

## VALIDATED ANTIANGIOGENIC ASSAY

### ■ Introduction

Biobide is a Biotechnological company offering Drug Discovery services to Pharmaceutical and Biotechnological companies. The service is based on the zebrafish model and the capability to offer highly efficient assays.

The zebrafish model is gaining relevance in pre-clinical trials due to its small size, transparency, ease to manipulate and rapid development. This model has a high genetic homology with humans (over 85%) as well as important parallels in organogenesis and functional mechanisms. These characteristics make this model an ideal candidate for large-scale trials. Drugs need to be tested in pre-clinical trials before they enter into clinical trials. In vitro pre-clinical assays offer little information and are less sensitive/specific while in vivo safety assays are expensive and time consuming. The assays in zebrafish have the benefit of being rapid and cost-effective and results highly transferable to other vertebrates and humans.

Biobide has developed a method to detect the capability of a compound to inhibit angiogenesis as part of efficacy pharmacology studies. Angiogenesis, the development of new blood vessels from existing vasculature, is essential in normal developmental processes and in numerous pathologies, including diabetic retinopathy, psoriasis and tumor growth and metastasis. Thus, the molecular dissection of angiogenic signaling is clinically relevant.

In the zebrafish embryo, blood flow begins at ~ 24 h postfertilization (hpf) and shortly after, the angiogenic vessels that perfuse the trunk of the embryo (intersegmental vessels) sprout from the vasculogenic vessels (dorsal aorta (DA) and posterior cardinal vein (PCV)). Furthermore, major molecular pathways regulating angiogenesis in mammalian systems (vascular endothelial cell growth factors, fibroblast growth factors, ephrin receptors, angiopoietins, etc...) are conserved in zebrafish [1].

Angiogenic vessels are easily monitored, thus, making them suitable for identification of angiogenesis inhibitors. Interestingly, different studies have shown that treatment of zebrafish embryos with clinical stage antiangiogenic compounds inhibits growth of angiogenic blood vessels [2].

In our assays, a transgenic zebrafish line with fluorescent (cop-GFP labelled) blood vessels is used in order to facilitate the visualization and analysis of the intersegmental vessels.



■ **Figure 1.** Bright field (left) and fluorescent (right) images of 48 hours post fertilization (hpf) embryos. Endothelial cell-specific expression of cop-GFP is driven by flk1 promoter.

### ■ Method Description

Embryos of the transgenic line obtained from crossing adult zebrafish are used under strict environmental conditions (temperature, humidity and photoperiod). The method consists on the following steps:

**Decoronation:** at 24 hpf, the embryos are treated with protease to obtain embryos out of the corions.

**Treatment:** The embryos are dispensed in 24 well-plates (5 per well) in a volume of 500  $\mu$ L of E3 medium. Depending on the previous knowledge and the number of the products to test, different assays are proposed:

- **Concentration-response curves:** Test compounds are added at five concentrations into the plate (depending on the previous knowledge of the compound, the concentration range is chosen).
- **Two steps assay:** In a first step, when the knowledge of an specific compound is limited, two different concentrations (1 and 20  $\mu$ M) are tested. In a second step, a concentration-response curve is carried out.
- **Screening of large number of compounds:** Test compounds are added at a single concentration (as default 20  $\mu$ M) into the plate.

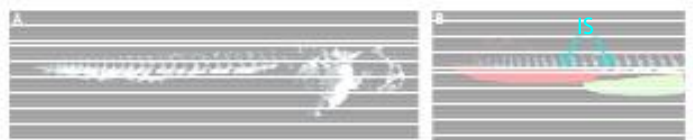
**Incubation:** The test plate is incubated for 24 h.

**Data collection:** after 24 h of treatment, embryos are anesthetized with tricaine and photomicrographs are taken.

**Analysis:** Pictures obtained are analyzed by counting two different parameters: total number of intersegmental vessels present in the trunk and number of intersegmental vessels that are complete, what means that they get to the DLAV (Dorsal Longitudinal Anastomotic Vessel).

Subsequently, statistical analysis is performed and data are interpreted.

The assay is under the scope of the Good Laboratory Practice (GLP) certification obtained by Biobide.



■ **Figure 2.** The figure shows a whole embryo (A) and the part of the trunk in which vessels are quantified (B) same picture but without the head and the yolk, (most bulging part). Intersegmental vessels (IS) are shown in blue.

## Results

To validate the Antiangiogenic Assay, the effects of five known antiangiogenic compounds are studied in zebrafish embryos. For this purpose, firstly, concentration-response curves are carried out to determine the range of compound action. Later, two different concentrations per compound are chosen (one inducing a moderate effect and the other inducing an acute response) and treatments are repeated three times in order to determine the repeatability of the assay.

### Table 1. Concentrations inducing different antiangiogenic effects in zebrafish.

Products tested: KRN633, VEGF receptor tyrosine kinase III inhibitor; AG1478, selective EGF receptor blocker; 2-Methoxyestradiol (2-ME), natural metabolite of 17 $\beta$ -estradiol that is devoid of estrogenic activity; Indirubin-3'-oxime, Cyclin-dependent kinase inhibitor; Curcumin, natural phenolic compound with potent anti-tumor, anti-inflammatory and anti-oxidant properties.

Compound	Concentration ( $\mu$ M)										
	0.001	0.01	0.02	0.05	0.1	0.5	1	5	10	20	40
KRN633											
AG1478											
2-ME											
Indirubin											
Curcumin											

Not tested  
  No effect  
  \*Moderate effect  
  \*\*Acute effect  
  Toxic/lethal

\*Moderate effect: the value of any of the two parameters analyzed is higher than half of the number found in control embryos (see AG1478 40  $\mu$ M).

\*\*Acute effect: when the value of the two parameters analyzed represents less than 50% of the control value (see KRN633 50 nM).

### Table 2. Compound's antiangiogenic activity in different systems.

Compound	Zebrafish	Cells	Animal models	Humans
	KRN633	Yes (10-50 nM)	ND	Yes
AG1478	Yes (10-40 $\mu$ M)	Yes (1 $\mu$ M)	Yes	ND
2-ME	Yes (5-20 $\mu$ M)	Yes (1-10 $\mu$ M)	Yes	Yes
Indirubin	Yes (0.5-10 $\mu$ M)	Yes (10-50 $\mu$ M)	ND	ND
Curcumin	Yes (5-10 $\mu$ M)	Yes (10-60 $\mu$ M)	Yes	Yes

ND: Not Described

### Table 3. Summary table of the antiangiogenic effects of tested products (n=3).

Summary of the results obtained from three independent experiments in which two concentrations of five selected products are tested. There are significant statistical differences (one-way analysis of variance, Dunnett's Multiple Comparison Test) in all treatments when compared with control embryos except when the lowest concentration of curcumin is tested.

Compound	TOTAL NUMBER OF VESSELS			COMPLETE VESSELS		
	MEAN	P value	CV	MEAN	P value	CV
Control	22.89 $\pm$ 0.12		1.16%	22,42 $\pm$ 0.14		1.37%
KRN633 20 nM	13.78 $\pm$ 1.14	p $\leq$ 0,01**	14.33%	7,78 $\pm$ 3.12	p $\leq$ 0,01**	69.50%
KRN633 50 nM	4.93 $\pm$ 0.87	p $\leq$ 0,01**	30.43%	0,33 $\pm$ 0.33	p $\leq$ 0,01**	NA
AG1478 20 $\mu$ M	16.60 $\pm$ 0.50	p $\leq$ 0,01**	5.25%	9,40 $\pm$ 2.003	p $\leq$ 0,01**	36.91%
AG1478 40 $\mu$ M	11.27 $\pm$ 1.39	p $\leq$ 0,01**	21.33%	1,27 $\pm$ 0.24	p $\leq$ 0,01**	32.87%
2-ME 10 $\mu$ M	18.87 $\pm$ 0.24	p $\leq$ 0,05*	2.21%	3,20 $\pm$ 0.76	p $\leq$ 0,01**	40.98%
2-ME 20 $\mu$ M	15.33 $\pm$ 0.29	p $\leq$ 0,01**	3.28%	0	p $\leq$ 0,01**	NA
Indirubin 0.5 $\mu$ M	15.00 $\pm$ 0.30	p $\leq$ 0,01**	3.53%	9.00 $\pm$ 0.72	p $\leq$ 0,01**	13.88%
Indirubin 10 $\mu$ M	9.13 $\pm$ 0.78	p $\leq$ 0,01**	14.91%	0,33 $\pm$ 0.18	p $\leq$ 0,01**	NA
Curcumin 5 $\mu$ M	20.67 $\pm$ 0.79	p $\leq$ 0,05	6.59%	18,07 $\pm$ 1.60	p $\leq$ 0,05	15.35%
Curcumin 10 $\mu$ M	3.72 $\pm$ 1.92	p $\leq$ 0,01**	NA	0,47 $\pm$ 0,47	p $\leq$ 0,01**	NA

CV: Coefficient of Variation.  
SE: Standard Error.

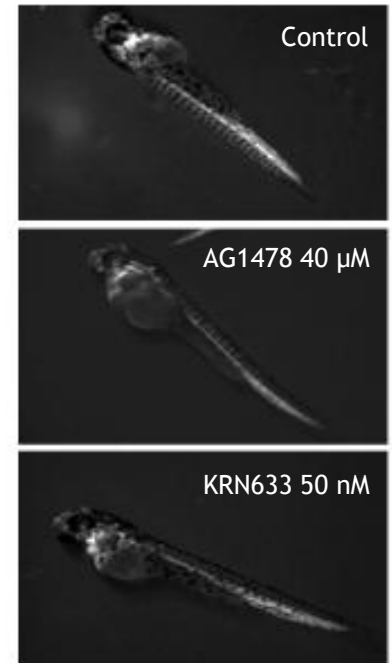


Figure 3. Examples of treated zebrafish embryos. While control embryos show regular intersegmental vessel pattern, embryos treated with AG1478 40  $\mu$ M show a decrease in the total number of vessels. In addition, most of them are incomplete. After the treatment with KRN633 50 nM, no intersegmental vessels are detected.

## Conclusions

Zebrafish embryos can be used to study the antiangiogenic activity of a compound since:

- The activity of five selected compounds with known antiangiogenic effects in other models is detected. The sensitivity of the method for the products selected is 100%.
- The antiangiogenic activity is consistently detected since there are significant statistical differences between treated and control embryos in three independent experiments (except the lowest curcumin concentration).

## Bibliography

- [1] Hanahan, 1997. Science (277), 48-50
- [2] Tran et al., 2007. Cancer Res 67 (23), 11386