. Our results of MTT assays showed that ZD1839 combined with TRAIL significantly inhibited the cell growth after 3 h treatment. Furthermore, the results of caspase-3 assay suggest that ZD1839 is a potent enhancer of TRAIL-induced apoptosis. Our results also showed that the combination treatment decreased Bcl-xL. In this context, it has been reported that overexpression of Bcl-xL in ESCC blocked TRAIL-induced apoptosis at mitochondria level [23]. Other investigators showed that EGFR inhibitors enhanced TRAIL-induced apoptosis following decrease of phosphorylated active Akt and Bcl-xL [41]. Based on these findings, we propose that stimulation of TRAIL-induced apoptosis by ZD1839 is mediated by caspase-9 activation via Bcl-xL down regulation following Akt dephosphorylation. Our future plan encompasses further examination of the mechanisms of ZD1839 and TRAIL action in TE8 cells. In summary, our results emphasized the anti-cancer properties of ZD1839 in esophageal cancer cells. Furthermore, we showed that ZD1839 augmented TRAIL-induced apoptosis of TRAIL-resistant ESCC cells by activating mitochondrial apoptotic pathway through inhibition of Akt phosphorylation. Based on these properties, both agents are potentially suitable adjuvant for the treatment of esophageal squamous cell carcinoma. Further studies on theirs efficacy and safety *in vivo* are required in order to

demonstrate potential clinical application.

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Figure Legends

Fig. 1 Effects of ZD1839 on cell growth in ESCC cells. **(A)** Detection of EGFR, phosphorylation of EGFR, HER2, and phosphorylation of tyrosine expression in human ESCC cells by Western blot analysis. Sub-confluent cells were treated with or without 10 ng/ml of EGF, and then collected after 30 min. Equivalent amounts (50 µg) of protein obtained from whole cell lysates were loaded in each lane, probed with anti-EGFR, HER2 and phosphorylation of EGFR and tyrosine antibody, and then visualized by using an ECL detection system. **(B)** Effect of ZD1839 on cell growth of human esophageal cancer cell lines. Cells were plated in each well of triplicate 96-well plates and cultured in the presence of indicated concentrations of ZD1839 for 72 h. Cell viability was determined by the MTT assay. Growth of control cells (not treated with ZD1839) was set at 100%. Each point represents the mean ± SD of three independent experiments.

Fig. 2 ZD1839 induced cell cycle arrest at G_1 phase in TE1 cells. **(A)** Western blot analysis for phosphorylation of EGFR. TE1 cells were treated with 1 µM ZD1839 for 24 h after stimulation with EGF (10 ng/ml). **(B)** Phase-contrast photomicrographs of TE1 cells treated with ZD1839 at the indicated concentrations or 0.01% DMSO were evaluated for 72 h after treatment. Note the significant and dose-dependent growth inhibition in ZD1839-treated cells. Magnification, x 100. **(C)** Effects of ZD1839 on cell cycle progression of TE1 cells. Flowcytometric analysis of

asynchronous TE1 cells treated with vehicle or the indicated concentrations of ZD1839 for 72 h. After treatment, the cells were harvested and fixed, and then stained with 10 µg/ml propidium iodide at room temperature for 30 min. DNA content and change in cell cycle parameter (G_0/G_1) were analyzed by FACScan flowcytometer, and the percentages of cells at G_0/G_1 phase were determined. Data are mean ± SD. **(D, E)**. Western blot analysis of G_0-G_1 regulatory proteins in TE1 cells. Cells were treated with the indicated concentrations of ZD1839 for 24 h **(D),** or treated with 1 µM ZD1839 for the indicated time intervals **(E)**. Equivalent amounts (50 µg) of protein obtained from the whole cell extracts were loaded in each lane, probed with antibodies as described in "Materials and methods" and then visualized by using an ECL detection system. β-actin was used to ensure equal protein loading.

Fig. 3   ZD1839 inhibits EGF-induced phosphorylation of Akt in TE8 cells. **(A)** Expression of Akt and phosphorylation of Akt in human esophageal cancer cells in the absence (mock) or presence of 10 ng/ml EGF (EGF). Phosphorylation of Akt was highly expressed in TE8 cells compared with another cell lines. **(B)** Effects of ZD1839 on EGF-stimulated EGFR phosphorylation and Akt activation in TE8 cells. EGFR and Akt were phosphorylated within 10 min of EGF treatment. The phosphorylation of EGFR and Akt was markedly inhibited by pretreatment with ZD1839 (1 µM) for 30 min. −, no pretreatment with ZD1839; +, pretreatment with ZD1839.

Fig. 4 Treatment with ZD1839 and TRAIL enhanced TE8 and TTn cell death.

(A) Effect of TRAIL on TE8 and TTn cell growth. Cells were plated in each well of

triplicate 96-well plates and cultured in the presence of various concentrations of

TRAIL for 24 h. Cell viability was determined by the MTT assay. Viability of cells of

the control group was set at 100%. Data are mean ± SD values of triplicate

determinations. **(B)** TE8 and TTn cells were plated in triplicate at a density of 3×10^3

cells/well in the 12-well culture plates, and treated with either 1 μM or 2 μM of

ZD1839 with or without TRAIL (100 ng/ml for TE8 and 500 ng/ml for TTn) for 24 h,

respectively. Cell viability was determined by MTT assay. Each point represents the

mean ± SD of three independent experiments. * $p < 0.01$, TRAIL alone versus

ZD1839 plus TRAIL; ** $p < 0.005$, ZD1839 alone versus ZD1839 plus TRAIL. **(C)**

Phase-contrast photomicrographs of TE8 cells treated with ZD1839 plus TRAIL was

described for 24 h after treatment. Cells treated with 0.01% DMSO alone were also

used as control. Note the cell death (floating cells) of ZD1839 plus TRAIL-treated

cells. Magnification, x 100. **(D)** Caspase-3 activity in TE8 cells, treated with ZD1839

(1μM) in the presence or absence TRAIL (100 ng/ml) for 3 h. Data are mean ± SD

values of triplicate determinations.

Fig. 5 ZD1839 combined with TRAIL activated caspase-3, caspase-9 and

PARP in TE8 cells. **(A)** TE8 cells were treated with ZD1839 (1 μM) and/or TRAIL

for the indicated time intervals. **(B)** Regulation of caspase-9 and Bcl-xL protein in TE8 cells treated with ZD1839 and/or TRAIL. TE8 cells treated with ZD1839 (1 μM) in the presence or absence TRAIL (100 ng/ml) for 3 h. Cell lysates were probed for both cleaved caspase-9 and Bcl-xL by in Western blot analysis.

Fig. 1

a

b

Fig. 2

a

phospho-EGFR

EGFR

ZD1839 (μM)

Control   0   0.1   1.0   10

EGF: 10 ng/ml

b

Control   ZD1839 (0.1 μM)   ZD1839 (1 μM)

c

Control

G0/G1   56.7%
S   18.1%
G2/M   25.2%

ZD1839 (0.1 μM)

G0/G1   67.0%
S   15.3%
G2/M   17.7%

ZD1839 (1 μM)

G0/G1   75.3%
S   13.0%
G2/M   11.7%

G0/G1 phase rate (%)

0.1 μM   1 μM   10 μM

Fig. 2

d

<u>24h after</u>

ZD1839(μM)

	0	0.1	1.0	10

p21

p27

Cyclin D1

cdk 4

β-actin

e

<u>ZD1839 1μM</u>

time (h)

	0	24	48	72

p21

p27

Cyclin D1

cdk 4

β-actin

Fig. 3

a

	TE1		TE6		TE8		TTn	
	Mock	EGF	Mock	EGF	Mock	EGF	Mock	EGF

phospho-Akt

Akt

β-actin

b

time (min)	0	10	30	60	10	30	60
ZD1839	−	−	−	−	+	+	+

phospho-EGFR

EGFR

phospho-Akt

Akt

β-actin

Fig. 4

a

b

Fig. 4

c

Control | ZD1839

TRAIL | ZD1839 + TRAIL

d

Fig. 5