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In vivo molecular validation of VEGF inhibitor extracted from marine Actinomycetes in zebrafish embryos

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Angiogenesis, the formation of new blood vessels by sprouting from pre-existing endothelium, is a significant component in tumor progression. Marine natural products have been proved to be a rich source of novel compounds in drug discovery especially in the area of small molecule based targeted anticancer chemotherapeutics identification. Symbiotic actinomycetes from marine tunicates are having unique environmental niche that arise as a new and valuable source of inhibitors of angiogenesis. In the present study, actinomycetes strains were isolated from the areas of Muttom, Kanyakumari, Tamil Nadu, India and it was screened for the production of bioactive compounds. The bioactive compounds obtained were treated on zebrafish embryos at 50 % epiboly stage and maintained for 72 hours post fertilization (hpf). RBC staining was done at 72 hpf to study the formation of blood vessel in embryos treated with biomolecules and RT-PCR was carried out to study mRNA expression of vascular endothelial growth factor (*vegfaa*) at 72 hpf. Biomolecules (T1 and T2) extracted from marine actinomyetes showed anti-angiogenic property by reducing the Intersegmental vessels (ISVs) (main angiogenic blood vessel) at 5 µM concentration. RT-PCR study reveals the reduction in mRNA expression of *vegfaa*. Our study emphasizes the importance of potential of anti-angiogenic compounds from marine tunicate symbiotic actinomycetes using zebrafish as *in vivo* model system.

[**Keywords**: Actinomycetes, Biomolecules, Tunicates, *vegfaa*, Zebrafish]

Introduction

Angiogenesis is a process of blood vessel formation from the pre-existing capillaries involved in physiological and pathological processes like embryo genesis, placentation, wound healing, inflammatory disorders and tumour growth[1,2]. Natural products play a crucial role in drug discovery and these are the sources for developing new drugs. Especially the marine actinobacteria are known to produce novel

themselves from predators. Studies by Folkman[3] in 1971 on angiogenesis research have paved the way for using marine bioactive molecules in the field of antiangiogenesis in the last three decades. Potential anti-angiogenic agents have been identified and extensively explored from marine organisms including sponges, sponge-associated bacteria, gorgonia, molluscs, soft coral and actinomycetes[4]. Bioactive compounds isolated from marine sources have become important in the study of antifungal, antibacterial, antiviral, anticancer, as well as anti-coagulant properties[5,6].

Zebrafish exhibit highly characteristic blood-vessel patterning and a short period of development of blood vessels [i.e. 96 hours post-fertilization (hpf)]. Vasculogenesis in zebrafish is initiated as early as 12 hpf, and by 24 hpf, a simple circulatory loop consisting of major vessel-like dorsal aorta and axial vein is established. By 24 hpf, development of angiogenic sprouts like the sub intestinal vessels (SIVs) is initiated to establish angiogenesis in the evaluation of anti-angiogenic agents[7]. Hence, zebrafish paves way to perform systemic angiogenic assays. Further, zebrafish as a model organism exhibit genetic and functional conservation across angiogenic pathways. The major modulators of angiogenesis, tyrosine-kinase domains of vascular endothelial growth factor receptor 2 (VEGFR2) and VEGF are also found to be conserved[8,9]. VEGF has been proved to be the fundamental mediator of physiological and pathological angiogenesis[10].

In the present study, zebrafish embryos were treated with bioactive compounds isolated from

marine actinomycetes to study the anti-angiogenic property. We observed the survival rates induced by bioactive compounds, blood vessel formation using RBC staining and also to evaluate their anti-angiogenic potential by reducing the expression of *vegfaa,* the major angiogenic determinant.

Materials and Methods

Zebrafish animal maintenance and embryo collection

A re-circulating stand-alone system (Aquaneering, USA) was used to maintain zebrafish (3-5 months old) at 28 ºC with 10:14 h dark: light cycle[11]. During normal period, zebrafish were fed with flakes foods and during breeding period they were fed with blood worms, and brine shrimps. All protocols were reviewed and approved by Institutional Ethical Committee of Centre for Molecular and Nanomedical Research Unit, Sathyabama Institute of Science and Technology.

Embryo collection was carried out using standard protocols from our previously published work[12]. Embryo medium (Hank's medium) were prepared as per standard protocol of Kimmel *et al.*[13], for culturing embryos. Embryos were collected at weekly intervals and various developmental stages were identified. The stages of development were fixed as hours of post fertilization (hpf)[13].

Separation of anti-angiogenic compounds

Tunicate samples were collected from Muttom, Kanyakumari, Tamil Nadu, India at 8°07′48.00″ N Latitude and 77°19′12.00″ E Longitude and was grown in lab condition to generate actinomycetes. Bioactive molecules from actinomycetes were obtained by solvent separation and Column and TLC fractionation of small molecules was done using standard protocols[14].

Pre-treatment of samples

Marine samples were rinsed with sterilized fresh seawater to remove loosely attached microorganisms and then subjected to dilution plate technique. Following this, the respective samples (tunicates) were cut down into small pieces (~1 cm X 3) and were homogenized in sterilized seawater.

Isolation of actinomycetes

One gram (1 g) of pre-treated samples were serially diluted in sterile sea water using serial dilution method and spread plated over the medium (Gause's inorganic agar medium No. 1 Gause GF) containing soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, $K2HPO_4$ 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 20 µM and agar 15 g in 1 L seawater. Plates were incubated at 28 ºC for 7-14 days. Dry colonies of actinomycetes were selected and isolated. All the colonies growing on the petriplates were purified by repeated streaking on actinomycetes isolation medium. Actinomycetes isolation medium (AIM) slants were used for short time preservation of purified actinomycetes[14]. For long term preservation, the purified actinomycetes were inoculated in AIM broth and were incubated at 28 ºC for 7-14 days. From this broth 20 % glycerol stocks were prepared and stored at -20 ºC.

Fermentation and extraction

Single colony of isolated actinomycetes cultures were streaked on the AIM medium and incubated for 7 days. After 7 days, the medium was cut into small pieces and were placed in 500 ml Erlenmeyer flask containing equal volume of ethyl acetate (cold percolation method). The flask was kept in the shaker for 24 hours at 220 rpm. The organic crude extract was filtered using Whattman No. 1 filter paper. The organic solvents were evaporated and the crude extract was concentrated in vacuum concentrator (Eppendorf 5301) at 45 ºC.

Treatment of biomolecules and morphological observations

The extracted biomolecules from actinomycetes (T1, T2) were dissolved in 0.1 % DMSO and treated with various concentrations (1 µg, 2.5 µg, 5 µg, 7.5 µg and 10 µg) to the embryo media where the embryos were staged and sorted at 50 % epiboly in a 6 well culture plate. The experiments were performed with 120 embryos (20 embryo × 6 wells) for each concentration of biomolecules and control embryos were treated with 0.1 % DMSO in 2 ml Embryo medium only. Embryos were visually inspected for viability/survival rate.

Anti-angiogenic activity of bioactive compound -RBC staining

Embryos treated with various concentrations of biomolecules (T1 and T2) and control at 50 % epiboly stage, were subjected to RBC staining at 72 hpf using standard protocols from previous published work[12,15].

Total RNA extraction and reverse transcription

RNA was isolated from embryos [treated with compounds (T1, T2) and control embryos] at 72 hpf and reverse transcribed using standard protocols from our previous research work[12]. Conditions for PCR are mentioned in Table 1 and primers sequences used in the study were obtained from Sigma (Table 2).

Results

Isolation and screening of Actinomycetes

Two different actinomycetes strains (T1, T2) were isolated from the marine tunicates using standard medium. The pure cultures of these strains were maintained as slants with starch casein agar medium and these slants were preserved at 40 °C. These two strains were screened for the production of bioactive compounds. The extracts were directly tested on zebrafish embryos for anti-angiogenic activity.

Morphological observations of zebrafish

The embryos of the control group developed normally in embryo medium, and hatching began at 48 hpf and completed at 72 hpf. The survival rates of zebrafish embryos treated with various concentrations of biomolecules (T1 & T2) for 24, 48 and 72 hpf were recorded (Fig. 1). The compounds didn't show any significant lethal effect on zebrafish embryos.

Blood vessel imaging

RBC staining at 72 hpf revealed the absence of major angiogenic vessels namely the Inter segmental vessels (ISVs) in developing embryos on treatment with biomolecule extracts namely T1, T2. Control embryos formed with normal vasculature is shown in Figure 2(A & C), whereas embryos treated with biomolecules developed with poor vasculature or moderated ISV formation is shown in Figure 2 (B & D). Magnified image of stained vessels is shown in Figure 3.

mRNA expression of VEGF when treated with biomolecules at 72 hpf

The potential of biomolecules extracted to decrease the expression of *vegfaa*, at its mRNA level was analyzed using Reverse Transcriptase PCR. Figure 4 depicts the PCR products of *vegfaa* when treated with Extract 1 (T1) and Extract 2 (T2) at various concentrations (1 µg, 2.5 µg, 5 µg, 7.5 µg and 10 µg) and control (0.1 % DMSO in EM). Results show that extracts have the potential to down regulate the level of mRNA expression of *vegfaa* at 5 µg and 10 µg of T1 and T2 treated embryos respectively. The RT-PCR analysis indicated that extracted biomolecules can inhibit new blood vessel formation by down regulating main angiogenic growth factor VEGF.

Table 1 — PCR condition for *vegfaa*

Condition	Temperature	Duration	
Initial denaturation	95 °C	4 minutes	
Denaturation	for 95 °C	30 seconds	35 cycles
Annealing	54 °C	30 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	7 minutes	

Discussion

In recent years, small molecule therapeutics design and development to inhibit angiogenesis has gained considerable importance in anti-angiogenesis research. Actinobacteria from marine environment are known to produce the most efficient groups of secondary metabolites and most of them are biologically active. Studies involving actinobacteria from terrestrial sources since 1950s have been screened

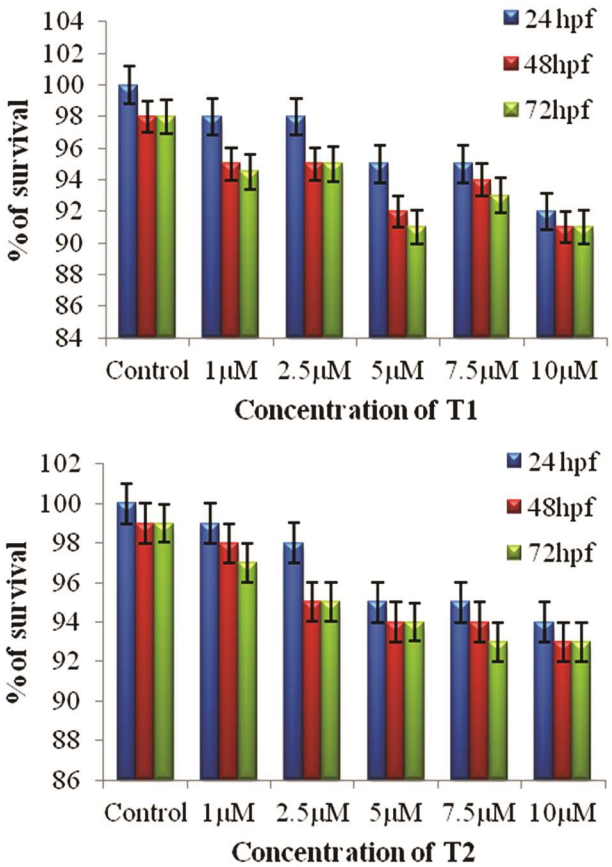

Fig. 1 — Survival rates of embryos treated with biomolecules extracted

Table 2 — Primers used in the study

Primer name	Forward	Reverse	Product size (bp)
vegfaa	5'-ctcctccatctgtctgctgtaaag- 3'	5'-ctctctgagcaaggctcacag-3'	398
β-actin	5'-tccccttgttcacaataacc-3'	5'-tctgttggctttgggattc-3'	223

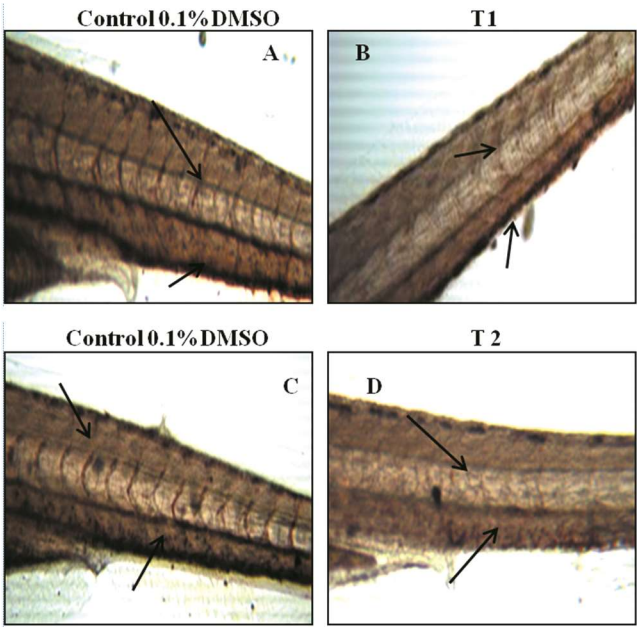

Fig. 2 — RBC staining: (A & C) Control embryos; and (B & C) Decreased ISV formation is observed at 5 µM of treated compounds (indicated by arrows) compared with control

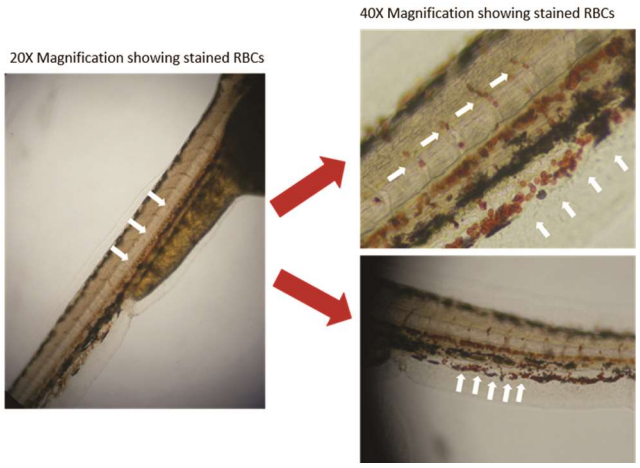

Fig. 3 — RBC staining: 20X and 40X magnification of stained RBCs

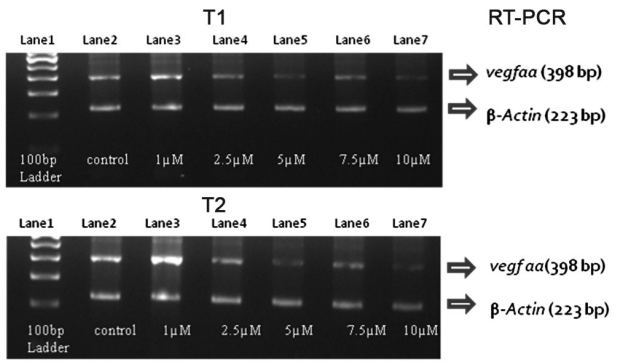

Fig. 4 — Expression of *vegfaa* after 72 hours incubation of extracts (T1, T2)

for many important antitumor, anticancer, antibiotics and immunosuppressive agents[16]. Many of the antitumor compounds from marine drugs are mainly derived from marine actinobacteria and these metabolites play an essential role in identification of pharmaceutical compounds[17]. The biomolecules (T1 and T2) extracted from marine actinomycetes in our study showed anti-angiogenic property by reducing the ISVs in 72hpf zebrafish embryos which was confirmed using RBC staining and the extracts T1 and T2 also reduced the mRNA expression of *vegfaa* at micromolar concentrations in zebrafish embryos. These results are correlated with our previous studies using methyxanthine pentoxifylline and resveratrol in zebrafish model[12,18].

Our work elucidates that the zebrafish is a suitable model for small molecules screening that can inhibit angiogenesis and it shares a high level of physiologic and genetic homology with humans, including digestive tract, brain, vasculature, musculature, and an innate immune system[19-24]. In the last decade, these advantages of zebrafish model in research have proved to be superior for use in cancer research. Long-standing methods for developing a zebrafish cancer model including transgenic regulation, carcinogenic treatment, and the transplantation of mammalian tumor cells[25] are well established in recent years. This made us to attempt to discover novel biomolecules from marine sources to study anti-angiogenic activity.

Summary and Conclusion

We investigated the anti-angiogenic potential of small molecules extracted from marine tunicates using zebrafish model. However, there is a need to develop assay/screening strategies. The identified small molecules coupled with biochemical and molecular methods in zebrafish would provide a platform to address various developmental and disease processes. Biomolecules from tunicates could possibly inhibit angiogenesis by decreasing the main angiogenic vessel namely the ISVs. Biomolecules extracted also found to suppress vegf mediated signaling pathway by down-regulating the gene expression of *vegfaa* at micromolar concentration. Further work has to be carried out to purify and predict the structural novelty of the compound(s).

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

JRN carried out the experimental part and drafted the manuscript, NS and BK made their contributions in sample (actinomycetes from Tunicates) collection and RRK designed the experiments and corrected the manuscript.

References

1 Folkman J, Angiogenesis in cancer, vascular, rheumatoid and other disease, *Nat Med*, 1 (1) (1995) 27-31.

2 Risau W, Mechanisms of angiogenesis, *Nature*, 386 (6626) (1997) 671-4.

3 Folkman J, Tumour angiogenesis: therapeutic implications, *N Engl J Med*, 285 (1971) 1182-1186.

4 Wang Y Q & Miao Z H, Marine-derived angiogenesis inhibitors for cancer therapy, *Mar drugs*, 11 (2013) 903-933.

5 Marderosian A D, Marine pharmaceuticals, *Pharama Sci*, 58 (1969) 1-30.

6 Ingber D, Fujita T & Kishimoto S, Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth, *Nature*, 348 (1990) 555–7.

7 Serbedzija G N, Flynn E & Willett C E, Zebrafish angiogenesis: a new model for drug screening, *Angiogenesis*, 3 (4) (1999) 353–359.

8 Lyons M S, Bell B, Stainier D & Peters K G, Isolation of the zebrafish homologues for the tie-1 and tie-2 endothelium-specific receptor tyrosine kinases, *Dev Dyn*, 212 (1) (1998) 133–140.

9 Liang D, Chang J R, Chin A J, Smith A, Kelly C, *et al*., The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development, *Mech Dev*, 108 (1–2) (2001) 29–43.

10 Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, *et al.*, The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing, *J Biol Chem*, 266 (1991) 11947–11954.

11 Kalaiarasi S, Arjun P, Nandhagopal S, Brijitta J, Iniyan A, *et al.*, Development of biocompatible nanogel for sustained drug release by overcoming the blood brain barrier in zebrafish model, *J Appl Biomed*, 14 (2) (2016) 157-169.

12 Nathan J R, Ganesh L, Felicia M M, Prakash S & Malathi R, Expression of adenosine receptors and vegf during angiogenesis and its inhibition by pentoxifylline — A study using zebrafish model, *Biomed Pharmacother*, 84 (2016) 1406–1418.

13 Kimmel C B, Ballard W W, Kimmel S R, Ullmann B & Schilling T F, Stages of embryonic development of the zebrafish, *Dev Dyn*, 203 (3) (1995) 253-310.

14 Kannan R R, Iniyan A M & Vincent S G P, Production of a compound against methicillin resistant *Staphylococcus aureus* (MRSA) from *Streptomyces rubrolavendulae* ICN3 and its evaluation in zebrafish embryos, *Indian J Med Res*, 139 (2014) 913–920.

15 Iuchi I & Yamamoto M, Erythropoiesis in the developing rainbow trout, Salmogairdneriirideus: histochemical and immunochemical detection of erythropoietic organs, *J Exp Zool*, 226 (1983) 409-417.

16 Panchanathan M, Jayachandran V, Kannan S & Se-Kwon K, Pharmaceutically active secondary metabolites of marine actinobacteria, *Microbiol Res*, 169 (2014) 262– 278.

17 Ravikumar S, Fredimoses M & Gnanadesigan M, Anticancer property of sediment acti-nomycetes against MCF-7 and MDA-MB-231 cell lines, *Asian Pac J Trop Biomed*, 2 (2) (2012a) 92–6.

18 Jhansi R N, Bakkiyanathan A & Malathi R, Resveratrol suppresses angiogenesis by down-regulating *Vegf/Vegfr2* in Zebrafish *(Danio rerio)* embryos, *J Chem Pharm Res*, 6 (12) (2014) 892-899.

19 Gore A V, Monzo K, Cha Y R, Pan W & Weinstein B M, Vascular development in the zebrafish, *Cold Spring Harb Perspect Med*, 2 (5) (2012) a006684.

20 Kanungo J, Cuevas E, Ali S F & Paule M G, Zebrafish Model in Drug Safety Assessment, *Curr Pharm Des*, 20 (34) (2014) 5416–29.

21 Kalueff A V, Stewart A M & Gerlai R, Zebrafish as an emerging model for studying complex brain disorders, *Trends Pharmacol Sci*, 35 (2) (2014) 63–75.

22 Guyon J R, Steffen L S, Howell M H, Pusack T J, Lawrence C, *et al.*, Modeling human muscle disease in zebrafish, *Biochim Biophys Acta*, 1772 (2) (2007) 205–15.

23 Weinstein B, Vascular cell biology in vivo: a new piscine paradigm, *Trends Cell Biol*, 12 (9) (2002) 439–45.

24 Lieschke G J, Oates A C, Crowhurst M O, Ward A C & Layton J E, Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish, *Blood*, 98 (10) (2001) 3087–96.

25 Mizgirev I & Revskoy S, Generation of clonal zebrafish lines and transplantable hepatic tumors, *Nat Protoc*, 5 (3) (2010) 383–94.