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Fishing anti(lymph)angiogenic drugs with zebrafish

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Zebrafish, an amenable small teleost fish with a complex mammal-like circulatory system, is being increasingly used for drug screening and toxicity studies. It combines the biological complexity of *in vivo* models with a higher-throughput screening capability compared with other available animal models. Externally growing, transparent embryos, displaying well-defined blood and lymphatic vessels, allow the inexpensive, rapid, and automatable evaluation of drug candidates that are able to inhibit neovascularisation. Here, 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.

Introduction

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 has key roles in development and growth. By contrast, in adults, it is only related to reproductive cycles, wound healing, or bone repair. Nevertheless, deregulated and persistent angiogenesis occurs in angiogenesis-related diseases, such as proliferative retinopathies, psoriasis, rheumatoid arthritis, and tumour growth or metastasis [1,2]. Angiogenesis is one of the hallmarks of cancer, where it has 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 many patients benefitting from their clinical use. Since bevacizumab, a humanised monoclonal antibody that blocks vascular endothelial growth factor A (VEGF), was approved for the treatment of metastatic colorectal cancer in 2004, an increasing number of antiangiogenic therapies for cancer, almost all of them blocking the activation of endothelial cells by VEGF, are gaining approval (Table S1 in the Supplemental material online) [4]. Nevertheless, limitations to the clinical success of anti-VEGF therapies, including intrinsic or acquired resistance after months of treatment, indicate the need to search for new antiangiogenic drugs as well as new therapy strategies based on the combined targeting of different pathways in tumour angiogenesis [5].

Q4 Recently, the lymphatic system, which also has a vital role in normal and pathological processes, has received significant interest. In healthy situations, the main functions of lymphatic vessels are to: (i) collect the excess of protein-rich fluid extravasated from blood vessels; (ii) transport this fluid back into the blood circulation; and (iii) 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 embryonic development but, under adult physiological conditions, is restricted to the endometrium during pregnancy. However, defective or excessive lymphangiogenesis can lead to diseases involving lymph accumulation in tissues, dampened immune responses, connec-

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tive tissue and fat accumulation, organ transplant rejection, and cancer or metastatic dissemination [7,8].

Given the role of excessive angiogenesis and lymphangiogenesis in tumour growth and metastasis, as well as in other diseases, the identification of new drugs that can inhibit these processes remains an urgent need. Thus, the development of new, reliable and accurate *in vitro* and *in vivo* models is required. Currently, different *in vitro*, *ex vivo*, and *in vivo* systems are being applied to the screening and characterisation of new lymph/angiomodulators, each with their own advantages and disadvantages. However their combination is necessary to gain insight into 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 Ref. [9]) offer information about the endothelial cell behavior 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 analyze vessel sprouting from vascular explants, although they do not allow the study of circulating endothelial progenitors recruited during the angiogenic process or the hemodynamic forces that have roles in angiogenesis, vascular remodeling, and maturation [10]. By contrast, *in vivo* models reproduce the cellular and molecular features involved in new vessel formation and the effect of modulators on the whole organism, giving a more-complete overview of the putative effects of the studied drug, compared with *in vitro* assays. However, a combination of cell- 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, the 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 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 ability 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 solubilising agents, such as 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 usually has 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 for either statistical calculations or high-throughput screening. Moreover, the availability of diverse molecular tools and transgenic zebrafish lines can provide clues for the mechanism of action and the therapeutic window of the drug candidates. Although the results obtained with zebrafish models would eventually have to be confirmed in a mammalian system (usually murine based), the incorporation of zebrafish *in vivo* assays in drug discovery appears to be a logical option to speed

TABLE 1

Q9 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–moderate amounts of test agents required	Nonmammalian: results must be validated in mammalian systems for potential clinical application; embryonic; difficult to evaluate (use of at least two blind evaluators is advisable); nonspecific inflammatory reactions can occur; tools unavailable to characterise molecular mechanism; actual concentrations of test compounds depend on diffusion from disc
 Mouse (Matrigel plug, sponge, corneal micropocket, disc assay, etc.)	Mammalian; tools to characterise molecular mechanism are available; some permit long-term monitoring; quantitative assays	Expensive; time consuming; ethically questionable on occasion; technically demanding; higher amounts of test agents required; nonsuitable for primary assays in medium–large-scale screening
 Zebrafish (ISV, SIV, caudal fin regeneration . . .)	Tools to characterise molecular mechanism available; quantitative; fast; suitable for high-throughput screening; automated in 96-well plates; simple manipulation; many transgenic zebrafish lines available; small amounts of test agents required; statistically significant numbers of embryos can be used for each assay; real concentrations of test compounds are known; yield useful information regarding pharmacological profile and toxicity of test agents (therapeutic windows)	Nonmammalian: results must be validated in mammalian systems for potential clinical application; embryonic (mostly); small size of embryos can make some observations challenging; specialised breeding conditions required; caudal fin amputation studies require higher working volumes and, therefore, higher amounts of test agents

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