ZEBRAFISH
a versatile *in vivo* model for drug safety assessment

Safety pharmacology has become an integral part of non-clinical safety assessment for new chemical entities in the past two decades[1]. The relative novelty of this discipline has granted it the flexibility to incorporate new experimental tools[2]. Telemetry has, for example, helped address the reduction of invasive methods in test animals and modern electrophysiological techniques have improved the assessment of cardiac safety[1]. Beside developments such as transgenic animals, the 'omics' technologies and novel biomarkers, *in vitro* testing is another evolving aspect of safety pharmacology. In addition to requiring a small amount of compounds, the time and cost effectiveness of *in vitro* assays have led to their use by the pharmaceutical industry for high or medium throughput safety screens early in the development process[3]. These assays are also of use at later stages development to dissect mechanisms of toxicity. One of the limitations encountered with *in vitro* studies is that they are not always fully predictive, as is the case with the patch clamp technology assessing the hERG channel outside its cellular environment where other ion channels could contribute to QT prolongation and lead to *torsades de pointes* (TdP)[4]. The more predictive *in vitro* assays, such as the Langendorff-perfused rabbit heart model are unfortunately labour and time intensive[5]. Therefore, there is a need in safety pharmacology for high content *in vivo* screening models with the capacity for higher throughput than is currently possible.

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The zebrafish model system is amenable to medium to high throughput screening because of numerous advantages, including (i) the relative ease of maintaining large stocks of animals; (ii) its high fecundity, which provides the investigator with large numbers of animals to analyse; and (iii) the rapid embryonic development *ex utero*, which facilitates experimental manipulation and allows the direct observation of tissue formation and organogenesis *in vivo* (**Figure 1**)[6]. The organisation of the genome and the genetic pathways controlling signal transduction and development are highly conserved between zebrafish and man[7]. These properties have established the zebrafish as an excellent model system that is relevant to studies of human diseases[8]. In addition,

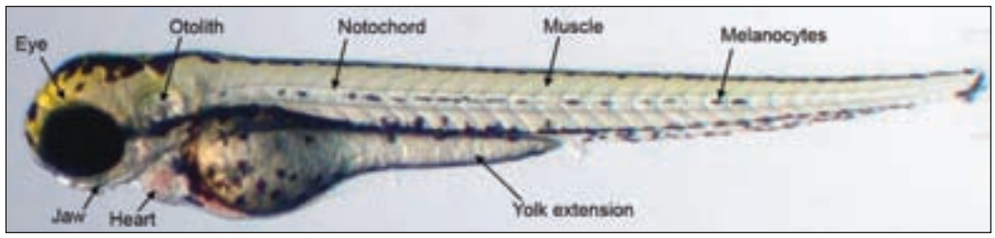

Figure 1
A zebrafish larva at three days post fertilisation. Organs such as the heart are clearly visible due to the optical clarity of the larvae at this age

since the larvae can live in as little as 200µl of fluid, only micrograms of compound are needed for screening, which may be undertaken in 96-well plates (**Figure 2**). Thus, *in vivo* analysis of the effects of compounds can be undertaken at much earlier stages in the drug development process, and at a higher throughput than hitherto possible. This is facilitated by the fact that zebrafish are DMSO tolerant and readily absorb compounds from the water in which they swim.

Safety pharmacology studies are performed before first exposure to man according to a hierarchy with: (i) the core battery assessing life sustaining respiratory, cardiac and central nervous (CNS) systems; (ii) supplemental studies performed as required for the other organs such as the gastrointestinal system; and (iii) follow-up studies will examine with more scrutiny one of the core battery system if necessary[9]. Here we review the role zebrafish can play in support of early discovery phases when pharmaceutical companies are looking for non-GLP technologies with medium throughput to provide drug safety assessment information for rapid decision making to select the best development candidates[10]. Whereas the respiratory system cannot be addressed directly in the zebrafish for obvious reasons, most of the other organ systems can be investigated. This review will focus primarily on the core battery with cardiac and CNS evaluation including vision but will also include on follow-up organs such as the intestine.

Figure 2
Zebrafish larvae at six days post fertilisation in a 96-well plate

Cardiac assessment

The potential of a drug intended for non-cardiovascular indications to cause TdP is a significant public health issue[11] and is also said to be the leading cause of attrition in drug development causing delays during clinical trials[3]. Various techniques, biomarkers and models are currently available or under evaluation as surrogates for the assessment of pro-arrhythmia risk factors in human[5,12]. The hERG gene (ether-a-go-go) and its zebrafish homolog (zERG) show high similarities suggesting an evolutionary conserved role. This is supported by the presence in zebrafish larvae of a specific atrioventricular block when the zERG gene is knocked down[13]. It has also been shown that the heart rhythm of zebrafish embryo in response to compounds that are known to prolong QT in man is very similar with a bradycardia preceding the 2:1 atrial to ventricular contraction ratio[13,14]. In addition, QT prolonging compounds have also been shown to increase corrected QT interval in adult zebrafish[15]. The distinctive embryonic 2:1 atrial to ventricular contraction ratio is also reported as a forme fruste in humans and can be considered a surrogate for QT prolongation in zebrafish. Therefore, it can be used to predict the potential of compounds to have an unwanted effect on QT. However, the zebrafish cardiac assay is not purely a 'hERG' predictor assay as other channels are concomitantly investigated in their native *in vivo* environment. **Figure 3** shows a graphical representation of the changes in heart rate using software analysis of high resolution video of a zebrafish heart. It has been shown that drugs which prolong QT interval both via hERG blockade, such as terfenadine, cisapride and pimozide, and compounds that do not act through hERG, such as YS035, have been identified in this assay[16]. Burns and colleagues[17] have now generated a transgenic zebrafish line expressing green fluorescent protein (GFP) in the myocardium and have used it to establish a high throughput methodology to screen small molecules for their effect on heart rate.

CNS assessment

Adverse effects on the nervous system have commonly been found and are said to account for 10% of the drug market withdrawals between 1960 and 1999[18]. Spontaneous locomotor activity (LA) is generally used in safety pharmacology as part of the functional observational battery (FOB) as a general starting point for CNS evaluation because it integrates function of several central nervous system structures[19]. Zebrafish have recently become the focus of neurobehavioural studies since they have a great potential to investigate behaviour and behavioural disorders[20]. Zebrafish display behavioural phenotypes that are quantifiable and relate to those seen in man such as learning and memory[21,22]. Adult and larvae zebrafish have also been shown to possess sleep patterns similar to those found in mammals[23,24]. In addition, it is known that zebrafish brain structure and function are similar to other vertebrates, lending further support to its use as a useful model for evaluating vertebrate behaviour[25]. Zebrafish behaviour can easily be observed and a high throughput screen has been developed that evaluates the effects of drugs on LA by recording and analysing the behaviour of larvae using live video-tracking in 96-well plates[26]. Using this approach it is possible to identify the hypoactive and hyperactive effects of common sedatives, such as diazepam and melatonin, and stimulants, such as caffeine and ethanol respectively.

The ability to examine zebrafish larval behaviour offers further opportunities in areas of safety pharmacology which are currently being developed. It is believed that insufficient CNS testing is currently performed to identify the proconvulsive potential and the abuse/dependency liability of compounds[10]. Seizures can be elicited in zebrafish larvae by exposure to commonly used convulsant agents like pentylenetetrazole[27]. Concentration-dependent epileptiform seizures induced by pentylenetetrazole result in stereotyped behaviour expressed by increased LA. It is therefore possible to identify the convulsant adverse effect of drugs. Neuropsychiatric effects, abuse liability and dependency disorders have been the major causes of drug withdrawal in the United States and European regulators will now require more information on the dependency potential of development compounds[10,28]. Following proposed *in vitro* studies and prior to challenging *in vivo* investigations involving higher vertebrates, zebrafish larvae could answer the need for a rapid dependency screening method. Zebrafish is a good candidate model because adults respond robustly in conditioned place preference tests to several addictive drugs (alcohol, cocaine and amphetamine) and the neurotransmitter cascade mediating the reward pathway is well conserved[29-31]. Such assays in larval zebrafish are currently under development to answer the need for low cost and time-effective higher throughput dependency screen[32].

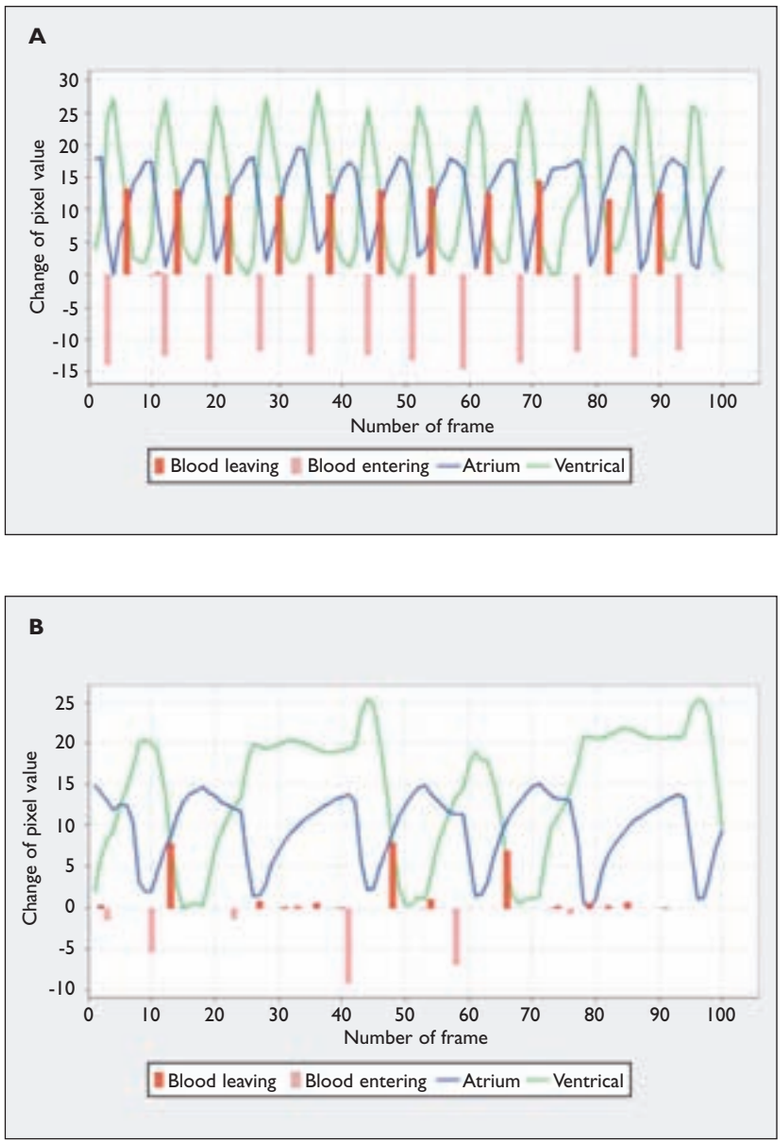

Figure 3: Data analysis of high resolution video of a three days post fertilisation zebrafish larvae heart where atrium beat (blue), ventricular beat (green) and outflow strength (red bars) are shown for: **A** normal heart rate; **B** 2:1 atrial/ventricular ratio arrhythmia in response to treatment with terfenadine (Berghmans et al, 2006)

Visual function assessment

Adverse ocular drug reactions are reported for a wide range of marketed drugs, including retinoids, vigabatrin, chloroquine, bisphosponates and

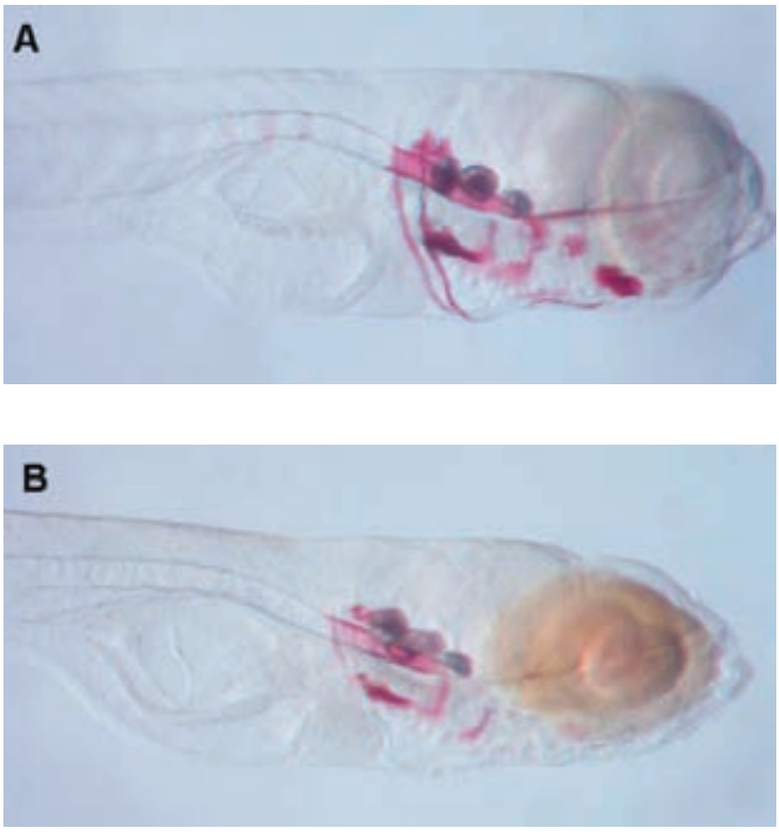

Figure 4: A Zebrafish larva at 10 days post fertilisation: many bones of the head skeleton are mineralised and these stain red with alizarin dye, **B** Larva exposed to 25μM prednisolone from 5 dpf to 10 dpf have a marked reduction in the stained mineralised tissue. Quantification of the staining showed a 55% reduction in mineralised area with steroid treatment. *(Reproduced with permission of the Biotechnology Journal)*

steroids[33]. Currently, ocular safety is assessed at a later stage in the development of a compound and convential studies are technically difficult and include the measurement of the electroretinogram (ERG). Zebrafish have a cone dense retina and thus, like humans, have rich colour vision and therefore provide a distinct advantage over nocturnal rodents. Visual development of zebrafish larvae is rapid to enable predator avoidance and feeding. By five days post fertilisation (dpf) the visual system is well developed by electrophysical, morphological and behavioural criteria[34]. Assays, such as the optokinetic and optomotor responses have been developed for measuring visual function that take advantage of visual reflexes[35] and are amenable to testing relatively low amounts of compound at an early stage in drug discovery than has previously been possible. The optokinetic response (OKR) assay is carried out with larvae immobilised in methylcellulose inside a striped drum, the rotation of which elicits a series of smooth pursuits followed by a rapid saccade as the eyes flick on to the next stripe. The movement of the large, pigmented eye can easily be followed under a microscope. Changing the stripe width, angular velocity and contrast also allows quantification of visual acuity, contrast sensitivity and light adaptation[36]. The optomotor response is the locomotor behaviour of an animal induced by moving a repetitive pattern and this can be elicited by moving horizontal stripes below long transparent chambers in which the larvae swim. The larvae follow the stripes and therefore those that cannot see are immobile. The testing of 10 drugs that cause adverse ocular events in humans in the OMR and OKR assays in zebrafish larvae has validated this approach to predicting possible effects on visual function[37]. Chlorpromazine, nicotine, chloroquine, ouabain, phenytoin and diazepam all showed inhibition in the assays after a five-day exposure of the larvae to compound. Atropine causes visual disturbance in humans by affecting accommodation and papillary dilation but was not detected as having an effect on zebrafish, which is not surprising since fish have fixed pupils and do not have binocular vision due to differences in the anatomical position of the eyes between fish and humans. It is also possible to measure ERG in zebrafish[38] to confirm specific effect on visual pathways but this does not offer the throughput desirable for screening at a preclinical stage.

Gastrointestinal assessment

Gastrointestinal adverse drug reactions are some of the most frequently reported in all phases of drug development accounting for approximately 18% of all reported clinical adverse events and 20-40% of those in hospitalised patients[39]. However, assessment of drug development candidates on the GI system is not a regulatory requirement prior to conducting Phase I trials. Spontaneous intestinal motility is observed in zebrafish at four days post fertilisation and regular contraction patterns, both anterograde and retrograde, are present[40]. The presence of functional cholinergic, tachykinergic and PACAP receptors suggests that gut motility in zebrafish is under the control of the enteric nervous system[41]. Furthermore, a direct effect of L-NAME on zebrafish peristalsis indicates that an endogenous nitrergic tonus is present in the larval gut[41,42]. Due to the transparency of the larvae, imaging of the gut inside the intact live animal are possible enabling drug-induced alterations of gut motility to be studied. The zebrafish has no stomach and

the anterior intestine secretes the corresponding gastric enzymes but not gastric acid[43], which prohibits the measurement of gastric emptying or gastric pH changes to be made.

Bone density assessment

Decreasing bone mineral density is a major side effect of several classes of drugs including long term glucocorticoid treatment for inflammatory conditions[44] and aromatase inhibitor therapy for breast cancer[45]. A 96-well plate assay for the assessment of the effects of compounds on bone demineralisation in zebrafish larvae has been developed[46]. Treatment for five days with the steroid prednisolone in the media in which the larvae swim was shown to reduce the mineralised area by 50%. **Figure 4** shows whole mount skeletal staining where the mineralised tissue is stained with alizarin red and the effects of steroid treatment are clearly visible. Such an assay could be applied to any class of compound which might be suspected to have an effect on bone demineralisation.

Blood coagulation

Zebrafish also offer the possibility of an assay to study blood coagulation at an earlier stage of the drug discovery process than that at which the *ex vivo* platelet aggregation test is usually employed. Analysis of the zebrafish thrombotic system demonstrated the presence of both the intrinsic and extrinsic pathways of coagulation, and also prothrombin, factor X, protein C, antithrombin and heparin cofactor II activity[47]. Thrombocytes, the equivalent of mammalian platelets in zebrafish, have nuclei in contrast to platelets but have been shown to form aggregates, filopodia and lipid rafts in response to platelet agonists[48]. Zygogen's Z-TagSM technology enables the expression of tissue specific fluorescent proteins in zebrafish. Thrombocytes have been fluorescently labelled and the optical clarity of the larvae allows visualisation of the movement of platelets in real time through the blood vessels. Clinical antithrombotics such as aspirin, ticlopidine and platelet glycoprotein IIb/IIIa inhibitors ReoPro, Aggrastat and Integrilin are effective in a zebrafish model of ADP induced aggregation[49].

Conclusion

As the cost of developing an innovative drug becomes higher so the importance of reducing the attrition rate of drugs in development is ever increasing. There is a need to improve the selection process of drugs in the late stages of discovery to limit the numbers of costly failures. The emergence of using zebrafish in non-GLP pre-clinical safety assessment is an exciting recent development that should allow rapid decision making in the early phases of the discovery process such as lead selection or lead optimisation. Outstanding questions on the use of zebrafish are; what is the predictability of zebrafish and what is the extent of the correlation with compound effects seen in man; can zebrafish pharmacokinetics methods be established that will allow PK-PD relationships to be understood? Studies are under way to understand issues raised by these questions. The advantages of the small compound requirements and DMSO tolerance of zebrafish can allow this technology platform to be applied at this critical phase of drug discovery to identify the chemical templates with the least side-effect liabilities. Zebrafish are also a suitable tool to address the areas in which the EMEA has currently issued guidelines on safety assessment of combinations of medicinal products and testing in juvenile animals of pharmaceuticals for pediatric indications. DDW

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