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SU5402, a pharmacological inhibitor of fibroblast growth factor receptor (FGFR), effectively hampers the initiation and progression of fin regeneration in teleost fish

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ABSTRACT

Tyrosine Kinase receptors (RTKs) are known to play a role in the regulation of number of important cellular activities. Growth factor signaling involves RTKs and mediates the proliferation of cells in various systems, making these receptors attractive targets for checking tumorous growth. The fibroblast growth factor (FGF) receptors are one such group of target receptors. Indolinone inhibitors such as SU5402, which bind irreversibly to these receptors, are being widely used to block FGF signaling and thereby curb uncontrolled proliferation. In the current study, we have used the regenerating fin of teleost fish Poecilialatipinna and studied the effect of SU5402 on this non-cancerous model of extensive cell proliferation. Our results indicate an adverse effect of SU5402 on regenerative outgrowth of fins and thereby reaffirm the anti-proliferative effect of this FGF receptor inhibitor. Morphometric, immunohistochemical, biochemical and histological observations all explicitly show a slowdown in the process of re-growth of amputated fins due to SU5402 treatment. The study also reveals a crucial role of FGF signaling in successful fin regeneration.

Keywords: SU5402, FGF, epimorphic regeneration, teleost

INTRODUCTION

It is well known that fibroblast growth factors (FGFs) play important roles in processes such as angiogenesis, wound healing and tumorigenesis. FGF signaling is seen to be upregulated in various types of cancers and also other systems of extensive cell proliferation such as regenerating tissue. They mediate their effects through a set of receptors, the FGF receptors (FGFRs), which are transmembrane tyrosine kinase receptors that belong to the immunoglobulin superfamily [1]. Protein tyrosine kinases and serine/threonine kinases are implicated in playing crucial roles in the cellular processes such as proliferation, differentiation and survival.

Chemically diverse small-molecule protein kinase inhibitors have been discovered to be potential therapeutic agents for various human diseases such as retinopathies, atherosclerosis, psoriasis, rheumatoid arthritis, endometriosis and solid tumor growth [2]. Based on the crystallographic studies of the catalytic domain of FGFR1 with indolinones[3, 4, 5]. Several classes of indolinones have emerged as inhibitors of various split kinases. SU5402 is one such indolinone that competes with ATP for binding to the catalytic domain and inhibits the tyrosine kinase activity of FGFR1[6]. Considering the fact that SU5402 does not inhibit the phosphorylation of insulin receptors and also exhibits no inhibitory effects on EGF receptor kinase, it is possible to hypothesize the use of SU5402 as an antiproliferative and/or antiangiogenic agent to counteract the uncontrolled proliferation and angiogenesis in cancer [3]. Our current work is one such study testing the antiproliferative effect of this drug. Here, we present the use of a

non-cancerous model of cell proliferation to test the use of antiproliferative drug SU5402. We have used the sailfin molly *Poecilialatipinna*, which can entirely regenerate its tail-fin, if amputated.

The regenerating fin of the teleost fish *P. latipinna* serves as a promising and convenient postembryonic developmental model that mimics most of the stages of tumorigenesis like rapid remoulding of the extracellular matrix followed by angiogenesis and proliferation of newly recruited pleuripotent cells before they differentiate. Based on the major cellular events, the regeneration of tail-fin in teleosts is divided into three discernible stages, *viz.*, wound healing, blastema formation and differentiation.

Following amputation of the tail-fin, the epidermal cells surrounding the injury site proliferate and migrate to cover the wound and form a *wound epidermis*. This stage is under the influence of various factors including COX-2, FGFs, Shh, BMPs, Activin-βA, anterior gradient (AG), and Wnts [7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. Among these, FGF-2 has been shown to have an indispensable role in the initiation of regeneration. Therefore, here we test the antiproliferative effects of anti-cancer drug SU5402 on a development model system, which is known to heavily depend on FGF signalling.

MATERIALS AND METHODS

Animals and Maintenance

Adult Sailfin Mollies, *Poecilialatipinna*(Lesueur, 1821), with a mean standard length of 5.5±0.2cm, procured from a local animal breeder, were maintained in aquaria containing dechlorinated and constantly aerated filtered fresh water. The daily photoperiod was kept at 12 hr of light and 12 hr of darkness, and the temperature range was 26 to 28°C. The fishes were fed with readymade fish food (White rose fish food, Mumbai, India) *ad libitum*. The animals were acclimatized for a week before the commencement of the experiment. The experimental protocols used in the current study were carried out in accordance to the ethical guideline of Drugs and Cosmetics Rules, 1945and, was reviewed and approved by the Institutional Animal Ethics Committee (No. ZL/IAEC/15-2010) prior to the commencement of experiment.

Experimental Setup and Drug dosage

A total of 60 fishes were selected and divided into 3 groups of 20 animals each. Group C was control, whereas LD and HD were treated groups. A stock solution of SU5402 (Calbiochem, EMD Biosciences Inc., US) in 1% DMSO was prepared and stored at 4ºC.

The treatment in each group was started a day before amputation as intramuscular injections in the tail tissue and continued till the completion of the experiment. Group C was injected with 1% DMSO alone, whereas groups LD and HD received injections of 1µg/gm body weight and 2µg/gm body weight of SU5402 respectively.

Caudal Fin Amputation and Measurements

30% of the total fin-length was amputated under hypothermic anaesthesia by means of sterile surgical blade. The time taken to reach various stages of fin regeneration, *viz.*, wound healing,blastema formation and differentiation were recorded. The fin measurements were taken using a digital vernier calliper on 5^{th}, 10^{th} and 15^{th} days post amputation (dpa), and the photographs were captured by a Canon PowerShot A1100 IS.

Localization of FGF2

Fresh frozen sections (8-10µm) of blastema stage tails from control as well as treated groups were processed as per standard protocol for HRP-DAB system and observed under a Leica DM2500 microscope. The images were captured using EC3 Camera (supported by LAS EZ software).

Cellular synthetic activities

The fins from each group were pooled, homogenized for 10% and then further processed for estimating the nucleic acids as well as the protein contents in the tissue sample. Nucleic acids were extracted by the method described by Schneider and the DNA and RNA levels were estimated by the DPA and Orcinol methods respectively [18, 19]. Protein was estimated using BCA (Bicinchoninic acid) assay kit (Genei Products, Merck, USA) which employs the method described by Smith*et al*.,[20].

Histological analysis
The regenerate was excised, fixed in Bouin's fixative for 12h, decalcified with 10% EDTA for 3 days and further processed for H-E staining.

Statistical analysis
The data sets with continuous variable were subjected to Bartlett test for homogeneity and the significance level of the treatment groups with control group was evaluated through Student's 't' test with 95% confidence limit. The values are expressed as either Mean ± SE or as Mode with range in parenthesis. All statistical analyses were done using a statistical program SPSS, 11.0 (SPSS Inc. Chicago, IL, US).

RESULTS

Expression of FGF-2
Firstly, an immunolocalization experiment was carried out to see the effect of SU5402 on the distribution of FGF-2 and its relative expression in fins. An observation of the fin sections revealed the presence of FGF-2 maximally in the growing regions of all the fins (**Figure 1**). However, greater immunoreactivity was seen in the control group fins for all three stages of regeneration as compared to the corresponding stages in the SU5402-treated groups. This difference in intensities was most prominent in the blastema (**Figure 1b**) and the differentiation (**Figure 1c**) stages and negligible in the initial wound healing stage (**Figure 1a**).

Morphogenesis of fins
A primary observation of the effect of SU5402 on the growth of amputated fish fins was taken by recording the length of the regenerate under treated and untreated conditions. Group C showed a normal pattern of development with the fins healing at 1dpa (**Figure 2a**), reaching blastema stage at 4dpa (**Figure 2b**) and entering the differentiation phase by 7dpa (**Figure 2c**). Fins from treated fish of groups LD and HD, however, failed to attain these stages at the same time points. The control group could complete the entire regeneration within 15-17 days and the fin length reached the initial fin length recorded before amputation. On the other hand, it was observed in the treated groups that the regeneration process was not completed in 15 days. As shown in **Table 1**, there was a definite delay in the appearance of structures that define the particular stages. Also, when the lengths of the regenerates were measured at specific time points, the treated group fins were seen to be lagging behind the control ones (**Table 2 and 3**).

Cellular synthetic activity
The determination of nucleic acid and protein contents in the fin regenerate of experimental groups showed that the amount of protein present in the treated groups decreased in a dose dependent manner (**Table 4**). The low concentration of protein in the fins of SU5402-treated animals indicates lower protein synthetic activities. Nucleic acid content was also seen to be reduced due to drug administration (**Table 5**). This coincides with the reduced proliferation of cells which is an evident cause of the treated fins regenerating only partially.

Histological analysis
The SU5402 treated group showed a very thin layer of epidermis covering the wound surface, as opposed to a thick epithelial cap seen in the control fin sections on the very first day after amputation. The epidermal layer and conjunctive tissues were all well formed and could be clearly observed in the control fishes, whereas the treated fishes showed poor formation of all these structures (**Figure 3a**).

By 5dpa, the control group showed better growth of the epidermis basal layer and membrane as compared to the treated fins (**Figure 3b**). A blastema could be localized in the interior of the conjunctive tissue of the distal extremity of the fin in regeneration. The blastema of the control showed a reduced intercellular space as compared to the treated fins.

7^{th}dpa marks the onset of the differentiation phase in the fin's regeneration.On this day, the healed part of the fin had grown showing the regenerative outgrowth in both the groups. However, there was a marked difference in the development of the connective tissue, formation of the bony rays and pigmentation of the regenerates.Each of these was observed at a much lower amount in the treated fins (**Figure 3c**). The connective tissue had not yet formed completely, leaving pronounced gaps in the intercellular spaces. The epidermis basal layer as well as the membrane had not shown much improvement from theblastemal stage.

Table 1: Days taken to reach various stages of regeneration in *P. latipinna*

Groups	Number of Days		
	WH	BL	DIFF
Control	[a]1 (1)	[a]4 (4)	[a]7(7-8)
Treated (LD)	[a]1(1-2)	[b]4(4-5) ***	[b]8(7-8) *
Treated (HD)	[b]2 (2) *	[b]5(5-6) ***	[b]9(8-9) *

*WH: Wound healing stage; BL: Blastema stage; DIFF: Differentiation stage; LD: Low-dose group; HD: High-dose group. Values are expressed in Mode and Range in Parenthesis;Data prefixed with different alphabets are statistically significant within the column; * p≤0.01; *** p≤0.001; n=5.*

Table 2: Progression of regenerate in Control and Treated fishes

Day	Length of regenerate (mm)		
	Control	Treated (LD)	Treated (HD)
5	[a]0.93±0.069	[b]0.63±0.033***	[c]0.37±0.040***
10	[a]1.93±0.058	[b]1.53±0.061***	[c]1.27±0.046***
15	[a]2.97±0.029	[b]2.00±0.066***	[c]1.53±0.024***

*LD: Low-dose group; HD: High-dose group. Values are expressed as Mean±SEM;Data prefixed with different alphabets are statistically significant within the row; *** p≤0.001; n=5.*

Table 3: Percentage growth rate of the regenerate during initial (between day 5 & 10) and final (between day 10 & 15) stages of growth phase

Day	Percentage growth rate		
	Control	Treated (LD)	Treated (HD)
0-5	[a]31.11±2.33	[b]18.89±2.94***	[c]12.22±1.13***
5-10	[a]33.33±1.60	[b]30.00±1.33	[c]21.30±1.12
10-15	[a]34.44±2.75	[b]15.56±1.86***	[c]8.89±1.05***
Overall	[a]98.89±3.44	[b]64.44±4.01***	[c]51.11±3.79***

*LD: Low-dose group; HD: High-dose group. Values are expressed as Mean±SEM; Data prefixed with different alphabets are statistically significant within the row; *** p≤0.001; n=5.*

Table 4: Protein content in the fin regenerates of control and SU5402 treated fish

Experimental Group	Wound Healing stage (mg/100mg tissue)	Blastema stage (mg/100mg tissue)	Differentiation stage (mg/100mg tissue)
Control	0.801±0.02[@]	0.900±0.012[@]	0.860±0.012[@]
Treated	0.712±0.012*	0.807±0.018*	0.767±0.007*

Table 5: Nucleic Acid levels in the fin regenerates of control and SU5402 treated fish

Experimental Group	Wound Healing Stage		Blastema stage		Differentiation stage	
	DNA (µg/100mg tissue)	RNA (µg/100mg tissue)	DNA (µg/100mg tissue)	RNA (µg/100mg tissue)	DNA (µg/100mg tissue)	RNA (µg/100mg tissue)
Control	18.233 ±0.145	28.167 ±0.167	5.033 ±0.033	3.510 ±0.006	22.333 ±0.167	4.590 ±0.038
Treated	11.867 ±0.186*	18.667 ±0.441*	4.100 ±0.058*	2.637 ±0.020*	15.333 ±0.333*	3.830 ±0.012*

Figure 1: Immunolocalization of FGF2 in the regenerates of control (C) and SU5402 (2µg/g) treated (HD) fish during defined stages of regeneration. CT-connective tissue; L-lepidotrichia

Figure 1a: Wound healing stage (1dpa)

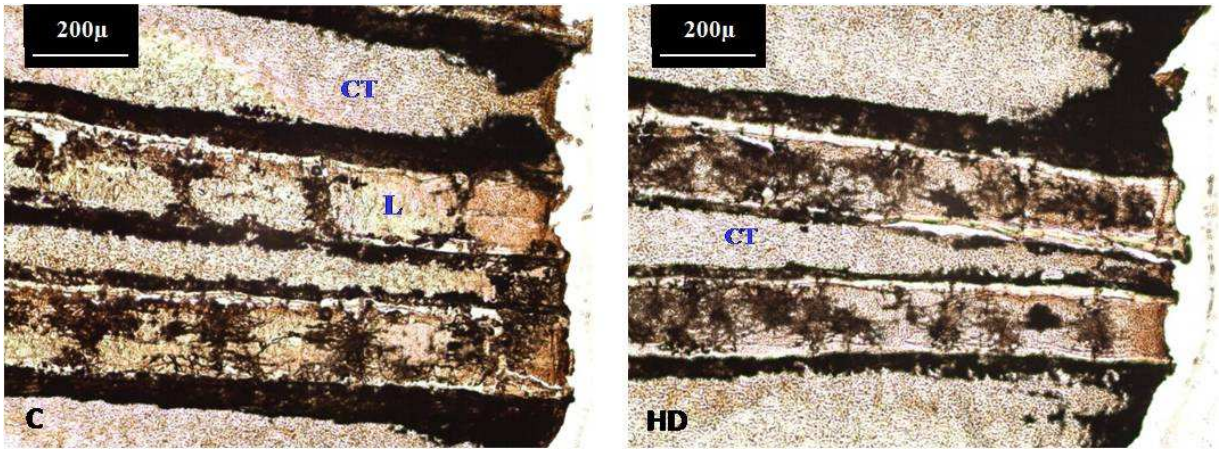


Figure 1b: Blastema stage (4dpa)

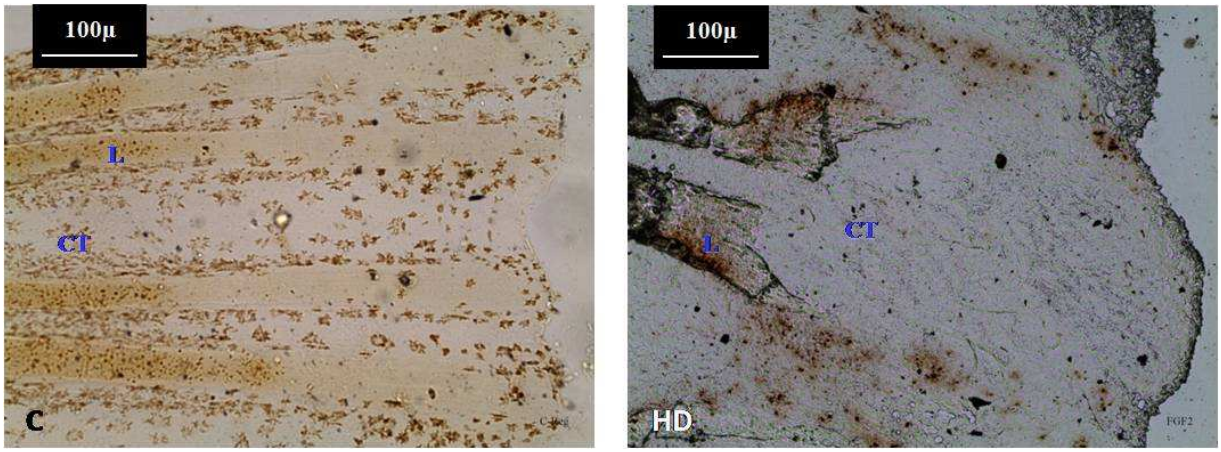


Figure 1c: Differentiation stage (7dpa)

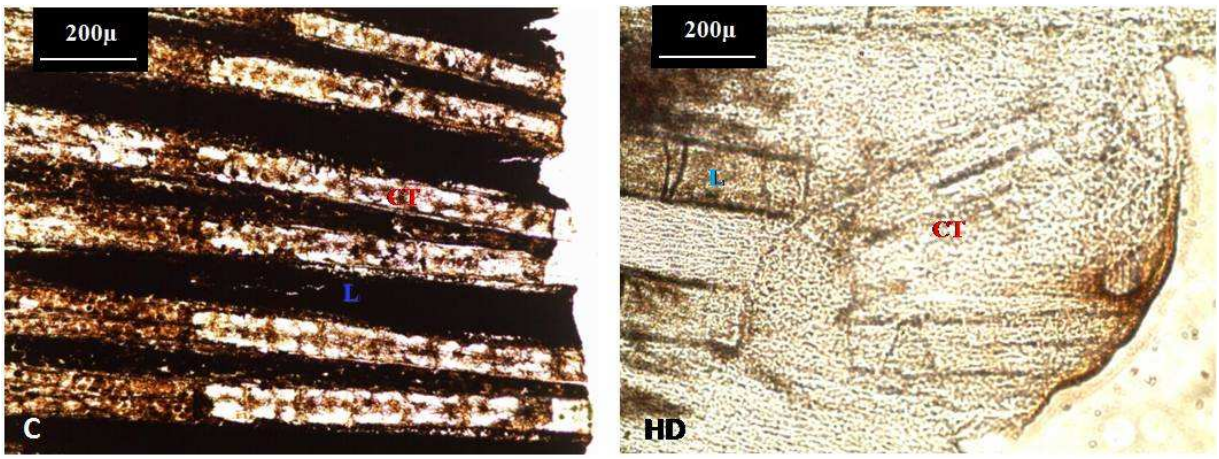


Figure 2: Morphometric analysis of regenerating tail fin treated with FGFR1 inhibitor SU5402

Figure 2a: Progress of regeneration in amputated fin at the wound-epithelium stage. C: Growing fin of control fish; LD: Growing fin of fish treated with low-dose of SU5402 (1µg/g); HD: Growing fin of fish treated with low-dose of SU5402 (2µg/g). Magnification: 4X

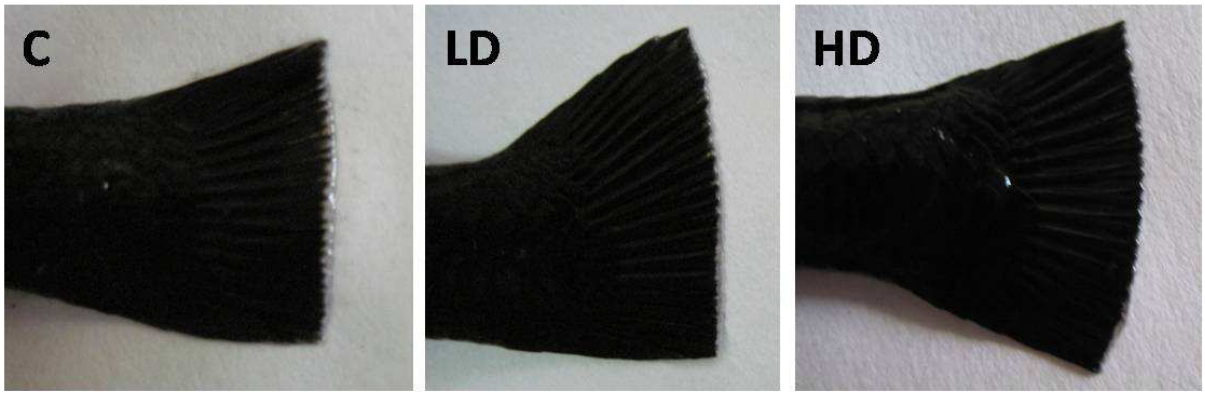


Figure 2b: Progress of regeneration in amputated fin at the blastema stage. C: Growing fin of control fish; LD: Growing fin of fish treated with low-dose of SU5402 (1µg/g); HD: Growing fin of fish treated with low-dose of SU5402 (2µg/g). Magnification: 4X

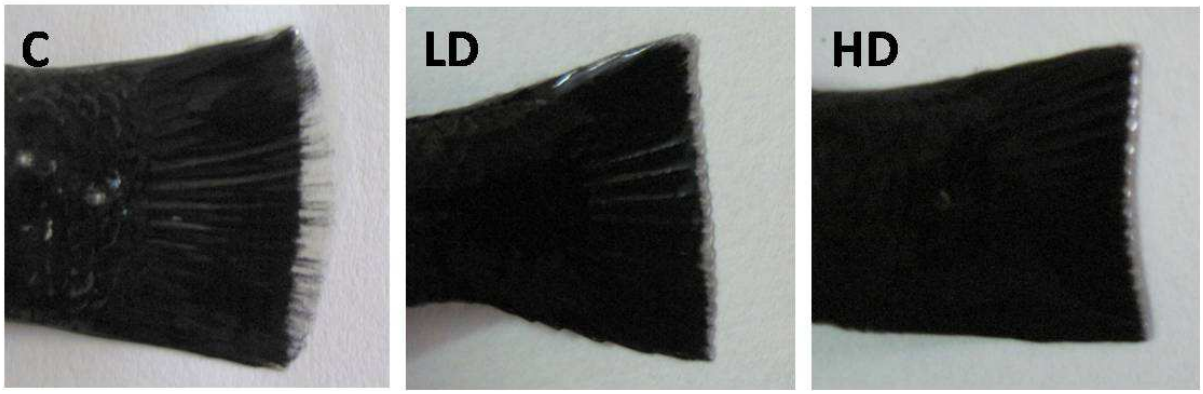


Figure 2c: Progress of regeneration in amputated fin at the differentiation stage. C: Growing fin of control fish; LD: Growing fin of fish treated with low-dose of SU5402 (1µg/g); HD: Growing fin of fish treated with low-dose of SU5402 (2µg/g). Magnification: 4X

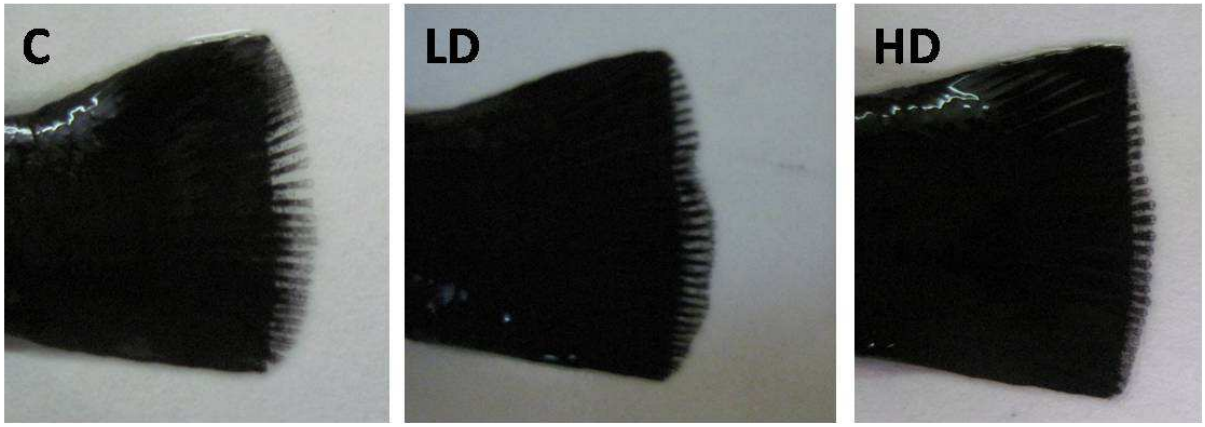


Figure 3: Effect of FGFR1 inhibitor SU5402 on the histology profiles of the regenerating fin at various stages. E: epidermis; BM: basal membrane; CT: connective tissue; AEC: apical epithelial cap; L: lepidotrichia; BL: blastema; ML: Melanocytes

Figure 3a: Histology profiles of tail fin regenerates at Wound-epithelium stage from C: control fish injected with 1% DMSO and T: test fish injected with 2µg/g body weight of SU5402

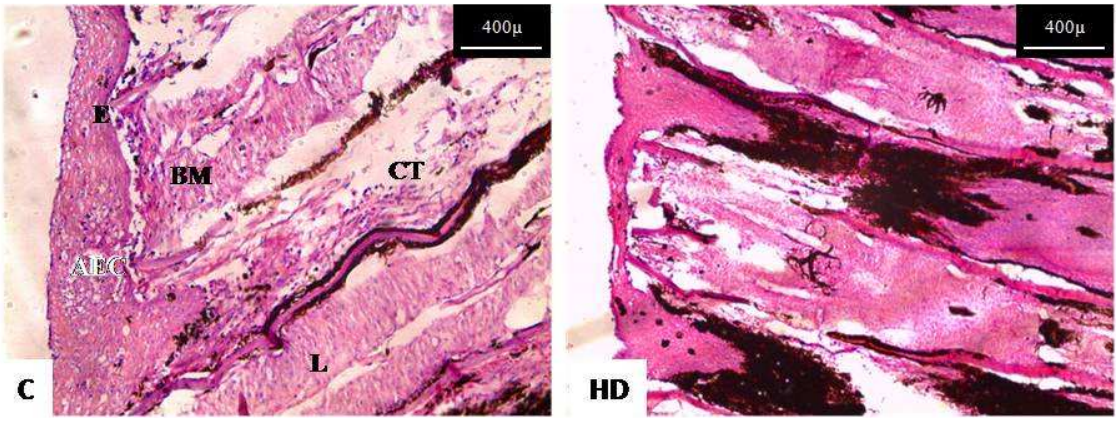


Figure 3b: Histology profiles of tail fin regenerates at Blastema stage from C: control fish injected with 1% DMSO and T: test fish injected with 2µg/g body weight of SU5402

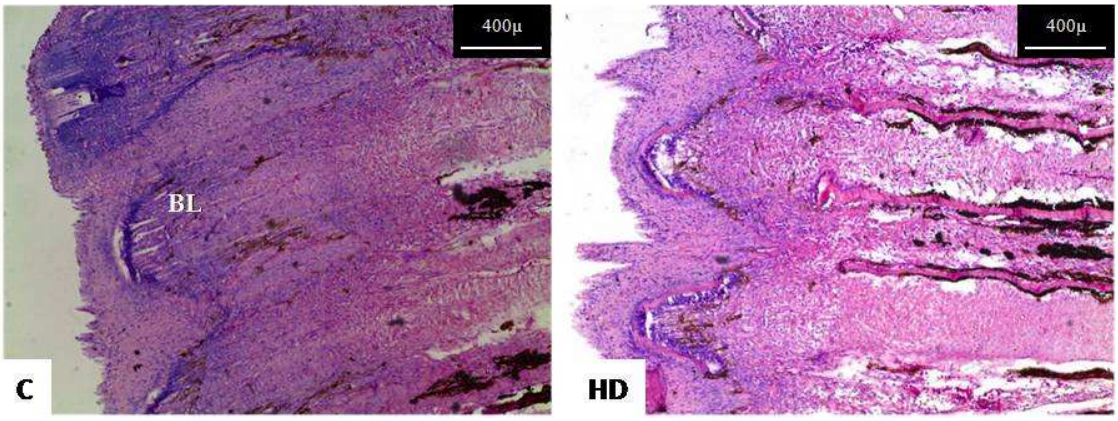
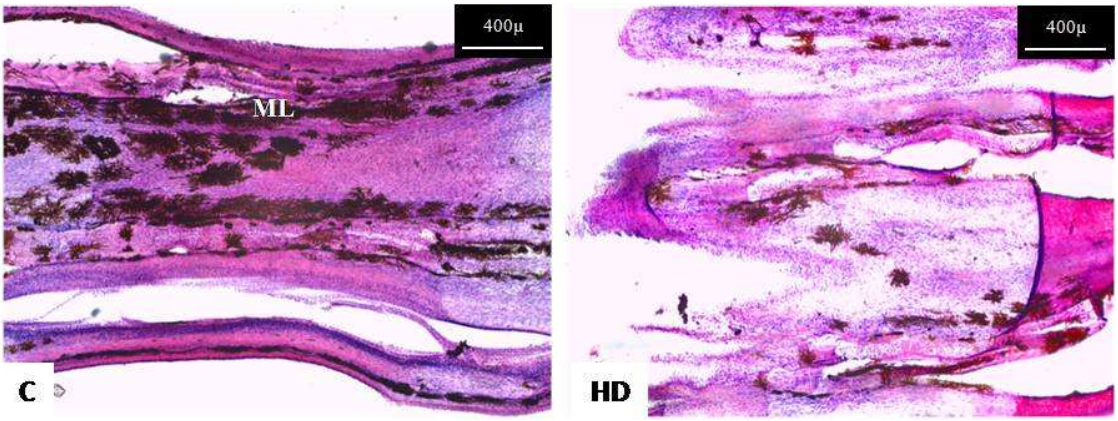


Figure 3c: Histology profiles of tail fin regenerates at Differentiation stage from C: control fish injected with 1% DMSO and T: test fish injected with 2µg/g body weight of SU5402



DISCUSSION

There is ample evidence suggesting that members of the fibroblast growth factor (FGF) family along with their tyrosine kinase receptors (FGFR1-4) act as autocrine as well as paracrine (angiogenic) growth factors in many solid tumors [21]. SU5402 is a potent anti-tumor compound of the current times. It has an indolin-2-one core and it inhibits FGFR1 tyrosine kinase by interacting with the ATP-binding site of the FGFR kinase domain. Because selectivity of indolin-2-one compounds against receptor tyrosine kinases is determined by substituents extending from the indolin-2-one core, SU5402 is a narrow-range tyrosine kinase inhibitor [22].

The complete process of restoration of tail fin in teleost fish, called epimorphic regeneration, occurs through the establishment of a balanced growth state, which involves specific mechanisms that temporally and spatially regulate cell proliferation [23]. Fibroblast growth factors (FGFs), which play an important role in a variety of biological processes, are known to be involved in the formation and organization of the extracellular matrix, in the production of growth factors as well as cytokines and chemokines[24], all of which are notable events of epimorphic regeneration. During this process, a blastema gives rise to all the mesenchymal cells, whereas definite areas of the epidermis proliferate leading to its extension, thus, allowing the enlargement of the whole structure. This occurs through strict growth controls and cell reprogramming occurring in adult tissues followed by sequential steps of cell differentiation and patterning leading to the faithful restoration of the lost parts [25]. This makes the regenerating tail fin of *Poecilialatipinna* an attractive model for studying SU5402, a potent inhibitor of FGF signalling.

When we studied the effect of this potential anti-tumor compound on the regenerating teleost fish fin, we found a profound negative impact on its growth. Firstly, immunolocalization of FGF-2 in the regenerating fin tissue suggested a reduction in its expression and change in its localization pattern upon treatment with SU5402. This observation finds support in a study by Hoffman and coworkers, which shows a decrease in expression of FGF-2 after SU5402 treatment in the developing submandibular glands of mice [26]. In our study, while the difference in expression as seen by immunohistochemistry was rather subtle during the wound healing stage, the reduction in expression during the subsequent stages was more pronounced. We hypothesize that since SU5402 is a receptor inhibitor, it does not affect the FGF-2 expression immediately after treatment. It however leads to accumulation of FGF-2 which, on failing to bind its receptors, eventually suppresses its own expression.

A record of the progress of growth of the fin over a period of 15 days, revealed a radical impact of the drug treatment. Control fins fared far better than the treated ones. SU5402 treatment caused a significant delay in attainment of the specific stages of epimorphic regeneration, *viz.*, wound healing, blastema and differentiation. Fischer *et al*., [27] had observed that treatment of non-small cell lung cancer cells with SU5402 inhibited proliferation of the cells in a dose-dependent manner, which helps explain our current results. The growth of regenerate in the case of our model of study naturally involves and in fact depends heavily on large scale proliferation of cells from the blastema stage up to the very end. Any negative effect that the drug in question has on proliferative activities of cells is bound to hamper the progression of tail re-growth.

Further, the biosynthesis of proteins is one of the most important biochemical processes during regeneration as reported by Thornton and Bromley [28]. Hence it was thought worthwhile to study the effect of FGF2 receptor inhibitor SU5402 on the protein content of the fins. Results showed a lower content of protein in the treated fins as compared to the control ones, supporting the known fact that FGF-2 signalling leads to activation of a number of genes and thereby *de novo* synthesis of a number of proteins. In absence of FGF signalling, the proliferative activities of blastemal cells are also hampered, which shows up in our results as reduced nucleic acid content.

Hematoxylin-eosin stained sections help us see the state of the progress of regeneration at the cellular level in a holistic way. Our results in this respect are concomitant with the observations of Kaftan and others, who have recently given histological evidence of SU5402 causing a dose-dependent delay in the healing of perforated tympanic membranes in rats [29].

CONCLUSION

The regenerating fin of teleost fish is a model of extensive cell proliferation and patterning. A major signal influencing this process is the ubiquitous fibroblast growth factor 2 that acts by binding to a specific Tyrosine Kinase Receptor, the FGFR1. By using this model, we hereby confirm the anti-proliferative effects of SU5402, a

specific pharmacological inhibitor of FGFR1, which was found to affect various key events of regeneration and hence altering the caudal fin regeneration at various defined stages.

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