INTRODUCTION

Tumor VEGF expression has been clinically associated with disease progression in a range of solid malignancies (1–6). This correlation is largely attributed to its ability to induce tumor angiogenesis by stimulating endothelial cell mitogen (7) and chemotaxis (8), increasing endothelial cell-associated protease activity (9–11), and elevating integrin expression in microvascular cells to augment extracellular matrix interactions (12, 13). These concordant activities facilitate vessel sprouting and capillary tube formation. In addition to a proangiogenic role, VEGF may also contribute to tumor progression through its profound permeabilizing effect on the vasculature and the induction of fenestrae (14, 15). A leaky tumor endothelium should enhance nutrient and catabolite exchange and represent less of a barrier to tumor cell intravasation during metastasis.

Diverse stimuli are suggested to elevate VEGF expression, including many growth factors and cytokines, such as platelet-derived growth factor, transforming growth factor β, and interleukin 6 (16–18), glucose deprivation (19), proto-oncogene activation (20–23), and the loss of tumor suppressor function (24). Rapid increases in VEGF expression also accompany the onset of hypoxia, which frequently arises in solid tumors because of inadequate perfusion. This hypoxic response is known to involve both transcriptional activation and stabilization of the VEGF mRNA (25, 26).

Two high-affinity receptors for VEGF with associated tyrosine kinase activity have been identified on human vascular endothelium: Flt-1 and KDR. Except for expression of Flt-1 on monocyte/macrophage lineages (27), pericytes (28), and smooth muscle cells (29), the receptors are endothelial specific and preferentially expressed at sites of active angiogenesis (30). The binding of VEGF as a disulfide-linked homodimer stimulates receptor dimerization (31) and activation of the RTK domain. The kinase autophosphorylates cytoplasmic receptor tyrosine residues, which then serve as binding sites for molecules involved in the propagation of a signaling cascade. Although multiple pathways are likely to be elucidated for both receptors, KDR signaling is most extensively studied, with a mitogenic response suggested to involve ERK-1 and ERK-2 mitogen-activated protein kinases (32), largely through activation of a PLC-γ-PKC-Raf-1-MEK (33), and cellular motility attributed to activation of the mitogen-activated protein kinase p38 and/or tyrosine phosphorylation of focal adhesion kinase and paxillin (34, 35).

Disruption of VEGF receptor signaling is a highly attractive therapeutic target, given the specificity of receptor expression, that angiogenesis is a prerequisite for all macroscopic solid tumor growth, and that the mature endothelium remains comparatively quiescent (with the exception of the female reproductive system and wound healing). A number of experimental approaches to inhibiting VEGF signal transduction have been examined, including use of neutralizing antibodies (36–38), receptor antagonists (39), soluble receptors (40), antisense constructs (41), dominant-negative strategies (42), and ribozymes (43). However, because continual abrogation of the VEGF pathway in tumor endothelium is likely to be required to constrain tumor growth, we aimed to produce a therapy compatible with chronic oral administration.

We have identified ZD4190, a novel p.o.-active tyrosine kinase inhibitor, and investigated its activity in vitro and in vivo. The results obtained are consistent with VEGF signaling blockade.

MATERIALS AND METHODS

ZD4190 and Recombinant Proteins. ZD4190 (Fig. 1) was synthesized as described by Hennequin et al. (44). RTKs used in isolated enzyme assays were lysates from insect cells infected with recombinant baculoviruses containing cytoplasmic receptor domains. VEGF165 and bFGF were similarly prepared using S. frugiperda 21 insect cells and Escherichia coli strain BL21(DE3)pLysS, respectively, and purified using a heparin-Sepharose column.3

Receptor Tyrosine Kinase Inhibition. A ELISA assay described previously (44) was used to determine the ability of ZD4190 to inhibit Flt-1, KDR, and FGFR1 RTK activity. Briefly, compounds were incubated (20 min at room temperature) with enzyme in a HEPES (pH 7.5) buffered solution containing 10 mM MnCl2 and 2 μM ATP in 96-well plates coated with a poly(Glu, Ala, Tyr) 6:3:1 random copolymer substrate (Sigma, Poole, United Kingdom). Phosphorylated tyrosine was then detected by sequential incubation with mouse IgG anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY), a horseradish peroxidase-linked sheep anti-mouse immunoglobulin antibody (Amersham, Little Chalfont, United Kingdom), and

Received 9/29/99; accepted 12/15/99.

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; ZD4190, N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[2-(1H)-1,2,3-triazol-1-yl]ethoxyquinazolin-4-amin; Flt-1, fms-like tyrosine kinase; bFGF, basic fibroblast growth factor; KDR, kinase insert domain-containing receptor; FGFR, fibroblast growth factor receptor; HUVEC, human umbilical vein endothelial cell; RTK, receptor tyrosine kinase; PLC-γ, phospholipase Cγ; PKC, protein kinase C; MEK, mitogen-activated ERK kinase.

3 J. O. Curwen, C. Grundy, R. Davies, P. Elvin, and D. J. Ogilvie. VEGF and bFGF induce blood pressure falls in anesthetized rats which are mediated by their individual receptors and are sensitive to the novel receptor tyrosine kinase inhibitors ZD4190 and ZM324573, submitted for publication.
Inhibition of Growth Factor-mediated HUVEC Proliferation. HUVEC proliferation in the presence and absence of growth factors was evaluated using [3H]thymidine incorporation (44). Briefly, HUVECs isolated from umbilical cords were plated (at passages 2–8) in 96-well plates (1000 cells/well) and dosed with ZD4190 (3 ng/ml) or bFGF (0.3 ng/ml). The cultures were then incubated for 4 days (37°C; 7.5% CO₂). On day 4, the cultures were pulsed with 1 μCi/well of [3H]thymidine (Amersham) and reincubated for 4 h. The cells were then harvested and assayed for the incorporation of tritium using a beta counter. IC₅₀ data were interpolated as described above.

Cytotoxicity Assay. Cells were plated in their respective media at predetermined densities that were known to enable logarithmic cell growth during the period of assay (PC-3 at 500 cells/well; all others at 1000 cells/well). Plates were incubated for 24 h (37°C; 7.5% CO₂) prior to the addition of ZD4190 (0.1–100 μM). Collectively, these data suggest that the ability of ZD4190 to inhibit VEGF-stimulated proliferation in endothelial cells at only 50 μM is via a specific effect on KDR or Flt-1-mediated signal transduction. IC₅₀ data for the inhibition of tumor cell growth in vitro were found to be >25 μM in each cell line examined.

ZD4190 Increases Epiphyseal Hypertrophy at the Femur Growth Plate. VEGF is known to be of importance in coordinating endochondral bone formation, which involves capillary invasion of cartilage as a prerequisite to the process of ossification (45). Chronic oral dosing of ZD4190 to young growing rats for 14 days produced a dose-dependent increase in the epiphyseal growth plate area (Fig. 2); increases of 22, 75, and 93% were observed, even at the maximum ZD4190 concentration examined in these assays (10 μM). Collectively, these data suggest that the ability of ZD4190 to inhibit VEGF-stimulated proliferation in endothelial cells at only 50 μM is via a specific effect on KDR or Flt-1-mediated signal transduction. IC₅₀ data for the inhibition of tumor cell growth in vitro were found to be >25 μM in each cell line examined.

Prolonged ZD4190 Treatment and Therapy Withdrawal. The effect of prolonged ZD4190 treatment was examined in the PC-3 human prostate xenograft model. Mice were housed in a barrier facility with 12-h light/dark cycles and provided with sterilized food and water ad libitum. All procedures were performed on mice of at least 8 weeks of age. PC-3, Calu-6, and SKOV-3 tumor xenografts were established in the hind flank by s.c. injection of 1 × 10⁶ cells in 100 μl of Matrigel (Fred Baker, Liverpool, United Kingdom) and the relevant serum-free media. MDA-MB-231 tumor xenografts were initially established by implantation of 1 × 10⁶ cells in serum-free DMEM, and cubic tumor fragments of 0.5–1 mm³ diameter were implanted for therapy experiments in mice receiving 100 μg of estradiol benzoate the day before tumor implant and an additional 50 μg at weekly intervals (50 μl, s.c.). Mice were randomized into groups of 10 prior to treatment at a point when tumors reached a volume of 0.3–0.55 cm³. Mice then received either ZD4190 or vehicle, administered once-daily at 0.1 ml/10 g body weight. Tumor volume was assessed twice weekly by bilateral Vernier caliper measurement, using the formula (length × width × height) / 2, where length was the longest diameter across the tumor, and width was the corresponding perpendicular. Growth inhibition from the start of treatment was calculated by comparison of the mean change in tumor volume for the control and treated groups, and statistical significance between the two groups was evaluated using a one-tailed t test. Linear regression analysis of log-transformed xenograft data was used to estimate the control tumor doubling time.

RESULTS

ZD4190 In Vitro Profile. ZD4190 is a submicromolar inhibitor of VEGF RTK activity in vitro with greatest effect against KDR (Table 1). Selectivity versus FGFR1 tyrosine kinase activity was demonstrated, with at least a 30-fold difference in the IC₅₀ required to inhibit HUVEC proliferation stimulated by VEGF or bFGF, respectively. No effects on basal HUVEC growth were observed, even at the maximum ZD4190 concentration examined in these assays (10 μM). Collectively, these data suggest that the ability of ZD4190 to inhibit VEGF-stimulated proliferation in endothelial cells at only 50 μM is via a specific effect on KDR or Flt-1-mediated signal transduction. IC₅₀ data for the inhibition of tumor cell growth in vitro were found to be >25 μM in each cell line examined.

Pan-Tumor Xenograft Activity. The antitumor activity of ZD4190 was examined in four histologically distinct human tumor xenograft models. Tumors were allowed to establish growth between 10 and 20 days prior to the start of treatment. Chronic oral dosing of ZD4190 produced a significant and dose-dependent inhibition of tumor xenograft growth in each model (Fig. 3). Treatment with 100 mg/kg/day of ZD4190 for 21 days conferred between a 79 and 95% inhibition of tumor growth, and statistically significant effects on tumor growth (one-tailed t test) were evident in two xenograft models (Calu-6 and PC-3) with only 12.5 mg/kg/day ZD4190 (Table 2).

ZD4190 inhibits VEGF receptor tyrosine kinase activity

**Table 1. In vitro profile of ZD4190**

<table>
<thead>
<tr>
<th></th>
<th>KDR</th>
<th>Fli-1</th>
<th>FGFR1</th>
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<tbody>
<tr>
<td>Growth factor stimulated</td>
<td>IC₅₀ (μM)</td>
<td>IC₅₀ (μM)</td>
<td>IC₅₀ (μM)</td>
</tr>
<tr>
<td>VEGF</td>
<td>29 ± 4</td>
<td>708 ± 63</td>
<td>5,300 ± 1,700</td>
</tr>
<tr>
<td>bFGF</td>
<td>50 ± 8</td>
<td>1,330 ± 260</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Basal</td>
<td>&gt;25,000</td>
<td>&gt;25,000</td>
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* a The ability of ZD4190 to inhibit Flt-1, KDR, and FGFR1 was examined by a 96-well ELISA assay with enzyme in 10 mM MnCl₂ with 2 μM ATP, using a poly(Glu, Ala, Tyr) 6:3:1 random copolymer substrate. Data represent the mean ± SE of between 9 and 12 (KDR and Flt-1) or 5 (FGFR1) separate experiments.

* b The effect of ZD4190 on growth factor-stimulated (VEGF or bFGF) or basal primary human umbilical vein endothelial cell (HUVEC) growth was examined using [3H]thymidine incorporation to assess proliferation. Data represent the mean ± SE of 5 separate experiments.

* c The effect of ZD4190 on Calu-6, PC-3, SKOV-3, and MDA-MB-231 cell proliferation in vitro was examined using [3H]thymidine incorporation to assess cell viability (two to four separate experiments).
prostate tumor xenograft model. Daily administration of ZD4190 (100 mg/kg/day) for 10 weeks produced a sustained inhibition of tumor growth (Fig. 4). When therapy was withdrawn, tumor growth resumed at a rate comparable with that of controls after a lag period of 10 days.

**DISCUSSION**

ZD4190 is a potent inhibitor of VEGF RTK activity (particularly KDR) and VEGF-stimulated endothelial cell proliferation in vitro.
when tumors reached a volume of 0.3–0.55 cm$^3$ and then treated with oral daily doses of ZD4190 (12.5, 25, 50, or 100 mg/kg/day) or vehicle [a 1% (v/v) solution of polyoxyethylene (20) sorbitan mono-oleate in deionized water] for 21 days. The percentage of tumor growth inhibition was calculated as the difference (T/C) between the change in control and ZD4190-treated tumor volumes over the period of treatment. Statistical significance was examined using a one-tailed test. Control tumor doubling times were estimated from linear regression analysis of log-transformed data.

Experiments in which the ATP concentration in the enzyme assay has been varied are consistent with an ATP competitive inhibition of kinase activity (data not shown). From a Flt-1 structural homology model, it has been hypothesized that the quinazoline ring of ZD4190 interacts with the adenine binding site of the kinase, while the anilino ring is buried in an adjacent hydrophobic pocket (44). Despite the fact that ZD4190 is thought to function as an ATP mimetic, selective inhibition of VEGF RTK activity was demonstrated versus FGFR1 tyrosine kinase (also implicated in angiogenesis) in both enzyme and cell assays.

The initial selection of ZD4190 for further preclinical evaluation was in part driven by the fact that it possessed pharmacokinetic properties compatible with chronic oral dosing. This criterion was seen as necessary to satisfy the perceived clinical requirements of an antiangiogenic compound (i.e., chronic for continual inhibition of tumor angiogenesis and once-daily oral administration for patient convenience and compliance). A retrospective analysis of acute plasma pharmacokinetic data and antitumor data in the Calu-6 xenograft model (after oral administration) indicated that for a series of 50 substituted 4-anilinoquinazolines with a similar molecular weight KDR RTK inhibitor dosed i.p. from the day of tumor implantation (50). This broad-spectrum antitumor profile contrasts with that of a direct antiproliferative effect on tumor cells (49), dominant-negative inhibition of KDR (42), and a small molecular weight KDR RTK inhibitor dosed i.p. from the day of tumor implantation (50). This broad-spectrum antitumor profile contrasts with that of tumor cell-directed therapies.

ZD4190 produced a dose-dependent increase in the femoral epiphyseal growth plate in growing rats, which is consistent with an ability to inhibit VEGF signaling and elicit an antiangiogenic effect in vivo. Angiogenesis is an essential event in endochondral ossification during long bone elongation (46), and vascular invasion of the growth plate has been suggested to depend upon VEGF production by hypertrophic chondrocytes (47). Expansion of the hypertrophic chondrocyte zone and inhibition of angiogenesis have also been demonstrated recently after treatment with agents that specifically sequester VEGF (45, 48). It is possible that in addition to direct effects on endothelial cell biology, the inhibition of VEGF signaling may also partly influence osteogenic remodeling through direct or indirect effects on osteoblast, chondrocyte, and chondroclast function (45).

Daily oral administration of ZD4190 was found to impart significant antitumor activity in histologically diverse human tumor xenograft models. This activity is attributed to inhibition of VEGF signaling in the tumor vasculature and not to a direct antiproliferative effect on tumor cells. The inhibition of tumor cell proliferation by ZD4190 in vitro occurs at concentrations that are >500-fold greater than those required to inhibit VEGF-stimulated HUVEC proliferation (comparison of IC$_{50}$, ZD4190 was found to be 97.5 ± 0.5% (mean ± SE, n = 5) protein bound in mouse plasma, and the free drug exposure produced by oral administration of 100 mg/kg/day ZD4190 (the maximum dose examined) is less than that required to produce a direct antiproliferative effect on tumor cells in vitro (data not shown). Although only four tumor types were studied, a prototype compound was found to confer significant antitumor activity in each of nine different tumor models examined, including a rhabdomyosarcoma, fibrosarcoma, and vulval and colon carcinomas. Other approaches aimed at inhibiting VEGF signaling have also produced broad spectrum antitumor activity, including use of a VEGF antibody (49), dominant-negative inhibition of KDR (42), and a small molecular weight KDR RTK inhibitor dosed i.p. from the day of tumor implantation (50). This broad-spectrum antitumor profile contrasts with that of tumor cell-directed therapies.

ZD4190 was found to be a significantly more potent (24-fold) inhibitor of KDR RTK activity in vitro than of that associated with Flt-1. KDR has a lower affinity for VEGF binding than Flt-1 but has been found to be more abundantly expressed on endothelial cells in culture (51, 52) and has a much greater signaling capacity (53). Although the relative contributions of KDR and Flt-1 signaling in mediating tumor progression have not been resolved, a
number of studies suggest that KDR may perform a predominant role. A KDR blocking antibody has been shown to disrupt tumor angiogenesis and invasion in a human malignant keratinocyte model (54), and activation of KDR alone with a selective agonist has been found to increase tumor vascularization and proliferation and induce angiogenesis in a corneal pocket assay (55). The Orf virus-derived NZ-7 VEGF gene product (VEGF-E), which can only bind to KDR, has also been found to elicit an endothelial mitotic and vascular permeabilizing response comparable with that of native VEGF (56), whereas placenta growth factor, which can only bind to Flt-1, has little appreciable effect on either (57). VEGF-E also promotes endothelial cell migration and tubule formation in vitro and angiogenesis in the rabbit cornea (58). In addition, vascular expression of KDR, but not Flt-1, has been found to be associated with the development of high-grade gliomas (59) and metastatic colon carcinoma (60).

Although ZD4190 may have some effect on Flt-1 signaling in vivo, it remains unclear as to whether inhibition of Flt-1 RTK activity would contribute significantly to the constraint of tumor angiogenesis. Experiments with deletion of the Flt-1 tyrosine kinase domain indicate that normal angiogenesis during development is not reliant on signaling from this receptor (61).

Homologous Flt-1 gene deletion confers an embryo lethal phenotype that results from the abnormal assembly of vascular vessels (62). Collectively, these studies suggest that Flt-1 may regulate matrix/vessel assembly in development through sequestration of VEGF. The role of Flt-1 signaling in endothelial cell migration is still equivocal (55, 63), although a confirmed functional role has been demonstrated in the promotion of monocye/macrophage migration and tissue factor production (64, 65), the stimulation of pericyte mitogenesis and migration (28), and the inhibition of functional dendritic cell maturation (66). It is therefore conceivable that inhibition of these effects could provide additional therapeutic benefit by reducing macrophage-mediated thrombolytic events, preventing blood vessel stabilization, or increasing the capacity to direct an antitumor immune response (in an immunocompetent host, respectively).

It has been suggested that because endothelial cells are of a stable genetic background, they may be less likely to acquire resistance to an angiogenic therapy, which is a common failure of many tumor cell-directed treatments (67). Prolonged dosing of ZD4190 to mice bearing PC-3 prostate tumor xenografts for 10 weeks was found to constrain tumor growth for the duration of dosing and was well-tolerated throughout. Regrowth of tumors after withdrawal of treatment was expected because removal of ZD4190 will enable tumor vasculature to respond to VEGF and thereby facilitate rapid tumor expansion through stimulation of angiogenesis.

Given that many factors have been suggested to have angiogenic activity, it is possible that alternative stimuli could eventually circumvent the constraint imposed by a VEGF signaling blockade. However, there is increasing evidence that VEGF may also function as a survival factor for newly formed vasculature (68, 69). This effect may be partly attributable to increased expression of the antiapoptotic protein Bcl-2 in endothelial cells, in response to VEGF (70). It is possible therefore, that if VEGF does play a major role in enhancing neovascular survival, inhibition of VEGF signaling may confer a therapeutic advantage regardless of the initial angiogenic stimulus. In addition, the antitumor activity of ZD4190 in histologically disparate tumor types may partly be attributable to a common effect on tumor vascular permeability. Acute dosing of ZD4190 to mice bearing PC-3 tumors has been found to reduce vascular permeability in xenografts using contrast medium-enhanced magnetic resonance imaging and at doses that elicit antitumor activity during chronic administration (71).

In comparison with the use of conventional cytotoxic agents, VEGF RTK inhibitors may provide a more tolerable cytostatic treatment with clinical utility in a wide range of solid tumor types, either as a monotherapy or in combination with radiation and/or additional chemotherapy. VEGF RTK inhibition may also have application in the treatment of other angiogenesis-dependent pathologies, such as rheumatoid arthritis (72) and diabetic retinopathy (73).


ZD4190: An Orally Active Inhibitor of Vascular Endothelial Growth Factor Signaling with Broad-Spectrum Antitumor Efficacy

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Cancer Res 2000;60:970-975.

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