A Zebrafish *In Vivo* Phenotypic Assay to Identify 3-Aminothiophene-2-Carboxylic Acid-Based Angiogenesis Inhibitors

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ABSTRACT

Small molecules that inhibit angiogenesis are attractive drug candidates for cancer, retinopathies, and age-related macular degeneration. *In vivo*, phenotypic screening in zebrafish (*Danio rerio*) emerges as a powerful methodology to identify and optimize novel compounds with pharmacological activity. Zebrafish provides several advantages for *in vivo* phenotypic screens especially for angiogenesis, since it develops rapidly, externally, and does not rely on a functional cardiovascular system to survive for several days during development. In this study, we utilize a transgenic line that allows the noninvasive monitoring of angiogenesis at a cellular level. The inhibition of angiogenesis can be observed under a fluorescent stereoscope and quantified. To exemplify the versatility and robustness of the zebrafish screen, we have employed a series of 60 novel compounds that were designed based on a potent VEGFR2 inhibitor. Herein, we report their structure-based design, synthesis, and *in vivo* zebrafish screening for optimal activity, toxicity, and off-target effects, which revealed six reversible inhibitors of angiogenesis.

INTRODUCTION

Angiogenesis refers to the formation of new vessels, developing from preexisting endothelial cells through a tubular structure formation. Its spatial and temporal control plays a crucial role not only in development but also in cancer, age-related macular degeneration, diabetic and prematurity retinopathies, wound healing, and tissue regeneration.¹ Identifying new molecules that control angiogenesis would be critical in developing new therapies for all these conditions.

As far as the molecular basis of angiogenesis is concerned, the vascular endothelial growth factor family (VEGFs) of ligands and the corresponding receptors represent the central regulators.² While there is some redundancy in function and ligand–receptor interactions, VEGFR2 represents the main receptor with a pivotal role in

signal transduction and in the promotion of angiogenesis. The growth factor binds to the extracellular domain of VEGFR2 and facilitates dimerization of the receptor. The intracellular part consists of the tyrosine kinase domain and the carboxyl terminus domain, which get autophosphorylated upon dimerization to initiate the signal transduction pathways that lead to angiogenesis. VEGFR2 is expressed as early as the first embryonic stages and persists until adulthood in endothelial cells. During development, VEGFR2 is responsible for the proliferation of endothelial cells and is also highly expressed in tumor vascular networks. Therefore, VEGFR2 has been proposed as a target to inhibit blood vessel formation in growing tumors.³

Zebrafish express four receptors that regulate angiogenesis: Flt1, Kdr, Kdr-like (kdrl), and Flt4 are highly homologous to the human receptors.⁴ Different combinations of these enzymes regulate the development of the various vessels in the developing embryo. The zebrafish angiogenesis pattern is highly stereotypical, tightly regulated, and well described at the cellular resolution during development.⁵ In addition, zebrafish embryos can survive and develop normally for the first 4 days even in the absence of a vascular system, as in the *cloche* mutant, or in the absence of circulation due to a noncontractile heart, as in the *silent heart* mutant.^{6,7} Other advantages of the zebrafish embryos include (a) small size, (b) rapid development, (c) the ability to conduct noninvasive observations due to their transparent nature, and (d) the availability of tissue-specific transgenic lines that assist in the examination of organogenesis. Overall, they represent an attractive model to gain insight into the molecular function of novel compounds, as in the case of Dorsomorphin.⁸ Several groups have previously used endothelial-specific transgenic lines to screen for novel antiangiogenic compounds and developed automated methodologies to identify and quantify the antiangiogenic activity of compound libraries.^{9–14}

Previous work from our laboratories generated and characterized *Tg(kdrl:gfp)*^{s843} currently used extensively to study angiogenesis *in vivo*. In addition, based on the anthranilamide scaffold AAL993 (*Fig. 1*), a highly potent and selective inhibitor of VEGFRs with good pharmacological properties and excellent oral bioavailability, we designed and synthesized a small library of inhibitors for VEGFR2.¹⁵ Several examples from that work, analogues [1–3] shown in *Figure 1*, demonstrated exceptional binding affinities for VEGFR2.¹⁵ Stimulated by the successful outcome of these efforts, we decided to enrich our chemical library with additional examples and expand our analysis to

Fig. 1. (A) Crystallographic structure of VEGFR2 kinase domain complex with a potent inhibitor (PDB ID: 3EWH). (B) Close-up view of the ATP-binding site showing the docked conformation of a hydrazine-based inhibitor (compound **140**) shown *inset*). Atom colors are *orange* for inhibitor C, *cyan* for protein C, *red* for O, *blue* for N, and *yellow* for S. Potential hydrogen bonds between the inhibitor and key catalytic residues are shown with *dashed lines*. (C) Previously identified VEGFR2 inhibitors AAL993 [**1–3**] and the general formula of the designed inhibitors.

include their evaluation *in vivo*. Herein, we report the design, synthesis, and *in vivo* biological evaluation of 60 new analogues based on 3-aminothiophene-2-carboxylic acid, which resulted in the identification of 6 novel molecules with antiangiogenic properties. We propose a simple methodology that can be used to quantify their activity, utilizing the ImageJ freeware.

MATERIALS AND METHODS

Computational Methods

The high-resolution crystallographic structure of the VEGFR-2 kinase domain in complex with a pyridyl-pyrimidine benzimidazole inhibitor (PDB accession code 3EWH) was employed for the docking calculations.¹⁶ All heteroatoms were removed, and the missing residues 858–860, 1052–1055, and 1062–1066 were modeled using Modeller9.1.¹⁷ The engineered residues at positions 817, 916, and 990 were changed to the native residues Cys817, Val916, and Glu990 and missing atoms were

added using the XLEaP module of AMBER v12.¹⁸ Subsequently, the protein was subjected to energy minimization using the *parm99SB* force field and an implicit generalized Born solvation model by applying positional restraints of $50 \text{ kcal} \times \text{mol}^{-1} \times \text{\AA}^{-2}$ on the crystallographic C α atoms.^{19,20}

The initial conformations of the ligands were generated from SMILES representations using the program Omega 2.3 with default parameters.²¹ For protein and ligands, all nonpolar hydrogen atoms were merged and Gasteiger charges were applied in AutoDockTools 1.5. The search space was defined by a grid box centered on the crystallographic inhibitor and comprised $81 \times 61 \times 61$ grid points of 0.375 Å spacing. For each complex, 100 docking rounds were calculated with AutoDock4.2 using the Lamarckian genetic algorithm with the default parameters from AutoDock3.^{22,23} The maximum number of energy evaluations was set to 10 million and the results were clustered using a tolerance of 2.0 Å. Visual examination of the results and rendering of the figures were performed using VMD 1.9.²⁴

Synthesis and Characterization of the Compounds

All solvents and reagents for organic synthesis were obtained from commercial suppliers and used without further purification. All reactions

were carried out under a dry Ar atmosphere with anhydrous freshly distilled solvents under anhydrous conditions. Yields refer to chromatographically and spectroscopically (¹H NMR) homogeneous materials. Reactions were monitored by thin-layer chromatography carried out on 0.25-mm silica gel plates (60 F254, E. Merck). Silica gel (60, particle size: 0.040–0.063 mm, E. Merck) was used for flash column chromatography. NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 298 K using deuterated solvents as internal standards: [CDCl₃, 7.26 ppm (¹H) and 77.2 ppm (¹³C)]; DMSO, 2.50 ppm (¹H) and 39.5 ppm (¹³C)]. Chemical shift values (δ) are given within an accuracy of 0.01 ppm for ¹H NMR and 0.1 ppm for ¹³C NMR data, while the coupling constants (J) are within 0.1 Hz. Multiplicities are designated as singlet (s), doublet (d), triplet (t), or multiplet (m). Representative characterization data from the synthesized compounds [**11a** to **14o**] are given in Supplementary Data (Supplementary Data are available online at www.liebertpub.com/adt).

Zebrafish Pharmacological Assays and Immunohistochemistry

Zebrafish were kept and raised according to the Zebrafish book guidelines and compounds were dissolved at the desired concentrations in embryo water at maximum 0.1% EtOH or DMSO.²⁵ Twelve embryos per well were transferred into a 12-well plate maintained at 28°C, with 3 mL solution per well. For the calculation of LC₅₀, we used twelve embryos per molecule in three different concentrations to roughly determine LC₅₀ during early development (4–24 hpf) and in three different concentrations in three independent experiments to calculate LC₅₀ (22–72 hpf). Values were rounded up to the nearest whole number. For the quantification of antiangiogenic activity of the compounds, we measured the length of 10 intersegmental vessels (ISVs) flanking the yolk extension level from twenty *Tg(kdrl:gfp)*⁸⁴³ embryos in three independent experiments. The images were acquired by a Leica DFC350-FX camera, under a Nikon SMZ2800 stereoscope, using the LAS V3.8.0 software. One-way ANOVA of Microsoft Excel was used to calculate the statistical significance.

RESULTS

Rational Design, Synthesis Characterization, and Computational Evaluation of Novel Thiophene Analogues

The thiophene analogues [2] and [3] served as the basis of the new design and were substantiated by computational modeling, which employed the crystallographic structure of VEGFR2 kinase domain in the inactive conformation (Fig. 1B). In particular, the aniline amide functionality in [2] and [3] was extended to include benzylic and acylhydrazone-based groups (X-Ar² in Fig. 1C). Based on our docking calculations, this modification would preserve the binding mode of the VEGFR2 inhibitors and provide the possibility of additional hydrogen bonding interactions in the case of the acylhydrazone-based compounds (Fig. 1B). In certain bound configurations, the thiophene's sulfur and carbonyl group is expected to interact with the side-chain amine of Lys868, while the hydrazine moiety is predicted to interact with the carboxylate group of Glu885. The aromatic Ar¹ moiety, pyridyl or nitro-substituted, could interact with the backbone of Cys919, while

sp P35968 VEGFR2_HUMAN 834-1162	LKLGLKPLGRGAFGQVIEADAFGIDKTATCCTVAV K MLKEGATHSEHRALM	883
sp P35918 VEGFR2_MOUSE 832-1160	LKLGLKPLGRGAFGQVIEADAFGIDKTATCCTVAV K MLKEGATHSEHRALM	881
sp Q5GIT4 VEGFR2_DANRE 843-1173	LKLGEPLGRGAFGQVVEATAYGIEKATTCTTVAV K MLKEGATSSEYRALM	892
	****:*****:***:***:***:***:*****:***:*****	
sp P35968 VEGFR2_HUMAN 834-1162	S ELKILIHIGHHLNVNLLGACTKPGGPLMVIV E CFKGNLSTYLRSKR	933
sp P35918 VEGFR2_MOUSE 832-1160	S ELKILIHIGHHLNVNLLGACTKPGGPLMVIV E SKFKNLSTYLGRKR	931
sp Q5GIT4 VEGFR2_DANRE 843-1173	S ELKILIHIGHHLNVNLLGACTKQGGPLMVIV E YCKHGNLSSYLKSKRG	942
	*****:*****:***:*****:***:*****:***:*****	
sp P35968 VEGFR2_HUMAN 834-1162	EFVYPYKTKGARFR-----QGKD-YVGAIPVDLKRRLDSITSSQSSASSG	976
sp P35918 VEGFR2_MOUSE 832-1160	EFVYPYKSKGARFR-----QGKD-YVGELSVDLKRRLDSITSSQSSASSG	974
sp Q5GIT4 VEGFR2_DANRE 843-1173	EYSPYKRTPRMPNRRVQQDEDPREGDLGLGTSTRLDICTGTAVCTRGT	992
	*:***:***:***:***:***:***:***:***:***:***:***:***:***	
sp P35968 VEGFR2_HUMAN 834-1162	FVEEKSLSDVVEEEEAPEDLYKDFTLEHLICYSFQVAKGMEFLASRKCIH	1026
sp P35918 VEGFR2_MOUSE 832-1160	FVEEKSLSDVVEEEEASEELYKDFTLEHLICYSFQVAKGMEFLASRKCIH	1024
sp Q5GIT4 VEGFR2_DANRE 843-1173	EQTYKTLQDEQESSD-----WDHLTMEDLISYSFQVAKGMEFLASRKCIH	1037
	*:***:***:***:***:***:***:***:***:***:***:***:***:***	
sp P35968 VEGFR2_HUMAN 834-1162	RDLAARNILLSEKNVVK I DFGLARDIYKDPDYVRKGDARLPLKWMAPET	1076
sp P35918 VEGFR2_MOUSE 832-1160	RDLAARNILLSEKNVVK I DFGLARDIYKDPDYVRKGDARLPLKWMAPET	1074
sp Q5GIT4 VEGFR2_DANRE 843-1173	RDLAARNILLSSENSVVK I DFGLARDVYKDPDYVRKGDARLPLKWMAPET	1087
	*****:*****:*****:*****:*****:*****:*****:*****	
sp P35968 VEGFR2_HUMAN 834-1162	IFDRVYTIQSDVWSFGVLLWEIFSLGASPYPGVKIDEEFCRRLKEGTRMR	1126
sp P35918 VEGFR2_MOUSE 832-1160	IFDRVYTIQSDVWSFGVLLWEIFSLGASPYPGVKIDEEFCRRLKEGTRMR	1124
sp Q5GIT4 VEGFR2_DANRE 843-1173	IFDRVYTTQSDVWSFGVLLWEIFSLGASPYPGVCIDESFCRRLKEGTRMR	1137
	*****:*****:*****:*****:*****:*****:*****:*****	
sp P35968 VEGFR2_HUMAN 834-1162	APDYTTPEMYQTMLDCWHGEPSQRPTFSELVEHLGN	1162
sp P35918 VEGFR2_MOUSE 832-1160	APDYTTPEMYQTMLDCWHEDPNQRPSFSELVEHLGN	1160
sp Q5GIT4 VEGFR2_DANRE 843-1173	APDYATPEIYQTMLDCWLDRLDRPTFTQLVEHLGN	1173
	****:***:*****:***:***:***:*****:***:*****	

Fig. 2. Sequence alignment of the VEGFR2 kinase domains from human (UniProt accession number: P35968), mouse (UniProt accession number: P35918), and zebrafish (UniProt accession number: Q5GIT4) indicating the position of the conserved Lys868, Glu885, Cys1045, and the semiconserved Cys919 (Ser917 in mouse) ATP-binding site residues shown in Figure 1B.

its amine functionality is anticipated to be hydrogen bonded with the thiol of Cys1045. Finally, the hydrophobic aromatic Ar² moiety would be buried within a lipophilic pocket defined by residues Ile888, Leu889, Ile892, Val898, Val899, Leu1019, and Ile1044. The sequence identity of the kinase domain between human and mouse is 96.1%, between human and zebrafish is 74.5%, and between mouse and zebrafish is 73.9%. The ATP-binding site residues Lys868, Glu885, Cys919, and Cys1045 are conserved between human and zebrafish (Fig. 2).

The targeted thiophene-containing analogues were generated according to the procedure presented in Figure 3, which represents an improved synthetic approach compared to our previously reported attempts.¹⁵ Specifically, methyl-3-aminothiophene-2-carboxylate [4] was hydrolyzed with KOH at 120°C producing quantitatively the corresponding amino acid [5]. The reaction needs to be instantaneously heated at that temperature otherwise decarboxylated by-products may be obtained. Treatment of [5] with phosgene, generated *in situ* from triphosgene and activated carbon in toluene, furnished the desired 2-thiaisatoic anhydride [6] in 56% overall yield. Alkylation of [6] with benzylic-type bromides A–D (Fig. 3) in the presence of K₂CO₃ yielded the corresponding bicyclic intermediates [7–10] that were regioselectively opened with a series of benzylic amines [a–h] and hydrazines [i–o] to furnish analogues [11–14].

In Vivo Estimation of LC₅₀ and Specificity of Small Compound Libraries

We calculated the LC₅₀ after the addition of the compounds in two time points: (a) at 4 h postfertilization (hpf) to test for potential teratogenicity and off-target effects and (b) at 22 hpf after dechorination of the embryos as a more specific antiangiogenic screen. The majority of the molecules have comparable LC₅₀ scores between these two stages, indicating that the small molecules tested are able to cross through the chorion of unhatched embryos. In addition, all the molecules range from 5 to 50 μM. Embryos treated with high doses of the molecules showed mild cardiac edema due to specific defects in angiogenesis similar to the published morpholino and mutant data of *kdrl* (Fig. 4a).²⁶ Eleven out of the sixty-four analogues were insoluble in water at μM concentrations and precipitated, not allowing further characterization (Table 1).

Identification of Novel Molecules Inhibiting Angiogenesis by a Phenotype-Based Screen

To test the ability and specificity of these novel analogues to inhibit angiogenesis *in vivo*, 20 hpf *Tg(kdrl:gfp)^{s843}* embryos were incubated with all of the compounds and in concentrations of 1, 10, and 60 μM. Inhibition of angiogenesis was scored on the development of ISVs at 50 hpf and we were able to identify six new molecules that display a specific antiangiogenic activity (Fig. 5). In particular, compound [1] that has been previously identified as a very potent inhibitor of VEGFR2¹⁵ displayed equal antiangiogenic activity with the parent compound ALL993 (Table 1). This was also the case for compounds [12f] and [12i] that exhibit >75% suppression of angiogenesis at 10 μM. The remaining three inhibitors showed

Fig. 3. Reagents and conditions: (a) [4] (1.0 equiv), KOH (1.5 equiv), H₂O (0.55M), 120°C, 20 min., quantitative; (b) triphosgene, activated carbon, toluene, 25°C, 45 min.; (c) [5] (1.0 equiv), phosgene (6.0 equiv), 0°C, 60 min., 56%; (d) [6] (1.0 equiv), Ar¹CH₂Br (1.1 equiv), K₂CO₃ (2.2 equiv), DMF (0.62 M), 2 h, 25°C, 50%–70%; (e) [7–10] (1.0 equiv), ArXNH₂ (1–1.5 equiv), TEA (1–1.5 equiv), DMF (0.62 M), 2–16 h, 25°C–60°C.

Fig. 4. An *in vivo* phenotypic screen reveals six new molecules that inhibit angiogenesis. **(A)** Brightfield image of a representative embryo treated with one of the active compounds tested. There is no gross morphology phenotype compared to the untreated embryos, besides the cardiac edema (arrow), due to the defect in angiogenesis and blood circulation. **(B–J)** Fluorescence microscopy images of 50 hpf embryos *Tg(kdrl:gfp)*^{S843} in embryo water **(B)** or treated from 20 to 50 hpf with 10 μ M **[12o]** **(C)**, **AAL993** **(D)**, **[1]** **(E)**, **[12n]** **(F)**, **[12i]** **(G)**, **[12c]** **(H)**, **[12f]** **(I)**, **[12m]** **(J)**. **[12o]** **(B)** is a representative example of compounds that did not show inhibition of angiogenesis at 10 μ M, as indicated by the wild-type ISV development. Scale bar 100 μ m.

25%–75% **[12m]** and **[12n]** and 5%–25% **[12c]** suppression of angiogenesis at the same concentration.

A Simple Way to Quantify Inhibition of Angiogenesis

We screened quantitatively 64 compounds and identified 6 novel ones with the antiangiogenic activity. Aiming to quantify this activity, we decided to follow a simple protocol. Previous methodologies included the measurement of ISV length as a percentage to the somite length or the vessel diameter in different positions of the embryo, which is very elaborate but also laborious requiring confocal

microscopy scanning images.^{27,28} We used the 10 μ M concentration to quantify the inhibition of angiogenesis, using ImageJ software on fluorescent stereoscope pictures (Fig. 5). We calculated the total length of the 10 ISVs flanking the end of yolk extension to have a representative and reproducible measurement. The six novel compounds had an antiangiogenic activity ranging from 10% to 100%. Three of them **[1]**, **[12f]**, and **[12i]** were comparable to the reference compound (AAL993) but had higher LC₅₀, indicating that they were less toxic than AAL993. In these compounds, we looked for off-target defects upon treatment with 10 μ M during development. We did not detect any arrhythmias or changes in the heart rate (Supplementary Movies S1, S2, 20–50 hpf treatment) or gross morphological defects in axis developmental patterning during gastrulation (not shown, 4–50 hpf). In this way, we identified six novel compounds and quantified their antiangiogenic activity.

The Novel Small Molecules Are Stable in an Aqueous Solution and Their Inhibitory Potential Activity Is Reversible

We conducted two additional tests so as to explore the mode of function of the newly identified angiogenesis inhibitors *in vivo*. We compared the inhibition of angiogenesis (6A) between treatment with the small molecules from 20 hpf until 72 hpf (6B, E) and when replacing the active compounds in the solution at 50 hpf (6C, F). We did not

observe a significant increase in the inhibition of angiogenesis between the two conditions (Fig. 6C, F compared to B, E), indicating that the inhibitors remain active and are stable for at least 48 h in an aqueous pH-neutral solution. We also withdrew the compounds and evaluated whether angiogenesis was reinitiated. In all cases shown for **[12f]** and **[12m]**, we observed novel ISVs sprouting from the pre-existing vessels of the treated embryos within 22 h (Fig. 6D, G compared to C, F). These data indicate that their specific inhibition of angiogenesis is reversible; a feature particularly desirable in a potential drug.

Table 1. Antiangiogenic Activity of the Compounds Tested

ID	Inhibition at 10 μ M ^(a)	LC ₅₀ (μ M) 4 hpf ^(b)	LC ₅₀ (μ M) (SD) 22 hpf ^(c)
AAL993	+++	5	5 (1)
1	+++	7	7 (1)
2	–	10	13 (2)
3	–	6	6 (1)
11a	–	12	14 (2)
11b	–	25	30 (4)
11c	–	12	9 (2)
11d	–	22	27 (3)
11e	–	22	31 (3)
11f	–	9	12 (1)
11g	–	18	19 (2)
11h	–	22	23 (2)
11i	–	20	22 (2)
11j	–	<i>n.d.</i>	<i>n.d.</i>
11k	–	22	23 (2)
11l	–	30	38 (3)
11m	–	45	45 (5)
11n	–	22	22 (3)
11o	–	22	27 (3)
12a	–	8	11 (2)
12b	–	20	23 (2)
12c	+	18	22 (2)
12d	–	12	12 (1)
12e	–	19	22 (2)
12f	+++	9	9 (2)
12g	–	12	16 (2)
12h	–	17	21 (2)
12i	+++	12	12 (1)
12j	–	13	15 (2)
12k	–	20	23 (2)
12l	–	22	25 (2)
12m	++	14	14 (2)
12n	++	10	11 (2)
12o	–	12	16 (2)

Table 1. (Continued)

ID	Inhibition at 10 μ M ^(a)	LC ₅₀ (μ M) 4 hpf ^(b)	LC ₅₀ (μ M) (SD) 22 hpf ^(c)
13a	–	<i>n.d.</i>	<i>n.d.</i>
13b	–	10	11 (1)
13c	–	10	6 (1)
13d	–	10	9 (2)
13e	–	32	40 (5)
13f	–	10	10 (1)
13g	–	10	6 (1)
13h	–	<i>n.d.</i>	<i>n.d.</i>
13i	–	30	35 (4)
13j	–	23	15 (2)
13k	–	50	50 (5)
13l	–	20	25 (3)
13m	–	<i>n.d.</i>	<i>n.d.</i>
13n	–	<i>n.d.</i>	<i>n.d.</i>
13o	–	<i>n.d.</i>	<i>n.d.</i>
14a	–	<i>n.d.</i>	<i>n.d.</i>
14b	–	8	11 (2)
14c	–	22	22 (2)
14d	–	50	50 (5)
14e	–	19	13 (2)
14f	–	5	7 (1)
14g	–	10	5 (1)
14h	–	<i>n.d.</i>	<i>n.d.</i>
14i	–	10	9 (2)
14j	–	<i>n.d.</i>	<i>n.d.</i>
14k	–	<i>n.d.</i>	<i>n.d.</i>
14l	–	13	8 (1)
14m	–	<i>n.d.</i>	<i>n.d.</i>
14n	–	5	5 (1)
14o	–	50	50 (5)

^aInhibition refers to suppression of angiogenesis as indicated by (–) for <5%, (+) for 5%–25%, (++) for 25%–75%, and (+++) for >75%. LC₅₀ was calculated at ^b24 hpf when molecules were added at 4 hpf based on single determination and ^cat 72 hpf when molecules were added at 22 hpf in three independent experiments (standard deviation in parenthesis).

n.d., not determined due to precipitation.

Fig. 5. Quantification of inhibition of angiogenesis. **(A)** The dotted yellow lines denote the ISVs that were measured with the help of ImageJ software. **(B)** The total length of 10 ISVs at the end of the yolk extension was measured from at least 20 embryos from three independent experiments and the percentage of inhibition of angiogenesis of each compound was estimated compared to the untreated age-matched controls. Scale bars: SD. ** $P < 0.05$; *** $P < 0.005$ versus untreated control.

DISCUSSION

In this report, six novel compounds with antiangiogenic activity based on 3-aminothiophene-3-carboxylic acid were identified by using an *in vivo* phenotypic screen. Zebrafish emerges as a powerful vertebrate model to test chemical compounds in large scale for a number of physiological processes. These include angiogenesis during development and tumor xenograft models.^{29,30}

From the previously identified potent VEGFR2 inhibitors [1] ($IC_{50} = 3$ nM), [2] ($IC_{50} < 1$ nM), and [3] ($IC_{50} = 70$ nM), only the parent compound (ALL993) and compound [1] displayed reversible suppression of angiogenesis $> 75\%$ at $10 \mu M$ (Table 1).¹⁵ Based on these results, a new series of thiophene-based analogues were designed based on either [1] with $Ar^1 = 3$ -pyridine or ALL993 with $Ar^1 = 4$ -pyridine (Fig. 1C). Surprisingly, none of the compounds with $Ar^1 = 3$ -pyridine [11a-11o], (Fig. 3) displayed any

Fig. 6. Fluorescent microscopy images of untreated **(A)**, treated with [12f] **(B-D)**, [12m] **(E-G)** larvae at 72 hpf ($63\times$ magnification). Inhibition of angiogenesis is visible when the compounds remain up to 72 hpf **(B, E)**. After refreshing the compounds at 50 hpf, no additional effect on the inhibition of angiogenesis was observed **(C, F)**, compared to **B, E)**. After withdrawing the compounds and replacing them at 50 hpf with embryo water, the ISVs started sprouting from the pre-existing vessels (arrows in **D, G** compare to **B, E)**.

antiangiogenic potential at the concentrations used. All inhibitors of angiogenesis were identified among the novel series with Ar¹ = 4-pyridine [12a–12o], (Fig. 3), with the most potent being [12f] and [12i] (Table 1). Substitution of the pyridine ring with either Ar¹ = 3-nitrophenyl [13a–13o] or Ar¹ = 4-nitrophenyl [14a–14o] groups resulted in not only inactive molecules but also in 10 out of 30 insoluble compounds.

The discovery of the novel and reversible angiogenesis inhibitors [12f] and [12i] supports our hypothesis that extension of the aniline amide functionality of ALL993 to include benzylic and acylhydrazide-based groups would produce equally potent inhibitors. In addition, our data indicate that the 3-trifluoromethylphenyl group is the optimal Ar² substituent, since 4-bromophenyl in [12c], 4-fluorophenyl in [12m], or 4-trifluoromethylphenyl in [12n] resulted in less potent inhibitors (5%–75% at 10 μM). On the other hand, bulkier Ar² substituents such as naphthalene, 3,4-dimethoxyphenyl, and 4-tert-butylphenyl groups do not exhibit any suppression of angiogenesis at the concentrations employed.

By employing a fast and robust zebrafish chemical screen in combination with a computational aided structure-based design, we have optimized the lead compound ALL993 to identify 6 novel reversible inhibitors of angiogenesis *in vivo*. Using *in vivo* phenotypic assays instead of *in vitro* biochemistry has provided more robust and relevant information on the activity of compounds, as well as in regard to their toxicity potential. The zebrafish phenotypic screen using the *Tg(kdrl:gfp)^{s843}* line incorporates a number of additional information in relation to an *in vitro* screening against a panel of kinases. More specifically, molecules that work on multiple targets (even on hitherto unidentified receptors) to exhibit inhibition of angiogenesis will be selected. However, any potential off-target effects and toxicity can be estimated during the development of zebrafish embryos *in vivo*, since the majority of signaling pathways are activated during the first 24 hpf.

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DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest to disclose.

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Abbreviations Used

ISV = intersegmental vessel

VEGF = vascular endothelial growth factor