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PRIMER

The zebrafish embryo as a model for assessing off-target drug effects

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Although first used experimentally for the genetic analysis of vertebrate development and neurobiology, the zebrafish has been adapted as a model for many human diseases. In recent years, the zebrafish embryo has increasingly attracted the attention of chemists and pharmacologists for its utility in identifying chemicals with pharmacological activity in a whole-animal context. Its experimental virtues make it an ideal system with which to identify new bioactive molecules, and to assess their toxicity and teratogenicity at medium-to-high throughput. More recently, the zebrafish embryo has been applied to identify off-target effects of drug candidates. Here, we discuss the value of the zebrafish embryo for detecting off-target effects, and propose that this model could be useful for improving the efficiency of the drug-development pipeline.

Introduction

An ideal drug is a compound that interacts with a single, well-defined molecular target, and that exerts the desired therapeutic effect with high affinity and specificity. Systematic attempts of the pharmaceutical industry to find or create such compounds have taught us that ideal drugs are in reality a great challenge to develop. Regardless of how carefully a given drug-target interaction has been assessed using in vitro tests, many drugs and drug candidates show off-target effects in a complex organism that need to be balanced against the potential therapeutic benefit for the patient. For example, drug-induced QT-interval prolongations as a consequence of drugs that affect cardiac repolarization still present unpredictable clinical problems (Camm et al., 2000; Keating and Sanguinetti, 2001). However, research has taught us that this is not unexpected. Most proteins have multiple functions and/or are structurally related members of protein families. In addition, compounds can interact with multiple, even structurally unrelated, target molecules.

The early phases of drug development involve in vitro assays that aim to determine drug-target affinity. Naturally, this does not enable an assessment of a compound's effective range of interactions nor its effects in the context of an intact organism; these evaluations are carried out in subsequent phases of drug development. Comprehensive tests in mammals are unrealistic for screening compounds on a large scale because they are laborious, time consuming and expensive. Hence, assays on mammals are usually employed only in late phases of the drug-development process. As a result, a high proportion of drug candidates fail at an advanced stage of drug development owing to the detection of intolerable toxic or off-target effects that were undetectable during earlier phases. It is therefore desirable to develop cheap, alternative models of sufficient complexity to enable systematic studies of a compound's mode of action and to understand the molecular nature of additional (unintended) targets at earlier stages of drug development. In this Primer article, we discuss the advantages of using the zebrafish embryo as an economical and physiologically relevant model to screen for off-target effects of drug candidates.

Advantages of the zebrafish embryo as a model

The zebrafish embryo offers an inexpensive system that combines many features that are desirable for the development of new approaches to drug development (Bowman and Zon, 2010). As a vertebrate, the zebrafish shares a high degree of conservation with mammalian systems: the genomes of zebrafish and humans are highly related and contain orthologous genes encoding enzymes and regulatory molecules that control similar aspects of development and body homeostasis. Moreover, many drugs used to treat human diseases have comparable effects in zebrafish embryos and humans (Peterson et al., 2004; Zon and Peterson, 2005; Barros et al., 2008). Human psychotropic drugs that are used to treat schizophrenia induce specific behavioral changes in zebrafish embryos, such as changes in the pattern of motility in response to a brief flash of light (Kokel et al., 2010). Thus, although it might not be possible to measure, for example, schizophrenia as such in a zebrafish embryo, the specific behavioral effects induced on exposure to a drug candidate might allow identification of new antipsychotic drug candidates in an efficient manner.

The technical aspects of working with zebrafish embryos also make them an attractive system for high-throughput screening approaches. Zebrafish embryos promptly develop ex utero into free-swimming, independently feeding larvae within 5 days post-fertilization. They are small and transparent and can be assayed in up to 384-multiwell plates, which permits the screening of compounds at a considerable scale at low cost. Promising developments in the automation of embryo handling and image acquisition should open up the prospects of screening on a scale of tens of thousands of molecules within a couple of weeks (Yang et al., 2009) (C.G. and Urban Liebel, unpublished results).

Over the last 20 years, the zebrafish research community has isolated and characterized several-thousand mutants. Many of these mutants mimic human diseases, including cancer, polycystic kidney diseases, myopathies, cardiomyopathies and neurodegeneration (Haffter et al., 1996; Wein-

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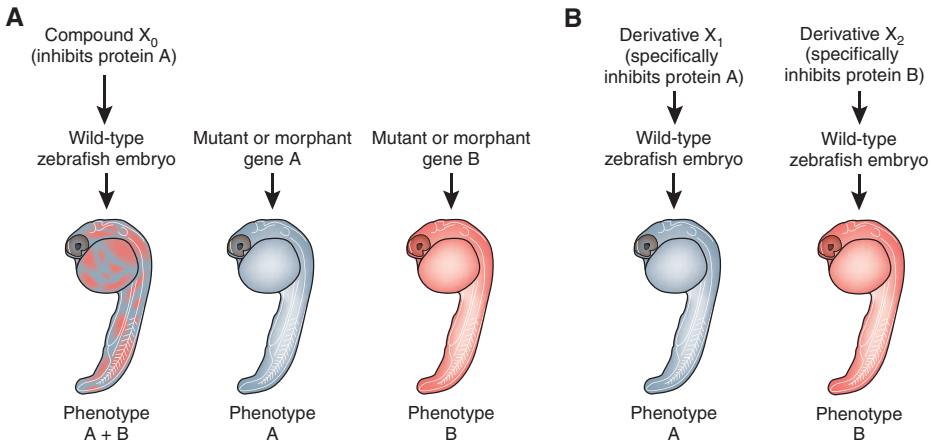

Fig. 1. The use of zebrafish embryos to assess off-target effects of drugs. Zebrafish mutants provide a blueprint for the effects of the loss of activity of proteins and, in combination with compound derivatization, can aid the development of drugs with greater specificity in whole animals. (A) Wild-type embryos treated with compound X_0, designed as an inhibitor of protein A, show phenotype B in addition to the phenotype A that is seen in embryos that are mutant for protein A. Phenotypic data of zebrafish mutants can be used to determine that the nature of the off-target effect is similar to that caused by a mutation in gene B. Therefore, the results of the assay indicate that the encoded protein B might be another target of the compound X_0. (B) Derivatization of compound X_0 yields specific compounds X_1, which induces a phenotype in zebrafish embryos resembling knockout of gene A only and X_2, which induces a phenotype resembling knockout of gene B only. This process, using an intact animal, can lead to the elimination of the drug's off-target effects by derivatization of compound X_0.

stein et al., 1996; Amsterdam et al., 1999; Wienholds et al., 2003). Efficient protocols for knocking down specific genes (including exon-specific deletions) in a transient manner using antisense morpholinos (Nasevicius and Ekker, 2000), as well as the generation of transgenic strains (Kawakami, 2005; Grabher and Wittbrodt, 2008), have further increased the spectrum of zebrafish models of human diseases (Amatruda and Patton, 2008; Sullivan and Kim, 2008; Dahme et al., 2009; Martin and Renshaw, 2009; Payne and Look, 2009).

Embryos of zebrafish mutants can be applied to rapid whole-animal drug-specificity assessments (Fig. 1). For example, if drug candidate X_0 is designed to inhibit protein A, drug candidate X_0 should induce a phenotype in wild-type zebrafish that is equivalent to that of a zebrafish mutant for protein A. Any effects induced by drug candidate X_0 that differ from those observed in the mutant for protein A indicate that compound X_0 might have additional targets. The detailed catalog of existing phenotypic descriptions of cloned zebrafish mutants or morphants (morpholino knockdown animals) can guide the identification of the molecular nature of secondary targets (www.zebrafish.org and www.zfin.org). Following such a strategy, the zebrafish can aid the development of single-target derivatives originating from a compound with multiple targets (Fig. 1). Several studies applying the zebrafish model in a similar manner have recently been reported in the literature (Ishizaki et al., 2010; Behra et al., 2004; Davidson et al., 2008; Molina et al., 2009; Arslanova et al., 2010; Buckley et al., 2010; Hao et al., 2010; Kokel et al., 2010; Rihel et al., 2010). In the sections below, we summarize some of these examples to illustrate the utility of the zebrafish embryo as a promising system for detecting off-target effects of drug candidates.

Inhibitors of acetylcholine esterase

Acetylcholine esterase (AChE) hydrolyzes the neurotransmitter acetylcholine (ACh) following ACh release from peripheral and central nerve terminals. In addition to being broadly used as insecticides, inhibitors of AChE have been used to treat human disorders, such as the autoimmune disease myasthenia gravis and the neurodegenerative Alzheimer's disease. The zebrafish genome encodes a single ACh-hydrolyzing enzyme. Loss-of-function mutations in the zebrafish *ache* gene therefore generate animals that are completely devoid of ACh-hydrolyzing activity (Behra et al., 2002). Lack of AChE activity causes progressive myopathy of skeletal muscles. Whereas initial formation of myofibrils is normal in homozygous *ache*-mutant zebrafish, muscle fibers disrupt over the course of a few days after fertilization, resulting in a gradual loss of motivity (Behra et al., 2002). It was shown that manifestation of AChE-dependent myopathy also requires the ACh receptor, because concomitant loss of both *ache* and the ACh receptor complex rescued myopathic disruption of myofibrils as seen in *ache* single mutants. Thus, removal of ACh-receptor activity leads to suppression of the myopathy (Behra et al., 2002).

This myopathy can be mimicked in wild-type zebrafish by the application of the AChE inhibitor galanthamin (Behra et al., 2004). Interestingly, another AChE inhibitor, physostigmine (eserine), did not cause disruption of myofibrils (although it did paralyze the animals). It was suggested previously that physostigmine interacts not only with AChE but also antagonizes ACh receptor (Kawai et al., 1999). This off-target effect thereby explained the lack of myofibrillar degeneration of skeletal muscles of physostigmine-treated zebrafish embryos and suggested that physostigmine has secondary-target effects in addition to inhibition of AChE activity. On the basis of these observations, it was proposed to use the *ache* myopathy phenotype as a comparative standard for AChE inhibitors (Behra et al., 2004).

Pifithrin-α, an inhibitor of p53, also acts on p73 in the zebrafish embryo

The proteins p53 and p73, both members of the p53 family of proteins, are implicated in sensitizing cells to ionizing radiation. p53 can act as a tumor suppressor and is mutated in 50% of human tumors (Hollstein et al., 1991). In studies aimed at elucidating the role of p53 in radiation resistance of zebrafish, it was discovered that genetic knockdown of p53 expression results in increased survival and fewer morphological alterations in response to ionizing radiation (Davidson et al., 2008). Davidson and colleagues observed a different effect when they treated embryos with pifithrin-α (PFT-α), a pharmacological inhibitor of p53. Embryos showed developmental abnormalities of the head, brain, eyes and kidney. These abnormalities were similar to those caused by morpholino knockdown of p73

expression, strongly suggesting that the p53 inhibitor PFT-α causes off-target effects related to inhibition of p73. As in the case of the AChE inhibitors, comparison of the phenotype of mutants (in this case, of morphants) with the effects of the drug can allow deductions on the nature of secondary target molecules.

The specificity of the BMP antagonist dorsomorphin and its derivatives

Dorsomorphin was identified as an inhibitor of the bone morphogenetic protein (BMP) signaling pathway by screening a library of compounds by using zebrafish embryos. Inhibition of BMP signaling during gastrulation results in strong dorsalization phenotypes that can easily be scored under a dissecting microscope. Using this approach, the Peterson laboratory screened over 7500 compounds and identified dorsomorphin as a compound that caused a dorsalized phenotype characteristic of mutations in components of the BMP pathway (Yu et al., 2008a). As it was the very first small molecule discovered that inhibited the BMP pathway, its application as a therapeutic agent in anemia and the rare debilitating disease fibrodisplasia ossificans progressiva were investigated (Yu et al., 2008b; Hao et al., 2010). However, dorsomorphin, as well as its analog LDN 193189, were found to cause considerable off-target effects: in zebrafish embryos, the drug caused a failure to form intersegmental vessels (Hao et al., 2010), suggesting that it inhibits the activity of the vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR2, also known as KDR and FLK1). In addition, it was found that these compounds inhibit the activity of the adenosine monophosphate (AMP)-dependent protein kinase complex (AMPK), which is involved in the regulation of energy metabolism. This latter off-target effect might account for the observed necrosis caused by dorsomorphin.

Charles Hong and colleagues undertook a structure-activity relationship (SAR) study of dorsomorphin-related compounds on the basis of the assumption that varying the structure of dorsomorphin might result in a compound with more-specific activities on one or the other of the three targets (the BMP pathway, VEGFR2 and AMPK) (Hao et al., 2010). This SAR study on zebrafish embryos enabled the simultaneous evaluation of on-target and off-target effects, as well as nonspecific lethal effects, of 63 structural variants in parallel. Indeed, by varying the structure of dorsomorphin, they generated a variant that had exquisite specificity for the BMP receptor Alk2 (also known as ACVR1) but had negligible interactions with the Alk2-related TGFβ receptor Alk5 (also known as TGFBR1), VEGFR2 or AMPK. Other dorsomorphin variants showed different activity profiles, some of which were highly specific for VEGFR2 (Hao et al., 2010). The effects determined in the embryo-based assay were subsequently verified by in vitro kinase assays.

Collectively, this work demonstrates that zebrafish embryos can be used to screen systematically for complex drug effects. In the study by Hong and colleagues, on-target and off-target effects could be compared to facilitate the selection of compounds with better target specificity than dorsomorphin (Hao et al., 2010). Although it is not yet clear whether these compounds will make it into the clinic, promising preliminary results with dorsomorphin (Yu et al., 2008b; Yu et al., 2008a) raise hopes that the variants of this drug could be developed into useful therapeutic agents.

Conclusions

The transparency, rapid extra-uterine development and small size of the zebrafish embryo are important characteristics that have made this organism so useful for genetic screens. The very same features enable the screening of bioactive compounds that influence complex cellular behaviors in an intact vertebrate organism. As outlined above, zebrafish embryos can facilitate the study of off-target effects of drugs and drug candidates. Many zebrafish mutants that have been generated thus far have well-documented phenotypic characteristics (www.zebrafish.org and www.zfin.org). As such, these phenotypes can be used as 'blueprints' for determining the effects of drug candidates on specific biological pathways and processes.

Many drug candidates approved for advanced phases of drug development on the basis of in vitro-based classical screens fail during in vivo testing in mammalian models as a result of poor target specificity, poor bioavailability or toxicity. We propose that high-throughput screening on zebrafish embryos will be an economical and physiologically relevant system for the identification of drug candidates with high specificity and minimal off-target effects. It remains to be determined whether drug candidates identified using such a system will make it into the clinic at higher frequency than those selected using currently used assays. The results of the few chemical screens carried out thus far in the zebrafish are promising (Milan et al., 2003; Peterson et al., 2004; Burns et al., 2005; Mathew et al., 2007; North et al., 2007; Yu et al., 2008b; Yu et al., 2008a; Loynes et al., 2009; Molina et al., 2009; Yeh et al., 2009; Durand and Zon, 2010; Kokel et al., 2010; Rihel et al., 2010). A complication could arise from differing secondary metabolism of the drugs, which might generate modifications with species-specific toxicity and off-target effects. However, in comparative studies reported thus far, this does not seem to be a problem. For example, cardiotoxic drugs that cause QT-interval prolongation (these arrhythmias are frequent secondary drug effects in humans) also caused cardiotoxicity in a zebrafish assay (Milan et al., 2003). The capacity of this system to assess the potential effect of drug candidates on heart physiology is therefore another feature of zebrafish physiology that is relevant for drug development and the detection of off-target effects (Dahme et al., 2009).

Efforts are currently underway to systematically knock out all genes in the zebrafish genome, providing a genome-wide resource that will enable screening for off-target effects of a wide range of drug candidates. Such a catalog of phenotypes, combined with the experimental advantages of the zebrafish embryo, has a high potential to improve the drug screening process: in addition to information gained through in vitro interaction studies with isolated target molecules or through biochemical analyses of cultured cells, assays carried out in zebrafish embryos will provide primary readout information regarding a chemical's mode of action in an intact animal. This strategy will allow for the screening of compounds that affect complex cellular behaviors – for example, that of stem cells in their native environment – and will simultaneously reveal information about general toxicity and off-target effects.

COMPETING INTERESTS

The authors declare no competing interests.

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