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Characterization of sequence-dependent synergy between ZD1839 ('Iressa') and oxaliplatin

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Abstract

ZD1839 ('Iressa'), a selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), is currently undergoing preclinical and clinical evaluation in several solid tumors. The present study aimed to assess the effect of ZD1839 in combination with oxaliplatin in the colon cancer cell lines HT-29 and LoVo. For *in vitro* chemosensitivity testing, cells were treated with serial dilutions of each drug sequentially at a fixed ratio of doses that corresponded to 1/20, 1/10, 1/5, 1/2, 1, 1.5 and 2 times the individual IC_{50} values. Oxaliplatin followed by ZD1839 produced a synergistic effect. In contrast, oxaliplatin following ZD1839 exhibited an additive effect at best. Mass spectrometry examination revealed that ZD1839 modestly enhanced cellular oxaliplatin accumulation and platinum-DNA (Pt-DNA) adducts ($P > 0.05$). In additional studies, high-performance liquid chromatography revealed that oxaliplatin had no effect on ZD1839 accumulation. In contrast, ZD1839 markedly inhibited removal of Pt-DNA adducts ($P < 0.05$). With oxaliplatin treatment (1 day) followed by ZD1839 (1 day), then incubation with drug-free medium (1 day), 90% of Pt-DNA adducts remained. Apoptosis examination revealed that oxaliplatin-induced apoptosis was prolonged by sequential oxaliplatin followed by ZD1839 treatment compared with oxaliplatin alone. Further experiments revealed that ZD1839 decreased cellular γ-glutamyltransferase activity.

Conclusions: The above observations provide a mechanistic explanation for the synergy of oxaliplatin followed by ZD1839, and suggest that this treatment combination warrants further preclinical and clinical investigation.

Keywords: Colon cancer; HT-29; LoVo; EGFR-TKI; ZD1839 ('Iressa'); Sequence-dependent

1. Introduction

EGFR is expressed in the majority of human colorectal cancers and high expression of this receptor is generally associated with resistance to cytotoxic agents and poor prognosis [1,2]. In this regard, the blockade of EGFR-mediated signaling pathways has been proposed as a potential therapeutic modality for colorectal cancer. More recently, a variety of strategies to target the EGFR have been developed and these include use of anti-EGFR mAbs and intracellular TKIs [3,4].

ZD1839, a low molecular weight, synthetic anilinoquinazoline, is an orally active, selective EGFR-TKI. This agent acts by competitively inhibiting ATP binding and results in marked inhibition of cellular proliferation and increased apoptotic events [5]. This drug has shown antitumor activity in a range of solid tumors [6–8]. Recently, some *in vitro* and *in vivo* studies have shown that coadministration with ZD1839 enhances the effect of cytotoxic agents [9–11]. Interestingly, the enhancement of antitumor efficacy seems to be independent of the mechanism of action of the chemotherapeutic agents used in combination [12]. A few studies have also evaluated the

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Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; CI, combination index; Pt, platinum; HPLC, high-performance liquid chromatography; γ-GT, γ-glutamyltransferase; GSH, glutathione; IC_{50} (IC_{90}), dose that reduces 50% (90%) of cell growth; 5FU, 5-fluorouracil; Topo-II, topoisomerase II; NER, nucleotide excision repair; OD, optical density; mAbs, monoclonal antibodies; DNA-PK, DNA-dependent protein kinase.

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optimal sequence of administration by analyzing the timing of receptor manipulation and exposure to cytotoxic agents. However, these results were inconclusive: Mendelsohn and Fan [13] reported that receptor blockade after chemotherapy is more effective than before chemotherapy in their A431 xenograft model, and raised the hypothesis that subsequent receptor blockade could interfere with repair of the damage by cytotoxic agents. However, in six head and neck cancer cell line models, Magne *et al.* [14] showed the optimal sequence is ZD1839 followed by chemotherapy (5FU and/or cisplatin).

Oxaliplatin is a third-generation platinum analog with single-agent activity in some tumors. Although it displayed activity in colorectal cancer that showed initial resistance to cisplatin, carboplatin, 5FU, and irinotecan [15], to date, the overall response rates of oxaliplatin alone were 20% in first-line chemotherapy and 10% in 5FU-resistant colorectal cancer, respectively [16,17]. *In vitro* studies [18–20] have described several potential mechanisms of oxaliplatin resistance, including reduced drug accumulation resulting in reduced Pt-DNA adduct formation, increased detoxification by γ-GT and GSH, increased repair of Pt-DNA adducts by a process that appears to involve NER apparatus. These findings lead us to investigate the preclinical rationale for combining ZD1839 with oxaliplatin in colorectal cancer.

This study was undertaken in two human colorectal cancer lines (HT-29 and LoVo) that express different levels of EGFR to evaluate the optimal combination schedule. Further experiments explored the cellular pharmacology of the synergistic effect with respect to modulation of EGFR phosphorylation, cell cycle distribution, cellular drug accumulation, Pt-DNA adducts and apoptosis level.

2. Methods

2.1. Materials

Oxaliplatin was purchased from Sanofi and dissolved in PBS to prepare a 10 mM stock solution. ZD1839 was kindly provided by AstraZeneca. A 10 mM working solution in DMSO was prepared before use. These drugs were diluted with culture medium immediately before use. *Hin*d III and RNase A were purchased from TaKaRa.

2.2. Cell lines

The colon cancer cell lines HT-29 and LoVo were obtained from American Type Culture Collection and were maintained at 37° in a 5% carbon dioxide incubator. HT-29 cells and LoVo cells were routinely cultured in McCOY's 5A medium (Biowhittaker Co.) and Ham's F12 medium (Sigma), respectively, supplemented with 10% calf serum (Life Technologies, Inc.). The cells were usually exposed to the drugs when they reached approximately 70% confluence.

2.3. Evaluation of cytotoxicity and combination effect

Cells in exponential growth phase were transferred to 96-well flat-bottomed plates. 100 μL cell suspensions containing 2×10^4 viable cells were plated into each well. After 24 hr, the cells were attached to the bottom of the plate and 100 μL of drug-containing medium were added. At the end of the indicated incubation period, growth inhibition was assessed by MTT assay [21]. Dose–response curves were generated. All of the experiments were performed in triplicate.

In order to determine the effects of sequencing, cells were treated with serial dilutions of each drug sequentially or both drugs simultaneously at a fixed ratio of doses that corresponded to 1/20, 1/10, 1/5, 1/2, 1, 1.5 and 2 times the individual IC_{50} values. Fractional survival (f) was calculated by dividing the mean OD value in the drug-treated group by the OD value in the control group. The data on the effect of the drug combinations were analyzed by the method of Chou and Talalay [22]. In brief, log $[(1/f) - 1]$ was plotted against log (drug dose). From the resulting median effect lines, the x intercept (log IC_{50}) and slope m were calculated for each drug and for the combination by the method of least squares. These parameters were then used to calculate the doses of individual drugs and the combination required to produce varying levels of cytotoxicity according to the following equation:

$$\text{Dose}_f = \text{Dose}_{\text{IC}_{50}} \left[\frac{1-f}{f}\right]^{1/m} \quad (1)$$

Because the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival f could be divided into the component doses $(D)_1$ and $(D)_2$ of drugs 1 and 2, respectively. For each level of cytotoxicity, the CI was then calculated according to following equation:

$$\text{CI} = \frac{(D)_1}{(D_f)_1} + \frac{(D)_2}{(D_f)_2} + \frac{\alpha(D)_1(D)_2}{(D_f)_1(D_f)_2} \quad (2)$$

where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce survival f, $(D_f)_1$ and $(D_f)_2$ are the concentrations of the individual drugs required to produce f, and $\alpha = 1$ or 0, depending on whether the drugs are assumed to be mutually non-exclusive or mutually exclusive, respectively. In this method, the CI was used to signify antagonism (CI > 1), additivity (CI = 1), or synergism (CI < 1).

Unless otherwise indicated, experiments were repeated until three replicates yielded coefficients $R > 0.9$ for all three median effect lines. Results of multiple experiments were summarized by indicating the mean ± SD of the CI of the indicated level of growth inhibition.

In all cases, cells were exposed to oxaliplatin for 1 day, and to ZD1839 for 5 days.

Sequence I: sequential ZD1839 5-day followed by oxaliplatin 1-day.

Sequence II: ZD1839 4-day followed by ZD1839 plus oxaliplatin 1-day.
Sequence III: sequential oxaliplatin 1-day followed by ZD1839 5-day.
Sequence IV: ZD1839 plus oxaliplatin 1-day followed by ZD1839 4-day.

2.4. Immunoprecipitation and Western blotting

Total cells lysates were prepared by homogenizing the cell pellets in lysis buffer (1% Triton, 50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulphonyl-fluoride, 2 mM Na-orthovanadate, 10 mM leupeptin, 100 mM Na-fluoride, 10 mM Na-pyrophosphate) as described previously [23] from cells that were treated with ZD1839 and/or oxaliplatin schedules. Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad Laboratories). For EGFR autophosphorylation analysis, protein extracts were immunoprecipitated for 6 hr with anti-EGFR monoclonal antibody and 20 μL of protein-A/G sepharose beads (Amersham Pharmacia). Precipitates were then separated on 8% SDS–PAGE and transferred onto nitrocellulose membrane. The filters were blocked in 5% skimmed milk for 1 hr before probing with P-Tyr monoclonal antibody at 1:200 dilution. For Western blot analysis of EGFR expression, aliquots containing 50 μg protein were loaded onto a 10% SDS–PAGE and probed with anti-EGFR monoclonal antibody at 1:200 dilution for 1 hr. After the primary monoclonal antibody incubations, the filter was washed and incubated with a 1:20,000 dilution of secondary antimouse IgG horseradish peroxidase-linked antibody (Sigma) for 1 hr. After additional washes, the blots were developed by an enhanced chemoluminescence detection system (ECL-plus, Amersham Pharmacia).

2.5. Flow cytometry analysis

Cells were trypsinized into single cells, washed twice in 1 × cold PBS, then fixed in 1 mL 70% ethanol. After treatment with 2 mg/mL RNase, the cells were stained with 1 mL of 500 μg/mL propidium iodide (Sigma) and analyzed using FACScan flow cytometer (Becton Dickson). Data were interpreted using the CellFit software provided by the manufacturer.

2.6. Measurement of cellular platinum accumulation and Pt-DNA adducts

The measurement of cellular platinum accumulation was performed as described by Bible *et al.* [24]. HT-29 cells grown to 70–80% confluence on duplicate 100-mm tissue culture dishes were treated with oxaliplatin and/or ZD1839 as indicated. After incubation for the indicated period of time, cells were washed four times with ice-cold PBS and solubilized by direct addition of 3 mL of 70% nitric acid to the first of each set of two dishes, followed by serial transfer of this solution to each additional dish in each set to solubilize the remaining cells. After a 24-hr incubation at 20–22°, aliquots were removed for estimation of protein and elemental platinum. To assess protein content, samples were diluted 1:4 in nitric acid and assayed for absorbance at 356 nm. Results were compared with absorbance of known amounts of BSA in the same diluent. To assess platinum content, aliquots containing 1 mL of nitric acid extract were heated to 100° for 5 min, combined with 1 mL of 30% H_2O_2, heated to 100° for 5 min, and subjected to mass spectrometry as described below.

Pt-DNA adducts was measured as described by Walker *et al.* [25]. HT-29 cells grown to 70–80% confluence on four 100-mm tissue culture dishes were treated with oxaliplatin alone or oxaliplatin plus ZD1839 as indicated. At the completion of the incubation, cells were trypsinized and sedimented at 200 g for 10 min, washed three times with ice-PBS, and lysed in 5 mL of TEN buffer (10 mM Tris–HCl [pH 7.4 at 21°], 10 mM EDTA, 150 mM NaCl) supplemented with 0.4% SDS and 1 mg/mL of proteinase K. After incubation at 50° for 16 hr, highly purified DNA samples were prepared by extraction with phenol/$CHCl_3$ and $CHCl_3$, ethanol precipitation, RNase A treatment, phenol/$CHCl_3$ and $CHCl_3$ extraction, and *Hin*d III digestion. After aliquots (2 μg of DNA) were subjected to eletrophoresis on agarose minigels to confirm complete removal of RNA and digestion of DNA, DNA was re-extracted with phenol-$CHCl_3$ and $CHCl_3$, ethanol precipitated, resuspended in 750 μL of 0.6 M HCl, and heated to 95° for 30 min. The DNA concentration was estimated by measuring absorbance at 260 nm; elemental Pt was assayed by inductively coupled plasma mass spectrometry as described previously [25]. In brief, a Gilson AS90 autosampler operating at a rate of 0.5 mL/min was used to infuse samples into a Perkin-Elmer Sciex 6000 mass spectrometry operating at the following settings: at nebulizer flow rate, 0.9 L/min; inductively coupled plasma RF power, 1200 W; lens voltage, 8.0 V; analog stage voltage, −2100 V; pulse stage voltage, 1700 V; dwell time, 100 ns/amu. Platinum was expressed as the sum of platinum species detected at 194 and 195 amu using a program that sweeps from 1 to 263 amu 50 times per reading. Platinum standards (0.2–20 ng/mL in 0.6 M HCl) were utilized to confirm the linearity of the assay ($R \geq 0.999$), and rhodium 103 served as an internal standard. Each experiment was performed minimum three times.

2.7. γ-GT activity assay

The γ-GT activity was determined as described previously [18]. Exponentially growing cells were harvested in 0.01 M Tris–HCl (pH 8.5) and sonicated. The reaction was performed at 37° for 20 min and used L-γ-glutamyl-*p*-nitroanilide as the substrate and glycylglycine as the receptor. One unit of γ-GT is defined as the amount of activity that liberates 1 nmol of *p*-nitroaniline per min, measured at 540 nm.

2.8. Determination of ZD1839 accumulation by HPLC

Six 175 mm flasks of cells were grown to 70% confluence for each data point. HT-29 cells were exposed to 3.8 μM ZD1839 for 1 day, or 3.8 μM ZD1839 plus 10 μM oxaliplatin 1-day, or 10 μM oxaliplatin 1-day, then 3.8 μM ZD1839 for 1 day. After the indicated incubation, cells were washed thoroughly with PBS, trypsinized and centrifuged into a pellet and lysed in 1 mL of −20° methanol. Aliquots of the supernatant were analyzed using HPLC. Because of the low concentration of ZD1839 expected when cells were treated with ZD1839 1-day, concentrated sample was required. Therefore, after centrifugation, supernatants were lyophilized and residues were dissolved in 60 μL of DMSO.

The HPLC equipment consisted of Waters 600 series with a UV detector (Waters 2487 Dual λ Absorbance Dector) and a PC-based data system (Millenium, Waters). Chromatographic separation was performed on a C18 analytical column (150 mm × 4.6 mm, 10 μm) protected by a C18 guard column (10 mm × 4.6 mm, 10 μm). The mobile phase was composed of 0.2 M phosphoric acid–triethylamine buffer solution (pH 3.0) and acetonitrile (76:24, v/v) with a flow rate of 1 mL/min under isocratic conditions. The UV detector was set at 330 nm, and injection volume was 20 μL.

2.9. Apoptosis analysis

Cells were plated at 5×10^4 cells per well in a 96-well microtiter plate and grown for 24 hr. After 24 hr, the cells were treated with ZD1839 and/or oxaliplatin as indicated, samples harvested and analyzed for histone-associated DNA fragment as per manufacturer's instructions in the Cell Death ELISA^{PLUS} kit (Roche Molecular Biochemical). The test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by biotinylated antihistone-coupled antibodies, and their enrichment in the cytoplasm is calculated as absorbance of sample cells/absorbance of control cells. Enrichment factor was used as a parameter of apoptosis and shown on the *y*-axis as mean ± SD.

2.10. Statistical analysis

Statistical analysis was performed using Student's *t*-test, and P values <0.05 were taken to indicate statistical significance.

3. Results

3.1. Cytotoxicity of ZD1839 and oxaliplatin

HT-29 cells were exposed to either ZD1839 or oxaliplatin alone. As shown in Fig. 1A, marked time-dependent activity of ZD1839 was observed, with the IC_{50} of 3-day exposure (23.6 μM) being six times higher than that of 5-day exposure (3.8 μM). However, as shown in Fig. 1B, the time-dependent cytotoxicity of oxaliplatin was less pronounced, with IC_{50} of 1-day exposure (15.1 μM) being approximately 3.8 times higher than that of 5-day exposure (4.0 μM).

Based on the above results, we fixed drug exposure time, with ZD1839 5-day and oxaliplatin 1-day, to evaluate the effects of combining these two drugs. When cells were exposed to a fixed 1:4 molar ratio of ZD1839 and oxaliplatin in sequence IV, more cells were inhibited than when cells were exposed to each drug alone (Fig. 1C and D). After the log of $[(1/f) - 1]$ vs. log (drug dose) was plotted for each treatment (Fig. 1E), the x intercept and slope were determined and then used to calculate the CI. When the two drugs are assumed to be mutually exclusive, results of this analysis are equivalent to the isobologram method.

When the effects of sequence I were analyzed with the assumption of mutually exclusive effects, the CI was 1.2 ± 0.1 ($N = 3$) at the IC_{50} and greater than 1 over much of the range of concentration examined (Fig. 2A, solid line). For the sake of completeness, the CI calculated with the assumption of mutually non-exclusive drug interactions is also shown (Fig. 2A, dotted line). However, the effect of sequence II was additive, with the CI of 0.95 ± 0.1 ($N = 3$) at the IC_{50} and approached or dropped below 1 over the range of concentration examined (Fig. 2B).

When cells were treated with sequences III and IV, slight to moderate synergisms were observed. The CIs were 0.8 ± 0.1 and 0.9 ± 0.1 ($N = 3$), respectively, at the IC_{50} and <1 over the concentrations examined (Fig. 2C and D), indicating that oxaliplatin followed by ZD1839 produces greater synergism.

To determine whether the schedule-dependent synergy was cell line-specific or not, another colon cancer cell line, LoVo, was treated with ZD1839 and oxaliplatin at a molar ratio of 1:17, respectively. Similarly, synergistic effects were obtained in sequence III ($CI = 0.7 \pm 0.2$ at the IC_{50}, $N = 3$) and in sequence IV ($CI = 0.8 \pm 0.1$ at the IC_{50}, $N = 3$). Less than additive and additive effects were observed in opposite sequences, with the CI in sequence I being 1.1 ± 0.2 at the IC_{50} but <1 at IC_{90}, and in sequence II being 1.0 ± 0.1 ($N = 3$) at the IC_{50} but <1 at IC_{90}. This suggested that the effects of ZD1839 followed by oxaliplatin were synergistic at higher levels of cytotoxicity.

3.2. Effects of ZD1839 and oxaliplatin on EGFR phosphorylation

To better understand the mechanistic basis of schedule-dependent synergy of oxaliplatin and ZD1839, we studied the effects of this combination on the tyrosine phosphorylation of EGFR in HT-29 cells. As shown in Fig. 3, treatment of cells with 3.8 μM ZD1839, which is the IC_{50} concentration of 5-day exposure, and 3.8 μM ZD1839 combined with 15.1 μM oxaliplatin, which is the IC_{50} concentration of

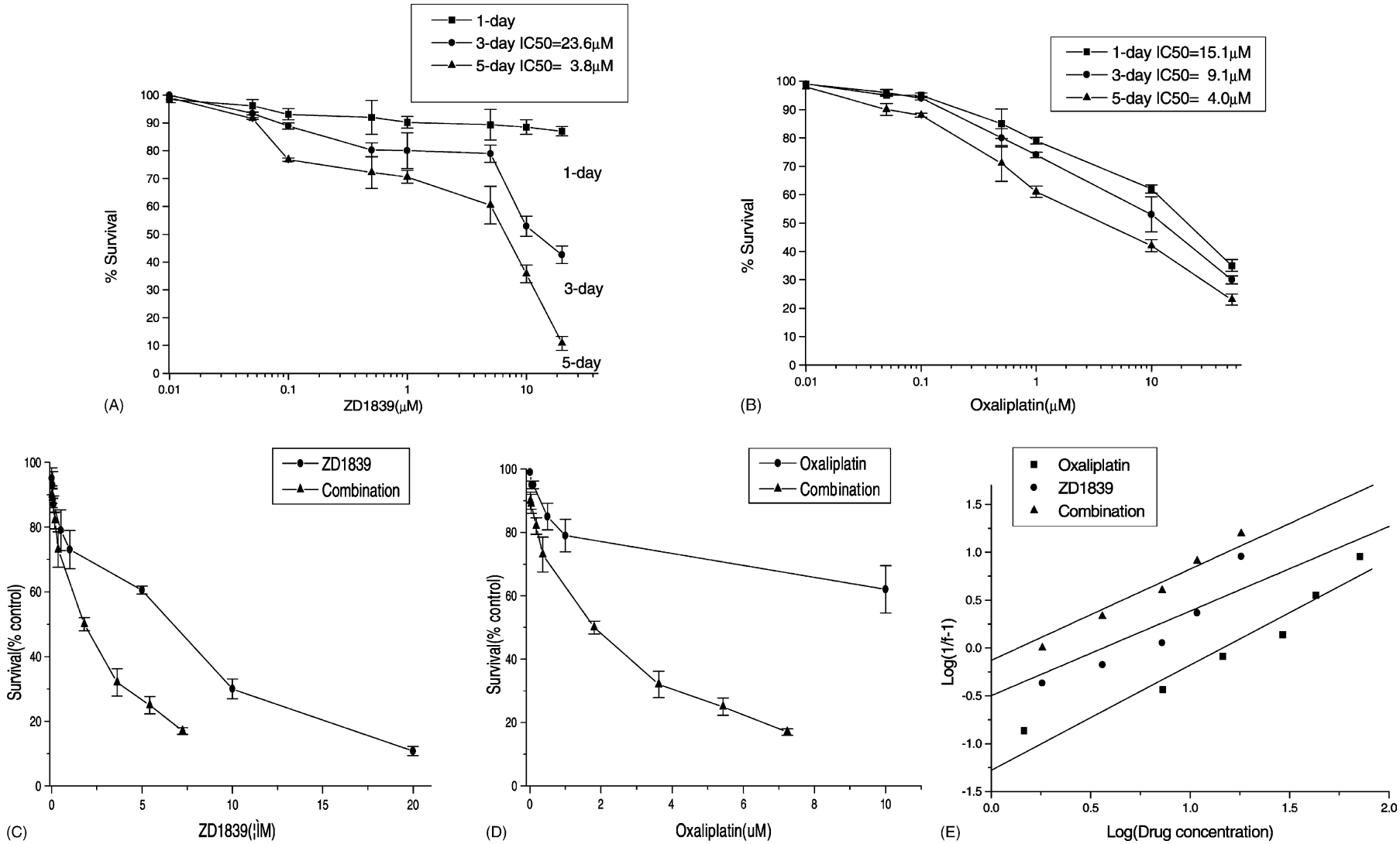

Fig. 1. Effect of treating HT-29 cells with ZD1839 and oxaliplatin. (A and B) Dose–response curves of cells treated for 1 day, 3 days and 5 days with ZD1839 alone, and oxaliplatin alone. (C and D) Cells were treated for 5 days with ZD1839 alone, for 1 day with oxaliplatin alone, or a 1:4 ratio of ZD1839 and oxaliplatin together for 1 day followed by ZD1839 for 4 days. (E) Median effect plot of data shown in C and D.

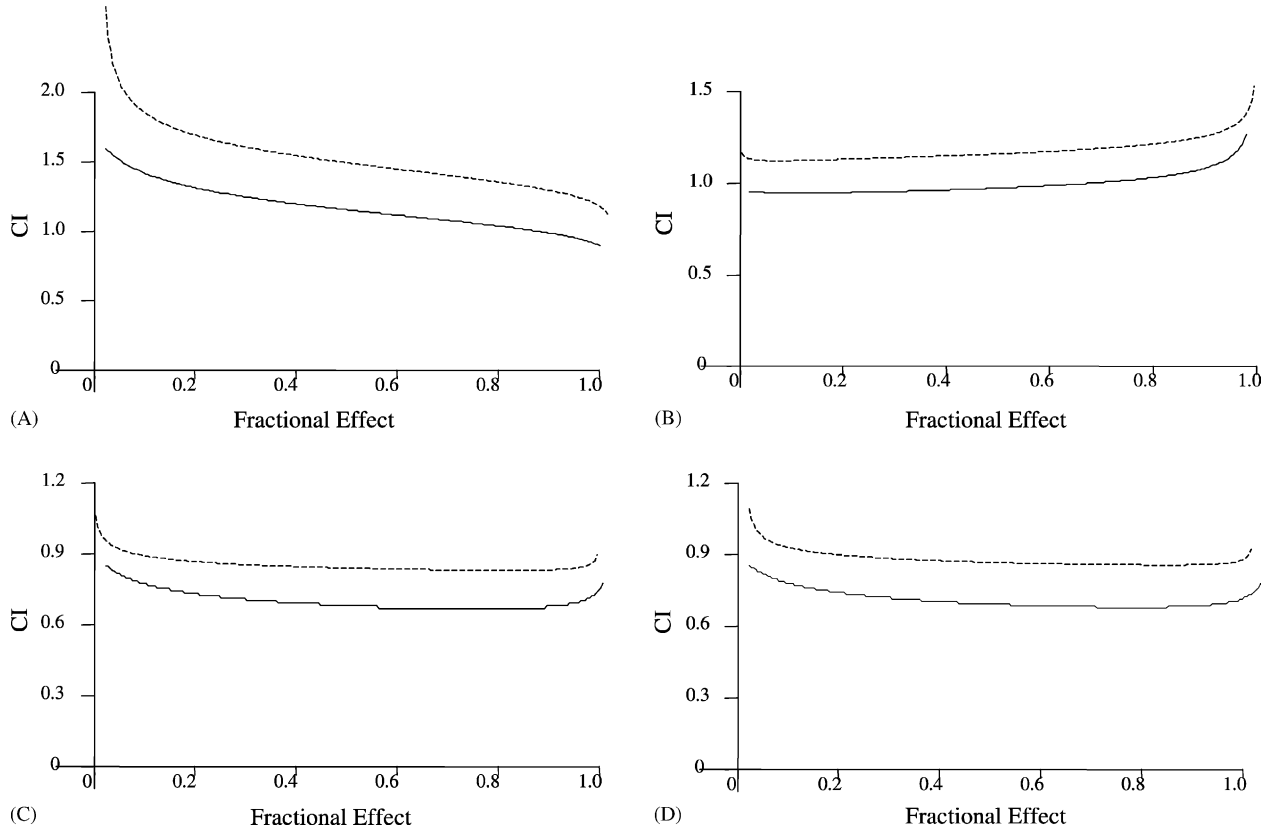

Fig. 2. Effect of sequential treatments with ZD1839 and oxaliplatin at a molar ratio of 1:4. (A–D) CI plots with the assumption that the effects of the drugs were mutually exclusive (solid line) or mutually non-exclusive (dotted line). HT-29 cells were treated with (A) sequential ZD1839 5-day followed by oxaliplatin 1-day (sequence I); (B) sequential ZD1839 4-day followed by ZD1839 plus oxaliplatin 1-day (sequence II); (C) sequential oxaliplatin 1-day followed by ZD1839 5-day (sequence III); or (D) ZD1839 plus oxaliplatin 1-day followed by ZD1839 4-day (sequence IV).

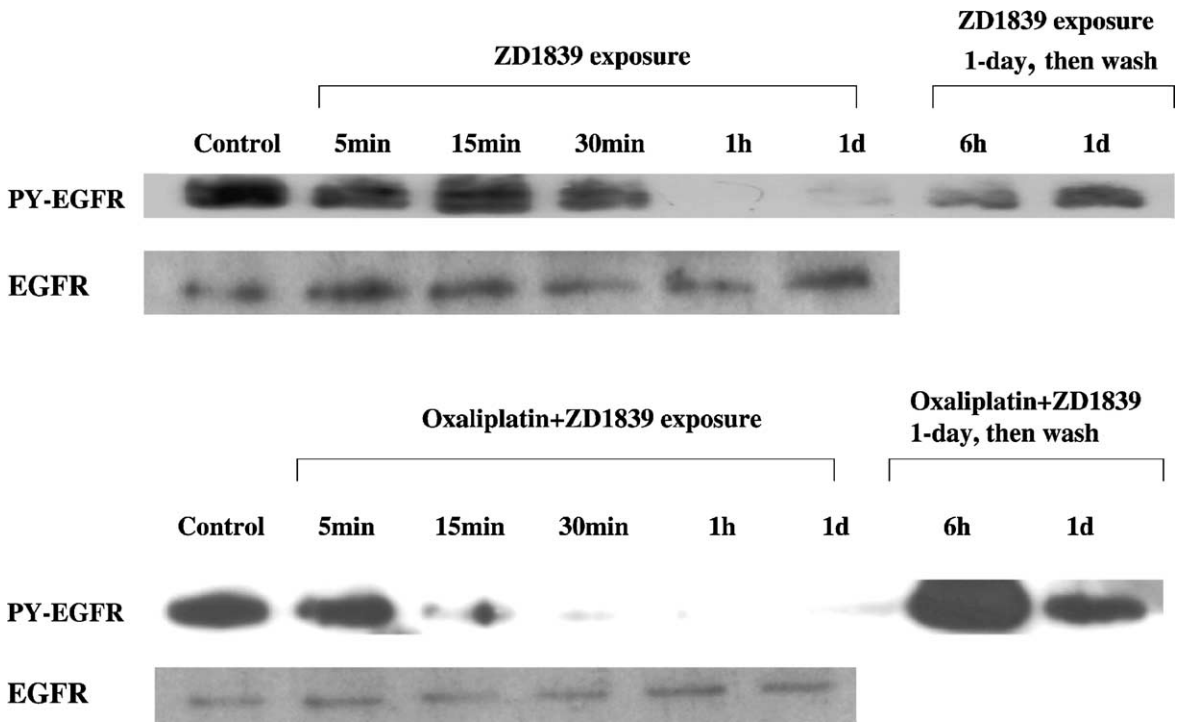

Fig. 3. Effects of ZD1839 and oxaliplatin on EGFR phosphorylation. HT-29 cells were treated for the indicated lengths of time with 3.8 μM ZD1839, 3.8 μM ZD1839 + 15.1 μM oxaliplatin; after 1-day incubation with ZD1839, and ZD1839 + oxaliplatin, cells were washed three times and then incubated with drug-free medium for the indicated time. At the completion of the incubation, samples were prepared for determination of PY-EGFR and total EGFR.

1-day exposure, resulted in a complete inhibition of EGFR phosphorylation in 1 hr. Moreover, ZD1839 combined with oxaliplatin did not alter EGFR expression. To further determine how long the inhibitory effects of ZD1839 and ZD1839 plus oxaliplatin lasted after their removal, we pretreated cells with 3.8 μM ZD1839 alone or ZD1839 plus oxaliplatin for 1 day, then washed three times and incubated cells with drug-free medium. Although in the case of simultaneous exposure to ZD1839 and oxaliplatin, the EGFR phosphorylation markedly increased after drug removal for 6 hr, in both the ZD1839 and ZD1839 plus oxaliplatin exposure settings, the inhibition of phosphorylation lasted for a similar period, with phosphorylation reaching a normal level by 24 hr after removal of ZD1839 or ZD1839 plus oxaliplatin. This observation argues against a role for oxaliplatin in potentiating the inhibition of EGFR phosphorylation by ZD1839.

3.3. Effects of ZD1839 and oxaliplatin on cell cycle distribution

The effects of ZD1839- and oxaliplatin-induced cell cycle distribution were evaluated by flow cytometry. Treatment with 3.8 μM ZD1839 or 15.1 μM oxaliplatin alone for 1 day resulted in G0/G1 phase (51.9%, $P = 0.067$) or G2/M phase (17.7%, $P = 0.071$) arrest, respectively, and a small decrease in S phase (36.8%, $P = 0.053$ and 35.2%, $P = 0.048$, respectively) compared with that in controls (G0/G1, 43.8%; S, 46%; G2/M, 9.7%). Simultaneous exposure to ZD1839 and oxaliplatin for 1 day led to marked G0/G1 phase (64.8%) ($P < 00001$) and G2/M phase (21.6%) ($P < 0.0001$) arrest, with a concurrent S-phase depletion (11.4%) relative to the controls (46%) ($P = 0.014$) (Fig. 4). These results provide a potential explanation for the observed potentiation of effects between ZD1839 and oxaliplatin.

When cells were exposed to sequential ZD1839 followed by oxaliplatin, pronounced G2/M (20.1%) phase arrest and S-phase depletion (33.8%) were observed. However, with the opposite sequence, G2/M phase arrest (30.2%), S-phase depletion (15.5%) and a concomitant increase in G0/G1 phase were more prominent. This indicated that the combination of oxaliplatin with ZD1839 is inhibiting the ability of cells to leave both G1 and G2 phase, and this inhibition is more pronounced with sequential oxaliplatin followed by ZD1839.

3.4. ZD1839 enhances the effect of oxaliplatin on total cellular platinum accumulation and platinum-DNA adducts

To evaluate the influence of ZD1839 on oxaliplatin effect, total cellular platinum content was measured by mass spectrometry after a 1-day exposure to 15.1 μM oxaliplatin in the absence and presence of 3.8 μM ZD1839. As shown in Fig. 5A, the exposure to oxaliplatin in the presence of ZD1839 and pre-exposure to ZD1839 led to a modest increase in platinum accumulation compared with that of

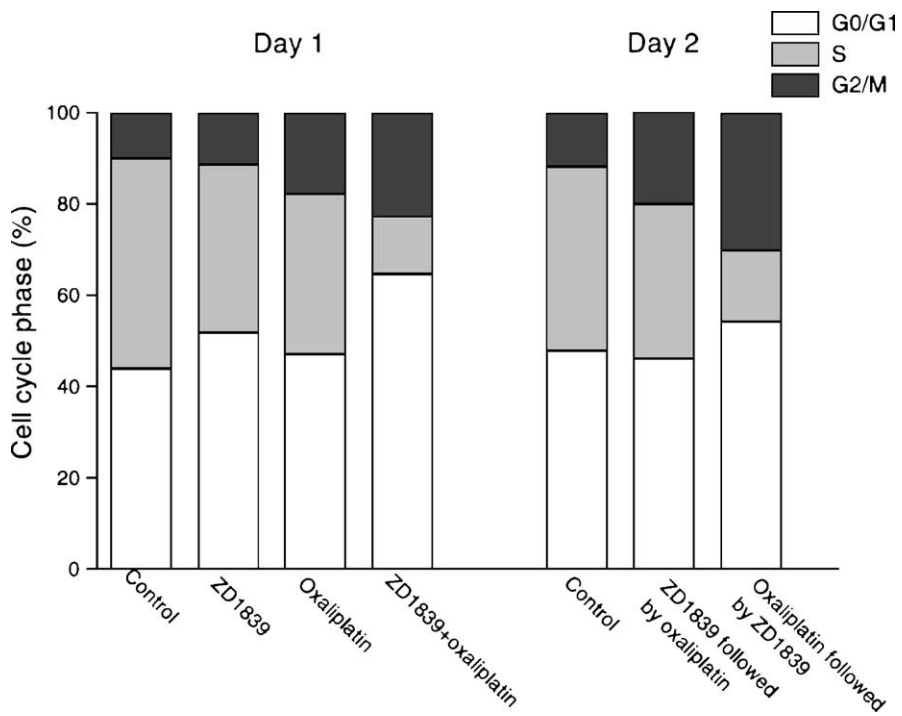

Fig. 4. Effects of ZD1839 and oxaliplatin on cell cycle distribution. HT-29 cells were treated with 3.8 μM ZD1839 alone, 15.1 μM oxaliplatin alone, ZD1839 plus oxaliplatin for 1 day, or sequential ZD1938 1-day followed by oxaliplatin 1-day, or the opposite sequence. Cells were harvested and fixed for flow cytometry analysis as described in Section 2. Columns: mean values of duplicate samples.

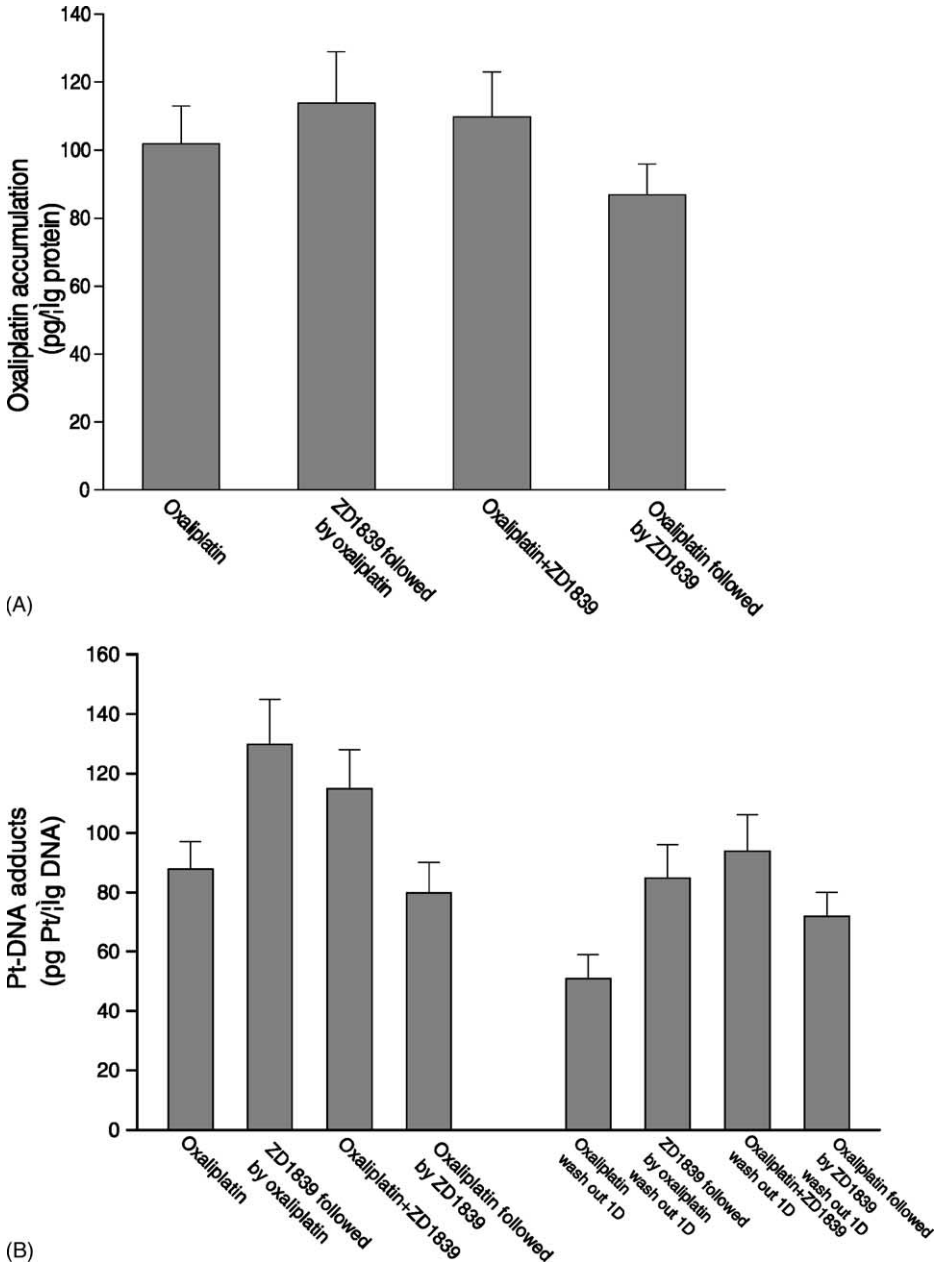

Fig. 5. Evaluation of platinum accumulation and Pt-DNA adducts in HT-29 cells. (A) Cellular platinum accumulation. Cells were exposed to 15.1 μM oxaliplatin alone for 1-day, 3.8 μM ZD1839 1-day followed by 15.1 μM oxaliplatin 1-day, oxaliplatin plus ZD1839 for 1-day, or 15.1 μM oxaliplatin 1-day followed by 3.8 μM ZD1839 1-day, then analyzed for total cellular platinum accumulation as described in Section 2. (B) Effects of ZD1839 on Pt-DNA adducts formation and on removal of Pt-DNA adducts. For Pt-DNA adducts formation, cells were treated with oxaliplatin alone, or sequential ZD1839 1-day followed by oxaliplatin 1-day, or oxaliplatin plus ZD1839 for 1 day. For removal of Pt-DNA adducts, after completion of 1-day exposure to oxaliplatin, cells were incubated in the absence or presence of 3.8 μM ZD1839 for 1-day and then harvested for measurement of Pt-DNA adducts. Error bars in A and B: +1 SD from three separate experiments.

oxaliplatin alone ($P = 0.131, 0.239$). However, when cells were exposed to oxaliplatin 1-day followed by ZD1839 1-day, the platinum content decreased compared with that of oxaliplatin alone 1-day (87 pg vs. 102 pg of platinum/μg of protein) ($P = 0.055$), suggesting an efflux of oxaliplatin after oxaliplatin removal for 1 day.

Because DNA is recognized as the primary cellular target of platinum, we also examined Pt-DNA adducts in the absence and presence of 3.8 μM ZD1839. As shown in Fig. 5B, ZD1839 had effects on the subsequent formation of Pt-DNA adducts (88 pg of platinum/μg of DNA in oxaliplatin alone, 130 pg in sequential ZD1839 followed by oxaliplatin, 115 pg in simultaneous oxaliplatin and ZD1839, and 80 pg in sequential oxaliplatin followed by ZD1839). Moreover, ZD1839 followed by oxaliplatin-induced higher Pt-DNA adducts ($P = 0.014$) than either simultaneous administration ($P = 0.042$) or sequential oxaliplatin followed by ZD1839 ($P = 0.361$). However, these effects of oxaliplatin-induced DNA damage cannot explain the maximal synergy of oxaliplatin followed by ZD1839.

These unexpected results led us to further evaluate the effect of ZD1839 on removal of Pt-DNA adducts.

3.5. ZD1839 inhibits removal of Pt-DNA adducts

For this experiment, after 1-day exposure to 15.1 μM oxaliplatin, cells were washed three times and incubated with oxaliplatin-free medium in the absence or presence of 3.8 μM ZD1839 for 1 day, and then harvested for measurement of Pt-DNA adducts. As shown in Fig. 5B, the removal of Pt-DNA adducts from single-agent oxaliplatin-treated cells is higher than from cells treated with the combination, with a 42.0% decrease in the number of adducts ($P < 0.05$). However, the schedule of oxaliplatin followed by ZD1839 led to a longer period Pt-DNA damage, with maintenance of 90% Pt-DNA adducts after oxaliplatin removal for 1 day ($P = 0.032$). This indicated that ZD1839 inhibits removal of Pt-DNA adducts, and the DNA repair process was schedule-dependent.

3.6. Effect of oxaliplatin on ZD1839 accumulation

To assess the possibility that oxaliplatin might influence cellular accumulation of ZD1839 in HT-29 cells, cellular ZD1839 levels were assayed by HPLC in the absence or presence of oxaliplatin. As shown in Fig. 6, exposure to 15.1 μM oxaliplatin for 1 day, either preceding ZD1839 or combining with ZD1839, did not increase cellular ZD1839 accumulation ($P = 0.357$, 0.248). These data rule out the possibility that oxaliplatin is enhancing cellular ZD1839 accumulation.

3.7. Effects of ZD1839 and oxaliplatin on γ-GT activity

To determine whether ZD1839 or oxaliplatin influenced the cellular γ-GT activity, HT-29 cells were treated with 3.8 μM ZD1839 alone, 15.1 μM oxaliplatin alone, or simultaneous exposure to ZD1839 and oxaliplatin for 1 day. Compared with control, the γ-GT activity was not markedly elevated by oxaliplatin ($P = 0.06$) (Fig. 7). Notably, a 25% decrease of γ-GT was observed after cells were treated with ZD1839 for 1 day ($P = 0.02$). However, this effect was counteracted when cells were treated with oxaliplatin in combination with ZD1839 ($P = 0.288$). These results raised the possibility that ZD1839 might be down-regulating γ-GT expression.

3.8. Effects of ZD1839 and oxaliplatin on apoptosis

Since the combination of ZD1839 with oxaliplatin resulted in schedule-dependent anti-proliferative effects, studies were performed to evaluate whether ZD1839 enhances apoptotic events induced by oxaliplatin. Cells were treated with 3.8 μM ZD1839 alone, 15.1 μM oxaliplatin alone for 1 day, or combination schedules as indicated for 2 days, then were incubated with drug-free medium for 1 day. As shown in Fig. 8, incubation with ZD1839 or oxaliplatin alone resulted in a small increase in apoptosis but combined treatment with ZD1839 and oxaliplatin produced a significant, synergistic increase in apoptosis. Notably, compared with oxaliplatin alone or ZD1839 followed by oxaliplatin, oxaliplatin followed by ZD1839 increased apoptotic maintenance. These results

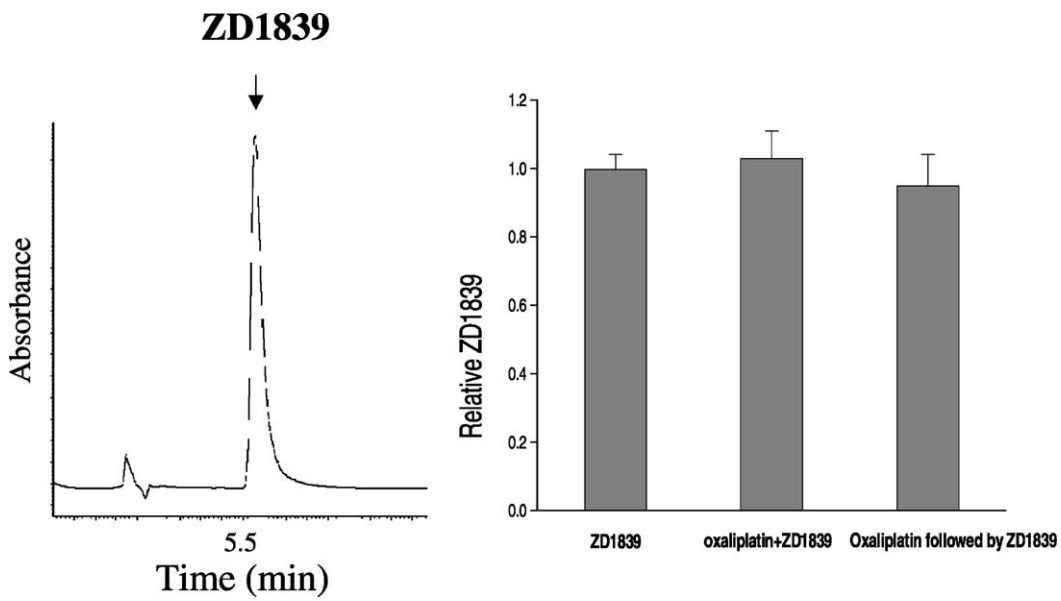

Fig. 6. Effect of oxaliplatin on ZD1839 accumulation. Left panel: HPLC chromatograph obtained when HT-29 cells were treated with 3.8 μM ZD1839 alone for 1 day, ZD1839 plus 15.1 μM oxaliplatin for 1-day, or oxaliplatin 1-day followed by ZD1839 1-day. Arrow: elution time of *bona fide* ZD1839. Extracts applied to the HPLC column were derived from equal cell numbers, based on an adjustment of extract volumes so that they corresponded to equal starting amounts of total cellular protein. Right panel: relative ZD1839 accumulation/mg protein. Results are representative of three independent experiments.

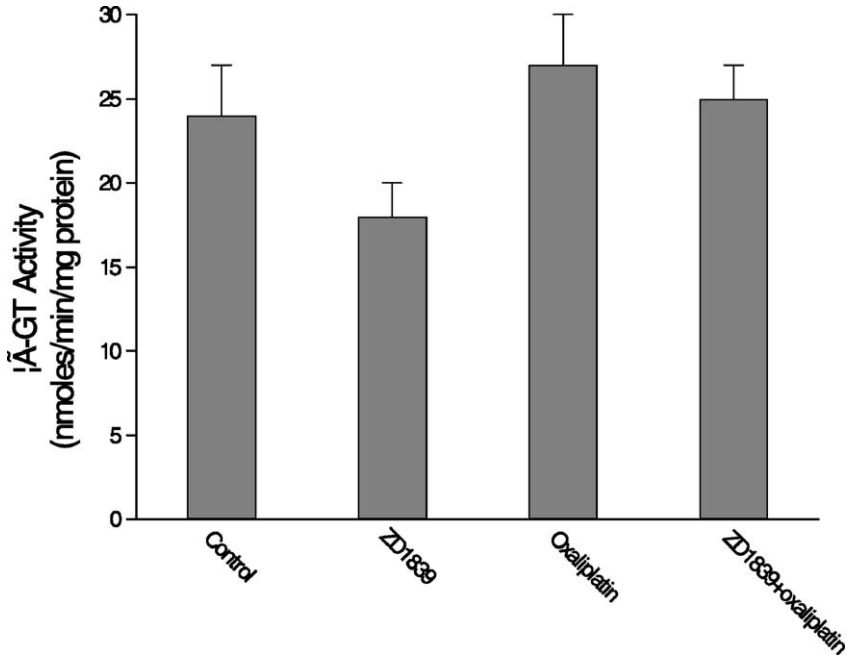

Fig. 7. Effect of ZD1839 and oxaliplatin on γ-GT activity. HT-29 cells were treated for 1 day with 3.8 μM ZD1839 alone, 15.1 μM oxaliplatin alone or ZD1839 plus oxaliplatin. γ-GT activity was then determined as described in Section 2. Results presented are averages from three separate experiments.

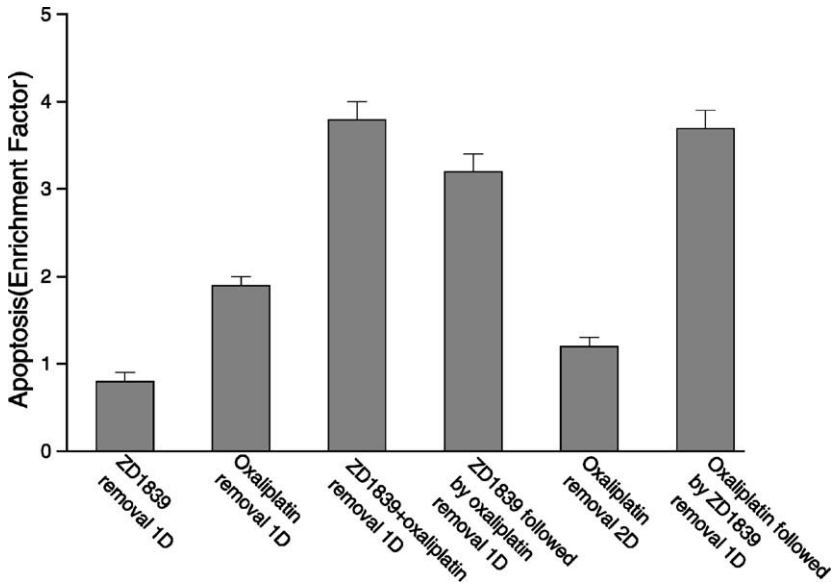

Fig. 8. Effect of ZD1839 and oxaliplatin on apoptosis. HT-29 cells were treated with 3.8 μM ZD1839 alone for 1-day, 15.1 μM oxaliplatin 1-day, or ZD1839 plus oxaliplatin 1-day, or with sequential ZD1839 1-day followed by oxaliplatin 1-day, or sequential oxaliplatin 1-day followed by ZD1839 1-day. After the completion of treatment as indicated, cells were washed and incubated for 1 or 2 days in drug-free medium and apoptosis was determined as described in Section 2. Results are presented as relative increase in apoptosis of treated cells compared with untreated control cells and represent the average of triplicate determinations.

provide a potential explanation for the schedule-dependent cytotoxicity of the combination.

4. Discussion

The combination of ZD1839 with chemotherapeutic agents is currently undergoing preclinical and clinical evaluation in several solid tumors [26–29]. The present report represents a preclinical study to investigate the effect of the combination of ZD1839 and oxaliplatin in colon cancer.

This study has demonstrated that ZD1839 administration, either simultaneous or following oxaliplatin, produced synergistic interaction in both HT-29 and LoVo cell lines (Fig. 2). The effect produced by sequential oxaliplatin followed by ZD1839 was slightly higher than that of simultaneous exposure to oxaliplatin and ZD1839

followed by ZD1839. These effects do not appear to reflect differences in cell line specificity. HT-29 cells, which express mutant P53 [30], and LoVo cells, which express wild-type P53 [31], both experienced greater than additive effects when exposed to ZD1839 simultaneously or following oxaliplatin. Furthermore, HT-29 cells express lower levels of EGFR than LoVo cells (our unpublished observation), confirming that factors other than simply the amount of EGFR may influence cancer cell sensitivity to EGFR-targeted therapy [9].

Investigation of the mechanism of schedule-dependent synergy in this study has led to a number of novel observations. Previous studies have indicated that oxaliplatin treatment induces a G2 arrest [32] that appears to afford oxaliplatin-treated cells an opportunity to repair DNA damage and to escape apoptosis before proceeding through the cell cycle. However, ZD1839, which induces G1 phase arrest and blocks cell entry into S phase, appears to enhance oxaliplatin-induced G2 arrest with a significant decrease in S phase. Notably, sequential oxaliplatin 1-day followed by ZD1839 1-day exposure induced more prominent G0/G1 phase, G2/M phase arrest and a concomitant decrease in S phase compared with simultaneous exposure or the opposite sequence (Fig. 4). The resulting decrease in S phase cells could be attributable to the apoptotic cell death in addition to the inhibition of cells leaving both G1 and G2 phase. However, the increase in G2/M phase suggests that cell cycle effects might not explain the additive or synergistic effects observed when oxaliplatin is combined with ZD1839.

HT-29 cells have been found to be inherently cisplatin-resistant with increased cellular GSH [19]. However, our results have shown that oxaliplatin alone has a modest effect on cellular accumulation (Fig. 5A), indicating that this cell line was not deficient in oxaliplatin accumulation. Additional experiments demonstrated that ZD1839 induced an increase of oxaliplatin accumulation and Pt-DNA adducts (Fig. 5B). These observations provide a potential explanation for the additive and synergistic effects observed when ZD1839 precedes or accompanies oxaliplatin. It is possible that the increase in oxaliplatin accumulation and oxaliplatin-derived Pt-DNA adducts by ZD1839 may have been due to oxaliplatin transportation.

Although the elevation of cellular oxaliplatin accumulation and Pt-DNA adduct might explain the effect of ZD1839 on the efficacy of oxaliplatin, these effects do not appear to explain the observed maximal synergy in sequential oxaliplatin followed by ZD1839. The observation that oxaliplatin neither inhibits EGFR autophosphorylation nor prolongs the inhibitory effect of EGFR autophosphorylation by ZD1839, argues against the possibility that oxaliplatin is potentiating the effects of ZD1839 by further inhibiting EGFR-mediated signaling (Fig. 3). Subsequent studies also indicated that oxaliplatin does not affect ZD1839 accumulation (Fig. 6), ruling out the possibility that oxaliplatin was directly enhancing the ZD1839 transportation. This prompted us to further examine the mechanism of schedule-dependent synergy.

Further experiments revealed that ZD1839 induces a decrease in cellular γ-GT activity (Fig. 7). γ-GT is a key enzyme in cellular GSH metabolism. Tumor use of GSH is mediated predominantly through γ-GT [33]. A previous study [18] has demonstrated that GSH appears to play an important role in tumor resistance to some chemotherapeutic agents. GSH may protect cells by binding to or reacting with chemotherapeutic agents, by preventing damage to protein or DNA, or by participating in repair processes [34]. These results are consistent with the fact that ZD1839 is augmenting the antitumor effect of oxaliplatin. It is possible that ZD1839, either accompanying or following oxaliplatin, might modulate oxaliplatin-induced DNA adducts by interfering with GSH action.

Previous studies [1,13] have indicated that the potentiation of chemosensitivity by EGFR blockade attributes to enhancement of apoptotic events. Similarly, in our study, an enhancement of oxaliplatin-induced apoptosis was observed when ZD1839 followed oxaliplatin (Fig. 8). Recent evidence has suggested a link between apoptosis and the repair of DNA damage [20]. In particular, the decrease of Pt-DNA adducts results from the increase of DNA repair [35]. Interestingly, we have shown that ZD1839 inhibited removal of Pt-DNA adducts (Fig. 5B). This effect might reflect inhibition of Topo-II. It has been suggested that Topo-II binds to platinated DNA and is involved in its repair [36]. Indeed, in agreement with this hypothesis, Braun *et al*. [37] have recently reported that ZD1839 significantly down-regulates Topo-II function in HCT/SN-38 cells, which is a SN-38-resistant colon cancer cell line. These observations raise the possibility that Topo-II might play a role in response to oxaliplatin-induced damage. It is likely that cellular Pt-DNA adducts will ''recruit'' Topo-II to DNA and increases the likelihood of ZD1839 and Topo-II interaction. On the other hand, repair of platinum-induced DNA damage appears to require NER and recombination apparatus [20,38]. It was suggested that recombination and NER pathways play independent roles in detoxification of platinum. In fact, Bandyopadhyay *et al*. [39] have demonstrated that anti-EGFR mAbs trigger a specific physical interaction between EGFR and DNA-PK in the cytosol in a variety of cell types, with DNA-PK level and activity significantly reduced in the nucleus and concurrently increased in the cytosol. Since one of the important functions of DNA-PK is to repair double-strand breaks, it implies, with a reduction of DNA-PK in the nucleus, anti-EGFR mAbs might result in the impairment of DNA activity in the nuclei by interference in EGFR signaling. However, whether ZD1839 is also involved in the modulation of DNA recombination remains to be determined.

This study indicates that ZD1839 enhances the antitumor effect of oxaliplatin in HT-29 and LoVo cell lines. The synergistic effect of oxaliplatin followed by ZD1839, is

associated with persistence of oxaliplatin-induced DNA damage by ZD1839. ZD1839 not only modestly enhances cellular Pt-DNA adducts but also markedly inhibits removal of Pt-DNA adducts, which, likewise, prolongs oxaliplatin-induced apoptosis. Previous *in vitro* studies have also shown enhanced effects when ZD1839 was combined with other cytotoxic agents, such as 5FU, and cisplatin [14]. Data on whether the favorable cytotoxic effects of sequential exposure to oxaliplatin or other cytotoxic agents followed by ZD1839 may be translated into benefits in clinical trials are awaited and further animal studies are warranted.

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