Sequence-dependent inhibition of human colon cancer cell growth and of prosurvival pathways by oxaliplatin in combination with ZD6474 (Zactima), an inhibitor of VEGFR and EGFR tyrosine kinases

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Abstract
To date, clinical studies combining the new generation of targeted therapies and chemotherapy have had mixed results. Preclinical studies can be used to identify potential antagonism/synergy between certain agents, with the potential to predict the most efficacious combinations for further investigation in the clinical setting. In this study, we investigated the sequence-dependent interactions of ZD6474 with oxaliplatin in two human colon cell lines in vitro. We evaluated the in vitro antitumor activity of ZD6474, an inhibitor of vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) and RET tyrosine kinase activity, and oxaliplatin using three combination schedules: ZD6474 before oxaliplatin, oxaliplatin before ZD6474, and concurrent exposure. Cell proliferation studies showed that treatment with oxaliplatin followed by ZD6474 was highly synergistic, whereas the reverse sequence was clearly antagonistic as was concurrent exposure. Oxaliplatin induced a G2-M arrest, which was antagonized if the cells were previously or concurrently treated with ZD6474. ZD6474 enhanced oxaliplatin-induced apoptosis but only when added after oxaliplatin. The sequence-dependent antitumor effects appeared, in part, to be based on modulation of compensatory prosurvival pathways. Thus, expression of total and active phosphorylated EGFR, as well as AKT and extracellular signal-regulated kinase, was markedly increased by oxaliplatin. This increase was blocked by subsequent treatment with ZD6474. Furthermore, the synergistic sequence resulted in reduced expression of insulin-like growth factor-I receptor and a marked reduction in secretion of vascular endothelial growth factor protein. ZD6474 in combination with oxaliplatin has synergistic antiproliferative properties in human colorectal cancer cell lines in vitro when oxaliplatin is administered before ZD6474.

Introduction
Colon cancer is the third most common type of cancer among men and women in the United States (1). The treatment of advanced colorectal cancer consisted of fluoropyrimidine-based chemotherapy for several decades. Fortunately, in the last few years, the standard care for patients with metastatic colon cancer has shifted to more active regimens consisting of combinations of irinotecan, oxaliplatin, and 5-fluorouracil. The addition of irinotecan or oxaliplatin to 5-fluorouracil/leucovorin resulted in improved response rates and progression-free survival in large, randomized trials; moreover, irinotecan-containing regimens resulted in improved overall survival (2–4). Although traditional chemotherapy has improved outcomes for patients with advanced colorectal cancer, these agents are still associated with limited efficacy, indicating that new therapies to combat this disease are urgently needed. Several novel targeted agents are being investigated both as single agents and in combination with chemotherapy to assess the potential for increased efficacy. Two of these, cetuximab, a monoclonal antibody (mAb) against the epidermal growth factor receptor (EGFR), and bevacizumab, a mAb against the vascular endothelial growth factor (VEGF) protein, have already shown clinical benefit and are commercially approved for use in advanced colorectal cancer. Thus, agents that target the EGFR and VEGF pathways have been clinically benchmarked in this disease.

The EGFR is overexpressed in many epithelial tumors, including 65% to 70% of colorectal cancer. Increased levels of the EGFR have been associated with more aggressive disease and a poor prognosis as manifested by reduced disease-free and overall survival. For these reasons, the EGFR is being exploited as an antitumor target (5–9). Recently, the results of a phase II trial revealed the significant clinical activity of cetuximab, a chimeric IgG1 mAb that binds competitively to the external domain of EGFR, in patients with advanced, irinotecan-refractory colorectal cancer. Patients who received combination...
therapy with cetuximab and irinotecan experienced a superior response rate (22.9% versus 10.8%) and prolonged time to tumor progression (4 versus 1.5 months) when compared with those who received monotherapy with cetuximab (10).

Another important pathway in the pathogenesis and progression of colorectal cancer is the VEGF pathway, which is associated with the induction of angiogenesis. Tumor-related neovascularization plays a critical role in colorectal cancer progression, and this has been well documented (11, 12). Several studies have shown that VEGF expression is increased in the progression from nonmalignant to malignant colon tumors (13–15). In fact, the expression of this growth factor was highest in carcinomas followed by adenomas and was lowest in normal mucosa (15). Both microvessel density and expression of VEGF have been associated with progression of colorectal cancer, development of metastases, and a poor prognosis (16–23). The approaches that have been proposed for blocking the VEGF receptor (VEGFR)/ligand system include a neutralizing mAb to VEGF (bevacizumab) and inhibitors of the VEGFR2 tyrosine kinase (SU11248, ZD6474, AZD2171, and PTK787/ZK22854). Many of these are currently in preclinical and clinical development, whereas bevacizumab, recently, received regulatory approval for the treatment of metastatic colorectal cancer (24–33). In fact, results from a randomized phase III trial showed that the addition of bevacizumab to irinotecan, 5-fluorouracil, and leucovorin yielded significant increases in the objective response rate (45% versus 35%) and in overall survival (20.3 versus 15.6 months) as well as improvement in progression-free survival (10.6 versus 6.2 months; ref. 34).

ZD6474 is an orally bioavailable, small-molecule inhibitor of VEGFR signaling (IC50 = 60 nmol/L for VEGF-dependent in vitro human umbilical vascular endothelial cell proliferation) and EGFR signaling (IC50 = 170 nmol/L for EGFR-dependent in vitro human umbilical vascular endothelial cell proliferation; refs. 35, 36). ZD6474 also has activity against both VEGFR3 tyrosine kinase and RET signaling (37). Chronic oral administration of ZD6474 has been shown to produce a dose-dependent inhibition of tumor growth against a histologically diverse panel of human tumor xenograft models (breast, colon, lung, and prostate vulva; ref. 35). In preclinical studies, the in vitro antitumor and antiangiogenic activity of ZD6474 is potentiated by its use in combination with paclitaxel (36). In phase I studies, daily oral ZD6474 was shown to be generally well tolerated at doses up to 300 mg/d. Common dose-related adverse events included diarrhea, rash, and asymptomatic QTc prolongation. Pharmacokinetic variables revealed that absorption and elimination of ZD6474 after a single oral dose was slow with a half-life of ~120 hours (38). ZD6474 is currently undergoing clinical evaluation and showed prolongation of progression-free survival in two recent phase II trials in non–small cell lung cancer (39, 40). Phase III trials of ZD6474 in non–small cell lung cancer have been initiated.

In certain preclinical and clinical scenarios, the addition of agents that target VEGFR or EGFR signaling pathways to standard chemotherapy seems to provide greater activity than standard chemotherapy alone. However, despite the extensive number of clinical trials conducted with combinations of chemotherapy and targeted agents, relatively little is known about the sequence-dependent antitumor effects of combining targeted therapies and chemotherapy. We hypothesized that sequence-dependent antitumor effects of combining ZD6474 with oxaliplatin may exist and that these effects may be, in part, based on modulation of the EGFR and its downstream effector pathways. A greater understanding of the combinatorial effects of chemotherapy and agents targeting specific survival pathways in cancer may lead to strategies of chemopotentiation that are distinct from those currently being tested in the clinic.

Materials and Methods

Drugs

ZD6474 [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[1-methylpiperidin-4-yl]methoxy]quinazolin-4-amine] was kindly provided by AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom). A 10-mmol/L working solution in DMSO was prepared and stored in aliquots at −20°C. Oxaliplatin was obtained from the pharmacy of the University of Colorado Health Science Center (Aurora, CO) and dissolved in water. C225 (cetuximab) anti-EGFR human-mouse chimeric anti-EGFR IgG1 mAb was supplied by ImClone Systems (New York, NY). UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), a selective inhibitor of mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2, was provided by Cell Signaling (Beverly, MA). Stock solutions were prepared at 10 mmol/L in DMSO and stored in aliquots at −80°C. These drugs were diluted in culture medium to obtain the desired concentrations immediately before use.

Cell Lines

The human colon cancer cell lines HCT-116 (p53 mutated; Ras wild-type) and HT29 (p53 wild-type; Ras mutated) were obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin and were maintained at 37°C in an incubator under an atmosphere containing 5% CO2. HT29-MEK1 (R4F) cells were kindly provided by Dr. Pamela L. Rice (University of Colorado Health Science Center, Denver, CO) and cultured in RPMI 1640 supplemented with 600 μg/mL hygromycin. The cells were routinely screened for the presence of Mycoplasma (MycopAlert, Cambrex Bioscience, Baltimore) and exposed to the drugs when they reached ~70% confluence.

Evaluation of Cytotoxicity and Combination Effects

Cellular cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, cells in the exponential growth phase were
transferred to 96-well flat-bottomed plates with lids. Cell suspensions (100 μL) containing 3,000 viable cells for HT29 and 2,000 for HCT-116 were plated into each well and incubated overnight before exposure with different concentrations of drugs. Initially, HT29 and HCT-116 cells were exposed to increasing concentrations of ZD6474 (1–21 μmol/L for 48 hours) and oxaliplatin alone (3–30 μmol/L for 24 and 48 hours) in addition to these three sequences: (a) oxaliplatin followed by ZD6474 (OXA-ZD6474), cells were exposed to oxaliplatin for 24 hours and then the medium containing drug was removed and cells were rinsed once with HBSS followed by exposure to ZD6474 for 48 hours; (b) ZD6474 followed by oxaliplatin (ZD6474-OXA), cells were exposed to ZD6474 for 48 hours and then the medium containing drug was removed and cells were rinsed once with HBSS followed by exposure to oxaliplatin for 24 hours; and (c) concurrent drug exposure (ZD6474 + OXA), cells were exposed to both ZD6474 and oxaliplatin for 48 hours.

Each drug sequence was seeded into three wells and was done in triplicate. At the end of each time point, 40 μL/well of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, of a tetrazolium compound, 3(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, were added (Promega, Madison, WI). Cells were then incubated at 37°C with 5% CO₂ for 4 hours. Plates were read on a plate reader set at 490 nm absorbance. The results of the combined treatment were analyzed according to the method of Chou and Talalay by using the CalcuSyn software program (Biosoft, Ferguson, MO). The resulting combination index (CI) is a quantitative measure of the degree of interaction between different drugs. When CI = 1, it denotes additivity; if CI >1, it denotes antagonism; if CI <1, it indicates synergism.

Flow Cytometric Analysis of Cell Cycle Distribution and of Induction of Apoptosis

Apoptosis was measured using YO-PRO-1/propidium iodide (Molecular Probes, Eugene, OR). YO-PRO-1 is a green fluorescent DNA dye that is incorporated into apoptotic cells, whereas propidium iodide is taken up only by necrotic cells. Cells stained with YO-PRO-1 and propidium iodide were analyzed by flow cytometry. Cells were cultured in T-75 flasks and, after 3 days, harvested by trypsinization and plated in six-well plates. After overnight incubation, the cells were exposed to each drug or drug combination sequence. At the end of each time point, both cells and medium were collected, washed once in Ca/Mg-containing PBS, and aliquoted for apoptosis and cell cycle analysis. For apoptosis measurements, cells were resuspended in 1 mL of Ca/Mg-containing PBS followed by the addition of 1 μL of each YO-PRO-1 and propidium iodide. Cells were placed on ice and analyzed by flow cytometry within 30 minutes. For cell cycle analysis, cells were resuspended in Krishan’s stain and allowed to incubate at 4°C for a minimum of 6 hours before analysis. Each experiment was done in triplicate.

Flow Cytometry of VEGFRs

Untreated cells were cultured in T-75 flasks and, after 3 days, harvested by trypsinization. HCT-116 and HT29 cells were washed once with wash buffer (1% fetal bovine serum, 0.1% azide in PBS). Cells were stained for 30 minutes on ice with one of the following primary antibodies: goat anti-human VEGFR1 (flt-1) at 5 μg/mL, mouse anti-human VEGFR2 (flk-1/KDR) mAb at 2.5 μg/mL, and mouse anti-human VEGFR3 biotinylated mouse mAb at 2.5 μg/mL (all from R&D Systems, Inc., Minneapolis, MN). Cells were then washed twice with wash buffer and incubated with the appropriate biotinylated secondary antibody on ice. After 30 minutes, the cells were again washed twice and all were incubated with streptavidin-phycocerythrin (BD Pharmingen, San Diego, CA) at 0.25 μg/mL for 30 minutes on ice. Cells were washed twice and then analyzed by flow cytometry. Each experiment was done in triplicate.

Flow Cytometry of EGFR Pathway Proteins

Cells were seeded into 100-mm tissue culture dishes 2 days before treatment and then exposed to drugs as described above. After treatment, cells were harvested and fixed with 0.5% formaldehyde for 10 minutes at 37°C, washed once in PBS, permeabilized with 90% methanol, and placed on ice for 30 minutes. The samples were then stored at −20°C and processed the following day. After one wash with 0.5% bovine serum albumin in PBS (wash buffer), the cells were incubated for 30 minutes at room temperature with one of the following antibodies: anti-EGFR rabbit polyclonal antibody at 1:200, anti-phosphorylated EGFR (Tyr1068) rabbit polyclonal antibody at 1:50, anti-ERK rabbit polyclonal antibody at 1:25, anti-phosphorylated ERK (Thr202/Tyr204) rabbit polyclonal antibody at 1:100, anti-phosphorylated AKT (Ser473) mouse monoclonal at 1:40, or anti-AKT polyclonal at 1:50 (all from Cell Signaling). Samples were then washed once with wash buffer and incubated with the appropriate FITC-conjugated secondary antibody diluted in wash buffer for 30 minutes at room temperature. Cells were then washed twice with wash buffer and analyzed by flow cytometry. Each experiment was done in triplicate.

Immunoblotting

Cells were seeded into six-well plates 24 hours before treatment with each drug or drug combination as described above. After treatment, cells were scraped into radioimmunoprecipitation assay buffer containing protease inhibitors, EDTA, NaF, and sodium orthovanadate. The total protein in samples was determined using the bicinchoninic acid total protein assay reagent from Pierce (Rockford, IL). Total protein (30 μg) per well was electrophoresed on a 7.5%, 10%, or 12% SDS-PAGE and electrophoretically transferred to Immobilon membrane (Hybond-P, Amersham Biosciences, Buckinghamshire, United Kingdom). Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in TBS containing 0.1% Tween 20 before overnight incubation at 4°C with one of the following antibodies: anti-EGFR rabbit polyclonal antibody at 1:2,000, anti-phosphorylated EGFR (Tyr1068) rabbit polyclonal antibody at 1:2,000, anti-p21 mouse mAb at 1:2,000, anti-cyclin D1 mouse mAb at 1:2,000, anti-phosphorylated Chk2 rabbit mAb at 1:2,000,
anti-Bax and anti-Bcl-xL rabbit polyclonal at 1:10,000, anti-cleaved poly(ADP-ribose) polymerase (Asp214) rabbit polyclonal at 1:1,000, anti-survivin mouse mAb at 1:1,000, anti-insulin-like growth factor-I receptor (IGF-IR) polyclonal antibody at 1:1,000, or anti-phosphorylated IGF-IR (Tyr 1131) polyclonal antibody at 1:1,000 (all from R&D Systems). After the primary antibody, blots were washed thrice for 15 minutes in TBS containing 0.1% Tween 20 and were incubated with the appropriate secondary anti-rabbit IgG1 horseradish peroxidase–linked antibody at 1:2,000 (Amersham Biosciences) or anti-mouse IgG1 horseradish peroxidase–linked antibody at 1:2,000 (Amersham Biosciences) for 1 hour at room temperature. After three additional washes, the blots were developed by an enhanced chemiluminescence detection system (ECL-plus, Amersham Biosciences). Each experiment was done in triplicate.

**Evaluation of VEGF-A Secretion**

HCT-116 and HT29 cells were plated in 24-well plates before the treatment with each drug or drug combination. The concentration of VEGF-A in the conditioned medium was measured using a commercially ELISA kit (R&D Systems) according to the manufacturer’s instructions. Assays were done in triplicate. Results were normalized for the number of producing cells and reported as pg of VEGF per 3 × 10^5 cells per 72 hours.

**Statistical Analysis**

We used a commercially available statistical program (InStat 3, GraphPad, San Diego, CA) to do ANOVA parametric analysis for comparing the means of different treatments.

**Results**

**EGFR and VEGFR Expression by HCT-116 and HT29**

Before carrying out the cytotoxicity assay, we characterized the EGFR and VEGFR expression of HCT-116 and HT29 cells. As depicted in Fig. 1A, the two colon cancer cell lines express higher amounts of EGFR/phosphorylated EGFR than the CEM cells (internal negative control). Although the HCT-116 cells express a slightly higher level of EGFR/phosphorylated EGFR than HT29 cells (1.75- and 2-fold higher, respectively), the proportions were similar. We also evaluated the expression of three VEGFR receptors (VEGFR1, VEGFR2, and VEGFR3) in the colon cell lines. Interestingly, both HT29 and HCT-116 showed expression of VEGFR1 and VEGFR3, whereas VEGFR2 expression was only detected in the human umbilical vascular endothelial cells (internal positive control; Fig. 1B). These results were confirmed by Western blot (data not shown).

**Sequence Dependence of Oxaliplatin and ZD6474 Interaction on Proliferation of Colorectal Cancer Cells**

To evaluate the antiproliferative effects of oxaliplatin or ZD6474 alone and in combination using three different treatment sequences, we first did a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Treatment with ZD6474 alone for 48 hours resulted in a dose-dependent inhibition of growth with an IC_{50} value of 16 µmol/L for both cell lines. Similarly, a dose-dependent inhibition of growth by oxaliplatin was observed with IC_{50} of 20 to 25 µmol/L and 7 to 14 µmol/L against HT29 and HCT-116 cells at 24 and 48 hours, respectively. We then assessed combinations of ZD6474 and oxaliplatin in the three different exposure sequences. As shown in Fig. 2, a 24-hour exposure to oxaliplatin followed by a 48-hour exposure to ZD6474 resulted in clear synergy with a CI between 0.2 and 0.5 in both HT29 and HCT-116 cells. This was significantly different (P < 0.001) to the reverse sequence that was clearly antagonistic in both cell lines, with CI between 1.5 and 1.9. Likewise, concurrent exposure was also antagonistic with CI between 1.3 and 1.6 (P < 0.001, compared with synergistic sequence).

**Cell Cycle Arrest by Oxaliplatin Is Modulated by Sequence-Dependent Combination with ZD6474**

To explore the mechanism of this sequence-dependent synergy, we investigated the effects of oxaliplatin and...
ZD6474 in the three different exposure sequences on cell cycle distribution. As depicted in Fig. 3A, in HT29 cells the treatment with oxaliplatin for 24 hours resulted in a G2-M phase arrest (39%) compared with controls (10%; \(P < 0.001\)). This effect was increased with a longer (48 hours) exposure to oxaliplatin (58%). As reported with other EGFR antagonists (41), the treatment with ZD6474 induced a small increase in G1 phase (70%) compared with controls (60%). Interestingly, the oxaliplatin-induced block in G2-M was maintained with the OXA-ZD6474 sequence (40%) but was antagonized in the reverse sequence (10%) or with concurrent exposure (22%). A similar, although less pronounced, effect was obtained with HCT-116 cells (Fig. 3B). Several proteins, such as Chk2, p21, and cyclin D1, are involved in the regulation of the cell cycle (42, 43). We next evaluated the effects of the three sequences on the expression of these proteins. The results depicted in Fig. 4 reflect that the expression of phosphorylated Chk2 in HT29 cells was markedly increased by treatment with oxaliplatin alone, although relatively unchanged by ZD6474 alone. The oxaliplatin-induced phosphorylation of Chk2 was maintained with the synergistic sequence (OXA-ZD6474) but reduced by the concurrent exposure (oxaliplatin + ZD6474) and completely blocked by the reverse sequence (ZD6474-OXA). Activation of Chk2 is known to lead to increased expression of cyclin-dependent kinase inhibitor p21 expression (42). Figure 4 shows that oxaliplatin induction of p21 was strongly potentiated by the OXA-ZD6474 sequence compared with the reverse sequence. As also depicted in Fig. 4, cyclin D1 expression was reduced by oxaliplatin and slightly by ZD6474, consistent with the G1 arrest we observed. The

**Figure 2.** Effect of the combination of ZD6474 and oxaliplatin on colon cell line. Approximately \(3 \times 10^5\) HT29 cells and \(2 \times 10^5\) HCT-116 cells were cultured in 96-well plates. Cells were treated with increasing concentration of ZD6474 and/or oxaliplatin using three different sequences: oxaliplatin for 24 h followed by ZD6474 for 48 h (A and B); ZD6474 for 48 h followed by oxaliplatin for 24 h (C and D), and simultaneous exposure of drugs for 48 h (E and F). CI values were calculated according to the Chou and Talalay mathematical model for drug interactions using CalcuSyn software. Columns, average of three independent experiments; bars, SD.
Oxaliplatin and ZD6474 Interaction Is EGFR Dependent

Because oxaliplatin seems to induce a prosurvival response in vitro, a setting in which the EGFR-based effects of ZD6474 would be expected to predominate, we next assessed the effect of the exposure sequences on EGFR expression of Bcl-xL, Bax, poly(ADP-ribose) polymerase, and survivin in HT29 cells (Fig. 6). We hypothesized that oxaliplatin would induce the expression of proapoptotic proteins because of DNA damage but at the same time produce a tumor survival response through increasing the expression of antiapoptotic proteins. Further, we hypothesized that ZD6474 would modulate the oxaliplatin-induced changes in expression of prosurvival and proapoptotic proteins in a sequence-dependent manner. Consistent with our hypothesis, poly(ADP-ribose) polymerase cleavage was strikingly induced by the OXA-ZD6474 sequence compared with the other exposure sequences and single-agent exposure. The proapoptotic protein Bax was induced by single-agent oxaliplatin, and this induction was maintained in OXA-ZD6474 and oxaliplatin + ZD6474 sequences. Notably, there was a reduction in Bax expression by the ZD6474-OXA sequence. Interestingly, the antiapoptotic protein Bcl-xL was induced by oxaliplatin alone, suggesting a prosurvival response, whereas this induction was markedly abrogated by the synergistic OXA-ZD6474 sequence and partially maintained by the other sequences. Interestingly, the antiapoptotic protein survivin was strongly induced by single-agent treatment with oxaliplatin or ZD6474, and although all three sequences led to a reduction in survivin expression compared with oxaliplatin alone, the reduction was most pronounced by the antagonistic ZD6474-OXA sequence. These results suggest that the sequence-dependent effects of the combination of oxaliplatin and ZD6474 may at least be partially explained by the ability of ZD6474 to block prosurvival responses through increasing the expression of antiapoptotic proteins. Further, we hypothesized that the synergistic OXA-ZD6474 sequence and partially reduced expression of p21, whereas the other two sequences seemed to antagonize the oxaliplatin effect on cyclin D1 expression.

Oxaliplatin-Induced Apoptosis of Colorectal Cancer Cells Is Potentiated by ZD6474

Next, we assessed the effect of these three sequences on apoptosis and apoptotic regulatory proteins. As expected, treatment with oxaliplatin for 24 or 48 hours resulted in an increase in apoptosis compared with ZD6474 for 48 hours (Fig. 5). In the OXA-ZD6474 sequence, a significant induction of apoptosis was observed, which was greater than either single-agent oxaliplatin or ZD6474 exposure as well as the other two exposure sequences. Notably, the antagonistic ZD6474-OXA sequence induced the induction of apoptosis observed with oxaliplatin at 24 hours. To elucidate further the mechanism of these proapoptotic effects by the synergistic sequence, we evaluated the synergistic OXA-ZD6474 sequence further reduced cyclin D1 expression, whereas the other two sequences seemed to antagonize the oxaliplatin effect on cyclin D1 expression.

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expression and activation as well as on the downstream effectors Akt, phosphorylated Akt, ERK, and phosphorylated ERK. As a consequence of prosurvival pathway activation, oxaliplatin treatment for 24 and 48 hours markedly increased the expression of total and phosphorylated EGFR in both cell lines as depicted in Fig. 7. In HT29 cells, this induction was blocked when oxaliplatin was combined with ZD6474 in all three sequences but with the greater reduction of EGFR activity being observed with the synergistic OXA-ZD6474 sequence. By contrast, in HCT-116 cells, the synergistic and concurrent treatments completely blocked the oxaliplatin-induced expression of total and activated EGFR, whereas the antagonistic sequence was without effect (Fig. 7). As expected, oxaliplatin exposure dramatically increased the expression of both total and phosphorylated ERK and AKT in HT29 and HCT-116 cell lines (Fig. 7). Subsequent but not prior treatment with ZD6474 significantly reduced the oxaliplatin-induced increase in the expression levels of total and phosphorylated ERK and AKT (Fig. 7). These data support the hypothesis that oxaliplatin promotes an EGFR-dependent cell survival response that is blocked by subsequent exposure to ZD6474, which may be responsible for the observed synergistic activity of this sequence. To support this hypothesis and to isolate further the EGFR dependence of these effects, we used two approaches. First, we showed in both HT29 and HCT-116 cells that similar sequence-dependent effects on cell cycle and apoptosis were obtained if oxaliplatin treatment is followed by C225 (an antibody-based inhibitor of EGFR) or U0126 (an inhibitor of MEK1; Fig. 8). Second, we made use of HT29 cells (R4F) that stably expressing a constitutively active form of MEK1 (44). The R4F cells when treated with the OXA-ZD6474 sequence both failed to undergo apoptosis (Fig. 9A) or a G2-M arrest (Fig. 9B). Together, these data suggest that the block of oxaliplatin-induced EGFR pathway by ZD6474 contributes functionally to the marked synergism exhibited by the OXA-ZD6474 treatment.

**Oxaliplatin and ZD6474 Interaction Involves Other Prosurvival Pathways**

IGF-IR-dependent signaling is another important prosurvival pathway in colorectal cancer, and activation of this receptor leads to the production of angiogenic factors, including members of the VEGF family (45). We therefore elucidated the involvement of the IGF-IR pathway in the sequence-dependent interaction of oxaliplatin and ZD6474. As depicted in Fig. 10, oxaliplatin clearly reduced the expression but not the activation of IGF-IR. Interestingly, ZD6474 alone reduced the expression of both total and phosphorylated IGF-IR (Fig. 10). Because activation of IGF-IR leads to the production of angiogenic factors, including VEGF-A, we measured VEGF-A secretion by ELISA from both HT29 and HCT-116 cells following treatment with single-agent oxaliplatin or ZD6474 and in the three sequences (Fig. 11). Oxaliplatin treatment alone inhibited VEGF-A secretion at 24 and 48 hours particularly in HCT-116 cells ($P < 0.05$ at 24 hours and $P < 0.001$ at 48 hours in HT29; $P < 0.001$ at 24 and 48 hours in HCT-116) as did ZD6474, which was more prominent in HT29 ($P < 0.001$). The block in VEGF-A secretion by single agents was clearly potentiated when the drugs were administrated in the synergistic sequence and produced an almost complete suppression (>98%) of VEGF-A secretion in both cell lines. By contrast, the other two sequences reduced VEGF secretion in a manner similar to single-agent oxaliplatin and ZD6474 (Fig. 11).

**Discussion**

The possibility of combining cytotoxic drugs with molecularly targeting agents that specifically interfere with key
pathways controlling proliferation, invasion, and/or metastatic spreading has generated wide interest. Activation of the EGFR signaling pathway is of known importance in the development and progression of human epithelial cancers. For this reason, several approaches to the inhibition of EGFR have been developed and many preclinical and clinical trials have been conducted with the aim of testing the antitumor activity of selective EGFR antagonists in combination with cytotoxic therapies. This class of targeted inhibitors has all shown successful preclinical activity, which, unfortunately, has been tempered by limited clinical success. In fact, several large phase III clinical trials of the EGFR tyrosine kinase inhibitors gefitinib (Iressa) or erlotinib (Tarceva) have failed to show any advantage of combining these agents with chemotherapy over single-agent therapy (46–48). A consideration that has arisen from

Figure 7. Effect of exposure sequences on EGFR pathway. HT29 and HCT-116 cells were treated as described previously, harvested and fixed with formaldehyde, and permeabilized with methanol. The following day, the cells were stained with EGFR, phosphorylated EGFR, ERK, phosphorylated ERK (pERK), AKT, and phosphorylated AKT (pAKT) polyclonal antibodies and analyzed by flow cytometry. Results are mean fluorescence intensity change from no primary antibody control (i.e., secondary antibody only). Columns, average of three independent experiments; bars, SD.
the “negative” results of these clinical trials is that the empirical combination of biological agents and chemotherapy may not yield positive results and that a better understanding of the mechanistic effects of these agents in combination is required. Because the focus of our group is on the development of targeted agents against gastrointestinal malignancies, we decided to evaluate in vitro the optimum way to combine oxaliplatin, a DNA-damaging agent, and ZD6474, an inhibitor of VEGFR and EGFR signaling, against two different human colon cell lines, HT29 and HCT-116. We first characterized the expression of EGFR and VEGFR on these cell lines. As previously reported, we found that both cell lines significantly express EGFR and phosphorylated EGFR (41). Although recent studies have suggested that VEGFRs are expressed not only on endothelial cells but also on tumor cells, including breast, colon, and lung adenocarcinoma, we found that the HT29 and HCT-116 cell lines were negative for VEGFR2 expression (49–51). Interestingly, they both showed expression of VEGFR1 and VEGFR3. The functional significance of the expression of these receptors on colorectal cancer is currently under investigation in our group, but it has been shown by others that VEGFR1 on colorectal cancer is coupled to cell invasion and migration but not to proliferation (49). We next evaluated the antiproliferative effect of oxaliplatin and ZD6474 using three different schedules. Our results provide evidence of a strong synergistic antiproliferative effect in both cell lines between the two drugs only when oxaliplatin treatment preceded ZD6474, whereas the reverse sequence and concurrent treatment were clearly antagonistic. Similar to our findings, Xu et al. have shown a sequence-dependent antiproliferative synergy between gefitinib and both oxaliplatin and irinotecan, a topoisomerase I inhibitor (41, 52). In addition, Morelli et al. (53) have shown in an in vitro model of an EGFR-positive human esophageal cancer epithelial cell line with a functional EGFR-dependent autocrine growth pathway that the potentiation of the antiproliferative activity of selected cytotoxic drugs, such as three platinum derivatives (cisplatin, carboplatin, and oxaliplatin) and taxanes (paclitaxel and docetaxel), by combined treatment with three EGFR inhibitors (gefitinib, cetuximab, or ZD6474) is schedule and sequence dependent.

To further investigate the mechanisms underlying the synergistic interaction of ZD6474 with oxaliplatin, we examined the effect of the three sequences on cell cycle phase progression and regulatory proteins. As previously reported, treatment of cells with oxaliplatin, a DNA-damaging agent, induced a G2-M arrest allowing for DNA repair before continued cell cycle progression (41). We observed that the oxaliplatin-induced G2-M phase block was maintained in the OXA-ZD6474 sequence, whereas antagonized in both reverse and concurrent treatments. The G2-M phase is regulated by various checkpoint proteins, including Chk2 and p21, a cyclin-dependent kinase inhibitor. Agents that induce DNA damage activate Chk2 by phosphorylation at Thr68, which leads to increased expression of p21 and ultimately a block in cell cycle progression (42). Consistent with this, we showed that oxaliplatin induced Chk2 phosphorylation and p21 expression and this was either maintained or potentiated in the OXA-ZD6474 sequence but antagonized in the other two sequences when ZD6474 preceded oxaliplatin or when both agents were given concurrently.

**Figure 8.** Effect of C225 and UO126 on cell cycle distribution. HT29 and HCT-116 cells were plated in six-well plates and treated with three different sequences: sequential oxaliplatin (20 and 15 μmol/L, respectively) 1 d followed by ZD6474 (13 μmol/L for both cell lines) 2 d; oxaliplatin (20 and 15 μmol/L, respectively) 1 d followed by C225 (20 μg/mL) or UO126 (10 μmol/L) for 2 d. After the treatment, cells were harvested and samples were stained for cell cycle and apoptosis analysis as indicated in Materials and Methods.
As reported with other EGFR signaling inhibitors, we found that ZD6474 alone increased the proportion of cells in G1, associated with a small decrease in cyclin D1 expression (43). These observations may explain, in part, the synergy and the antagonism observed. Induction of G1 arrest by pretreatment with ZD6474 before oxaliplatin may limit the DNA damage induced by oxaliplatin. Conversely, treatment with ZD6474 following oxaliplatin would be expected to potentiate the G2-M phase arrest caused by oxaliplatin and further limit the ability of the cells to repair damaged DNA and progression through the cell cycle. In support of this, we found that the synergistic sequence led to a clear potentiation of oxaliplatin-induced apoptosis, suggesting irreparable DNA damage, whereas the other two sequences, particularly the reverse sequence, antagonized this activity. The apoptosis data were then confirmed by monitoring the expression of proteins involved in mediating cell death. Thus, poly(ADP-ribose) polymerase cleavage was strongly induced only in the OXA-ZD6474 sequence, whereas oxaliplatin-induced expression of the proapoptotic protein Bax was maintained by the synergistic sequence but completely blocked by the reverse sequences. A key observation in the present study was that oxaliplatin also induced a prosurvival response as reflected by an increase in the expression of antiapoptotic proteins Bcl-xL and survivin. The oxaliplatin-induced expression of Bcl-xL was completely blocked by OXA-ZD6474 sequence. Interestingly, although oxaliplatin-induced survivin expression was reduced by all three sequences, the largest reduction was observed with ZD6474-OXA sequence. Collectively, these data suggest that treatment with ZD6474 following oxaliplatin alters the balance of proapoptotic and prosurvival proteins, ultimately leading to potentiation of oxaliplatin-induced apoptosis in human colorectal cancer cells in vitro. In these in vitro studies, we hypothesized that the prosurvival response induced by oxaliplatin was mediated by EGFR pathway. Indeed, other studies have shown activation of this pathway in response to treatment with DNA-damaging agents, such as doxorubicin and cisplatin, but not with microtubule-binding agents, such as paclitaxel and vinblastine (54). Consistent with this, oxaliplatin produced a marked increase in both the expression and the activation of EGFR and the downstream effectors AKT and ERK that was antagonized by subsequent treatment with ZD6474. This was then substantiated by the finding that similar synergistic effects were obtained when the cells were exposed to oxaliplatin followed by C225 (an antibody inhibitor of EGFR) or UO126 (an inhibitor of MEK1). In addition, maintenance of the G2-M block and potentiation of apoptosis by the OXA-ZD6474 sequence were completely blocked in HT29-MEK1 (R4F) cells expressing a constitutively active form of MEK (44). Together, these data strongly suggest that the antitumor synergistic interaction between oxaliplatin and ZD6474 depends, in large part, on reversal of ZD6474-mediated antagonism of the activation of the EGFR prosurvival pathway by oxaliplatin. EGFR activation is likely serving as a cell survival response in colorectal cancer cells exposed to DNA-damaging chemotherapeutic agents, such as oxaliplatin.

By examining IGF-IR expression and activation, we further assessed stimulation of prosurvival pathways in...
colorectal cancer cells exposed to oxaliplatin. A variety of tumors, including colon, show altered expression of IGF-I and its receptor, IGF-IR. Recently, it has been shown that IGF-I, acting through its receptor, plays an important role in multiple mechanisms that mediate human colon cancer growth, including regulation of VEGF-A secretion and angiogenesis (45). We showed that IGF-IR expression, but not activation, was down-regulated by oxaliplatin treatment. This effect was strongly potentiated only when oxaliplatin preceded ZD6474. Moreover, the modulation of IGF-IR expression and activation by the synergistic sequence paralleled regulation of VEGF-A secretion. The marked block of VEGF-A production by the synergistic OXA-ZD6474 sequence is particularly interesting as this factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005;5:1011–27. 18. Amaya H, Tanigawa N, Lu C, et al. Association of vascular endothelial growth factor expression with tumor angiogenesis, survival, and thymidine phosphorylase/platelet-derived endothelial cell growth factor expression in human colorectal cancer. Cancer Lett 2001;174:227–35. 19. Tokunaga T, Oshika Y, Abe Y, et al. Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. Br J Cancer 1998;77:998–1002.

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