Automated Z-Tag^{SM} Zebrafish Assay for Anti-Angiogenic Compound Screening

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Background

Pathological angiogenesis contributes to over 70 diseases, including cancer, age-related macular degeneration and rheumatoid arthritis. Current *in vitro* angiogenesis models lack biological complexity, and existing *in vivo* systems can be time-consuming and costly. This assay demonstrates the use of Z-Tag^{SM} zebrafish to screen compounds for anti-angiogenic potential, thus providing a whole animal model that is more rapid and less expensive than other *in vivo* models.

Z-Tag^{SM} *TG(VEGFR2:GRCFP)* Transgenic Zebrafish

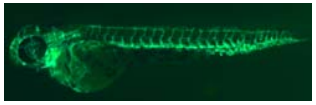


Figure 1. Transgenic zebrafish expressing green reef coral fluorescent protein (GRCFP) driven by the vascular endothelial growth factor receptor 2 (VEGFR2) promoter for blood vessel-specific expression.

Zebrafish Angiogenesis Assay Workflow

Figure 2. The LOPAC^{1280} compound library was screened in a collaboration between Zygogen and Emory University's Molecular Libraries Screening Center to identify anti-angiogenic compounds using *TG(VEGFR2:GRCFP)* zebrafish.

Zebrafish embryos were collected and dechorionated. At 1 day post fertilization (dpf), embryos were arrayed into multi-well plates

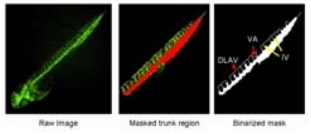
Sciclone ALH 3000

1 dpf zebrafish embryos (5 per condition) were treated with 30 µM of each drug using the Sciclone ALH3000 (Caliper LifeSciences). PTK787, A known anti-angiogenic compound (10µM), was the positive control, while 1% DMSO was the negative control.

640 compounds → Discovery-1

640 compounds → ImageXpress 5000

At 2dpf, the embryos were imaged using the Discovery-1^{TM} high content imaging system (at Zygogen) or the ImageXpress 5000^{TM} (at Emory University).



After image acquisition, the number of angiogenic blood vessels in the trunk of each zebrafish was quantified using automated algorithms developed by Zygogen. Compounds causing an inhibition of blood vessel growth without any observable toxic effects were considered positive hits.

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Results of Zebrafish Anti-Angiogenic Compound Screen

Zygogen and Emory University successfully completed a screen of the LOPAC^{1280} library. Three compounds, representing 0.23% of the library, were classified as hits based on their strong inhibition of blood vessel growth. Two of these hits, SU4312 (VEGF receptor inhibitor) and Compound 1127 (epidermal growth factor receptor inhibitor), were previously reported to have anti-angiogenic activity. The third hit, Compound 619, is a novel anti-angiogenic compound. Hits were identified as those compounds whose mean value fell more than 3 standard deviations from the mean value for the entire plate (Figure 3). Follow-up experiments demonstrated that the three hit compounds reduce blood vessel growth in a dose-dependent manner (Figure 4). The Z'-factor was 0.5, suggesting that the zebrafish angiogenesis screen is a robust assay for detecting anti-angiogenic compounds.

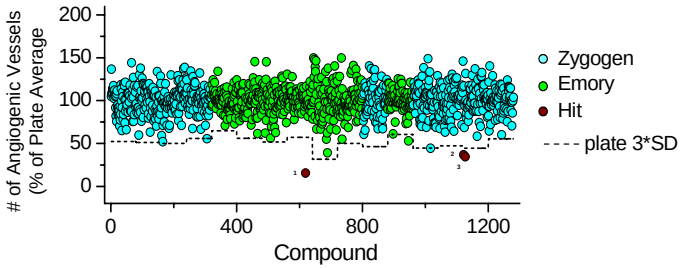

Figure 3: **Zebrafish LOPAC^{1280} library screen**. Blue points represent compounds tested at Zygogen and green points represent compounds tested at Emory. Red points represent the hit compounds identified in the screen.

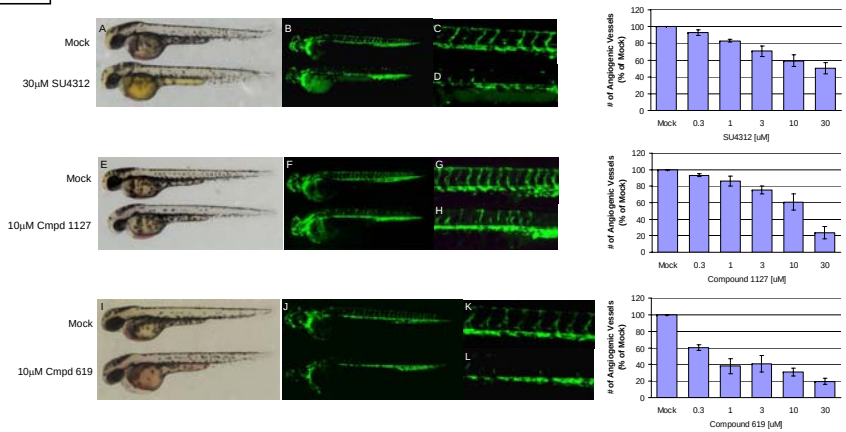

Figure 4: Anti-angiogenic hit compounds identified in LOPAC^{1280} library screen. Images of embryos treated with SU4312 (A-D), Compound 1127 (E-H) and Compound 619 (I-L). C, D, G, H, K and L show inhibition of intersegmental vessel growth in the zebrafish trunk. The graphs show the dose dependent inhibition of vessel growth for each compound. Data are given as mean +/- SEM of three independent experiments for each compound. Total "n" was 21-33 embryos per compound.

Conclusion

The Z-Tag^{SM} zebrafish assay was used to successfully screen a 1,280 compound library and to identify known and novel anti-angiogenic hits. The novel hit, Compound 619, subsequently showed anti-angiogenic activity in *in vitro* endothelial cell assays. To our knowledge, this is the first demonstration of the ability of a hit compound arising from an *in vivo* zebrafish screen to show therapeutic activity in human cell-based assays. We are continuing to investigate Compound 619 function in mammalian tumor models.

In Vitro Angiogenesis testing of Novel Hit Compound

It is critical to demonstrate that some novel hit compounds identified in the zebrafish angiogenesis assay are also active in mammalian models. We therefore tested Compound 619 for activity in human endothelial cell culture based angiogenesis assays using human umbilical vein endothelial cells (HUVECs). Cell proliferation and viability were assessed using the thymidine incorporation and CellTiterGlo assays, respectively. Endothelial cell migration was assessed using a trans-well migration assay and endothelial tube formation was tested by culturing HUVECs onto growth-factor reduced matrigel in 96-well plates. Compound 619 inhibited endothelial cell proliferation (Figure 5A), decreased cell viability (Figure 5B) and reduced endothelial tube formation (Figure 6) in a dose-dependent manner, but had no significant effect on endothelial cell migration (Figure 7).

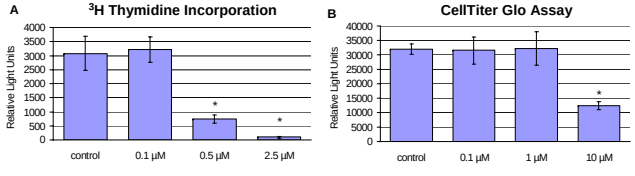

Figure 5: Compound 619 inhibits endothelial cell proliferation and viability. HUVECs were seeded into 96 well plates. After 24 h, the cells were incubated with Compound 619 at the indicated concentrations. Thymidine uptake (Panel A) and ATP levels (Panel B) were measured at 3 days. Data are shown as mean +/- S.D. Five to eight replicates were analyzed for each condition. * indicates Statistical significance by Student's *t* test ($p < 0.05$).

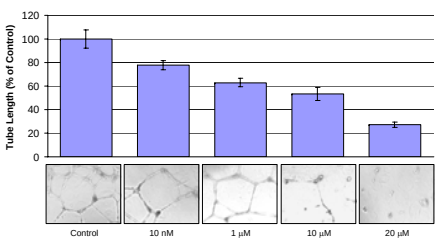

Figure 6: Compound 619 inhibits endothelial tube formation. HUVECs were plated on matrigel in complete media containing the indicated concentrations of Compound 619. Tubes were allowed to form for 18 h. Tube length was quantified using Image Pro Plus software. Data are shown as average tube length per field of view +/- SEM. Each of the drug-treated conditions significantly inhibited tube formation as determined by Student's *t* test ($p < 0.05$).

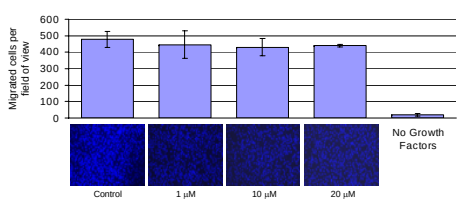

Figure 7: Compound 619 does not affect endothelial cell migration. Serum-starved HUVECs were seeded into the top chamber of a trans-well migration insert at the indicated concentrations of Compound 619. Cells were allowed to migrate for 22 h. Data are shown as mean of each filter +/- S.D. Pictures show stained migrated cells.