Three stories on Eph kinase inhibitors: from *in silico* discovery to *in vivo* validation

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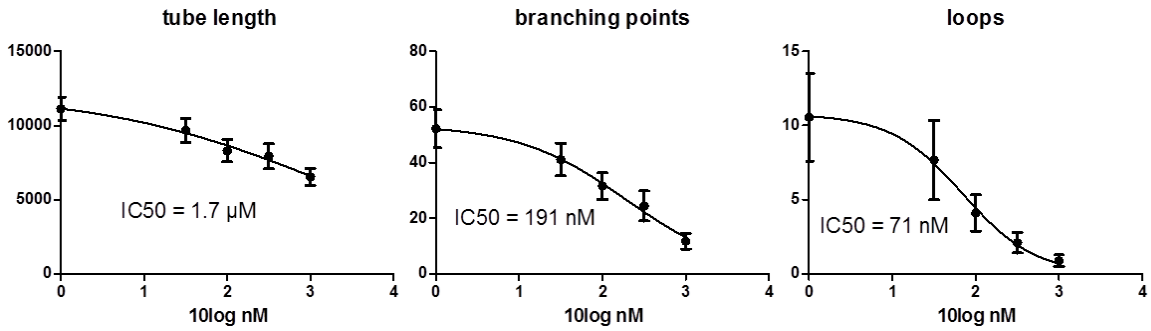
1. Angiogenesis assays

1.1 Cell Proliferation

HUVEC (human umbilical vein endothelial cells) were purchased from Promocell (Heidelberg, Germany) and cultivated in endothelial cell growth medium (Promocell) for two passages. 1.5 x 10^3 cells/well were seeded in a 96-well plate. After 24h, cells were treated with the indicated concentrations of compounds and incubated for 72h. Finally, cells were washed with PBS, incubated with 100 µL/well crystal violet solution (0.5% crystal violet, 20% methanol in H_2O) for 10 min, washed and dried. For solvation of crystal violet, 100 µl/well ethanol/Na-citrate solution (50% ethanol, 50% 0.1 M Na-Citrate in H_2O) were added, incubated for 5 min and measured at 540 nm using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland). Proliferation is indicated as % of untreated controls. No anti-proliferative effects were obtained for any of the inhibitors tested (data not shown). N = 6-9.

1.2 Tube formation

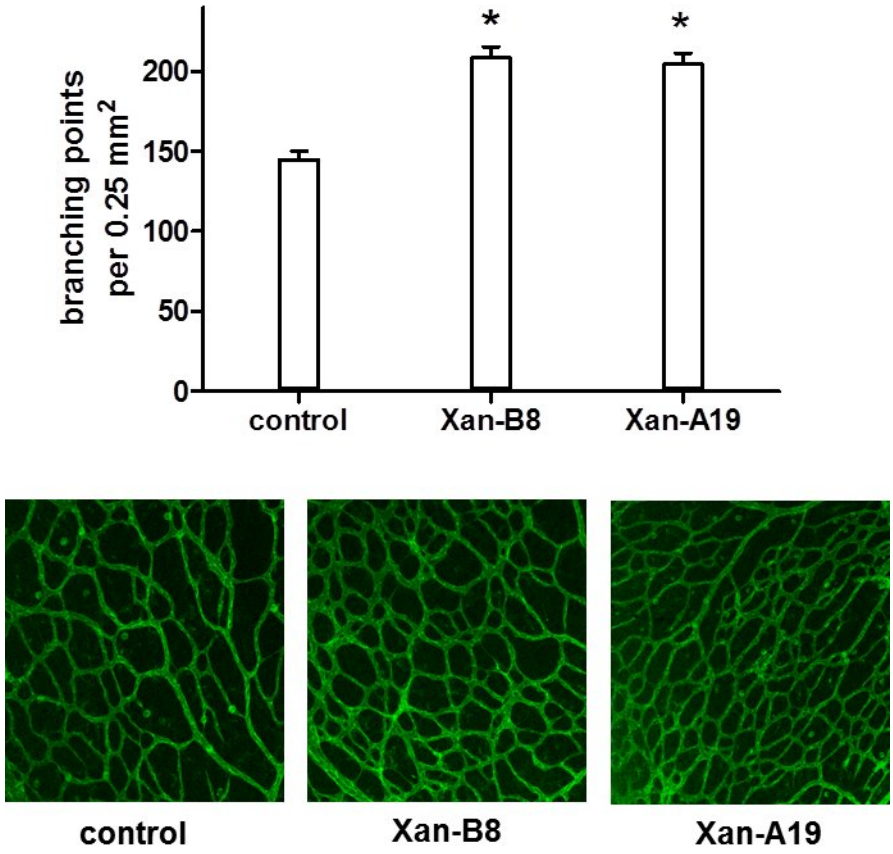
11 x 10^3 HUVEC were seeded on matrigel (Matrigel™, Schubert&Weiss-OMNILAB, Munich, Germany) in an angiogenesis slide from ibidi (Munich, Germany), treated as indicated and incubated for 15h. Images were taken using the TILLvisION system. Analysis of images was performed by Wimasis GmbH (Munich, Germany). As parameters of tube formation, tube length, number of branching points and number of loops were analyzed. N = 6-9



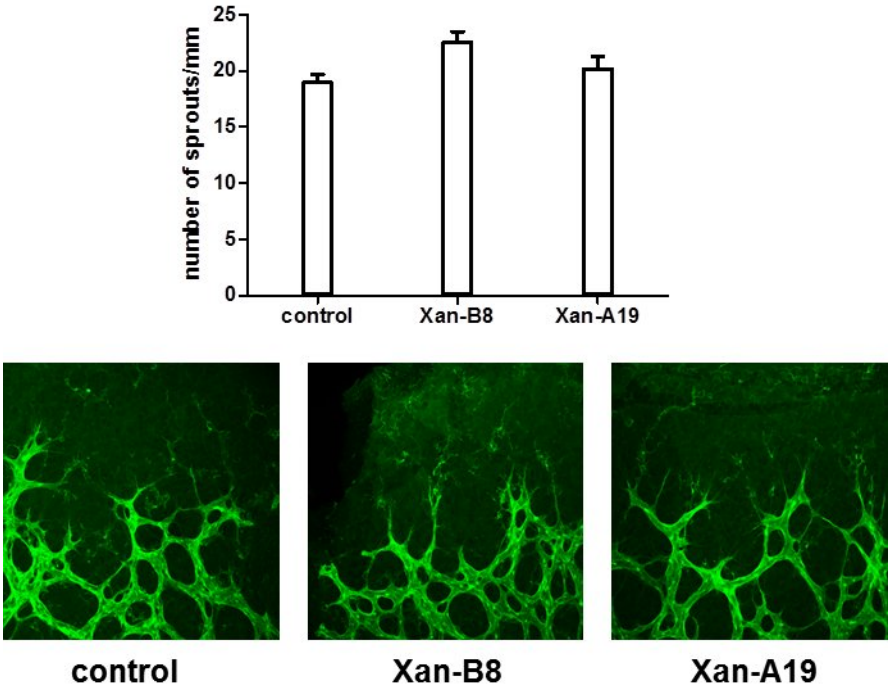
Supplementary Figure 1: Tube formation of HUVECs on Matrigel was dose dependently inhibited by **Xan-B8**. Formation of loops turned out to be the most sensitive morphometric parameter.

1.3 Mouse retina

Compounds (1 mg/kg) or DMSO equivalent was injected *i.p.* in pups of Black/6 mice at day P1, P2 and P3. On day P4 pups were sacrificed by decapitation, retinas were prepared and stained according to Pitulescu et al.[1] In brief, eyes were removed, fixed in 4% (v/v) paraformaldehyde (2 h, RT) and retinae were prepared. After blocking (2 h, RT in PBS containing 10% Triton X100 and 1% BSA), retinae were stained for isolectin B4 (IB4, Alexa 488 conjugated, Millipore). Pictures were taken with a.Leica TCS SP 8 SMD confocal microscope. The numbers of vascular sprouts per 1,000 µm perimeter of the vascular front were counted by using Image J. The number of vascular branching points per 0.25 mm^2 of tissue area was also quantified with ImageJ as previously described (Pitulescu et al.). All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life. N = 3.



Supplementary Figure 2: Vascular branching in the developing mouse retina was increased by both, **Xan-B8** and **Xan-A19**.



Supplementary Figure 3: The density of vascular sprouts per mm of vascularization front was not altered by the tested compounds.

2. Selectivity profile of Pyr-B1

The selectivity profiling was performed at DiscoveRx in a library of 456 kinases at a concentration of 1 µM. Briefly, kinases were mostly expressed as fusion proteins to T7 phase and grown in 24-well blocks in *E. Coli* (derived from the BL21 strain). The rest of the kinases were expressed in HEK-293 cells and tagged with DNA for qPCR detection. Affinity resins were generated by mixing streptavidin-coated magnetic beads with biotinylated small molecule ligands for 30 minutes at 25 °C, followed by biotin and blocking buffer addition (SeaBlock (Pierce), 1 % BSA, 0.05 % Tween 20, 1 mM DTT). Inhibitors were kept at 40x stocks in 100 % DMSO and added to the corresponding 384-well plates (40 µL). After 1 hour of incubation at 25 °C while shacking, affinity beads were washed with 0.05% Tween 20 in PBS. The beads were re-suspended in PBS buffer containing 0.05 % Tween 20 and 0.5 µM non-biotinylated affinity ligand, to then be incubated at 25 °C for 30 minutes while shacking. The kinase concentration present in the eluate was determined by qPCR.[2]

3. Fluorescence assays

3.1 Fluorescence measurements

Absorption and fluorescence measurements were recorded on a SpectraMax M5 using a quartz cuvette (1 cm). A 5 mM solution of inhibitor (in 100% DMSO) was diluted in acetonitrile and its optical density at 370 nm was adjusted to 0.075 ± 0.01. Fluorescence was measured upon excitation at 370 nm. Quantum yields were calculated using quinine hemisulfate monohydrate (φ_R = 0.53) in 1 M H_2SO_4 as a reference and according to:

$$\varphi = \varphi_R \frac{F}{F_R} \frac{A_R}{A} \frac{n^2}{n_R^2}$$

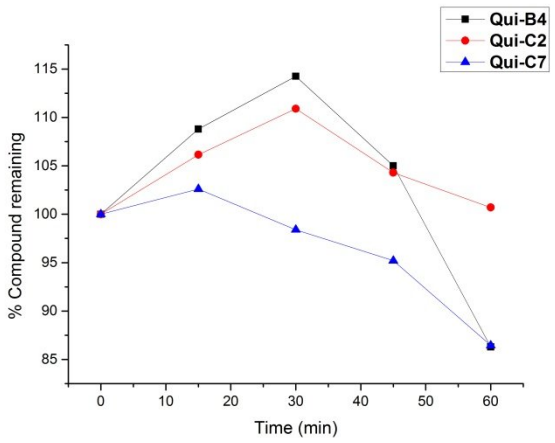
φ_R corresponds to the quantum yield of the reference. n and n_R correspond to the refractive index of the solvents used for the inhibitor and for the reference. A and A_R are the optical densities of the inhibitor and of the reference. F and F_R correspond to the integrated emission spectra.

3.2 Confocal microscopy

MDA-MB-231 cells were plated at 20,000 cells per well in height-well Lab-Tek chamber slides and allowed to incubate overnight. A 5 mM solution of **Qui-B4** (in 100% DMSO) was serially diluted in the culture media and added to the cells. After 24 h of incubation, cells were washed with PBS and fixed with 4% formaldehyde for 10 minutes at room temperature. Actin filaments were stained for 1 h using a 0.1 µM solution of tetramethylrhodamine isothiocyanate (TRITC)-phalloidin. After washing with PBS, images were taken using a 40 × 1.25 oil objective in a Leica wide field microscope.

4. Metabolic stability assays

The metabolic stability assays were performed using liver microsomes at Cerep. Compounds were stored as 10 mM stock solutions in DMSO and tested at a concentration of 0.1 µM in duplicates. The compounds were incubated at 37 °C for 0, 15, 30, 45 and 60 minutes with human liver microsomes (0.1 mg/mL) and detected using HPL-MS/MS.[3] Metabolic stability, expressed as percent of the parent compound remaining, was calculated by comparing the peak area of the compound at the time point relative to that time 0.



Supplementary Figure 4: *In vitro* metabolic stability of compounds **Qui-B4**, **Qui-C2** and **Qui-C7** determined in a liver microsome assay at Cerep. The data points represent the average of two independent measurements.

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