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Zebrafish based small molecule screens for novel cardiovascular drugs

Peter Novodvorsky[1,2], Marc MJ Da Costa[1,2], Timothy JA Chico[1,2]

[1]MRC Centre for Developmental and Biomedical Genetics, University of Sheffield

[2]Department of Cardiovascular Sciences, University of Sheffield

[1]MRC Centre for Developmental and Biomedical Genetics, NIHR Cardiovascular Biomedical Research Unit, Sheffield Teaching Hospitals NHS Trust, [2]Department of Cardiovascular Science, University of Sheffield

Lab D38, University of Sheffield, Firth Court, Sheffield S10 2TN, UK.

t.j.chico@sheffield.ac.uk

Tel 00 44 114 222 2396

Fax 00 44 114 276 5413

Abstract

The zebrafish is increasingly being adopted as an in vivo model of high throughput drug screening. In this brief review we outline the advantages and disadvantages of this approach and summarize recent screens that have attempted to identify novel small molecules with activity on the cardiovascular system.

Introduction

The zebrafish (*Danio rerio*) is a popular tropical pet fish that has been exploited for many years as a genetically tractable model of developmental biology [1]. Since the egg is fertilized after laying, zebrafish embryos develop externally to the mother, making them accessible for injection with genetic constructs or exposure to chemical treatment from the earliest stages. The near transparency of early embryos allows observation of organ development without the need for invasive manipulation. Tissue specific transgenics that express fluorescent reporters in the heart [2], endothelium [3,4] or blood [5] have allowed cardiovascular researchers to visualize cardiovascular development in a manner impossible using mammalian models. More recently, the zebrafish has been applied to drug discovery in attempts to identify small molecules with potential therapeutic effects. The first zebrafish based small molecule screen was published in 2000 [6], since when the results of at least fifty subsequent screens have been described. As with any model system, the zebrafish possesses advantages and disadvantages that must be appreciated when considering a zebrafish based small molecule screen, and we outline these below.

Advantages of zebrafish screens

The zebrafish boasts many advantages for drug screening. Fertile females lay several hundred eggs per week, which simply require incubation in medium to allow development until the onset of feeding at around 5 days post fertilization (dpf). Embryonic development proceeds rapidly; by 24 hours post fertilization (hpf) a recognizable vertebrate body plan is in place, with a primitive vasculature and a two chambered beating heart [7]. Embryos can be automatically dispensed into 96 well plates (generally 3-5 embryos/well) and survive in low volumes of liquid (100μl). Robotic platforms allow rapid application of precise concentrations of small molecule libraries to the medium [8]. These generic features are common to almost all zebrafish-based screens, varying only in the developmental stage of embryo used, the libraries tested, and the zebrafish strain used. These may be transgenics, expressing a tissue specific fluorescent reporter in an organ of interest, mutants with a phenotype the screen aims to rescue or influence, or wildtype embryos, depending on the drug effect being sought. If modulation of expression of a known gene is the effect sought, whole mount in situ hybridization can be performed in an automated manner to assess expression of the target gene in hundreds of embryos in parallel. **Figure 1** shows a typical workflow for a zebrafish-based screen. Once a possible "hit" is identified, rapid rescreening in different doses allows assessment of the drug effect in a more rigorous manner.

Disadvantages of zebrafish screens

Each screen needs a specific assay, and the nature of this is critical to the screen's ability to identify true "hits" without an unacceptable number of false positives

and false negatives. A good screen needs a reproducible, rapidly measurable endpoint with minimal variability, and ideally will include positive and negative controls that allow assessment of technical reliability. These considerations limit screens for physiological parameters such as blood flow that are highly variable in early embryos. Although the nature of the screen to some extent determines the throughput, in general at most only a few thousand molecules are tested per screen. This limitation means that most studies use libraries of molecules known to have some bioactive properties, rather than a truly unbiased screen as can be applied to *in vitro* screening efforts. Once a hit is identified in a zebrafish screen, its mechanism of action is not known, and this can cause difficulty in lead optimization and prediction of other, potentially harmful effects. Most screens test only a single concentration and this, coupled with unpredictable drug penetration, is likely to lead to a significant false negative rate. Although vascular delivery of drugs is possible via microinjection, this is technically challenging and unlikely to be scalable to a level that allows high throughput screening. Although the zebrafish and mammalian genomes are similar, levels of protein homology are generally low, and genome duplication in the fish has led to multiple zebrafish homologues of many human genes [9]. These features raise the possibility that a drug with effect in zebrafish may have no effect on the human homologue due to protein dissimilarity, while a drug that targets a human protein may have no effect on a duplicated zebrafish gene product due to duplication. It remains to be seen to what extent these disadvantages limit the ability of zebrafish -based drug discovery to identify therapeutic compounds for human use.

Screening for drugs with novel cardiovascular effects in zebrafish

Below, we summarize some recent screens that illustrate features of screen design and that have identified promising lead compounds; these are also summarized in **Table 1**.

A zebrafish screen to identify anti-arrhythmics

Long QT syndrome (LQTS) in humans predisposes to lethal cardiac arrhythmias and can be caused by genetic diseases or factors such as drugs. Such effects are a common cause of drug withdrawal after marketing when pro-arrhythmic effects become manifest [10].

To identify small molecules that shorten the QT interval, Peal et al exploited the *breakdance* zebrafish mutant [11]. This mutant, previously generated in a forward mutagenesis screen [12], suffers 2:1 AV block caused by QT prolongation induced by a mutation in the potassium channel KCNH2. Visual assessment of the embryos was used to assess whether this AV block was reversed by any of the small molecules examined. From 1200 drugs, Flurandrenolide and 2-methoxy-N-(4-methylphenyl) benzamide (2-MMB) rescued the 2:1 AV block phenotype. Both exert their function in a dose-dependent manner by shortening repolarization time as evidenced by voltage-sensitive optical mapping of ventricular action potential duration. These agents therefore may represent potential therapeutic strategies for the treatment of LQTS.

Zebrafish screens to identify drugs for hyperlipidemia

To identify compounds that inhibit dietary absorption of lipid, zebrafish embryos were fed the fluorescent phospholipid reporter PED-6, which is normally taken up and can be visualized in the gall bladder [13]. The ability of 3840 compounds to prevent this uptake was tested in 5dpf embryos exposed to test compounds prior to feeding. Embryos were examined by fluorescent stereomicroscopy for quantification of gall bladder fluorescence, and compounds that reduced this were considered as primary hits. This screen suffered from a significant false positive rate (1.1%) but was able to identify a number of compounds (whose identities were not revealed) that reliably prevented lipid uptake in rescreening and secondary screens.

Zebrafish screens to identify drugs that modulate angiogenesis

The earliest cardiovascular drug screens in zebrafish sought to identify agents that influence angiogenesis [14], and this remains the most frequent assay in more recent screens. The availability of endothelial specific transgenics, and the stereotypical and easily observed vascular development in zebrafish has made screening for such molecules particularly successful. Although early screens aimed to identify pan-angiogenesis inhibitors, later studies often aim to find drugs that preferentially affect specific vascular beds (see below).

Xyloketals are natural compounds isolated from an endophytic fungus *Xylaria* sp. from the South China Sea [15] with vasorelaxant and angiogenic activities [16,17]. Xu et al. synthesised 21 novel xyloketal derivatives and tested their effect on angiogenesis in *Fli1:GFP* transgenic zebrafish that express GFP in the endothelium [17]. When the effect on the subintestinal vein was examined,

several of these compounds induced increased branching of this vascular plexus, suggesting that they may promote angiogenesis.

Arterial and venous angiogenesis are differentially regulated during embryogenesis. A recent screen aimed to identify compounds that selectively regulate these two processes [18]. The formation of the caudal vein plexus (CVP) in *Fli1:GFP* transgenics was used as a model for venous angiogenesis and the formation of primary intersegmental vessels (ISV) was used as the readout for arterial angiogenesis. From 469 compounds, the investigators found that aplexone preferentially suppresses venous angiogenesis while having no effect on primary ISV formation. Aplexone was found to exert its effects via the HMG-CoA pathway as evidenced by increased expression of several enzymes of this pathway and decreased cholesterol levels in aplexone-treated embryos. As a result of this, venous ECs exhibit defective posttranslational protein modification geranylgeranylation when treated with aplexone [19,20].

In keeping with the observation that some agents are able to disrupt angiogenesis in specific parts of the vasculature while having little effect on others, another group attempted to discover drugs that alter development of the embryonic zebrafish retinal vasculature while sparing angiogenesis elsewhere [21]. From 2000 molecules tested, five reproducibly caused specific changes in retinal vasculature; enalapril maleate, zearalenone, pyrogallin, albendazole and mebendazole.

Supporting the important role of the HMG-CoA pathway in angiogenesis, a separate screen for small molecules that prevents ISV angiogenesis identified a number of HMG-CoA inhibitors (statins) as anti-angiogenic, leading to the authors' suggestion that these agents may be able to reduce tumour angiogenesis

as an adjuvant to cancer therapy [22]. This is intriguing, given the epidemiological evidence suggesting that statins when taken for prevention of cardiovascular disease may confer some protection from cancer, although a definite link remains unproven [23].

A recent small screen of eleven compounds known to influence angiogenesis in other systems (recombinant vascular endothelial growth factor (VEGF), Tumour necrosis factor, Adalimumab, Infliximab, Bevacizumab, Oncostatin, Interleukin 1β, Nacyselyn and LY294002) also used retinal angiogenesis as its primary assay [24]. Although the selective PI3 Kinase inhibitor LY294002 was shown to inhibit retinal angiogenesis in a dose-dependent manner with no effect on trunk vasculature, the other agents tested had no effect. Although this may seem surprising given the clear role of VEGF in angiogenesis, and the clear reduction in embryonic vascular development seen with VEGF knockdown or small molecule inhibition [25,26], peptides such as monoclonal antibodies or growth factors are unable to penetrate the embryo sufficiently to influence vascular development, and as such the zebrafish is unsuited for screening the effects of such molecules.

Zebrafish screens are particularly well suited to assessment of natural products, which contain a diverse and poorly characterized combination of potentially bioactive molecules. In a screen of over 80 East African plant derived natural produces, extracts from *Oxygonum sinuatum* and *Plectranthus barbatus* were identified to have anti-angiogenic effect on ISV formation [27]. Once such an effect is detected, fractionation is required to identify which constituent is responsible for the biological effect.

Zebrafish screens to identify drugs that modulate cardiac development

Fibroblast growth factors (FGFs) play an important role in regulation of proliferation and embryonic development, exerting their effects via various signalling cascades including MAP/ERK kinases [28,29]. A transgenic reporter line for FGF that expresses GFP at sites of FGF activity was used in a small molecule screen to identify novel activators of FGF signalling [30]. From 5000 compounds tested, one ((E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one, or BCI) increased fluorescence in a concentration-dependent manner. In keeping with the central role for FGF in cardiac development, BCI treatment led to an expansion of cardiac progenitor cells with expanded cardiac tissue. The expansion of cardiac progenitors was accompanied by diminished expression of blood and vascular markers [30].

Conclusion

The slowing pace of new drug discovery has been well publicized [31], and remains in need of a solution. Until a drug that has been discovered in a zebrafish screen has been shown to possess clinical utility, the potential of zebrafish based screens to identify novel drugs remains unproven. Even if zebrafish screens are able to identify therapeutic moieties, given the relative infancy of this approach it is unrealistic to expect translation to clinical application for at least five years. Nevertheless, even without such a breakthrough, the screens so far conducted have allowed novel insights into disease related mechanisms and pathways. The increasing rate of publication of zebrafish-based screens suggests a growing acceptance of the technology and increasing momentum for its utilization in drug discovery efforts.

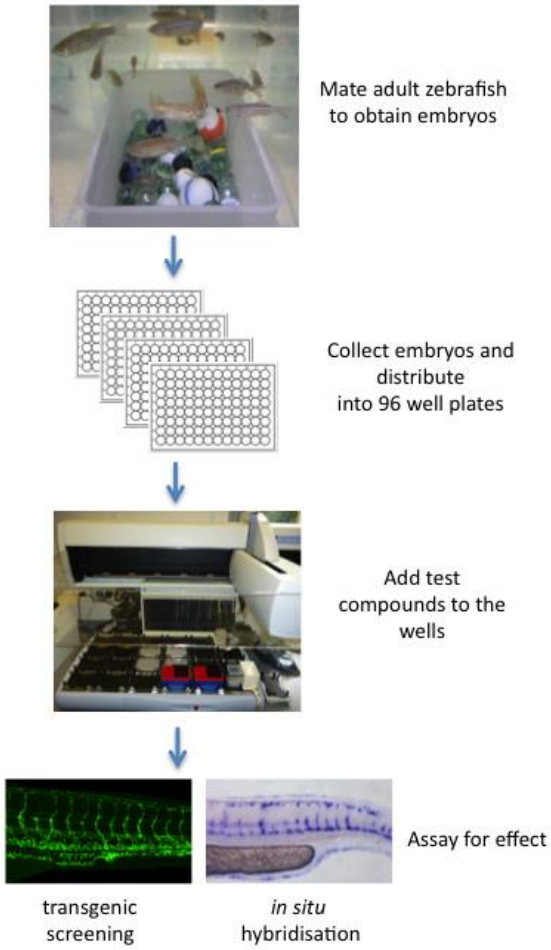

Figure 1

Generic workflow for small molecule screen in zebrafish showing the major steps involved from mating the adult zebrafish and collecting the embryos, followed by distribution of the embryos into 96 well plates and the addition of libraries of small molecules to screen. The end assay varies according to the effect sought, but frequently examines vascular development using transgenic embryos, or specific vascular gene expression assessed by in situ hybridization.

Reference	Effect sought	Drug libraries used	Drugs tested	Conc^{n} used	Zebrafish strains used	Stage drug added	Stage screened	Assay	Compounds identified
[11]	chemical suppressors of LQT Syndrome	Prestwick, Illkirch, France and Chembridge	1200	10ng/µL	*Breakdance*	48hpf	72hpf	visual screen for the presence of 2:1 AV block	Flurandrenolide and 2-methoxy-N-(4-methylphenyl) benzamide (2-MMB)
[13]	inhibitors of lipid absorption	MLSCN	3840	25µM	WT	5dpf	6dpf	qualitative visual assessment of gallbladder fluorescence	Not revealed
[17]	angiogenic activities of newly designed xyloketal derivatives	Newly synthesized xyloketal derivatives (1-21)	21	100µM	*Tg(fli1:EGFP)*	6hpf	78hpf	visual screen for the presence of ectopic subintestinal veins (SIV) branches	Xyloketals 2,3,4,5,8,9,12-16,19,20 and 22
[18]	compounds selectively suppressing arterial and venous angiogenesis	Biomol International (300 compounds), synthetic chemical library	468	10µM	*Tg*(*kdrl:GFP*)^{la116}	4-24hpf	48hpf	visual assay (confocal imaging) of ISV and caudal vein plexus angiogenesis	aplexone
[21]	compounds affecting vascular development in zebrafish retina	Spectrum Collection	approx. 2000	10µM	*Tg(fli1:EGFP)*	24hpf corresponding to 20 somite stage (19hpf) and 68hpf corresponding to pectoral fin stage (68hpf) respectively	1-6dpf	visual screen for morphological abnormalities in retinal vessel development	enalapril maleate, zearalenone, pyrogallin, albendazole and mebendazole
[22]	Angiogenesis inhibitors	Spectrum Collection	2000	10µM	*Tg(flk1:EGFP)*	20hpf	52hpf	quantitative visual screen for inhibition of ISV angiogenesis	simvastatin, mevastatin, lovastatin, rosuvastatin, isorotenone, dihydromunduletone and aristocholic acid

[24]	inhibitors of retinal angiogenesis	compounds known to affect angiogenesis	11	various	*Tg(flk1:EGFP)*	24hpf	5dpf	quantitative visual screen for the number of primary branches of hyaloid retinal vessels	LY294002 (PI3 kinase inhibitor)
[27]	medicinal plants for angiogenesis inhibitors	methanolic extracts from east African medicinal plants	over 80	various	*Tg(fli1:EGFP)*	16hpf	40hpf	visual screen for the inhibition of ISV angiogenesis	6-methyl-1,3,8-trihydroxyanthraquinone (emodin) and coleon A lactone ($C_{20}H_{19}O_6$)
[30]	activators of FGF signalling cascade, to study the role of FGF signalling in embryonic heart development	NCI diversity set (NCI/NIH), Natural Products library (MicroSource Discovery Systems Inc.),Phosphatase targted set (ChemDiv Inc.)	5000	10µM	*Tg*(*dusp6:EGFP*)^{pt6}	24hpf	30hpf	visual screen for reporter fluorescence	BCI ((*E*)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1*H*-inden-1-one)
[32]	biological activity of newly synthesized retinoic acid analogues	22 novel retinoid acid analogues	22	10µM	hybrid of AB and TU WT	5hpf	24hpf	visual screen for morphological alterations such as disruption of the body axis	retinoic acid analogue BT10

Table 1. Summary of recent cardiovascular screens. WT: wildtype, hpf: hours post fertilization, dpf: days post fertilization, ISV: intersegmental vessels, AV block: atrioventricular block, LQT Syndrome: long QT Syndrome,

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