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Abstract

Zebrafish, an amenable small teleost fish with a complex mammal-like circulatory system, is being increasingly employed for drug screening and toxicity studies. It combines the biological complexity of in vivo models with a much higher-throughput screening capability than those of other available animal models. Externally-growing transparent embryos, displaying well defined blood and lymphatic vessels, allow an inexpensive, rapid and automatable evaluation of drug candidates that are able to inhibit neovascularization. In this article, we briefly review zebrafish as a model for the screening of anti(lymph)angiogenic drugs, with emphasis on the advantages and limitations of the different zebrafish-based in vivo assays.

Keywords	angiogenesis; antiangiogenic drugs; lymphangiogenesis; antilymphangiogenic drugs; screening; zebrafish

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HIGHLIGHTS

- Zebrafish-based *in vivo* assays are being popularized for anti(lymph)angiogenic drug screening.
- These assays show advantages when compared to chick or mouse-based models.
- They contribute to speed up the screening and to reduce the number of sacrificed mice.
- They can provide relevant information regarding the mechanism of action and toxicity of the tested agents.
- Their use in the early phases of drug discovery may bias the mechanism of action of the hits identified, with a preferential selection of anti-VEGF agents.

Fishing anti(lymph)angiogenic drugs with zebrafish

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Keywords: angiogenesis; antiangiogenic drugs; lymphangiogenesis; antilymphangiogenic drugs; screening; zebrafish

Teaser: *In vivo* assays that use the *Tg(fli1a:EGFP)y1* transgenic zebrafish line are becoming increasingly popular to test anti(lymph)angiogenic drugs. Although their potential for drug discovery is unquestionable, their selection is biased.

Abstract

Zebrafish, an amenable small teleost fish with a complex mammal-like circulatory system, is being increasingly employed for drug screening and toxicity studies. It combines the biological complexity of *in vivo* models with a much higher-throughput screening capability than those of other available animal models. Externally-growing transparent embryos, displaying well defined blood and lymphatic vessels, allow an inexpensive, rapid and automatable evaluation of drug candidates that are able to inhibit neovascularization. In this article, we briefly review zebrafish as a model for the screening of anti(lymph)angiogenic drugs, with emphasis on the advantages and limitations of the different zebrafish-based *in vivo* assays.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is controlled by a sensitive interplay of stimulators and inhibitors. This tightly regulated process shows key roles in development and growth. In contrast, in adults it is only related to reproductive cycles, wound healing or bone repair. Nevertheless, a deregulated and persistent angiogenesis occurs in so-called angiogenesis-related diseases, such as proliferative retinopathies, psoriasis, rheumatoid arthritis, tumour growth or metastasis [1,2]. Angiogenesis is considered to be one of the hallmarks of cancer, where it plays a pivotal role in tumour progression and metastasis dissemination [3]. Therefore, targeting angiogenesis has attracted extensive attention in the field of pharmacological research in recent years. The search for new angiogenesis inhibitors is a hot topic, with hundreds of thousands of patients benefitting from their clinical use. Since bevacizumab, a humanized monoclonal antibody that blocks vascular endothelial growth factor A (VEGF), was approved for the treatment of metastatic colorectal cancer in 2004, a continuous increasing number of antiangiogenic therapies for cancer, virtually all of them blocking the activation of endothelial cells by VEGF, are gaining approval (see Table S1 in Supplementary material) [4]. Nevertheless, the limitations in the clinical success of anti-VEGF therapies, including intrinsic or acquired resistance appearance after months of treatment, indicates the need to search for new antiangiogenic drugs as well as new therapy strategies based on the combined targeting of different pathways in the tumour angiogenesis scenario [5].

Recently, the lymphatic system, which also plays a vital role in normal and pathological processes, has become a subject of great interest. In normal situations, the main functions of lymphatic vessels are: to collect the excess of protein-rich fluid that has been extravasated from blood vessels; to transport this fluid back into the blood circulation; and to absorb intestinal dietary fat and vitamins. The lymphatic system is also essential for the trafficking of immune cells and immune surveillance [6]. The formation of new lymphatic vessels, named lymphangiogenesis, is active during the embryonic development, but under adult physiological conditions this process is restricted to the endometrium during pregnancy. However, a defective or excessive lymphangiogenesis can lead to severe diseases involving lymph accumulation in tissues, dampened immune responses, connective tissue and fat accumulation, organ transplant rejection and cancer or metastatic dissemination [7,8].

Taking into account the role played by excessive angiogenesis and lymphangiogenesis in tumour growth and metastasis, as well as in other diseases, the identification of new drugs

that are able to inhibit these processes remains an urgent need. For this purpose, the development of new reliable and accurate *in vitro*, and especially, *in vivo* models, is demanded. Currently, different *in vitro*, *ex vivo* and *in vivo* systems are being applied to the screening and characterization of new lymph/angiomodulators. All these different models have their own advantages and disadvantages, their combination being mandatory to get insight on the impact of a given compound in the global process, therefore increasing the chances of success in preclinical and clinical development. In general, *in vitro* assays of angiogenesis (reviewed in [9]) offer information about the endothelial cell behaviour under drug exposure, but they do not consider the entire microenvironment. *Ex vivo* angiogenesis assays include the mouse or rat aortic ring, the lymphatic ring and the retinal explant assays, among others. These *ex vivo* assays are useful to analyse vessel sprouting from vascular explants, although they do not allow the study of the circulating endothelial progenitors recruited in the angiogenic process or the hemodynamic forces that play a relevant role in angiogenesis, vascular remodelling and maturation [10]. In contrast, *in vivo* models reproduce the cellular and molecular features involving the complete process of new vessel formation and the effect of modulators in the whole organism, giving a more complete overview about the putative effect of the studied drug, when comparing with *in vitro* assays. However, a combination of cell-based and organism-based chemical screens can complement each other, and eventually provide additional information. Traditionally, antiangiogenic compounds have been tested *in vivo* by means of either the chick chorioallantoic membrane (CAM) [11], or by several mouse models, including the Matrigel plug, sponge implant and disc assays, among others [12]. Moreover, in previous years zebrafish embryo has emerged as a promising *in vivo* model that can throw light on the biology of physiological and tumour angiogenesis at the whole organism level, allowing a cost-effective high throughput chemical screening. Interestingly, there is evidence revealing that drug targets are well conserved between zebrafish and humans. Therefore, lead compounds identified in zebrafish-based chemical screens are likely to have similar activities in humans [13]. In Table 1, the main strengths and weaknesses of the use of chick, mouse or zebrafish *in vivo* models to assay angiogenesis are listed. Among the strengths of zebrafish embryo-based *in vivo* assays are the simple manipulation, economy of the tested agents, which can be assayed at a known concentration, and the possibility to obtain relevant quantitative information in a short time. The absorption and bioavailability of a compound in these lower vertebrate animal models depend on its molecular weight, hydrophobicity, and number of hydrogen bond donors and acceptors [14], although solubilizing agents like dimethyl sulfoxide (DMSO) can be added to the screening

media to ensure solubility and drug penetration. Furthermore, zebrafish embryos are generally permeable to small molecules dissolved in the swimming medium, allowing drug administration by immersion. However, the drug concentration for waterborne treatment of zebrafish embryos has usually to be increased by an order of magnitude above the effective concentrations required for cell culture experiments. The use of zebrafish embryos facilitates the performance of the high number of experiments needed either for statistical calculations or for high throughput screening. Moreover, the availability of very diverse molecular tools and transgenic zebrafish lines may give clues for the mechanism of action and the therapeutic window of the drug candidates. Although the results obtained with zebrafish models would have to be finally confirmed in a mammalian system (usually, a murine-based one), the incorporation of zebrafish *in vivo* assays in the drug discovery way seems to be a logical option to speed up the screening process and to reduce the number of mice sacrificed. The advantages of zebrafish embryo models may explain the observed increased popularity of zebrafish-based angiogenesis assays. In the last fifteen years, an outstanding inflation of articles dealing with (lymph)angiogenesis can be found in scientific literature. Bibliometric search in Scopus or PubMed using combinations of terms, such as <<angiogenesis>> and each model species including humans, provide data to follow a rise and fall trend in the field. After a constant increase for more than ten years, an important recent decay in the relative number of published angiogenesis studies in all species, including humans, is indeed found, potentially due to the global economic crisis. During this period, zebrafish studies are unique as they show a stable maintenance in both general scientific production and that on angiogenesis in particular (see Figure S1 in Supplementary material). Further bibliometric analyses of this phenomenon support the view that development of new antiangiogenic drugs using transgenic zebrafish *in vivo* assays is at the heart of this statistical behaviour. In this review, we focus our attention on this effect trying to highlight underlying experimental reasons.

Zebrafish-based *in vivo* angiogenesis assays

Zebrafish have a closed circulatory system. The molecular processes underlying their vessel formation, the anatomic mechanisms for the developing vasculature and the process used to assemble vessels, are highly similar to those occurring in humans [15]. Importantly, early hematopoietic and endothelial cells in mammals and zebrafish express a common set of genes [16]. Early zebrafish embryonic vascular development begins at around 12 hour-post-

fertilization (hpf), when angioblasts originate in the lateral plate mesoderm. Later at around 24 hpf the development of dorsal aorta (DA) and dorsal vein (DV) forms the first circulation loop [17]. Once the primitive zebrafish vasculature is formed by vasculogenesis, most of the subsequent vessels, including the development of intersegmental vessels (ISV, blood vessels in between each two neighbouring myomeres) and sub-intestinal veins (SIV), occur by angiogenesis [18]. Thus, for the formation of the ISV a set of new sprouts emerges from the dorsal part of the DA and grows dorsally along vertical somite boundaries to interconnect and form the dorsal longitudinal anastomotic vessel (DLAV) [13]. On the other hand, SIV are originated from the posterior cardinal vein (PCV) and developed later than the ISV (between 48 and 72 hpf, Figure 1A) [19]. Nonetheless, it is worth mentioning that ECs in different vascular beds use different molecular cues and morphogenetic mechanisms to form the vessels. Detailed information on the complex regulatory signalling pathways involved in zebrafish angiogenesis is provided in Supplementary material.

The scientific community is paying increasing attention to several transgenic zebrafish lines as *in vivo* live fluorescent models for the study of angiogenesis modulation [20]. The ability to produce tissue-specific germ line transgenic fish expressing enhanced green fluorescent protein (EGFP) has made this organism an ideal system to visualize the formation of embryonic and adult structures. *Tg(fli1a:EGFP)y1* is a popular transgene in zebrafish that drives EGFP expression under endothelium-specific fli1a promoter. This promoter is specifically activated in endothelial cells along the complete zebrafish embryo, juvenile and adult (see Figure 1A-B). This specific expression leads to green *in vivo* fluorescence in all endothelial cells (see also Figure S2 in Supplementary material). This permits observation of bright blood and lymphatic vessels at all stages of embryogenesis (see Figure S2 B-E in Supplementary material). During late development and in adult specimens, EGFP is also observed in transparent organs, such as the fins (see Figure S2 F-G in Supplementary material). In embryos, ISV, SIV, the supra-intestinal artery (SIA), and the hyaloid vessels over the ocular lenses have been used to evaluate angiogenesis inhibition or promotion by chemical reagents added to water [21,22]. Thoracic duct formation (see Figure S2 D-E in Supplementary material) has also been used to study the effect of compounds over lymphangiogenesis during transgenic zebrafish embryogenesis [23]. This will be further discussed below in the section "Zebrafish as a tool for studying lymphangiogenesis". On the other hand, new vessels associated with the outgrowing blastema can also be observed during the regeneration of the fin induced after cutting. This regeneration process, that has been

classically studied to analyse organ morphogenesis [24], has also been used in biomedical [25,26] or toxicology studies [27] induced after fin clips in embryos [28], larvae [29] and adults (see Figure S2 F-G in Supplementary material). These studies have been successfully used to establish the importance of VEGFR in this regeneration process, allowing the detection of chemical agents that are able to inhibit regenerative angiogenesis [30].

Although many other alternative endothelial-driven transgenic lines have been produced, the most frequently used one is *Tg(fli1a:EGFP)y1*, accounting for more than 85% of total citations. Table S2 (Supplementary material) lists the most popular available endothelial-driven transgenic lines, with indication of their gene expression, the number of research articles citing them at ZFIN, suppliers and references.

Comparison of the different zebrafish-based assays for the screening of antiangiogenic drugs

As mentioned above, different approaches are being used in the screening of antiangiogenic compounds in zebrafish models. They include the intersegmental vessel (ISV) formation, the development of subintestinal vessels (SIV), the thoracic duct formation (TDF) assay, the adult caudal fin regeneration (ACFR) test, the hyaloid vessels (HV) formation assay, the blood flux videos, the central nervous system (CNS) vascular development assays, the tumour-xenograft-induced angiogenesis assays, the retinal angiogenesis assays in hypoxia-exposed adult zebrafish and the coronary angiogenesis assay upon cardiac amputation or cryoinjury in adults. A literature review of the published articles that used any of those models to test the activity of antiangiogenic drugs reveals that ISV is largely the most widely used zebrafish-based *in vivo* assay, followed by SIV and ACFR assays (see histogram in Figure 1C). Reasons for the prevalence of ISV use include the easy-handling, fastness and economy of the tested agent, since it is performed in small volumes [31]. The use of transparent embryos facilitates the visualization of the drug effect and allows an easy automatization, so that in a few days a number of embryos can be treated, yielding abounding results in a short time [20,32]. A high content screening assay [33] and a high throughput strategy [34] are good examples of this extensive attempt to establish an efficient drug pipeline using zebrafish.

Among the limitations of ISV assay, the possible false positive results arising from an effect on embryo development rather than on angiogenesis inhibition should be considered. This

limitation could be somehow superseded by complementing this assay with the information obtained by recording the blood flux in short videos (see Supplementary videos S1-S7 in Supplementary material). Sometimes, drugs do not inhibit vessel formation, but blood circulation through ISV is compromised or abolished. These effects on blood circulation are easily observed in the videos. The number of odd results can also be minimized by using the ACFR assay, from which clear conclusions are normally derived (see Figure S3 and Table S3 in Supplementary material). Furthermore, this assay provides a better approximation to the toxicity of tested reagents, with lower effective doses being needed [30]. A limitation of ACFR assay relies on the need of higher amounts of the tested agents, derived from the use of larger working volumes. This would be partially overridden by the use of young zebrafish, which could be assayed in smaller volumes. Moreover, the interruption of the fin regeneration by potential antiangiogenic drugs could be caused by a direct teratogenic effect of the reagent over osteoblasts [35]. Alternative wound healing assays are being proposed in embryos [36] and adults (MMB, manuscript in preparation) to discriminate between these potential effects of reagents. Table S4 in Supplementary material summarizes the advantages and disadvantages observed in the three different zebrafish angiogenesis assays herein discussed, namely, the ISV assay, the video visualization of blood flux through ISV and the ACFR assay.

A comment regarding the sensitive zebrafish embryo retina neovascularization assays deserves to be underscored. Inhibitors of angiogenesis are here to stay for the therapy of irreversible causes of blindness such as macular degeneration, macular edema and some retinopathies [37]. Although at present anti-VEGF therapies are the standard of care for some of these diseases, the results obtained are far from perfect, the development of new antiangiogenic drugs being an urgent need also in ophthalmology. Consequently, zebrafish embryos may be used as a novel and cost-effective tool for the screening of antiangiogenic drugs for the eye [38-40]. Despite the anatomical and developmental differences between ocular vasculatures in teleosts and humans, most of the pathologies related to a deregulated ocular angiogenesis share common molecular and cellular mechanisms [41]. Indeed, several models of vascular ocular disorders (retinopathies and macular degeneration, among others) have been developed using zebrafish embryos and is widely accepted that eye neovascularization in zebrafish embryos may represent a novel target for the identification of new angiogenesis inhibitors [38,42].

The table within Figure 2 shows the information regarding the antiangiogenic activity

exhibited by several inhibitors of angiogenesis when these zebrafish assays were applied (Figure S4 in Supplementary material and the accompanying reference list is a version of Figure 2 including the bibliographic references). They have been gathered from a bibliography survey as well as from some of our own results, also shown in Figure S3 (Supplementary information). Data of *in vivo* activity in chick or mouse-models have also been included, when available. Some interesting conclusions can be extracted from the contents of Table 2. Firstly, ISV and SIV assays yield similar results for all the tested agents, in terms of angiogenesis inhibition. This could facilitate the interlab comparison, since the choice of one or another assay may largely depend on the researcher's skills and preferences. Secondly, this model emerges as an excellent tool for the screening of VEGF/VEGFR inhibitors, easily detected by any of the zebrafish-based assays (with the exception of the human antibody bevacizumab, by a limitation of interspecies recognition of the zebrafish VEGF). Moreover, this homogeneity of results could help to decipher these drugs mode of action and to resolve an effective pipeline for an ever-increasing number of agents, some of them already reaching full approval or advanced clinical trials. Taking this into account, it has been suggested that comparison of the susceptibility of embryonic and adult zebrafish angiogenesis to a given VEGFR inhibitor could provide an indication of the drug selectivity [30]. Furthermore, comparison of the effect of compounds on angiogenesis and toxicity parameters in zebrafish embryos, in an efficacy-toxicity approach, may help to stratify antiangiogenic drug candidates based on their action mechanism, estimate their therapeutic window, and establish a prediction of their possible clinical outcome [43].

Data from Table within Figure 2 (Figure 2A) show a clear bias in the results obtained by using zebrafish-based assays, given that they do not appear to allow the detection of agents acting on some non-VEGF targets. It should be kept in mind that limitations of clinical anti-VEGF trials, including moderate benefits, or the appearance of toxicity or resistance mechanisms could be overcome by compounds targeting alternative angiogenic pathways, which could prolong the duration of anti-VEGF treatments and extend their clinical benefits [44]. This bias, perceived as a limitation of these zebrafish-based assays, has been observed in the case of erlotinib (EGFR inhibitor, approved by FDA for the treatment of non-small cell lung cancer), CAS 948557-43-5 (a Tie 2 kinase inhibitor), aeroplysinin-1 (a sponge derived compound that inhibits the angiogenic activation downstream the receptor), paclitaxel (a cytoskeletal drug that targets tubulin) and the endothelial proliferation inhibitors targeting methionine-aminopeptidase 2 (MetAP2) fumagillin and TNP470, among others. However, all

these compounds can be readily identified with chick or mouse-based assays. This bias is probably due to the preponderance of VEGF signalling in the control of zebrafish developmental and regenerative angiogenesis (Figure 2B) [45]. The case of FGF inhibitors deserves a special mention, since they seem to be detected by means of the ACFR, but not by the ISV or SIV assays (Figure 2B). This may be explained by the relevant role played by FGF in the regenerative angiogenesis after amputation (Figure 2B) [46]. Something similar occurs with genistein and LY294002, inhibitors of PI3K that can inhibit, at least partially, angiogenesis in regenerating fin or hyaloid angiogenesis, but not ISV (Figure 2B) [39,47]. This is in agreement with the importance of PI3K/AKT/mammalian target of rapamycin (mTOR) signalling pathway in ocular neovascularization and fin regeneration [47,48]. All these commented data show that like Janus, the ancient Roman god of duality, the zebrafish-based *in vivo* assays have two faces: one looking towards an effective, fast and easy tool for the selection of new antiangiogenic drugs, and the other looking to those other missed compound that could help to increase the clinical efficacy of the anti-VEGF therapies. In other words, although zebrafish is an attractive tool for the drug discovery, it has some limitations related with the detection of other non-VEGF targeted compounds, which could help to overcome the problems of toxicity or resistance mechanisms already seen with anti-VEGF treatments [44].

Zebrafish for the screening of antilymphangiogenic drugs

The role of the lymphatic vessels in different human pathological conditions has already been well documented and recently reviewed [49]. This will be further discussed below in the section "The role of lymphatic vessels in human diseases" (Supplementary material). In the past, the understanding of biological and functional aspects of the human lymphatic system in health and disease has been overshadowed by the enormous emphasis and progress obtained on angiogenesis. This fact has been partly due to the difficulties found in the identification of these translucent vessels in tissues, mainly because of the lack of well-defined lineage-specific markers. Recently this scenario has rapidly changed with the discovery of useful transcription factors and proteins expressed by lymphatic endothelial cells (LECs), such as Prox-1, podoplanin, LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) and VEGFR-3 [50]. Furthermore, advances in imaging technologies together with the new molecular tools and genetics models have contributed to accelerate the pace of lymphatic

system research.

Zebrafish embryo is becoming an emerging and fascinating model to facilitate the progress on the lymphangiogenesis field and to provide a powerful tool for the screening of lymphangiogenic antagonists/agonists [51]. Zebrafish embryo lymphatic vascular system shares molecular, morphological and functional characteristics with the lymphatic vessels present in higher vertebrates [15]. Lymphatic development starts after the establishment of a functional cardiovascular system, when LEC progenitors appear to originate from the cardinal vein (2 dpf) [52]. At this stage, jugular lymphatic sacs originate rostral lymphatic vessels and sprouts forming the parachordal vessels emerge from the PCV. Then, the arterial intersegmental vessels serve as guidance routes for the dorsal and ventral navigation of lymphatic progenitors, which form the rest of the trunk lymphatic network, including the dorsal longitudinal lymphatic vessel, the intersegmental lymphatic vessels and the thoracic duct. Around 3.5-4 dpf, the formation of the thoracic duct takes place by lymphangiogenesis when ventrally-migrating LECs branch rostrally and caudally between the DA and PCV, and finally this duct expands and forms lymph sacs at later stages [53]. Detailed information on the complex regulatory signalling pathways involved in zebrafish lymphangiogenesis is provided in Supplementary material.

One of the advantages offered by zebrafish embryo as a model to study lymphangiogenesis is the possibility of applying a modified version of the lymphangiography, the traditional method used in humans to visualize the lymphatic vasculature. It is worth mentioning that in this technique, fluorescent spheres conjugated with high molecular weight particles are injected subcutaneously into the tail region or directly into the thoracic duct, allowing the rapid detection of structural aberrations in the lymphatic vessels upon drug treatment. Nonetheless, these methods are considered too complex to be included in a drug screening platform and currently, the most promising strategy is the use of transgenic zebrafish. Indeed, as mentioned in the introduction, several zebrafish transgenic lines that express fluorescent reporters under vascular specific promoters have been instrumental in making zebrafish a favourite model for *in vivo* screening and functional analysis of the vascular system. In addition, there are available transgenic lines with specific expression in lymphatic vessels that facilitate the visualization of the lymphatic system for scientists [54]. See the Supplementary material section "Transgenic zebrafish lines available to study lymphangiogenesis" for additional details on this issue.

The use of zebrafish embryo models for studying lymphangiogenesis primarily focuses on the thoracic duct development, which can be quantified by counting the number of somites with thoracic duct or parachordal lymphangioblasts. Results can be represented as the developed or inhibited thoracic duct length. Another approach (used with transgenic lines that express nuclear fluorophores under the fli1a promoter) is the direct counting of LECs within the zebrafish thoracic duct. Although the thoracic duct is regarded as the common site, additional lymphatic vessels, including a complex network of blind-ended lymphatic capillaries, can be identified in the zebrafish trunk using lymphangiography [53].

Zebrafish embryo phenotype-based chemical screening has been used to identify the antilymphangiogenic properties of different drugs previously approved for human use [55]. In this chemical battery, kaempferol, cinnarizine, flunarizine and leflunomide have shown anti-lymphatic activity by inhibiting the thoracic duct formation in zebrafish [55]. They confirmed their data in a murine *in vivo* lymphangiogenesis Matrigel plug assay, in which kaempferol, leflunomide and flunarizine prevented lymphatic growth. On the other hand, zebrafish exposed to MAZ51 (VEGFR-3 inhibitor) have shown a significant reduction in the number of lymphatic capillaries [56], and rapamycin (antiproliferative compound) has suppressed the thoracic duct development in zebrafish [57]. Other experiments have revealed that CI-1040 (MAPK inhibitor), BEZ235 (dual PI3K and TOR inhibitor) and PTK787/ZK222584 (VEGFR-1,-2,-3 inhibitor) impaired thoracic duct formation in zebrafish [58]. Interestingly, our research using the zebrafish as a tool for lymphangiogenesis analysis has shown that bathing exposure with toluquinol and AD0157, two marine-derived compounds, reduces the thoracic duct formation in zebrafish embryos. These compounds have shown anti-lymphangiogenic properties in a wide experimental battery of *in vitro*, *ex vivo* and *in vivo* models [23,59]. Figure 3 shows the use of the zebrafish thoracic duct lymphangiogenesis assay to identify toluquinol as an antilymphangiogenic compound.

Nonetheless, as can be noticed in this review, the use of zebrafish embryo as a model to search for new lymphangiogenesis inhibitors is still far from its use in the antiangiogenic drugs screening.

In spite of the potential of the zebrafish model as an attractive approach for the identification of anti(lymph)angiogenic drugs, this model has a number of limitations, recently reviewed [60]. Therefore, the combination of different *in vivo* models in order to get insight into the properties and action mechanisms of new potential anti(lymph)angiogenic drugs is highly recommended. In this context, mouse and zebrafish embryo can be considered as

complementary models for the study of lymphangiogenesis and lymphatic vessel-related diseases [53].

Concluding remarks

In general, the efficacy of specific chemicals in *in vitro* assays is far away from optimal when compared to their clinical use. This type of bias might be related to either the potentiality of treatment (*in vivo* assays are far more complex than *in vitro* ones), or particularities in the effective concentration in the final preclinical assays. In this cell transition between cell culture and higher vertebrate *in vivo* assays, zebrafish-based assays appear to be potentially useful, given their advantages as compared to chick and mouse-based models. Data available in bibliography, including our own research, show that zebrafish provides effective experimental models not only for anti(lymph)angiogenic drug screening but also for drug pharmacological characterization and optimization. Zebrafish angiograms, both in embryos and adults, are easy-handling assay procedures that may permit scientist to analyse hundreds of compounds in a short time, which is essential for a high throughput screening. Moreover, besides the different effects of those compounds on (lymph)angiogenesis, the zebrafish embryo assays allow the identification of side effects, including toxicity, developmental delay, tissue malformations or brain haemorrhage, and provide a valuable prediction of the therapeutic window of the new drug candidates. The combination of the information derived from the *in vitro*, *ex vivo* and *in vivo* assays will help to better evaluate the pharmacological potential of the new anti(lymph)angiogenic agents, therefore increasing the chances to succeed in preclinical and clinical trials.

A final reflection on drawbacks in zebrafish assays in the early steps of drug discovery should be added. When "fishing" new drug candidates by means of blind screening strategies, guided by activity assays, the size of the mesh will determine the size of the fish caught in the net. Concern that zebrafish-based *in vivo* assays could induce a bias in the action mechanism of the hits selected, probably discarding compounds that could inhibit angiogenesis by acting on non-VEGF relevant targets, should be kept in mind. In any case, as stated in the old Irish saying 'May the holes in your net be no larger than the fish in it', evidence that zebrafish is an excellent system to easily identify new anti-VEGF agents and the fact that virtually all the approved antiangiogenic therapies target VEGF, ensure the zebrafish-based *in vivo* assay application in several steps of the drug discovery process.

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Figures S2 A and C were obtained in Marie Andrée Akimenko´s lab in Ottawa, Canada.

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Author contributions

M.G.C. carried out experiments to illustrate the performance of the assays, collaborated in updating bibliography and writing the manuscript. A.R.Q. oversaw the experiments and contributed to the final version of the manuscript. M.A.M. oversaw the experiments and contributed to the final version of the manuscript. M.M.B. optimized the maintenance and manipulation of fishes and embryos, oversaw all the experimental procedures included as supplementary material, contributed studying bibliography and writing the final version of the manuscript.

Disclosure Statement

The authors state that no competing financial interests exist.

References

1. Rodríguez-Caso, L. *et al.* (2012) What is known of angiogenesis-related rare diseases? A systematic review of literature. *J. Cell Mol. Med.* 16, 2872-2893
2. Carmeliet, P. (2005) Angiogenesis in life, disease and medicine. *Nature* 438, 932-936
3. Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646-674
4. Ramijawan, R.R. *et al.* (2017) Anti-angiogenesis for cancer revisited: is there a role for combinations with immunotherapy? *Angiogenesis* 20, 185-204
5. Quesada, A.R. *et al*. (2007) Playing only one instrument may be not enough: limitations and future of the antiangiogenic treatment of cancer. *BioEssays* 29, 1159-1168.
6. Schulte-Merker, S., Sabine, A. and Petrova, T.V. (2011) Lymphatic vascular morphogenesis in development, physiology, and disease. *J. Cell Biol.* 193, 607-618.
7. Alitalo, K. (2011) The lymphatic vasculature in disease. *Nat. Med.* 17, 1371-1380.
8. Karaman, S. and Detmar, M. (2014) Mechanisms of lymphatic metastasis. *J. Clin. Invest.* 124, 922-928.
9. Sanz-Nogué, C. and O'Brien, T. (2016) In vitro models for assessing therapeutic angiogenesis. *Drug Discov. Today* 21, 1495-1503
10. Chávez, M.N. *et al*. (2016) Zebrafish as an Emerging Model Organism to Study Angiogenesis in Development and Regeneration. *Front. Physiol.* 7, 56
11. Ribatti, D. (2016) The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model. *Mech. Dev.* 141, 70-77
12. Eklund, L. *et al*. (2013) Mouse models for studying angiogenesis and lymphangiogenesis in cancer. *Mol. Oncol.* 7, 259-282
13. Wheeler, G.N. and Brändli, A.W. (2016) Simple vertebrate models for chemical genetics and drug discovery screens: lessons from zebrafish and Xenopus. *Dev Dyn.* 238, 1287-1308

14. Lipinski, C.A. *et al.* (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 1, 3-26

15. Isogai, S., Horiguchi, M. and Weinstein, B.M. (2001) The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol*. 2, 278-301

16. Abrams, E.W. and Mullins, M.C. (2009) Early zebrafish development: it's in the maternal genes. *Curr. Opin. Genet. Dev*., 4, 396

17. Gore, A.V. *et al.* (2012) Vascular development in the zebrafish. *Cold Spring Harb Perspect Med*. 2:a006684

18. Schuermann, A., Helker, C.S. and Herzog, W. (2014) Angiogenesis in zebrafish. *Semin Cell Dev Biol*. 31, 106-114

19. Koenig, A.L. *et al.* (2016) Vegfa signaling promotes zebrafish intestinal vasculature development through endothelial cell migration from the posterior cardinal vein. *Dev Biol* 411, 115-127

20. Tran, T.C. *et al.* (2007) Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res* 67, 11386-11392

21. Sulaiman, R.S. *et al.* (2016) A novel small molecule ameliorates ocular neovascularisation and synergises with anti-VEGF therapy. *Sci. Rep.* 6:25509

22. Reynolds, A.L., *et al.* (2016) Phenotype-Based Discovery of 2-[(E)-2-(Quinolin-2-yl)vinyl]phenol as a Novel Regulator of Ocular Angiogenesis. *J. Biol. Chem*. 291, 7242-7255

23. García-Caballero, M., *et al.* (2016) Novel application assigned to toluquinol: inhibition of lymphangiogenesis by interfering with VEGF-C/VEGFR-3 signalling pathway. *Br. J. Pharmacol*. 173, 1966-1987

24. Marí-Beffa, M. and Murciano, C. (2010) Dermoskeleton morphogenesis in zebrafish fins. *Dev Dyn* 239, 2779-2794

25. Marí-Beffa, M. *et al.* (2007) Zebrafish fins as a model system for skeletal human studies. *Scientific World Journal* 7, 1114-1127

26. Oppedal, D. and Goldsmith, M.I. (2010) A chemical screen to identify novel inhibitors of fin regeneration in zebrafish. *Zebrafish* 7, 53-60

27. Mathew, L.K., Andreasen, E.A. and Tanguay, R.L. (2006) Aryl hydrocarbon receptor activation inhibits regenerative growth. *Mol Pharmacol* 69, 257-265

28. Macdonald, N.P. *et al.* (2015) Assessment of biocompatibility of 3D printed photopolymers using zebrafish embryo toxicity assays. *Lab Chip* 16, 291-297

29. Mariappan, D., *et al.* (2009) Somitovasculin, a Novel Endothelial-Specific Transcript Involved in the Vasculature Development. Arterioscler. *Thromb. Vasc. Biol.* 29, 1823-1829

30. Bayliss, P.E. *et al.* (2006) Chemical modulation of receptor signaling inhibits regenerative angiogenesis in adult zebrafish. *Nat Chem Biol* 2, 265-273

31. Tobia, C. *et al.* (2015) Zebrafish embryo intersegmental vessels: a tool for investigating sprouting angiogenesis. *Methods Mol Biol*. 1214, 173-184

32. Spaink, H.P., *et al.* (2013) Robotic injection of zebrafish embryos for high-throughput screening in disease models. *Methods* 62, 246-254

33. Leet, J.K. *et al.* (2014) High-Content Screening in Zebrafish Embryos Identifies Butafenacil as a Potent Inducer of Anemia. *PLoS One* 9:e104190

34. Letamendia, A., *et al.* (2012) Development and validation of an automated high-throughput system for zebrafish in vivo screenings. *PLoS One* 7:e36690

35. Pruvot, B.*, et al.* (2014) Developmental defects in zebrafish for classification of EGF pathway inhibitors. *Toxicol. Appl. Pharmacol*. 274, 339-349

36. Cheng, J., *et al.* (2011) Nanotherapeutics in angiogenesis: synthesis and in vivo assessment of drug efficacy and biocompatibility in zebrafish embryos. *Int. J. Nanomedicine* 6, 2007-2021

37. Ferrara, N. and Adamis, A. P. (2016) Ten years of anti-vascular endothelial growth factor therapy. *Nat. Rev. Drug Discov*. 15, 385-403

38. Kitambi, S.S. *et al*. (2009) Small molecule screen for compounds that affect vascular development in the zebrafish retina. *Mech Dev.* 126, 464-477

39. Rezzola, S. *et al.* (2014) In vitro and ex vivo retina angiogenesis assays. *Angiogenesis* 17, 429-442

40. Rezzola, S. *et al.* (2016) Zebrafish (Danio rerio) embryo as a platform for the identification of novel angiogenesis inhibitors of retinal vascular diseases. *Biochim Biophys Acta* 1862, 1291-1296

41. Alvarez, Y. *et al.* (2007) Genetic determinants of hyaloid and retinal vasculature in zebrafish. *BMC Dev Biol.* 7, 114

42. Cao, R. *et al.* (2008) Hypoxia-induced retinal angiogenesis in zebrafish as a model to study retinopathy. *PLoS One.* 3:e2748

43. Chimote, G. *et al.* (2014) Comparison of effects of anti-angiogenic agents in the zebrafish efficacy-toxicity model for translational anti-angiogenic drug discovery. *Drug Des Devel Ther*. 8, 1107-1123

44. Quesada, A.R. *et al.* (2010) Do not say ever never more: the ins and outs of antiangiogenic therapies. *Current Pharmaceutical Design*. 16, 3932-3957

45. Betz, C. *et al.* (2016) Cell behaviors and dynamics during angiogenesis. *Development* 143, 2249-2260

46. Shibata, E. *et al.* (2016) Fgf signalling controls diverse aspects of fin regeneration. *Development* 143, 2920-2929

47. Sasore, T. and Kennedy, B. (2014) Deciphering combinations of PI3K/AKT/mTOR pathway drugs augmenting anti-angiogenic efficacy in vivo. *PLoS One* 9:e105280

48. Rojas-Muñoz, A. *et al.* (2009) ErbB2 and ErbB3 regulate amputation-induced proliferation and migration during vertebrate regeneration. *Dev Biol*. 327, 177-190

49. Venero Galanternik, M. *et al.* (2016) Building the drains: the lymphatic vasculature in health and disease. *Wiley Interdiscip Rev Dev Biol.* 5, 689-710

50. Zheng, W., Aspelund, A. and Alitalo, K. (2014) Lymphangiogenic factors, mechanisms, and applications. *J Clin Invest.* 124, 878-887

51. Okuda, K.S. *et al.* (2012) lyve1 expression reveals novel lymphatic vessels and new mechanisms for lymphatic vessel development in zebrafish. *Development* 139, 2381-2391

52. Tammela, T. and Alitalo, K. (2010) Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* 140, 460-746

53. Kim, J.D. and Jin, S.W. (2014) A tale of two models: mouse and zebrafish as complementary models for lymphatic studies. *Mol Cells*. 37, 503-510

54. Wilkinson, R.N. and van Eeden, F.J. (2014) The zebrafish as a model of vascular development and disease. *Prog Mol Biol Transl Sci*. 124, 93-122.

55. Astin, J.W. *et al.* (2014) An in vivo antilymphatic screen in zebrafish identifies novel inhibitors of mammalian lymphangiogenesis and lymphatic-mediated metastasis. *Mol Cancer Ther.* 13, 2450-2462

56. Hoffman, S.J. *et al.* (2012) An in vivo method to quantify lymphangiogenesis in zebrafish. *PLoS One* 7:e45240

57. Flores, M.V. *et al.* (2010) Visualization of embryonic lymphangiogenesis advances the use of the zebrafish model for research in cancer and lymphatic pathologies. *Dev Dyn.* 239, 2128-2135

58. Fevurly, R.D. *et al.* (2012) Novel zebrafish model reveals a critical role for MAPK in lymphangiogenesis. *J Pediatr Surg.* 47, 177-182

59. García-Caballero, M. *et al.* (2017) Targeting VEGFR-3/-2 signaling pathways with AD0157: a potential strategy against tumor-associated lymphangiogenesis and lymphatic metastases. *J Hematol Oncol.* 10, 122.

60. Okuda, K.S. *et al.* (2016). Utilizing Zebrafish to Identify Anti-(Lymph)Angiogenic Compounds for Cancer Treatment: Promise and Future Challenges. *Microcirculation* 23, 389-405

Additional contents

This short review is accompanied by:

*Table 1. Strengths and weaknesses of the major model species used to evaluate antiangiogenic drugs *in vivo.*

*Three figures.

*Supplementary material containing three text sections, 4 supplementary tables, 4 supplementary figures, additional bibliography and seven short videos.

*Highlights

Figure legends

Figure 1. *Tg(fli1a:EGFP)y1*/AB zebrafish *in vivo* assays to screen (lymph)angiogenic modulators.

(A) Scheme of a 48 hpf embryo showing *in vivo* assays. Green lines are transgenic endothelial cells. Red rectangle shows the region observed in the right fluorescence figure. Vertical discontinuous line is caudal fin cut section. Bar is 1 mm. CNV, TX, PAC, SIV, DA, DLAV, ISV and PCV are central nervous system, tumour xenograft assays, parachordal vessels, subintestinal vessels, dorsal aorta, dorsal longitudinal anastomosis vessels, intersegmental vessels and posterior cardinal vein, respectively. (**B)** Scheme of an adult zebrafish with regenerating caudal fin. Grey arrow shows fluorescence image of regenerating vessels in the caudal fin blastema (right of fin cut white discontinuous line). RV and CV represent retinal and coronary vessels (**C)** Histogram showing number of reviewed articles using zebrafish *in vivo* assays described in the main text. A complete list of the bibliographic references used to build the histogram is provided in Supplementary material.

Figure 2. Comparison of the activity of angiogenesis inhibitors demonstrated by using different *in vivo* assays.

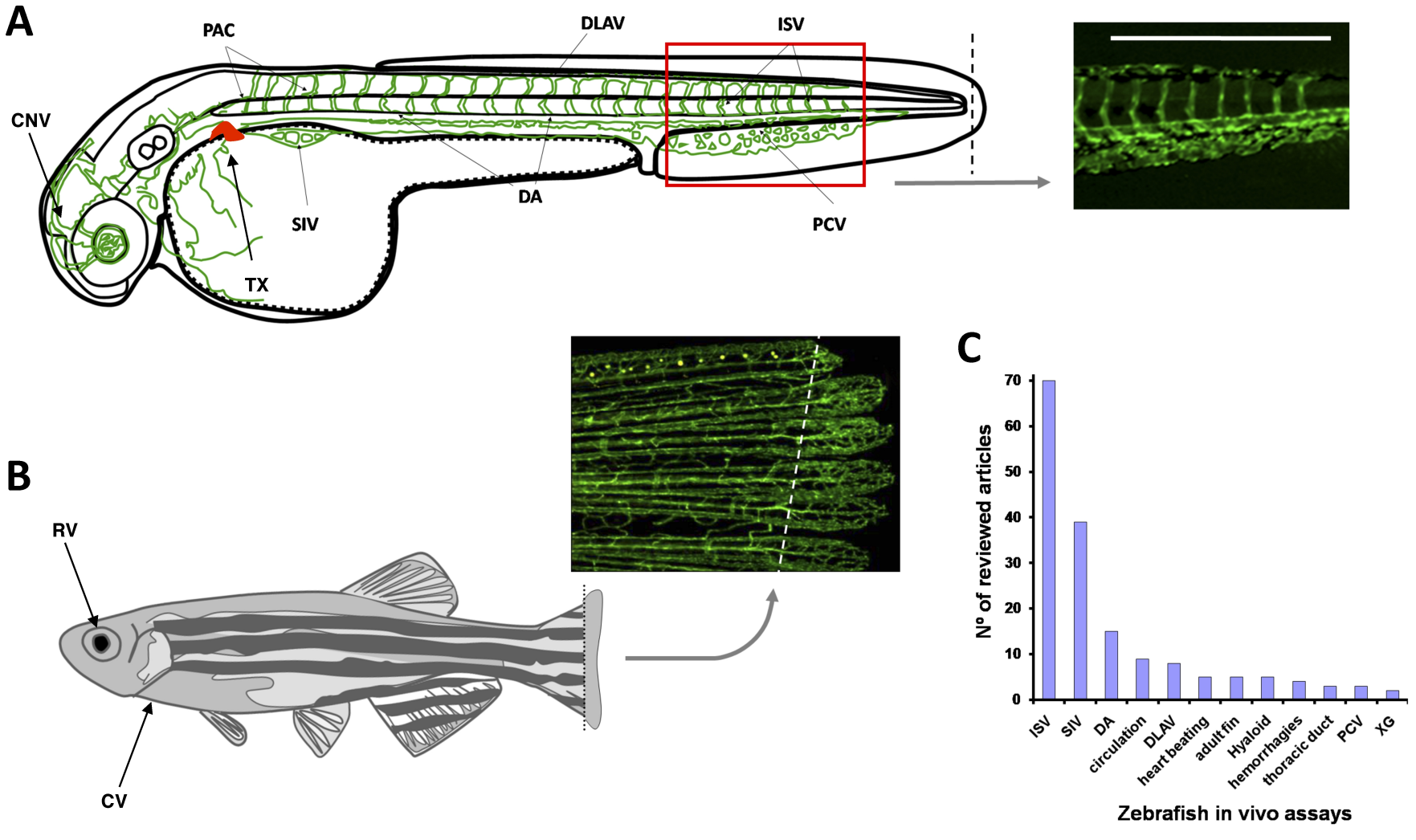
(A) Table showing the information regarding the antiangiogenic activity exhibited by several inhibitors of angiogenesis when these *in vivo* assays were applied (A version of this table including the bibliographic references is available in Supplementary material). They have been gathered from a bibliography survey as well as from some of our own results, also shown in Figure S3 (Supplementary information). Colour code: brown: negative; pale brown: very moderate; green: positive; pale green: partial inhibition. **(B)** Scheme summarizing the results presented in the upper table. A free arrow indicates that compounds acting on a given target show a positive inhibition in a particular zebrafish-based *in vivo* assay. A crossed arrow indicates that no antiangiogenic activity is detected by means of that assay.

Figure 3. Toluquinol abolishes thoracic duct development in the lymphangiogenesis zebrafish assay.

Transgenic *Tg(fli1:eGFP)y1* zebrafish embryos were incubated in zebrafish water with the indicated concentrations of the tested compound at 28.5 °C for 4 days and then, thoracic duct length was analysed in the anesthetized embryos. **(A)** Representatives

pictures of untreated and treated zebrafish. White arrowheads indicate the thoracic duct in zebrafish and the red square includes the area at higher magnification (bar=100 μm and 70 μm on higher magnification). **(B)** Quantification of the defective thoracic duct formation at 5 dpf determined by the percentages of embryos with severe (no vessels), drastic (5-25% of TD), moderate (25-90% of TD) and no lymphatic defects (100% of TD). A total of 50 embryos were analysed in each experimental condition.

Figure 1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hemorrhagies
thoracic duct
PCV
XG
Zebrafish in vivo assays

Figure 2

A

Compound	Target	ISV	SIV	Fin regeneration	Mouse	Chick
Bevacizumab	VEGF					
Sorafenib	VEGFR-2, PDGFRβ					
Sunitinib	VEGFR-1-2, PDGFRσ/β					
Pazopanib	VEGFR-1-3, PDGFRβ, FGFR-1-2					
SU5416 (semaxinib)	VEGFR2, PDGFR, Flt-1, Flt-4, c-kit					
PTK787 (vatalanib)	VEGFR-2, VEGFR-1, PDGF, Flt-4, and c-Kit					
SU4312	VEGFR2, PDGFR					
DMF	VEGFR2 and HIF1a expression, AKT, Erk					
Toluquinol	VEGFR3, AKT					
AD0157	VEGFR2-3 and downstream mediators					
Damnacanthal	VEGFR1-3, FGFR1, 2 and 4,c-Met and EGFR.					
Kahweol	VEGFR2?					
QODG (Quercetin derivative)	VEGFR2					
2-Methoxyestradiol	Apoptosis, HIF1a					
Genistein	PI3K/AKT/HIF−1α/VEGF and NF−κB/COX−2					
LY294002	PI3K					
SU5402	FGFR2					
Suramin	FGF					
PD173074	FGFR1					
CAS 948557-43-5	Tie2 Kinase Inhibitor					
Erlotinib	EGFR					
TNP470	MetAP2 (Endothelial proliferation)					
Fumagillin	MetAP2 (Endothelial proliferation)					
Paclitaxel	destabilization of microtubules					
Aeroplysinin-1	Angiogenic growth factor signaling, AKT					

B

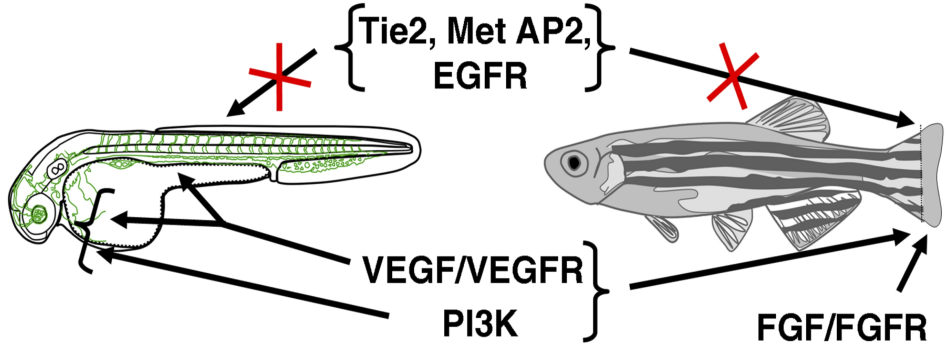
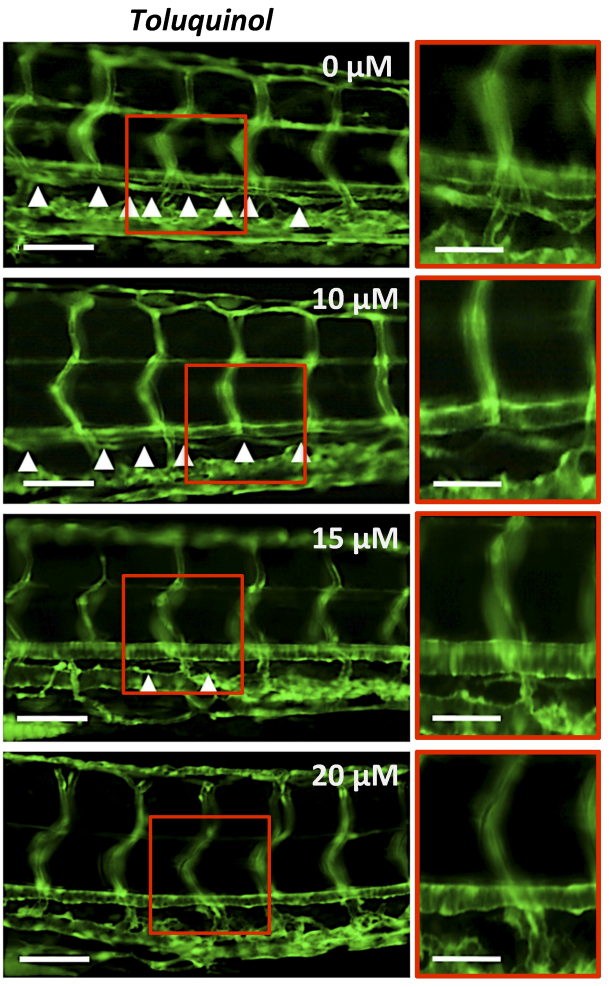


Figure 3

A



B

Phenotype	*DMSO*	*10 µM Toluquinol*	*15 µM Toluquinol*	*20 µM Toluquinol*
No vessels	4% (2 embryos)	4% (2 embryos)	6% (3 embryos)	20% (10 embryos)
5-25 % of TD	6% (3 embryos)	10 % (5 embryos)	20% (10 embryos)	22% (11 embryos)
25-90 % of TD	8% (4 embryos)	16% (8 embryos)	18% (9 embryos)	24% (12 embryos)
100 % of TD	82% (41 embryos)	70% (35 embryos)	56% (28 embryos)	34% (17 embryos)

Table 1. Strengths and weaknesses of the major model species used to evaluate antiangiogenic drugs *in vivo*

Model species *(Typical assays)*	**Strengths**	**Weaknesses**
 **Chick** *(CAM)*	• Inexpensive • Suitable for medium-scale screening • Simple manipulation • Low to moderate amounts of test agents are required	• Non-mammalian: results must be validated in mammalian systems for potential clinical application • Embryonic • Difficult to be evaluated (the use of at least two blind evaluators is advisable) • Nonspecific inflammatory reaction may appear • Unavailable tools to characterize molecular mechanism • Actual concentrations of the test compounds depend on the diffusion from the disc
 **Mouse** *(Matrigel plug, sponge, corneal micropocket, disc assay…)*	• Mammalian • Tools to characterize molecular mechanism are available • Some of them permit long-term monitoring • Quantitative assays	• Expensive • Time consuming • Ethically questionable in occasions • Technically demanding • Higher amounts of test agents are required • Non suitable for primary assay in medium to large-scale screening
 **Zebrafish** *(ISV, SIV, caudal fin regeneration…)*	• Tools to characterize molecular mechanism are available • Quantitative • Fast • Suitable for high throughput screening • Automated in 96 well plates • Simple manipulation • Many transgenic zebrafish lines are available • Small amounts of test agents are required • Statistically significant numbers of embryos can be used for each assay • Real concentrations of the test compounds are known • Yields useful information regarding pharmacological profile and toxicity of the test agents (therapeutic windows)	• Non-mammalian: results must be validated in mammalian systems for potential clinical application • Embryonic (mostly) • The small size of embryos can make some observation challenging • Specialized breeding conditions are required • Caudal fin amputation studies require higher working volumes and therefore higher amounts of test agents

Supplementary material for

Fishing anti(lymph)angiogenic drugs with zebrafish

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Contents

Angiogenesis and lymphangiogenesis signalling and regulation in zebrafish

Angiogenesis signalling

Early expression of stem cell leukemia (scl) and fetal liver kinase-1/vascular endothelial growth factor receptor 2 (flk1/vegfr2) is required for endothelial and hematopoietic lineage formation in both mouse and zebrafish [1,2]. It has also been reported that ETS factors function synergistically with other factors, including members of the Forkhead (FOX) family of transcription factors [3] and KLF genes [3,4] to specify endothelial cell fate.

Ephrin B2 is expressed in arterial endothelial cells although absent in veins, and its receptor EphB4, is mainly expressed in venous endothelial cells [5]. Likewise, *sonic hedgehog (shh)* induces the expression of *vegf*, and the VEGF-VEGFR2/Nrp1 pathway activates Notch signalling (Notch, Delta, Jagged, etc.) during arterial specification [6,7]. Phospholipase C (PLC)-γ1, an immediate downstream component of the VEGF receptors, is required for transducing VEGF signalling [8,9], while COUP-TFII is required for venous differentiation [10]. Interestingly, selective cell segregation is controlled by different signalling pathways, including VEGF-A and VEGF-C [5]. Moreover, the phosphoinositide 3-kinase (PI3K) and the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling pathways, antagonistically regulate arterial-venous fate determination during zebrafish development [11-13], and Dep1 acts upstream of PI3K in the arterial differentiation [14]. In addition, lack of Sox7/18 in zebrafish results in loss of arteries, showing that are important actors in the arterial-venous morphogenesis [15-17].

Several studies have shown that VEGF-A is produced by the somites among which the ISV sprouts migrate, and VEGF-A/VEGFR-2, as well as Notch and Semaphorin/PlexinD1 signalling pathways, promote the growth of these ISV [18-20]. The inhibition of ISV formation upon VEGFR-2 function loss confirms its critical role in this process [19]. Of note, the VEGFR-1 receptor also plays a crucial role during angiogenesis in zebrafish [21]. Specification of tip and stalk cells in these sprouts is controlled by the mentioned signalling cascades [22]. On the other hand, SIV are originated from the PCV and developed later than the ISV (around 30 hpf) [23]. Nonetheless, it is worth mentioning that ECs in different vascular beds use different molecular cues and morphogenetic mechanisms to form the vessels. For instance, the BMP signalling pathway is the major player in the initial stage of the subintestinal

plexus formation, although Notch, VEGF and Semaphorin/PlexinD1 signallings are required for proper formation of functional arterial SIVs. Flt1 participates as well in the remodeling of the subintestinal plexus, involving retraction of venous leading buds [24]. In conclusion, angiogenic factors mediating ISV development such as PDGFs and SHH are not required for SIV development, whereas BMP are required for SIV but not for ISV.

Interestingly, the *Msx* and *BMP* genes are activated in the zebrafish caudal fin regeneration [25]. Tail fin vessel regeneration is sensitive to VEGFR inhibition and several molecular markers have been identified in this process, including the transcription factor *msxb*, the fibroblast growth factor receptor 1, FGFR-1 as well as the SHH [26].

Although the aforementioned molecules and signalling cascades are the most relevant in the angiogenic process in zebrafish, others such as survivin and Nogo-B have been recently described as angiogenesis regulators [27-30].

Lymphangiogenesis signalling

Prox1 is a master regulator in the lymphatic specification from a subpopulation of zebrafish PCV endothelial cells, and VEGFC-VEGFR3 signalling is also likely to induce budding and migration of the Prox1-postive secondary sprouting cells in zebrafish [31]. However, it is also possible a contribution to lymphatic progenitors from mesenchyme [32]. In the embryonic zebrafish, LECs express Neuropilin-2 [33] and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) [34], and in the adult stage, angiopoietin 2 is expressed in those lymphatic vessels as well [35]. Up to now several signalling pathway has been described to modulate the lymphatic development [36], although VEGF-C appears to be the main chemoattractant cue for migrating LECs [37] through the VEGFR-3 cascade activation. Other signalling cascades, such as G Protein Coupled Receptor and BMP/TGFβ signalling, and Notch pathways are known to modulate distinct aspects of lymphatic development [38]. Furthermore, VEGF-D, cooperatively with VEGF-C, Alk3/Alk3b, Smad5 and Apelin regulates lymphangiogenesis during zebrafish embryonic development [39-41].

The role of lymphatic vessels in human diseases

Lymphedema, characterized by the presence of localized fluid retention and excessive tissue swelling caused by a compromised/damaged lymphatic system unable to return interstitial fluid to the thoracic duct and bloodstream, affects 100 million people around the world [42]. On the other hand, human lymphatic syndromes, such as Milroy disease (a congenital form of lymphedema), lymphedemadistichiasis syndrome (LDS) (lymphedema onset at adolescence) and Hennekam syndrome (a generalized lymphatic dysplasia) are caused by mutations in crucial players of the lymphatic vasculature (VEGFR-3 *FOXC2* and *CCBE1,* respectively) [43-45]. Crohn's disease and ulcerative colitis, the two most commonly occurring inflammatory bowel diseases (IBDs) are developed when intestinal lymphatics are obstructed due to the lymphoid aggregates formation [46]. Moreover, the lymphatic vasculature may have a potential role in guarding cardiovascular health by facilitating the proper excretion of cholesterol from the extravascular tissues and avoiding atherosclerosis [47].

Metastatic dissemination of tumor cells can occur via lymphatics, especially in breast, colon, prostate and melanoma cancer [48-49]. Importantly, the presence of metastatic cells in lymph nodes indicates bad prognosis for patients since tumors cells from primary solid tumors are gaining access to the lymphatic vessels, invading lymph nodes and they can spread to distant organs [50,51].

Considering the increasing number of human lymphangiogenesis-related disorders is not surprising that many investigations are focusing on this topic. Numerous efforts are being made to find effective therapeutic strategies based on the identification and characterization of new and promising antilymphangiogenic drugs [49,52]. Additionally, the screening and discovery of potent lymphangiogenesis modulators is regarded as a new strategy to overcome the development of drug resistence frequently seen in the treatment with antitumor-associated angiogenesis compounds [20]. Given the complexity and redundancy of the VEGF signalling network in promoting angiogenesis and lymphangiogenesis the blockade of either VEGFR-3 or VEGFR-2 alone might not be sufficient and, multitargeting by small molecules may be an appropriate strategy for effective inhibition of (lymph)angiogenesis [20].

Although the FDA has clinically approved different multi-kinase inhibitors, such as sunitinib, that also target the lymphangiogenic process, there are not approved therapies specifically targeting lymphangiogenesis [49,53]. Some antilymphangiogenic drugs

(monoclonal antibodies VGX-100 and IMC-3C5) have undergone phase I clinical trials for advanced and metastatic solid tumors [54,55], and some others are under preclinical trials.

Transgenic zebrafish lines available to study lymphangiogenesis

Currently, there is a number of transgenic zebrafish lines available to study lymphangiogenesis. Among these transgenic lines, is worth to mention the Tg(fli1a:egfp)^{y1}, with expression in both lymphatic and blood vessels, with non-vascular expression in neural crest derived tissues in the head) [7]; the Tg(stab1^{BAC}:yfp), with weak expression in veins and lymphatics [31]; Tg(sagff27c;uas:egfp), expressed in trunk lymphatics, weak expression in PCV, with non-vascular expression in other organs, including lens, intestine and cardiac muscle [31]; Tg(-5.2lyve1b:egfp)^{nz150} and Tg(-5.2lyve1b:dsred)^{nz101}, expressed in major axial veins and lymphatics, with weak non-vascular expression in fins [34]; the Tg(-6.6flt4:YFP)^{hu488}, with expression in DA, PCV and ISVs at 24 hpf, although expression becomes weak from 48 hpf [45]; the Tg(flt4^{BAC}:mCitrine)^{hu7135}, expressed in blood vessels initially and increasingly in venous from 26 hpf and by 5 dpf, with similar expression pattern to *lyve1b*) [56]; the Tg(prox1a^{BAC}:KalTA4;4xUASE1b:unctagRFP)^{nim5}, expressed in arterial and venous vessels before 24 hpf, although vascular expression becomes restricted to venous cells by 32 hpf and is lymphatic-specific by 5 dpf, with expression in a number of non-vascular tissues such as the myotome, liver, neuromasts, lens and retina [57]; the Tg(prox1a^{BAC}:Citrine)^{zf33}, expressed in lymphatic vessels and lymphatic precursor cells on the posterior cardinal vein [34]. Lymphatic experimental assays in these transgenic lines primarily focus on the thoracic duct development, which can be quantified by counting the number of somites with thoracic duct or parachordal lymphangioblasts, and results can be represented as the developed or inhibited thoracic duct length. Another approach used with transgenic lines that express nuclear fluorophores under the fli1a promoter consists on the counting of LECs within the zebrafish thoracic duct [58]. Although the thoracic duct is regarded as the common site, additional lymphatic vessels, including a complex network of blind-ended lymphatic capillaries, can be identified in the zebrafish trunk using lymphangiography [41].

References

1. Gore, A.V. *et al.* (2012) Vascular development in the zebrafish. *Cold Spring Harb Perspect Med.* 2:a006684
2. Kabrun, N. *et al.* (1997) Flk-1 expression defines a population of early embryonic hematopoietic precursors. *Development* 124, 2039-2048
3. Meadows, S. *et al*. (2011) Regulation of endothelial cell development by ETS transcription factors. *Semin Cell Dev Biol*. 22, 976-984
4. De Val, S. *et al.* (2008) Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell* 135, 1053-1064
5. Herbert, S.P. *et al.* (2009) Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science* 326, 294-298
6. Lawson, N.D. and Weinstein, B.M. (2002) Arteries and veins: making a difference with zebrafish. *Nat Rev Genet.* 3, 674-682
7. Lawson, N.D., Vogel, A.M. and Weinstein, B.M. (2002) Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell,* 3, 127-136
8. Covassin, L.D. *et al.* (2009) A genetic screen for vascular mutants in zebrafish reveals dynamic roles for Vegf/Plcg1 signalling during artery development. *Dev Biol.* 329, 212-226
9. Lawson, N.D. *et al.* (2003) phospholipase C gamma-1 is required downstream of vascular endothelial growth factor during arterial development. *Genes Dev.* 17, 1346-1351
10. You, L.R. *et al.* (2005) Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* 435, 98-104
11. Hong, C.C., Kume, T. and Peterson, R.T. (2008) Role of crosstalk between phosphatidylinositol 3-kinase and extracellular signal-regulated kinase/mitogen-activated protein kinase pathways in artery-vein specification. *Circ. Res.* 103, 573-579
12. Kume, T. (2010) Specification of arterial, venous, and lymphatic endothelial cells during embryonic development. *Histol Histopathol.* 25, 637-646
13. Peterson, R.T. (2004) Discovery of therapeutic targets by phenotype-based zebrafish screens. *Drug Discov Today Technol.* 1, 49-54

14. Rodriguez, F. *et al.* (2008) The receptor protein-tyrosine phosphatase, Dep1, acts in arterial/venous cell fate decisions in zebrafish development. *Dev Biol.* 324, 122-130
15. Cermenati, S. *et al.* (2008) Sox18 and Sox7 play redundant roles in vascular development. *Blood* 111, 2657-2666
16. Herpers, R. *et al.* (2008) Redundant roles for sox7 and sox18 in arteriovenous specification in zebrafish. *Circ Res.* 102, 12-15
17. Pendeville, H. *et al.* (2008) Zebrafish Sox7 and Sox18 function together to control arterial-venous identity. *Dev Biol.* 317, 405-416
18. Siekmann, A.F. and Lawson, N.D. (2007) Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* 445, 781-784
19. Habeck, H. *et al.* (2002) Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr Biol.* 12, 1405-1412
20. Okuda, K.S. *et al.* (2016) Utilizing Zebrafish to Identify Anti-(Lymph)Angiogenic Compounds for Cancer Treatment: Promise and Future Challenges. *Microcirculation* 23, 389-405
21. Chappell, J.C. *et al.* (2016) Flt-1 (VEGFR-1) coordinates discrete stages of blood vessel formation. *Cardiovasc Res.* 111, 84-93
22. Isogai, S., Horiguchi, M. and Weinstein, B.M. (2001) The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol*. 2, 278-301
23. Koenig, A.L. *et al.* (2016) Vegfa signalling promotes zebrafish intestinal vasculature development through endothelial cell migration from the posterior cardinal vein. *Dev Biol.* 411, 115-127
24. Hen, G. *et al.* (2015) Venous-derived angioblasts generate organ-specific vessels during zebrafish embryonic development. *Development* 142, 4266-4278
25. Nechiporuk, A. and Keating, M.T. (2002) A proliferation gradient between proximal and msxb-expressing distal blastema directs zebrafish fin regeneration. *Development* 129, 2607-2617
26. Bayliss, P.E. *et al.* (2006) Chemical modulation of receptor signalling inhibits regenerative angiogenesis in adult. *Nat Chem Biol.* 2, 265-273
27. Ma, Ach. *et al.* (2007) The role of survivin in angiogenesis during zebrafish embryonic development. *BMC Dev Biol.* 7, 50
28. Zhao, B. *et al.* (2010) Nogo-B receptor is essential for angiogenesis in zebrafish via Akt pathway. *Blood* 116, 5423-5433

29. Delvaeye, M. *et al.* (2009) Role of the 2 zebrafish survivin genes in vasculo-angiogenesis, neurogenesis, cardiogenesis and hematopoiesis. *BMC Dev Biol.* 9, 25
30. Han, H.W. *et al.* (2014) The Nogo-C2/Nogo receptor complex regulates the morphogenesis of zebrafish lateral line primordium through modulating the expression of dkk1b, a Wnt signal inhibitor. *PLoS One* 9:e86345
31. Bussmann, J. *et al.* (2010) Arteries provide essential guidance cues for lymphatic endothelial cells in the zebrafish trunk. *Development* 137, 2653-2657
32. Yaniv, K. *et al.* (2006) Live imaging of lymphatic development in the zebrafish. *Nat Med.* 12, 711-716
33. Marcelo, K.L., Goldie, L.C. and Hirschi, K.K. (2013) Regulation of endothelial cell differentiation and specification. *Circ Res.* 112, 1272-1287
34. Okuda, K.S. *et al.* (2012) lyve1 expression reveals novel lymphatic vessels and new mechanisms for lymphatic vessel development in zebrafish. *Development* 139, 2381-2391
35. Pham, V.N., Roman, B.L. and Weinstein, B.M. (2001) Isolation and expression analysis of three zebrafish angiopoietin genes. *Dev Dyn*. 221, 470-474
36. Pichol-Thievend, C., Hogan, B.M. and Francois, M. (2014) Lymphatic vascular specification and its modulation during embryonic development. *Microvasc Res.* 96, 3-9
37. Küchler, A.M. *et al.* (2006) Development of the zebrafish lymphatic system requires VEGFC signalling. *Curr Biol.* 16, 1244-1248
38. Coso, S., Bovay, E. and Petrova, T.V. (2014) Pressing the right buttons: signalling in lymphangiogenesis. *Blood* 123, 2614-2624
39. Bower, N.I. *et al.* (2017) Vegfd modulates both angiogenesis and lymphangiogenesis during zebrafish embryonic development. *Development* 144, 507-551
40. Kim, J.D. *et al.* (2014) Essential role of Apelin signalling during lymphatic development in zebrafish. *Arterioscler Thromb Vasc Biol.* 34, 338-345
41. Kim, J.D. and Jin, S.W. (2014) A tale of two models: mouse and zebrafish as complementary models for lymphatic studies. *Mol Cells*. 37, 503-510
42. O'Donnell, T.F., Rasmussen, J.C. and Sevick-Muraca, E.M. (2017) New diagnostic modalities in the evaluation of lymphedema. *J Vasc Surg Venous Lymphat Disord.* 5, 261-273

43. Balboa-Beltran, E. *et al.* (2014) A novel stop mutation in the vascular endothelial growth factor-C gene (VEGFC) results in Milroy-like disease. *J Med Genet.* 51, 475-478
44. Fang, J. *et al.* (2000) Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary. *Am J Hum Genet.* 67, 1382-1388
45. Hogan, B.M. *et al.* (2009) Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. *Nat Genet.* 41, 396-398
46. D'Alessio, S., Tacconi, C. and Danese, S. (2015) Targeting lymphatics in inflammatory bowel disease. *Oncotarget* 6, 34047-34048
47. Lim, H.Y. *et al.* (2009) Hypercholesterolemic mice exhibit lymphatic vessel dysfunction and degeneration. *Am J Pathol*. 175, 1328-1337
48. Paduch, R. (2016) The role of lymphangiogenesis and angiogenesis in tumor metastasis. *Cell Oncol (Dordr).* 39, 397-410
49. Dieterich, L.C. and Detmar, M. (2016) Tumor lymphangiogenesis and new drug development. *Adv Drug Deliv Rev.* 99, 148-160
50. Ji, R.C. (2017) Lymph Nodes and Cancer Metastasis: New Perspectives on the Role of Intranodal Lymphatic Sinuses. *Int J Mol Sci.* 18, 51
51. Sleeman, J.P. (2015) The lymph node pre-metastatic niche. *J Mol Med.* 93, 1173-1184
52. Dunworth, K.S. *et al.* (2016) Utilizing Zebrafish to Identify Anti-(Lymph)Angiogenic Compounds for Cancer Treatment: Promise and Future Challenges. *Microcirculation* 23, 389-405
53. Nagahashi, M. *et al.* (2010) Lymphangiogenesis: a new player in cancer progression. *World J Gastroenterol.* 16, 4003-4012
54. Falchook, G.S. *et al.* (2014) A first-in-human phase I study of vgx-100, a selective anti-vegf-c antibody, alone and in combination with bevacizumab in patients with advanced solid tumors. *J. Clin. Oncol.* 32, 2524
55. Saif, M.W. *et al.* (2016) Phase 1 study of the anti-vascular endothelial growth factor receptor 3 monoclonal antibody LY3022856/IMC-3C5 in patients with advanced and refractory solid tumors and advanced colorectal cancer. *Cancer Chemother Pharmacol.* 78, 815-824
56. van Impel, A. *et al.* (2014) Divergence of zebrafi sh and mouse lymphatic cell fate specification pathways. *Development* 141, 1228-1138

57. Dunworth, W.P. *et al.* (2014) Bone morphogenetic protein 2 signalling negatively modulates lymphatic development in vertebrate embryos. *Circ Res.* 114, 56-66
58. Hoffman, S.J. *et al.* (2012) An in vivo method to quantify lymphangiogenesis in zebrafish. *PLoS One* 7:e45240

Additional bibliography used for the histogram contained in Figure 1

Alex, D. *et al*. (2010) Indirubin shows anti-angiogenic activity in an *in vivo* zebrafish model and an *in vitro* HUVEC model. *J. Ethnopharmacol*. 131, 242-247

Alex, D. *et al*. (2010) Resveratrol derivative, trans-3,5,4'-trimethoxystilbene, exerts antiangiogenic and vascular-disrupting effects in zebrafish through the downregulation of VEGFR2 and cell-cycle modulation. *J. Cell. Biochem*. 109, 339-346

Alvarez, Y. *et al*. (2009) Selective inhibition of retinal angiogenesis by targeting PI3 kinase. *PLoS One* 4, e7867

Astin, J.W. *et al*. (2014) An *in vivo* antilymphatic screen in zebrafish identifies novel inhibitors of mammalian lymphangiogenesis and lymphatic-mediated metastasis. *Mol. Cancer Ther*. 13, 2450-2462

Bansode, R.R. *et al*. (2011) Coupling *in vitro* and *in vivo* paradigm reveals a dose dependent inhibition of angiogenesis followed by initiation of autophagy by C6-ceramide. *Int. J. Biol. Sci*. 7, 629-644

Bohni, N. *et al*. (2013) Integration of microfractionation, qNMR and zebrafish screening for the *in vivo* bioassay-guided isolation and quantitative bioactivity analysis of natural products. *PLoS One* 8, e64006

Bonventre, J.A. *et al*. (2013) Manipulation of the HIF-Vegf pathway rescues methyl tert-butyl ether (MTBE)-induced vascular lesions. *Toxicol. Appl. Pharmacol*. 273, 623-634

Buchanan, C.M. *et al*. (2011) DMXAA (Vadimezan, ASA404) is a multi-kinase inhibitor targetting vegf-r2 in particular. *Clin. Sci. (Lond.)* 122, 449-457

Camus, S. *et al*. (2012) Identification of phosphorylase kinase as a novel therapeutic target through high-throughput screening for anti-angiogenesis compounds in zebrafish. *Oncogene* 31, 4333-4342

Cannon, J.E. *et al*. (2010) Intersegmental vessel formation in zebrafish: requirement for VEGF but not BMP signalling revealed by selective and non-selective BMP antagonists. *Br. J. Pharmacol*. 161, 140-149

Cárdenas, C. *et al*. (2011) Anti-angiogenic and anti-inflammatory properties of kahweol, a coffee diterpene. *PLoS One* 6, e23407

Chan, J.Y. *et al*. (2011) Polyphyllin D, a steroidal saponin from *Paris polyphylla*, inhibits endothelial cell functions in vitro and angiogenesis in zebrafish embryos in vivo. *J. Ethnopharmacol*. 137, 64-69

Chang, D.J. *et al*. (2012) design, synthesis and biological evaluation of novel Deguelin-based heat shock protein 90 (HSP90) inhibitors targeting proliferation and angiogenesis. *J. Med. Chem*. 55, 10863-10884

Chang, G.H. *et al*. (2014) ARHGAP18: an endogenous inhibitor of angiogenesis, limiting tip formation and stabilizing junctions. *Small GTPases* 5, 1-15

Chen, T. *et al*. (2012) Synthesis and antiangiogenic activity of novel gambogic acid derivatives. *Molecules* 17, 6249-6268

Chen, Y.H. *et al*. (2015) Pro-angiogenic effects of chalcone derivatives in zebrafish embryos *in vivo*. *Molecules* 20, 12512-12524

Cheng, J. *et al*. (2011) Nanotherapeutics in angiogenesis: synthesis and *in vivo* assessment of drug efficacy and biocompatibility in zebrafish embryos. *Int. J. Nanomedicine* 6, 2007-2021

Crawford, A.D. *et al*. (2011) Zebrafish bioassay-guided natural product discovery: isolation of angiogenesis inhibitors from East african medicinal plants. *PLoS One* 6, e14694

Crucke, J. and Huysseune, A. (2014) Blocking VEGF signaling delays development of replacement teeth in zebrafish. *J. Dent. Res*. 94, 157-165

Delov, V. *et al*. (2014) Transgenic fluorescent zebrafish *Tg(fli1:EGFP)*^{y1} for the identification of vasotoxicity within the zFET. *Aquat. Toxicol*. 150, 189-200

Donnini, S. *et al*. (2010) Abeta peptides accelerate the senescence of endothelial cells *in vitro* and *in vivo*, impairing angiogenesis. *FASEB J*. 24, 2385-2395

Farooq, M. *et al*. (2014) Biological screening of novel derivatives of valproic acid for anticancer and antiangiogenic properties. *Asian Pac. J. Cancer Prev*. 15, 7785-7792

Feng, N. *et al*. (2016) HIF-1α and HIF-2α induced angiogenesis in gastrointestinal vascular malformation and reversed by thalidomide. *Sci. Rep*. 6, 27280

Galvin, O. *et al*. (2016) A sustained release formulation of novel quininib-hyaluronan microneedles inhibits angiogenesis and retinal vascular permeability in vivo. *J. Control Release* 233, 198-207

García-Caballero, M. *et al*. (2011) Dimethylfumarate inhibits angiogenesis *in vitro* and *in vivo*: a possible role for its antipsoriatic effect? *J. Invest. Dermatol*. 131, 1347-1355

García-Caballero, M. *et al*. (2013) Toluquinol, a marine fungus metabolite, is a new angiossuppresor that interferes the AKT pathway. *Biochem. Pharmacol*. 85, 1727-1740

García-Caballero, M. *et al*. (2014) The marine fungal metabolite, AD0157, inhibits angiogenesis by targeting the Akt signaling pathway. *Mar. Drugs* 12, 279-299

García-Caballero, M. *et al*. (2016) Novel application assigned to toluquinol: inhibition of lymphangiogenesis by interfering with VEGF-C/VEGFR-3 signalling pathway. *Br. J. Pharmacol*. 173, 1966-1987

Garkavtsev, I. *et al*. (2011) Dehydro-α-lapachone, a plant product with antivascular activity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11596-11601

Guan, Y.Y. *et al*. (2014) Raddeanin A, a triterpenoid saponin isolated from *Anemone raddeana*, suppresses the angiogenesis and growth of human colorectal tumor by inhibiting VEGFR2 signaling. *Phytomedicine* 22, 103-110

Hao, J. *et al*. (2010) *In vivo* structure-activity relationship study of Dorsomorphin analogs identifies selective VEGF and BMP inhibitors. *ACS Chem. Biol*. 5, 245-253

He, Z.H. *et al*. (2010) Anti-angiogenic effects of the fruit of *Alpinia oxyphylla*. *J. Ethnopharmacol*. 132, 443-449

He, Z.H. *et al*. (2011) Anti-angiogenic effect and mechanism of rhein from *Rhizoma rhei*. *Phytomedicine* 18, 470-478

He, Z.H. *et al*. (2012) Antiangiogenic effects and mechanisms of *trans*-ethyl *p*-methoxycinnamate from *Kaempferia galanga* L. *J. Agric. Food Chem*. 60, 11309-11317

Heinke, J. *et al*. (2013) Antagonism and synergy between extracellular BMP modulators Tsg and BMPER to balance blood vessel formation. *J. Cell Sci*. 126, 3082-3094

Hermans, K. *et al*. (2010) Role of synectin in lymphatic development in zebrafish and frogs. *Blood* 116, 3356-3366

Hong, S.J. *et al*. (2009) Angiogenic effect of saponin extract from *Panax notoginseng* on HUVECs *in vitro* and zebrafish *in vivo*. *Phytother. Res*. 23, 677-686

Hu, F. *et al*. (2013) Angiogenic effects of danshen and gegen decoction on human endothelial cells and zebrafish embryos. *Am. J. Chin. Med*. 41, 887-900

Huang, W. *et al*. (2016) Anti-angiogenic activity and mechanism of kaurane diterpenoids from *Wedelia chinensis*. *Phytomedicine* 23, 283-292

Incardona, J.P. *et al*. (2011) Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. *Toxicol. Appl. Pharmacol*. 257, 242-249

Jia, Y. *et al*. (2010) Thiolutin inhibits endothelial cell adhesion by perturbing Hsp27 interactions with components of the actin and intermediate filament cytoskeleton. *Cell Stress Chaperones* 15, 165-181

Kim, N.H. *et al*. (2015) The small molecule *R*-(-)-β-*O*-methylsynephrine binds to nucleoporin 153 kDa and inhibits angiogenesis. *Int. J. Biol. Sci*. 11, 1088-1099

Kuo, M.W. *et al*. (2011) Soluble THSD7A is an N-glycoprotein that promotes endothelial cell migration and tube formation in angiogenesis. *PLoS One* 6, e29000

Lai, P.K. *et al*. (2014) Induction of angiogenesis in zebrafish embryos and proliferation of endothelial cells by an active fraction isolated from the root of *Astragalus*

membranaceus using bioassay-guided fractionation. *J. Tradit. Complement. Med.* 4, 239-245

Lam, H.W. *et al.* (2008) The angiogenic effects of *Angelica sinensis* extract on HUVEC *in vitro* and zebrafish *in vivo*. *J. Cell Biochem.* 103, 195-211

Lam, K.H. *et al.* (2011) Nobiletin, a polymethoxylated flavonoid from citrus, shows anti-angiogenic activity in a zebrafish *in vivo* model and HUVEC *in vitro* model. *J. Cell Biochem.* 112, 3313-3321

Lam, S.H. *et al.* (2011) Toxicogenomic and phenotypic analyses of bisphenol-A early-life exposure toxicity in zebrafish. *PLoS One* 6, e28273

Lam, I.K. *et al.* (2012) *In vitro* and *in vivo* structure and activity relationship analysis of polymethoxylated flavonoids: Identifying sinensetin as a novel antiangiogenesis agent. *Mol. Nutr. Food Res.* 56, 945-956

Lambert, E. *et al.* (2011) Netrin-4 acts as a pro-angiogenic factor during zebrafish development. *J. Biol. Chem.* 287, 3987-3999

Leet, J.K. *et al.* (2014) High-content screening in zebrafish embryos identifies butafenacil as a potent inducer of anemia. *PLoS One* 9, e104190

Letamendia, A. *et al.* (2012) Development and validation of an automated high-throughput system for zebrafish *in vivo* screenings. *PLoS One* 7, e36690

Li, Y.B. *et al.* (2013) IC-4, a new irreversible EGFR inhibitor, exhibits prominent anti-tumor and anti-angiogenesis activities. *Cancer Lett.* 340, 88-96

Li, K.K. *et al.* (2014) *In vitro* and *in vivo* mechanistic study of a novel proanthocyanidin, GC-(4'8)-GCG from cocoa tea (*Camellia ptilophylla*) in antiangiogenesis. *J. Nutr. Biochem.* 25, 319-328

Li, S. *et al.* (2015) Formononetin promotes angiogenesis through the estrogen receptor alpha-enhanced ROCK pathway. *Sci. Rep.* 5, 16815

Li, Q. *et al.* (2016) Proanthocyanidins, isolated from *Choerospondias axillaris* fruit peels, exhibit potent antioxidant activities *in vitro* and novel anti-angiogenic property in vitro and in vivo. *J. Agric. Food Chem.* 64, 3546-3556

Lin, C. *et al.* (2012) Quercetin-4'-*O*-beta-D-glucopyranoside (QODG) inhibits angiogenesis by suppressing VEGFR2-mediated signaling in zebrafish and endothelial cells. *PLoS One* 7, e31708

Lin, S. *et al.* (2014) Anti-oxidant and anti-angiogenic properties of phenolic extract from *Pleurotus tuber-regium*. *J. Agric. Food Chem.* 62, 9488-9498

Lin, S.W. *et al.* (2015) Coral-derived compound WA-25 inhibits angiogenesis by attenuating the VEGF/VEGFR2 signaling pathway. *Mar. Drugs* 13, 861-878

Lintnerová, L. *et al*. (2014) A development of chimeric VEGFR2 TK inhibitor based on two ligand conformers from PDB: 1Y6A complex - Medicinal chemistry consequences of a TKs analysis. *Eur. J. Med. Chem*. 72, 146-159

Liu, C.L. *et al*. (2014) Molecular mechanisms of angiogenesis effect of active sub-fraction from root of *Rehmannia glutinosa* by zebrafish sprout angiogenesis-guided fractionation. *J. Ethnopharmacol*. 151, 565-575

Liu, J.X. *et al*. (2014) Marine compound catunaregin inhibits angiogenesis through the modulation of phosphorylation of Akt and eNOS *in vivo* and *in vitro*. *Mar. Drugs* 12, 2790-2801

Liu, L. *et al*. (2015) A novel engineered VEGF blocker with an excellent pharmacokinetic profile and robust anti-tumor activity. *BMC Cancer* 15, 170

Liu, G. *et al*. (2016) A novel polysaccharide from *Sargassum integerrimum* induces apoptosis in A549 cells and prevents angiogensis *in vitro* and *in vivo*. *Sci. Rep*. 6, 26722

Lu, X.L. *et al*. (2012) Marine Cyclotripeptide X-13 promotes angiogenesis in zebrafish and human endothelial cells via PI3K/Akt/eNOS signaling pathways. *Mar. Drugs* 10, 1307-1320

Lu, X.L. *et al*. (2014) Protective effects of puerarin against Aß40-induced vascular dysfunction in zebrafish and human endothelial cells. *Eur. J. Pharmacol*. 732, 76-85

Ma, L. *et al*. (2011) Structural modification of honokiol, a biphenyl occurring in *Magnolia officinalis*: the evaluation of honokiol analogs as inhibitors of angiogenesis and for cytotoxicity and their structure-activity relationship. *J. Med. Chem*. 54, 6469-6481

Mahony, C. *et al*. (2013) Pomalidomide is nonteratogenic in chicken and zebrafish embryos and nonneurotoxic *in vitro*. *Proc. Natl. Acad. Sci. U.S.A*. 110, 12703-12708

Metelo, A.M. *et al*. (2015) Pharmacological HIF2α inhibition improves VHL disease–associated phenotypes in zebrafish model. *J. Clin. Invest*. 125, 1987-1997

Murphy, E.A. *et al*. (2010) Disruption of angiogenesis and tumor growth with an orally active drug that stabilizes the inactive state of PDGFRβ/B-RAF. *Proc. Natl. Acad. Sci. U.S.A*. 107, 4299-4304

Muthukumarasamy, K.M. *et al*. (2015) Identification of noreremophilane-based inhibitors of angiogenesis using zebrafish assays. *Org. Biomol. Chem*. 14, 1569-1578

Oggier, D.M. *et al*. (2011) Effects of the protein kinase inhibitor PKC412 on gene expression and link to physiological effects in zebrafish *Danio rerio* eleuthero-embryos. *Toxicol. Sci*. 119, 104-115

Pandey, R. *et al*. (2015) Cyclosporin A disrupts notch signaling and vascular lumen maintenance. *PLoS One* 10, e0119279

Patra, C.R. *et al*. (2011) Reactive oxygen species driven angiogenesis by inorganic nanorods. *Nano Lett*. 11, 4932-4938

Pruvot, B. *et al*. (2014) Developmental defects in zebrafish for classification of EGF pathway inhibitors. *Toxicol. Appl. Pharmacol*. 274, 339-349

Radi, M. *et al*. (2012) A combined targeted/phenotypic approach for the identification of new antiangiogenics agents active on a zebrafish model: From *in silico* screening to cyclodextrin formulation. *Bioorg. Med. Chem. Lett*. 22, 5579-5583

Raghunath, M. *et al*. (2009) Pharmacologically induced angiogenesis in transgenic zebrafish. *Biochem. Biophys. Res. Commun*. 378, 766-771

Reynolds, A.L. *et al*. (2016) Phenotype-based discovery of 2-[(*E*)-2-(Quinolin-2-yl)vinyl]phenol as a novel regulator of ocular angiogenesis. *J. Biol. Chem*. 291, 7242-7255

Sasore, T. and Kennedy, B. (2014) Deciphering combinations of PI3K/AKT/mTOR pathway drugs augmenting anti-angiogenic efficacy *in vivo*. *PLoS One* 9, e105280

Shao, X. *et al*. (2012) Taurine protects methamphetamine-induced developmental angiogenesis defect through antioxidant mechanism. *Toxicol. Appl. Pharmacol*. 260, 260-270

Shen, M. *et al*. (2013) Discovery of Rho-kinase inhibitors by docking-based virtual screening. *Mol. Biosyst*. 9, 1511-1521

Soomro, S. *et al*. (2011) Design of novel artemisinin-like derivatives with cytotoxic and anti-angiogenic properties. *J. Cell Mol. Med*. 15, 1122-1135

Sulaiman, R.S. *et al*. (2016) A novel small molecule ameliorates ocular neovascularisation and synergises with anti-VEGF therapy. *Sci. Rep*. 6, 25509

Tse, H.Y. *et al*. (2012) Angiogenic efficacy of simplified 2-herb formula (NF3) in zebrafish embryos *in vivo* and rat aortic ring *in vitro*. *J. Ethnopharmacol*. 139, 447-453

Vogt, A. *et al*. (2009) High-content analysis of cancer-cell-specific apoptosis and inhibition of *in vivo* angiogenesis by synthetic (-)-pironetin and Analogs. *Chem. Biol. Drug Des*. 74, 358-368

Vollmer, L.L. *et al*. (2011) A simplified synthesis of novel dictyostatin analogs with in vitro activity against epothilone B resistant cells and antiangiogenic activity in zebrafish embryos. *Mol. Cancer Ther*. 10, 994-1006

Weng, W.T. *et al*. (2014) α-Melanocyte-stimulating hormone inhibits angiogenesis through attenuation of VEGF/VEGFR2 signaling pathway. *Biochim. Biophys. Acta* 1840, 1850-1860

Wrasidlo, W. *et al*. (2008) The marinelipopeptide somocystinamide A triggers apoptosis via caspase 8. *Proc. Natl. Acad. Sci. U.S.A*. 105, 2313-2318

Wu, F. *et al*. (2015) Irisin induces angiogenesis in human umbilical vein endothelial cells *in vitro* and in zebrafish embryos *in vivo* via activation of the ERK signaling pathway. *PLoS One* 10, e0134662

Yang, X. *et al*. (2014) A synthetic *dl*-nordihydroguaiaretic acid (nordy), inhibits angiogenesis, invasion and proliferation of glioma stem cells within a zebrafish xenotransplantation model. *PLoS One* 9, e85759

Yang, B.R. *et al*. (2016) Amelioration of acute myocardial infarction by saponins from flower buds of *Panax notoginseng* via pro-angiogenesis and anti-apoptosis. *J. Ethnopharmacol*. 181, 50-58

Yozzo, K.L. *et al*. (2013) High-Content Screening Assay for identification of chemicals impacting cardiovascular function in zebrafish embryos. *Environ. Sci. Technol*. 47, 11302-11310

Yu, X. *et al*. (2013) Anti-angiogenic activity of *Herba epimedii* on zebrafish embryos *in vivo* and HUVECs *in vitro*. *Phytother. Res*. 27, 1368-1375

Yue, G.G. *et al*. (2013) Anti-angiogenesis and immunomodulatory activities of an anti-tumor sesquiterpene bigelovin isolated from *Inula helianthus-aquatica*. *Eur. J. Med. Chem*. 59C, 243-252

Yue, G.G. *et al*. (2015) Novel PI_3K/AKT targeting anti-angiogenic activities of 4-vinylphenol, a new therapeutic potential of a well-known styrene metabolite. *Sci. Rep*. 5, 11149

Zhang, W. *et al*. (2013) Toxic effects of copper ion in zebrafish in the joint presence of CdTe QDs. *Environ. Pollut*. 176C, 158-164

Zhang, Z.R. *et al*. (2014) *In vivo* angiogenesis screening and mechanism of action of novel tanshinone derivatives produced by one-pot combinatorial modification of natural tanshinone mixture from *Salvia miltiorrhiza*. *PLoS One* 9, e100416

Zhong, Z.F. *et al*. (2012) Anti-angiogenic effect of furanodiene on HUVECs *in vitro* and on zebrafish *in vivo*. *J. Ethnopharmacol*. 141, 721-727

Zhou, Z. *et al*. (2015) A VEGFR1 antagonistic peptide inhibits tumor growth and metastasis through VEGFR1-PI3K-AKT signaling pathway inhibition. *Am. J. Cancer Res*. 5, 3149-3161

Zhu, W. *et al*. (2011) 5-Formylhonokiol exerts anti-angiogenesis activity *via* inactivating the ERK signaling pathway. *Exp. Mol. Med*. 43, 146-152

Table S1. Antiangiogenic drugs approved by FDA/EMA for the treatment of solid tumors

Drug	Type	Molecular target	Malignancies
Bevacizumab	Humanized monoclonal antibody	VEGF	MCRC, NSCLC, OC, MBC, glioblastoma, metastatic RCC, endometrial cancer, mesothelioma and cervical cancers
Ramucirumab	Human monoclonal antibody	VEGFR2	MCRC, NSCLC, gastric adenocarcinoma
Aflibercept (VEGF-Trap)	Fusion protein (VEGFR chimera)	VEGF-A/B, PlGF	MCRC
Sunitinib	TKI	VEGFR-1-2, PDGFRσ/β	Metastatic RCC, gastrointestinal stromal tumors, pancreatic neuroendocrine tumors
Sorafenib	TKI	VEGFR-2, PDGFRβ	Advanced RCC, metastatic differentiated TC, unresectable HCC
Pazopanib	TKI	VEGFR-1-3, PDGFRβ, FGFR-1-2	Metastatic STC and advanced RCC
Vandetanib	TKI	VEGFR-2	Unresectable or metastatic TC
Axitinib	TKI	VEGFR-1-3, PDGFRβ	Advanced RCC
Regorafenib	TKI	VEGFR-1-3, PDGFRβ, FGFR-1-2	Chemo-refractory MCRC, unresectable HCC and GIST
Cabozantinib	TKI	VEGFR-2, Tie2	Refractory advanced RCC, metastatic medullary TC, pancreatic neuroendocrine tumors
Levantinib	TKI	VEGFR-1-3, PDGFRα, FGFR-1-4	TC, HCC and RCC
Cediranib	TKI	VEGFR-1-3	OC

TKI (tyrosine kinase inhibitor), MCRC (Metastatic colorectal carcinoma), NSCLC (non small cell lung cancer), OC (ovarian cancer), MBC (metastatic breast cancer), RCC (renal cell carcinoma), HCC (hepatocellular carcinoma), TC (thyroid carcinoma), STC (soft tissue carcinoma).

Additional bibliography reviewed in Table S1

The antiangiogenic drugs approved by FDA/EMA for the treatment of solid tumors have been recently reviewed in:

Jayson, G.C. *et al*. (2016) Antiangiogenic therapy in oncology: current status and future directions. *Lancet* 388(10043), 518-529

Lin, Z. *et al*. (2016) Angiogenesis inhibitors as therapeutic agents in cancer: Challenges and future directions. *Eur. J. Pharmacol*. 793, 76-81

Ramjiawan, R.R. *et al*. (2017) Anti-angiogenesis for cancer revisited: Is there a role for combinations with immunotherapy? *Angiogenesis* 20, 185-204

Table S2. Usage of endothelial-driven transgenic lines in reviewed articles

Transgenic line	Driving gen/expression	ZFIN ref	Supplier	References
Tg(fli1a:EGFP)y1 (y2-y5)	Fli-1/Endothelial cells, cytoplasmic	888	Weinstein/ZIRC	1
Tg(fli1:neGFP)y7	Fli-1/Endothelial cells, nuclear	107	Weinstein/ZIRC	2
y1; Tg(lfabp:RFP; elaA:EGFP)	Fli-1/liver fatty acid binding protein/pancreas elastase A/liver and exocrine pancreas	63	Gong lab/CZRC	3-5
y1, Tg(elavl3:GCaMP5G)a4598	Fli-1/neural-specific RNA-binding protein/Neural specific- calcium indicator	30	Schier lab	6-8
y1, Tg[lyve1:DsRed2/Flt1:YFP]nz150	Fli-1/Vegfc/Lymphatic-specific marker	4 (14/20)	Crosier/Schulte-Merker/EZRC	9-11
y1, Tg(NBT:MAPT-GFP)zc1	Fli-1/Microtubule-associated protein tau/Primary motor neuron marker	11	Chien lab	12
y1, Tg(gata1:RFP)sd2	Fli-1/GATA-1/Endothelial cells and blood circulation	40	Zon lab	13-16
Tg(gata1a:dsRed)sd2	GATA-1/Blood cells	201	EZRC-ZIRC	15, 17
Tg(gata1:GFP)la781	GATA-1/Erythroid lineage	45	Shuo Lin lab	18
Tg(gata2:eGFP)la3	GATA-2/Blood cells	6	Zon lab	17
Tg(-7.8gata4:GFP)ae1 (ae2-3)	GATA-4/Endocardial and myocardial cells	14	Evans lab	19-21
Tg(0.8flt1:RFP)hu5333	Flt1/arterial ISV	20	Schulte-Merker lab	22
Tg(efnb2a:EGFP)y77	Ligand of Eph-receptor/ Artery	1	Weinstein lab	23
Tg(nkx2.3:efnb2a,myl7:EGFP)el589	Ligand of Eph-receptor/Artery	1	Crump lab	24
Tg(dll4:EGFP)lcr1	Notch ligand/ Endothelial cells	1	De Val lab	25
TgBAC(dll4:GAL4FF)hu10049	Notch ligand/Endothelial cells	2	Schulte-Merker lab	26
Tg(hsp70l:canotch3-EGFP)co17	Notch3 intracellular domain/Perivascular	1	Appel lab	27
TgPAC(tal1:d2eGFP)hkz08t	Tal-1/Endothelial cells	2	Wen lab	28-29
TgPAC(tal1:d2eGFP; tal1:DsRed)hkz06t	Tal-1/Endothelial cells	1	Wen lab	28

Tg(Tie2:eGFP)s849	Tie-2 receptor tyrosine kinase/Endothelial cells	20	Stainier lab	30-31
Tg(5xUAS:cdh5-EGFP)ubs12	VE-cadherin/Pan-endothelial	7	Affolter Lab	32-34
TgBAC(cdh5:Citrine)mu102	VE-cadherin/Pan-endothelial	2	Siekmann lab	35
TgBAC(cdh5:GAL4FF)mu101	VE-cadherin/Pan-endothelial	8	Siekmann lab	22, 34
Tg(kdrl:G-RCFP)zn1 (zn10)	Vegfr2/flk1/kdr/Angioblast/endothelial precursors	53	Zygogene Research Department	36
Tg(kdrl:RFP)la4	Vegfr2/flk1/kdr/Angioblast/endothelial precursors	14	Shuo Lin/CZRC	37
Tg(kdrl:EGFP)s843	Vegfr2/flk1/kdr/Vegfr4/Angioblast/endothelial precursors	320	Stainier/CZRC,EZRC,ZIRC	38-39
Tg(kdrl:nlsmCherry)is4	Vegfr2/flk1/kdr/Endothelial cells	6	Essner lab	40-42
Tg(kdrl:mCherry)ci5 (is5) (fli1a:negfp)y1 (y7)	Fli-1,vegfr2/flk1/kdr/Endothelial marker, green nuclei and red cytoplam	3 (23/13)	Sumanas/Essner labs	43-44
TgBAC(flt4:Citrine)hu7135	Vegfr3/Pan-endothelial	7	Schulte-Merker lab	45-47
Tg(flt4:YFP)hu4881	Vegfr3/Pan-endothelial	3	Schulte-Merker lab	48
Tg(myl7:eGFP)twu277	Cardiac myosin light chain 2/Myocardial cells	193	Tsai lab	49-52
Tg(myl7:Gal4-VP16)f15	Atrial myosin regulatory light chain 2/heart	2	Randal Patterson lab	53-54

List of references numbered in Table S2

1. Lawson, N.D. and Weinstein, B.M. (2002) *In vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol,* 248, 307-318

2. Roman, B.L. *et al.* (2002) Disruption of *acvrl1* increases endothelial cell number in zebrafish cranial vessels. *Development* 129, 3009-3019

3. Her, G.M. *et al.* (2003) In vivo studies of liver type fatty acid binding protein (L-FABP) gene expression in liver of transgenic zebrafish (*Danio rerio*). *FEBS Lett.* 538, 125-133

4. Wan, H. *et al.* (2006) Analyses of pancreas development by generation of gfp transgenic zebrafish using an exocrine pancreas-specific elastaseA gene promoter. *Exp. Cell Res.* 312, 1526-1539

5. Farooq, M. *et al.* (2008) Histone deacetylase 3 (hdac3) is specifically required for liver development in zebrafish. *Dev. Biol.* 317, 336-353

6. Antinucci, P. and Hindges, R. (2016) A crystal-clear zebrafish for in vivo imaging. *Sci. Rep.* 6, 29490

7. Auer, T.O. *et al.* (2015) Deletion of a kinesin I motor unmasks a mechanism of homeostatic branching control by neurotrophin-3. *Elife* 4:e05061

8. Campbell, P.D. *et al.* (2014) Unique function of kinesin Kif5A in localization of mitochondria in axons. *J. Neurosci.* 34, 14717-14732

9. Astin, J.W. *et al.* (2014) An *in vivo* antilymphatic screen in zebrafish identifies novel inhibitors of mammalian lymphangiogenesis and lymphatic-mediated metastasis. *Mol. Cancer Ther.* 13, 2450-2462

10. Coxam, B.*et al.* (2014) Pkd1 regulates lymphatic vascular morphogenesis during development. *Cell Rep.* 7, 623-633

11. Chang, G.H. *et al.* (2014) ARHGAP18: an endogenous inhibitor of angiogenesis, limiting tip formation and stabilizing junctions. *Small GTPases* 5, 1-15

12. Bansode, R.R. *et al.* (2011) Coupling *in vitro* and *in vivo* paradigm reveals a dose dependent inhibition of angiogenesis followed by initiation of autophagy by C6-ceramide. *Int. J. Biol. Sci.* 7, 629-644

13. Bolcome, R.E. 3rd *et al.* (2008) Anthrax lethal toxin induces cell death-independent permeability in zebrafish vasculature. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2439-2444

14. Mariappan, D. *et al.* (2009) Somitovasculin, a novel endothelial-specific transcript involved in the vasculature development. *Arterioscler. Thromb. Vasc. Biol.* 29, 1823-1829

15. Hermans, K. *et al*. (2010) Role of synectin in lymphatic development in zebrafish and frogs. *Blood* 116, 3356-3366

16. Oggier, D.M. *et al*. (2011) Effects of the protein kinase inhibitor PKC412 on gene expression and link to physiological effects in zebrafish *Danio rerio* eleuthero-embryos. *Toxicol. Sci*. 119, 104-115

17. Traver, D. *et al*. (2003) Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat. Immunol*. 4, 1238-1246

18. Long, Q.M. *et al*. (1997) GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development* 124, 4105-4111

19. Heicklen-Klein, A. and Evans, T. (2004) T-box binding sites are required for activity of a cardiac GATA-4 enhancer. *Dev. Biol*. 267, 490-504

20. Gupta, V. *et al*. (2013) An injury-responsive Gata4 program shapes the zebrafish cardiac ventricle. *Curr. Biol*. 23, 1221-1227

21. Han, P. *et al*. (2014) Hydrogen peroxide primes heart regeneration with a derepression mechanism. *Cell Res*. 24, 1091-1107

22. Bussmann, J. *et al*. (2010) Arteries provide essential guidance cues for lymphatic endothelial cells in the zebrafish trunk. *Development* 137, 2653-2657

23. Swift, M.R. *et al*. (2014) SoxF factors and Notch regulate *nr2f2* gene expression during venous differentiation in zebrafish. *Dev. Biol*. 390, 116-125

24. Choe, C.P. and Crump, J.G. (2015) Eph-Pak2a signaling regulates branching of the pharyngeal endoderm by inhibiting late-stage epithelial dynamics. *Development* 142, 1089-1094

25. Sacilotto, N. *et al*. (2013) Analysis of Dll4 regulation reveals a combinatorial role for Sox and Notch in arterial development. *Proc. Natl. Acad. Sci. U.S.A*. 110, 11893-11898

26. Hermkens, D.M. *et al*. (2015) Sox7 controls arterial specification in conjunction with *hey2* and *efnb2* function. *Development* 142, 1695-1704

27. Wang, Y. *et al*. (2014) Notch3 establishes brain vascular integrity by regulating pericyte number. *Development* 141, 307-317

28. Zhen, F. *et al*. (2013) Hemogenic endothelium specification and hematopoietic stem cell maintenance employ distinct Scl isoforms. *Development* 140, 3977-3985

29. Jing, L. *et al*. (2015) Adenosine signaling promotes hematopoietic stem and progenitor cell emergence. *J. Exp. Med*. 212, 649-663

30. Motoike, T. *et al*. (2000) Universal GFP reporter for the study of vascular development. *Genesis* 28, 75-81

31. Jin, S.W. *et al*. (2007) A transgene-assisted genetic screen identifies essential regulators of vascular development in vertebrate embryos. *Dev. Biol*. 307, 29-42

32. Lenard, A. *et al*. (2013) In Vivo analysis reveals a highly stereotypic morphogenetic pathway of vascular anastomosis. *Dev. Cell* 25, 492-506

33. Kochhan, E. *et al*. (2013) Blood flow changes coincide with cellular rearrangements during blood vessel pruning in zebrafish embryos. *PLoS One* 8, e75060

34. Hultin, S. *et al*. (2014) AmotL2 links VE-cadherin to contractile actin fibres necessary for aortic lumen expansion. *Nat. Commun*. 5, 3743

35. Bussmann, J., and Schulte-Merker, S. (2011) Rapid BAC selection for tol2-mediated transgenesis in zebrafish. *Development* 138, 4327-4332

36. Cross, L.M. *et al*. (2003) Rapid analysis of angiogenesis drugs in a live fluorescent zebrafish assay. *Arterioscler. Thromb. Vasc. Biol*. 23, 911-912

37. Huang, H. *et al*. (2005) NXT2 is required for embryonic heart development in zebrafish. *BMC Dev. Biol*. 5, 7

38. Jin, S.W. *et al*. (2005) Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* 132, 5199-5209

39. Zhou, Y. *et al*. (2015) UXT potentiates angiogenesis by attenuating Notch signaling. *Development* 142, 774-786

40. Wang, Y. *et al*. (2010) Moesin1 and Ve-cadherin are required in endothelial cells during in vivo tubulogenesis. *Development* 137, 3119-3128

41. Zygmunt, T. *et al*. (2012) In parallel interconnectivity of the dorsal longitudinal anastomotic vessels requires both VEGF signaling and circulatory flow. *J. Cell Sci*. 125, 5159-5167

42. Vanhollebeke, B. *et al*. (2015) Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/β-catenin pathway during brain angiogenesis. Elife 4:e06489

43. Nguyen, P.D. *et al*. (2014) Haematopoietic stem cell induction by somite-derived endothelial cells controlled by meox1. *Nature* 512, 314-318

44. Marín-Juez, R. *et al*. (2016) Fast revascularization of the injured area is essential to support zebrafish heart regeneration. *Proc. Natl. Acad. Sci. U.S.A*. 113, 11237-11242

45. Gordon, K. *et al*. (2013) Mutation in Vascular Endothelial Growth Factor-C, a ligand for Vascular Endothelial Growth Factor Receptor-3, is associated with autosomal dominant Milroy-like primary lymphedema. *Circ*. *Res*. 112, 956-960

46. van Impel, A. *et al*. (2014) Divergence of zebrafish and mouse lymphatic cell fate specification pathways. *Development* 141, 1228-1238

47. Kok, F.O. *et al*. (2015) Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev*. *Cell* 32, 97-108

48. Hogan, B.M. *et al*. (2009) Vegfc/Flt4 signalling is suppressed by Dll4 in developing zebrafish intersegmental arteries. *Development* 136, 4001-4009

49. Ho, Y.L. *et al*. (2007) *In vivo* assessment of cardiac morphology and function in heart-specific green fluorescent zebrafish. *J*. *Formos*. *Med*. *Assoc*. 106, 181-186

50. de Pater, E. *et al*. (2009) Distinct phases of cardiomyocyte differentiation regulate growth of the zebrafish heart. *Development* 136, 1633-1641

51. Liu, J. *et al*. (2010) A dual role for ErbB2 signaling in cardiac trabeculation. *Development* 137, 3867-3875

52. Hinits, Y. *et al*. (2012) Zebrafish Mef2ca and Mef2cb are essential for both first and second heart field cardiomyocyte differentiation. *Dev*. *Biol*. 369, 199-210

53. Steinbicker, A.U. *et al*. (2011) Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. *Blood* 117, 4915-4923

54. Muthukumarasamy, K.M. *et al*. (2015) Identification of noreremophilane-based inhibitors of angiogenesis using zebrafish assays. *Org*. *Biomol*. *Chem*. 14, 1569-1578

Table S3. Results obtained with the tested compounds in three different zebrafish angiogenesis assays

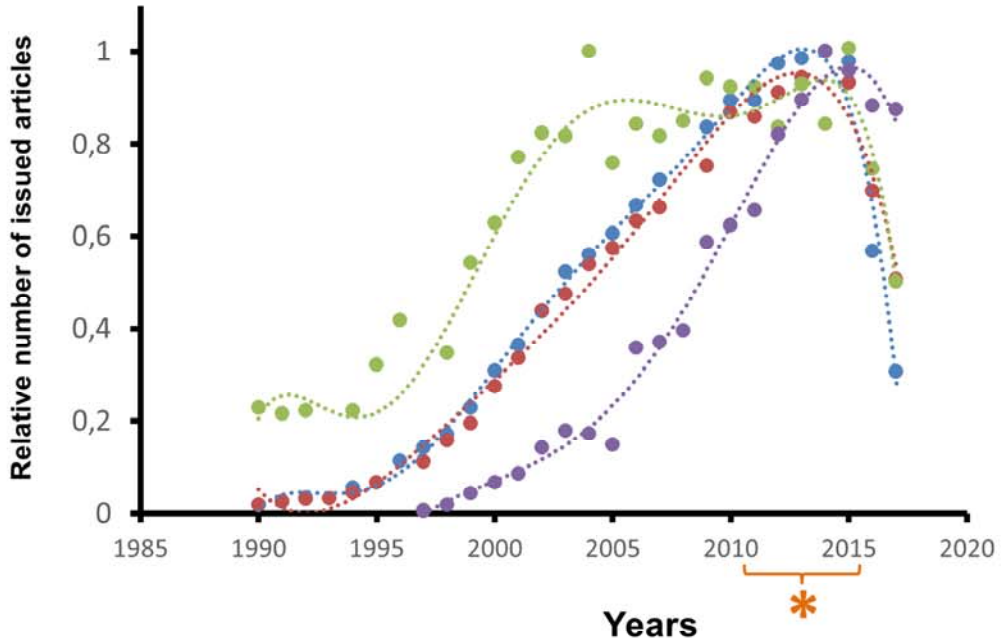
Compound / Assay	*Control (DMSO)*	*Genistein*	*Kahweol*	*2-ME*	*MMF*	*Sunitinib*	*SU4312*
Intersegmental vessels	-	-	±	+	-	+	+
Videos	-	-	+	+	-	+	+
Fin regeneration	-	±	+	+	-	+	+

(+) clear antiangiogenic effect; (-) clear non-antiangiogenic effect; (±) partial antiangiogenic effect

Table S4. Advantages and disadvantages observed in the three different zebrafish angiogenesis assays

Intersegmental vessels assay		Videos		Fin regeneration assay	
Advantages	*Disadvantages*	*Advantages*	*Disadvantages*	*Advantages*	*Disadvantages*
• Zebrafish embryos are an easy-handling. • Since embryos are transparent, the visualization of the drug effects is easy. • It only requires small compound concentrations. • This assay can be automated. • It is a fast assay. • In few days, many embryos can be treated and many results can be obtained in a short time.	• Although this assay is good for the identification of VEGF/VEGFR inhibitors, antiangiogenic compounds acting on different molecular targets may render false negatives .	• Those derived from the use of zebrafish embryo. • It complements the information obtained with the intersegmental vessels assay. Sometimes, drugs do not inhibit vessel formation, but blood circulation through intersegmental vessels is compromised or abolished. These effects on blood circulation are easily observed in the movies.	• Inadequate tricaine concentration used for zebrafish immovilization can affect the cardiac rate and the blood circulation through the intersegmental vessels.	• This assay provides clear results, with low frequency of odd results. • It provides a better approximation to the toxicity of the tested reagents.	• Use large working concentrations and subsequent agents quantity. This can be partially reduced if using juvenile zebrafish. • Absence of blood vessel formation is always associated to regeneration outgrowth arrest. This precludes any direct conclusion over angiogenesis.

Figure S1. The rise and fall of the use of different model species in research within the area of angiogenesis.



Violet = zebrafish
Green = chick
Red = mouse
Blue = human
Asterisk: years of depletion

Figure S2. Morphology of the embryonic tail bud and the caudal fin of the Tg(fli1a:EGFP)y1 zebrafish line.

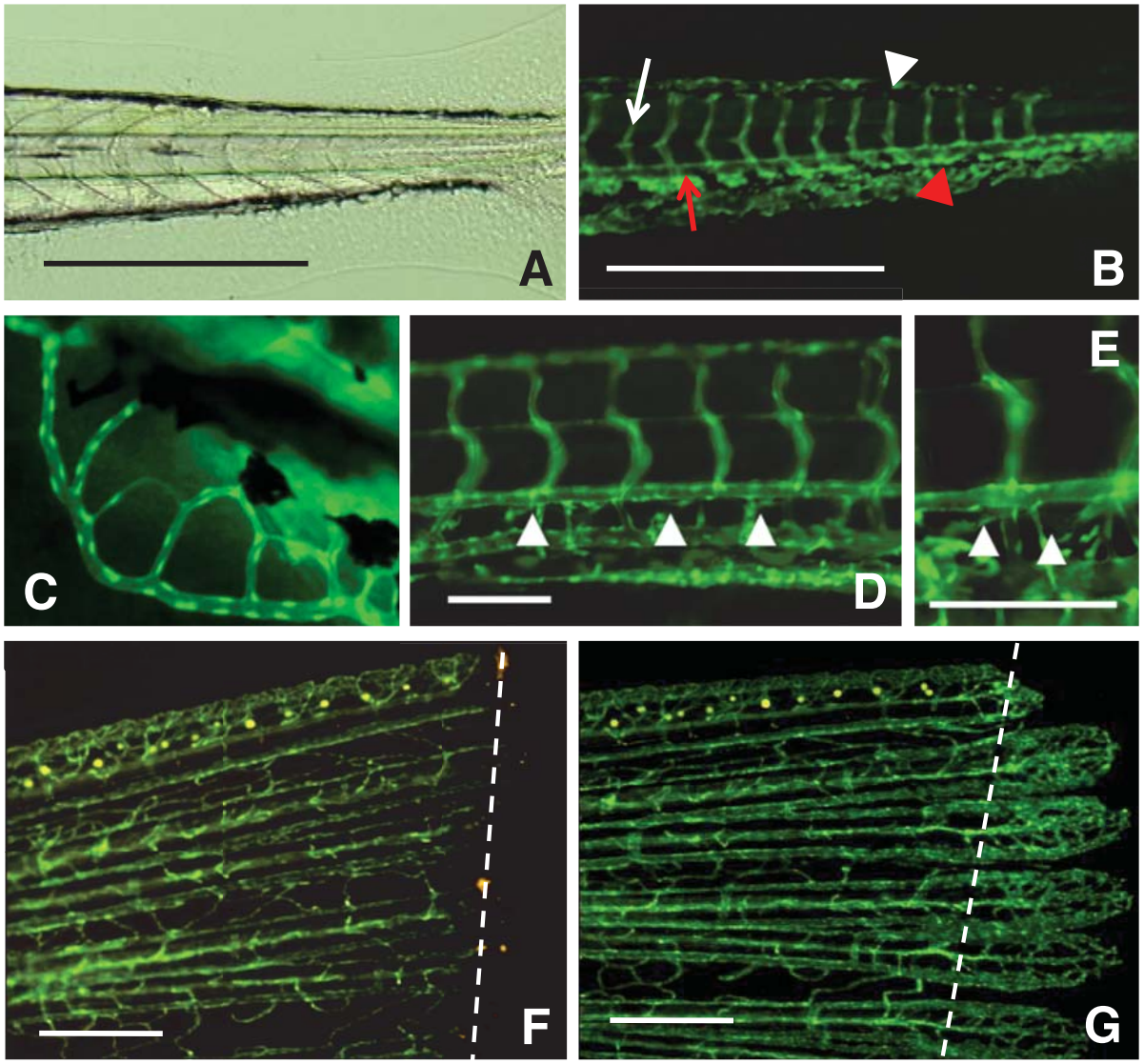
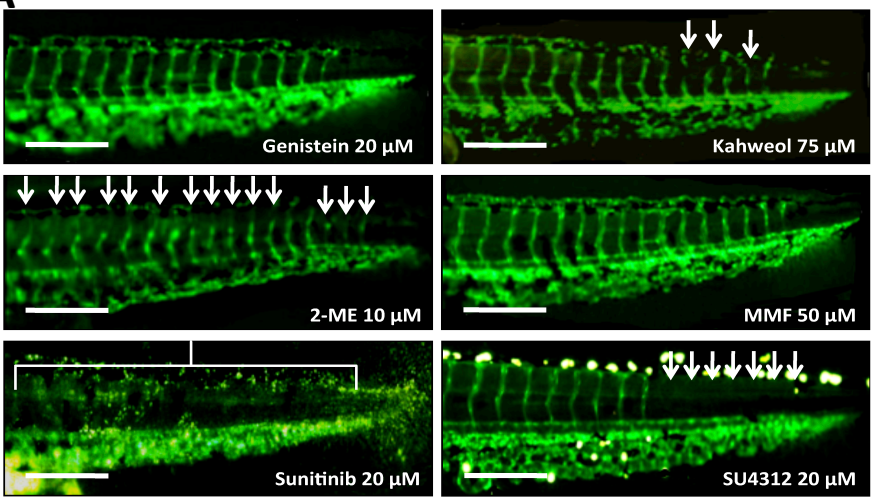


Figure S3. Use of three different assays to screen antiangiogenic compounds in zebrafish.

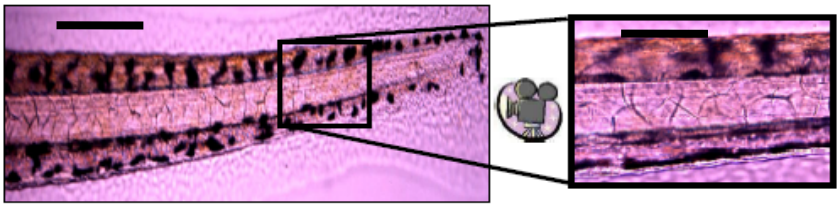
A



B

Compounds	ISV assay	
	Positive/ Total	% inhibition
DMSO	1/50	2
Genistein 20 µM	3/45	6.7
Kahweol 75 µM	17/32	53.1
2-ME 10 µM	25/30	83.3
MMF 50 µM	1/35	2.9
Sunitinib 20 µM	31/31	100
SU4312 20 µM	30/30	100

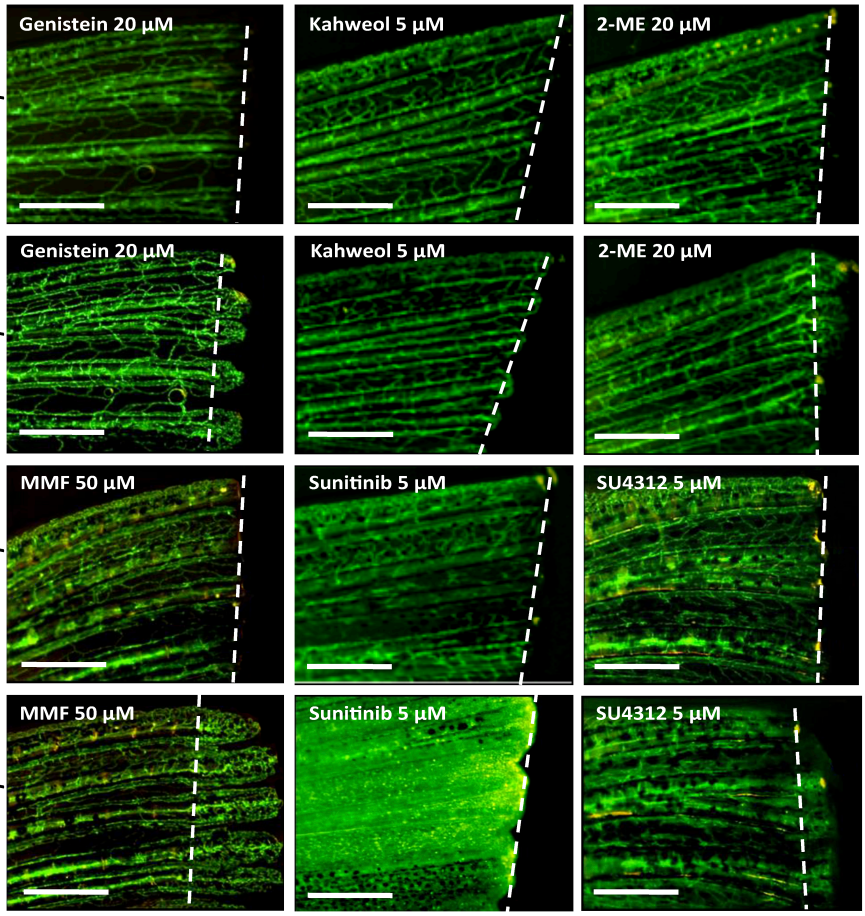
C



D

Compounds	Blood circulation movies	
	Positive/ Total	% inhibition
DMSO	0/25	0
Genistein 20 µM	8/24	33.3
Kahweol 75 µM	13/20	65
2-ME 10 µM	21/25	84
MMF 50 µM	0/20	0
Sunitinib 20 µM	22/22	100
SU4312 20 µM	20/20	100

E



F

Compounds	Caudal fin regeneration assay	
	Positive/ Total	% inhibition
DMSO	0/5	0
Genistein 20 µM	2/5	40
Kahweol 75 µM	4/5	80
2-ME 10 µM	4/5	80
MMF 50 µM	0/5	0
Sunitinib 20 µM	5/5	100
SU4312 20 µM	5/5	100

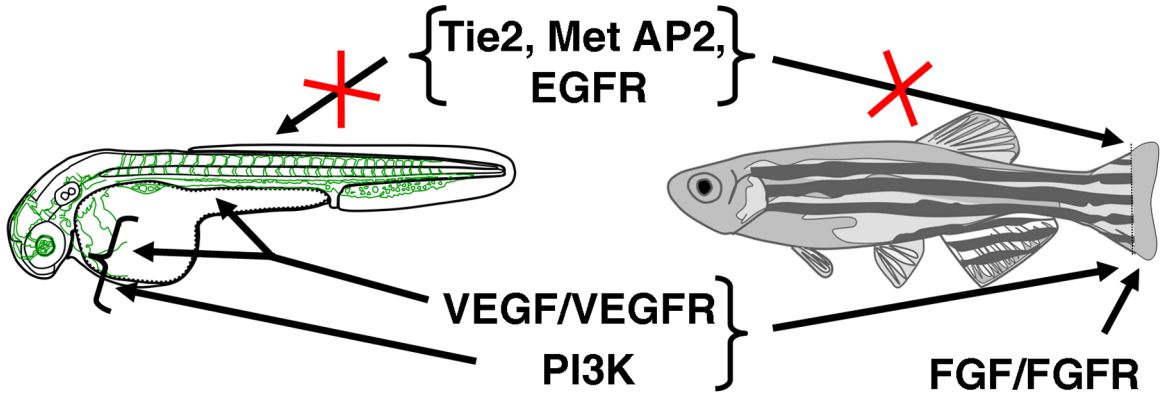
Figure S4. A version of Figure 2 including bibliographic references.

A

Compound	Target	ISV	SIV	Fin regeneration	Mouse	Chick
Bevacizumab	VEGF		(1)		(2)	(3)
Sorafenib	VEGFR-2, PDGFRβ	(4)			(5)	
Sunitinib	VEGFR-1-2, PDGFRσ/β	(4,6)*	(1)	*		
Pazopanib	VEGFR-1-3, PDGFRβ, FGFR-1-2	(6)	(1)			
SU5416 (semaxinib)	VEGFR2, PDGFR, Flt-1, Flt-4, c-kit	(7)	(1)		(8)	(9)
PTK787 (vatalanib)	VEGFR-2, VEGFR-1, PDGF, Flt-4, and c-Kit	(10)	(1)	(11)	(12)	(13)
SU4312	VEGFR2, PDGFR	*		*		
DMF	VEGFR2 and HIF1a expression, AKT, Erk	(14)		(14)	(14)	(14)
Toluquinol	VEGFR3, AKT	(15)		(15)	(15)	(15)
AD0157	VEGFR2-3 and downstream mediators	(16)			(16)	(16)
Damnacanthal	VEGFR1-3, FGFR1, 2 and 4,c-Met and EGFR.	(17)			(17)	(18)
Kahweol	VEGFR2?	(18)*		*	(18)	(18)
QODG (Quercetin derivative)	VEGFR2	(19)		(19)		
2-Methoxyestradiol	Apoptosis, HIF1a	*		*	(20)	(20)
Genistein	PI3K/AKT/HIF-1α/VEGF and NFI-κB/COX-2	*		*	(21)	(22)
LY294002	PI3K	(23)		(24)		
SU5402	FGFR2	(25)		(26)		
Suramin	FGF		(1)		(27)	(28)
PD173074	FGFR1	(4)			(29)	
CAS 948557-43-5	Tie2 Kinase Inhibitor	(4)			(30)	
Erlotinib	EGFR	(31)	(1)		(31)	(32)
TNP470	MetAP2 (Endothelial proliferation)	(6)	(1)		(33)	(34)
Fumagillin	MetAP2 (Endothelial proliferation)	(4)			(34)	(34)
Paclitaxel	destabilization of microtubules	(4)			(35)	(36)
Aeroplysinin-1	Angiogenic growth factor signaling, AKT	*			(37)	(37)

- Our own results, as shown in figure **S3.**

B



Additional bibliography cited in Table within Figure S4

1) Chimote, G. *et al*. (2014) Comparison of effects of anti-angiogenic agents in the zebrafish efficacy-toxicity model for translational anti-angiogenic drug discovery. *Drug Des Devel Ther*. 8, 1107-1123

2) Lee, S.Y., *et al*. (2008) Inhibitory effect of bevacizumab on the angiogenesis and growth of retinoblastoma. *Arch Ophthalmol*. 126, 953-958

3) Nowak-Sliwinska, P. *et al*. (2010) Processing of fluorescence angiograms for the quantification of vascular effects induced by anti- angiogenic agents in the CAM model. *Microvasc Res*. 79, 21-28

4) Letamendia, A. *et al*. (2012) Development and validation of an automated high-throughput system for zebrafish in vivo screenings. *PLoS One*. 7, e36690

5) Murphy, D.A. *et al*. (2006) Inhibition of tumor endothelial ERK activation, angiogenesis, and tumor growth by sorafenib (BAY43-9006). *Am J Pathol*. 169, 1875-1885

6) Beedie, S.L. *et al*. (2016) Shared mechanism of teratogenicity of anti-angiogenic drugs identified in the chicken embryo model. *Sci Rep*. 6:30038

7) Crucke, J. and Huysseune, A. (2015) Blocking VEGF signaling delays development of replacement teeth in zebrafish. *J Dent Res*. 94, 157-165

8) Litz, J. *et al*. (2004) The multi- targeted kinase inhibitor SU5416 inhibits small cell lung cancer growth and angiogenesis, in part by blocking Kit-mediated VEGF expression. *Lung Cancer*. 46, 283-291

9) Wang, L.L. (2004) Antitumor activities of a novel indolin-2-ketone compound, Z24: more potent inhibition on bFGF-induced angiogenesis and bcl-2 over-expressing cancer cells. *Eur J Pharmacol*. 502, 1-10

10) Chan, J. *et al*. (2002) Dissection of angiogenic signaling in zebrafish using a chemical genetic approach. *Cancer Cell*. 3, 257-267

11) Bayliss, P.E. et al. (2006) Chemical modulation of receptor signaling inhibits regenerative angiogenesis in adult zebrafish. *Nat Chem Biol*. 2, 265-273

12) Qian, D.Z. *et al*. (2004) The histone deacetylase inhibitor NVP-LAQ824 inhibits angiogenesis and has a greater antitumor effect in combination with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584. *Cancer Res*. 64, 6626-6634

13) Cruz, A. *et al*. (2001) Endothelin-1, a regulator of angiogenesis in the chickchorioallantoic membrane. *J Vasc Res*. 38, 536-545

14) García-Caballero, M. *et al*. (2011) Dimethylfumarate inhibits angiogenesis in vitro and in vivo: a possible role for its antipsoriatic effect? *Invest Dermatol*. 131, 1347-1355

15) García-Caballero, M. *et al*. (2013) Toluquinol, a marine fungus metabolite, is a new angiosuppresor that interferes with the Akt pathway. *Biochem Pharmacol*. 85, 1727-1770

16) García-Caballero, M. *et al*. (2014) The marine fungal metabolite, AD0157, inhibits angiogenesis by targeting the Akt signaling pathway. *Mar Drugs*. 12, 279-299

17) García-Vilas, JA. *et al*. (2017) The noni anthraquinone damnacanthal is a multi-kinase inhibitor with potent anti- angiogenic effects. *Cancer Lett*. 385, 1-11

18) Cárdenas, C. *et al*. (2011) Anti-angiogenic and anti-inflammatory properties of kahweol, a coffee diterpene. *PLoS One*. 6, e23407

19) Lin, C. *et al*. (2012) Quercetin-4'-O-β-D-glucopyranoside (QODG) inhibits angiogenesis by suppressing VEGFR2-mediated signaling in zebrafish and endothelial cells. *PLoS One*. 7, e31708

20) Fotsis, T. *et al*. (1994) The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. *Nature*. 368, 237-239

21) Wietrzyk, J. *et al*. (2001) Antiangiogenic and antitumour effects in vivo of genistein applied alone or combined with cyclophosphamide. *Anticancer Res*. 21, 3893-3896

22) Kiriakidis, S. (2005) Novel tempeh (fermented soyabean) isoflavones inhibit in vivo angiogenesis in the chicken chorioallantoic membrane assay. *Br J Nutr*. 93, 317-323

23) Sasore, T. and Kennedy, B. (2014) Deciphering combinations of PI3K/AKT/mTOR pathway drugs augmenting anti-angiogenic efficacy in vivo. *PLoS One*. 9, e105280.

24) Alvarez, Y. *et al*. (2009) Selective inhibition of retinal angiogenesis by targeting PI3 kinase. *PLoS One*. 4, e7867

25) Zhang, Z.R. *et al*. (2014) In vivo angiogenesis screening and mechanism of action of novel tanshinone derivatives produced by one-pot combinatorial modification of natural tanshinone mixture from Salvia miltiorrhiza. *PLoS One*. 9, e100416

26) Kawakami, A. *et al*. (2004) Early fin primordia of zebrafish larvae regenerate by a similar growth control mechanism with adult regeneration. *Dev Dyn*. 231, 693-699

27) Pesenti, E. *et al*. (1992) Suramin prevents neovascularisation and tumour growth through blocking of basic fibroblast growth factor activity. *Br J Cancer*. 66, 367-372

28) Danesi, R. *et al*. (1993) Suramin inhibitd bFGF-induced endothelial cell proliferation and angiogenesis in the chick chorioallantoic membrane. *Br J Cancer*. 68, 932-938

29) Dimitroff, C.J. *et al*. (1999) Anti-angiogenic activity of selected receptor tyrosine kinase inhibitors, PD166285 and PD173074: implications for combination treatment with photodynamic therapy. *Invest New Drugs*. 17, 121-135

30) Semones, M. *et al*. (2007) Pyridinylimidazole inhibitors of Tie2 kinase. *Bioorg Med Chem Lett*. 17(17), 4756-4760

31) Pan, Y. *et al*. (2015) A preclinical evaluation of SKLB261, a multikinase inhibitor of EGFR/Src/VEGFR2, as a therapeutic agent against pancreatic cancer. *Mol Cancer Ther*. 14(2), 407-418

32) Berndsen, R.H. *et al*. (2017) Combination of ruthenium(II)-arene complex [Ru(η6-p- cymene)Cl2(pta)] (RAPTA-C) and the epidermal growth factor receptor inhibitor erlotinib results in efficient angiostatic and antitumor activity. *Sci Rep*. 7, 43005.

33) Yoshida, T. *et al*. (1998) Suppression of hepatoma growth and angiogenesis by a fumagillin derivative TNP470: possible involvement of nitric oxide synthase. *Cancer Res*. 58, 3751-3756

34) Kusaka, M. *et al*. (1991) Potent anti- angiogenic action of AGM-1470: comparison to the fumagillin parent. *Biochem Biophys Res Commun*. 174, 1070-1076

35) Belotti, D. *et al*. (1996) The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res*. 2, 1843-1849

36) Vacca, A. *et al*. (2002) Docetaxel versus paclitaxel for antiangiogenesis. *J Hematother Stem Cell Res*. 11, 103-118

37) Rodríguez-Nieto, S. *et al*. (2002) Antiangiogenic activity of aeroplysinin-1, a brominated compound isolated from a marine sponge. *FASEB J*. 16, 261-263

Supplementary figure legends

Figure S1. The rise and fall of the use of different model species in research within the area of angiogenesis. Bibliometric search in Scopus were carried out using combinations of terms, such as <<angiogenesis>> and each model species including humans. The temporal evolution of the relative number of references is depicted. For each species, the number of references published in each year is relativized taking as relative value 1 the maximum number of articles published within a year. Asterisk indicates the years of the global economic crisis.

Figure S2. Morphology of the embryonic tail bud and the caudal fin of the *Tg(fli1a:EGFP)y1* zebrafish line. **(A-B)** Tail bud of 10 dpf (A) and 3 dpf (B) zebrafish embryos. The V-shaped muscle segments are the myomeres (A). (B) White and red arrows are intersegmental vessels and dorsal aorta, respectively. White and red arrowheads indicate dorsal longitudinal anastomotic vessel and caudal vein plexus, respectively. **(C)** Subintestinal vessels in a 3 dpf embryo (Reprinted from Journal of Ethnopharmacology, 151, Liu et al., Molecular mechanisms of angiogenesis effect of active sub-fraction from root of Rehmannia glutinosa by zebrafish sprout angiogenesis-guided fractionation, 565–575., Copyright (2014), with permission from Elsevier). **(D-E)** Embryonic thoracic duct (white arrowheads) after 4 days of incubation with DMSO (lymphagiogenesis analyses). **(F-G)** 0 (F) and 3 (G) dpa fin regenerates showing the formation of blood vessels in the fin blastema under DMSO treatment (G). White dashed line indicates amputation level. Bars represent 500 (A-B), 100 (D-E) and 150 (F-G) μm.

Figure S3. Three different assays to screen antiangiogenic compounds in zebrafish. **(A)** Intersegmental vessels assay in zebrafish embryos incubated with six different compounds and **(B)** table with the results obtained with each compound: 20 μM genistein do not inhibit the formation of intersegmental vessels, 75 μM kahweol slightly inhibits intersegmental vessels formation (arrows), 10 μM 2-methoxy-estradiol (ME) interrupts the growth of all the intersegmental vessels (arrows), 50 μM monomethlylfumarate (MMF) do not inhibit intersegmental vessels formation, 20 μM sunitinib removes all intersegmental vessels (bracket) and 20 μM SU4312 removes about seven caudal vessels (arrows). Bars represent 100 μm. Table shows the number of positive fishes over the total and the calculated percentage (% inhibition) affected by each treatment. **(C)** Embryonic regions observed in the videos and **(D)** table with the

results obtained with the different compounds. Bars represent 50 in low and 150 μm in high magnification. **(E)** Photographs of fin cuts showing the effects of six compounds on the formation of blood vessels in the caudal fin regeneration assay and **(F)** table with the results of these assays: 20 μM genistein slightly inhibits vessels and fin regeneration, 5 μM kahweol completely inhibits blastema formation, 20 μM 2-ME significantly impairs blood vessel and 3 dpa blastema formation, 50 μM MMF does not inhibit either vessels formation or early caudal fin regeneration, 5 μM sunitinib completely suppresses blastema formation, and 5 μM SU4312 affects fin regeneration almost completely, and new blood vessels are not well formed. White dashed line indicates the cut level at 0 dpa. Bars represent 100 μm.

Figures S4. This is a version of Figure 2 that contains the bibliographic references in which its contents is based. **(A)** Table containing the information regarding the antiangiogenic activity exhibited by several angiogenesis inhibitors. **(B)** Scheme summarizing the results presented in the upper table. A free arrow indicates that compounds acting on a given target show a positive inhibition in a particular zebrafish-based in vivo assay. A crossed arrow indicates that no antiangiogenic activity is detected by means of that assay.