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Your Zebrafish Partner

ANGIOGENESIS INHIBITION ASSAY

Biobide is a biotechnology company offering drug discovery services to Pharma, Biotech, Chemical, Cosmetic and Nutraceutical companies. Our services are based on the **zebrafish model** and the capacity to offer highly efficient tailor made 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.

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 inhibit growth of angiogenic blood vessels [2]. In our automated assay, a transgenic zebrafish line with fluorescent (cop-GFP labelled) blood vessels (Figure 1) is used in order to facilitate the visualization and analysis of the intersegmental vessels (Figure 2).

METHOD DESCRIPTION

The method consists on the following steps:

Treatment: 24 hpf embryos are dispensed 1 per well in a 96 well assay plate. Then, the test compounds at the desired concentration are dispensed into each well of the assay plate containing the embryos. Depending on the previous knowledge and the number of products to test, different assays are proposed:

- Concentration-response curves: an initial curve of six concentrations is proposed: 0.01, 0.1, 1, 10, 30 and 100 µM. For IC50 calculation, a second appropriate curve will be done if necessary.
- Screening: only one concentration (around 20-30 µM) per compound will be tested.
- Assays opened to client interest.

Incubation: The test plate is incubated for 24 h.

Data collection: After 24 hours of treatment, embryos are anesthetised with tricaine to prevent their movement and the assay plate is placed in the microscope where pictures are taken.
Image analysis: Image analysis is done semi-automatically in two different steps:

First step: the fluorescent image is processed automatically to determine a region of interest containing the tail of the embryo. Fluorescent area present in this region is measured and when significant statistical differences are found between the means of control and treated embryos, next step in the analysis is carried on.

Second step: two different parameters are manually quantified from the images obtained:
1. The total number of intersegmental vessels.
2. Number of intersegmental vessels that are complete.

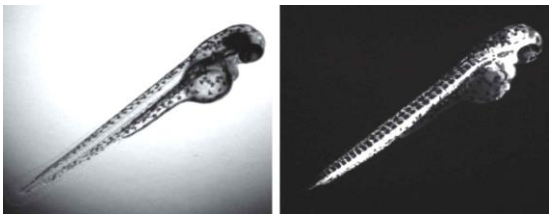

Figure 1. Bright field (left) and fluorescent (right) images of 48 hours post fertilization (hpf) embryos. Endothelial cell-specific expression of cop-GFP is driven by flk1 promoter.

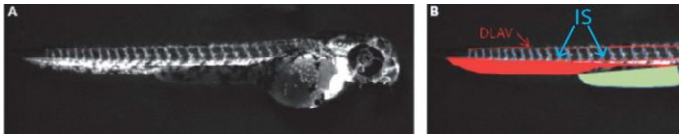

Figure 2. The figure shows a whole embryo (A) and the part of the trunk in which vessels are quantified. (B) Same picture but without the head and the yolk, (most bulging part). Intersegmental vessels (IS) are shown in blue and DLAV in red.

VALIDATION RESULTS

To validate the angiogenesis assay, the effect of 18 compounds with capacity of inhibiting angiogenesis and 10 compounds lacking effect in previous angiogenesis screenings (3,4) were tested (Figure 3, 4). For this purpose, a concentration response curve (0.01, 0.1, 1, 10, 30 and 100 µM) was developed. Furthermore, IC50 value was calculated for the positive compounds detected in the first round.

Figure 3. Summary of the results obtained during the validation of antiangiogenic assay

COMPOUND	TARGET	ANGIOGENESIS INHIBITION	TOTAL VESSELS	COMPLETE VESSELS	CONCENTRATION (µM)
KRN633	VEGFR 1-2 and 3	YES	0.035	0.026	-
ZD6474 (Vandetanib)	VEGFR-1-2-3 and EGFR	YES	-	-	100
Sunitinib malate	VEGFR-1-2-3, PDGFRb, c-kit,FLT3,CSF1-R and RET	YES	2.6	1.7	-
Sorafenib Tosylate	VEGFR-2-3,PDGFR, c-kit and Raf	YES	0.78	0.53	-
PD173074	FGFR1 and 3	YES	-	-	100
PD166866	FGFR1	YES	43.9	16	
AG-1296	PDGFRα and β c-kit receptor.	YES	-	-	20
PDGFR tyr kin inhibitor	PDGFR α and β	YES	0.19	0.14	-
Tie 2 Kinase Inhibitor	Tie 2	NO	-	-	-
Bosutinib	Abl and Scr	YES	-	-	50
AG 1478	EGFR	YES	22.8	13	-
Indirubin-3´-oxime	Cyclin-dependent kinase and GSK-3β	YES	18.3	4.2	-
Fumagillin	Methionine aminopeptidase	NO	-	-	-
NS-398	Cicloxigenase 2(COX-2)	YES	-	-	30
HIF-1 inhibitor	HIF-1	NO	-	-	-
NVP-BEZ235	PI3K y mTor	YES	-	-	10
2-Methoxyestradiol	Endothelial cells	YES	30.6	10.2	
Paclitaxel	Antimicrotubule agent	YES	-	-	?
Tyrphostin AG490	JAK-2	NO	-	-	-
Bestatin	Aminopeptidase	NO	-	-	-
Thioacetamide	-	NO	-	-	-
E64	Cysteine proteases	NO	-	-	-
O6-benzylguanine	O(6)-alkylguanine DNA alkyltransferase	NO	-	-	-
Cyclosporine A	Calcineurin	NO	-	-	-
4-Methylpyrazole hydrochloride	Alcohol dehydrogenase	NO	-	-	-
N-Acetyl-L-cysteine	Antioxidant	NO	-	-	-
Amiodarone hydrochloride	Non-selective ion channel blocker	NO	-	-	-
Cis-Diammineplatinum(II) dichloride	Forms cytotoxic adducts with DNA	NO	-	-	-

Compounds with known antiangiogenic effect

Compounds with lacking antiangiogenic effect

Specificity: 100%

Sensitivity: 83%

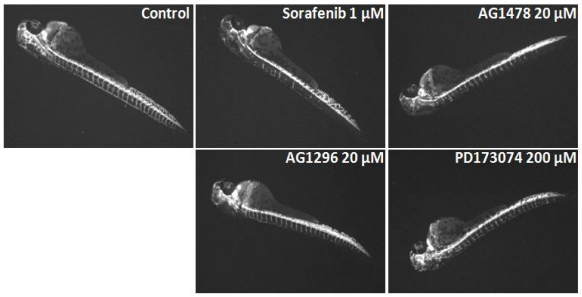

Figure 4. Figure shows pictures of 48 hpf embryos treated with the indicated compounds as representatives of the phenotypes found.

Figure 5. CV values for the complete vessels parameter is always lower than 15% with one exception (<20%) .

COMPOUND	CONCENTRATIOON TESTED(µm)	CV TOTAL VESSELS
KRN633	0.03	13.6
ZD6474	100	13.9
Sunitinib malate	2	2.2
PD173074	200	12.2
PD166866	30	12.4
AG-1296	20	2.4
Bosutinib	60	0.7
AG1478	20	11.2
Sorafenib Tosylate	1	3.3
Indirrubin-3´-oxime	10	19.3
NS-398	40	3.2
NVP-BEZ235	30	4.0
2-Methoxyestradiol	10	2.6

- The antiangiogenic activity of a compound can be studied through this automated method since 100% specificity and 83% sensibility was obtained after the validation.

[1] Hanahan, 1997. Science (277), 48-50
[2] Tran et al., 2007. Cancer Res 67 (23), 11386
[3] Kalén, 2009. Chemistry and Biology 16, 432-441
[4] Kälin, 2009. Blood 114 (5), 1110-1122