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

Stephen R. Wedge, Donald J. Ogilvie, Michael Dukes, Jane Kendrew, Jon O. Curwen, Laurent F. Hennequin, Andrew P. Thomas, Elaine S. E. Stokes, Brenda Curry, Graham H. P. Richmond, and Peter F. Wadsworth

ABSTRACT

There is evidence that vascular endothelial growth factor (VEGF) contributes to solid tumor growth through the promotion of both angiogenesis and tumor vascular permeability. To abrogate VEGF signaling, we developed a small molecular weight inhibitor of VEGF receptor tyrosine kinase (RTK) activity that was compatible with chronic oral administration. ZD4190, a substituted 4-anilinoquinazoline, is a potent inhibitor of KDR and Flt-1 RTK activity, and VEGF stimulated HUVEC proliferation in vitro. Chronic once-daily oral dosing of ZD4190 to young rats produced a dose-dependent increase in the femoral epiphyseal growth plate area, which may be attributed to the inhibition of VEGF signaling in vivo because vascular invasion of cartilage is a prerequisite to the process of ossification. Once-daily oral dosing of ZD4190 to mice bearing established (0.5 cm³) human tumor xenografts (breast, lung, prostate, and ovarian) elicited significant antitumor activity and at doses that would not be expected to have any direct antiproliferative effect on tumor cells. Prolonged tumor cytostasis was further demonstrated in a PC-3 xenograft model with 10 weeks of ZD4190 dosing, and upon withdrawal of therapy, tumor growth resumed after a short delay. These observations are entirely consistent with the proposed mode of action. ZD4190 is one of a series of VEGF RTK inhibitors that may have utility in the treatment of a range of histologically diverse solid tumor types.

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 mitogenesis (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 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 lyases from insect cells infected with recombinant baculoviruses containing cytoplasmic receptor domains. VEGF soluble 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 Flk-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 horseradish peroxidase-dependent chemiluminescence (Perkin Elmer, Boston, MA). The results obtained are consistent with VEGF signaling blockade.

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; ZD4190, N-(4-bromo-2-thienyl)-phenyl-6-methoxy-7-[2-(1H)-1,2,3-triazol-1-ylthoxy]quinazolin-4-amine; 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.

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2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer, Lewes, United Kingdom). IC_{50} data were interpolated by nonlinear regression (four-parameter logistic equation) using Microcal Origin software (version 3.78; Microcal Software Inc., Northampton, MA).

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 densities that were known to enable logarithmic cell growth during the period of assay (2–8) in 96-well plates (1000 cells/well) and dosed with ZD4190 ± VEGF (3 ng/ml) or bFGF (0.3 ng/ml). The cultures were then incubated for 4 days (37°C; 7.5% CO_{2}). 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 assessed for the incorporation of thymidine using a beta counter. IC_{50} data were interpolated as described above.

Tumor Cell Lines. Four human tumor cell lines were used: PC-3 (prostate adenocarcinoma), Calu-6 (lung carcinoma), MDA-MB-231 (mammary gland adenocarcinoma), and SKOV-3 (ovarian adenocarcinoma), each of which were obtained from the American Type Culture Collection (Manassas, VA). All cell culture reagents, where not specified, were obtained from Life Technologies, Inc. (Paisley, United Kingdom) prior to routine use in vitro.

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% CO_{2}) prior to the addition of ZD4190 (0.1–100 μM) or vehicle (0.1% DMSO in medium), and reincubated for an additional 2 h. Cell proliferation was assessed by [3H]thymidine incorporation as described in HUVEC experiments.

Episphyal Growth Plate Hypertrophy Studies. For all in vivo work, ZD4190 was suspended in a 1% (v/v) solution of polyoxyethylene (20) sorbitan mono-oleate in deionized water and administered by oral gavage. Young female Alderley Park rats (Wistar derived, 150 g in weight, 4–8 weeks of age) were dosed daily for 14 days with ZD4190 (at 0.25 ml/100 g body weight) or vehicle. Histological paraffin wax sections of the femoro-tibial joints were produced by standard histological techniques and stained with H&E. The sections were examined by light microscopy, and the area of the epiphyseal growth plate was measured using morphometric image analysis (Joyce-Loebl Magiscan Image Analyser; Applied Imaging Ltd.).

Tumor Xenograft Models. Female Swiss athymic (nu/nu genotype) mice were bred and maintained at Alderley Park in negative pressure isolators (PFI Systems Ltd., Oxon, United Kingdom). 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 × 10^6 cells in 100 μl of 50:50 Matrigel (Fred Baker, Liverpool, United Kingdom) and the relevant serum-free media. MDA-MB-231 tumor xenografts were initially established by implantation of 1 × 10^6 cells in serum-free DMEM, and cubic tumor fragments of 0.5–1 mm^3 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 cm^3. 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)^3, 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 IC_{50} 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 nM is via a specific effect on KDR or Flt-1-mediated signal transduction. IC_{50} 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 182% were evident after treatment with 15, 50, and 150 mg/kg/day ZD4190, respectively.

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).

Prolonged ZD4190 Treatment and Therapy Withdrawal. The effect of prolonged ZD4190 treatment was examined in the PC-3 human

Table 1  In vitro profile of ZD4190

<table>
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<tr>
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<th>KDR</th>
<th>Fli-1</th>
<th>FGFR1</th>
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<tr>
<td>IC_{50}</td>
<td>29 ± 4</td>
<td>708 ± 63</td>
<td>5,300 ± 1,700</td>
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<td></td>
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<tr>
<td>Growth factor stimulated</td>
<td>VEGF</td>
<td>bFGF</td>
<td>Basal</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>50 ± 8</td>
<td>1,330 ± 260</td>
<td>&gt;10,000</td>
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*IC_{50} for inhibition of isolated enzyme activity (μM) 

*IC_{50} for inhibition of HUVEC growth (μM) 

The ability of ZD4190 to inhibit Flt-1, KDR, and FGFR1 was examined by a 96-well ELISA assay with enzyme in 10 mM MnCl_{2} 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 4 (FGFR1) separate experiments.

The effect of ZD4190 on KDR function-stimulated (3 ng/ml VEGF or 0.3 ng/ml 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 five separate experiments.

The effect of ZD4190 on Calu-6, PC-3, SKOV-3, and MDA-MB-231 cell growth in vitro was examined using [3H]thymidine incorporation to assess cell viability (two to four separate experiments).

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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.

Fig. 2. A. Histological sections (×2.5) of proximal tibia of young rats treated for 14 days with (i) vehicle or (ii) 150 mg/kg/day ZD4190. Sections were stained with H&E. A marked increase in the epiphyseal growth plate zone of hypertrophy (indicated by the region between the arrowheads) was produced by ZD4190. B, morphometric image analysis of sections taken after 14 days of dosing indicated that ZD4190 produced a dose-dependent increase in the epiphyseal growth plate (five rats per group; *, P < 0.04 and **, P < 0.001 by one-tailed t test). Bars, SE.

Fig. 3. Effect of ZD4190 (□, 12.5 mg/kg/day; ▽, 25 mg/kg/day; △, 50 mg/kg/day; ○, 100 mg/kg/day) or vehicle (■) on growth of human tumor xenografts [(a) PC-3; (b) Calu-6; (c) MDA-MB-231; and (d) SKOV-3]. Xenografts were established s.c. in athymic mice and allowed to reach a volume of 0.3–0.55 cm³ prior to treatment. Oral once-daily treatment with ZD4190 or vehicle then commenced and was continued for the duration of the experiment. ZD4190 produced a dose-dependent inhibition of tumor xenograft growth in each of the models examined. Data points represent means for 10 mice, with errors being illustrated in one direction; bars, SE.

Fig. 4. Effect of ZD4190 (M, 12.5 mg/kg/day; ƒ, 25 mg/kg/day; ′, 50 mg/kg/day; E, 100 mg/kg/day) or vehicle (f) on growth of human tumor xenografts [(a) PC-3; (b) Calu-6; (c) MDA-MB-231; and (d) SKOV-3]. Xenografts were established s.c. in athymic mice and allowed to reach a volume of 0.3–0.55 cm³ prior to treatment. Oral once-daily treatment with ZD4190 or vehicle then commenced and was continued for the duration of the experiment. ZD4190 produced a dose-dependent inhibition of tumor xenograft growth in each of the models examined. Data points represent means for 10 mice, with errors being illustrated in one direction; bars, SE.

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when tumors reached a volume of 0.3–0.55 cm³ 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 t 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 in vitro profile, greatest antitumor activity correlated with sustained plasma levels of compound (>0.5 μM at 24 h) rather than total area under the plasma concentration versus time curve or peak plasma levels (data not shown). This correlation was not merely a consequence of compound accumulation on repeat dosing, suggesting that sustained inhibition of VEGF signaling is indeed required for optimal therapeutic effect in a human tumor xenograft model.

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 IC₅₀,). 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 also been found to be associated with the development of high-grade glioma (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). Homozygous 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 monocyte/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 antiangiogenic 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 recast 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 INHIBITS VEGF RECEPTOR TYROSYNE KINASE ACTIVITY


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