Supplementary Data

Supplementary Methods

Kinase inhibition

The cytoplasmic tyrosine kinase domain of VEGFR-2 (residues 797 to 1355 according to sequence deposited in databank SWISS-PROT P35968) was cloned into pFastBac fused to Glutathione-S-transferase (GST). The GST-fusion protein was expressed in SF-9 insect cells and extracted with HEPEX (20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM ß-glycerophosphate, 10 mM para-nitro-phenylphosphate, 30 mM NaF, 5 mM EDTA, 5% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 0.1% SDS, 0.5 µg/ml pepstatin A, 2.5 µg/ml 3,4-dichloroisocoumarin, 2.5 µg/ml trans-epoxysuccinyl-L-leucyl-L-amido butane, aprotinin 20 KIU/ml, leupeptin 2 µg/ml, benzamidine 1 mM and 0.002% PMSF). A random polymer POLY (Glu, Tyr 4:1) from SIGMA was used as substrate. Each 50 µl-reaction contained 5 % DMSO, 40 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mg/ml POLY (Glu, Tyr), 0.05% Triton X-100, 100 µM ATP, 1 µCi [γ-33P]ATP and 10 µl of enzyme preparation. Assays were carried out at room temperature for 20 minutes and terminated by the addition of 10 µl of 5 % H_3PO_4. Precipitated substrate was then trapped onto GF/B filters (Packard, 6005177) using a 96 well filter mate universal harvester (Packard, C961961). Incorporated radioactivity was determined by scintillation counting using a Microbeta Counter™ (Top Count). All measurements were performed in triplicates. For all other kinase assays the entire cytoplasmic domains of the receptors (from the end of the transmembrane region to the C-terminus) were cloned into pfastBac vector containing a GST. Fusion proteins were expressed in SF-9 insect cells and extracted with HEPEX buffer. The enzymatic reactions were performed in the presence of 1

µg/ml biotinylated PolyEY, 500 µg/ml unbiotinylated PolyEY (Sigma P-0275), 500 µM ATP (Sigma A-3377), 15 mM MgAc2, 40mM HEPES pH 7.4 for 30 min in 100 µl total volume. Reaction was stopped after 60 min by adding 50 µl of stop mix (250 mM EDTA, 20 mM HEPES pH 7.4); 100 µl of the solution was transferred to streptavidin-coated 96 well microtiter plates (Roche Streptawell #1989 685). After 30 min, the plates were washed with PBS in a Skatron Skanwasher 300b washer and 200 µl of a 250 ng/ml dilution of PY20 antibody (Wallac AD0039) in dilution buffer (Wallac 1244-111) was added. Excess antibody was removed (Wash Concentrate, Wallac 1244-114) and the reaction was developed by adding 200 µl enhancement solution (Wallac 1244-105). Time resolved fluorescence was measured in a Wallac Victor V multilabel counter, and the data were computed using the standard Graphpad software. Experiments were performed in duplicate.

Preparation of bovine retinal pericytes

The retinas from bovine eyes were mechanically dissociated followed by enzymatic digestion with 0.375 % collagenase type II in PBS with 0.25 % BSA for 30 minutes at 37°C. Afterwards the tissue was sieved through a 110 µm nylon mesh and washed with Dulbecco's Modified Eagles Medium (DMEM; BioWhittaker, catalogue #BE12-604F) containing 4.5 g/L Glucose and supplemented with 10 % fetal calf serum (FCS, PAA, catalogue #A15-043, Lot #A01128-405) and a standard complement of antibiotics and L-glutamine (both GibcoBRL). Immunopanning was performed in petri dishes coated with polyclonal goat-anti rabbit IgG antibody (DAKO, Z0421) and polyclonal rabbit anti-human von Willebrand factor (DAKO, A0082) as the second antibody for 1h at room temperature. The supernatants were transferred to tissue culture flasks (NUNC) and incubated with DMEM, 10 % FCS. Further passaging in uncoated tissue culture flasks favored the growth of pericytes. Immunological staining

with antibodies against smooth muscle alpha-actin (DAKO, M0851) and CD31 (DAKO, JC70A) for pericytes and endothelial cells respectively was performed to determine the uniformity of the pericyte culture and residual endothelial cells in the preparation. Carbocyanine 3 (Cy3) labeled secondary α-mouse IgG+IgM antibody (DAKO) was used for detection.

***In vivo* tumor models.**

Administration of BIBF 1120: For all *in vivo* experiments the test compound was suspended in a 0.5 % Natrosol solution (Hydroxyethylcellulose) and administered intragastrally by gavage needle (Infusionskanüle Olive A, Acufirm, No. 14 64 ll-1). The administration volume was 10 ml per kg body weight. Administration was once every 24 h. To prepare the suspension, compound was added to the Natrosol solution and the mixture was stirred overnight. Sometimes ultrasonication was needed. Solutions were kept at room temperature in the dark for a maximum of one week.

Monitoring tumour growth and side effects: Tumour diameters were measured three times a week (Monday, Wednesday and Friday) with calipers. Volumes [in mm3] were calculated according to the formula (tumour volume = length * diameter^2 * ?π?/6). To monitor side effects of treatment, mice were inspected daily for abnormalities and weighed three times a week (Monday, Wednesday and Friday). Animals were sacrificed when control tumours had grown to sizes of > 1250 mm3.

Evaluation of tumour responses: Therapeutic efficacy was determined based on the tumour growth in control vs. treated tumours and expressed as the T/C value.

The statistical evaluation was performed for the parameters tumour volume and body weight at the end of the experiment at day 20. For the tumour volume absolute values and for the body weight the percentage change referred to the initial weight of

day 1 was used. Due to the observed variation nonparametric methods were applied. For descriptive considerations the number of observations, the median, the lower (25%) and the upper (75%) quartile, the minimum and the maximum was calculated. For a quick overview of possible treatment effects the median of each treatment group T was referred to the median of the control C (100*T/C) for the tumour volumes. Each treatment group was compared with the vehicle control group in a one-sided (decreasing) exact Wilcoxon test. The p values for the tumour volume (efficacy parameter) were adjusted for multiple comparisons according to Bonferroni-Holm whereas the p values of the body weight (tolerability parameter) remained unadjusted in order not to overlook a possible adverse effect. The level of significance was fixed at α=5 %. An (adjusted) p-value of less than 0.05 was considered to be statistically significant. Differences between treatment groups were seen as indicative whenever $0.05 \leq$ p-value < 0.10. The statistical evaluation was prepared using the software package SAS version 8.2 (SAS Institute Inc., Cary NC, USA) and Proc StatXact (Cytel Software Corporation, Cambridge MA, USA).

Supplementary Table

Table S1. *In vivo* anti-tumor effects of BIBF 1120 in human cancer xenograft models.

Model	Derivation	Dose [mg/kg/d]	T/C value [%]
FaDu	HNSCC	100	11*
		50	14 / 27[#]
		2 x 50	15
		25	46
		10	82
Caki-1	Kidney	100	16
		50	25
		10	71
GS-9L	Glioma	50	30
		25	45
		10	74
HT-29	Colon	100	16
SKOV-3	Ovary	50	19
Calu-6	Lung	50	24
PAC-120	Prostate	100	34

*Nude mice bearing established human xenografts (0.05 - 0.1 cm^3 volume) were treated once or twice daily with BIBF 1120 p.o. (10 - 100mg/kg) or vehicle control. For T/C values, the median tumor volumes of each treatment group T was compared to the median of the control group at the end of the experiment, depending on tumor growth kinetics (see Materials and Methods).

[#]Results of two separate experimental series.

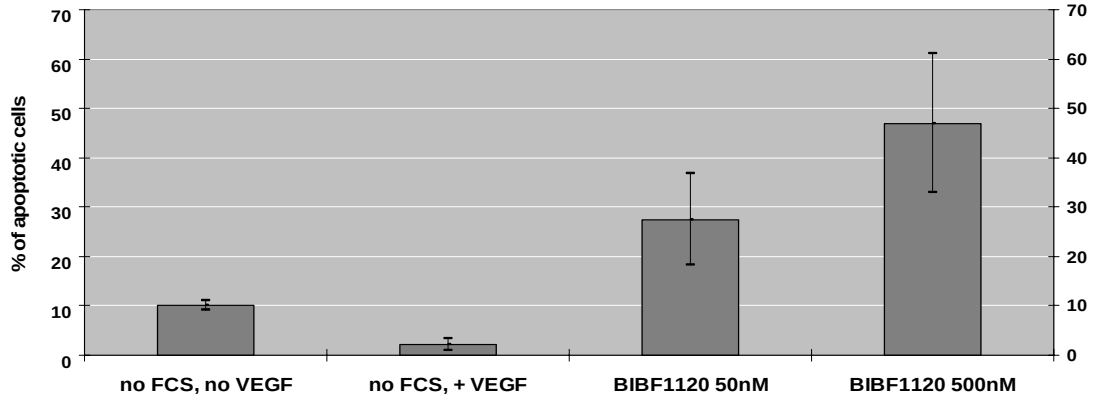
Supplementary Data Figure Legends

Figure S1. BIBF 1120 induces apoptosis in human endothelial cells (A) and shows sustained inhibition of the VEGFR-2 phosphorylation in NIH3T3 cells transfected with KDR (B). VEGFR-2 was immunoprecipitated with SC-315 (Santa Cruz Biotechnology) and the blot was stained with the anti-PY antibody #9411B from Cell Signaling.

Supplementary Data

Figure S1

A



B