EMORY | CHEMICAL BIOLOGY DISCOVERY CENTER

Probe Report
20 September 2007

Project Title: In vivo zebrafish angiogenesis assay
Grant Number: 1-X01-MH077629-01

Screening Center Name: Emory Chemical Biology Discovery Center
Principal Investigator of Screening Center: Ray Dingledine

Assay Submitter & Institution: Thanh Doan, Zygogen Inc

Assay or Pathway Target: This is a phenotypic assay that measures the extent of angiogenesis in 1 day old zebrafish embryos during overnight exposure to compounds**.**

Probe PubChem Compound Identifier (CID): Indirubin-3'-monoxime (5326739)

Assay provider information
Specific Aim: To discover novel angiogenesis inhibitors using live zebrafish embryos.

Significance: Angiogenesis is an important process to target for the treatment of a variety of diseases, including cancer, macular degeneration, psoriasis, and diabetic retinopathy (reviewed by Carmeliet and Jain, 2000). For example, solid tumors require an adequate supply of blood vessels in order to survive, grow and metastasize (Hanahan and Folkman, 1996; Li et al., 2000). Recently, a link between angiogenesis and Alzheimer's disease has also been postulated (Vagnucci and Li, 2003), possibly opening another large market for anti-angiogenesis drugs. Several in vitro and in vivo angiogenesis assays are currently being used for drug discovery. Each of these assays has distinct advantages and disadvantages (Jain et al., 1997; Auerbach et al., 2000). For instance, in vitro endothelial cell proliferation and apoptosis models are rapid and amenable to screening hundreds and thousands of compounds for their angiogenic potential. However, most in vitro models only address the effects of compounds on endothelial cells and not other tissues in the vascular bed, such as smooth muscle cells, fibroblasts and progenitor angiogenesis cells.

Angiogenesis involves the proliferation and migration of endothelial cells in the context of their environment but current in vitro assays do not address environmental influence on endothelial cells. In vivo angiogenesis models include mouse corneal angiogenesis assays, chick embryo chorioallantoic membrane (CAM) assays, matrigel plug and/or branching of blood vessels after ligation of the femoral artery. These models enable the study of endothelial cells in the context of their native environment but the throughput is very low and costly. In addition, genetic manipulation, such as gene knockout/knockdown and over-expression, is not feasible for some in vivo assays. Therefore, there is demand for a novel angiogenesis assay that combines the relevance of an in vivo environment with the potential for high throughput drug screening and rapid gene knockdown.

Rationale: Current in vitro models employed in screening compounds for effects on angiogenesis lack the biological complexity of in vivo systems. Zygogen, LLC developed a rapid in vivo compound screening assay to identify anti-angiogenic compounds. The zebrafish is a well-accepted model for the study of vertebrate development (Doan et al., 2004). The advantages of this system include the optical clarity of the embryos, the development of the embryos outside of their mother and the ease with which large numbers of embryos can be obtained and manipulated. Anti-angiogenic compounds cause a reduction in the growth of blood vessels in the trunk of the zebrafish embryo.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: In Vivo Angiogenesis Assay for HTS

PubChem bioassay identifiers generated for this screening project (AIDS): AID732 for primary screen, AID for dose-response curves in progress

Primary Assay Description as defined in PubChem: Transgenic blood vessel-specific fluorescent zebrafish, TG(VEGFR2:GRCFP), were mated with wild-type zebrafish (Tubingen strain). Eggs were collected the following morning and treated with pronase (1 mg/ml) to remove the chorions. Fertilized embryos were then placed into Petri dishes containing Holtfreter's solution (60 mM sodium chloride, 2.4 mM sodium bicarbonate, 0.8 mM calcium chloride, 0.67 mM potassium chloride, 10 mM HEPES, pH 7.0) and incubated at 28 degree C. Embryos were allowed to develop until they had reached the late somite stage of development (about 24 hours post-fertilization), when vasculogenic blood vessels as well as some angiogenic sprouts have formed. Embryos with fluorescent blood vessels were then arrayed into black 384-well polystyrene clear bottom plates (Greiner) with one embryo per well in 30 uL of Holtfreter's solution. A schematic of the assay is shown in Figure 1.

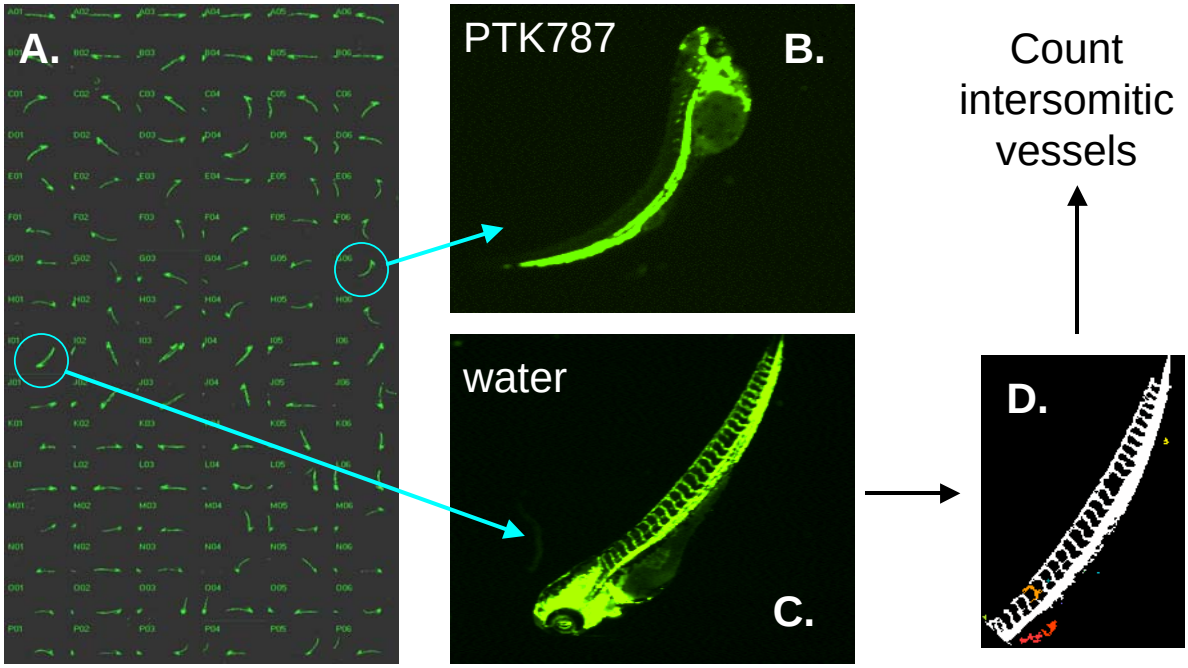

Fig 1. Assay for angiogenesis in zebrafish embryos expressing GFP in the endothelial cells that line blood vessels. A. Partial view of a 384-well plate with embryos arrayed one per well. B. After overnight treatment with a VEGF receptor antagonist, there are no observable vessels outside the spinal area. C. Embryo treated with water overnight shows robust angiogenesis. D. The spinal area is isolated with a box cursor and the number of intersomitic vessels is counted as a measure of angiogenesis.

The screen was performed using five zebrafish per compound, with each location (Zygogen and Emory) responsible for screening half of the LOPAC library (640 compounds each).

Compound stocks were diluted with media to a working concentration of 60 uM and 30 uL of each compound was added to one day post fertilization (dpf) zebrafish embryos using the Caliper Sciclone ALH 3000 (Zygogen) or Biomek NX (Emory) robotic liquid handlers. The final screening concentration was 30 uM in 60 uL of media (1% DMSO). The anti-angiogenic compound Tyrphostin AG 1478 (PTK787 (10 uM) was used as the positive control, while 1% DMSO in Holtfreter's solution was used as the negative control. After the compounds were added, plates were centrifuged at 32xg for 60 sec and incubated at 28 degree C overnight. At 2dpf, zebrafish embryos were anesthetized with 0.016% tricaine and incubated for 30 min at room temperature before acquiring images using the Discovery-1 (Zygogen) or ImageXpress 5000 (Emory) high-content imaging systems.

Data analysis: In each image, the trunk of the zebrafish was manually selected (Fig 1D) and the number of angiogenic blood vessels (intersegmental vessels and their branching arteries) was quantified using automated algorithms developed by Zygogen. Screening data for each test compound were normalized as percent of plate average and actives were defined as compounds that reduced angiogenic vessel counts more than 3 standard deviations from the plate mean.

% Control = (Mean (compound) - Mean (PTK787)/ (Mean (plate) - Mean(PTK787)) x 100
where:

Mean (compound) is the average blood vessel count determined from up to five embryos for each test compound treatment

Mean (PTK787) is the average blood vessel count of PTK787 treated embryos from the plate being analyzed

Mean (plate) is the average blood vessel count of the test compound-treated embryos from the plate being analyzed

Images of zebrafish treated with actives were assessed visually to confirm the anti-angiogenic activity of the hits; actives that were not confirmed visually or that caused toxicity were not analyzed further.

Center Summary of the Primary Screen:
The LOPAC-1280 library was screened and measurements from 5 embryos were averaged for each compound. There were 3 hits identified in this screen. Two are known angiogenesis inhibitors (SU4312 and AG1478). The third is novel and identified as a probe in this project. This project has been accepted for publication (Tran et al., 2007).

<u>Probe Optimization</u>
Chemical name of probe compound: indirubin-3'-monoxime

*Draw probe chemical structure and show stereochemistry if known:*see Figure 2.

Describe mode of action for biological activity of probe: The probe inhibits proliferation of endothelial cells at 25-fold lower concentration than it causes cellular toxicity. It also inhibits tubule formation but not migration of HUVECs.

Has this compound been provided to the MLSMR: It is commercially available through Sigma/Aldrich.

Description of secondary screens used to optimize probe structure: We obtained dose-response curves for anti-angiogenesis in zebrafish. We also measured [3H]-thymidine uptake, tubule formation, migration and toxicity (ATP levels) in human vascular endothelial cells.

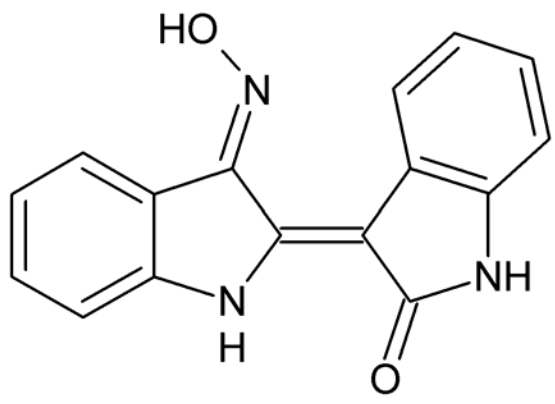

Figure 2. Structure of indirubin-3'-monoxime (IRO).

Center comments on chemistry strategy leading to probe identification:
This is a commercially available probe.

Detailed synthetic pathway for making probe:
The probe is available from Sigma/Aldrich as well as other suppliers.

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:
Molecular weight 277.3 g/mol. Solubility: soluble in DMSO and ethanol. soluble in DMSO at 18mg/ml. soluble in water at 0.25mg/ml. Appearance: Dark red solid. Chemical formula C16H11N3O2. Purity > 98%. XLogP= 1.9 Hydrogen Bond Donor Count: 3, Hydrogen Bond Acceptor Count: 4, Rotatable Bond Count: 2

Appendices

Comparative data on probe, similar compound structures and prior probes

IRO shows a monotonic inhibition of angiogenic vessel growth in zebrafish embryos with IC50 = 0.31 μM (Fig 3). The maximum inhibition is similar to that of the positive control, PTK787, a known inhibitor of the VEGF receptor.

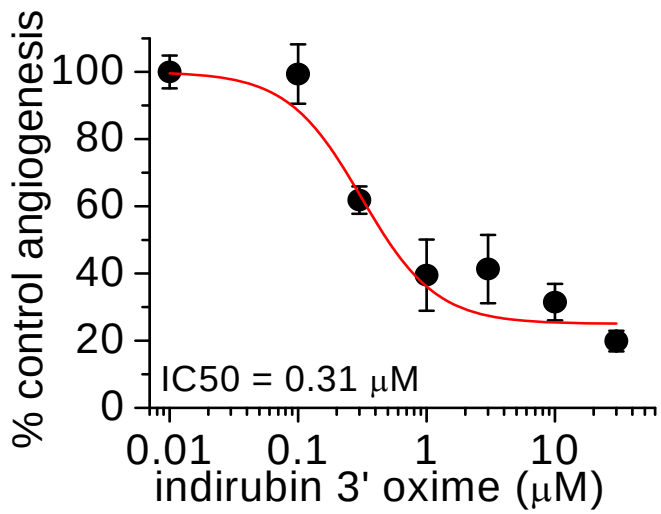

Fig. 3. IRO inhibits angiogenesis in zebrafish embryos during overnight exposure.

A total of 34 IRO analogs were synthesized and an additional 43 compounds purchased for testing in the zebrafish angiogenesis assay. The purpose was to explore the structural features of IRO that are important for

anti-angiogenic activity. Indirubin itself (Fig 4) was inactive up to 30 μM. Some additional SAR are shown in Figure 5. Similar to the observation that indirubin is inactive, the same substitution of ketone for oxime in SID 26676125 vs 26676136 lost activity (not shown) However, in other pairs of compounds (eg, SID 26676129 vs 26676134 in fig 5) the effect of the ketone substitution was less marked. Adding a Bromo group (SID 26676122) on the 5'-position also reduced activity compared with IRO itself. In the meantime, the substitution of methyl group on 7-position (SID 26676134) and fluoride on the 5-position (SID 26676136) of indirubin-3'-monoxime exhibited similar level of activity compare to IRO itself. Introducing other functional groups such as chloride, nitro, sulphonic acid on the same 5- or 7-position results in reduced inhibition activity.

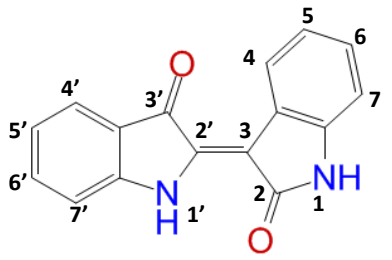

Fig. 4. Indirubin (SID 7990213)

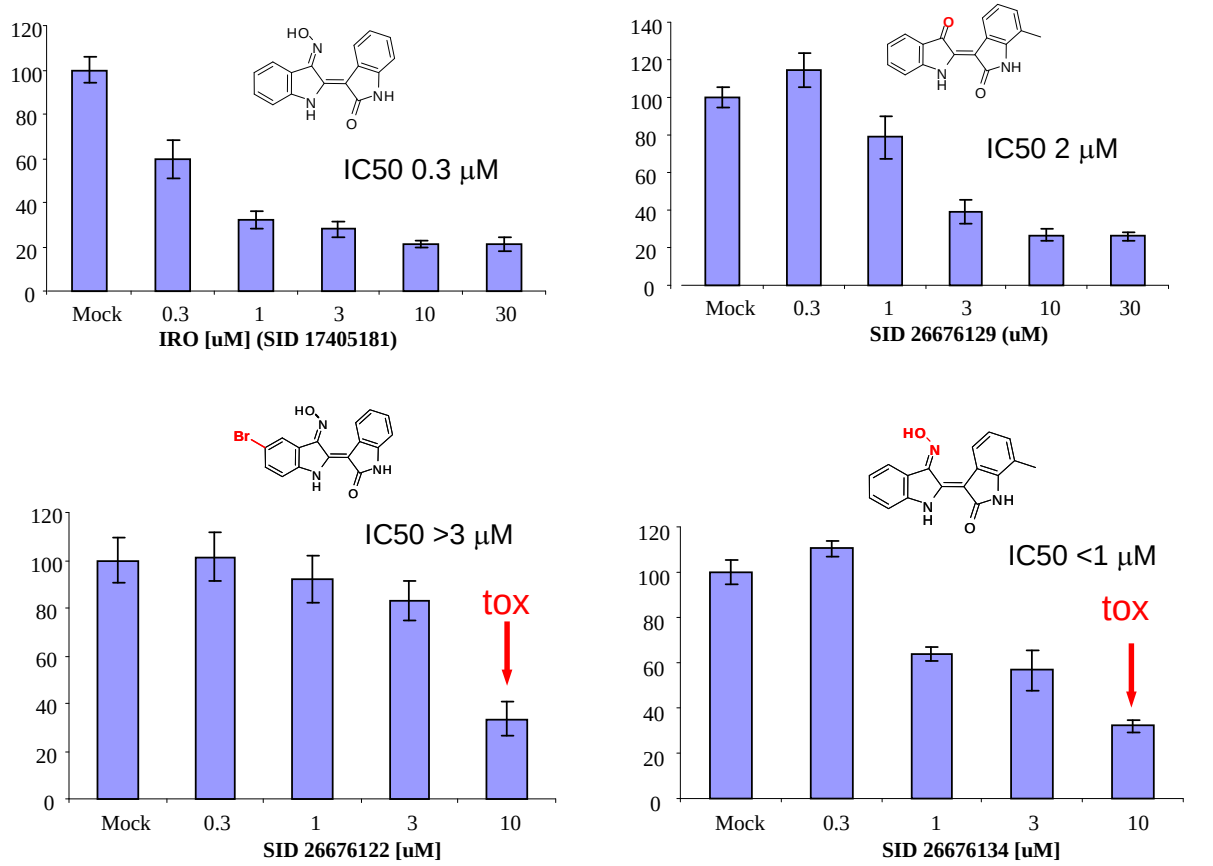

Fig 5. Structural features of IRO that promote anti-angiogenesis in zebrafish embryos.

Comparative data showing probe specificity for target

IRO does not inhibit vasculogenic blood vessel development nor cause regression of existing vasculature in the zebrafish. The parent compound, indirubin, and some of its analogs are reported to be inhibitors of glycogen synthase kinase 3 beta (GSK-3beta), cyclin-dependent

kinases Cdk1 and Cdk5 with in vitro IC50 values of 22, 80 and 100 nM, respectively (Hoessel et al., 1999; LeClerc et al., 2001).

To determine if IRO is anti-angiogenic in human endothelial cells, and to elucidate its mechanism of action on endothelium, we assessed IRO in human umbilical cord vein endothelial cell (HUVEC) assays. HUVEC proliferation was assessed using the ^{3}H thymidine incorporation assay. Endothelial cells were seeded into a 96-well plate, treated with different concentrations of IRO, and ^{3}H thymidine incorporation was determined. IRO inhibited endothelial cell proliferation with an IC_{50} of 0.36 μM (Table 1). Proliferation was inhibited at a greater than 20 fold lower concentration than that needed to produce cytotoxicity (IC_{50} = 8.7 μM), estimated by reduced cellular ATP levels.

Endothelial tube formation was assessed using HUVECs cultured on extracellular matrix. Full-growth media was used to stimulate tube formation. IRO was added to the growth media and tube formation was allowed to proceed for 18 hours. Tube length was measured using Image Pro Plus software. IRO inhibited endothelial tube formation with an IC_{50} of 6.5 μM.

Finally, endothelial cell migration was assessed using a trans-well migration assay (BD Biosciences). HUVECs were serum starved for 4 hours, then seeded into the top wells of migration chambers in endothelial basal media (without growth factors). Complete media containing growth factors was placed in the bottom well as a chemoattractant. Migration was allowed to proceed for 22 hours, at which point migrated cells were fixed, stained, and quantified. IRO did not significantly affect endothelial cell migration. Collectively, the data in Table 1 show that IRO inhibits two major components of the angiogenic process, endothelial tube formation and cell proliferation, but does not significantly affect endothelial cell migration.

Table 1. Biological effects of indirubin-3'-monoxime related to angiogenesis

IC50 (μM)	Effect
0.31	Angiogenesis in zebrafish
>30	Vasculogenesis in zebrafish
>30	regression of existing angiogenic vessels in zebrafish
0.36	HUVEC: proliferation (inhibition of [3H]-thymidine incorporation)
8.7	HUVEC: reduction of ATP levels (Cell-titre Glow assay)
6.5	HUVEC: inhibition of tubule formation
>30	HUVEC: inhibition of migration

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