Investigation of Two Dosing Schedules of Vandetanib (ZD6474), an Inhibitor of Vascular Endothelial Growth Factor Receptor and Epidermal Growth Factor Receptor Signaling, in Combination with Irinotecan in a Human Colon Cancer Xenograft Model

Teresa Troiani, Natalie J. Serkova, Daniel L. Gustafson, Thomas K. Henthorn, Owen Lockerbie, Andrea Merz, Michael Long, Mark Morrow, Fortunato Ciardiello, and S. Gail Eckhardt

Abstract Purpose: This in vivo study was designed to determine the optimal doses and schedules of vandetanib, a dual epidermal growth factor receptor (EGFR)-vascular endothelial growth factor receptor tyrosine kinase inhibitor, in combination with irinotecan in a murine xenograft model of human colon cancer.

Experimental Design: HT-29 tumor-bearing nude mice were treated with two doses of vandetanib (12.5 and 25 mg/kg/d) with or without irinotecan (100 mg/kg) using either sequential or concurrent schedules for 30 days. Tumor size was measured using standard variables, whereas the antiangiogenic response was evaluated using dynamic contrast-enhanced magnetic resonance imaging. Additionally, effects on EGFR-dependent signal transduction pathways and proliferation were assessed using immunohistochemistry. These pharmacodynamic end points were then evaluated for associations with antitumor efficacy and/or to plasma/tumor concentrations of vandetanib.

Results: The greatest antitumor efficacy was observed in the groups receiving the highest dose of vandetanib given continuously (concurrent schedule), alone or in combination with irinotecan. These dosing schedules resulted in significant effects on tumor vasculature, with decreased volume transfer constants, area under the curve, and permeability surface factor as well as increased gadolinium clearance after 30 days of treatment. In addition, these groups showed the greatest inhibition of EGFR signaling. Interestingly, tumor concentrations of vandetanib were increased by irinotecan in the concurrent schedule, possibly due to decreased tumor perfusion in this group.

Conclusions: These data suggest that higher, sustained concentrations of vandetanib (versus intermittent), alone and in combination with irinotecan, result in optimal antitumor efficacy in this model and may have implications for the design of future clinical studies with this drug.

Colorectal cancer is the second leading cause of cancer-related death in the United States. Overall survival is highly dependent on the stage of disease at diagnosis. Estimated 5-year survival rates range from 85% to 90% for patients with stage I disease to <5% for patients with stage IV disease (1). Recent advances in the field of colorectal cancer chemotherapy have resulted in increased survival and improved quality of life in patients affected by this disease (2). In addition to cytotoxic drugs, such as 5-fluorouracil/leucovorin, irinotecan, and oxaliplatin, which represent the foundation of treatment, even greater efficacy has been achieved with combinations of these standard agents with drugs that target molecules involved in the mechanisms of colorectal cancer growth and progression.

Based on the role of the epidermal growth factor receptor (EGFR) in many critical points of colon cancer biology, such as proliferation, apoptosis, angiogenesis, and metastasis, the EGFR pathway seems to be an important target for new therapeutic approaches. In colorectal cancer, the EGFR pathway, including...
the EGFR and its ligands, EGF, transforming growth factor-α, and heparin-binding EGF, is important in tumor growth and progression (3, 4). EGFR overexpression has been shown in 25% to 82% of human colorectal cancer and correlates with more aggressive disease, increased risk of metastasis, advanced tumor stage, and lymph node involvement (5–7). Recently, results from a phase II randomized clinical trial have been published comparing the efficacy of cetuximab alone, a chimeric IgG1 monoclonal antibody that binds competitively to the external domain of EGFR, in combination with irinotecan, as a second- or third-line therapy in patients with advanced, irinotecan-refractory colorectal cancer. The addition of cetuximab to irinotecan conferred a significant improvement in time to progression by reversing cellular resistance to irinotecan. Based on these results, cetuximab in combination with irinotecan has been approved by several regulatory agencies worldwide for this indication (8).

Angiogenesis is a dynamic process by which the blood supply of a tumor is provided by preexisting blood vessels and endothelial precursor cells (9, 10). Vascular endothelial growth factor (VEGF) plays a crucial role in endothelial cell proliferation, migration, invasion, and differentiation during angiogenesis by binding to the endothelial cell receptors VEGFR-1 and VEGFR-2 (KDR; refs. 10–12). Increased levels of VEGF expression have been found in a variety of human solid tumors, including colorectal cancer (13). A large number of clinicopathologic studies showed VEGF overexpression in 40% to 60% of colorectal cancer, a feature that is associated with increased vascular density and poor survival (14, 15). Moreover, several studies have shown that VEGF expression is increased in the progression from nonmalignant to malignant colon tumors and also serum VEGF levels in colorectal cancer patients are higher than those in controls and correlate with advanced stage and poor outcome (15). In preclinical studies, agents that selectively target VEGF and its receptors in colorectal cancer have shown significant antitumor effects, confirming that this ligand/receptor system is a valid target for colon cancer therapy (16–18). This approach was clinically benchmarked with the regulatory approval of bevacizumab for the treatment of metastatic colorectal cancer. Results from a phase III study showed that the addition of bevacizumab to irinotecan, 5-fluorouracil, and leucovorin in untreated patients with metastatic colorectal cancer conferred a significant improvement in both progression-free and overall survival (19).

Vandetanib (Zactima, ZD6474) is a once-daily, orally available agent that inhibits VEGFR-EGFR–dependent signaling as well as the rearranged-transfection receptor tyrosine kinase (16, 20, 21). Vandetanib has potent antitumor activity by a direct antiangiogenic mechanism (i.e., the blockade of VEGFR-2 signaling in endothelial cells) but can also directly inhibit cancer cell growth by blocking the EGFR autocrine pathway. The agent inhibits tumor growth in a broad spectrum of established human cancer xenografts in nude mice, including colorectal cancer. Preclinical studies with vandetanib have shown that this agent potentiates the antitumor activity of standard cytotoxic agents and radiotherapy, causing a marked antiproliferative and antiangiogenic activity (22). Chemopotentiation and/or radiopotentiation strategies are based on the hypothesis that the cellular damage induced by chemotherapy or by ionizing radiation can convert EGFR and/or VEGF ligands from growth factors into survival factors for cancer cells. In this situation, the blockade of these pathways in combination with cytotoxic drugs or with radiotherapy could cause irreparable cancer cell damage leading to increased programmed cell death. Furthermore, vandetanib could block neoangiogenesis more effectively than treatment with a selective anti-VEGFR-2 agent because, in addition to a direct inhibitory effect on VEGFR-2 signaling, it also could have an indirect effect on angiogenesis via blockade of EGFR-induced paracrine production of angiogenic growth factors, such as VEGF, basic fibroblast growth factor, and transforming growth factor-α, by cancer cells (20, 22).

In the last few years, an increasing number of clinical trials have been conducted combining molecularly targeted agents and conventional chemotherapy with mixed results. In fact, both cetuximab and bevacizumab do seem to potentiate the antitumor activity of standard chemotherapy regimens in metastatic colorectal cancer, whereas similar results have not been observed with combinations of gefitinib or erlotinib, two EGFR-selective tyrosine kinase inhibitors, with chemotherapy in non–small cell lung cancer (8, 19, 23, 24). Recently, it has been suggested that the identification of optimal dosing and scheduling of targeted agents is extremely important and may represent a significant challenge in combination strategies. In fact, we and other groups have shown that the combination of cytotoxic drugs and anti-EGFR or VEGFR-2 agents caused different antiproliferative effects in vitro depending on the treatment schedule (25, 26). In this context, our group has shown that vandetanib possesses antiproliferative antitumor activity in vitro, which acts in a sequence-dependent manner with chemotherapeutic agents, such as oxaliplatin and irinotecan, in two human colon cancer cell lines (27). In that study, treatment with chemotherapeutic agents followed by vandetanib resulted in enhanced antitumor activity in vitro. This effect was completely abrogated by the reverse sequence and/or the concurrent schedule of treatment with chemotherapy and vandetanib (27).

For the reasons outlined above, well-designed preclinical studies should be conducted to optimize combinations that proceed to clinical trials. Based on our previous results, in the present study, we evaluated the effects of vandetanib in a human colon carcinoma model in vivo. For this purpose, different sequences of treatment and doses of vandetanib alone or in combination with irinotecan were assessed to maximize antitumor activity. Furthermore, we sought to determine whether the incorporation of functional imaging, immunohistochemical markers, and pharmacokinetic studies would be important tools in translating these preclinical results to therapeutic strategies for patients.

**Materials and Methods**

**Drugs.** Vandetanib [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine] was kindly provided by Dr. Anderson Ryan (AstraZeneca Pharmaceuticals). This compound was made as a suspension in sterile filtered 1% Tween 80 by gentle mixing with 4-mm borosilicate glass beads immediately before use. Irinotecan was obtained from the pharmacy of the University of Colorado Health Science Center (Aurora, CO) and dissolved in sterile saline and kept at room temperature.

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Unpublished data.
Cell lines. The human colon cancer cell line HT-29 (p53 mutated; Kras wild-type) was obtained from the American Type Culture Collection. Cells were maintained on tissue culture plates in RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (HyClone) and 100 units/mL penicillin/100 µg/mL streptomycin (Invitrogen). Cells were kept in a humidified atmosphere of 95% air and 5% CO₂ at 37°C and routinely screened for the presence of Mycoplasma (MycoAlert, Cambrex Bioscience).

Tumor xenografts in nude mice. Four- to six-week-old athymic BALB/c nu/nu female mice were purchased from Simonsen Laboratories. The research protocol was approved by the University of Colorado Health Science Center Animal Care review board. Mice were caged in groups of five and kept on 12-h light/dark cycle and provided with sterilized food and water ad libitum. All of the studies were conducted in accordance with the NIH guidelines for the care and use of laboratory animals, and animals were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were allowed to acclimate for 7 days before any handling. HT-29 cells were harvested in exponential phase growth and resuspended in a 3:1 mixture of serum-free RPMI 1640 and Matrigel (BD Biosciences). Five million cells per mouse were injected s.c. into the left flank using a 23-gauge needle. The body weights were monitored ad libitum. All of the studies were conducted in accordance with the NIH guidelines for the care and use of laboratory animals, and animals were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were allowed to acclimate for 7 days before any handling. HT-29 cells were harvested in exponential phase growth and resuspended in a 3:1 mixture of serum-free RPMI 1640 and Matrigel (BD Biosciences). Five million cells per mouse were injected s.c. into the left flank using a 23-gauge needle. The body weights were monitored ad libitum.

Dynamic contrast-enhanced magnetic resonance imaging. To evaluate in vivo tumor vascularity, the animals from treatment groups 1 to 6 were assessed by magnetic resonance imaging (MRI; n = 4 for each group): group 1, vehicle; group 2, irinotecan only (100 mg/kg); group 3, vandetanib sequential (25 mg/kg); group 4, irinotecan + vandetanib sequential (100 and 25 mg/kg, respectively); group 5, vandetanib concurrent (25 mg/kg); and group 6, irinotecan + vandetanib concurrent (100 and 25 mg/kg, respectively; Fig. 1). The gadolinium-based dynamic contrast-enhanced MRI (DCE-MRI; for tumor perfusion) scans were done at baseline and after the first and third cycle. Animals were weighed, anesthetized by i.p. injection of 60 mg/kg xylazine and 10 mg/kg ketamine, and placed on the heating pad. A tail vein catheter was inserted immediately before imaging. In our mouse model, 0.1 mmol/kg gadolinium (Omniscan; 287 mg/mL), previously diluted in bacteriostatic 0.9% sodium chloride as 1:10 (v/v), was given i.v. in the tail vein of the mouse during fast T1-MRI scan series (DCE-MRI, see below). The dilution was done before each experiment and the total volume of i.v. injection did not exceed 200 µL, with 120 µL heparin-containing flush solution injected before 80 µL gadolinium solution to avoid preenhancement. The animals were then fixed in a mouse animal bed and inserted into a Bruker volume coil (36 mm diameter), tuned to the 1H frequency of 200 MHz, which was used for radiofrequency transmission and reception. The proton density and T1-weighted breath-gated MR images were done at a Bruker PharmaScan animal scanner (Bruker Medical) at 4.7 T. First, a series of fast spin-echo rapid acquisition with relaxation enhancement scans were done for tumor localization and dimension. Subsequently, a series of fast gradient-echo T1-weighted-modified driven equilibrium Fourier transform pulses were applied for total acquisition time of 15 min. The scan variables were as follows: field of view, 4.00 cm; slice thickness, 1.50 mm; interslice distance, 1.80 mm; echo time/repetition time, 9.8/120 ms; number of slices, 4; number of averages, 1; matrix size, 128 × 256; number of evolutions, 180; resolution time, 5 s; total acquisition time, 15 min. After 1 min of precontrast images, 0.1 mmol/kg Omniscan was injected using tail vein catheter. T1-weighted gadolinium-enhanced MRI scans were continuously taken for another 14 min.

All images were processed using Bruker ParaVision software. For proton density–weighted MRI, the total tumor volume was determined by multiplying the pixel volume by the number of pixels within the tumor area by hand-drawing the region of interest with “track” command from each set of slices. For DCE-MRI, the T1 signal voxel intensities were calculated for tumor core and tumor rim, adjunct...
muscle tissue, and bladder (to assess gadodiamide excretion rates and estimate gadodiamide blood levels) from each image. A compartmental pharmacokinetic model, using SAAM program (version 2; University of Washington, Seattle, WA), in which the tissue clearance of gadolinium was related to its transcapillary exchange in tumor and adjacent muscle tissue, was constructed based on T1 signal intensities. The volume transfer constants (Ktrans), the permeability surface factors, gadodiamide clearance from tumor (Q), and the areas under the T1 intensity curves (AUC; total and for the first 90 s) were reported as a quantitative assessment of tumor perfusion and permeability for gadolinium uptake.

**Immunohistochemistry.** Two frozen tumors from each group were sectioned (8-9 μm thick) and stained by immunohistochemistry as described previously (28). Briefly, the procedure generally consisted of (a) 20-min antigen retrieval using DAKO target retrieval solution, (b) 10-min peroxide block in water, (c) 1-h primary antibody incubation, (d) 30-min secondary antibody incubation with Envision+ anti-mouse or anti-rabbit peroxidase (3,3′-diaminobenzidine)-conjugated antibody (DAKO), (e) 5- to 7-min substrate incubation with 3,3′-diaminobenzidine + 20 μL chromogen (DAKO), and (f) 25-s counterstain using Hematoxylin Gill II. Antibody-specific alterations included (a) 8-min antigen retrieval step using Digest-All 2 (trypsin) solution (Zymed) for phosphorylated EGFR, (b) peroxide block in methanol for Ki-67, and (c) an additional binding block step between the peroxide block and primary antibody incubation using Biogenex power block for phosphorylated EGFR, phosphorylated AKT, and Ki-67. Primary antibodies used were as follows: phosphorylated EGFR (Thr1068, 1:25; ZyMed) rabbit polyclonal antibody, phosphorylated mitogen-activated protein kinase (MAPK; 1:50; Cell Signaling) clone 0.25 mg/mL; Zymed) rabbit polyclonal antibody, and Ki-67 (1:100, Cell Signaling) rabbit polyclonal antibody, and Ki-67 (1:100, 70 μg/mL; DAKO) clone MB-1 BM28 mouse monoclonal. To determine the percentage of Ki-67-positive cells, 1,000 cancer cells per field were counted and three fields were analyzed.

**Pharmacokinetic analysis and liquid chromatography/tandem mass spectrometry analysis of vandetanib.** Two mice from each group treated with vandetanib were used for pharmacokinetic analyses. Plasma and tumor samples were collected at the end of cycles 1 and 3, rinsed in PBS, and immediately frozen in liquid nitrogen and stored at -80°C before sample preparation for drug analysis. In the continuously dosed vandetanib groups, samples were collected - 4 h after the last dose. Analysis of vandetanib in mouse plasma and tumor was carried out using liquid chromatography/tandem mass spectrometry analysis as described previously (29). In brief, 50 μL plasma samples were mixed with 50 μL of 10 mmol/L ammonium acetate (pH 9.6) and 50 μL of 1 μg/mL trazodone (internal standard) followed by extraction with 1 mL of 1:1 ethyl acetate/pentane. For tumors, samples were homogenized using a Potter-Elvehjem tissue grinder with a polytetrafluoroethylene pestle bottom in 10 mmol/L ammonium acetate (pH 9.6) at ~ 100 mg/mL (w/v); 100 μL aliquots were transferred to another tube containing 50 ng trazodone followed by extraction with 1 mL of 1:1 ethyl acetate/pentane. The organic layer was collected following extraction, evaporated to dryness, and reconstituted in 1 mL of acetonitrile/water (1:1, v/v). Samples were analyzed with an API-3000 triple quadruple mass spectrometer (PE Sciex) with a turbo ionspray source interfaced to a PE Sciex 200 high-performance liquid chromatography system. The mobile phase was isocratic with 80% acetonitrile containing 10 mmol/L ammonium acetate and 0.1% acetic acid at a flow rate of 200 μL/min, and a Discovery HS F5, 5 μm, 120 Å, 50 × 2 mm column (Supelco), was used. Samples were quantitated by internal standard reference in multiple reaction monitoring mode by monitoring the transition m/z 475→112 for vandetanib and the transition m/z 372→176 for the internal standard (trazodone).

**Statistical analysis.** All statistical analyses were carried out using the Prism version 4.02 program (GraphPad Software, Inc.). One-way ANOVA test was done to evaluate statistically significant changes between the study groups. The correlation analysis was applied to calculate correlation coefficients between DCE-MRI end points, tumor growth, EGFR inhibition, and vandetanib tumor levels. P values of <0.05 were considered statistically significant. Significance levels were estimated using post hoc pairwise multiple comparison (Sidak or Tukey) method.

### Results

**Optimal schedule and dose of vandetanib in combination with irinotecan.** Based on our previous study (30), we used two

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**Table 1. Tumor growth characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Days 1-56</th>
<th>TTR 1,000 mm³</th>
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</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td>8.9 ± 1.2</td>
<td>17.5 ± 3.0</td>
</tr>
<tr>
<td><strong>Irinotecan</strong></td>
<td>19.8 ± 2.5</td>
<td>24.0 ± 3.1</td>
</tr>
<tr>
<td><strong>12.5-Vandetanib-Seq</strong></td>
<td>12.7 ± 1.6</td>
<td>21.3 ± 4.0</td>
</tr>
<tr>
<td><strong>12.5-Combo-Seq</strong></td>
<td>25.8 ± 5.5</td>
<td>38.8 ± 8.6</td>
</tr>
<tr>
<td><strong>12.5-Vandetanib-Conc</strong></td>
<td>19.8 ± 3.2</td>
<td>35.4 ± 7.5</td>
</tr>
<tr>
<td><strong>12.5-Combo-Conc</strong></td>
<td>30.1 ± 9.1*</td>
<td>42.5 ± 5.1*</td>
</tr>
<tr>
<td><strong>25-Vandetanib-Seq</strong></td>
<td>20.6 ± 2.5</td>
<td>29.0 ± 4.6</td>
</tr>
<tr>
<td><strong>25-Combo-Seq</strong></td>
<td>20.3 ± 4.4*</td>
<td>25.8 ± 5.7</td>
</tr>
<tr>
<td><strong>25-Vandetanib-Conc</strong></td>
<td>28.8 ± 5.1*</td>
<td>39.8 ± 6.3*</td>
</tr>
<tr>
<td><strong>25-Combo-Conc</strong></td>
<td>38.5 ± 3.3*</td>
<td>51.9 ± 1.8*</td>
</tr>
</tbody>
</table>

**NOTE:** Values are expressed as mean ± SE for each group over the given time frame.

Abbreviations: TDT, tumor doubling time; TTR 1,000 mm³, time to reach 1,000 mm³; Seq, sequential schedule; Conc, concurrent schedule.

*Significantly different (P < 0.05) from the vehicle-treated controls by ANOVA analysis with Tukey’s multiple comparison test between groups.
doses of vandetanib (12.5 and 25 mg/kg) that reflect drug exposures that could be achieved in humans. Further, to determine whether a sequence-dependent interaction between vandetanib and a standard chemotherapy, such as irinotecan, exists, we used two different treatment schedules in which vandetanib was given after irinotecan for 2 days (sequential schedule) or simultaneously (concurrent schedule; Fig. 1). Note that, to avoid confusion, we have referred to the two single-agent vandetanib schedules in the same manner as the combinations because they provided the appropriate controls: vandetanib sequential single-agent group and vandetanib concurrent single-agent group. As depicted in Fig. 2, a significant inhibition of tumor growth was obtained with irinotecan as a single-agent treatment ($P < 0.0001$ versus vehicle). Concurrent treatment with vandetanib at 12.5 mg/kg/d induced a more significant tumor growth inhibition than the sequential schedule ($P = 0.05$), although these effects were more pronounced using the higher dose of vandetanib (25 mg/kg; $P < 0.001$; Fig. 2). The combination of 12.5 mg/kg vandetanib on either schedule with irinotecan resulted in tumor growth inhibition that was significantly greater than the single agents ($P < 0.001$ for all comparisons of the combinations of irinotecan with concurrent or sequential schedule of vandetanib versus irinotecan alone or the respective schedule of vandetanib alone). Table 1 depicts the tumor doubling time and the time to reach 1,000 mm$^3$ volume of all groups of mice, which were determined by daily measurements of tumor volumes. Tumor doubling times for the entire experimental period (56 days) were the highest in the concurrent combination, high-dose vandetanib (25 mg/kg) group ($38.5 \pm 3.3$ days), which also showed the longest time to reach a 1,000 mm$^3$ tumor volume ($51.9 \pm 1.8$ days).

**Assessment of the effects of vandetanib and/or irinotecan on tumor vasculature using DCE-MRI.** To evaluate the effects of vandetanib (25 mg/kg dose groups only) in combination with irinotecan on tumor angiogenesis, HT-29 tumor-bearing mice were subjected to DCE-MRI analysis. Sequential gadolinium-enhanced images were obtained before, during, and after gadolinium injection (Fig. 3A). The tumor rims showed stronger enhancement compared with the cores (Fig. 3A). Due to this heterogeneity of tumor perfusion, gadolinium kinetics were calculated for the tumor rims only, as recommended in the literature (31). When compared with the normal adjacent muscles, tumor tissues exhibited increased...
vascularization as depicted in Table 2. Specifically, increased $K_{\text{trans}}$, initial 90-s gadodiamide AUC, and total AUC indicated increased perfusion of the tumor tissue, whereas decreased gadolinium clearance ($Q$) and increased permeability surface factors reflected increased tumor permeability. Posttreatment changes in enhancement were different between groups (Fig. 3A and B). As depicted, lower T1 intensities (black and red spectrum) were observed after gadodiamide injection in the vandetanib single-agent concurrent and concurrent combination groups compared with the hyperintensive tumor area (red, blue, and green spectrum) in the vehicle and irinotecan single-agent groups (Fig. 3A). This was also dynamically assessed on T1 intensity curves in Fig. 3B. The T1 curves after cycle 3 (green) were significantly lower in the vandetanib concurrent and combination concurrent groups compared with the other groups. Not only were the maximum intensities decreased, leading to decreased volume transfer constants $K_{\text{trans}}$ and initial AUCs (suggesting a decrease in perfusion), but also gadolinium washout was increased (increased gadolinium clearance from the tumor; Fig. 3B; Table 2). Only these two groups had significantly decreased permeability surface factors (Table 2), resulting in largely decreased total gadolinium AUC (Table 2).

By contrast, in the vehicle- and irinotecan-treated groups, there were no differences in the mean T1 intensity enhancement by the end of cycle 3 (Fig. 3B). In fact, some of the irinotecan-treated tumors showed an elevated enhancement (Fig. 3A), suggesting well-perfused and permeable tissues even at the core of the tumor. Interestingly, the effects on tumor vasculature were more pronounced in the group of animals at the core of the tumor. Interestingly, the effects on tumor (Fig. 3A), suggesting well-perfused and permeable tissues even at the end of cycle 3 (green T1 curve, Fig. 3B). This may have been related to a decrease in vandetanib levels in the tumors from the sequential groups after cycle 3, as noted below in Fig. 4B. From all quantitative end points of antiangiogenic response, the initial AUC in the first 90 s after gadolinium injection showed the best correlation with tumor size ($r = 0.823$).

**Effects of sequences on EGFR pathway activation and tumor cell proliferation.** Immunohistochemical analysis was done to evaluate the effects of the vandetanib high dose (25 mg/kg) alone, and in combination with irinotecan, using the two different sequences, on EGFR pathway activation. Two mice per group were sacrificed at the end of cycle 3 and tumors were stained for phosphorylated EGFR, phosphorylated MAPK, and phosphorylated AKT. As expected, no changes of phosphorylated EGFR levels and on phosphorylation of downstream effectors, such as MAPK and AKT, were observed in the irinotecan group compared with vehicle (Table 3). Likewise, the vandetanib single-agent sequential and sequential combination schedule had no effect on EGFR pathway activation. Inhibition of EGFR phosphorylation was observed only in the vandetanib concurrent single-agent and combination regimens and was enhanced with the combination. These effects were paralleled by phosphorylated MAPK and phosphorylated AKT inhibition in both of these groups (Table 3). To determine sequence effects on cancer cell proliferation, tumor samples were analyzed by immunohistochemistry for Ki-67. Table 3 illustrates that the reduction in the percentage of Ki-67–stained cancer cells was observed mainly in the vandetanib concurrent single-agent and combination groups.

**Pharmacokinetic analysis of vandetanib plasma and tumor concentrations.** Plasma and tumor concentrations of vandetanib in tumor-bearing mice were evaluated at the end of the first and third cycle of treatment to assess potential interactions between vandetanib and irinotecan using the different doses and schedules. Within groups, there was no difference in drug concentrations between the first and second cycle and thus these values were combined for each group. As depicted in

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### Table 2. Quantitative variables of tumor perfusion and permeability calculated from DCE-MRI

<table>
<thead>
<tr>
<th></th>
<th>IAUC (arbitrary units)</th>
<th>AUC (arbitrary units)</th>
<th>$K_{\text{trans}}$ (min$^{-1}$)</th>
<th>PS factor</th>
<th>Q (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.20 ± 0.05</td>
<td>18.17 ± 455</td>
<td>17.2 ± 8.8</td>
<td>2.703 ± 1.805</td>
<td>0.07 ± 0.04</td>
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<tr>
<td>Irinotecan</td>
<td>0.22 ± 0.05</td>
<td>19.44 ± 431</td>
<td>16.6 ± 5.2</td>
<td>2.781 ± 1.862</td>
<td>0.07 ± 0.03</td>
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<tr>
<td>25-Vandetanib-Seq</td>
<td>0.26 ± 0.13</td>
<td>23.47 ± 1133</td>
<td>19.4 ± 10.8</td>
<td>3.399 ± 2.483</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>25-Combo-Seq</td>
<td>0.20 ± 0.04</td>
<td>2062 ± 521</td>
<td>10.4 ± 1.1</td>
<td>1.911 ± 1.583</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>25-Vandetanib-Conc</td>
<td>0.16 ± 0.03</td>
<td>962 ± 405*</td>
<td>7.9 ± 2.9</td>
<td>1.915 ± 3.79*</td>
<td>0.14 ± 0.05*</td>
</tr>
<tr>
<td>25-Combo-Conc</td>
<td>0.11 ± 0.06*</td>
<td>726 ± 198*</td>
<td>5.3 ± 0.4</td>
<td>1.756 ± 1.53*</td>
<td>0.19 ± 0.03*</td>
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<tr>
<td>Muscle tissue</td>
<td>0.06 ± 0.04</td>
<td>435 ± 214</td>
<td>5.2 ± 1.1</td>
<td>1.431 ± 2.37*</td>
<td>0.23 ± 0.06</td>
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<tr>
<td>R with tumor size</td>
<td>0.023</td>
<td>0.647</td>
<td>0.657</td>
<td>0.747</td>
<td>-0.619</td>
</tr>
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</table>

**NOTE:** Quantitative variables of tumor perfusion and permeability after 30 d of treatment (end of the third cycle) were calculated from DCE-MRI scans using a compartmental gadolinium kinetic model. All variables were calculated for the tumor rim only (first six rows) and the adjacent nonaffected muscle tissue (as a baseline for normal tissue gadolinium kinetics; bottom row).

Abbreviations: IAUC, initial area under the curves (first 90 s); $K_{\text{trans}}$, transcapillary exchange rate; PS, permeability surface factor; Q, gadolinium clearance; $R$, correlation factor with tumor size.

* $P < 0.02$, compared with control ($n = 4$ per group).

* $P < 0.05$, compared with control ($n = 4$ per group).

* $P < 0.01$, compared with control ($n = 4$ per group).
Fig. 4A, plasma concentrations of vandetanib were significantly higher in the concurrent treatment groups in a dose-dependent manner compared with the sequential schedule. However, vandetanib was still detectable in the sequential groups with concentrations of 260 ± 170 and 630 ± 438 ng/mL, respectively, for the 12.5 and 25 mg/kg doses 6 days after dosing. Although the addition of irinotecan did not appreciably affect vandetanib plasma concentrations in any group, it did increase tumor vandetanib concentrations in both the 12.5 and 25 mg/kg concurrent combination groups (Fig. 4B).

Discussion

The present study was designed to assess two doses and schedules of vandetanib in combination with irinotecan in an in vivo model of colon cancer. The design was based on previous preclinical data conducted by us and others, indicating that there may be sequence-dependent effects with combinations of EGFR-targeted agents and cytotoxic chemotherapy (25–27). To be able to interpret the results within an appropriate biological context, we incorporated functional imaging (DCE-MRI), immunohistochemistry of signaling pathways and proliferation, and pharmacokinetic sampling of vandetanib, along with end points of antitumor efficacy.

The results of this study showed that irinotecan alone had a modest effect on tumor growth delay, whereas in the groups treated with vandetanib alone, the cumulative dose of vandetanib was the primary determinant of antitumor efficacy. The combination of irinotecan and vandetanib resulted in essentially additive effects. The DCE-MRI results suggested greater effects in the irinotecan concurrent combination schedule, and the immunohistochemical results were consistent with those observed on tumor growth inhibition, with the greatest effect on EGFR, MAPK, AKT signaling, and tumor cell proliferation occurring in the high-dose (25 mg/kg) vandetanib concurrent schedule. Taken together, these results suggest that the higher dose of vandetanib, given continuously with irinotecan, is the most effective schedule in this model of colorectal cancer.

These results did not recapitulate our previous in vitro studies of vandetanib and chemotherapy, which showed that vandetanib followed by chemotherapy was synergistic, whereas either concurrent exposure or the reverse sequence (vandetanib followed by chemotherapy) was antagonistic (27). In those in vitro studies, the observed sequence-dependent effects seemed to be mediated through EGFR-dependent interaction with chemotherapy, resulting in differential modulation of oxaliplatin-induced prosurvival and proapoptotic pathways. The in vivo results are consistent with VEGFR-mediated effects attenuating EGFR-based sequencing events, which may have implications for clinical studies that combine EGFR, VEGFR-based agents, and chemotherapy. For example, could the largely unimpressive results of EGFR-based tyrosine kinase inhibitors and chemotherapy in patients be improved by the addition of agents that target the VEGF pathway? Lastly, another important issue when comparing the in vitro and in vivo results is the ~5-fold higher vandetanib concentrations used in vitro (10 μmol/L) than those achieved in plasma in the current study, indicating that the sequencing effects were concentration dependent.

DCE-MRI was used as a pharmacodynamic end point in this study based on its prior validation as a noninvasive imaging biomarker in preclinical and clinical studies of agents that inhibit VEGF-driven tumor angiogenesis (32–36). DCE-MRI is sensitive to differences in blood volume and vasculature permeability and is based on repetitive, rapid T1-weighted MRI scans before, during, and following the gadolinium-containing i.v. injection. The quantitative assessment of the T1-weighted signal from DCE-MRI is used to derive estimates of factors related to blood volume (transcapillary exchange rate ktrans) and permeability (permeability surface factors and gadolinium clearance; ref. 37) that are hallmarks of the
angiogenic phenotype associated with most cancers. Our hypothesis was that antitumor efficacy would correlate with the noninvasive measures of blood volume and vascular permeability provided by DCE-MRI. We anticipated that our preclinical results would be best interpreted within the context of this biological end point. In fact, these expectations were met. There was a dose- and schedule-dependent effect of single-agent vandetanib on DCE-MRI that reflected its ability to reverse VEGF-driven vascular effects in this colon cancer xenograft model. The greatest effects on DCE-MRI variables were observed in the concurrent single-agent and combination groups, which also represented the groups with the greatest antitumor efficacy. Intriguing also was the fact that the concurrent combination group had the most substantial changes in DCE-MRI from baseline and, although not reflected in greater tumor growth inhibition, did seem to be associated with induction of tumor growth stability over time (Table 1: TDT and TTR 1,000 mm³). Intermittently dosed vandetanib, either alone or in combination with irinotecan, showed lesser effects on tumor growth and DCE-MRI, suggesting that discontinuous dosing of VEGF-targeted inhibitors may undermine their ability to affect tumor growth kinetics and maintain inhibition of VEGF signaling.

The plasma and tumor concentrations of vandetanib were measured in this study to determine whether irinotecan had any effect on vandetanib concentrations and to confirm that vandetanib was eliminated from the tumor and plasma before irinotecan in the sequential combination groups. As expected, the concentrations of vandetanib were dose dependent and higher in the concurrent schedules where the drug was given continuously. What was revealing, however, was the fact that in the sequential combination groups, at both dose levels, there was detectable vandetanib 6 days after dosing with plasma and tumor concentrations of 700 ng/mL and 50 μg/g respectively, at the 25 mg/kg dose level. Therefore, it is unlikely that true sequential dosing was achieved in this study, except in cycle 1, although the immunohistochemical results done at the end of cycle 3 indicate phosphorylated EGFR levels similar to vehicle and single-agent irinotecan. Interestingly, the concurrent combination groups showed an increase in tumor vandetanib concentrations. Although the mechanism by which irinotecan increased vandetanib tumor concentrations is unknown, it most likely relates to tumor physiology rather than a pharmacologic interaction because irinotecan had been given 7 days previously. Further, unlike gefitinib and imatinib (55, 56), vandetanib does not alter the plasma or tissue pharmacokinetics of irinotecan, suggesting a lack of drug interaction at the level of ATP-binding cassette transporters. A possible explanation for this result is the decreased tumor perfusion, noted in the concurrent combination schedule on DCE-MRI. Altered tumor perfusion would alter the dynamics of drug accumulation and elimination for a lipophilic drug such as vandetanib that has shown flow-limited distribution (30). These results have implications for subsequent studies, such as the use of agents that have relatively short elimination half-lives for assessing sequence-dependent effects, and the potential effect of combinations of chemotherapeutic and VEGF-based inhibitors on intratumoral concentrations of drugs.

At the level of tumor tissue, effects on activation of EGFR, MAPK, and AKT indicated that the greatest inhibition was observed when vandetanib was given continuously (concurrent schedule), alone or in combination with irinotecan. Single-agent irinotecan had no effects on these pathways nor did the intermittent (sequential schedule) administration of vandetanib. These results indicate that optimal biological effects of vandetanib on EGFR and downstream signaling pathways require prolonged exposure, again instilling a note of caution when attempting schedules that require interrupted dosing. As anticipated, the index of proliferation we used, Ki-67, paralleled the effects of the dosing groups on tumor growth inhibition.

The results of this study have important implications for the design, analysis, and application of dosing schedules of agents that target tumor and endothelial cell growth factor pathways, alone and in combination with chemotherapy. First, although in vitro studies may lead to important insights into the mechanisms of chemotherapy potentiation by targeted agents, these should be followed by rationally designed in vivo studies. Second, preclinical in vivo studies must incorporate a constellation of clinically relevant end points that can be used in interpreting the results (i.e., putting the results into a biological/pharmacologic/physiologic context) and may be used as “benchmarks” in the clinical studies to assess whether these effects are recapitulated in patients. Clearly, the translation of laboratory science into clinical treatment strategies for cancer patients is imperfect, yet the profusion of novel agents and combinations mandates that we prioritize regimens that are tested in the clinic. This can only be achieved through the continual refinement and iterative process that results from rationally designed informative preclinical studies. Vandetanib

Table 3. Immunohistochemical evaluation of EGFR-dependent signal translation pathways and tumor cell proliferation in HT-29 xenografts

<table>
<thead>
<tr>
<th>pEGFR</th>
<th>pMAPK</th>
<th>pAKT</th>
<th>Ki-67 (% positive cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.6 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>1.8 ± 0.3</td>
<td>0.7 ± 1.2</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>25-Vandetanib-Seq</td>
<td>1.5 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>1.3 ± 1.2</td>
</tr>
<tr>
<td>25-Combo-Seq</td>
<td>1.5 ± 0.4</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>25-Vandetanib-Conc</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>25-Combo-Conc</td>
<td>0.3 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

NOTE: Mice bearing HT-29 tumor xenografts were treated as described above and immunohistochemical analysis was done on two mice per group after 30 d of treatment (end of the third cycle).

Abbreviations: pEGFR, phosphorylated EGFR; pMAPK, phosphorylated MAPK; pAKT, phosphorylated AKT.

Gustafson, unpublished data.
is currently undergoing clinical evaluation and, in phase II trials, has shown improvement in progression-free survival in patients with advanced non–small cell lung cancer (28, 29). Phase III trials of vandetanib in non–small cell lung cancer have been initiated.

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References

Investigation of Two Dosing Schedules of Vandetanib (ZD6474), an Inhibitor of Vascular Endothelial Growth Factor Receptor and Epidermal Growth Factor Receptor Signaling, in Combination with Irinotecan in a Human Colon Cancer Xenograft Model

Teresa Troiani, Natalie J. Serkova, Daniel L. Gustafson, et al.


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