PTK 787/ZK 222584, a Tyrosine Kinase Inhibitor of all Known VEGF Receptors, Represses Tumor Growth with High Efficacy

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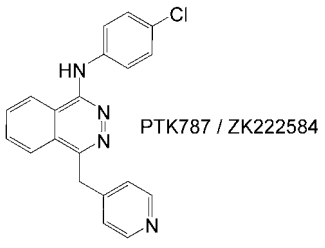
The angiogenesis inhibitor PTK 787/ZK 222584 (PTK/ZK) blocks all known VEGF receptor (VEGFR) tyrosine kinases, including the lymphangiogenic VEGFR3, in the lower nanomolar range. From a panel of 100 kinases only PDGFR, c-kit, and c-fms are inhibited beyond those in the nanomolar range. PTK/ZK functions as a competitive inhibitor at the ATP-binding site of the receptor kinase as shown here in kinetic experiments. The VEGF signal blockade in microvascular endothelial cells (MVEC) results in a blockade of MVEC proliferation ($IC_{50} = 30$ nM), without affecting the proliferation of normal tissue cells and tumor cells. The efficacy of PTK/ZK depends on its continuous presence within the endothelial target cells. Early removal attenuates its antiproliferative activity in vitro. Growth inhibition of endothelial cells is fully reversible as demonstrated by "washout" experiments. Without inhibiting tumor cell proliferation directly, PTK/ZK results in a significant retardation of tumor growth in a number of experimental tumor models of different tissue origin. Combination of PTK/ZK with an antiandrogen revealed additive effects on tumor-growth inhibition. Treatment efficacy was monitored both by tumor weight and by the determination of serum concentrations of the surrogate marker PSA. PTK/ZK is currently being investigated in patients with different solid tumor types for its therapeutic utility. Preliminary data from phase I/II clinical trials of PTK/ZK as a monotherapy suggested a positive safety and tolerability profile, which we interpret to be a consequence of the high selectivity of the drug for a limited number of kinases. Preliminary response, time to progression, and overall survival data were promising.[1] Based on these encouraging results, PTK/ZK is currently in Phase III clinical trials for metastatic colorectal cancer.

Introduction

From the time of the discovery of ATP in 1929 until the mid-1980s, most ideas about the potential of ATP-binding enzymes as a therapeutic target were hampered by concerns over possible negative consequences on energy metabolism and cell-signaling pathways. Targeting the ATP-binding pocket was also considered problematic due to its relative conservation in many enzymes. It was not until the discovery of natural molecules with the properties of specific kinase activation (e.g. phorbol esters[2,3]) and inhibition (e.g. staurosporine[4]) that a systematic conception of kinase-inhibitor synthesis began.[5] Currently, rationally designed small-molecule therapies are able to target the deep ATP-binding groove with high affinity and some binding specificity due to subtle differences within the groove. Given that many mutated, and thereby constitutively activated, cellular kinases are known to be oncogenic, these kinases are now relevant therapeutic targets for small molecules, with the hope that blocking their activity might result in tumor-growth inhibition.[6] In this respect, two small-molecule kinase inhibitors have recently entered the cancer drug market, constituting a breakthrough for this kind of therapeutic approach: Gleevec,[7] a bcr/c-abl and c-kit inhibitor, and Iressa,[8] an EGFR inhibitor.

Antiangiogenic therapy is an emerging field in tumor biology in which kinase inactivation plays an important role. Angiogenesis in adults is a physiological process in reproductive tissues and a pathophysiological one in wound healing, ocular disease, inflammation, and tumor growth. Inhibition of angiogenesis was therefore considered more than thirty years ago as a specific target for tumor therapy.[9] Blood-vessel growth is induced by binding of VEGF-A isoforms to their receptors and progresses along a VEGF gradient established in hypoxic tissues. VEGF receptors are almost exclusively located on the surface of endothelial cells.[10] The growth of lymphatic vessels is rather dependent on VEGF-C and VEGF-D.[11] To interfere with VEGF signaling is a valid strategy for blocking angiogenesis.[12,13] This can be done by means of humanized monoclonal antibodies against VEGF-A[14] or by sequestering VEGF-A with a soluble chimeric protein consisting of the extracellular domain of VEGFR1 and the Fc portion of immunoglobulins, which traps VEGF-A.[15] PTK/ZK,[16] a small-molecule tyrosine kinase in-



PTK787 / ZK222584

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hibitor, interferes with the kinase activity of all three VEGF receptors (VEGFRs). The kinase domains of VEGFRs are located intracellularly and are activated upon extracellular binding of secreted VEGF family members followed by receptor dimerization. Here we show that VEGFR inhibition by PTK/ZK is achieved by competitive binding of the small molecule at the ATP-binding site within the kinase domains of VEGFR, and that the enzyme inhibition is reversible. Furthermore, we show that the enzyme inhibition is highly selective for VEGFRs and translates to growth inhibition of experimental tumors. We will discuss these findings in the light of early clinical experience with this compound.

Results

Kinase selectivity

Highly selective inhibitors are demanded for therapeutic use, since kinases are rather ubiquitous regulators of cell function. Therefore approximately 100 different kinases were analyzed for their inhibition by PTK/ZK at a concentration of 10 μM (Table 1). Inhibition in the nanomolar concentration range was found only for the angiogenesis-regulating kinases VEGFR1 ($IC_{50} = 54 \pm 46$ nM, $n = 90$), VEGFR2 ($IC_{50} = 39 \pm 21$ nM, $n = 264$), VEGFR3 (see cellular assays), and to a lesser extent PDGFRβ ($IC_{50} = 567 \pm 504$ nM, $n = 39$; Table 2a). The hematopoiesis-directed kinase receptors c-kit ($IC_{50} = 364 \pm 173$ nM, $n = 60$) and c-fms (600 nM, $n = 2$) were the only other kinases that were inhibited in this range. These findings are in accordance with those of Wood et al.[16] and Bold et al.[17] Lyn ($IC_{50} = 1.8$ μM), a member of the src kinase family, and c-raf ($IC_{50} = 3.6$ μM) are intracellular kinases and the only angiogenesis-irrelevant kinases found to be inhibited below 10 μM (Table 2b). For all other kinases, inhibition was weaker or even absent (Table 2c).

Table 1. Inhibition of kinases by PTK/ZK (double determinations); results are given as % inhibition of the respective kinase activity at 10 μM PTK/ZK, negative values represent measurement artifacts.

Abl(h)	36	Fms(h)	99	PKBβ(h)	6
Abl(T315I)(h)	2	Fyn(h)	−8	PKBγ(h)	−4
ALK(h)	68	GSK3α(h)	10	PKCα(h)	8
AMPK(r)	3	GSK3β(h)	6	PKCβII(h)	3
Arg(m)	35	IGF-1R(h)	7	PKCγ(h)	−4
Aurora-A(h)	79	IKKα(h)	1	PKCδ(h)	22
Axl(h)	12	IKKβ(h)	−6	PKCε(h)	−6
Blk(m)	32	IR(h)	6	PKCη(h)	8
Bmx(h)	5	JNK1α1(h)	−22	PKCι(h)	−6
BTK(h)	10	JNK2α2(h)	8	PKCμ(h)	7
CaMKII(r)	−10	JNK3(h)	−31	PKCθ(h)	3
CaMKIV(h)	−8	Lck(h)	30	PKD2(h)	5
CDK1/cyclinB(h)	4	MAPK1(h)	2	PRAK(h)	20
CDK2/cyclinE(h)	−1	MAPK2(h)	−1	PRK2(h)	20
CDK3/cyclinE(h)	5	MAPKAP-K2(h)	12	Ros(h)	−5
CDK5/p35(h)	64	MEK1(h)	−12	Rsk1(h)	−10
CDK6/cyclinD3(h)	8	Met(h)	14	Rsk2(h)	4
CDK7/cyclinH/MAT1(h)	7	MKK4(m)	−3	Rsk3(h)	10
CHK1(h)	-8	MKK6(h)	22	SAPK2a(h)	2β
CHK2(h)	2	MKK7β(h)	−35	SAPK2b(h)	10
CK1δ(h)	−14	MSK1(h)	1	SAPK3(h)	13
CK2(h)	8	MST2(h)	9	SAPK4(h)	−5
CSK(h)	23	NEK2(h)	−9	SGK(h)	44
c-SRC(h)	26	p70S6K(h)	5	Syk(h)	28
EGFR(h)	−18	PAK2(h)	−4	TrkB(h)	26
EphB2(h)	5	PAR-1Bα(h)	−4	Yes(h)	13
EphB4(h)	−10	PDGFRα(h)	76	ZAP-70(h)	25
Fes(h)	−13	PDK1(h)	−4	PRAK(h)	20
FGFR3(h)	14	PKA(h)	7		
Flt3(h)	7	PKBα(h)	3		

Table 2. Inhibition of kinases by PTK/ZK as shown by concentration/response curves.

	Dose response IC_{50} [nM]	Number of experiments
a) $IC_{50} < 1$ μM		
VEGFR1	54f ± 46	90
VEGFR2	39 ± 21	264
c-kit	364 ± 173	60
c-fms	600	2
PDGFRβ	567 ± 504	39
b) $IC_{50} = 1$–5 μM		
Lyn	1800	2
c-raf	3600	2
c) $IC_{50} > 10$ μM		
CDK1	> 10000	2
CDK2	> 10000	4
CDK4	> 10000	2
EGFR	> 10000	4
c-fyn	> 10000	2
GSKβ3	> 10000	1
InsR	> 10000	4
MAP	> 10000	2
PKCα(h)	> 10000	1
Plk-1	> 10000	1
c-src	> 10000	2
Tie2(h)	> 10000	5

Mechanism of kinase inhibition

To investigate the mechanism by which PTK/ZK inhibits the signaling of VEGFRs, PTK/ZK was used in the presence of increasing amounts of ATP in a VEGFR-2 kinase inhibition assay with a chimera of the GST-VEGFR kinase domain as enzyme. It was found that PTK/ZK exhibited reaction kinetics consistent with competitive inhibition to block ATP binding. In Figure 1, the results are presented as a Dixon plot. The regression lines, representing the reciprocal reaction velocities versus the inhibitor concentrations, intersect at one point for all inhibition curves as expected for a competitive mode of inhibition. Their increasingly flatter slopes are a sign of the decreasing apparent $K_{M\text{-app}}$ with increasing ATP concentrations.

Inhibition of cellular kinases VEGFR-2 and VEGFR-3

The inhibition of VEGFR kinases requires the permeation of the drug into the cell. When microvascular endothelial cells (MVECs) were incubated with PTK/ZK for 5 min followed by the addition of VEGF-A, we observed a concentration-dependent inhibition of VEGFR-2 autophosphorylation. In Figure 2, a Western blot of VEGFR-2 receptor autophosphorylation is

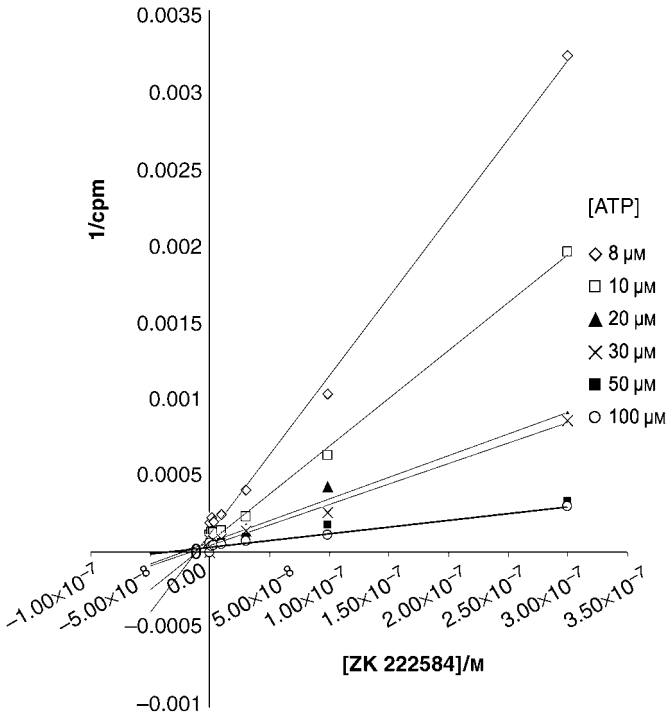

Figure 1. *Competitive inhibition of VEGFR2-kinase by PTK/ZK. Concentration-dependent inhibition of GST-VEGFR kinase activity was investigated as described in the Experimental Section in the presence of increasing concentrations of ATP. The results are given as a Dixon representation of the inverse of reaction velocity versus concentration of PTK/ZK (data points and linear trend lines).*

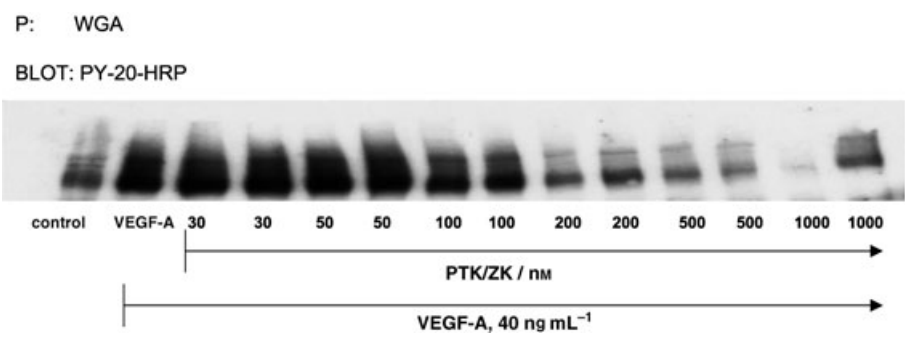

Figure 2. *Inhibition of VEGFR2 autophosphorylation by PTK/ZK. MVECs were cultured in 24-well plates and maintained overnight in low-serum medium before PTK/ZK was added in increasing concentrations followed by stimulation with VEGF-A at 40ng mL^{-1}. Cell lysates were treated as indicated in the Experimental Section.*

shown. When MVECs were stimulated with VEGF-C, similar results were seen for the inhibition of VEGFR-3 receptor autophosphorylation tested either by Western blot or by ELISA. In Figure 3 a concentration-dependent VEGFR-3 signaling decrease is shown in the presence of PTK/ZK. The IC_{50} of 30 nM is in the same concentration range as that for VEGFR-2.

Duration and reversibility of inhibition

The inhibition of VEGFR kinases in endothelial cells by PTK/ZK prevented VEGF-dependent cell proliferation (IC_{50} = 30 nM; Figure 4). It is worth mentioning that at a concentration of 3 μM there was complete inhibition of cellular proliferation to levels similar to that of the unstimulated control cells, while no cytotoxicity was observed for tumor cells (data not shown). This assay was then used to assess the onset and reversibility of the inhibitory action of PTK/ZK. We incubated endothelial cells under assay conditions as described above and added the inhibitor PTK/ZK either at the beginning or at later time points. Belated addition of PTK/ZK to cell preparations led to a shift in apparent IC_{50} values, with a complete lack of inhibition observed when PTK/ZK was added later than 32 h from the start of the proliferation assay (Figure 5). Furthermore, we examined the reversibility of the inhibitory action of PTK/ZK on endothelial cells. Figure 6 shows the effect on endothelial cell proliferation after simultaneous addition of PTK/ZK with VEGF-A, followed by the removal of PTK/ZK from the medium at various time points by exchange of medium plus VEGF. When PTK/ZK was removed directly after the start of growth stimulation with VEGF-A or for up to 8 h following, almost no endothelial cell growth inhibition was observed compared to controls. However, when the removal was made 48 h from the start of the experiment, the inhibition of cell proliferation reached its maximum and could no longer be remedied by the removal of PTK/ZK.

To further show the reversibility of PTK/ZK inhibition of proliferation, we performed a proliferation experiment in which we incubated MVEC for 24 h with PTK/ZK or not, washed it out and then added medium or medium plus VEGF (Figure 7). The first observation is that the number of cells does not increase very much in the culture period. Cells cultured in low-serum medium did not proliferate much either within one day or four days. Treatment with PTK/ZK for one day reduces the cell number slightly both after one or four days of culture. If, however, VEGF is present beginning with day 2 for three days, the endothelial cell proliferation can be stimulated similarly, both in cells that never experienced PTK/ZK or those treated with 3 μM of PTK/ZK for 24 h. Due to the interjacent treatments and washings, the VEGF stimulation ratio for both groups is not as high as with previous or parallel proliferation assays (cf Figure 4).

Inhibition of tumor growth

PTK/ZK has a high oral bioavailability (65 μM after 0.5 h; 75 μM after 1 h; 50 μM after 4 h in female NMRI nu/nu mice after application of 50 mg kg^{-1}) and its efficacy has been analyzed in a number of different syngeneic and xenograft tumors. Table 3 shows a summary of the *T/C* ratios of several tumor models after treatment with PTK/ZK. In most cases, an approximately 50% inhibition of the tumor growth was found, independently

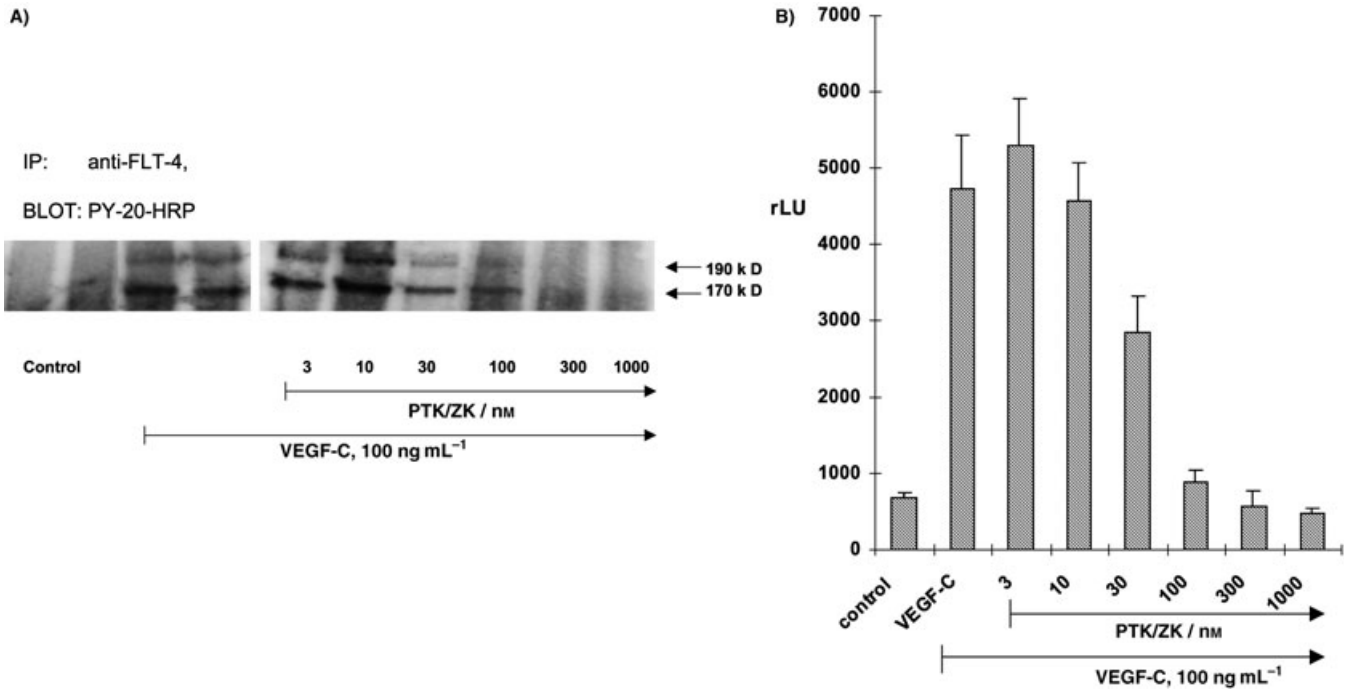

Figure 3. *A) and B) Inhibition of VEGFR3 autophosphorylation by PTK/ZK. MVECs were stimulated with 100 ng mL^{-1} VEGF-C in the presence of increasing amounts of PTK/ZK, and the lysates were investigated by A) Western blot or B) ELISA.*

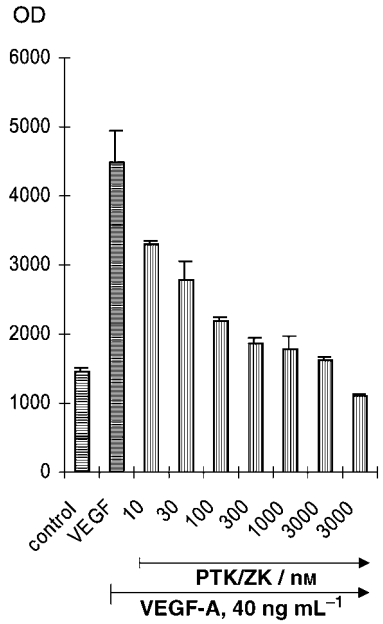

Figure 4. *Inhibition of microvascular endothelial cell proliferation by PTK/ZK. MVECs were cultured for 3 days in the presence of 1 nM VEGF-A and different concentrations of PTK/ZK. The cell number was determined as described in the Experimental Section.*

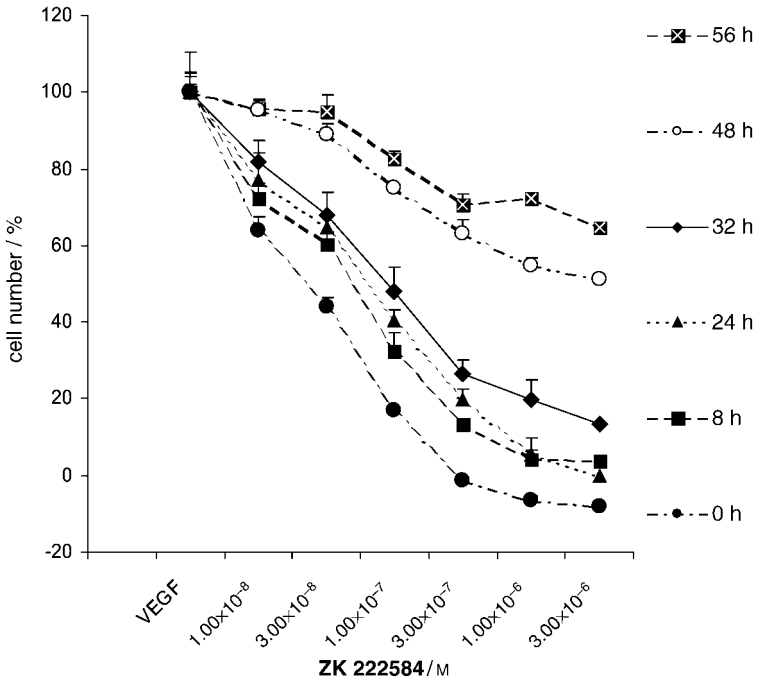

Figure 5. *Inhibition of VEGF-A-stimulated MVEC proliferation by PTK/ZK is dependent on the time of compound addition. MVEC were treated with 40 ng mL^{-1} VEGF-A, and PTK/ZK was added after increasing periods of time. The amount of endothelial cells was quantified after 3 days of culture. The graph shows the set of curves obtained for the different time points of PTK/ZK addition and demonstrates the apparent increase in IC_{50} values upon belated addition.*

of the origin of the tumor. The animal weights developed in parallel in treated and untreated groups; this indicated that no acute toxicity occurred (data not shown). The effects of increasing doses of PTK/ZK on tumor growth inhibition were investigated in the B16F10 melanoma model, starting with daily doses of 25 mg kg^{-1} up to 100 mg kg^{-1} and also with dosing twice daily. As illustrated in Figure 8a, the tumor growth in C57/Bl6 mice was inhibited in a dose-dependent manner. Twice daily dosing improved effica-

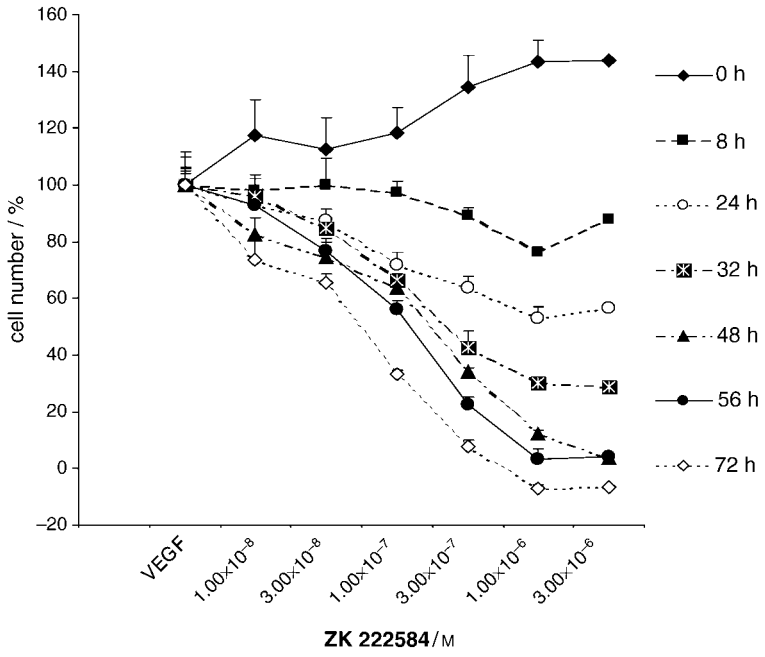

Figure 6. *Washout of PTK/ZK is possible and relieves inhibition of proliferation. MVECs were treated with 40 ng mL^{-1} VEGF-A in the presence of PTK/ZK. Then the medium was removed, followed by two washes with medium before VEGF-A was added, without inhibitor. The apparent changes in IC_{50} are depicted. The set of curves representing the different time points of washout demonstrate that early removal of PTK/ZK abrogates the inhibitory action of PTK/ZK.*

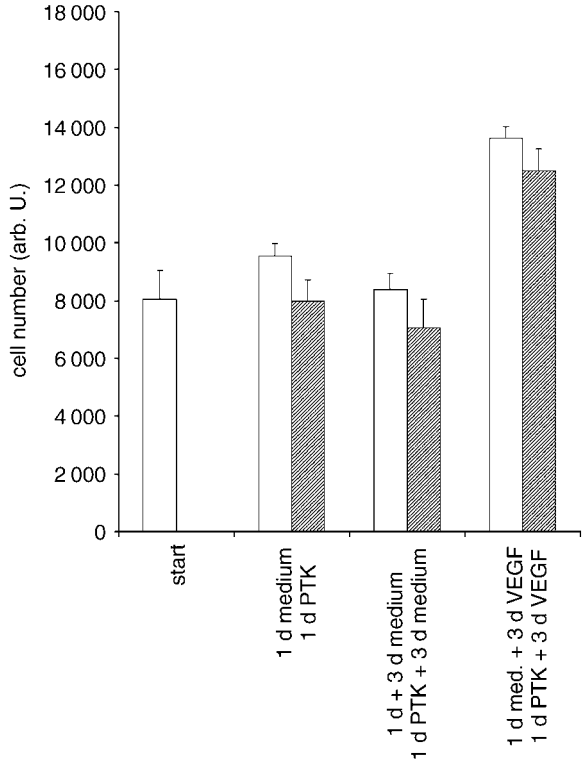

Figure 7. *MVEC cell number after various times of culture. MVECs were added to culture dishes, and cell number was determined after 16 h in serum-poor medium (start). Then cells were cultured for 24 h with (hatched) or without PTK/ZK, washed and quantified. Parallel cultures, pretreated or not for 24 h, were then cultured either in low-serum medium or in VEGF containing low-serum medium for another 3 days and quantified.*

cy even further (Figure 8b). In Figure 9a, the inhibitory time course of tumor growth was investigated for the human prostate hormone-sensitive xenograft CWR22. Effective tumor-growth inhibition was also observed in this model. The efficacy could be increased by a combination of PTK/ZK (100 mg kg^{-1} perorally (p.o.)) and the antiandrogen cyproterone acetate (CPA; 30 mg kg^{-1} subcutaneously (s.c.)). In addition, serum concentrations of prostate-specific antigen (PSA) of the animals were measured; this is used as a surrogate marker in the adjuvant therapy with antiandrogens (Figure 9b). We found that tumor size and plasma PSA concentrations decreased in direct correlation with the treatment success (4.8 μg L^{-1} for the combination versus 52 μg L^{-1} in untreated control animals at day 34; Figure 9b).

Discussion

The identification and characterization of PTK/ZK is an example of a selective, potent, and well-tolerated kinase inhibitor that is suitable for tumor therapy. Almost 100 out of the 518 putative and postulated cellular kinases were tested[18] and no other kinase than those described here was found to be inhibited in a nanomolar range by PTK/ZK. All three VEGFR tyrosine kinases (1–3) were inhibited efficiently. The observed selectivity of PTK/ZK and its lack of effects on tumor-cell proliferation support the assertion that the inhibition of VEGFR kinases is the relevant mechanism by which tumor-growth inhibition in animal models is achieved. Compounds with a broader kinase-inhibition profile, such as small-molecule inhibitors of VEGFRs and EGFR, are known to cause additional side effects, such as rashes[19] and damage of the intestinal mucosa resulting in serious diarrhea.[20] In animals, damage in the eye and buccal mucosa was observed after long-term treatment with compounds that show EGFR-blocking efficacy (~1 month; unpublished results by M.H.). By contrast, the weaker inhibition of PDGFR-β by PTK/ZK may further enhance its antiangiogenic activity as PDGF signaling has been shown to be important in the interaction of pericytes with endothelial cells and thus secures the stabilization of developing blood vessels.[21] c-kit plays a role in hematopoietic cell development; however, in animal experiments, no changes in leukocyte patterns were observed.[16] Hypertension is a common side effect for VEGFR kinase inhibitors, including PTK/ZK. This reaction is possibly due to the blocking of VEGF signaling in its function as an endothelial permeability enhancer[22] and results in a generalized fluid retention within the vascular bed. Hypotension is known as a NO-mediated side effect of VEGF action[23,24], and VEGF signal blocking can thus result in hypertension.[25] Clinically, this is generally manageable by antihypertensive medication.[26]

The competitive inhibition of ATP binding by PTK/ZK to the kinase domain resulted in a blockade of VEGFR autophosphorylation and in inhibition of endothelial-cell proliferation. The reversibility of PTK/ZK action is shown by the resumption of

Table 3. *Antitumor efficacy of PTK/ZK in various experimental human tumor models. T/C is defined as the ratio of the tumor weights of treated (T) and untreated (C) animals.*

Model	Origin	Dose of PTK/ZK [mg kg^{-1} d^{-1} p.o.]	Duration of experiment [d]	Weight of control tumors [mg ± SD]	Weight of PTK/ZK-treated tumors [mg ± SD]	T/C
A375	melanoma	50	14	641 ± 454	301 ± 335	0.47
HCT116	colon	25 twice daily	19	599 ± 128	292 ± 151	0.49
HCT116	colon	50 twice daily	19	599 ± 128	308 ± 67	0.51
MaTu/ ADR	mamma	50	21	663 ± 366	372 ± 237	0.56
DU145	prostate	50	35	408 ± 83	254 ± 128	0.61
CWR22	prostate	50	33	1106 ± 601	509 ± 256	0.46

MVEC growth after removal of the agent without inducing cellular toxicity. The reversible inhibition of proliferation is in contrast to mechanisms proposed for other VEGFR inhibitors, such as SU5416, that appear to bind irreversibly[27] (a finding that was questioned, however, see review [28]). A consequence of the reversible binding of PTK/ZK is the requirement for a continuous presence of PTK/ZK to exert its activity, as evidenced by a loss of its inhibitory effect following washout of the agent. The reversibility of action in combination with a lack of accumulation of the drug may well be an advantage in the clinic, allowing a rapid interruption of dosing in case of surgery and after accidents, thus avoiding adverse effects on wound healing. Plasma concentrations of PTK/ZK in patients that were 5–10 times higher than the

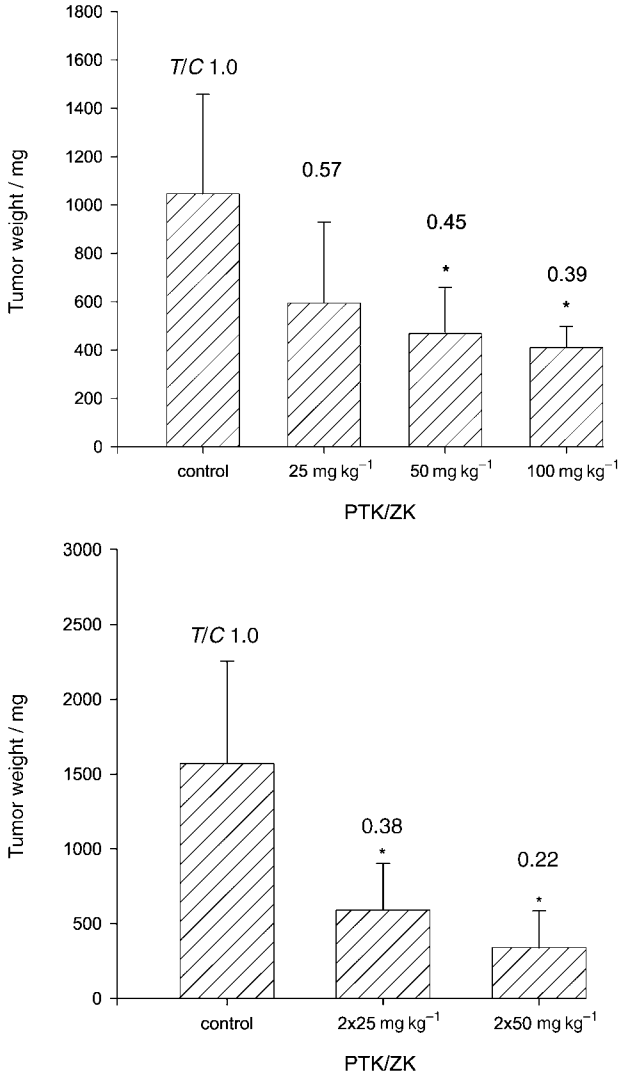

Figure 8. *A) and B) Dose dependency of syngeneic tumor-growth inhibition by PTK/ZK and the effect of dosing twice daily. Mouse B16F10 melanoma cells were grafted onto C57/Bl6 mice. Animals were treated during the entire period of growth (12 d) with oral doses A) once or B) twice a day.*

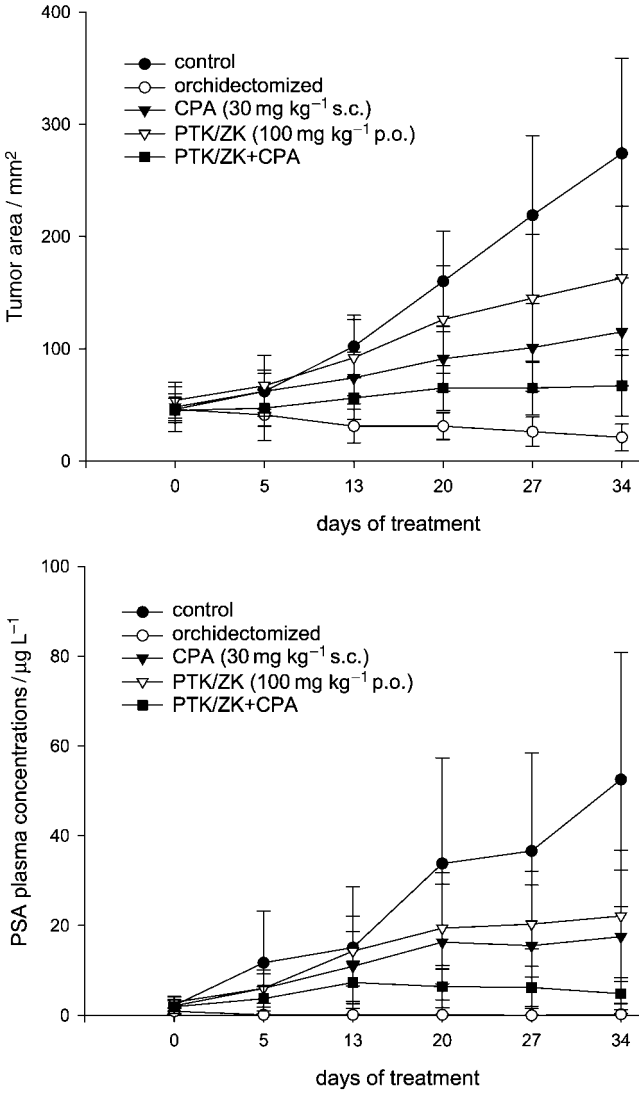

Figure 9. *A) and B) Growth inhibition in the human hormone-dependent prostate tumor xenograft model CWR22 by PTK/ZK is accompanied by a reduction in PSA plasma concentrations. A) Tumor cells were grafted on nude mice and treated after establishment with 100 mg kg^{-1} PTK/ZK daily p.o. or 30 mg kg^{-1} cyproterone acetate (CPA) s.c. three times a week (Monday, Wednesday, Friday) or a combination of both. B) PSA concentrations in serum samples were measured regularly.*

IC_{50} concentration necessary for inhibition of in vitro cellular functions were maintained with therapeutic doses for 24 h (for details see ref. [1]). It is assumed therefore that VEGF signaling is blocked completely for most of the time. However, even a partial signal blockade should be effective in angiogenesis inhibition because VEGF action is subject to dosing effects, as shown previously with VEGF-producing myoblasts implanted into mice[29] and heterozygous VEGF gene-ablated animals, which die at mid-gestation due to incomplete vessel formation.[30]

In cellular assays, PTK/ZK inhibited VEGFR-3 with the same potency as VEGFR-2 and VEGFR-1. As this receptor is described as a major regulator of lymphangiogenesis, one might expect that PTK/ZK has an effect on tumor metastasis.[31] There are some hints in the literature that an increase in the formation of lymphatic vessels accompanying tumor growth causes increased metastases.[32] It is not clear, however, if lymphatic vessel growth is a prerequisite for tumor metastases.[33]

PTK/ZK, being highly selective and relatively nontoxic, resulted in vivo in the inhibition of tumor growth in a variety of different experimental tumor models. The effect was largely independent of the origin of the tumor. Tumor regression was generally not found, but rather an attenuation of tumor growth. These findings confirm results of recently published data from other models.[16] Taken together, it is tempting to speculate that PTK/ZK may be effective in the clinic in a number of solid-tumor indications. This assumption is supported by the first results from clinical phase I/II studies, in which PTK/ZK showed preliminary evidence of antitumor activity in patients with different types of advanced solid tumors. Morgan et al.[1,26] correlated dynamic contrast-enhanced magnetic resonance imaging parameters with therapeutic efficacy in patients suffering from advanced colorectal cancer and liver metastases. These results prompted the initiation of two clinical phase III studies (CONFIRM I/II), in which patients with metastatic colorectal cancer were treated either with a combination regimen of oxaliplatin/leucovorin/5-FU (FOLFOX4) and daily oral PTK/ZK or with chemotherapy alone.

We interpret the favorable safety profile of PTK/ZK as evident from preclinical results as well as from the preliminary clinical data as a consequence of its kinase-selectivity pattern. It can be speculated that, in future, antiangiogenic treatment, like cytostatic therapy, might consist of a combination of several drugs targeting different angiogenic signaling mechanisms. Those might be applied transiently or continuously to obtain more complete effects on the growing vessel system, such that the therapy may result in tumor-size reduction, an effect not observed regularly if VEGF signaling is blocked alone.

Experimental Section

Methods

Endothelial-cell isolation: Isolation from foreskin, cell culture, proliferation assays, and VEGFR2-receptor autophosphorylation was performed as previously described.[34]

VEGFR-3 autophosphorylation: MVECs (1.5×10^6 per well) of low passage number were plated on collagen G-coated 48-well plates in endothelial cell basal medium (EBM) and endothelial cell growth medium (EGM) complete medium (including EGM-2, BD-Clonetech). After 5 h, medium was exchanged for EBM-2 without EGM-2 but containing 0.2% bovine serum albumin (BSA; EBM meager). After another 12 h, the medium was removed, EBM-2 meager (250 μL) and the respective compound dilutions were added in EBM-2 meager (50 μL). Solutions were carefully mixed and left for 5 min at 4 °C before the addition of EBM-2 meager (200 μL) containing VEGF-C (final concentration in the assay was 5 nM; Reliatech, Braunschweig). The solution was then carefully mixed and incubated for 15 min at room temperature. The medium was removed, and cells were washed twice with cold phosphate-buffered saline/2 mM vanadate. Cells were then lysed with Duschl buffer (100 μL; 50 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 1.5% Triton X-100, 10 mM Na pyrophosphate, 100 mM NaF, 10% glycerol, and (freshly added before the experiment) 2 mM orthovanadate and 1 tablet Complete (Roche # 1836145) per 50 mL).

For the ELISA, Fluoronic MaxiSorp—microtiter plates (# 3204006 Zinser)—were coated overnight at 4 °C with Flt-4 antibody (Flt-4 (C-20) # sc-321, Santa Cruz; 1 $\mu g\,mL^{-1}$) in coating buffer ($NaCO_3$, pH 9.6, 100 μL per well). After washing with washing buffer (3×, 0.1% Tween 20 in Na_2HPO_4, pH 7.4), the wells were incubated with blocking buffer (250 μL, Roti Block 1/10 from Roth, Karlsruhe for 1 h at RT). Washing with washing buffer (3×) was followed by addition of cell lysates and incubation overnight at 4 °C. Then wells were washed with buffer (3×), anti-phosophotyrosine antibody coupled to HRP (16–105; UPSTATE; dilution 1:20000 in TBST + 3% Top Block # 37766, Fluka) was added, and the plates were incubated overnight at 4 °C. Washing with washing buffer (6×) preceded the addition of BM chemoluminescence ELISA reagent # 1582950 (Roche) and measurement of luminescence.

Kinase assays: Inhibition of VEGFR-1, VEGFR-2, c-kit, c-fms, c-raf, lyn, and PDGFRβ activity: Kinase activity was measured with GST-kinase domain fusion constructs of the respective kinases according to the following protocol to obtain concentration response curves. Components were added to a microtiter plate in the following sequence: inhibitor in threefold final concentration (10 μL; 3% DMSO in buffer (40 mM TrisCl, pH 7.5, 1 mM DTT, 1 mM $MnCl_2$, 10 mM $MgCl_2$, 0.2% poly(ethylene glycol) 20000)) and substrate mixture (10 μL; 24 μM ATP containing 500–1000 cpm per pmol γ^{33}P-ATP, 24 $\mu g\,mL^{-1}$ poly(Glu_4Tyr) in buffer). Reaction was started by adding enzyme preparation (10 μL) diluted appropriately in buffer that contained vanadate (10 μM). After incubation for exactly 10 min, the reaction was stopped by addition of stop solution (10 μL, 250 mM EDTA). Aliquots (10 μL) of the reaction mixture were transferred to phosphocellulose filters. The filters were washed in 0.1% phosphoric acid and dried before meltilex scintillator was applied (Wallac, Perkin–Elmer) and the radioactivity was counted. For PDGFR-β and c-kit, final ATP concentrations were reduced to 100 nM. For c-fms, the following buffer system was used: 20 mM TRIS-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μM orthovanadate, 1 mM DTT, 250 $\mu g\,mL^{-1}$ poly(ethylene glycol) 20000.

Inhibitory action upon other kinases were determined at Upstate Inc, Charlottesville, in the presence of PTK/ZK at a 10 μM concentration. For lyn, concentration-dependent inhibition curves were prepared by Upstate Inc. In brief, in a final reaction volume of 25 μL, Lyn (h) (5–10 mU) was incubated with poly(Glu_4Tyr) (0.1 $mg\,mL^{-1}$; in 50 mM Tris, pH 7.5, 0.1 mM EGTA, 0.1 mM, Na_3VO_4, 0.1% β-mercaptoethanol, 10 mM Mg acetate) and γ-33P-ATP mix (specific activity ca. 500 $cpm\,pmol^{-1}$, 100 μM ATP (in 4 mM MOPS buffer, pH 7.2, containing 0.2 mM DTT, 0.2 mM orthovanadate, 1 mM EGTA, 5 mM α-glycerol phosphate, 15 mM $MgCl_2$)). The reac-

tion was initiated by the addition of the Mg-ATP mix. After incubation for 40 min at RT, the reaction was stopped by the addition of a 3% phosphoric acid solution (5 μL). 10 μL of the reaction was then spotted onto a Filtermat A and washed (3× 5 min) in phosphoric acid (75 mM) and methanol (1×) prior to drying and scintillation counting. (For details see http://www.upstate.com/img/coa/14-510-25281U.pdf)

Experimental tumor models: Tumor cells were implanted by a single subcutaneous injection of $0.5–1.5\times10^6$ cells, dependent on the tumor type (1:1 with Matrigel), in Earl's solution (0.1 mL). Treatment with PTK/ZK started after establishment of the tumors (tumor size ~20–40 mm^2). The treatment in the B16F10 model started one day after injection of the tumor cells. The parameters evaluated were tumor area (tumor size) and tumor weight compared to control as indicated by the *T/C* ratio. Tumor area was measured by using a caliper and calculated by multiplication of the longest diameter and its perpendicular. Determination of plasma PSA concentrations was performed according to the manufacturer's protocol (DRG International Inc., USA).

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