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Review Article

CGP 79787D (PTK787/ZK222584), CGP 84738, NVP-AAC789, NVP-AAD777 and related 1-anilino-(4-pyridylmethyl)phthalazines as inhibitors of VEGF- and bFGF-induced angiogenesis

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Introduction

Angiogenesis is a process of sprouting, growth and maturation of new capillaries from existing blood vessels that occurs as a response of both neoplastic and stromal cells to hypoxia/acidosis. The induction and promotion of this process is mediated by several cytokines such as VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor) and PDGF (platelet-derived growth factor) (1, 2), which are produced by tumor cells and are upregulated in many other disease states where changes in vessel permeability and increased vessel formation play a role in the symptoms and progression of the disease. Besides cancer, other diseases include rheumatoid arthritis, diabetic retinopathy and macular degeneration. VEGF appears to be the key mediator of the process of neovascularization and also contributes to progression of several diseases by increasing vascular permeability. As a mediator of vascular permeability, VEGF is also known as VPF (vascular permeability factor) (3). The binding of VEGF to the endothelial cell receptors KDR (kinase insert domain-containing receptor or VEGFR-2) and Flt-1 (Fms-like tyrosine kinase or VEGFR-1) induces their homo- or heterodimerization and triggers an intracellular autophosphorylation in their kinase domain (1, 4-7). This event ultimately leads, through a cascade of signal transmissions, to the growth message in the nucleus. Whereas this angiogenic process appears essential during embryogenesis and embryonic development, it is tightly controlled in adult tissues and only regularly occurs during the reproductive cycle and tissue repair. Therefore, modulation of angiogenesis through blockade of the kinase functionality of either KDR or Flt-1 or both appears to be an attractive therapy for treating neoangiogenesis-related diseases in adults.

Various approaches have been investigated to interfere with the VEGF/VEGF receptor (VEGFR) system in animals in order to understand the role of either KDR or Flt-1 in the various pathological states. Besides biopharmaceutical approaches (8, 9), several small molecules are known to target the VEGF/VEGFR system and, among them, the class of 1-anilino-(4-pyridylmethyl)-phthalazines described in this article presents a promising pharmacological profile.

Tumor angiogenesis

The concept of antiangiogenesis therapy in tumor treatment was already proposed in the early seventies by Folkman (10), based on the observation that most solid tumors cannot grow beyond a certain critical size unless

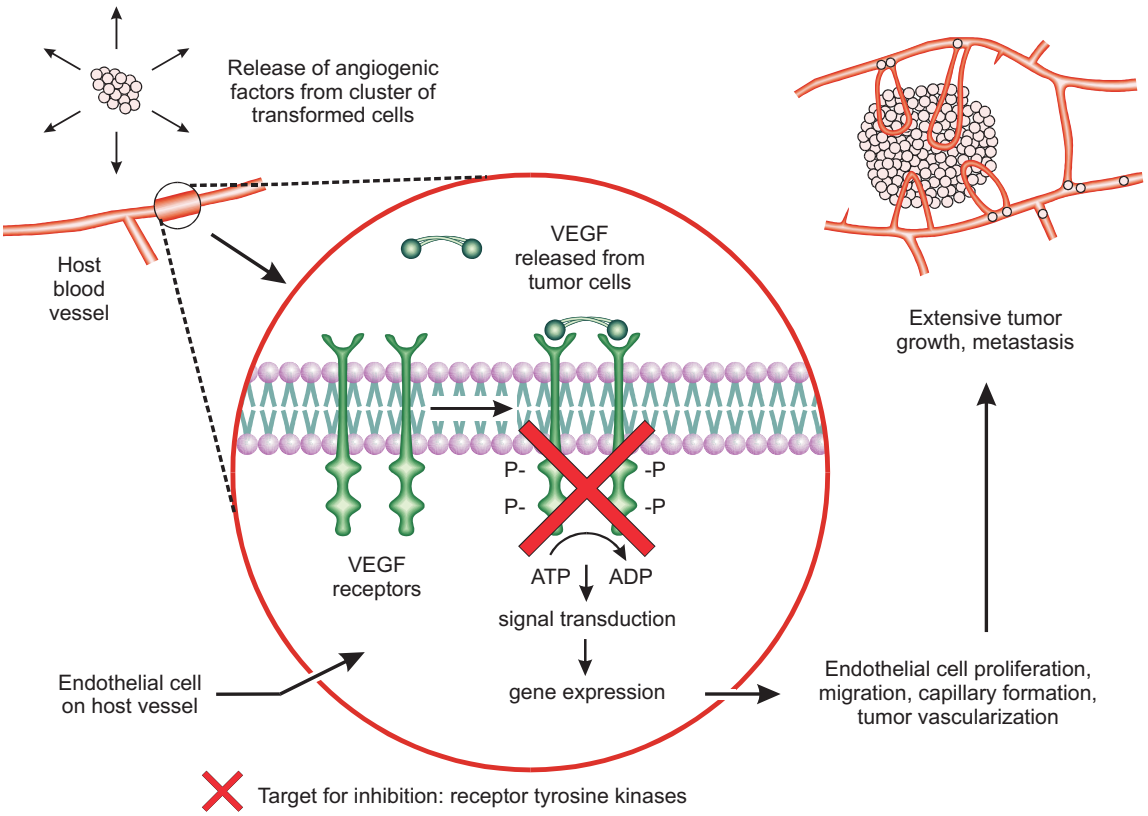


Fig. 1. The concept is that most solid tumors cannot grow beyond a certain critical size unless they establish their own blood supply by inducing formation of new vessels (10). Tumors induce this process by secreting cytokines like VEGF. VEGF then ligates to the endothelial cell receptor KDR and initiates the autophosphorylation of the kinase domain. This event then ultimately leads, through a cascade of signal transmissions, to the formation of new blood vessels. Blocking this process by inhibiting KDR therefore should stop tumor growth.

they establish their own blood supply by inducing the formation of new vessels sprouting from existing host capillaries to secure their nutrition and oxygen supply and waste removal (11) (Fig. 1). In addition, growth of blood vessels also promotes metastasis by providing a route for transmission of tumor cells to other sites within the body (12, 13). A major hurdle for cancer therapy is the fact that tumor cells are prone to mutate frequently, thus inducing resistance formation. However, interactions with VEGF receptors target the genetically more stable host endothelial cells instead of labile tumor tissues. This may provide a cancer treatment modality that is less prone to resistance development (14, 15). Finally, since growth of tumor tissue is generally angiogenesis-dependent, inhibition of tumor-induced angiogenesis is expected to be effective irrespective of tumor type (16-22).

Among the several possible pathways to modulate tumor angiogenesis (23, 24) inhibition of the VEGF/VEGFR pathway has been proven to be a valid target using several approaches including VEGF (25-28) or VEGFR antibodies, or most recently, low molecular kinase inhibitors (29) (Fig. 2).

VEGF receptor tyrosine kinase inhibitors as angiogenesis modulators

Several kinase inhibitors are currently undergoing clinical trials (30-32). Among them the bcr-abl inhibitor STI571 (Glivec®; CGP 57148B) was recently introduced for the treatment of leukemias. These tyrosine kinase inhibitors competitively bind to the ATP-binding sites of their respective receptors and block the autophosphorylation of the intracellular tyrosine kinase region, thus interrupting the stimuli to the nucleus.

Several VEGFR tyrosine kinase inhibitors have been reported for the treatment of tumor angiogenesis (29) (Fig. 2). 3-Substituted indolin-2-ones were disclosed as inhibitors of the mouse VEGFR tyrosine kinase Flk-1 (fms-like kinase) (33). The most advanced VEGFR tyrosine kinase inhibitor, SU 5416 (34), potently blocked the human VEGFR tyrosine kinases KDR and Flt-1. SU 5416 (35, 36) is undergoing clinical trials as an i.v. formulation and recently, SU 6668 was disclosed as a follow-up of the same structural class showing good oral bioavailability (37, 38). AstraZeneca disclosed ZD 4190 and ZD 6474, both orally active 4-aminoquinazolines, as KDR inhibitors with a broad spectrum of antitumor efficay (39, 40).

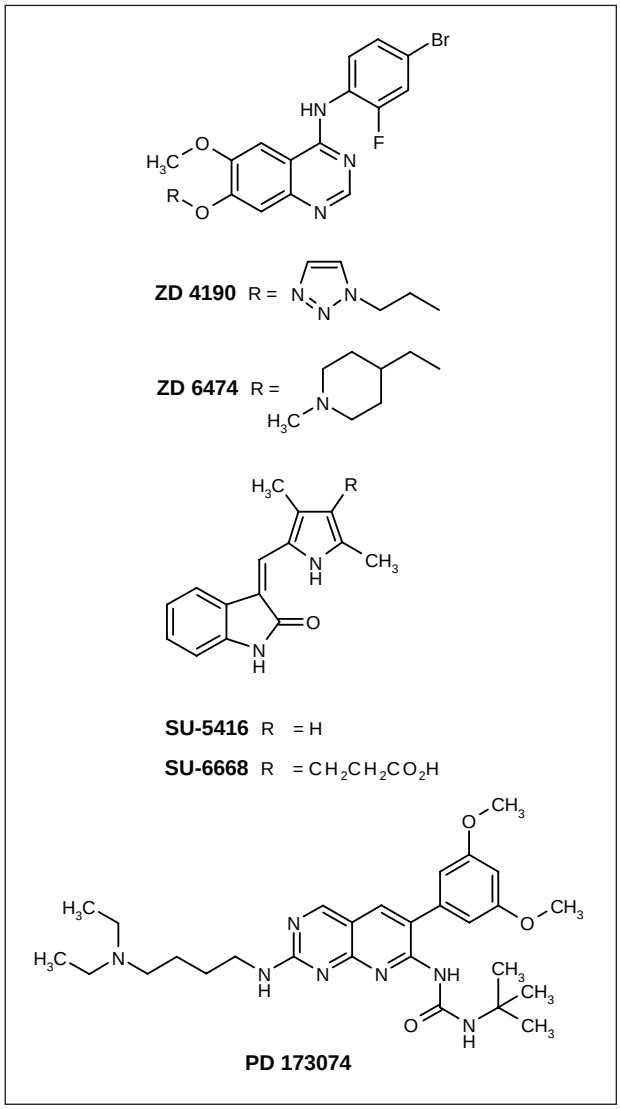


Fig. 2. VEGF receptor tyrosine kinase inhibitors with antitumor activity.

PD 173074 was synthesized by Parke-Davis as a dual inhibitor of both angiogenically active tyrosine kinases, KDR and FGFR1 (fibroblast growth factor receptor 1) (41, 42).

During our screening for Flt-1 inhibitory activity we discovered the 1-anilino-(4-pyridylmethyl)phthalazine structural class (Fig. 3) as potent and selective VEGFR tyrosine kinase inhibitors (43, 44). This class of kinase inhibitors had selectivity for the PDGF receptor (PDGFR) subfamily (45, 46) in enzymatic as well as in cellular assays. Besides inhibiting KDR, Flt-1 and Flk-1 they also blocked PDGFR-β, c-Kit (receptor for stem cell factor) and Flt-4 (lymphatic endothelial cell receptor tyrosine kinase or VEGFR-3 [46-50]) activity. However, they did not inhibit structurally unrelated receptor tyrosine kinases, such as the EGF receptor (epithelial growth factor receptor), c-abl (virally transduced Abelson oncogene), c-src (protooncogene) and serine-threonine kinases like PKC-α (protein kinase C-α). *In vivo*, PTK787 (CGP 79787D or ZK222584) (Fig. 3), a representative of this series, showed excellent oral bioavailability and induced dose-dependent reduction of VEGF- and PDGF-induced angiogenesis in a murine growth factor implant model (51). It also inhibited the growth of several human carcinomas grown s.c. in nude mice, as well as a murine renal carcinoma and its metastases in a syngenic, orthotopic model (52). PTK787 did not impair wound healing in an incisional wound healing model in rats, another angiogenesis driven process. Even though it potently inhibited c-Kit, PTK787 did not have any significant effects on circulating blood cells or bone marrow leukocytes as a single agent or impair hematopoetic recovery after challenge with concomitant cytotoxic anticancer agents. In patients, PTK787 was well tolerated, had a favorable pharmacokinetic profile and could be administered on a continuous basis (53). Therefore, PTK787 has therapeutic potential for the treatment of solid tumors and is successfully undergoing clinical trials in tumor patients in a joint collaboration between Schering AG and Novartis.

Chemistry of 1-anilino-(4-pyridylmethyl)phthalazines

The 1-anilino-(4-pyridylmethyl)phthalazines were synthesized from 2-(4-pyridyl)-3-hydroxy-indene-1-one (**6**) (54) by condensation with hydrazine hydrate, activation and substitution with the appropriate aniline according to Scheme 1. Thus, base catalyzed condensation of phthalide with 4-pyridinecarboxaldehyde led to the intermediate **A** which rearranged to **6** (55). Condensation of **6** with hydrazine hydrate then yielded 4-(4-pyridyl)methyl-2*H*-phthalazin-1-one (**7**). Introduction of the 1-anilino-group on the phthalazine core was achieved via two methods. First, in a one step procedure described by Andersen and Pedersen (56), a mixture of **7**, 4-chloraniline, phosphorous pentoxide and triethylamine hydrochloride was melted to give 1-(4-chloranilino)-(4-pyridylmethyl)phthalazine (CGP 79787) directly. Alternatively, **7** was activated as the imidoyl chloride **8** and then reacted with the appropriate anilines to afford the 1-anilino-phthalazines as shown in Scheme 1 (57).

Considering that the available X-ray crystal structures of protein kinases indicate that usually no other binding pockets except the ATP cleft exist in their catalytic domains, we modeled the binding mode for 1-anilino-(4-pyridylmethyl)phthalazines. CGP 79787 was docked in a model of the ATP binding site of KDR constructed using the available X-ray structures (58) of the kinase domain of the FGFR1, another structurally related growth factor receptor. The putative binding mode (Fig. 4) results from extensive docking analyses aimed at identifying a model consistent with the structural complementarity between the inhibitor and the cleft as well as the available structure-activity relationships (44). Our hypothesis proposes that, in contrast to ATP and many reported kinase inhibitors, CGP 79787 does not form direct hydrogen

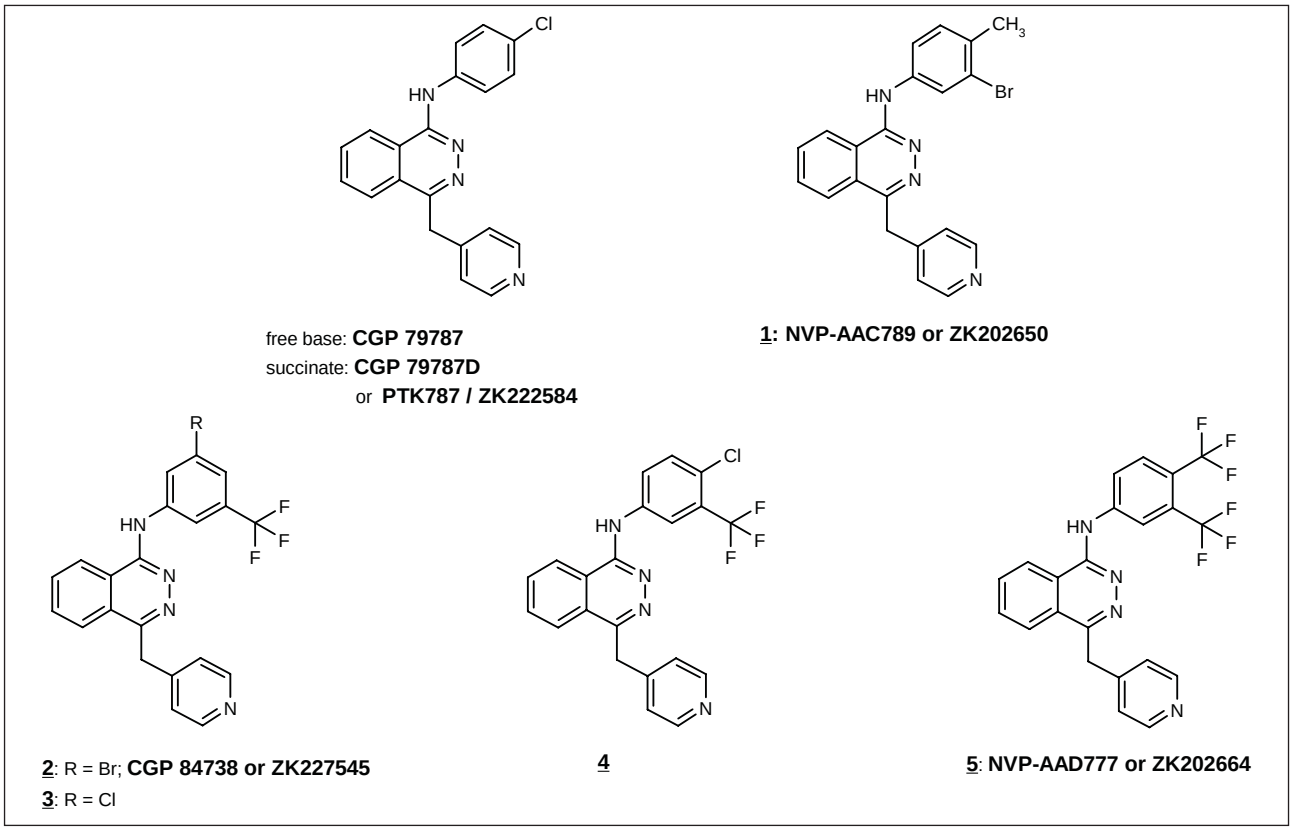


Fig. 3. 1-Anilino-(4-pyridylmethyl)phthalazines as angiogenesis inhibitors.

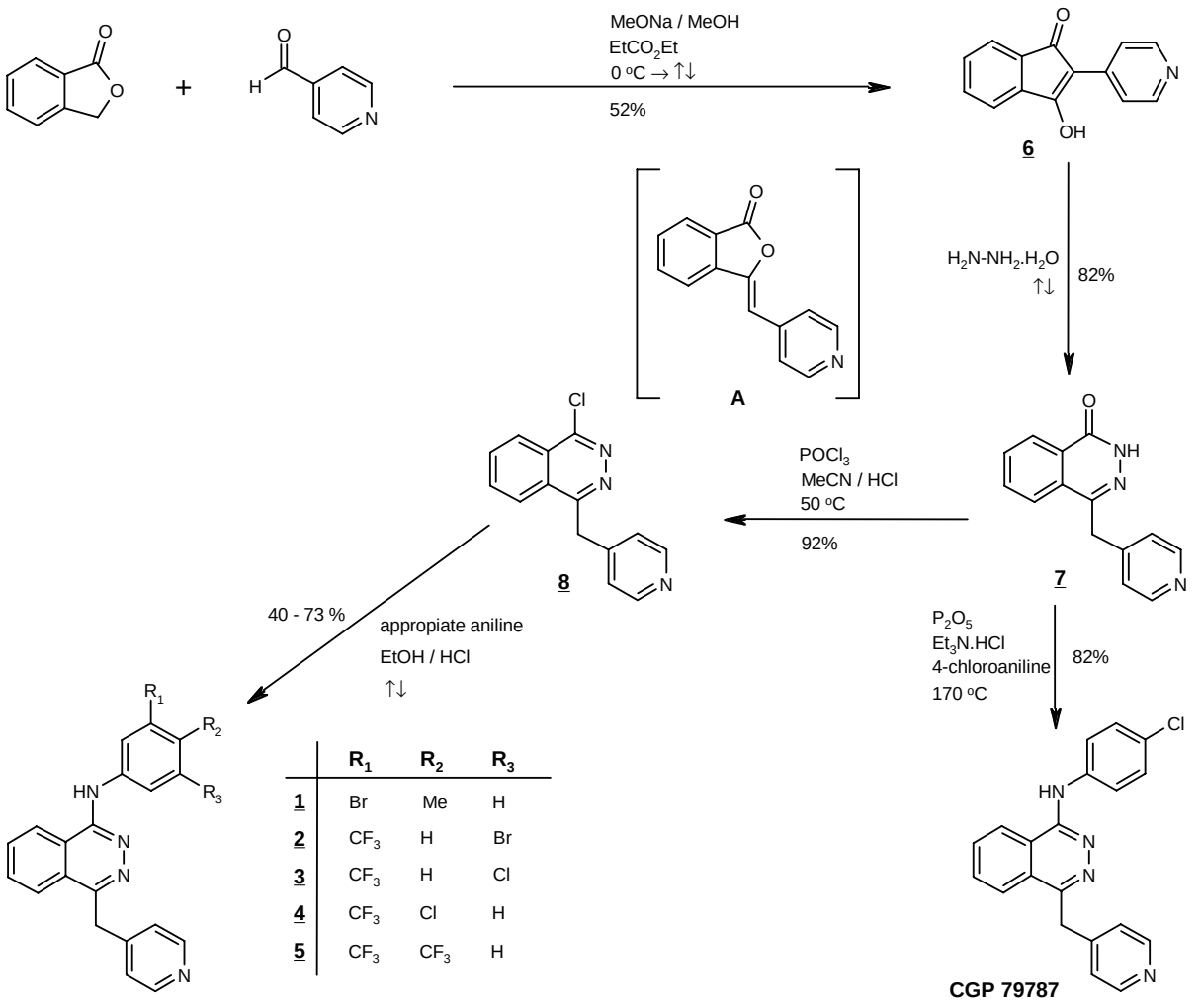
bonds with the peptide backbone of the hinge region but rather optimally occupies the hydrophobic regions of the binding site. The 4-chlorophenyl moiety is located in the hydrophobic pocket formed by residues Val 914, Val 912, Val 897, Leu 887, Cys 1043, Phe 1045 and the hydrocarbon part of the side chain of Lys 866, while the phthalazine bicyclic core makes hydrophobic contacts with Leu 1033, Gly 920 and Leu 838. Although no direct hydrogen bonds with the hinge region are possible in this binding orientation, the anilino NH group of the inhibitor is located at distances from the backbone of Glu 915 and Cys 917 allowing water-mediated hydrogen bonds to be formed (Glu 915 and Cys 917 are the residues of the hinge region that should be involved in bidentate hydrogen bonding with the adenine ring of ATP). In addition, the pyridyl nitrogen of the inhibitor is assumed to be engaged in a hydrogen bond with the side chain of Lys 1060, a residue belonging to the activation loop of the kinase. Lys 1060 is not conserved outside the tyrosine kinases of the PDGF family (PDGF-R, c-Kit, KDR, Flt-1, Flk-1) and therefore may contribute to the selective recognition of CGP 79787 by the members of this family. However, based on the model one cannot completely rule out an alternative hydrogen bond with the proximal Asn 1031 or a residue of the glycine-rich loop which may be prone to conformational rearrangement (59).

In vitro profile of 1-anilino-(4-pyridylmethyl)-phthalazines

Using enzymatic assays with recombinant GST-fused kinase domains and synthetic substrates, 1-anilino-(4-pyridylmethyl)phthalazines have been shown to be potent inhibitors of VEGFR tyrosine kinases, being active in the submicromolar range (44). Among the derivatives depicted in Table I, PTK787 and **1** were the most potent inhibitors of KDR. The sterically more demanding disubstituted aniline-derivatives exemplified by **2-5** were weaker inhibitors for KDR, however. This tendency was also obvious in their inhibition potential towards other VEGFR tyrosine kinases, like Flt-1 or Flk or other kinases of the PDGFR subfamily. In contrast to the other compounds, PTK787 and **1** also inhibited c-Kit and PDGFR-β in the submicromolar concentration range. However, none of them was active against kinases from other receptor families such as FGFR-1, EGFR, Tek and c-Met or intracellular kinases like c-src, c-abl and PKC-α.

Since a kinase inhibitor must enter cells in order to inhibit the kinase domain of the receptor, the effects of 1-anilino-(4-pyridylmethyl)phthalazines were tested in cell-based receptor autophosphorylation assays using KDR-transfected CHO cells (Table II). They potently inhibited VEGF-induced autophosphorylation of KDR with an ED_{50} in the range of 9-39 nM. 1-Anilino-(4-pyridylmethyl)phtha-

Scheme 1: Synthesis of 1-anilino-(4-pyridylmethyl)phthalazines



	R_1	R_2	R_3
1	Br	Me	H
2	CF_3	H	Br
3	CF_3	H	Cl
4	CF_3	Cl	H
5	CF_3	CF_3	H

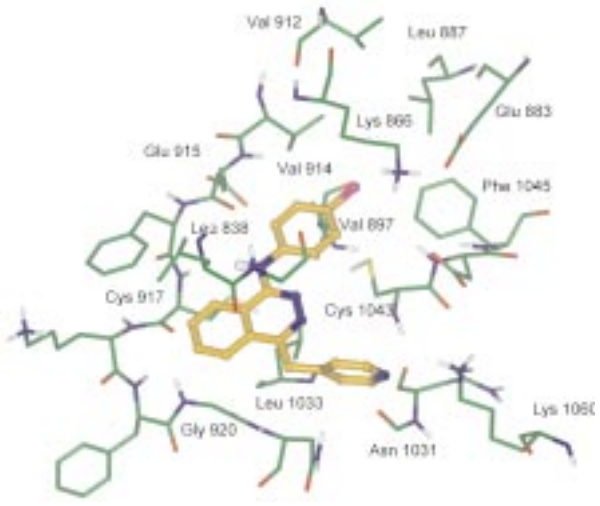


Fig. 4. Modeling of CGP 79787 in the active site of KDR.

lazines also selectively inhibited VEGF-mediated endothelial cell functions including proliferation, migration and survival in the nanomolar range. Potential antiproliferative effects unrelated to VEGF inhibition were tested using cells that do not express the VEGF receptors. In concentrations up to 1 μM, no cytotoxic or antiproliferative effect on these cells could be observed (51). To test the ability of the 1-anilino-(4-pyridylmethyl)phthalazines to inhibit the functional response to VEGF and bFGF as compared to reference compounds, an endothelial cell proliferation assay in HUVECs (human umbilical vein endothelial cells) based on BrdU incorporation was used (Table II). Consistent with the fact that none of these compounds inhibited FGFR-1 kinase (see Table I), they selectively blocked VEGF-induced proliferation of HUVECs but not that induced by bFGF (EC_{50} >1 μM).

Table I: Inhibitory activity of 1-anilino-(4-pyridylmethyl)phthalazines against receptor tyrosine kinases.

IC_{50} (μM)	PTK787	NVP-AAC789 **1**	CGP 84738 **2**	**3**	**4**	NVP-AAD777 **5**
KDR	0.037 ± 0.002 n = 20	0.048 ± 0.012 n = 21	0.44 ± 0.15 n = 3	0.76 ± 0.22 n = 5	0.30 ± 0.044 n = 3	0.77 ± 0.037 n = 9
Flt-1	0.077 ± 0.012 n = 8	0.24 ± 0.069 n = 14	0.63 ± 0.015 n = 3	0.89 ± 0.26 n = 5	0.57 ± 0.074 n = 3	3.0 ± 0.41 n = 5
Flk	0.27 ± 0.04 n = 6	0.21 ± 0.054 n = 6	1.1 ± 0.25 n = 4	1.8 ± 0.34 n = 5	1.3 ± 0.11 n = 3	2.6 ± 0.43 n = 4
c-Kit	0.73 ± 0.05 n = 8	0.74 ± 0.14 n = 9	2.5 ± 0.29 n = 3	> 10 n = 4	2.4 ± 0.28 n = 3	5.7 ± 0.96 n = 3
c-Fms	1.4 ± 0.1 n = 9	1.2 ± 0.19 n = 12	4.0 ± 0.46 n = 4	5.8 ± 1.2 n = 5	3.9 ± 0.31 n = 4	>10 n = 3
PDGFR-β	0.58 ± 0.08 n = 8	0.96 ± 0.35 n = 9	6.6 ± 2.9 n = 5	3.6 ± 0.96 n = 4	3.0 ± 0.73 n = 4	4.5 ± 0.74 n = 3
FGFR-1	>10 n = 4	>10 n = 4	>10 n = 3	>10 n = 4	>10 n = 4	6.3 ± 0.35 n = 3
EGFR	>10 n = 4	>10 n = 4	>10 n = 4	>10 n = 4	>10 n = 3	>10 n = 4
Tek	>10 n = 4	>10 n = 5	>10 n = 3	>10 n = 5	>10 n = 3	>10 n = 3
c-Met	>10 n = 5	>10 n = 4	>10 n = 3	>10 n = 4	>10 n = 4	>10 n = 4
c-src	>10 n = 4	>10 n = 4	>10 n = 3	>10 n = 4	>10 n = 4	>10 n = 4
c-abl	>10 n = 4	4.4 ± 1.7 n = 3	>10 n = 4	>10 n = 4	>10 n = 3	>10 n = 4
PKC-α	>10 n = 5	>10 n = 3	>10 n = 4	>10 n = 4	>10 n = 4	>10 n = 4

The kinase assays were performed as filter binding assays, using recombinant GST-fused kinase domains of the receptors expressed in baculovirus and purified over glutathione sepharose. [^{33}P)-ATP was used as the phosphate donor and polyGluTyr(4:1) peptide was used as the acceptor with the exception of PDGF-β activity, where autophosphorylation was measured instead. Assays were performed under conditions optimized for each kinase and at the following ATP concentrations: 1.0 μM (c-Kit, c-Met, c-Fms), 2.0 μM (EGFR), 5.0 μM (c-abl), 8.0 μM (Flt-1, Flk, KDR, FGFR-1, Tek), 10.0 μM (PDGFR-β, PKC-α), 20.0 μM (c-src). IC_{50} (± SEM; n: repeats) values were calculated by linear regression analysis of the percentage inhibition of each compound (for details see refs. 44, 51).

Table II: Inhibition of VEGF-induced KDR autophosphorylation in KDR-transfected CHO cells and VEGF- and bFGF-induced proliferation of HUVECs (ED_{50} ± SEM; n ≥ 3; for detals see ref. 51).

ED_{50} (nM)	Inhibition of receptor autophosphorylation in KDR-transf. CHO cells	Inhibition of HUVEC proliferation VEGF-induced	bFGF-induced
PTK787	34 ± 2	6 ± 1	>5 x 10^3
1	9 ± 1	1.6 ± 0.9	>5 x 10^3
2	39 ± 3	7 ± 5	>5 x 10^3
3	26 ± 2	12 ± 5	>5 x 10^3
4	27 ± 1	13 ± 7	>10^3
5	31 ± 3	20 ± 5	>5 x 10^3
SU 5416		16 ± 7	>10^3
SU 6668		792 ± 132	>10^3
ZD 4190		16 ± 6	>5 x 10^3
PD 173074		361 ± 6	20 ± 2

In vivo profile of 1-anilino-(4-pyridylmethyl)-phthalazines

Since antiangiogenic drugs have to be given chronically, our aim was to develop a compound that would inhibit VEGF-induced angiogenesis after oral administration. Therefore we tested whether 1-anilino-(4-pyridylmethyl)phthalazines are absorbed orally in mice. After oral administration (50 mg/kg) to mice, plasma concentrations of PTK787 remained above 1 μM for more than 8 h (51). Furthermore, the compound showed excellent oral bioavailability in rats, dogs and humans.

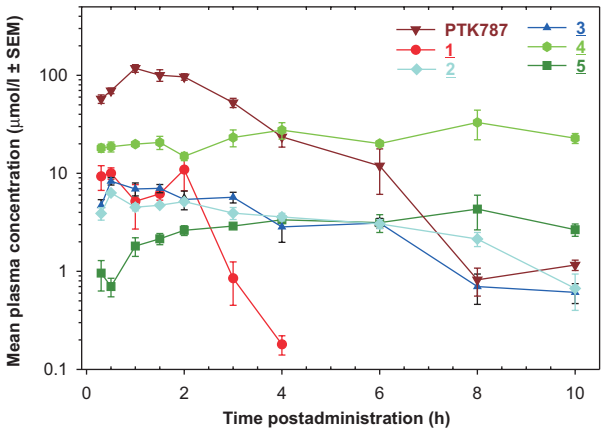


Fig. 5. Mean plasma concentrations of 6 compounds of the 1-anilino-(4-pyridylmethyl)phthalazines class in mice after oral administration of 30 mg/kg. Female OF1 mice received an oral dose of 30 mg/kg. The compound was formulated in 5% DMSO/0.5% Tween 80. At the allotted times the groups of mice (n = 4) were sacrificed, blood removed and the concentration in the plasma determined by reversed-phase HPLC-UV. Bars are SEM (n = 4).

Table III: Pharmacokinetic parameters of 1-anilino-(4-pyridylmethyl)phthalazines after oral administration of 30 mg/kg to mice (c_{max} ± SEM; n ≥ 3).

	$AUC_{0\text{-}24\ h}$ [a] (h·µmol/l)	c_{max} ± SEM[b] (µM)	t_{max} [c] (h)
PTK787	336	118 ± 11	1.0
1	21	11 ± 4	2.0
2	32	6.3 ± 0.2	0.5
3	34	8.3 ± 0.7	0.5
4	237	na[d]	na[d]
5	30	4.3 ± 1.7	8.0

[a]$AUC_{0\text{-}24h}$: area under the plasma concentration *versus* time curve from 0-24 h, calculated by linear trapezoidal rule; [b]c_{max}: maximum plasma concentration; [c]t_{max}: time at which c_{max} attained; [d]na: not applicable (see graph in Fig. 5).

Figure 5 and Table III compare plasma levels of 1-anilino-(4-pyridylmethyl)phthalazines after oral administration of 30 mg/kg to mice. All derivatives were well absorbed orally. The main differences were observed in the corresponding AUC values and the duration of exposure of the drug attained over time after oral administration. With respect to $AUC_{0\text{-}24h}$, PTK787 and **4** showed 10 times higher exposure than the other derivatives. The highest C_{max} was observed for PTK787 1 h after administration. Compounds **4** and **5** gave very long exposure times, attaining their peak drug concentrations only after more than 8 h posttreatment. Whether this long exposure period was due to slow absorption by the gastrointestinal tract or a result of slow metabolism and/or excretion was addressed in the following experiment. In rats, 20 mg/kg of **5** given intravenously, formulated in 1-methyl-2-pyrrolidone/polyethylenglycol 300, produced an $AUC_{0\text{-}24h}$ of 115 ± 17 h·µmol/l with an extremely long terminal half-life of 8.8 ± 1.5 h. This indicates that the observed effect may have been due to a prolonged elimination phase resulting from slow metabolism and/or excretion of the parent drug.

To determine whether the orally well absorbed KDR inhibitors block VEGF-mediated angiogenesis *in vivo*, we tested the effects of 1-anilino-(4-pyridylmethyl)phthalazines on the angiogenic response induced by VEGF in a growth factor implant model in mice (Fig. 6). To test the specificity of the response, the effects on bFGF-induced angiogenesis were also tested. In this model, subcutaneous implants containing VEGF or bFGF in normal mice induced the growth of vascularized tissue around the implant. This neovascularization process can be inhibited by antiangiogenic drugs. Selective inhibitors of these growth factors and/or their signaling pathways specifically blocked these responses in a concentration-dependent manner. The inhibition, expressed by ED_{50} values (Table IV), was quantified by measuring the weight of the newly formed tissue and the amount of hemoglobin (blood content) in the tissue. Consistent with their *in vitro*

a b c

Fig. 6. a: empty teflon chamber; b: teflon chamber filled with agar after implantation for 5 days; c: teflon chamber filled with agar containing VEGF after implantation for 5 days.Teflon chambers (a) filled with agar (0.8%) alone (b) or agar containing human VEGF (1 µg/0.5 ml) (c) or human bFGF (0.15 µg/0.5 ml) were implanted s.c. on the dorsal flank of C57/C6 mice. Mice were treated once a day with an oral dose of kinase inhibitor (12.5, 25 or 50 mg/kg) starting 1 day before implantation and continuing for 5 days after. One group of mice were treated with MAb-4301-42-35 (26), a monoclonal antibody against human VEGF (10 µg/mouse i.p. once daily). The mice were sacrificed on the 6th day of treatment and the chambers removed. The vascularized tissue growing around the chamber (c) was removed and weighed and the blood content assessed by measurement of hemoglobin.

Table IV: Inhibition of growth factor mediated angiogenesis in vivo *(for details see ref. 51).*

ED_{50} (mg/kg/day)	Inhibition of angiogenesis induced by VEGF	bFGF
PTK787	27	>50
1	26	9
2	13	>50
3	<50[a]	≈ 50
4	19	≈ 50
5	6	23
SU 6668	18	21
ZD 4190	7	≈ 30
PD 173074	>50	31

[a]78% inhibition of response at 50 mg/kg/day

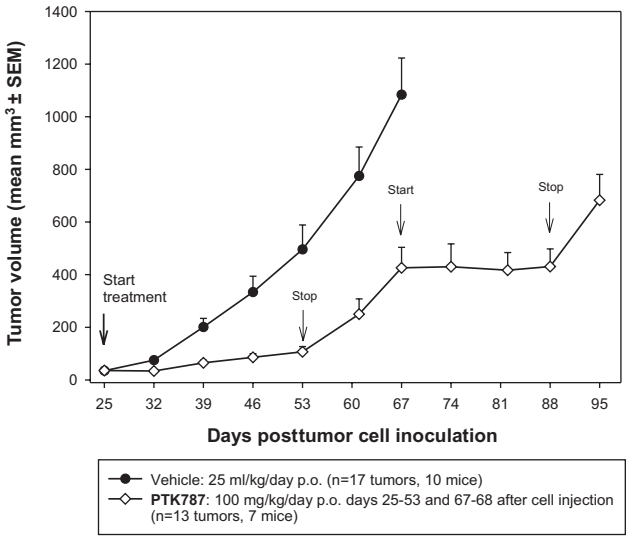


Fig. 7. Effects of intermittent therapy with PTK787 on the growth of DU145 prostate carcinoma.

inhibitory profile, the 1-anilino-(4-pyridylmethyl)phthalazines induced dose-dependent inhibition of VEGF driven angiogenesis in this growth factor implant model. In sharp contrast to their *in vitro* profile, where none of the compounds inhibited FGFR-1 tyrosine kinase in enzymatic assays with a recombinant kinase or bFGF-mediated HUVEC proliferation, some of them potently blocked bFGF-induced angiogenesis *in vivo*. At doses of 9 and 23 mg/kg, respectively, **1** and **5** inhibited 50% of the bFGF response in this system, being as potent as the true FGF inhibitor PD 173074 (Table II), which in contrast did not significantly reduce the VEGF response. The fact that 1-anilino-(4-pyridylmethyl)phthalazines did not inhibit bFGF-mediated events *in vitro* suggests that the *in vivo* response to bFGF might have been mediated by the endogenous VEGF/VEGFR system.

Effects of 1-anilino-(4-pyridylmethyl)phthalazines on tumor growth and the development of metastasis

PTK787 inhibited tumor growth in several different tumor models and metastasis development in a syngeneic, orthotopic model of renal carcinoma (51). The best effects were seen against prostate tumors. The growth of the CWR-22 human prostate and DU145 carcinomas, very slow growing tumors, was completely inhibited in some mice. Figure 7 shows the effects of PTK787 on the growth of DU145 prostate carcinoma applying the drug in cycles interrupted and followed by a recovery period. The drug significantly arrested tumor growth during continuous therapy over 4 weeks. Upon stopping treatment, tumor growth resumed at the same rate as in the vehicle-treated control mice. Upon continuation of therapy 2 weeks later, when tumors had reached a size of about 400 mm^3, tumor growth was again arrested. After cessation of therapy, the tumors again began to grow at the same rate as in the control mice. These observations, together with the reduced microvessel formation in tumors of PTK787-treated mice (51), are in agreement with the expected effects of a selective inhibitor of the VEGF/VEGFR system on tumor growth, interfering only via inhibition of neovascularization of newly formed tumor mass.

In contrast to the efficacy observed against prostate tumors, the growth of some other tumors was only partially inhibited or slowed and there was no tumor regression observed. The fact that not all tumors responded to the same extent to treatment with PTK787 indicates that once vessels are formed they are not affected by this inhibitor. Alternatively, remaining vessels may be the result of different angiogenesis stimulating pathways. Even though a large proportion of the investigated tumors secreted VEGF, many carcinomas also released bFGF (60). We analyzed and characterized our tumor models by real time-PCR according to their growth factor spectra. Whereas the DU145 carcinoma secreted large amounts of VEGF and no bFGF, the B16/BL6 melanoma upregulated both VEGF and bFGF *in vivo*.

The B16/BL6 melanoma system is one of the models where PTK787 only moderately reduced tumor growth. In this model, B16/BL6 melanoma cells are injected intradermally in the ear of C57BL/6 mice leading to the formation of a primary tumor and metastases in the regional lymph nodes. Having compounds expressing different profiles in the growth factor-driven implant model for angiogenesis inhibition *in vivo* available (Table IV) offers a good tool to address the question of whether additional inhibition of pathways stimulated by growth factors other than VEGF is beneficial in regard to efficacy. Furthermore, the bFGF secreting B16/BL6 melanoma model

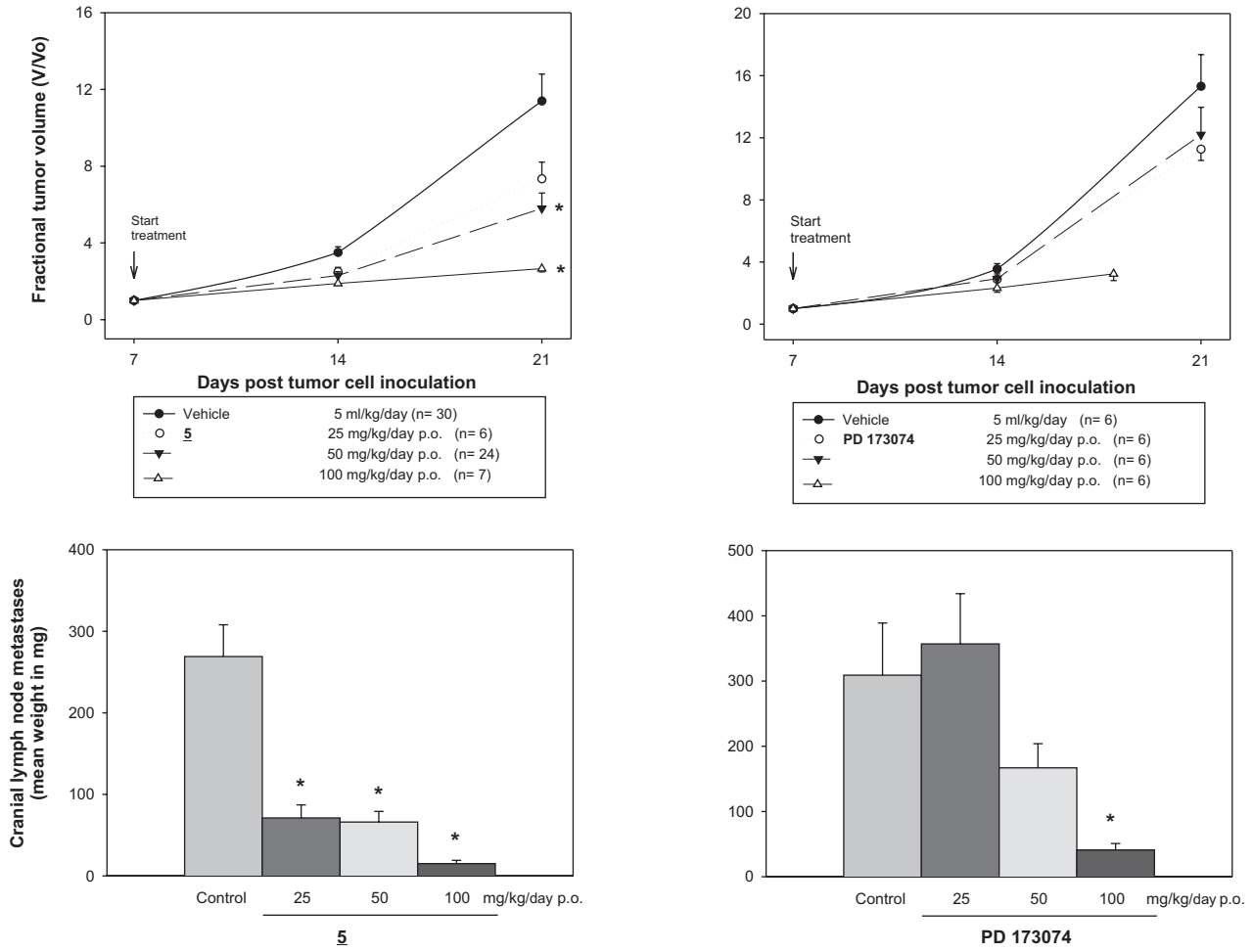


Fig. 8. Comparison of the effects of **5** and PD 173074 on tumor growth and tumor metastasis in the B16/BL6 melanoma model in mice. Intradermal injection of B16/BL6 melanoma cells into the ear of C57BL/6 mice leads to the formation of a primary tumor and metastases in the regional lymph nodes. Both the primary tumor and the metastases produce VEGF and bFGF. The size of the primary tumor was quantified microscopically with a computerized imaging system and the effect of drug substances on the formation of metastases was quantified by the weight of surgically removed cervical lymph nodes.

offers the possibility to compare the efficacy of 1-anilino-(4-pyridylmethyl)phthalazines with the true FGF inhibitor PD 173074.

Figure 8 summarizes the effects of **5** in the B16/BL6 melanoma model in comparison with PD 173074. On the primary tumor, **5** at oral doses of 25-100 mg/kg/day was at least as potent as PD 173074 in stopping primary tumor growth. Especially at lower doses, **5** reduced the formation of lymph node metastasis more potently than PD 173074. These effects were also superior to the results obtained with PTK787. With respect to tolerability, the more effective **5** was as well tolerated in mice in doses up to 100 mg/kg/day as PTK787. The effect of **5** on the lymph node metastasis was even greater than the effects on the primary tumor growth. This inhibition of metastasis formation may have been due to both the decreased vascularization of a primary tumor leading to reduced escape routes for metastatic cells, as well as the decreased vascularization of metastasis restricting their growth. For mimicking the clinical situation, the effect on the development of spontaneous metastasis in the lymph nodes observed in the B16/BL6 melanoma model is probably more relevant than the growth of the artificially developed primary tumor.

Correlation of the high potency of some of the angiogenesis inhibitors in the VEGF and bFGF secreting B16/BL6 melanoma model with their inhibition of VEGF- and bFGF-induced angiogenesis is shown in Figure 9. Whereas all of the described 1-anilino-(4-pyridylmethyl)-phthalazines potently inhibited VEGF-induced angiogenesis in the chamber model, only some of them showed antitumor activity in the bFGF secreting tumor model. Interestingly, there was a clear correlation between inhibition of bFGF-induced angiogenesis in the growth factor model and the effect in the B16/BL6 melanoma model. According to this observation, MAb-4301-42-35 (26), a

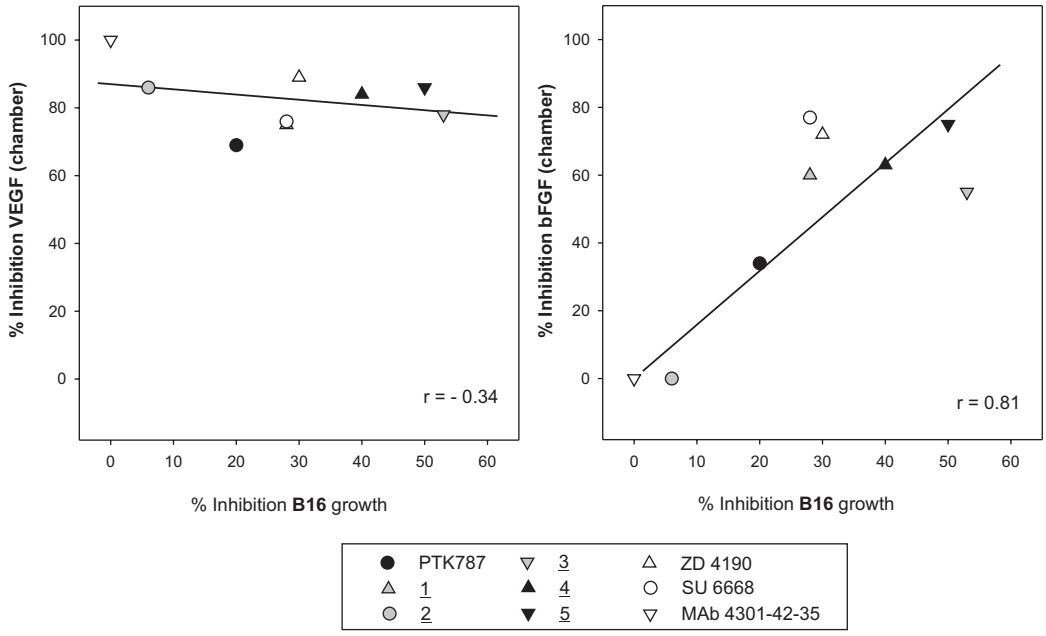


Fig. 9. Comparison of the effects of 1-anilino-(4-pyridylmethyl)phthalazines on tumor growth in the orthotopic B16/BL6 melanoma model with their *in vivo* antiangiogenic effects. The indicated inhibition of VEGF- or bFGF-induced angiogenesis refers to a dose of 50 mg/kg/day. Intradermal injection of B16/BL6 melanoma cells (5 x 10^4) into the ear of C57BL/6 mice leads to the formation of a tumor that produces both VEGF and bFGF (ELISA and real time-PCR). Mice were treated once a day with an oral dose of 50 mg/kg of VEGF receptor tyrosine kinase inhibitors starting 7 days after cell injection and continuing for 2 weeks. One group of mice were treated with MAb-4301-42-35 (26), a monoclonal antibody against human VEGF (10 μg/mouse i.p. once daily). The size of the tumor was quantified microscopically with a computerized imaging system.

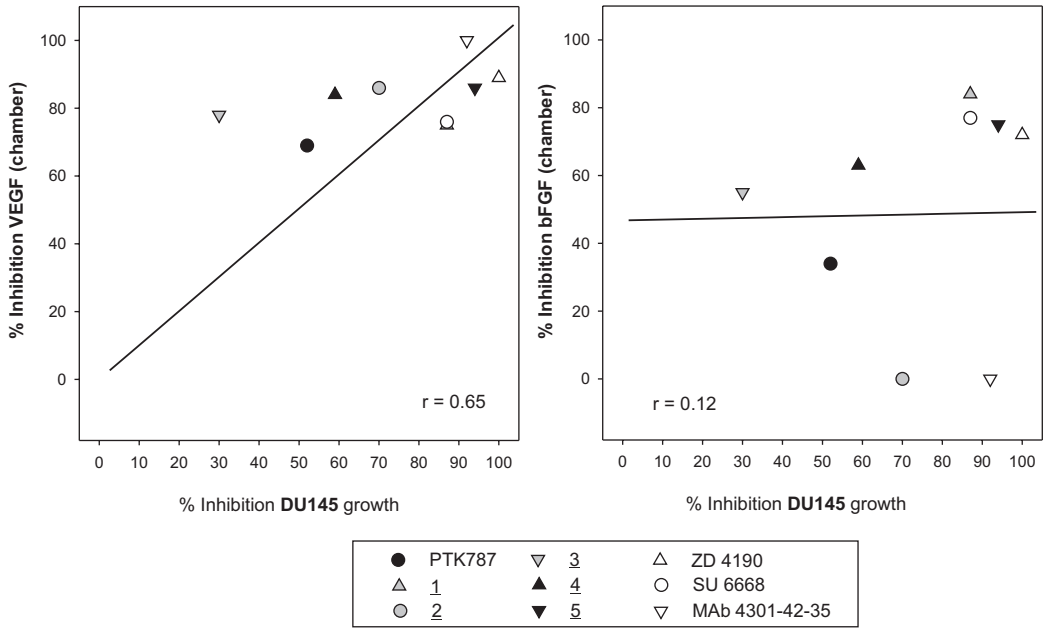


Fig. 10. Comparison of the effects of 1-anilino-(4-pyridylmethyl)phthalazines on tumor growth in DU145 prostate carcinoma with their *in vivo* antiangiogenic effects. The indicated inhibition of VEGF- or bFGF-induced angiogenesis refers to a dose of 50 mg/kg/day. Tumors were generated from the human prostatic carcinoma cell line, DU145, by intradermal injection of 10^6 cells in both flanks of nude mice. This tumor produces large amounts of VEGF but not bFGF (ELISA and real time-PCR). Twenty-five days after injection (tumor size 30-60 mm^3) mice were treated once a day with an oral dose of 50 mg/kg of VEGF receptor tyrosine kinase inhibitors for 4 weeks. One group of mice was treated with MAb-4301-42-35 (26), a monoclonal antibody against human VEGF (10 μg/mouse i.p. once daily). Tumor size was measured weekly with calipers.

monoclonal antibody selective against human VEGF, did not inhibit tumor growth. In DU145 prostate carcinoma (Fig. 10), however, which strongly secretes VEGF but not bFGF, all tested VEGFR tyrosine kinase inhibitors as well as the anti-human VEGF antibody MAb-4301-42-35 worked reasonably well in correlation with their potency in the growth factor model against VEGF. No correlation between inhibition of bFGF-induced angiogenesis and antitumor activity was observed in this model. This clearly shows that, depending on their *in vivo* angiogenesis inhibition profile, some compounds are effective against VEGF secreting tumors only, whereas others also potently inhibit the strong bFGF producer like B16/BL6 melanomas.

Other indications

Inhibition of VEGF/VEGFR-induced angiogenesis by KDR inhibitors has shown promising effects in the therapy of solid tumors and other neovascularization-related diseases. VEGF in its function as vascular permeability factor increases vascular permeability in ovarian cancer patients, leading to peritoneal dissemination. These metastatic lesions can cause formation of malignant ascites (61) and hence poor survival. Fidler *et al*. evaluated the effects of PTK787 on an ovarian cancer model expressing low levels of VEGF (Hey-A8 cells) and one strongly expressing VEGF (SKOV 3 i.p.1 cells) (62). Whereas no effect of treatment was observed for the Hey-A8 tumor, PTK787 significantly inhibited the growth of the VEGF secreting SKOV i.p.1 cells and ascites formation, resulting in an increased survival of mice with the implants. As expected, tumor-induced vascular hyperpermeability in the peritoneum of tumor-bearing mice was inhibited by the drug, which accounted for its inhibition of ascites formation.

The same group evaluated the effects of PTK787 on a second vascular permeability factor-induced symptom, malignant pleural effusion associated with advanced-stage lung cancer (63), an effect indicating poor prognosis and causing chest pain. Drainage followed by instillation of sclerosing agents is useful to improve patients' quality of life. Malignant pleural effusion is most often caused by lung adenocarcinoma. Fidler's group established a model for pleural effusion caused by human lung adenocarcinoma cells, in which they proved that oral treatment with PTK787 suppressed the formation of malignant pleural effusion by inhibiting vascular permeability. This suggests that KDR or Flt-1 inhibitors could be useful for controlling malignant pleural effusion in the lungs of cancer patients.

Hypoxia-induced upregulation of VEGF has also been recognized as an inducer of retinal neovascularization in the adult eye (64). Among the effects, proliferative diabetic retinopathy is the most common cause of severe visual loss in people under 60 years of age in industrialized countries. Having available a new model of retinal neovascularization in transgenic mice with photoreceptor-specific expression of VEGF, Campochiaro *et al*. studied the effects of PTK787 treatment on retinal neovascularization (65). Oral administration of the drug resulted in complete blockade of neovascularization, confirming that PTK787 is very effective in inhibiting VEGF signaling effects in the retina. The drug also completely blocked retinal neovascularization in the less selective oxygen-inducible ischemic retinopathy model. These findings suggest that inhibition of VEGFR signaling is sufficient to completely inhibit retinal neovascularization.

The VEGF/VEGFR system also drives inflammatory rheumatic and rheumatoid diseases such as rheumatoid arthritis (66, 67). In this context a recently published patent application (57) claims that 1-anilino-(4-pyridylmethyl)phthalazines are useful to treat rheumatoid arthritis and/or the related symptoms such as pain.

Summary and conclusions

1-Anilino-(4-pyridylmethyl)phthalazines are potent, selective and orally well absorbed inhibitors of vascular endothelial growth factor (VEGF) receptor tyrosine kinases. *In vitro* they block VEGF-stimulated autophosphorylation of KDR expressing cells, leading to the inhibition of survival effects of VEGF on endothelial cells. They also block PDGF-mediated effects at slightly higher concentrations but do not affect other pathways such as the bFGF receptor. To prove their efficacy *in vivo*, the effects of 1-anilino-(4-pyridylmethyl)phthalazines on cytokine-induced angiogenic responses were studied in a growth factor implant model in normal mice. In sharp contrast to their *in vitro* profile, *in vivo* 1-anilino-(4-pyridylmethyl)-phthalazines do not only inhibit VEGF signaling but in some cases they also interfere with the bFGF-induced angiogenesis pathway. In studies with tumor models expressing VEGF and bFGF in different ratios, it was demonstrated that inhibition of both VEGF- and bFGF-induced neovascularization increases the spectrum of antitumor efficacy. In regard to tolerability, no differences were observed. Inhibition of tumor growth was accompanied by profound effects on tumor vascularization and also reduction in vascular permeability. In a collaboration between Schering AG and Novartis, PTK787, a representative of the structural class of 1-anilino-(4-pyridylmethyl)phthalazines, is currently in clinical trials in cancer patients (53). Exciting results with PTK787 in other areas indicate that these compounds provide a novel therapeutic approach, not only for the treatment of cancer but also for the treatment of other diseases characterized by aberrant vascular permeability and neovascularization.

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