Novel tools in drug discovery: optimising the use of zebrafish for assessing drug safety and antitumoral efficacy

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In appreciation

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Abstract

High drug attrition rate during clinical and post market phases is one of the major factors contributing to the pharmaceutical industry productivity crisis. This problem is especially worrisome in the cancer field, where it is two to four times higher than in other health sectors. Most of the drugs are discarded due to safety (mainly cardio-, neuro-, and hepato-toxicities) and efficacy issues, which reflect the limitations of current preclinical models in anticipating such drawbacks. In this context, new models are needed in order to tackle these problems and to accomplish with the new demands (higher throughput and predictivity) of the modern research and development (R&D) processes. Zebrafish is a vertebrate with elevated homology to humans and unique biological properties, which make it suitable for high throughput studies. The final objective of my thesis is to improve the use of this animal model in an attempt to ameliorate the overall R&D process efficiency and thus, ease the productivity crisis. First, a semi-high throughput methodology has been generated for the assessment of cardio-, neuro- and hepato-toxicities in the same animal, thus, impacting the 3Rs principle. Second, xenografts of human cancer cells into zebrafish larvae for the study of anti-tumour drug efficacy have been standardised, validated and automated. Results obtained help to consolidate and validate the use of the zebrafish in the R&D process of new drugs, as a bridge between *in vitro* models and *in vivo* mammalian models.

Resumen

La alta tasa de deserción de medicamentos durante fases clínicas y posteriores a la comercialización es uno de los factores principales que contribuyen a la crisis de productividad que afecta a la industria farmacéutica hoy en día. Este problema es especialmente preocupante en el sector del cáncer, donde es de dos a cuatro veces mayor que en otros sectores de la salud. La mayoría de estos medicamentos son descartados debido a problemas de seguridad (principalmente cardio, neuro, y hepatotoxicidad) y de eficacia, lo que reflejan las limitaciones de los modelos preclínicos actuales para anticipar tales inconvenientes. En este contexto, se necesitan nuevos modelos para abordar este problema y cumplir con las nuevas demandas (mayor rendimiento y predictividad) de los procesos de investigación y desarrollo (I+D). El pez cebra es un vertebrado con alta

homología con los humanos y propiedades biológicas únicas, que lo hacen adecuado para estudios de alto rendimiento. El objetivo final de mi tesis es mejorar el uso de este modelo animal en un intento de mejorar la eficiencia general del proceso de I+D y, así, aliviar la crisis de productividad. Primero, se ha generado una metodología de rendimiento medio para la evaluación in vivo de las toxicidades cardíaca, neuronal, y hepática en un mismo animal, en línea con en el principio de las 3Rs. En segundo lugar, se ha estandardizado, validado y automatizado, el xenotrasplante de células tumorales humanas en larvas de pez cebra para el estudio de la eficacia de fármacos antitumorales. Los resultados obtenidos ayudan a consolidar y validar el uso del pez cebra en el proceso de I+D de nuevos fármacos, como puente entre los modelos *in vitro* y los modelos *in vivo* de mamíferos.

Preface

The pharmaceutical industry is facing a productivity crisis in which, despite the increasing expenditures in drug R&D, there is no clear correlation between investments and number of new drugs entering the market. The inefficiency of the process leads to extremely high investments costs, which are then reflected in the high prices of the few successful drugs that reach the market. In this context, the productivity crisis has not only negative consequences for the pharmaceutical sector, but it also harms patients that cannot access or afford effective treatments, and society, which has to deal with the elevated costs. The reasons behind this problem are multiple, complex and they involve factors related to the R&D, regulatory and business processes. One major issue is the high drug attrition rate during the clinical and post market phases, which is two to four folds higher in the oncology field than in other therapeutic areas. In this regard, safety and efficacy matters are the two main reasons for promising compounds to be finally discarded. Thus, high drug attrition rate echoes predictivity deficiencies in preclinical models. One solution might be represented by the use of alternative and complementary tools to improve the overall predictive output and so, reduce the posterior drug attrition rate. Zebrafish has emerged as a really promising model since it shows unique biological properties for a vertebrate, in addition to have high genetic and physiologic homology to humans. It is characterized by small size, fast life cycle, large progeny, transparency, ease of maintenance and genetic manipulation, and the ability to absorb molecules from the surrounding water. All these peculiar aspects, allow its utilisation in high throughput studies.

On these bases, during my PhD project, I improved the use of zebrafish for the evaluation of drug safety and antitumoral efficacy. Two different methodologies have been developed and validated: the **ZeGlobalTox** and the **ZeOncoTest**.

ZeGlobalTox is a middle-high throughput system in which zebrafish larvae are used to sequentially evaluate the three most important organ toxicities that are the main cause of drug attrition: cardio-, neuro-, and hepato-toxicities. Results have demonstrated high sensitivity, specificity, and accuracy of the model, compared to human data. Furthermore, our assay integrates for the first time the analysis of these three organ-toxicities in the same larvae; reducing animal usage, experimental time and costs, and quantity of

compound needed. In a context where animal utilisation in research is being contested, such an alternative fulfilling the 3Rs principles (Replacement, Reduction, and Refinement), is very welcomed.

ZeOncoTest is a methodology for the evaluation of antitumoral drug efficacy through an optimised zebrafish larvae xenograft model. Previously published reports presented contrasting data probably due to differences in incubation times and temperatures, image acquisition and analysis, cell labelling methods prior to transplantation and site of injection. With the ZeOncoTest we to optimised, standardised and automated the zebrafish larvae xenograft assay for anti-cancer drug discovery. Our results demonstrate that with this methodology, human tumour cells are able to engraft, grow and disseminate into zebrafish larvae and that they respond to known drugs as expected. Also, we showed that our system is suitable to investigate drugs mode of action.

Our work helps to validate zebrafish as a promising preclinical model, bridging the gap between high throughput but low predictive *in vitro* models and more predictive but low throughput *in vivo* mammalian models. We propose it as a filter of molecules coming from *in vitro* models and entering *in vivo* studies, thus improving the rationale of selection. Finally, it is envisioned that zebrafish consolidation in R&D processes will improve drug safety and efficacy predictions during preclinical phases, therefore reducing the high drug attrition rates and ameliorating the productivity crisis.

List of abbreviations

- **2D** → 2 Dimensions
- **3D** → 3 Dimensions
- **3Rs principle** → Replace, Reduce and Refine
- **6-OHDA** → 6-hydroxydopamine
- **ADMET** → Absorption, Distribution, Metabolism, Elimination and Toxicity
- **aSCs** → adult Stem Cells
- **BBB** → Blood Brain Barrier
- **CNS** → Central Nervous System
- **CRISPR** → Clustered Regularly Interspaced Short Palindromic Repeats
- **DMBA** → Dimethylbenzanthracene
- **DNT** → Developmental Neurotoxic
- **DNT-DIVER** → Developmental Neurotoxicity Data Integration and Visualization Enabling Resource
- **DSB** → Double Strand Break
- **EGFR** → Epithelial Growth Factor Receptor
- **EMEA** → European Medicines Agency
- **ENU** → N-ethyl-N-nitrosourea
- **ERG** → Ethera-go-go-Related Gene
- **ESCs** → Embryonic Stem Cells
- **FDA** → Food and Drug Administration
- **FOLFIRI** → 5-fluorouracil+irinotecan+folinic acid
- **FOLFOX** → 5-fluorouracil+oxaliplatin+folinic acid
- **GABA** → Gamma-Aminobutyric Acid
- **GFP** → Green Fluorescent Protein
- **hERG** → Human Ethera-go-go-Related Gene
- **HGF** → Hepatocyte Grow Factor
- **Hpf** → Hours post fertilisation
- **HTS** → High-Throughput Screenings
- **IATA** → Integrated Approaches to Testing and Assessment
- **IND** → Investigational New Drug

- **iPSCs** → induced Pluripotent Stem Cells
- **MIPDD** → Mechanistically Informed Phenotypic Drug Discovery
- **MNNG** → N-methyl-N^1-nitro-N-nitrosoguanidine
- **MOA** → Mechanism Of Action
- **MPTP** → 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
- **NDA** → New Drug Application
- **NDMA** → N-nitrosodimethylamine
- **NMEs** → New Molecular Entities
- **PARP** → Poly (ADP-Ribose) Polymerase
- **PD** → Pharmacodynamics
- **PDD** → Phenotypic Drug Discovery
- **PDX** → Patient Derived Xenograft
- **PK** → Pharmacokinetics
- **Pvs** → Perivitelline space
- **R&D** → Research & Development
- **SAR** → Structure-Activity Relationship
- **SEAZIT** → Systematic Evaluation of the Application of Zebrafish in Toxicology
- **TALENs** → Transcription Activator-Like Effector Nucleases
- **TDD** → Targeted Drug Discovery
- **TEAZ** → Transgene Electroporation in Adult Zebrafish
- **VEGF** → Vascular Endothelial Grow factor
- **WHO** → World Health Organisation
- **ZFNs** → Zing-Finger Nucleases

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1. INTRODUCTION

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1.1. Drug discovery and development

1.1.1. Historical perspective

Historically, drug discovery can be divided into three main periods. The first one dates back to early times of human beings. Humans have always used supplies obtained from nature to produce food, shelters, clothing, means of transportation, fertilizers, flavours and fragrances, and also, medicines. The first records of plants used as therapy date back to 2600 BC and they were written by the Mesopotamians. Furthermore, there is evidence that also Egyptians, Chinese, Greeks and Romans used natural products for medicinal purposes (Newman, Cragg and Snader, 2000). In the following centuries, primary compounds from plant sources were refined and led to the development of chemical substances. In the early 1800s, improved purification strategies allowed the isolation of the active principles of commonly used plants as strychnine, morphine, atropine and colchicine. The idea of "pure" compounds as drugs had appeared and was the base of what could be considered the first commercial pure natural product, morphine, isolated by Sertüner in 1815 and commercialised by E. Merck in 1826 (Figure 1) (Drews and Drews, 2000a; Pina, Hussain and Roque, 2010a).

The second period commenced around the early twentieth century, when drugs chemical structures started to be studied, allowing its modification, in order to improve its efficacy. Furthermore, it represented the beginning of a novel era of antibiotics discovery such as Penicillin (Newman, Cragg and Snader, 2000). The emergence of recombinant DNA technology, which is the insertion into a host organism of a pair of DNA molecules from two different species producing new genetic combinations (e.g. molecular cloning), made it possible to develop potential drugs target candidates. Finally, towards the end of the 20th century, drug discovery was revolutionized by the development of powerful new techniques such as molecular modelling, combinatorial chemistry, and automated high-throughput screening (Figure 1) (Drews and Drews, 2000b).

The third period started in the 21st century with the publication of the complete mapping of the human genome and the onset of the "Omics" revolution (Figure 1). These advances and developments allowed increased knowledge on drug toxicity, disease and its related biochemical pathways recognition, and target identification. Finally, a multifaceted drug discovery approach, in which different scientific disciplines collaborate with the pharmaceutical industry, led to in an increase in biopharmaceutical drugs approved by the FDA/EMEA for therapeutic use (Pina, Hussain and Roque, 2010b).

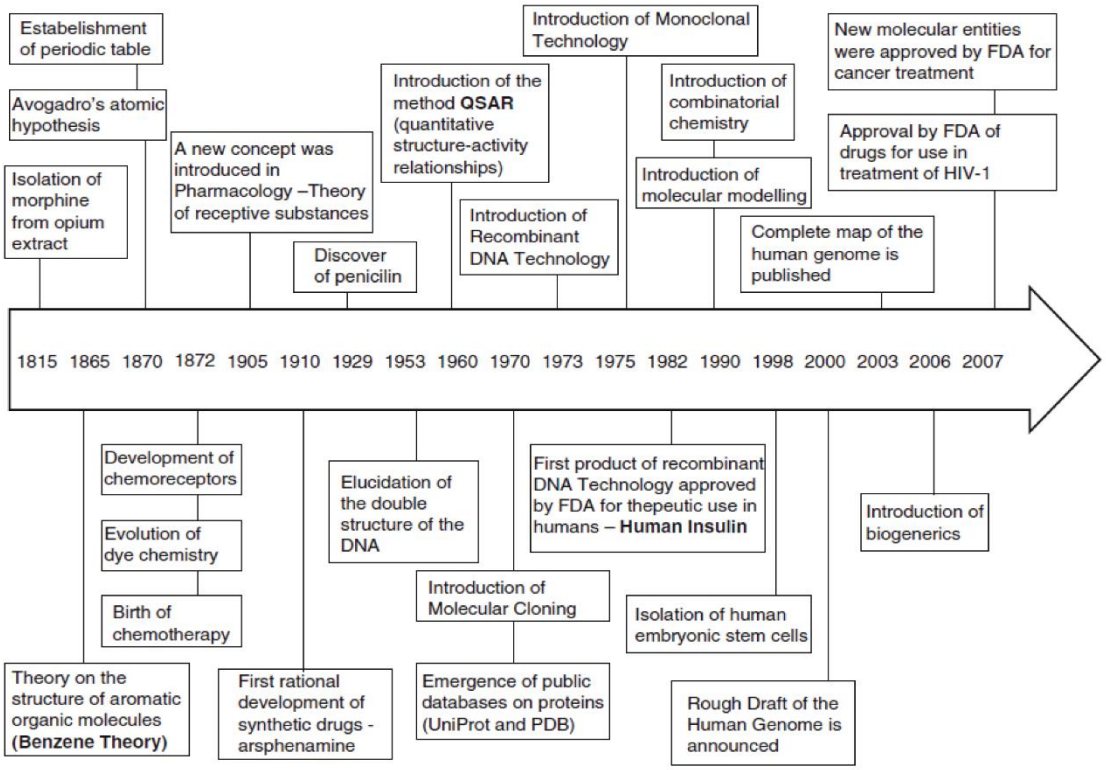

Figure 1. Timeline showing important events in drugs discovery. Obtained from: (Pina, Hussain and Roque, 2010a).

Nowadays, there are lots of different synthetic chemical entities, with thousands of variants and derivatives. However, compounds isolated from natural sources such as plants, micro-organisms, invertebrates, and vertebrates, continue to play an essential role in human health. The World Health Organisation (WHO) has estimated that approximately 80% of the world inhabitants rely mainly in traditional medicines as primary health care (Pina, Hussain and Roque, 2010b). Countries such as China and India still depend on plants as the basis for their traditional medicine systems (Miller, 2001). In addition, for the remaining 20% of the world population, mainly residing in developed

countries, natural products are still present as sources, derivatives or models in more than 50% of new chemical entities (Pina, Hussain and Roque, 2010b).

1.1.2. Approaches

The drug discovery process has changed from the times when serendipity played an important role in drug development, and creativity and intuition of the medicinal chemist was the basis for the success of new compounds. In modern days, drug discovery is more structured and rational. It can be divided into three different approaches (Figure 2):

- Phenotypic drug discovery (PDD): the molecular target of the drug remains unknown, and the compounds with the desired activity (hits) are selected based on the observation of phenotypic eventual changes in cells or whole organisms screenings (Lee *et al.*, 2012).
- Targeted drug discovery (TDD): the molecular target or MOA is known and it is selected from the literature (Hoelder, Clarke and Workman, 2012). Hits are chosen through physical interaction with the target, but no information on efficacy or safety is inferred.
- Mechanistically informed phenotypic drug discovery (MIPDD): the target of the compound is known or it has been identified during the drug discovery process, and phenotypic screening assays are performed to select hits (Moffat, Rudolph and Bailey, 2014). It is a combination of the previous two approaches.

Together with these three approaches, modern drug development incorporates rational drug design methods through the use of computational modelling and physiochemical properties databases (Rodenhizer *et al.*, 2018).

1.1.3. Process

As previously mentioned, the current drug R&D process comprises a series of steps and checkpoints that rely on the expertise in a wide range of disciplines such as biology, biochemistry, pharmacology, mathematics, computing, and molecular modelling (Figure 2). To obtain a successful drug, three important issues are addressed: the molecular target,

the compound, and its delivery into the market. This process takes approximately 12 years, and it is divided in four phases subsequently reported:

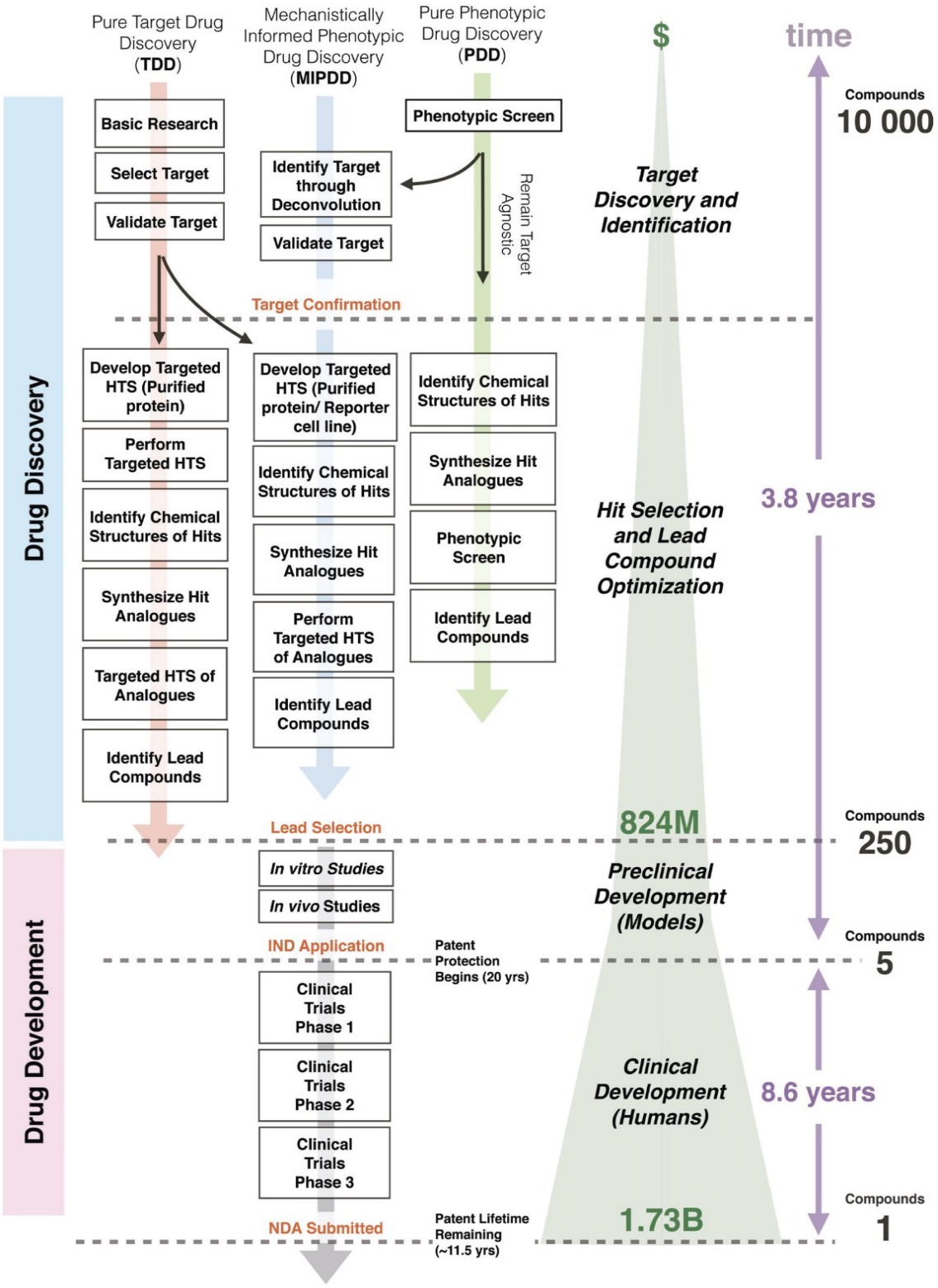

Figure 2. Schematic representation of the key steps in the drug discovery and development process. The three main approaches (TDD, PDD & MIPDD) are shown, together with the principal experimental assays for each stage. Approximated costs and timelines for key segments of the pipeline are also indicated. Phase IV of the clinical trial, which is done postmarked, is not showed. Abbreviations: HTS stands for High-Throughput Screenings, IND stands for Investigational New Drug, and NDA stands for New Drug Application. Obtained from: (Rodenhizer *et al.*, 2018).

1) Target identification and validation, and hit selection

If a TDD approach is followed, the drug development process begins with the target/s identification from the literature or public databases. In this regard, TDD strategies have substantially benefited from the emergence of the "Omics" techniques, which led to the discovery of new molecules susceptible to be targets (Lindsay, 2003). Once the target has been identified, experiments are performed in order to link it to *in vitro* or *in vivo* phenotypes and disease conditions. This process is called target validation and it often involves the use of genetic methods, such as knock-down, knock-out and over-expression models (Lindsay, 2003). Finally, the validated target serves to create a relevant system-based assay, in which vast libraries of compounds are screened in order to find a hit or lead, a hit worth to be further investigated (Figure 2).

If the PDD or MIPDD approaches are followed, an *in vitro* or *in vivo* assay is validated for biological relevance and then used to screen compounds altering specific phenotypes (Moffat *et al.*, 2017). Compounds producing the desired effect are selected as hits or leads. Afterwards, in MIPDD approaches, the target is identified by deconvolution techniques such as affinity chromatography, microarray and expression-cloning, or through computational techniques like virtual screening (Hoelder, Clarke and Workman, 2012; Rodenhizer *et al.*, 2018). Targets are finally validated as in TDD approaches (Figure 2).

2) Lead compound optimisation

Once lead compounds have been selected, they are optimised in order to increase target binding affinities, by establishing structure-activity relationships, and favour drug-like properties. Thus, those characteristics improving compounds pharmacodynamics (PD) and pharmacokinetics (PK) are optimised (Colombo and Peretto, 2008). This is achieved by the iteratively rescreening of synthetic analogue molecules derived from the original

hits. The process involves the outlining of molecular modifications and an extensive computational and experimental profiling (Magalhaes, Ferreira and Andricopulo, 2018) (Figure 2).

3) Preclinical development

Optimized compounds are then evaluated using preclinical *in vitro* and *in vivo* models of the disease to filter for toxicity and select for favourable pharmacodynamics properties and efficacy parameters. The best candidates are further characterized to determine the safe dose for the initial trials in humans. Once gathered preclinical data qualify the compound as a clinical candidate, an application for the investigation of a new drug is submitted to the appropriated regulatory body (FDA/EMEA) to request for the initiation of clinical trials (Rodenhizer *et al.*, 2018) (Figure 2).

4) Clinical development

Drugs experimentation in humans is typically conducted in four phases (Figure 2). Each phase is considered a separate independent trial and researchers must submit the resulting data gathered in each of them to the corresponding regulatory body for approval, (e.g., Spanish Agency of Medicines and Medical Devices in Spain) before continuing to the next stage. The four phases of a human clinical trial are the following:

- **Phase I** studies are designed to evaluate absorption, metabolization and excretion of potential drugs in humans. Side effects are also assessed, as a consequence of increasing doses. This phase can last few months and involves a small number of healthy volunteers (20 to 100). About 70% of experimental compounds passes this step (Center watch, 2017).

- **Phase II** studies are meant to test the therapeutic efficacy of potential drugs (proof of concept). Several hundred patients are involved in randomized blinded trials where an experimental group is treated with the candidate drug, and a control group is given the conventional treatment or a placebo. The term “blinded” means that neither the patients nor the investigators know who received the experimental compound. To complete this second stage, several months to two year are needed. Around 30% of potential drugs successfully go through Phase II (Center watch, 2017).

- **Phase III** studies provide the pharmaceutical company and the regulatory agency an accurate understanding of the effectiveness, the benefits and the range of possible adverse reactions associated with the potential drug. Similar to Phase II studies, Phase III trials involve randomized and blind testing. However, they are multicentre large-scale projects that may involve hundreds to thousands of patients and they can last several years. If completed successfully, which occurs for the 70% to 90% of the cases, the pharmaceutical company can then request marketing approval to the corresponding regulatory body (Center watch, 2017).

- **Phase IV** studies are conducted after the drug has reached the market. They are often called Post Marketing Surveillance Trials and they are used to compare the long-term benefit and cost-effectiveness of the new therapy, in comparison to other treatments already available in the market. The results of this phase could imply the restriction of the drug usage or even its removal from the market (Center watch, 2017).

1.1.4. Problems

As depicted in the previous section, drug R&D is a long, complex, and expensive process that results in more failures than successes. Indeed, only 10% of compounds entering Phase I clinical trials are ultimately approved by regulatory agencies (Hay *et al.*, 2014). This divergence in R&D spending and new drugs approvals has been referred as the pharmaceutical R&D "productivity crisis". One of its consequences is the high cost of bringing a new drug into the market, which can reach up to US$ 2.6 billion (DiMasi, Grabowski and Hansen, 2016). In addition, high costs discourage pharmaceuticals from focusing on several areas with unmet medical needs but not profitable enough (Blomme and Will, 2016).

Possible reasons for this productivity crisis are complex. Multiple factors may contribute to the decision of terminating the development of a compound: unbalanced risk-benefit assessment, high regulatory efficacy hurdles, challenging reimbursement and payer environment, increased complexity of the treatments for challenging illnesses, requirement of substantial improvements on existing therapies that already have a certain

effectiveness, and elevated cost of clinical trials (DiMasi *et al.*, 2009; Kaitin and Dimasi, 2011).

In particular, attrition due to toxicity in clinical phases represents a major concern. Also, lack of efficacy due to deficient compounds physicochemical properties significantly contributes to the failure of drug in Phase II, which is the stage with the highest attrition rates (see section 1.1.3). This situation reflects shortcomings in the data gathered during the early stages of drug discovery(Cook *et al.*, 2014; Waring *et al.*, 2015). In particular, the success of a potential drug in clinical trials is determined by how well efficacy and toxicity were predicted in preclinical screening phases. Therefore, the choice of adequate models is a fundamental determinant in the success of a drug.

1.1.5. Preclinical models

Since it is not possible to carry out primary screening of molecules in humans due to ethical and monetary reasons; model systems, including *in silico* biological models, cell- and tissue-based systems, and laboratory animals, are central to the discovery and development of new and better drugs for the treatment of human diseases.

In this time of productivity crisis, *in silico* models are gaining importance as they could potentially reduce R&D costs, time and animal usage. Such tools are normally employed in early phases, in order to predict deficiencies in absorption, target organ concentration, clearance, efficacy and toxicity. Specifically, computer-aided techniques are used for docking, structure and ligand-based virtual screening, pharmacophore and homology modelling, molecular dynamics assessment, two- and three-dimensional quantitative structure-activity relationship determination and ADMET profiles prediction (L Romero and Vela, 2014a). Furthermore, several large collaborative programs are underway to develop disease-specific and patient-specific *in silico* tools. However, these models have the important limitation that their predictions are based on published and not direct experimental data. Therefore, all the underlying mathematical models and parameters are only as good as the experimental data on which they were based (Sceats, 2011). The results obtained serve to pre-select the most promising potential drugs, that need then to be validated using direct *in vitro* and *in vivo* assays.

The most common *in vitro* models for initial evaluation of biological activity of the compounds are 2D cell cultures. The output is the activity of the potential drug on different cellular features, such as cell death, proliferation, gene expression, protein profile and cell cycle, among others (Andrade *et al.*, 2016). More complex 3D cell cultures, organoids and innovative organ-on-a-chip technologies have also been developed in an attempt to better mimic tissues and organs structures and function, and to study more challenging aspects, such as cell-matrix interactions, spatiotemporal gradients of chemicals or mechanically active microenvironments (L Romero and Vela, 2014b). However, the complex biology of a whole living organism cannot be faithfully recreated *in vitro*. Therefore, animal models have been traditionally used to fully understand how the potential drug would work *in vivo*.

The selection of appropriate animal models is one of the most important steps in the different experimental phases of the drug R&D process. They play a pivotal role in target validation, efficacy studies, PK and PD correlation and drug safety and tolerability assessment (Wang, 2012). Generally, disease animal models can be broadly divided into three categories subsequently reported:

1) Physiological models, in which the disease is induced by an invasive procedure. For example, zebrafish myocardial infarction model generated through heart cryoinjury (Chablais and Jaźwińska, 2012; González-Rosa and Mercader, 2012).
2) Pharmacological models, in which the condition is induced by the administration of a certain substance. For example, heart failure in larval and adult zebrafish caused by doxorubicin treatment (Liu *et al.*, 2014; Ma *et al.*, 2018).
3) Genetically modified models, in which a modification is introduced in the genome of the animal to generate the pathology. For example, disruption of the *tnnt2* gene inducing cardiomyopathy in zebrafish (Becker *et al.*, 2011).

Moreover, *in vivo* models can be subdivided into acute or chronic, depending on the duration of the disease. Classically, mammals, such as rats, mice, dogs, and rabbits are the gold standard to test the effects of potential drugs before moving to clinical trials with humans (Wang, 2012). However, the current tendency is to replace, reduce or refine the use of animals in experimentation (3Rs principle). Replacement can be absolute, such as with *in silico* and *in vitro* studies, or relative, through the avoidance of the use of protected

animals (i.e. all the vertebrates and cephalopods). In this regard, invertebrates species such as *Drosophila melanogaster* and *Caenorhabditis elegans*, or immature forms of vertebrates like *Danio rerio* embryos below five days post fertilisation (dpf), are increasingly being employed in different phases of the drug R&D, (Luz Romero and Vela, 2014).

All the models here mentioned have unique advantages that make them excellent in specific cases. However, they have limitations too (Table 1). Given the pharmaceutical productivity crisis previously explained, the lack of translatability of efficacy, safety and bioavailability data from animal studies to humans is particularly worrisome (Andrade *et al.*, 2016). Moreover, due to the recent economic crisis, the budget allocated for R&D has also been reduced. In conclusion, the poorly translatable value of preclinical data, the costs associated with current mammalian *in vivo* experiments, the ethical concerns, the legislative changes regulating animal tests and the 3Rs principle are driving researchers and pharmaceutical companies to consider the use of alternative and complementary animal models, such as zebrafish.

Table 1: Advantages and limitations of the different models in drug discovery and development.

	In silico	***In vitro***	***In vivo***
Advantages	- Animal replacement - Very cheap - Very high throughput - Controlled experimental environment - Human based biology	- Animal replacement - Cheap - High throughput - Controlled experimental environment - Well characterised - Human based biology	- Whole organism: more biologically relevant - High anatomical, molecular, genetic, and pathological similarities with humans - Evaluation of ADMET profiles
Limitations	- Very reductionist model - Built from indirect experimental data - Based on predictions - Lack of physiologic structures	- Reductionist model - Not holistic - Lack of physiologic structures - Results need to be validated *in vivo*	- Ethical considerations - Expensive - Low throughput - Less controlled experimental environment

1.2. Zebrafish as an innovative animal model

1.2.1. General information

Zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) is a small (3-5 cm length) benthopelagic cyprinid freshwater fish native to South Asia, where it is found in India, Pakistan, Bangladesh, Nepal and Bhutan (Braunbeck and Lammer, 2006). It typically inhabits moderately flowing to stagnant clear water of quite shallow depth in streams, canals, ditches, ponds and rice paddies (Engeszer *et al.*, 2007). The water is near-neutral to somewhat basic pH and the temperature ranges from 16 to 34ºC (Engeszer *et al.*, 2007). However, the optimal temperature for zebrafish is 28ºC, condition at which they grow quickly and can reach sexual maturity within two-three months (Halder *et al.*, 2010). Zebrafish are omnivorous, primarily eating zooplankton, phytoplankton and insects, although in scarcity times, they can also eat a variety of other foods such as worms and small crustaceans. In research laboratories, adults are often fed with artemia, or paramecia (Spence *et al.*, 2008). They can be easily maintained in aquariums filled with charcoal filtered tap water and an oxygen saturation of more than 80%. One female spawns between 50 and 200 eggs on a daily basis. They are telolecithal, non-adherent and fully transparent, with a meroblastic and discoidal cleavage (Figure 3) (Embry *et al.*, 2010). Embryo development is fast (Figure 3), with precursors to all major organs appearing within 36 hours post fertilisation (hpf) and hatching occurring at 48-72 hpf. By 120 hpf, zebrafish has developed organs and tissues, including brain, heart, liver, pancreas, kidneys, intestines, bone, muscles, nerve systems, and sensory organs. Independent feeding occurs by 5 days postfertilization, when all the nutrients stored in the yolk sac are consumed. Nevertheless, larvae, which are only 1–5 mm long, can live without feeding for 8 days in standard 96-well microplates.

Zebrafish genome is fully sequenced and it contains approximately 26.000 protein coding genes over 1,4 billion base pairs on 25 pairs of chromosomes (Howe *et al.*, 2013). The number of protein coding genes is the same as for the human genome in about half the size, and over a similar number of chromosomes. Finally, there is at least one orthologue for approximately 70% of human genes. This percentage is even higher, reaching the 82%, when only disease-related genes are taken into account (Howe *et al.*, 2013).

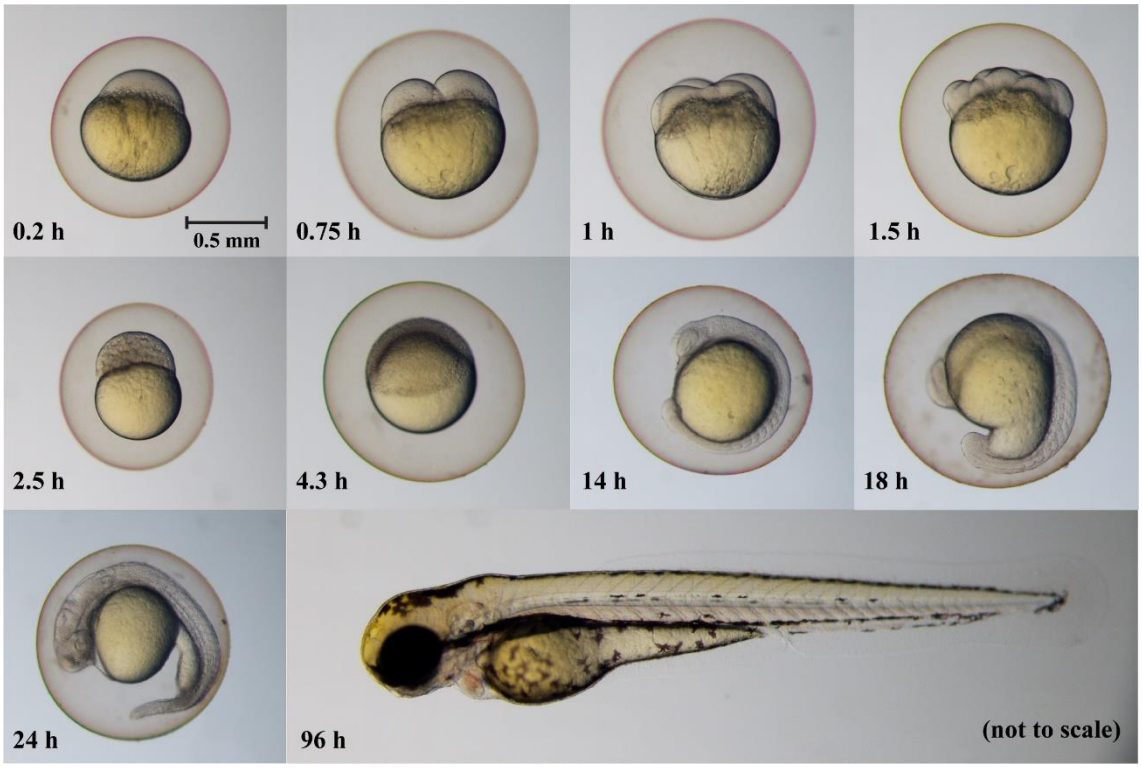

Figure 3. Zebrafish developmental stages. Photos to scale except the photo at 96 h.

1.2.2. Zebrafish in scientific research

Zebrafish ease of care, prolific breeding, transparency, and rapid external development made it an increasingly popular model organism in many fields of biology. Its use as a vertebrate model extends back to the 1930s, thanks to Charles Creaser, who began to introduce it in student laboratories as well as for experimental research (Creaser, 1934). In the following decades, several groups used zebrafish to study development, toxicology, neurobiology and cancer (Battle and Hisaoka, 1952; Marrable, 1965; Stanton, 1965; Endo and Iingalls, 1968). In 1980s, George Streisinger finally propelled its use as a laboratory animal model, elaborating the groundwork for many key experimental settings. He optimized zebrafish care and breeding, and developed tools for genetic and clonal analysis (George *et al.*, 1981; Walker and Streisinger, 1983; Streisinger, G., Singer, F., Walker, C., Knauber, D., & Dower, 1986). Indeed, zebrafish clones by Streisinger are among the earliest successful ones generated in vertebrates. As a consequence of it, the number of laboratories using zebrafish increased. This fact allowed a key initiative, the so called Big Screen or Tübingen/Boston screen, which boosted and consolidated the use of zebrafish in research by the end of 1990s (Meyers, 2018). The study was led by Christiane Nüsslein-

Volhard and Wolfgang Driever and consisted in the generation of stochastic mutation in the male sperm, by using the chemical mutagen N-ethyl-N-nitrosourea (ENU). The phenotypic analysis of the mutant progeny allowed to correlate phenotypes and genotypes to identify novel players in vertebrate development and behaviour. This ground breaking effort demonstrated that zebrafish are amenable to large-scale forward genetic screens, previously limited only to invertebrates such as flies, worms and yeast. It finally lead to the description of ~4000 zebrafish mutant lines with altered development or behaviour (Haffter *et al.*, 1996).

The success of this initiative opened the door to the generation of many different genetic assays. The use of the Tol2 and Sleeping Beauty transposon systems in zebrafish embryos allowed the generation of multiple transgenic lines expressing fluorescent proteins or other proteins involved in different cellular processes (Davidson *et al.*, 2003; Kawakami, 2007; Kwan *et al.*, 2007). Furthermore, the injection of morpholinos in embryos permitted to temporally knock-down the expression of a particular gene to study its function (Nasevicius and Ekker, 2000). More recently, several direct genome editing strategies based on zing-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and mostly CRISPR/Cas have been used to target genes-of-interest (Foley *et al.*, 2009; Huang *et al.*, 2011; Cornet, Di Donato and Terriente, 2018).

Since the Big Screen, zebrafish has increasingly been used in behavioural, disease and toxicology studies, besides in developmental biology. Finally, it is also a valuable regenerative model, due to its notable regenerative capabilities. It is indeed, able to quickly repair or replace many cells types and tissues including heart, brain, spinal cord, appendages and sensory cells within the eye and ear (Gemberling *et al.*, 2013).

1.2.3. Zebrafish as a tool for drug discovery and development

Zebrafish larvae absorb and respond to small molecules diluted in the surrounding water through their skin and gills. In addition, their small size allows the use of low amount of compounds. These aspects, together with zebrafish larvae unique biological properties, such as fast life cycle, large progeny, transparency, ease of maintenance, and high genetic and physiologic homology to humans, make the zebrafish a promising animal model for different phases of drug discovery and development processes (MacRae and Peterson,

2015). In particular, zebrafish is an excellent tool for small-molecule discovery, optimization and preclinical development of hit compounds.

Screening of lead compounds

As outlined above, zebrafish have several inherent advantages that make them suitable for high-throughput drug screenings (Delvecchio, Tiefenbach and Krause, 2011; Lessman, 2011). Most zebrafish-based screenings are performed following the PDD approach. Thus, libraries of compounds are usually filtered based on the promotion or reversion of specific phenotypes in wild type or genetically modified larvae. From there, the leads are identified. These screens have focussed on different aspects ranging from embryo morphology to cardiac physiology and sleep, and have identified not only novel classes of molecules, but also repurposing opportunities for existing drugs (MacRae and Peterson, 2015; Rennekamp and Peterson, 2015).

Once the most promising lead compounds are identified, PDD screening is usually followed by secondary assays designed to identify the targets and optimise the efficacy of the molecules.

Study of structure-activity relationship (SAR)

The optimisation of the lead molecule to a drug candidate is generally performed through structure-activity relationship (SAR) studies. The aim is to assess the relationship between a chemical structure and its biological activity. Related entities are generated with different side groups in order to confer greater potency, and minimal off-target effects (Patani and LaVoie, 1996). Zebrafish provide a powerful *in vivo* model to compare the capacity of structurally related molecules of causing defined phenotypes and modulating specific pathways (Hao *et al.*, 2010). In addition, they allow to simultaneously assess the effects of chemical modifications on both efficacy and toxicity of the compounds. This is an important advantage compared to traditional *in vitro* SAR models that serves to study the consequences of structural changes on the potency of the molecules, but not on the ADMET profiles (Luz Romero and Vela, 2014).

Target identification and validation

Identification of novel targets is a bottleneck in drug discovery. To date, approximately 2% of predicted proteins have been targeted with small molecules, while the estimated fraction of druggable proteins is approximately 15% (Das *et al.*, 2013). Another substantial hurdle is target validation, i.e. identify the functional role, the MOA of the target in the disease. There is a rich variety of computational, biochemical and genetic techniques for MOA determination. They are based on initial clues provided by structural or phenotypic effect comparison with known drugs, binding and site of action of each compound (Rennekamp and Peterson, 2015). However, these approaches are challenging and new alternatives are welcome. There is a vast arsenal of molecular biology and genetic approaches available for rapid target identification and validation through phenotypic effect comparison in zebrafish (Ito *et al.*, 2010). Importantly, the large collection of phenotypes associated with specific gene mutations and knock-downs can be used to identify similarities between drug-induced and genetic alteration-derived compositions. This approach is much more powerful than any other performed in culture, because a whole organism recreates all the interactions at the cellular and tissue level, as well as the metabolic aspects, in a way that a more reductionist *in vitro* model is not able to recapitulate. In addition, there are a much higher number and variety of phenotypes that can be distinguished (MacRae and Peterson, 2015).

Moreover, the emergence of the CRISPR-Cas technology has allowed to easily generate targeted loss-of-function mutants and knock-in lines (Chang *et al.*, 2013; Auer *et al.*, 2014). Thus, it is possible to obtain a vast repertoire of human disease-associated alleles in zebrafish (Liu *et al.*, 2017; Lu *et al.*, 2017), and the challenge now is to figure out their effect and to translate this knowledge into new therapies. In this regard, an additional interest that I pursued during my PhD studies, has been to review the use of the CRISPR-Cas9 technology in zebrafish to streamline the drug discovery process. As a result, a review paper in Frontiers of pharmacology was published (see Annex). Also, I collaborated in a study on the genetic and behavioural characterization of a zebrafish model for leukodystrophy, headed by the group of Dra. Aurora Pujol from Idibell (Pant *et al.*, 2019).

Drug toxicity

Previously listed zebrafish unique characteristics confers the possibility to efficiently perform toxicity screens of whole libraries of compounds. More importantly, zebrafish has been shown to have comparable targets, physiology, drug metabolism and pharmacology to humans (MacRae and Peterson, 2015). On these bases, they have been increasingly used for general toxicology assessment and, particularly, for cardiotoxicity, neurotoxicity and hepatotoxicity evaluation. Remarkably, these three types of toxicity are among the most common reasons for drug attrition in clinical phases and post market withdrawal (Cook *et al.*, 2014; Waring *et al.*, 2015; Blomme and Will, 2016).

Potential drugs are usually screened for **cardiotoxicity** through electrophysiological activity measurement in cells or in mammals. Zebrafish represents an interesting alternative animal model as heart is fully functional, with a complex repertoire of ion channels, and a completely develop vascular system by 96 hpf (Leong *et al.*, 2010). Furthermore, cardiac functions and hemodynamic parameters, such as heart rate, contractility, rhythmicity, gross morphology and blood flow, can be visually assessed in living animals thanks to embryos transparency and to the different fluorescent transgenic lines available (Chico, Ingham and Crossman, 2008). Finally, zebrafish heart, although differing in structure from mammalian hearts, exhibits similar functional characteristics: rhythm is regulated by an electrical system and heart beat is associated with a pacemaker activity, with pacemaker cells generating impulses that set the pace for blood pumping. Indeed, human cardiac electrophysiology is more similar to zebrafish than to rodents (MacRae and Peterson, 2015). One of the major components participating in this heart electrophysiology is the ion channel codified by the ethera-go-go-related gene (*ERG*). It is among the most important effectors in cardiotoxicity, as drugs-interaction with the ERG protein can lead to severe cardiac arrhythmia and death. Indeed, it is a common practice in the early phases of the drug R&D process, to test drugs cardiotoxicity effects by evaluating human ERG (hERG) channel blockade (Priest, Bell and Garcia, 2008). Zebrafish Erg is already present early in development and has a 99% conserved amino acid sequence with the hERG in the pore-forming domain. Accordingly, hERG inhibitors and other drugs, which have been associated with prolonged QT intervals in humans, consistently cause bradycardia and atrioventricular block in zebrafish embryos (Milan *et al.*, 2003; Wen *et al.*, 2012). In another study, molecules with toxic effects on the cardiac

activity and circulatory system in humans similarly recapitulate them in zebrafish (Schwerte and Pelster, 2000). During my PhD project, I was part of a recently published study, in which we used zebrafish embryos to test 92 compounds with known molecular targets and cardiotoxic activity in humans. Results show better sensitivity than rodents, 55.3 versus 3%, and only slightly lower specificity, 86.7% versus 91%. Moreover, zebrafish has a predictive performance similar to dogs (62% specificity, 87% sensitivity), which is the standard preclinical regulatory model for addressing cardiotoxicity (Dyballa *et al.*, 2019).

Zebrafish are also a promising *in vivo* model for **neurotoxicity** screens. Current approaches mainly rely on labour-intensive behavioural and morphological assays in mammals (Luz Romero and Vela, 2014). By five dpf, zebrafish central nervous system (CNS) is fully functional and they possess all the senses (vision, olfaction, taste, tactile, balance and hearing) (De Esch *et al.*, 2012). Sensory pathways share an overall homology with humans, and zebrafish possess complex CNS-driven behaviours such as memory and learning. Moreover, the blood brain barrier (BBB) development and functionality is similar to other vertebrates (Eliceiri, Gonzalez and Baird, 2011), and zebrafish present mammalian neurotransmitter systems such as GABA, dopamine, glutamate, serotonin, noradrenalin, acetylcholine and histamine, although differences in expression patterns are observed (Panula *et al.*, 2006, 2010). The small size of larvae allows to perform assays in a 96-well microplates, which can be coupled to a system that simultaneously tracks and records larvae movement, enabling high-throughput neurotoxicity screenings (Kokel *et al.*, 2010; Ingebretson and Masino, 2013). Based on this, several sensorimotor studies have been performed in this animal model, in which deviations from normal behaviours can be assessed as an indirect measurement of neurotoxicity. Drug-induced visual impairment, ototoxicity, olfactory toxicity, alterations in locomotor activity, anxiety, seizures, impaired memory and learning have been evaluated (Baraban *et al.*, 2007; Clark, Boczek and Ekker, 2011; De Esch *et al.*, 2012; Bailey, Oliveri and Levin, 2015; Niihori *et al.*, 2015). Zebrafish has shown behavior alterations similar to humans when treated with neuroactive compounds (e.g., ethanol, *d*-amphetamine, caffeine or cocaine), sedative molecules (e.g., citalopram, tramadol or diazepam) or neurotoxins (e.g., MPTP or 6-OHDA) (Anichtchik *et al.*, 2004; Lam, Korzh and Strahle, 2005; Irons *et al.*, 2010; Tran *et al.*, 2017; Bachour *et al.*, 2019). In addition, they respond to subtle and complex stimuli,

such as those produced by psychotropic drugs (Kokel and Peterson, 2011; Neelkantan *et al.*, 2013; Kyzar and Kalueff, 2016). Also, zebrafish is a good model for the detection of developmental neurotoxic (DNT) compounds (Nishimura *et al.*, 2015; d'Amora and Giordani, 2018), and a recent study shows that seventeen of the eighteen compounds known to be DNT in mammals, were also DNT in zebrafish (Hagstrom *et al.*, 2019). Finally, embryos transparency, together with the vast number of available transgenic lines, allows the visualization of the entire nervous system in living animals and specific neuronal, apoptotic and neurotoxicity markers can be analyzed in fixed intact zebrafish by immunohistochemistry or in situ hybridization.

Hepatotoxicity is the most common adverse drug response that leads to the failure of otherwise promising drug candidates (Navarro and Senior, 2006). Drug-induced hepatotoxicity is usually assessed *in vitro* by evaluation of biomarkers, such as cytochromes, in organelles (e.g. liver microsomes), or in hepatocytes. It is also evaluated *in vivo* by serum enzyme and hepatic excretory tests, assessment of alterations in the chemical constituents of the liver, and histological analysis (Luz Romero and Vela, 2014). However, *in vitro* assays have less than 25% sensitivity for the detection of hepatotoxins (O'Brien, P. J., Slaughter, M. R., Biagini, C., Diaz, D., Gao, B., Irwin, 2003), and among all the organ toxicities evaluated *in vivo*, hepatotoxicity is the one showing the poorest correlation with humans (Olson *et al.*, 2000; O'Brien *et al.*, 2006). Zebrafish offers an interesting alternative to conventional rodent models, as its liver, which is fully functional from 72 hpf, is highly similar to the mammalian in terms of biological function (Vliegenthart *et al.*, 2014). Indeed, defensive mechanisms against xenobiotic chemicals, such as enzyme and oxidative stress induction, are equivalent in zebrafish and mammals, indicating that they may have analogous detoxification pathways (McGrath and Li, 2008). Moreover, many homologs of mammalian genes codifying for drug metabolizing enzymes are expressed in the zebrafish liver, including various members of the cytochrome P450 3A family such as CYP3A, CYP1A, CYP19, and CYP26 (Carney, Peterson and Heideman, 2004; Bresolin, De Freitas Rebelo and Dias Bainy, 2005; Tseng *et al.*, 2005). Taking advantage of this functional similarity and of larvae transparency, several hepatotoxicity high-throughput screens have been performed based on morphological, chromogenic and fluorescent changes detection (Hill *et al.*, 2012; He *et al.*, 2013). Thus, liver degeneration, changes in size and shape, and yolk sac lipid retention

have been visually assessed either in wild type zebrafish or in transgenic lines expressing fluorescent proteins in hepatocytes (Zhang, Li and Gong, 2014). Yolk sac lipid retention is an indirect endpoint of liver function, as 70% of the yolk is composed by neutral lipids that are mostly metabolised through the liver. Therefore, diminished lipid-metabolization is indicative of impaired liver function (Jones *et al.*, 2008). In addition, enzymes such as biotin and carboxylase are found in zebrafish and the assessment of their levels and activity could be used to infer liver functionality (Zhang, Willett and Fremgen, 2004). Finally, histology can be performed on large numbers of larvae to give support to the readouts obtained through the above described screens (Hill, Howard and Cossins, 2002).

In conclusion, zebrafish is becoming an increasingly popular toxicology model in the drug R&D process as it is the only vertebrate with a real capacity to be used in high-throughput screens. On the other side, results obtained in zebrafish, as an *in vivo* model, are more predictive than the ones obtained *in vitro*, as they involve ADME properties. Thus, many of the *in vitro* screens may fail to detect compounds that are only toxic or active after metabolic conversion or, alternatively, they may fail to identify compounds that are inactive and/or secretable after being metabolically converted.

On these bases, the first part of my thesis project consisted in generating a middle-high throughput platform for the evaluation in zebrafish larvae of the three organ toxicities reported above. The objective was to sequentially integrate cardio-, neuro-, and hepato-toxicity independent analysis in the same larva, reducing the number of animals used and understanding the correlation between the three different toxicities. As a result of this work, we developed and validated the ZeGlobalTox assay, which resulted in a publication in the International Journal of Molecular Sciences (Results, Part 1).

Disease modelling and drug efficacy studies

Zebrafish genetic and physiological homology to humans, together with the emergence of gene editing techniques, particularly CRISPR-Cas9 technology, allowed to easily recreate human disease in this model (Liu *et al.*, 2017; Cornet, Di Donato and Terriente, 2018)(Annex). Examples of modelled disorders include developmental, neurological and cardiovascular pathologies, pigmentation defects, metabolic conditions and cancer (Kari, Rodeck and Dicker, 2007; Lieschke and Currie, 2007; Luz Romero and Vela, 2014).

Clearly, zebrafish models of human diseases are used in drug efficacy studies. An example of a compound discovered with such methodologies is prohema, which increases the number of haematopoietic stem cells and is currently in Phase II trials in patients undergoing umbilical cord blood transplantation for leukaemia and lymphoma (North *et al.*, 2007). Another successful drugs is dorsomorphin, used for the treatment of fibrodysplasia ossificans progressiva and anaemia of inflammation (Yu *et al.*, 2008). Finally, PROTO-1 is currently in development for preventing antibiotic-induced hearing loss (Owens *et al.*, 2008). With advances in genetics, imaging and automation, more diseases will be soon amenable to be modelled in zebrafish.

One of the most concerning disease worldwide is cancer. It is the second leading cause of death worldwide, after cardiovascular disease, and it was responsible for an estimated 9.6 million deaths in 2018. It means that globally, about 1 out of 6 deceases is due to cancer. Therefore, better disease understanding and therapies are necessary to alleviate its burden. In this regard, another main focus of my thesis was the establishment of a zebrafish larvae model for cancer disease, useful for the discovery of new better therapies.

1.3. Cancer

1.3.1. Epidemiology

Cancer is defined by the WHO as the generic term for “a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs” (WHO-cancer). The most commonly affected organs are: lung, breast, colon, prostate, skin (non-melanoma tumours) and stomach. The highest mortality rates are reported for: lung (18.4% of the total cancer deaths), colon (9.2%), stomach (8.2%) and liver (8.2%) cancers (Bray *et al.*, 2018). Besides its huge social and medical impact, this pathology represents a significant and increasing economic burden. Indeed, neoplasms are the principal indication for medical care expenditure and pharmaceutical investment nowadays (Stewart and Wild, 2014).

During the last decades, multiple actions have been taken in order to bring social awareness, identify risk factors and implement existing evidence-based prevention

strategies to reduce the cancer load. Also, huge efforts have been done to promote early diagnosis, in order to reduce the disability, suffering and deaths caused by this condition. A better understanding of tumour biology has allowed the development of multiple therapies that are currently in use (Figure 4). Normally, if a tumour mass is localized, it is removed through surgery (e.g., polypectomy in colorectal cancer). Chemotherapy, and for some types of cancer radiotherapy, is mostly used to treat tumours that have already disseminated or for which surgery is not possible, due to the position of the primary mass (e.g. most brain neoplasms). Although these treatments have long been the gold standards, they generate serious side effects, as the administration is systemic and they target all proliferating cells in the body, not discriminating between transformed cells and normal cells in highly regenerating tissues (Brower, 2013). Furthermore, such therapies have little or no effect on quiescent cells, which have been proposed to include cancer stem cells, pointed out as the responsible for drug resistance, relapse and metastatic dissemination in some types of neoplasia (Visvader, 2011; Shibue and Weinberg, 2017). Therefore, more precise target therapies have been developed to avoid drug resistance and diminish side effects, such as imatinib for the treatment of BCR-ABL positive chronic myelogenous leukaemia patients (Hochhaus *et al.*, 2017) (Figure 4). An interesting strategy is the so-called synthetic lethality, which consist in the combination of two or more genetic lesions that are solely no lethal, but together result deadly to the cell. One successful example is the treatment with PARP inhibitors of tumours carrying germline mutations in either *BRCA1* or *BRCA2* genes (Lord and Ashworth, 2017). BRCA1 and BRCA2 are proteins involved in double-strand DNA breaks (DSB) repair, whereas the PARP1 protein is involved in the repair of single-strand breaks. Drugs inhibiting PARP1 trap the protein on DNA and block its catalytic activity, causing multiple DSB that cannot be efficiently repaired in tumour cells with *BRCA* genes mutated, leading to their death (Lord and Ashworth, 2017). Finally, one of the most promising approaches is immunotherapy, which aim to boost the body natural defences to fight cancer (e.g., T-cell adoptive transfer for the treatment of advanced melanomas) (Morgan *et al.*, 2006). These different strategies, alone or in combination, together with early tumour detection, have helped to improve overall patient survival. However, while some types of cancer can be efficiently treated, others remain more challenging and deadly (Bray *et al.*, 2018). Thus, despite the development of novel drugs with different MOA, the benefits for patients are

still modest in general (Fojo, Mailankody and Lo, 2014). Indeed, the average gain in progression-free survival and overall survival are as low as 2.3 and 2.1 months respectively (Fojo, Mailankody and Lo, 2014). This fact reflects the need of more precise, safe and efficacious therapies, as well as a better and faster detection and diagnosis tools (Toniatti *et al.*, 2014). Particularly, the ultimate goal is the development of precision medicine treatments in which each patient, with his known individual genetic and environmental variability, will be treated with the most appropriate therapy.

1.3.2. Drug discovery and development in cancer

Drug discovery in cancer did not become a subject of intense research until the end of World War II. Interestingly, it was a compound derived from a chemical gas used during the war that became the first anti-cancer agent. Nitrogen mustard (Goodman *et al.*, 1984) was effectively used in the treatment of non-Hodgkin's lymphoma (Gilman and Philips, 1946), after the observation of lymphatic suppression in soldiers who died from an accidental release of stockpiled mustard gas. Similar fortuitous discoveries followed for other anti-cancer drugs, which were used based on observed exposure-depending effects without having a precise understanding of the mode of action. Posteriorly, retrospective studies identified the molecular mechanisms of these drugs. Nitrogen mustard, for example, was found to be an alkylating agent irreversibly binding to the alkyl group on DNA, generating interstrand crosslinks and eliciting a strong cytotoxic effect in proliferating cells (Connors and Double, 1970; Nicholson *et al.*, 1970; Scott, 1970). Since then, several improved alkylating compounds with stronger potency have been generated (Gilman and Philips, 1946). Nowadays, there are more than 150 anti-cancer drugs available for clinical use, however, the vast majority of them have not been discovered following such fortuitous approaches (Sun *et al.*, 2017).

Modern drug development process in the cancer field is initiated by the use of fast *in vitro* models for high throughput screenings. A wide range of culture assays has been developed to evaluate different tumoral features, such as sustained proliferation, invasion, genome instability, deregulated cellular energetics, evasion of growth suppressors and resistance to cell death (Figure 4). However, phenotypes involving cell-microenvironment interactions, not only cell autonomous processes (i.e. evasion of

immune response, induction of angiogenesis and inflammation), are difficult or impossible to study with *in vitro* models (Figure 4) (Moffat, Rudolph and Bailey, 2014; Ediriweera, Tennekoon and Samarakoon, 2019).

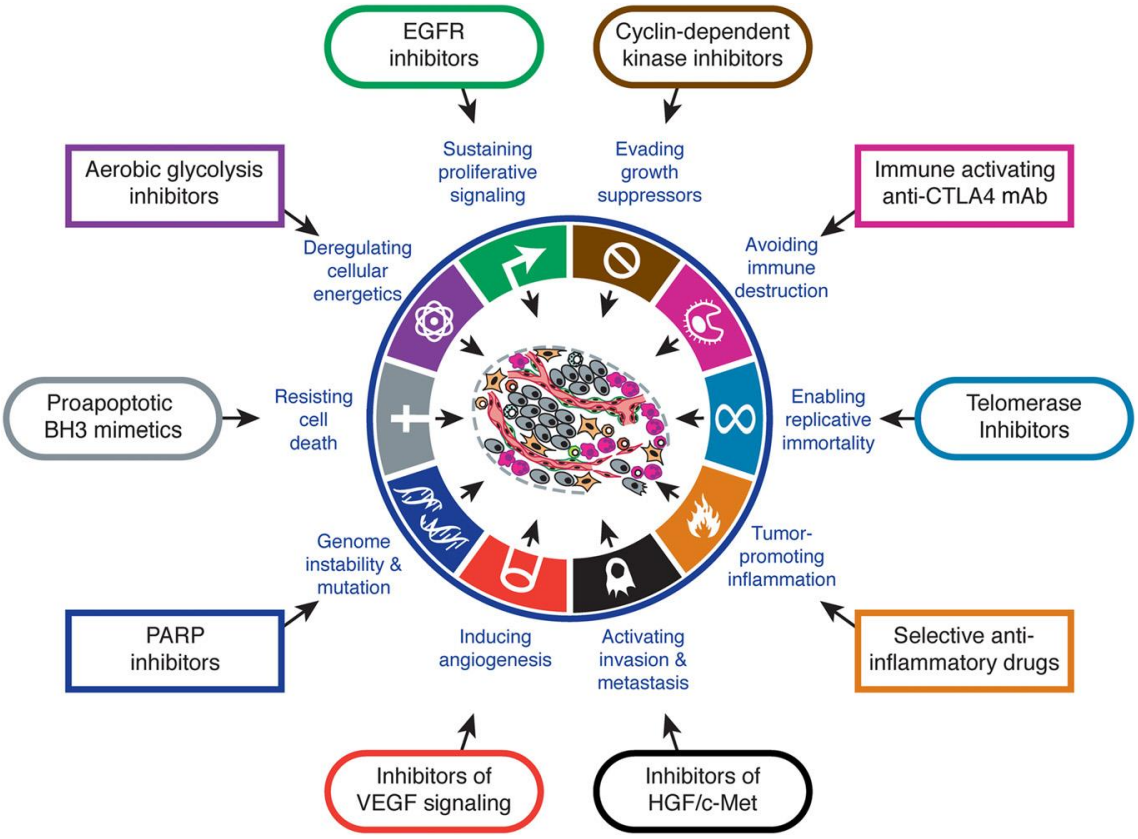

Figure 4. Examples of target therapies affecting different cancer hallmarks. Abbreviations: EGFR stands for Epithelial Growth Factor, HGF stands for Hepatocyte Grow Factor and VEGF stands for Vascular Endothelial Grow Factor. Extracted from: (Hanahan and Weinberg, 2011).

As previously explained for other areas, once a hit has been identified *in vitro*, it is subsequently optimised to a lead compound. So, promising molecules are then tested *in vivo* in order to obtain more precise pharmacokinetics, toxicity and efficacy data.

Mouse xenografts, with either human cancer cell lines or patient tumour samples, are the most prominent *in vivo* models for such studies in cancer (Sia *et al.*, 2015). Mice present a high degree of genetic similarity to humans (~85%, National Human Genome Research Institute) and xenografts are experimentally reproducible. However, they present several disadvantages, such as the low throughput, medium-high costs and the requirement for immunosuppression. Moreover, patient-derived xenografts (PDX) show high

resemblance to human tumours, but the low availability of material from biopsies (0.5-1 million human tumour cells are needed for each xenograft) can be a major issue (Zhang, Moore and Ji, 2011; Sia *et al.*, 2015). In order to circumvent some of these limitations, such as requirement for immunosuppression and sample availability for the generation of PDXs, mouse genetic cancer models can be used instead. They are useful for understanding the effect of different mutations on the biology and progression of tumours. However, derived cancers are normally less complex than the original ones in patients. Furthermore, murine genetic cancer models can be even more time consuming and expensive than the corresponding xenograft and they present a lot of variability in tumour incidence, latency and growth capability (Becher and Holland, 2006).

In conclusion, both type of mouse models (genetic and xenograft) have their own advantages and disadvantages, which may be complemented with the use of other less common *in vivo* models, such as *Drosophila melanogaster, Caenorhabditis elegans* or zebrafish. As previously pointed out, the selection of suitable *in vivo* models in the preclinical phase is especially important, as it evaluates the toxicity, efficacy and PK and PD profiles, which determines the entry subsequent clinical phases (Talmadge *et al.*, 2007).

1.3.3. Problems in the anti-cancer drugs R&D process

As above depicted, drug R&D is a challenging process. Moreover, the generation of novel anti-cancer treatments has its specific hurdles associated with the complexity of the disease. Indeed, cancer encompasses more than 100 distinct malignancies with diverse risk factors and epidemiology (Stratton, Campbell and Futreal, 2009). The heterogeneity of the pathology and the associated high risks and costs of the R&D phases of drug discovery could explain why anti-tumour treatments prices are rising faster than in other sectors of health care, drawing concerns from patients, physicians, and researchers (Bach, 2009; Mailankody and Prasad, 2014). The expenses associated with the development of a new anti-cancer drug reach $648 million to $2,6 billion and the all process can take 6 to 15 years. Furthermore, the annual cost of a new cancer drug exceeds $100.000, some even nearing $200.000 (Mailankody and Prasad, 2015; DiMasi, Grabowski and Hansen, 2016; Prasad and Mailankody, 2017; Prasad, De Jesús and Mailankody, 2017). These

aspects make anti-cancer compounds unavailable and unaffordable for most patients (Sullivan and Aggarwal, 2016).

One element that significantly contributes to high costs in the R&D phases is the high attrition rate of cancer medications. It exceeds two to four times the one for non-oncology drugs (Hay *et al.*, 2014; Nixon *et al.*, 2017). Indeed, the approval percentage for anti-cancer drugs is only 7% and surprisingly, they are more likely to fail during Phase III randomized controlled trial rather than in early efficacy studies (Phase II). Low Phase III success rates are worrisome, as 65% of all R&D cost has already been spent before this stage, which also account for 60% of all clinical trial expenditure (Hay *et al.*, 2014). The elevated failure degree specifically at this late step also suggests that the bottleneck in cancer drug development lies in the efficacy evaluation of novel compounds (Seruga *et al.*, 2015).

Taking into account the previously mentioned limitations of current *in vitro* and *in vivo* cancer models, together with the extremely high anti-cancer drug attrition rates, it is clear that there is a lack of translatability and better preclinical tools are needed in order to predict the most promising compounds in reasonable times. This would allow narrowing down the number of molecules entering expensive clinical phases with a better rationale and, therefore, decreasing their attrition rates and unsustainable prices. In this regard, zebrafish could be a useful complementary model, as it is more physiologically relevant than *in vitro* systems and, at the same time, cheaper and faster than *in vivo* mice models.

1.3.4. Zebrafish in cancer

Zebrafish have been found to spontaneously develop tumours, which are similar to human malignancies in genetics, morphology, histology and signalling pathways (Patton *et al.*, 2005; Langenau *et al.*, 2007; Basten *et al.*, 2013; Yan *et al.*, 2019). These neoplasia commonly originate in testis, gut, thyroid, liver, peripheral nerve, connective tissue, and ultimobranchial gland, but also in blood vessels, brain, gill, nasal epithelium, and lymphomyeloid system (Smolowitz, Hanley and Richmond, 2002; Matthews, 2004). Moreover, the vast majority of human cancers can be reproduced in zebrafish through chemical treatment, genetic technologies, and tumour cell transplantation.

Mutant lines

Zebrafish have been used in cancer studies since their beginnings in research (Stanton, 1965; Pliss and Khudoley, 1975), when they were shown to develop tumours upon treatment with carcinogen (Pliss, G.B., Zabezhinski, M.A., Petrov, A.S., and Khudoley, 1982). In addition, exposure to mutagenic compounds, such as dimethylbenzanthracene (DMBA), N-nitrosodimethylamine (NDMA), ENU, and N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG), has led to the generation of a wide variety of tumour types (Beckwith *et al.*, 2000; Spitsbergen *et al.*, 2000a, 2000b; Mizgireuv *et al.*, 2004). Most of the induced tumours were found in the digestive system (i.e. liver, pancreas, and intestinal canal), skin, muscle, vasculature, and testis. Several studies took advantage of zebrafish small size, large progenies, fast life cycle, easy manipulation and high genetic and physiologic homology with humans to perform forward genetic screens for the identification of genes and drugs with an impact on cancer biology (Amsterdam *et al.*, 2004; Stern *et al.*, 2005; Moore *et al.*, 2006; Shepard *et al.*, 2007). These methods have the advantage to be unbiased, allowing the identification of previously unknown genes or new functions for already known ones. However, they are time-consuming and present limitations such as the difficulty to effectively isolate mutations, due to functional redundancy, and the need of measurable phenotypes. Furthermore, mutations are generated randomly, not allowing the control of where and how they are produced (Lawson and Wolfe, 2011).

These disadvantages were solved with the emergence of reverse genetic approaches, which allow the introduction of mutations specifically in the gene of interest. Several types of tumours have been generated using such strategies and, particularly, genome editing techniques like ZFNs, TALENS and mostly CRISPR/Cas. Mutations have been introduced in main tumour suppressor genes, such as: *p53*, *apc*, *nf1*, *ptenb*, (Faucherre *et al.*, 2008; Phelps *et al.*, 2009; Shin *et al.*, 2012; Ignatius *et al.*, 2018) or mismatch DNA repair genes, like: *mlh1*, *msh2*, *msh6* (Feitsma *et al.*, 2008).

Zebrafish cancer models can also be generated through transgenesis. Several systems have been used, ranging from the commonly used promoter-oncogene Tol2 or Sleeping Beauty transposon constructs, to inducible (e.g., heatshock) and bipartite expression systems like Gal4/*UAS*, Cre/*loxP*, and lexA/*lexAOP* (Langenau *et al.*, 2003; Patton *et al.*,

2005; He *et al.*, 2016; Nguyen *et al.*, 2016; Mayrhofer *et al.*, 2017). An advantage of these last strategies and combinations of the two (e.g., Tet-ON, Cre^{ERT2}/*loxP*) is their ability to circumvent oncogene-related lethality prior to sexual maturity, due to temporal and spatial control of gene expression.

Transgenic zebrafish lines have also been established through the use of sophisticated vector systems. An example is represented by the miniCoopR, which carries a *mitf* (melanocyte inducing transcription factor) minigene and a cassette where the gene (oncogene) of interest is allocated under the control of the *mitf* promoter. This vector has been introduced in a triple mutant Tg (*mitf:*$BRAF^{V600E}$*); p53*$^{-/-}$*; mitf*$^{-/-}$ zebrafish strain. As *mitf* expression is required for the generation of melanocyte progenitors and mature cells, the inability of generating such melanoma prone melanocytes (express oncogenic $BRAF^{V600E}$ and carry a p53 loss-of-function mutation), protects mutant fish from developing melanoma. However, the introduction of the miniCoopR vector rescues the expression of *mitf* and allows the study of the effect of the gene (oncogene) of interest in melanoma formation (Ceol *et al.*, 2011; Iyengar, Houvras and Ceol, 2012). Furthermore, adaptations of the miniCoopR vector in combination with the CRISPR/Cas9 technology have allowed to study the activity of several tumour suppressor genes (Ablain *et al.*, 2018). Finally, there is a novel system, called TEAZ, which allows the electroporation of the DNA constructs into adult fish, at a specific location and time (Callahan *et al.*, 2018).

All these zebrafish genetic cancer models have been shown useful to study processes of tumour initiation and development, as well as the interactions between malignant and normal cells, tissues and structures. However, as previously explained for mouse genetic cancer models, limitations are represented by the time required for tumour formation and the variability in cancer incidence and growth.

Transplantation of tumour cells

Another approach to study cancer in zebrafish is the transplantation of tumour cells. This strategy circumvents some of the previously mentioned genetic models limitations, as well as the existing differences between zebrafish and human tumours, when xenografted cells are of human origin. In this regard, depending on the origin of the cells, two types of transplantations can be distinguished: allotransplantation and xenotransplantation

(Figure 5). Allogeneic transplantation is the transfer of cells, tissues, or organs to a recipient from a genetically non-identical donor of the same species, whereas xenotransplantation or heterologous transplant is the process of implanting living cells, tissues or organs from one species to another (Figure 5).

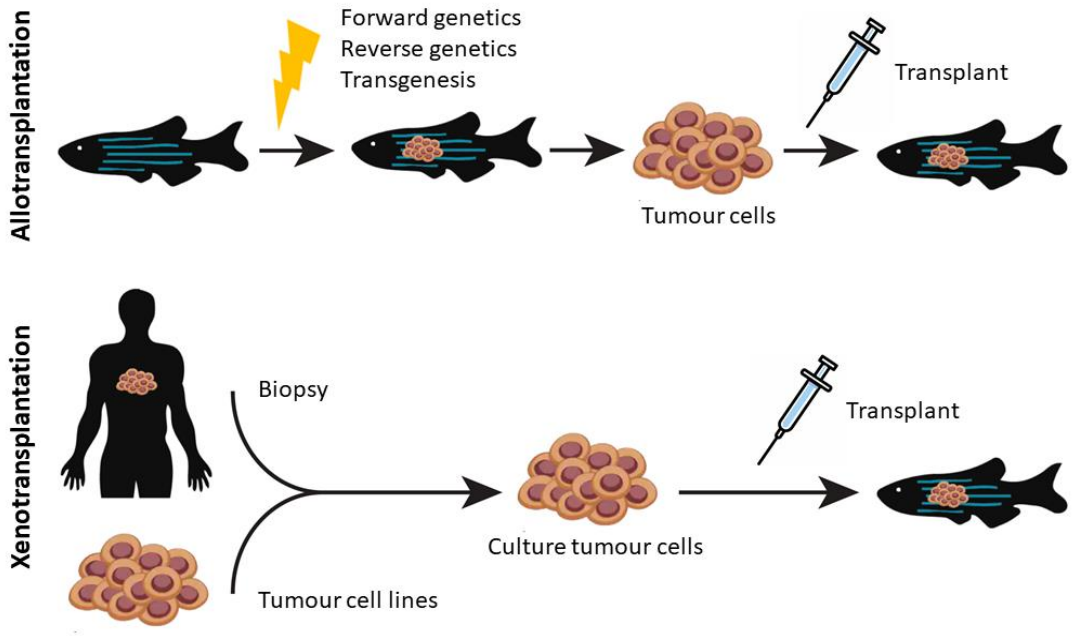

Figure 5. Types of tumour transplantation in zebrafish depending on the origin of the tumour.

Due to the small size, fast life cycle and transparency, zebrafish larvae represent an ideal tool to directly visualize the processes of angiogenesis, intravasation, extravasation, migration and metastasis formation, in a large number of animals, favouring higher throughput and statistical robustness (Nicoli and Presta, 2007; Lee *et al.*, 2009; Chen *et al.*, 2015). Moreover, zebrafish embryos do not fully develop their adaptive immune system until four weeks of life (Trede *et al.*, 2004), and they lack innate immune defence until day three or four (Lam *et al.*, 2004). Therefore, there is no need of immunosuppression in case of performing xenotransplantations at early larval stages. In addition, due to the small size, few cells are needed for the successful engraftment.

Another zebrafish advantage is represented by the rapid development of most organs, being fully functional already at early stages (five dpf). This is an important feature, as allows for orthotopic transplantations, which is the transfer of tumour cells to a recipient into the same organ in which cancer has developed in the donor, already at early

developmental timepoints. Most studies report orthografts in larval organs such as brain and eyes (Lal *et al.*, 2012; Jo *et al.*, 2013; Chen *et al.*, 2015; Welker *et al.*, 2016). A main advantage of orthotopic models is the fact that tumour cells are inserted into a similar microenvironment as in their original site and they are, therefore, deemed to resemble more closely the natural tumorigenesis in human. Nevertheless, some organs and tissues are not fully developed in larvae (e.g., mesonephros in kidneys or the BBB in the brain, that are formed around the tenth-twelfth day of life) and zebrafish lack breast, lungs and prostate (Diep *et al.*, 2015; Quiñonez-Silvero, Hübner and Herzog, 2019). This implies the impossibility of generating some orthotopic models. Interestingly, human breast, lung and prostate tumours, as well as other cancer cell types, have been shown to successfully engraft in alternative sites, such as the perivitelline space (pvs) or the yolk sac, where they grow and respond to specific chemotherapeutics (Mercatali *et al.*, 2016; W. Xu *et al.*, 2018; Li *et al.*, 2019). Moreover, it might be possible to add human tissue-specific hormones, as supplement in the water or co-inject them with the cells. Also, specific transgenic zebrafish lines could be generated to produce factors that may favour implantation and growth of tumour cells.

Adult zebrafish can also be employed as hosts for transplantation (Stoletov *et al.*, 2007; Smith *et al.*, 2010; Tang *et al.*, 2014). Adult models are more complex than larval, as they require immunosuppression, present cell tracking imaging limitations and have lower throughput and higher cost (Eden *et al.*, 2015). The methods applied to achieve immunosuppression are similar to the ones used in mice: sublethal radiation (Zon *et al.*, 2004), treatment with dexamethasone (Soza-Ried *et al.*, 2010) or use of immune-compromised lines such as the *rag1*-/-, *rag2*-/- or *myb*-/- mutants (Wienholds *et al.*, 2002; Soza-Ried *et al.*, 2010; Tang *et al.*, 2014). However, immune-compromised zebrafish are not commonly used in transplant experiments because they display other associated diseases and are difficult to maintain. One strategy to avoid immunosuppression consist in syngeneic transplantation of tumour cells from a genetically identical donor fish (Smith *et al.*, 2010). Another more complex method consists in transplanting irradiated human cancer cells into zebrafish embryos, and 3 months later, performing another graft of non-irradiated cells into the same vaccinated fish (Zhang *et al.*, 2016). Finally, adults are not transparent as larvae (not possible to image tumour cells inside the body), with the

exception of specific lines, such as the casper that is not pigmented due to the lack of melanocytes and iridophores (White *et al.*, 2008).

Based on all the above considerations, the most common way to model cancer in zebrafish by transplantation is the generation of larvae xenografts.

Xenotransplantation in larvae

As previously mentioned, murine tumour xenotransplantation studies remain the gold standard for tumour studies and anti-cancer drugs efficacy evaluation. However, the long time required, the high cost and the complexity of these systems foresee the emergence of alternative complementing models. In this regard, zebrafish larvae xenografts have grown in popularity in the last decade since Lee and colleagues performed the first ones to reproduce melanoma (Lee *et al.*, 2005). Nowadays, this is the most common strategy to evaluate cancer progression in zebrafish, as it provides a good compromise between high-throughput and inexpensive but poorly predictive *in vitro* assays and more predictive but low throughput and costly mice xenografts. Zebrafish larvae xenografts are commonly used to assess proliferation, migration, and neovascularization, and to test drugs affecting these cancer hallmarks. Most assays use two dpf embryos, as by this stage, larvae already present most of the precursors to all major organs and have not developed the innate immune system yet.

Zebrafish larvae optical transparency is a great advantage for xenografts assays, as it allows fluorescently labelled transplanted human tumour cells to be easily visualized inside them. Fluorescent cell labelling is achieved through two different strategies: via cell infection with viral vectors coding for fluorescent proteins or, more commonly, through the use of fluorescent membrane and cytoplasmic dyes (Corkery, Dellaire and Berman, 2011; Hohn and Petrie-Hanson, 2012; Yang *et al.*, 2013; Bentley *et al.*, 2015; De Boeck *et al.*, 2016; Cabezas-Sainz *et al.*, 2018).

The selection of the injection site is important because it can influence how tumours engraft and disseminate, as well as the efficiency of drug administration and delivery and, subsequently, the therapeutic response. In larvae xenograft assays, the yolk sac is the most common site of injection, since it is easily accessible and can accommodate large volumes of cells (Figure 6) (Marques *et al.*, 2009; Eguiara *et al.*, 2011; Veinotte, Dellaire and

Berman, 2014; Cabezas-Sainz *et al.*, 2018). More technically challenging injections have been performed in the hindbrain ventricle (Haldi *et al.*, 2006), heart (intracardiac/pericardiac injections), and into the vasculature, through the caudal vein or the large ducts of Cuvier (DoC) (Figure 6) (Zhao, Yang, *et al.*, 2011; He *et al.*, 2012; Kanada *et al.*, 2014; De Boeck *et al.*, 2016; Sacco *et al.*, 2016). Transplants into the blood circulation allows the study of extravasation and micrometastasis formation, skipping the earlier metastatic events of invasion and intravasation (He *et al.*, 2012). Finally, an increasingly used injection site is the perivitelline space (Figure 6) (Nicoli and Presta, 2007; Lee *et al.*, 2009; Zhao, Wang, *et al.*, 2011; Fior *et al.*, 2017). This region between the skin and the outer membrane of the yolk sac is suitable for the study of primary tumour growth and the all metastatic process, since it provides a suitable microenvironment for the cancer cells to engraft and it is reasonably close to major vessels, facilitating their dissemination.

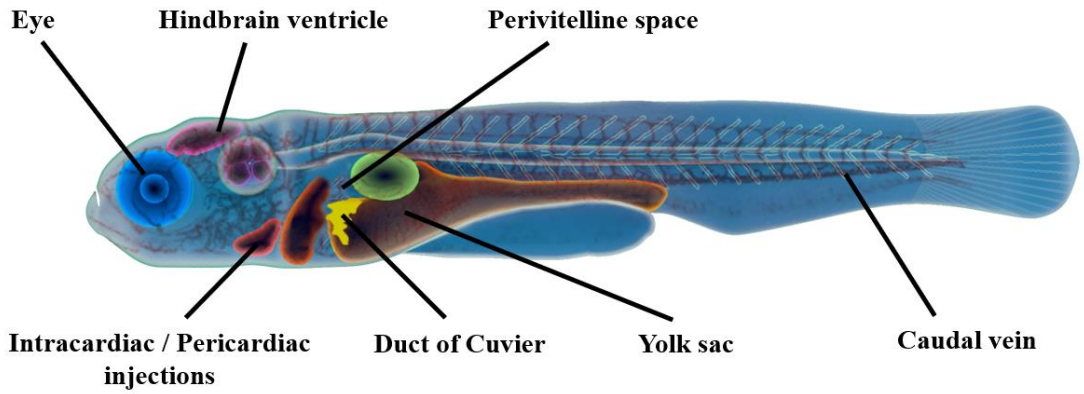

Figure 6: Common injections sites of tumour cell transplantation into zebrafish larvae or adult.

The study of other tumour aspects is facilitated by the availability of different zebrafish transgenic lines. For example, transplantation of human tumour cells into transgenic zebrafish larvae with fluorescent vasculature has been used to specifically investigate angiogenesis, as well as interactions of cancer cells with endothelial cells, intravasation and extravasation (Zhao, Wang, *et al.*, 2011; C Tulotta *et al.*, 2016; Brown *et al.*, 2017; Drabsch, Snaar-Jagalska and Ten Dijke, 2017). Also, xenotransplantation into transgenic zebrafish embryos with fluorescent immune cells, can be used to study the interaction of

human tumour cells with the innate immune system (He *et al.*, 2012; Van Der Ent *et al.*, 2014; Claudia Tulotta *et al.*, 2016; Britto *et al.*, 2018).

Finally, an emerging tendency in translational cancer research is the use of PDX in zebrafish embryos. Tumour cells from primary or metastatic human biopsies are collected by surgery to be transplanted into zebrafish. This field was pioneered by Marques and colleagues who transplanted pancreas, stomach, and colon primary tumours into the yolk of two dpf larvae (Marques *et al.*, 2009). More recently, xenografts of primary cultures of gastric, breast and neuroendocrine cancers have been performed (Mercatali *et al.*, 2016; Gaudenzi *et al.*, 2017; Wu *et al.*, 2017). Interestingly, it has been shown that there are comparable responses to chemotherapy (e.g. FOLFOX and FOLFIRI) and biological therapies (e.g. Cetuximab) among patients and zebrafish PDXs (Fior *et al.*, 2017). Thus, this approach could be used to provide specific information about the effectiveness of a treatment for the development of personalised medicine. Although PDXs have been broadly developed in mouse models, they need a big amount of biopsic material from patients and long incubation periods for tumours to develop (Astone *et al.*, 2017). Therefore, mouse PDXs might not be useful for decision making, as some patients might not be in time to benefit of the experimental findings. Zebrafish could be used as a powerful alternative model, as zebrafish larvae-PDXs require only a small number of cells from biopsies and tumour development is accomplished within few days. This enables a throughput high enough to test different possible therapies with still time for patient information.

The facts presented here foresaw a promising future for zebrafish cancer xenotransplantation studies. However, although really encouraging results have been published, its use is still scarce in the preclinical phases of drug discovery. This might be explained by lack of standardisation in experimental conditions. Important differences have been reported regarding site of injection, cell labelling methods and incubation temperatures, and they often result in contrasting outputs. In this context, the second part of my PhD thesis project consisted in the generation and validation of a standardized automated zebrafish larvae xenotransplantation system, the ZeOncoTest, for the consolidation of the zebrafish model as a powerful tool for the preclinical phase of anti-cancer drug discovery (Results part 2).

2. OBJECTIVES

2. OBJECTIVES

Zebrafish are increasingly being used as a high throughput *in vivo* preclinical tool to assess the toxicity and efficacy of novel drugs. Their high homology with humans, elevated number of progeny, fast life cycle and small size allows physiologically relevant and high throughput studies. In the context of a productivity crisis for the pharmaceutical industry and high drug attrition rates, zebrafish could be used as an alternative model bridging the gap between preclinical *in vitro* studies, which are high throughput but low predictive, and *in vivo* mammalian studies that show more predictivity to humans, but are expensive and time-consuming.

The **general aim** of my **PhD thesis projec**t has been to validate and consolidate the use of zebrafish in the pharmaceutical R&D process, as a tool for the assessment of toxicity and antitumoral efficacy of novel drugs.

Specifically, the **objective** of the **first part** of the project has been to generate a middle-high-throughput platform, for the evaluation of the cardio-, neuro-, and hepato-toxicities in the same zebrafish larvae, reducing the number of animals used in toxicology studies, in agreement with the 3R principles. We called the resulting assay ZeGlobalTox.

The antitumoral drug discovery field is specially affected by the productivity crisis, as anti-cancer drugs attrition rate is two to four times higher compared to other drugs. As a consequence, the median annual cost to launch a new antitumoral compound exceeds $150.000. In addition, despite new treatments in the last decades have significantly improved patient health and survival for some types of malignancies, their effect is still moderate or null for others. Indeed, cancer is the second leading cause of death worldwide.

Zebrafish has been shown to develop neoplasms as a result of exposure to carcinogenic substances or genetic mutations. Furthermore, they are used in xenograft studies to evaluate complex tumour behaviours, such as cell dissemination and colonization. In this context, the **objective** of the **second part** of my thesis project has been to standardize, automate and validate a zebrafish xenotransplantation system, to be used in the preclinical phases of anti-cancer drug discovery. We named our newly developed assay ZeOncoTest.

3. RESULTS

3. RESULTS

3.1. Part 1: ZeGlobalTox

The first part of my PhD thesis project consisted in the improvement and validation of the use of zebrafish larvae as a middle-high-throughput preclinical model for toxicity assessment. Cardio-, neuro-, and hepato-toxicities, which are the main responsible for safety drug attrition in clinical phases and post market withdrawal, have been assessed in the same larvae. Although previous reports have shown the suitability of zebrafish larvae to evaluate these three toxicities independently, none has evaluated them in the same individuals. In this line, our method represents an important innovation in reducing the number of animals, in agreement with the 3Rs principle, as well as the amount of compound used. We finally validated the procedure with drugs with known toxicology in humans. Our results provide a reliable proof of principle of the assay translatability.

Cornet C, Calzolari S, Miñana-Prieto R, Dyballa S, van Doornmalen E, Rutjes H, Savy T, D'Amico D, Terriente J. ZeGlobalTox: An Innovative Approach to Adress Organ Drug Toxicity Using Zebrafish. International Journal of Molecular Sciences. 2017 Apr 19;18(4). DOI: 10.3390/ijms18040864.

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ZeGlobalTox. An Innovative Approach to Address Organ Drug Toxicity Using Zebrafish.

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Abstract: Toxicity is one of the major attrition causes during the drug development process. In that line, cardio-, neuro- and hepatotoxicities are among the main reasons behind the retirement of drugs in clinical phases and post market withdrawal. Zebrafish exploitation in high-throughput drug screening is becoming an important tool to assess the toxicity and efficacy of novel drugs. This animal model has, from early developmental stages, fully functional organs from a physiological point of view. Thus, drug-induced organ-toxicity can be detected in larval stages, allowing a high predictive power on possible human drug-induced liabilities. Hence, zebrafish can bridge the gap between preclinical in vitro safety assays and rodent models in a fast and cost-effective manner. ZeGlobalTox is an innovative assay that sequentally integrates in vivo cardio-, neuro-, and hepatotoxicity assessment in the same animal, thus impacting strongly in the 3Rs principles. It Reduces, by up to a third, the number of animals required to assess toxicity in those organs. It Refines the drug toxicity evaluation through novel physiological parameters. Finally, it might allow the Replacement of classical species, such as rodents and larger mammals, thanks to its high predictivity (Specificity: 89%, Sensitivity: 68%, and Accuracy: 78%).

Keywords: ZeGlobalTox; Zebrafish; high-throughput; adverse drug reaction; drug toxicity; cardiotoxicity; neurotoxicity; hepatotoxicity.

1. Introduction

The direct costs of bringing a new drug to the market are continuously increasing. Nowadays, the estimated costs are higher than US $1 billion per drug, and most of that is spent in the clinical phases [1]. On the other hand, the pharmaceutical industry has

multiplied its investments in R&D. However, this increase does not correlate well with an increased success rate in marketing new drugs. This is partly due to the high rate of compound failure during clinical trials, where only around 10% of the molecules entering phase 1 clinical trials are ultimately approved by the United States Food and Drug Administration (FDA) [2,3]. Lack of efficacy or drug safety (toxicity) are the major factors in drug attrition, with the lack of efficacy being the leading cause of drug attrition during clinical trials and unanticipated toxicity being the most common cause of post market withdrawal [3–6]. To reduce the large costs of drug development, and streamline the whole process, the need arises to identify potential adverse drug response (ADR) as earlier as possible, and, definitely before entering costly regulatory preclinical and clinical phases [7]. In that sense, toxicity affecting liver, heart and/or central nervous system (CNS) in humans are among the most common toxic effects induced by drugs [1,8–10].

During the drug discovery process (candidate selection and lead optimization), the traditional first step in safety understanding is to perform enzymatic or cell culture-based in vitro screenings [11]. These high-throughput assays require small compound quantities and reduce later animal testing, in line with the Replacement, Reduction and Refinement (3R) principles. Although useful as a first indication of putative drug toxicities (e.g., assays for interaction with the human Ether-a-go-go Related Gene (hERG) channel), they often have low predictions of the final human organ toxicity outcomes, which result from complex Absorption, Distribution, Metabolism and Excretion (ADME) mechanisms and cell and tissue interactions, which are biological processes difficult to mimic by in vitro approaches. On the other hand, mammalian toxicity studies remain the gold standard for risk prediction in humans, but they are highly expensive, time-consuming, require large amounts of test compound, and they are not always predictive. Therefore, they are not suitable for early stage toxicology screenings of medium-large compound libraries.

In order to speed up the drug development pipeline, prioritize drug candidates for animal testing, and reduce unnecessary costs in later mammalian studies, academic and pharmaceutical industry researchers are showing an increasing interest in the zebrafish model. Given the high degree of conservation among species, the effects observed in zebrafish-based experiments are considered representative for other higher vertebrate species, including humans. Unlike in vitro models, zebrafish embryos represent a

complex organism where metabolic pathways and other physiological reactions are already established and functional, allowing the evaluation of toxicity, while considering uptake, metabolic reactions, and excretion. Therefore, its use provides a closer scenario to human biology than in vitro systems. Moreover, zebrafish larvae provide several technical and economic advantages, due to its unique properties (extensively reviewed in [12,13]), for developing high-throughput drug screenings. Hence, their exploitation results in a reduction of time and cost, when compared with rodent studies, while providing higher informative value than in vitro studies [13–15]. Furthermore, according to international ethical regulations [16], zebrafish larvae up to 5 days post fertilization (dpf) are considered *in vitro* models and are accepted as an alternative to animal testing [17,18]. Therefore, their use is in accordance with the 3Rs principle.

Based on these facts, we have developed the ZeGlobalTox assay, an innovative experimental procedure that addresses organ-specific toxicity of different drugs on zebrafish larvae (up to 5 dpf). Thus, the proposed approach allows the independent analysis of cardio-, neuro-, and hepatotoxicity effects in the same animal (Figure 1A,B), as a proxy to predict their possible impact in human organs. This allows reducing the amount of larvae used, experimental time, and costs and quantity of tested molecule. Furthermore, since the procedure uses whole animals, it has the advantage of addressing tested compounds' bioavailability, if necessary. We will show that by using ZeGlobalTox, a high toxicity predictivity is achieved – specificity (89%), sensitivity (68%) and accuracy (78%). These results reinforce the validation of zebrafish, as a suitable model for pre-mammalian studies to reduce and/or replace mammalian vertebrate usage, experimental time, and cost during the process of drug discovery and development.

2. Results

2.1 Experimental Work frame

The ZeGlobalTox assay has been designed as a medium-throughput platform to detect, in the same animal, the three most concerning toxicities causing drug attrition, namely cardio-, neuro- and hepatotoxicity. Several issues were considered when planning the experimental protocol. The main concern was that drug-induced mortality and/or developmental toxicity (teratogenicity) could mask possible organ-toxicities appearing later in development. To counteract this prospect, we included a preliminary Acute

Toxicity assay performed with five logarithmic concentrations which follows Organisation for Economic Cooperation and Development (OECD) guidelines (OECD 236) (Fig. 1A). This preliminary assay allowed the identification of non-mortal/non-teratogenic concentrations -no observed effect concentration (NOEC)- to use in the following assays. The hypothesis is that NOEC could affect organ physiology (organ toxicity), while uncoupled from putative developmental toxicity side-effects affecting organ development or function. Another consideration was to start drug incubations at 96 hours post fertilization (hpf), when the analysed organs are close to or already developed (Fig. 1B). The overall aim is to understand the drug impact in organ physiology and function rather than early embryogenesis. The third consideration was to organize the sequential organ evaluation during the experimental and drug incubation time.

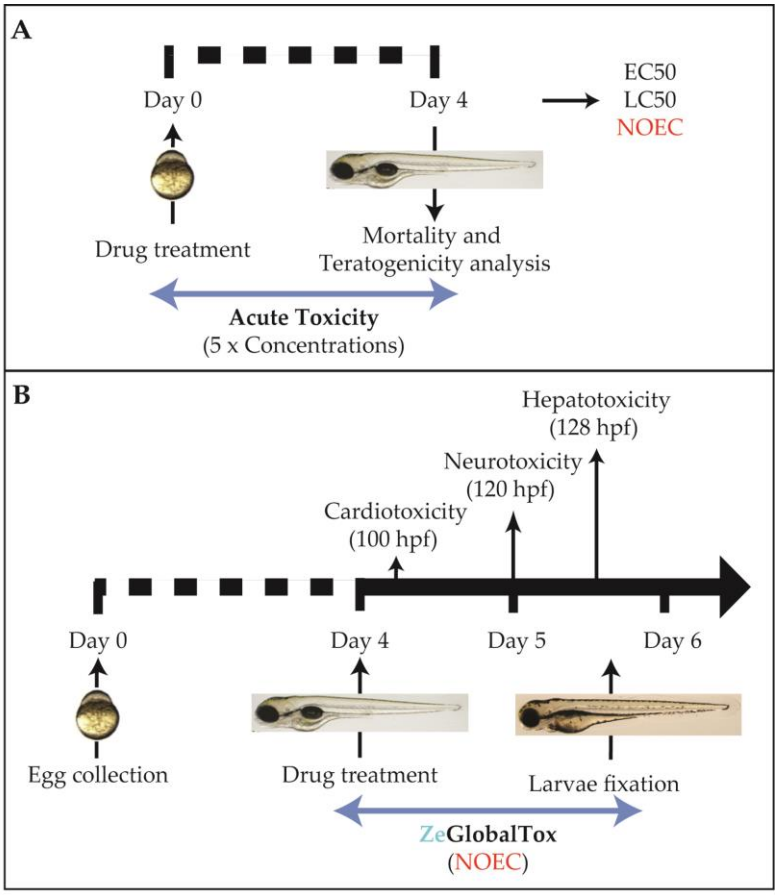

Figure 1. Complete ZeGlobalTox experimental setup. (**A**) Acute Toxicity experimental pipeline; (**B**) ZeGlobalTox experimental pipeline. Drugs are added from 96 hpf. Cardiotoxicity is evaluated at 100 hpf, neurotoxicity at 120 hpf and hepatotoxicity at 132 hpf. Abbreviations: NOEC (no observed effect concentration).

Among the three organs, the heart is developed earlier. In addition, cardiotoxic effects are observable shortly after compound incubation. Thus, cardiotoxicity evaluation was chosen first. Neurotoxicity was analysed second through the drug impact on motor behaviour (locomotion), which is a fundamental readout of CNS function. Although both autonomous swimming and liver development are completed by 5 dpf, phenotypes promoted by hepatotoxic effects require a longer drug exposure. In addition, part of the hepatotoxicity evaluation required fixed larvae. Then, hepatotoxicity was the final parameter evaluated. The integrated experimental pipeline is displayed in Figure 1.

2.2 Test Compounds.

In order to validate our ZeGlobalTox platform, 24 compounds were evaluated; including four drugs used as positive toxic controls. To calculate ZeGlobalTox predictive potential, compounds were chosen according to their know toxicity in humans, as displayed in known molecule toxicity databases such as TOXNET (Hazardous Substances Data Bank, HSDB), Side effects (EMBL), drugs.com, ema.europa. The four selected control drugs have been selected due to their reported toxicities both in humans and zebrafish. Haloperidol, a known anti-dopaminergic antipsychotic drug, has been used as our positive cardiotoxic drug because it has been described to produce hERG blockade, QT interval prolongation, and arrhythmias both in humans and zebrafish [19–21]. MPTP, a prodrug to 1-methyl-4-phenylpyridinium (MPP^+) first synthesized as an analgesic, has been shown to cause permanent Parkinson's symptoms by destroying dopaminergic neurons in the substantia nigra [22]. Indeed, it has been used to model Parkinson's disease in various animal models including zebrafish [23]. Hence, we used MPTP as the neurotoxic positive control drug. Finally, ethanol and acetaminophen (APAP, paracetamol) were used as our positive hepatotoxicity drugs. Both are well known molecules producing liver injury (extensively reviewed in [24,25]), with steatosis as a major side-effect of ethanol and liver malfunction and necrosis of liver tissue as the main toxic effect from paracetamol. Both hepatotoxic effects have also been reported in zebrafish larvae [26–29] (Table 1).

Table 1. Selected compounds and their toxicity in humans. White background: tested compounds. Grey background: compounds used as controls. Abbreviations: HCL (hydrochloride), NaCl (sodium chloride), MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

Drug	*Cardiotoxicity*	*Neurotoxicity*	*Hepatotoxicity*
Acetaminophen	Toxic [1, 2, 3]	Toxic [2, 3]	Toxic [1, 2, 3], (Hinson, Roberts and James, 2011)
Ethanol	Toxic [1]	Toxic [1]	Toxic [26,27]
Haloperidol	Toxic [1, 2, 3], [19–21]	Toxic [1, 2, 3]	Safe
MPTP	*A*	Toxic [22,30,31]	*A*
(±)-Epinephrine HCL	Toxic [1, 2, 3]	Toxic [1, 2, 3]	Safe
Ciprofloxacin	Toxic [1, 2, 3]	Toxic [1, 2, 3]	Toxic [1, 2, 3]
Cisapride	Toxic [1, 3]	Safe	Safe
D-(+)-glucose	Safe	Safe	Safe
Digoxigenin	Toxic [1]	Safe	Safe
Docetaxel	Toxic [2, 3]	Toxic [1, 2, 3]	Toxic [1, 2, 3]
Dofetilide	Toxic [1, 2, 3]	Safe	Safe
Finasteride	Safe	Safe	Safe
Flupirtine	Safe	Safe	Toxic [4]
Fusidic Acid	Safe	Safe	Toxic [32,33]
Isoniazid	Safe	Toxic [1, 2, 3]	Toxic [1, 2, 3]
L-Cysteine	Safe	Safe	Safe
L-Glutamine	Safe	Safe	Safe
Methyldopa	Safe	Toxic [1, 3]	Toxic [1, 2, 3]
NaCl	Safe	Safe	Safe
Pindolol	Toxic [1, 2, 3]	Toxic [2, 3]	Safe
Riluzole	Toxic [2, 3]	Safe	Toxic [2, 3]
Suramin	Safe	Toxic [34,35]	Safe
Trifluoperazine HCL	Toxic [1, 2, 3]	Toxic [1, 2, 3]	Toxic [1, 2, 3]
Vincristine	Toxic [1, 2, 3]	Toxic [1, 2, 3]	Safe

[1] TOXNET (Hazardous Substances Data Bank, HSDB); [2] Side effects (EMBL; European Molecular Biology Laboratory); [3] drugs.com; [4] ema.europa, *A* effects in humans not known.

2.3 Acute Tox Analysis

As explained above, the Acute Tox test was performed to determine the maximum drug concentration in which no mortality or gross teratogenic effects were observed (Non Observed Effect Concentration; NOEC). As a positive toxic drug, we chose Diethylaminobenzaldehyde (DEAB), a Retinoid Acid inhibitor that promoted mortality and teratogenicity in a reproducible concentration dependent curve. 1% DMSO, which is

the constant solvent concentration in all conditions, was used as the negative control. Mortality curves for all compounds at 96 hpf (blue line; Figure. 2A-T), compared with DEAB curves (red line; Figure. 2A-T), are shown in Figure 2. Additionally, the results from this analysis are displayed in Table 2.

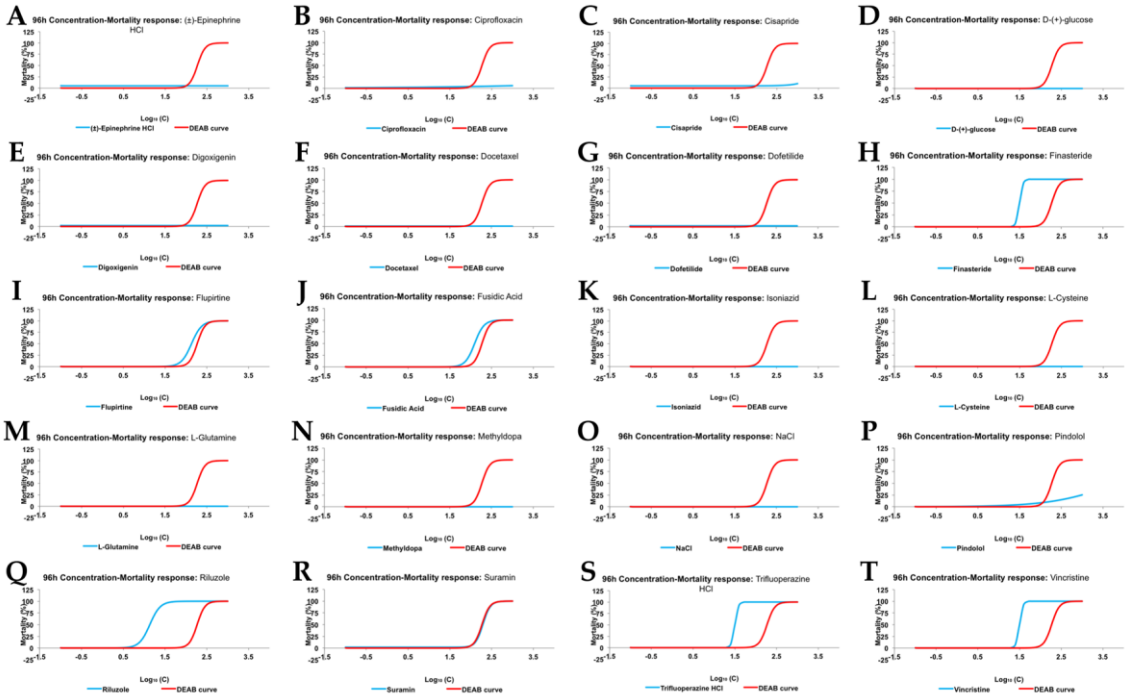

Figure 2. 96 hpf mortality concentration response curve (red line), compared to DEAB (Diethylaminobenzaldehyde)(blue line), for (**A**) (±)-Epinephrine hydrochloride; (**B**) Ciprofloxacin; (**C**) Cisapride; (**D**) D-(+)-glucose; (**E**) Digoxigenin; (**F**) Docetaxel; (**G**) Dofetilide; (**H**) Finasteride; (**I**) Flupirtine; (**J**) Fusidic Acid; (**K**) Isoniazid; (**L**) L-Cysteine; (**M**) L-Glutamine; (**N**) Methyldopa; (**O**) NaCl; (**P**) Pindolol; (**Q**) Riluzole; (**R**) Suramin; (**S**) Trifluoperazine hydrochloride; and (**T**) Vincristine.

Table 2. NOEC, LOEC and LC50 of selected compounds at 96 hpf. White background: tested compounds. Grey background: DEAB, used as positive toxic control. Abbreviations: NaCl (sodium chloride), MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), DEAB (Diethylaminobenzaldehyde).

Drug	***96h NOEC (µM)***	***96h LOEC (µM)***	***96h LC50 (µM)***
DEAB	1.00	10.00	185.99
(±)-Epinephrine hydrochloride	1000.00	N.A.	3.11E+10
Ciprofloxacin	1000.00	N.A.	7.01E+09
Cisapride	1000.00	N.A.	2935.74
D-(+)-glucose	1000.00	N.A.	N.A
Digoxigenin	100.00	1000.00	N.A

Drug	***96h NOEC (µM)***	***96h LOEC (µM)***	***96h LC50 (µM)***
Docetaxel	10.00	100.00	N.A
Dofetilide	10.00	100.00	N.A
Finasteride	10.00	100.00	31.62
Flupirtine	10.00	100.00	136.05
Fusidic Acid	10.00	100.00	124.15
Isoniazid	1000.00	N.A.	N.A
L-Cysteine	1000.00	N.A.	N.A
L-Glutamine	1000.00	N.A.	N.A
Methyldopa	1000.00	N.A.	N.A
NaCl	1000.00	N.A.	N.A
Pindolol	100	1000	6608.09
Riluzole	1	10	13.71
Suramin	100	1000	196.56
Trifluoperazine hydrochloride	10	100	31.62
Vincristine	10	100	31.62

2.4 Cardiotoxicity Analysis

Four cardiac parameters were evaluated to assess whether a compound was cardiotoxic: heart rate (beats per minute, BPM), QTc prolongation, ejection fraction (EJF), and cardiac arrest. Haloperidol has been described as cardiotoxic in humans and zebrafish [19] and was used as our cardiotoxic control. 1% DMSO was used as negative control.

Cardiotoxicity evaluation was performed from 96 hpf, when zebrafish heartbeat is already stabilised [36,37], so the analysis might not be altered by unstable beating. Zebrafish hearts were video-recorded at 4h after drug incubation (100 hpf) and analysed using the ZeCardio® β software (Figure 3A).

Eight compounds – haloperidol, cisapride, docetaxel, dofetilide, pindolol, riluzole, trifluoperazine HCL, and vincristine – decreased heart rate when compared to DMSO-only (Figure 3B). Longer cardiac arrest was promoted by the same compounds as well (Figure 3E). Inversely, zebrafish larvae treated with ciprofloxacin and D-(+)-glucose showed increased heart rates but no differences were observed in the duration of the cardiac arrest, when compared to DMSO-only treated larvae (Figure 3B,E).

QTc interval prolongation was detected in larvae treated with haloperidol and pindolol, while ciprofloxacin and D-(+)-glucose showed shorter QTc interval than the DMSO

treated group (Figure 3C). Finally, no differences in ejection fraction were detected in any of the 24 compounds tested (Figure 3D).

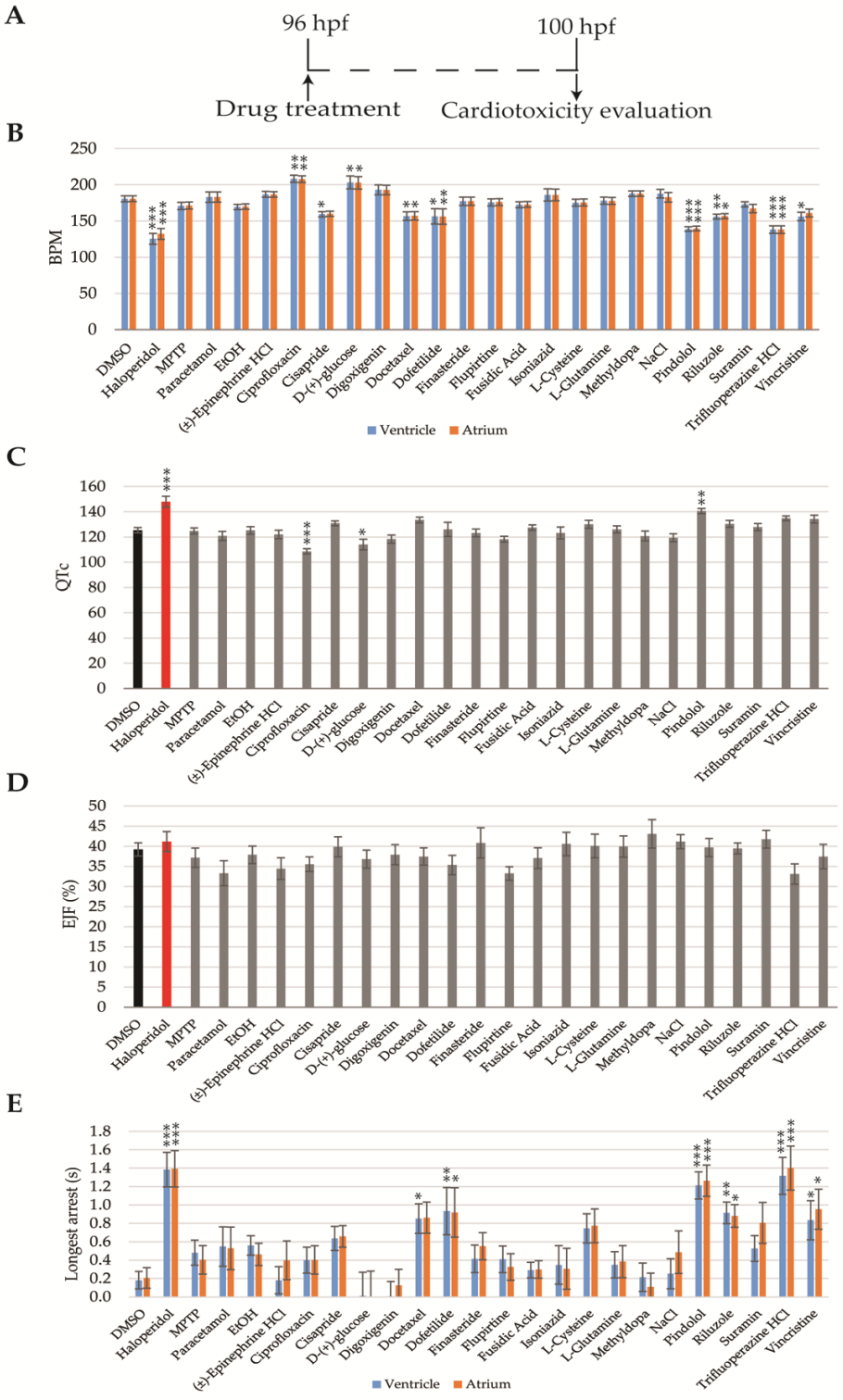

Figure 3. Cardiotoxicity evaluation results. (**A**) Scheme of the experimental procedure; (**B**) Bar graphs showing heart beat frequency in beats per minute (bpm); (**C**) QT corrected interval (QTc);

(**D**) Ejection fraction (EJF); (**E**) and longest cardiac arrest of 100h old zebrafish larvae. Asterisks indicate statistical significance after a One-way ANOVA: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Black bar: negative control. Red bar: positive control. $n = 16$ but for DMSO $n = 46$.

2.5 Locomotor Activity Analysis

By 5 dpf, zebrafish larvae perform spontaneous swimming and their visual system is fully developed [31,38,39]. Therefore, behavioural experiments were performed from this time point (Figure 4A). Deviations in total distance moved, in response to photo-visual stimulation, were analysed as a direct measurement of neurotoxicity. Thus, drugs increasing or decreasing total distance moved when compared to the DMSO-only group were considered neurotoxic. As the positive neurotoxic control, we used MPTP, which has been identified as a neurotoxic drug in humans and zebrafish [40].

Decreased motility was detected in MPTP, paracetamol and trifluoperazine-HCL treated larvae, while (±)-epinephrine HCL, docetaxel, pindolol and vincristine groups showed increased motility when compared to the DMSO (Figure 4B).

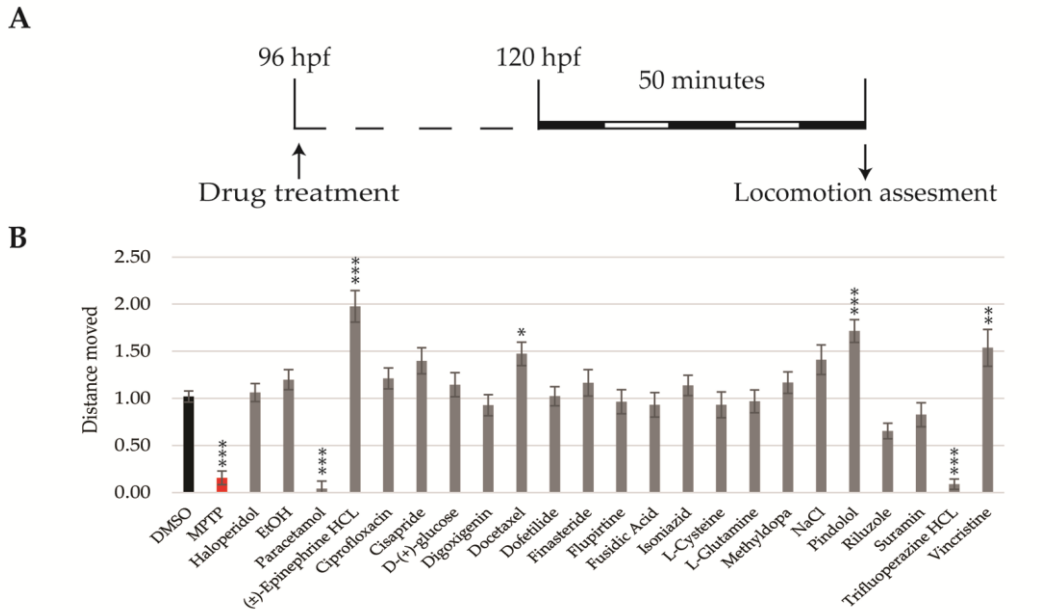

Figure 4. Locomotion results. (**A**) Scheme of the experimental procedure; (**B**) Bar graphs showing total distance moved corrected to the DMSO group. Asterisks indicate statistical significance after a One-way ANOVA * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Black bar: negative control. Red bar: positive control. Experiment performed once with 16 larvae per condition. n=43 for the DMSO.

2.6 Hepatotoxicity Analysis

Zebrafish liver development is fast and can be divided in three main stages: specification, differentiation, and hepatic outgrowth (reviewed in [41]). By 5 dpf, the liver is fully functional and consists of two lobes, with an overall oblong shape [42]. Since hepatotoxic effects are mainly due to metabolic processes, which require certain time to be executed, experiments were performed at 132 hpf. As the positive control we used paracetamol and ethanol, which have been shown to produce hepatotoxicity in humans and zebrafish [43].

2.6.1 Hepatomegaly and Liver Necrosis Evaluation

Zebrafish larvae were fixated and photographed after 36 h of drug incubation (96-132 hpf; Figure 5A). The transgenic zebrafish line *Tg(cmlc2:GFP; fabp10:RFP; ela31:EGFP)* expressed RFP protein in all liver cells. The analysis of fluorescence intensity allowed for the detection of drugs affecting liver size or the number of hepatocytes [44]. Thus, drugs reducing the number of hepatocytes (necrosis) translated into reduced RFP area, whereas drugs increasing liver size (hepatomegaly) corresponded with increased RFP area. In that regard, liver areas of the 24 compounds were analysed and compared with those obtained using the DMSO-only group. Three drugs including paracetamol, flupirtine and methyldopa showed decreased RFP area signal, whereas finasteride and fusidic acid treatments increased the area of the RFP signal (Figure 5B).

2.6.2 Steatosis and Yolk Lipid Accumulation Evaluation

During the first week of development, the unique source of energy for the zebrafish embryo and larva is the yolk sac. Zebrafish yolk consists of 70% neutral lipid, which is metabolized mainly in the liver [45]. Thus, yolk lipid accumulation can be used as an endpoint for liver function since, if impaired, the yolk metabolism and absorption is delayed, which results in higher lipid retention [46]. On the other hand, drug-induced steatosis (hepatocyte lipid accumulation) is an off-target liver effect which can be used to prioritize compounds for development [47,48]. Hence, drugs affecting lipid metabolism in human hepatocytes might be identified by using zebrafish livers [26,49].

In order to assess drugs producing steatosis and yolk accumulation, and subsequent to the RFP filtered images being acquired, zebrafish larvae were stained with Oil Red O. Larvae were sorted into steatosis positive or negative, yolk lipid accumulation or both (see

materials and methods). Percentages were calculated for each drug and compared with those obtained in the DMSO-only group (Figure 5D).

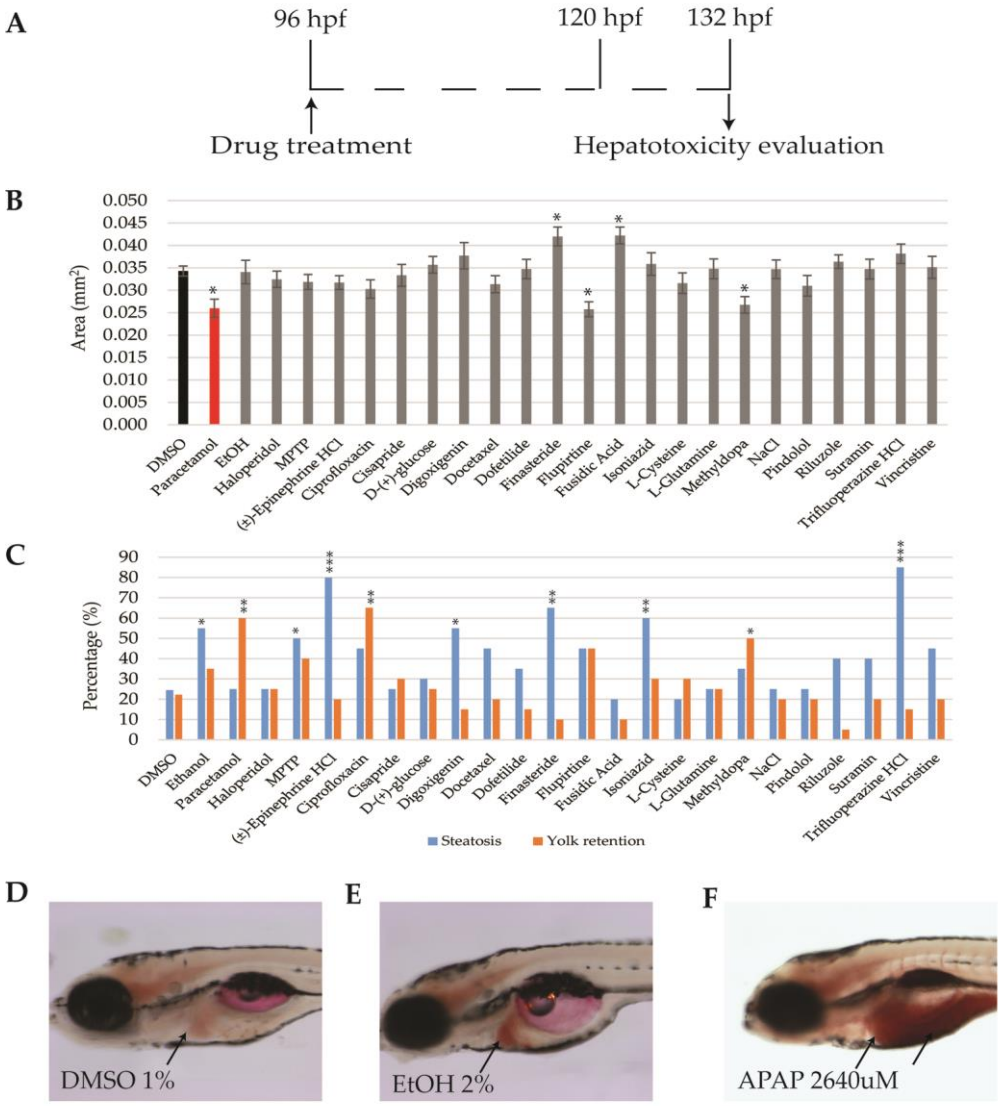

Figure 5. Hepatotoxicity results (**A**) Scheme of the experimental procedure (**B**). Bar graphs showing average liver area in mm. (**C**) Bar graphs showing the percentage of larvae presenting steatosis or yolk lipid accumulation after oil red O stain (**D-F**) Representative oil red O whole mount staining images of (**D**) DMSO, (**E**), EtOH and (**F**) APAP; black arrows point at non-affected liver (**D**), liver with steatosis (**E**) and yolk lipid retention (**F**), respectively. Asterisks indicate statistical significance after One-way ANOVA (liver area) or Fisher's exact test (steatosis and yolk lipid retention): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Black bar: negative control (**B**). Red bar: positive control (**B**). $n = 20$ but DMSO $n = 45$.

Seven out of 24 drugs were considered to be positive for steatosis (a representative image is displayed in Figure 5E): EtOH (55%), MPTP (50%), (±)-epinephrine HCL (80%), digoxigenin (55%), finasteride (65%), isoniazid (60%) and trifluoperazine HCL (85%).

Three drugs were considered to produce yolk lipid retention (a representative image is displayed in Figure 5F): paracetamol (60%), ciprofloxacin (65%) and methyldopa (50%). DMSO percentages for steatosis and yolk lipid accumulation were 24.44% and 22.22%, respectively (Figure 5C,D).

3. Discussion

Cardio-, neuro- and hepatotoxicity are the most relevant organ-toxicities promoting drug attrition during preclinical, clinical, and post market stages [10]. Previous studies have shown the relevance of using the zebrafish model for predicting the possible impact of drugs in those three organs individually [21,29,50–56]. However, no previous studies have integrated the analysis of these three organ-toxicities in the same animal; a procedure that reduces animal usage, experimental time and costs, and quantity of tested compound. Results obtained through the ZeGlobalTox assay show high sensitivity, specificity, and accuracy values when we compare the zebrafish experimental data with known human toxicity outputs (Table 3). This is indeed a promising conclusion, given the need for predictive and cost-effective procedures required to narrow down the number of compounds reaching expensive and time-consuming mammalian and clinical studies. Altogether, we propose ZeGlobalTox could be used to reduce time and costs of drugs for being approved, together with improving 3Rs policies during the whole drug discovery process. Nonetheless, we will discuss below a number of aspects to be considered in order to improve this approach.

Table 3. Zebrafish *versus* Human predictive assessment of Cardiotoxicity, Neurotoxicity, Hepatotoxicity and ZeGlobalTox. White background: tested compounds. Grey background: Positive toxic controls. Abbreviations: TN: true negative, TP: true positive, FN: false negative, FP: false positive, PPV: positive predictive value, NPV: negative predictive value. Specificity: TN/(TN+FP); Sensitivity: TP/(TP+FN); Accuracy: (TP+TN)/(TP+TN+FP+FN); PPV: TP/(TP+FP), NPV: TN/(TN+FN);

Drug	**Cardiotoxicity**	**Neurotoxicity**	**Hepatotoxicity**
(±)-Epinephrine HCL	FN	TP	FP
Ciprofloxacin	TP	FN	TP
Cisapride	TP	TN	TN
D-(+)-glucose	FP	TN	TN
Digoxigenin	FN	TN	FP
Docetaxel	TP	TP	FN

Drug	Cardiotoxicity	Neurotoxicity	Hepatotoxicity	
Dofetilide	TP	TN	TN	
Finasteride	TN	TN	FP	
Flupirtine	TN	TN	TP	
Fusidic Acid	TN	TN	TP	
Isoniazid	TN	FN	TP	
L-Cysteine	TN	TN	TN	
L-Glutamine	TN	TN	TN	
Methyldopa	TN	FN	TP	
NaCl	TN	TN	TN	
Pindolol	TP	TP	TN	
Riluzole	TP	TN	FN	
Suramin	TN	FN	TN	
Trifluoperazine HCL	TP	TP	TP	
Vincristine	TP	TP	TN	
Acetaminophen	FN	TP	TP	
Ethanol	FN	FN	TP	
Haloperidol	TP	FN	TN	
MPTP	-	TP	-	**ZeGlobalTox**
Specificity	90%	100%	77%	89%
Sensitivity	69%	54%	80%	68%
Accuracy	78%	75%	82%	78%
PPV	90%	100%	73%	88%
NPV	69%	65%	83%	72%

Four endpoints were analysed for cardiotoxicity evaluation – BPM, QTc, EJF and cardiac arrest. A drug was considered cardiotoxic when one of these parameters was found statistically different when compared to the DMSO-only group. Our analysis has detected cardiotoxic end-phenotypes in 9 out of 12 human cardiotoxic compounds present in the study. However, from 6 drugs reported to produce QTc prolongation in humans – haloperidol, (±)-epinephrine HCL, ciprofloxacin, cisapride, dofetilide and trifluoperazine HCL – only haloperidol treated larvae displayed that phenotype. Furthermore, pindolol, not reported to produce QTc prolongation in humans, showed increased QTc in zebrafish larvae. This latter phenotype might be explained by pindolol non-selective blockage of heart ß-receptors. Interestingly, bradycardia was detected in 4 out of 6 drugs producing QTc prolongation in humans. This is consistent with results presented by Wen et al. [57],

which showed a correlation between drugs producing QTc prolongation (in dogs) and bradycardia in zebrafish. On the other hand, tachycardia was observed in D-(+)-glucose treated zebrafish larvae. Although glucose is generally innocuous for humans, cardiotoxicity has been reported in hyperglycaemic patients and patients suffering from diabetes [58–60]. A correlation between high blood glucose levels and poorer outcomes after cardiac arrest has also been described [61]. Therefore, high doses of glucose might also be related to increased cardiotoxicity risk in humans. We hypothesize tachycardia detected in zebrafish might be due to the need for eliminating/compensating high glucose concentrations as fast as possible. Cardiotoxic false negatives (FN) such as paracetamol, ethanol and (±)-epinephrine HCL could be explained by differences among human and zebrafish physiology or by the ZeGlobalTox procedure, where cardiotoxic effects are analysed only 4h after drug incubation. Most cardiotoxic effects can be detected shortly after compound incubation; however, these three compounds might require a longer exposure to reproduce their known cardiotoxic effects. This seems certain for ethanol and paracetamol, since their human cardiotoxic impact is observed as a late effect after drug poisoning. In fact, paracetamol has been reported to have no impact on the heart rate in zebrafish larvae [50,57], to the point that Wen et al. [54] included this drug as a negative cardiotoxic drug [57]. In summary, we support zebrafish as a powerful tool for predicting drug-induced cardiotoxic liabilities in humans, including typical repolarization and depolarization end-phenotypes such as Qtc or EJC. However, our experimental methodology – drug exposure timing, chosen drug concentration, image acquisition, and image analysis – might require further improvement to facilitate a more accurate detection of some of the analysed parameters.

Regarding neurotoxicity assessment, motor behaviour might be affected by neurotoxic, but also by non-neurotoxic compounds that affect the function of the nervous system, such as hypnotic or neuroactive drugs [52,54,60]. This ambivalence could have promoted the identification of larger percentage of false positives (FP). However, we observed high specificity, since no false positives have been detected. On the other hand, better sensitivity is indeed required because five compounds known to produce some kind of neurotoxicity in humans did not alter larvae locomotion significantly. Thus, we suggest locomotion results should be interpreted cautiously. Indeed, we propose drugs altering zebrafish locomotion should be tagged with a red-flag, since they could signal a possible

Central Side Effect impact. However, drugs not influencing larvae locomotion cannot be tagged safe for neurotoxicity, since they might be neurotoxic without affecting locomotor neural pathways. In that regard, future ZeGlobalTox experimental versions might include a more comprehensive assessment of neural tissue after drug incubation – neuronal mortality, axonal growth defects, etc.

Previous studies have shown the robustness of zebrafish for hepatotoxicity prediction [43,44]. This robustness is supported by a high degree of genetic conservation for the enzymes and pathways required in drug metabolism, such as ARH receptors, CYP enzymes, or Adh isoenzymes, which are present, and functional, from early developmental stages, including our experimental window [62–64]. Three phenotypic endpoints were analysed for hepatotoxicity evaluation: liver area, steatosis, and yolk lipid retention. A drug was considered hepatotoxic when at least one of these parameters was statistically different when compared to the DMSO-only group. Consistent with previous studies, we show that paracetamol reduces liver size and increased yolk lipid accumulation. Thus, by reducing the hepatocyte number and/or viability, paracetamol was reducing zebrafish liver size and impairing its function, which led to a decreased lipid metabolism and therefore, its accumulation in the yolk. On the other hand, larvae treated with 2% ethanol showed steatosis but no impact on the liver size or yolk lipid accumulation. Steatosis promoted by 2% ethanol has been extensively reported [26,49,65]. However, there are controversial results regarding ethanol impact in liver size. Gong et al. [44] identified a reduction in liver size, but Sadler et al. showed hepatomegaly [26,27,49]. In our hands, 2% ethanol did not significantly affect the liver area. However, we detected more rounded livers (shape differences). This phenotype agreed with [26] and might be indicative of an inflammatory process, which later leads to hepatomegaly. Larvae treated with (±)-epinephrine HCL, digoxigenin and finasteride were found to produce significant higher percentages of hepatic steatosis when compared to the DMSO-only treated group. (±)-epinephrine is not reported to be hepatotoxic in humans. However, is known that high levels of epinephrine stimulate lipolysis in adipose tissue liberating free fatty acids to the blood, which are then absorbed by the liver that converts them to triglycerides [64,66]. Furthermore, (±)-epinephrine stimulates the breakdown of glycogen in the liver releasing glucose [67]. Glucose can also be converted to fatty acids and finally into triglycerides [68]. Thus, high (±)-epinephrine concentrations might lead to an

excessive accumulation of triglycerides in hepatocytes producing, as a side effect, hepatic steatosis in zebrafish larvae. Finasteride and digoxigenin are both extensively metabolized in the liver. Finasteride is a 5-alpha reductase inhibitor that is metabolized via the cytochrome P450 system (CYP 3A4). No severe hepatotoxicity or clinical liver injury has been reported. However, some publications report a mild transient serum aminotransferases elevation occurring during finasteride therapy [69]. Digoxigenin is a steroid that when attached to sugars form glycosides. Digoxigenin is metabolized in the liver via the human liver alcohol dehydrogenase [70]. Thereby, hepatic steatosis, observed after digoxigenin treatment might be originated by a similar mechanism to that seen after ethanol 2% treatment. Consistent with that, in our approach both treatments cause steatosis in the same percentage (Figure 5C). Finally, regarding MPTP, its hepatotoxicity in humans is not known, however it has been reported to be hepatotoxic in rat livers or isolated hepatocytes [71,72]. Consistent with these studies, steatosis was observed in MPTP treated larvae. All in all, predictive power of zebrafish hepatotoxicity assessment, is greater than most in silico or in vitro approaches that are traditionally used [73].

Overall, our results show ZeGlobalTox to be a reliable method to red flag a toxic compound according to its putative general organ liability. On its current methodological version – preliminary AcuteTox, drug concentration, drug exposure timing, and typology of end-phenotypes – it yields an overall high sensitivity, specificity, and accuracy at identifying specific organ toxicities. However, we acknowledge some adjustments need to be implemented to more accurately segment the general organ-toxicities into specific end-phenotypes (i.e.: General cardiotoxicity *vs* specific QTc prolongation). Moreover, the exposure to NOEC might yield some false negatives. In that sense, testing more than one concentration might provide a better understanding of a possible drug-induced organ liability, if that phenotype requires a higher than NOEC concentration to be triggered. In spite of those possible drawbacks, we expect our results will further support the use of zebrafish as an appropriate model to be exploited in early phases of drug discovery/development. In that regard, zebrafish could become the chosen model to bridge the gap between low predictive but high throughput in vitro studies and high predictive but expensive and time-consuming in vivo mammalian studies.

4. Materials and Methods

4.1 Materials and Chemicals

The 20 chemicals used in the present study were chosen and kindly provided by Pivot Park Screening Centre (Oss, The Netherlands) to be tested in a single-blind test: ciprofloxacin, cisapride, L-cysteine, digoxigenin, docetaxel, dofetilide, (±)-epinephrine hydrochloride, finasteride, flupirtine, fusidic acid, D-(+)-glucose, L-glutamine, isoniazid, methyldopa, pindolol, riluzole, sodium chloride (NaCl), suramin, trifluoperazine hydrochloride, and vincristine. The sic chemicals used as controls were purchased from Sigma-Aldrich (Sant Louis, MO, USA): dimethyl sulfoxide (DMSO) (D8418), ethanol (EtOH) (02860-1L), haloperidol (H1512), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (M0896-10MG), paracetamol (APAP, acetaminophen Bioxtra) (A7085-100G) and 4-Diethylaminobenzaldehyde (DEAB) (D86256).

4.2 Zebrafish Maintenance.

Zebrafish embryos were obtained by mating adult fish through standard methods. All experiments were performed on zebrafish larvae from 4 dpf until 5.5 dpf, with the exception of the Acute Toxicity test (see "zebrafish exposure conditions*"* below). Transgenic zebrafish (*Danio rerio*) *Tg(cmlc2:GFP;fabp10:RFP;ela31:EGFP)* was obtained by crossing individual transgenic lines and were kept according to established standard procedures. *Tg(cmlc:GFP)* [50] expresses Green Fluorescent Protein (GFP) in cardiomyocytes, *Tg(fabp10:RFP)* [74] expresses Red Fluorescent Protein (RFP) in hepatocytes, and *Tg(ela31:EGFP)* [75] expresses enhanced Green Fluorescent Protein (EGFP) in pancreatic cells. In the present study, pancreatic toxicity was not analysed, but since it was not affecting the current image analysis, and it might become useful in future experiments, the pancreatic reporter line was kept inside the complete transgene line.

4.3 Drug Exposure Conditions.

Mortality and Developmental toxicity were assessed through an Acute Toxicity test**,** adapted from specific OECD guidelines (FET: Fish Embryo Toxicity; OECD 236). Thus, 20 wild type (wt) zebrafish embryos per condition were incubated with tested compounds from 3 to 96 hpf. The test was performed in five logarithmic concentrations per drug (from 0.1µM to 1mM). Each larva was analysed for mortality, body deformity, oedema,

tail detachment, pigmentation, heart activity, heart oedema and motor activity. For every compound, a no observed effect concentration (NOEC) was identified to use in following experiments. The concentrations for drugs used as organo-toxic positive controls were obtained from previous publications or in-house validation: paracetamol (2600 µM; [46]), EtOH (2%; [26]), MPTP (100 µM; [30], and haloperidol (10 µM; [21]). DMSO 1% was used as negative control in all experiments.

For the ZeGlobalTox assay, fertilized *Tg(cmlc2:GFP; fabp10:RFP; ela31:EGFP)* zebrafish embryos were collected in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM, $MgSO_4$) in Petri dishes. At 3, 24 and 48 hpf, dishes were observed and all not fertilized, abnormal or coagulated eggs were discarded. At 96hpf, 20 larvae per condition were incubated with the NOECs from the different drugs and allowed to develop until 132 hpf at 28.5ºC.

4.4 Cardiotoxicity Evaluation in Zebrafish Larvae.

After 4 hours of drug incubation (100hpf), zebrafish larvae were anesthetized by immersion in 0.7µM tricaine methanesulfonate (A4050, Sigma-Aldrich, Saint Louis, MO, USA)/E3 solution. 10 µM haloperidol treated embryos were used as positive cardiotoxic controls. The 1% DMSO treated embryos were used as negative cardiotoxic controls. Embryos were positioned in an agarose based mold to allow their appropriate orientation under the fluorescence stereo microscope (Olympus MVX10). Individual fluorescent hearts were recorded during 60 s each (Fig. 6A). Videos were acquired with a high-speed recording camera (Hamamatsu C11440 ORCA-flash 2.8) and analysed with the ZeCardio® β software to extract different cardiac parameters – heart rate, cardiac arrest, QTc prolongation and Ejection Fraction (EJF) (Figure 6B).

ZeCardio® β software, developed by ZeClinics and currently in β status, provides a graphical user interface (GUI) that facilitates the semi-automatic analysis of living heart videos. Interactive analysis of the different parameters functions as follows: The user draws a line along the heart axis, from ventricle to atrium, to initiate the calculation. At the ventricle and atrium, an additional line perpendicular to the heart axis (first line) is automatically displayed (Figure 6C). All lines can be subjected to modification of their angles and lengths. From the line selections, two outputs are generated: (i) A kymograph for each of the lines that allows, on one hand, the visual inspection and easy

identification/validation of phenotypes (Figure 6D) and, on the other hand, it is used for individual beat detection (Figure 6E); (ii) A numerical output that is displayed in the ZeCardio® GUI.

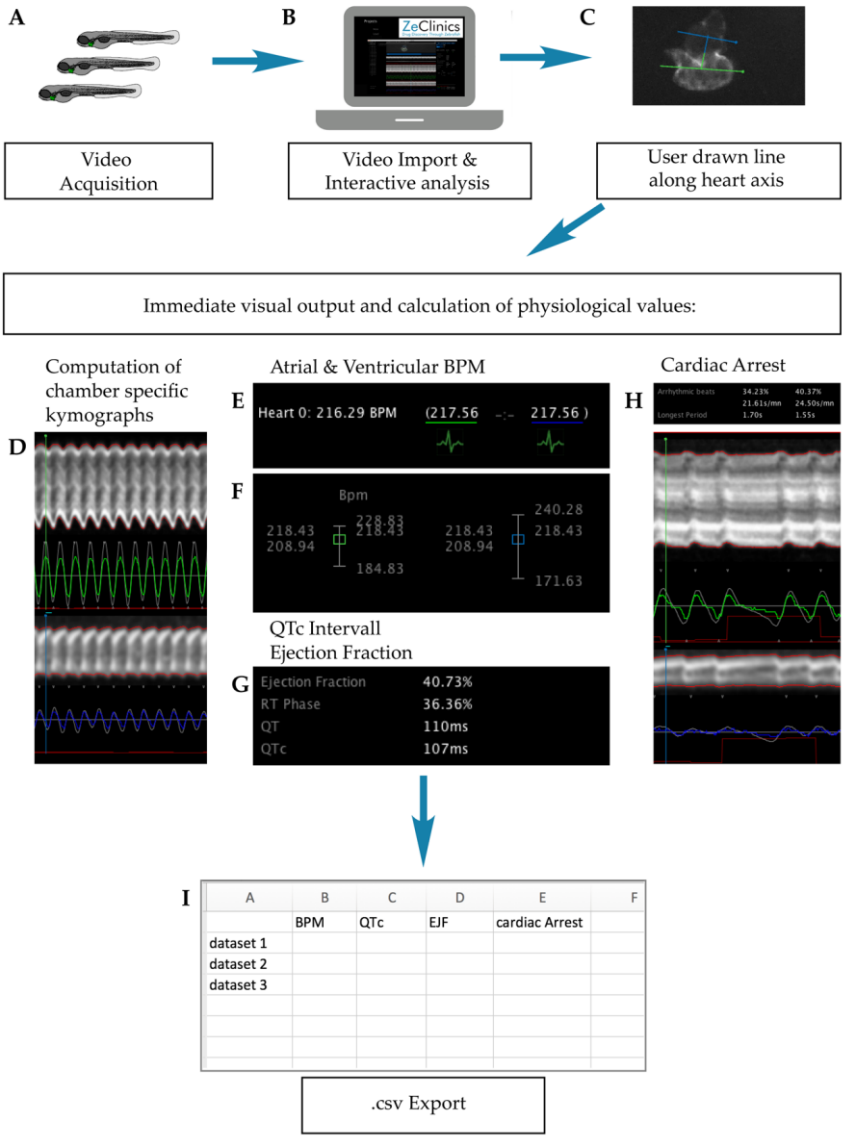

Figure 6. ZeCardio β software user pipeline. (**A**) Video acquisition of larvae incubated with candidate drug; (**B**) Video import into the software; (**C**) User drawn line acres heart axis; (**D**-**H**) GUI (Graphical User Interface) display of (**D**) Chamber kymographs; (**E**) atrial and ventricular BPM (Beats Per Minute) values; (**F**) Distribution plot over time of atrial and ventricular BPM; (**G**) QTc interval and EJF values and (**H**) Cardiac arrest events; (**I**) Output values are presented in .csv format. Kymographs and measurements are displayed in green or blue for ventricle or atrium respectively.

Heart beat frequency for each chamber is detected and frequencies presented in the GUI as a mean. A plot distribution is used for assessing beating stability over time (Figure 6F).

In the same fashion, chamber specific cardiac arrest is measured as the longest beating pause (Figure 6H). No beating chambers and/or incorrect bpm can be manually flagged when detected.

For calculation of QTc interval (linearly corrected QT interval) the Framingham formula (QTc = QT + 0.154 (1 – RR), adjusted for zebrafish as QTc = QT + 0.154 (2.66 – RR). RR = 6.6ms/measured bpm is applied (Figure 6G). Finally, Ejection Fraction, calculated as the maximal dilatation (the diastolic diameter, DD) versus the maximal contraction (systolic diameter, SD) is measured in % as in EF% = (DD-SD)/DD*100 (Figure 6G). Computed values for the described parameters were exported in .csv format (Figure 6H). Computed values for the described parameters were exported in .csv format (Figure 6H).

4.5 Neurotoxicity evaluation in zebrafish larvae.

Immediately after heart video acquisition, larvae are washed with E3 medium to remove tricaine methanesulfonate from the solution. Fresh drug solution is added and larvae are transferred individually in a volume of 150µL to 96 wells plates.

Neurotoxicity is analysed at 120hpf by locomotion assessment using the EthoVision XT 11.5 software and the DanioVision device from Noldus Information Technologies, Wageningen, The Netherlands. This closed system consists of a camera placed above a chamber with circulating water and a temperature sensor set at 28 ºC. The 96-wells plate are placed in the chamber, which can then be illuminated with white light using the software. Larvae are then left for 20 min under these conditions and with the lights on to help their acclimation. Finally, larvae locomotion is measured during 50’ under the following light/dark conditions: 10’ darkness-10’ Light-10’ darkness-10’ Light-10’ darkness. Total distance moved (in mm) is acquired under this light/dark trial. Due to circadian rhythms, all locomotion assays were performed from 13:00pm onwards to ensure steady activity of the zebrafish [76].

Neurotoxicity is assessed by comparing locomotion differences among tested compounds (solved in DMSO) and negative control group (DMSO 1%-only).

4.6 Hepatoxicity evaluation in zebrafish.

After 36h of drug incubation (132hpf), embryos are fixed in 4% paraformaldehyde (158127-500G, Sigma-Aldrich, Saint Louis, MO, USA) for 2-4h at room temperature (RT) and then 3x washed with PBS.

4.6.1 Liver area analysis

Fixed larvae are observed under an Olympus MVX10 fluorescent stereo microscope and photographed with a digital camera (Olympus DP71) and the cell'D software. RFP filtered images of the liver were taken and their areas analysed using the FIJI software for hepatomegaly and necrosis detection.

4.6.2 Oil Red O staining

Oil Red O is a lysochrome dye used for the staining of neutral triglycerides and lipids. In order to detect the presence of steatosis and yolk lipid retention, zebrafish larvae were stained with Oil Red O (O0625-25G, Sigma-Aldrich, Saint Louis, MO, USA) as described in [77]. Briefly, the skin pigment from fixed larvae is removed incubating with bleaching solution (for 10mL: 6 ml H_2O, 0.25 ml 20X SSC, 0.5 ml formamide, 3.3 ml H_2O_2) during 20 minutes at RT. Then, larvae are 5x washed with PBS. Bleached embryos are first submerged in 85% Propylene glycol (PG) (134368-1L, Sigma-Aldrich, Saint Louis, MO, USA) for 10 minutes and then in 100% PG for another 10 minutes before staining them with Oil Red O 0,5% in 100% PG (overnight, at RT and with gentle rocking). Oil Red O stained embryos are washed in 100% PG for 30 minutes, 50 minutes in 85% PG, and 40 minutes in 85% PG with an equal volume of PBS. Finally, embryos are washed 1x with PBS before adding 80% glycerol (G7757-500ML, Sigma-Aldrich, Saint Louis, MO, USA). Bright field images are taken to detect both steatosis and yolk lipid accumulation. For steatosis, larvae are considered positive when 3 or more round lipid droplets are visible within the hepatic parenchyma (Figure 5E and Figure 7; [77]). Yolk lipid retention is considered positive when red strong signal is observed in the yolk area (Figure 5F).

Embryos were incubated with ethanol 2% as positive controls for steatosis [26,49] and with APAP 2600µM as the positive control for necrosis and yolk lipid accumulation [46]. DMSO 1% treated larvae were used as a negative control group.

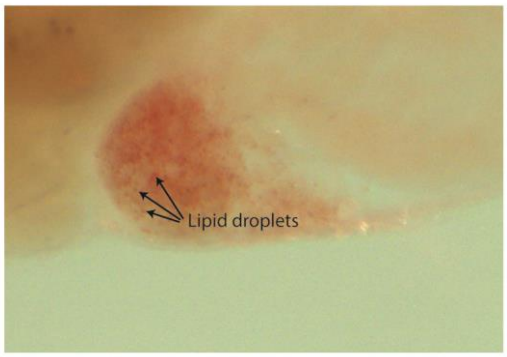

Figure 7. Lipid droplets on a zebrafish liver stained with Oil Red O. Steatosis is considered when three or more droplets are seen within the liver area.

4.7 Statistical analysis

Data were analysed using the IBM SPSS Statistic version 20.0 software (Armonk, NY, USA). Data are presented as mean ± standard error (SE). Prior to the analyses, the Shapiro–Wilk test was used to assess the normality of the distribution of the dependent variables. Not normally distributed variables were transformed using Templeton's two-step method for transforming continuous variables to normal [78]. Statistical analysis of the data for the cardiotoxic and neurotoxic parameters as well as for liver size measurements were performed using One-way ANOVA followed by Dunnett test. Fisher's exact test was used for data analysis of the steatosis and yolk lipid retention. Results were statistically compared between drug-treated groups and untreated (DMSO) group. Differences were considered statistically significant when $p < 0.05$.

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Author Contributions: Carles Cornet designed the project, performed the experiments, analyzed the data, and contributed to the manuscript preparation. Simone Calzolari conceived and designed the project, performed the experiments, and contributed to the manuscript preparation. Rafael Miñana-Prieto performed the experiments. Sylvia Dyballa designed the ZeCardio software, contributed to its development, and contributed to writing the manuscript. Els van Doornmalen. contributed to the experimental design and analysis of results. Helma Rutjes provided reagents, and contributed the project design and data analysis. Thierry Savy developed the ZeCardio

software. Davide D'Amico and Javier Terriente conceived of the project, coordinated the work and contributed to the manuscript preparation.

Conflicts of Interest: The authors declare the following conflict of interest: All authors, except Els van Doornmalen, Helma Rutjes and Thierry Savy, are currently employed by Zeclinics.

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3.2. Part 2: ZeOncoTest

The second part of my PhD thesis consisted in the standardisation, automation and validation of an optimized tumour cell xenograft assay for drug discovery and target validation in zebrafish larvae. We performed a thorough bibliographic search and subsequently tested several experimental settings, such as different cell labelling techniques, injection sites, image acquisition and analysis methods, in order to choose the best existing experimental conditions and develop new better ones. We finally proved the reliability and robustness of our system through the evaluation of tumour growth and metastatic potential of three tumour cell lines and the effect of known anti-cancer drugs on them. Furthermore, we show that our method can also be used to elucidate drugs mechanism of action. Results of this work have been summarised in a manuscript that has been sent to the journal Pharmaceuticals for its publication, and is currently under revision.

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Article

ZeOncoTest: refining and automating the zebrafish xenograft model for drug discovery in cancer

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Abstract: Human cancer xenografts have become a powerful tool in cancer drug discovery. Mice xenografts represent a widely validated tool, but their use is limited by elevated costs and low throughput. To overcome these restrictions, zebrafish larvae have been established as an alternative xenograft host. Their small size and optic transparency allow the tracking of transplanted cancer cells *in vivo* across the body with high resolution. Therefore, primary tumor growth and early steps of metastasis, such as intravasation and extravasation, which are difficult to evaluate in mice, can be addressed in zebrafish larvae. In spite of its advantages, the use of zebrafish larvae xenografts has been hindered by lack of experimental standardization and validation. In this context, our aim has been to standardize, validate and automate this model to increase its biological translatability and drug screening throughput. To this end, we have developed the ZeOncoTest, a highly reliable assay in which optimized experimental conditions are combined with automated image acquisition and analysis. This method has been validated with human cancer cell lines and known chemotherapeutics. The results recapitulate growth and metastatic behavior of different tumor cells, along with the expected efficacy of the known compounds. Finally, the methodology has proven useful for understanding drugs mode of action. The insights gained bring us a step further for the zebrafish larvae xenograft model to enter the regulated preclinical drug discovery path.

Keywords: Zebrafish larvae; cancer; xenograft; drug discovery; automation; throughput.

1. Introduction

Cancer is the second leading cause of death worldwide, accounting for 9.6 million estimated deceases in 2018 [1]. It is also the primary indication for pharmaceutical investment and medical care expenditure. During the last decades, a better understanding of tumor biology led to the development of therapies that increased the survival rate for multiple cancer types [2]. However, most common treatments consist in systemic administration of chemotherapeutics that target proliferating cells in neoplasms, but also in normal tissues with high regeneration capacity, causing severe side effects. Moreover,

chemotherapies do not affect quiescent cells (i.e. cancer stem cells; CSCs), which may play an important role in drug resistance, relapse and metastatic dissemination [3]. Finally, some types of cancer remain deadly because not responsive to available treatments. All these drawbacks reflect the need for discovering more precise, safe and efficacious therapies [1].

The drug discovery process in oncology is traditionally initiated by the use of fast *in vitro* systems for high throughput screenings. However, cell cultures are oversimplified tools due to the absence of tumor heterogeneity, microenvironment components and anatomical structures for proper growth and metastatic potential evaluation [4]. In order to validate and filter out *in vitro* results, drugs are tested in more biologically relevant *in vivo* models before entering clinical phases. Mouse xenografts of human cancer cells have become the prevailing translational tool in preclinical studies [5]. The use of rodents is recommended based on the high resemblance of transplanted tumors to the original ones. Also, chick chorioallantoic membrane xenografts might be useful, although poorly validated, for studying metastatic events [6]. Despite their translational value, the high cost and low throughput of these models hamper their use for screening large numbers of possible therapies. Moreover, individual cancer genotype is emerging as a crucial factor leading therapeutic decisions. Thus, patient derived xenografts (PDXs) are potentially powerful tools, but the high amount of tissue needed for transplants in rodents is a major issue against its widespread use [7]. These flaws in the current preclinical models might partially explain the high costs and elevated drug attrition rate (95%) during the progression of anti-cancer treatments through the subsequent clinical phases [8,9]. Therefore, the use of innovative screening systems, including alternative animal models, could reduce costs and time and allow assessing more compounds. This would indeed increase the chances of success in clinical trials and, hence, reduce drug attrition rates.

Zebrafish is an established animal model for academic research, but a more recent addition to the pharmaceutical drug development toolbox. However, its use is expanding as a fast and economical alternative to rodents for disease understanding, target validation and drug discovery in multiple indications [10–14]. In regard to oncology, zebrafish displays a variety of features of great translational value. First, it develops tumors if exposed to carcinogenic substances [15–17]. Second, oncogenes, tumor suppressors and the main molecular pathways involved in cancer progression are highly conserved in

zebrafish, in comparison to humans [18]. In this context, embryos can be genetically manipulated at ease for understanding the role of specific genes in diseases and, in particular, in tumor development [19–21]. Third, the larvae immature immune system allows transplantation and survival of human cancer cells with no need of immunosuppression [22,23]. Moreover, larvae optical transparency grants *in vivo* tracking of xenotransplanted fluorescent cells through standard and confocal imaging, for measuring tumor growth and detecting early metastatic events such as intravasation and extravasation, which are difficult to uncover in murine xenografts [24]. Based on these translational advantages, human tumor cells xenograft in zebrafish larvae has been implemented as a potentially useful tool for drug discovery [25–27]. This method allows comparing cancer development with or without candidate therapies in just a few days, at a lower cost and higher throughput than equivalent murine experimental assays. Moreover, the low amount of material needed for the transplants makes the use of zebrafish larvae possibly amenable for PDXs and precision medicine [28,29]. Despite these benefits, previous reports displayed contrasting results due to technical differences mainly in incubation times, image acquisition and analysis methods, cell labeling and site of injection. This lack of experimental standardization and validation might have led to a lower-than-expected exploitation of this screening tool in the pharma industry.

In this context, our aim was to establish a standardized xenograft system in zebrafish larvae, the ZeOncoTest. In order to increase drug screening throughput in the preclinical phase, while providing robust biological translatability, we reviewed and tested different available procedures and implemented robust settings for cell staining and injection into larvae. Additionally, we developed streamlined automated techniques for imaging and image analysis. To validate the accuracy of our method, we evaluated the behavior of a battery of tumor cell lines and the impact of different chemo-therapeutics.

2. Results

To define the best methodology, we reviewed the close to 250 articles showing the use of cancer xenografts on zebrafish larvae (Supplementary table 1). The majority of the articles were consistent about the cell injection time, with 86% of the reports injecting at 48 hours post fertilization (hpf) larvae. However, we detected a great amount of

variability in most of the other experimental settings and conditions. As such, the incubation temperature for xenotransplanted larvae ranged from 28.5ºC, standard for fish development, to 36ºC, closer to the ideal temperature for human cells standard growth of 37ºC. Individualization of animals throughout the experiment was only performed in 10% of the reports. The most common cell labelling methods were represented by fluorescent dyes, in 70% of the studies, compared to 30% using cells stably expressing fluorescent proteins. 82% of the dyes employed were membrane-specific. Among those, CMDiI was utilized in 64% of the cases. As for the injection site, the yolk was the standard choice in half of the reports, followed by the perivitelline space (pvs) used in the 25% of the studies. Injections in the vasculature, brain ventricle and heart were performed in the remaining 25% of the cases. Finally, the use of the xenotransplant test to address drugs mechanism of action (MoA) was only shown in half of the reports (Supplementary table 1). Based on these reports, we tested and compared different experimental conditions to define the most adequate xenotransplant methodology conforming the ZeOncoTest.

2.1 ZeOncoTest: general experimental setup and workflow

Basic conditions, such as stage of injection and incubation temperature, were set to guarantee the survival of injected animals and xenotransplanted human cancer cells. As shown in Figure 1, we chose to inject larvae at 48 hpf and grow them at 35ºC, which are the best settings reported in previous studies (Supplementary table 1) [30–32]. Due to different growth and invasion capabilities of distinct cancers, we decided to define specific experimental time frames for every tumor cell type of interest, by injection and live imaging observation till a maximum of 144 hours post injection (hpi). In order to evaluate the anti-tumor effect of compounds, we chose to incubate injected larvae between two defined time points within 0 and 144 hpi, tp1 and tp2, with candidate drugs at the No Observed Effect Concentration (NOEC). As for NOECs determination, we set up an acute toxicity test, in which larvae are exposed to a dose range treatment at the same conditions – stage, temperature – established for the subsequent efficacy assay. Finally, we determined to infer the anti-cancer effect of candidate drugs by comparing tumor growth and dispersion between untreated and treated xenografted animals (Figure 1).

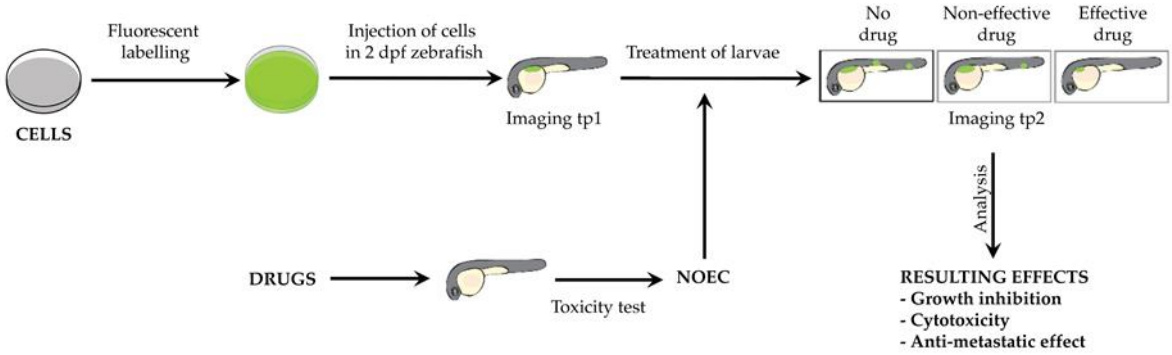


Figure 1. ZeOncoTest pipeline. Fluorescently labelled cells are injected in 48 hpf larvae, subsequently imaged at tp1. After the first imaging, xenotransplanted larvae are incubated at 35ºC with the NOEC of candidate drugs, previously calculated. Subsequent imaging is performed at tp2. The evaluation of drug impact on tumour growth and metastatic potential is given by the calculation and comparison of tumour mass and dispersion of cells at the 2 time points, in treated and untreated animals.

2.2 Improved and new methodologies

2.2.1 3D imaging, automation and throughput

A main source of potential bias in previous zebrafish xenograft reports resulted from estimating tumour areas from images acquired through stereo or widefield microscopies [33–35]. In our experience, when larvae were not equally oriented between different time-points, results based on areas measurements were inconsistent (data not shown). To overcome this limitation, we decided to use confocal microscopy and acquire z stacks to extrapolate three-dimensional images of tumour masses and calculate their volumes at the different time points. In our hands, volumes comparison provided a more reliable estimation of the growth of irregularly shaped tumour masses, as exemplified in Supplementary Video 1.

In addition, 90% of the previous reports measured tumour progression through comparing averages of whole populations between time points (Supplementary table 1). An important improvement is represented by the fact that we chose to keep the larvae individualized in 96 well plates. This approach allows to compare images from the same larvae at different times and to measure individual tumour growth and invasion. In contrast to group quantifications, singular estimations have an enormous impact on the statistical robustness of the method, since every larva can be considered a biological replicate. Moreover, we elected to analyse volumes ratios between time points, instead of

absolute values, allowing the normalization with respect to the original tumour size. The result is a lower variability and, hence, a more accurate quantification.

To further increase the screening throughput, we developed an imaging system in 96 well plates through an automated microscopy platform. Finally, we generated an automated image analysis pipeline to streamline the quantification of tumour volume and cell dispersion parameters in the same animals across different time points. The implementation of this workflow allows the screening of 80 individualized fish per experiment, a throughput that, to our knowledge, has never been reached before.

2.2.2 Choice of a suitable cell labelling method

In order to track cancer cells in grafted larvae, it was necessary to consider different factors regarding their labelling. First, we needed to select a method not causing cell toxicity throughout the duration of the experiment. Moreover, labelling was required to be homogeneous. Finally, we had to ensure a rapid loss of fluorescence following tumour cell death. The achievement of these purposes would guarantee a reliable evaluation of tumour volumes and dispersion across time and under different drug treatments.

As mentioned above, the use of fluorescent dyes was a common method to label cells prior to injection, as shown in 70% of the previous zebrafish larvae xenografts studies (Supplementary table 1) [26,31,36,37]. Based on this, we tested the most widely used lipophilic membrane dye CMDiI and cytoplasmic dye CFSE in the breast cancer cell line MDA-MB-231. Both dyes were used at 3 concentrations: 1, 2 and 5 µM. Toxicity was detected at 2 µM for CMDiI and at 5 µM for CFSE at 96 hours post staining (hps), by estimation of cell numbers through hexosaminidase assay. Counts were compared to unstained cells (Figure 2A, B). In addition, at non-toxic concentrations of both CMDiI (1 µM) and CFSE (2 µM), we detected non-homogeneous cell labelling (Figure 2C, D), although CMDiI displayed better cell retention than CFSE.

Once we defined the maximum tolerated concentration for the cell dyes, we proceeded to test the fluorescence loss following cell death. As a positive control, we used MDA-MB-231 cells stably infected with a vector coding for the green fluorescent protein (GFP). It is understood that physiological protein degradation, and resulting loss of GFP fluorescence, occurs following cell death. These fluorescent cells were labelled with 1

µM CMDiI and exposed to a high dose of DMSO (10%) to achieve cell death [38,39]. In order to detect dead cells, DAPI staining was performed after 24 hours. DAPI only permeates dead or dying cells through their disrupted plasma membranes, but cannot penetrate living cells, in which membranes are still intact [40]. Interestingly, 95.7% of DAPI positive dead cells displayed CMDiI labelling retention, whereas only 5.4% of them kept GFP expression (Figure 2E). This striking outcome was validated through the measurement of tumour masses in the zebrafish larvae in vivo setting. The yolk was chosen as injection site for simplicity. As previously shown in other xenografts settings, we expected cell death to be significant shortly after transplantation, with the most resistant cells adapting to the new microenvironment and then expanding [41,42]. In accordance with the in vitro observations, the CMDiI positive cell masses were significantly higher than the GFP positive ones in the same xenografts at the second time point, indicating that dead cells retained the dye staining, while losing GFP protein expression. The average tumour mass variation between the two time points was of 9.9 taking into account CMDiI labelled cells, and 0.6 by analysing GFP expressing cells (Figure 2F, G). Both sets of data *in vitro* and *in vivo* suggest that the use of membrane (CMDiI) and cytoplasmic (CFSE, data not shown) dyes does not allow a proper discrimination and exclusion of dead cells from the tumour mass imaging and analysis. Therefore, their use results in a biased over-estimation of tumour masses and growth.

Based on these observations, commonly-used dyes were discarded for the ZeOncoTest, and stable cell infection with retroviral vectors coding for fluorescent proteins was chosen as the most suitable method to label tumour cells before transplantation. Indeed, fluorescent proteins expression guarantees non-toxic, steady and homogeneous cell labelling, fluorescence transmission to daughter cells and its dynamic loss at death by physiological protein degradation. As such, this labelling option warrants a proper tracking of grafted cells and an accurate estimation of tumour volumes and dispersion across different time points.

2.2.3 Establishment of an appropriate injection site

Half of the previous studies reported tumour cell injections in the yolk and 25% in the pvs [29,35,36,43,44]. The main advantage of injecting intra-yolk is its accessibility. On the other hand, the yolk is a syncytium that might not provide an ideal microenvironment

for the attachment and growth of solid tumour cells [26,30–36,45,46]. According to our experience, transplantation in the pvs requires greater technical skills than injection into the yolk. This might be a reason behind the lower number of xenografts reported to use this injection site [29,44,47–49], despite the fact that the pvs displays better features for anchorage-dependent cell growth and greater accessibility to the vascular system. Both aspects are likely to be crucial in primary solid tumour growth and metastases occurrence.

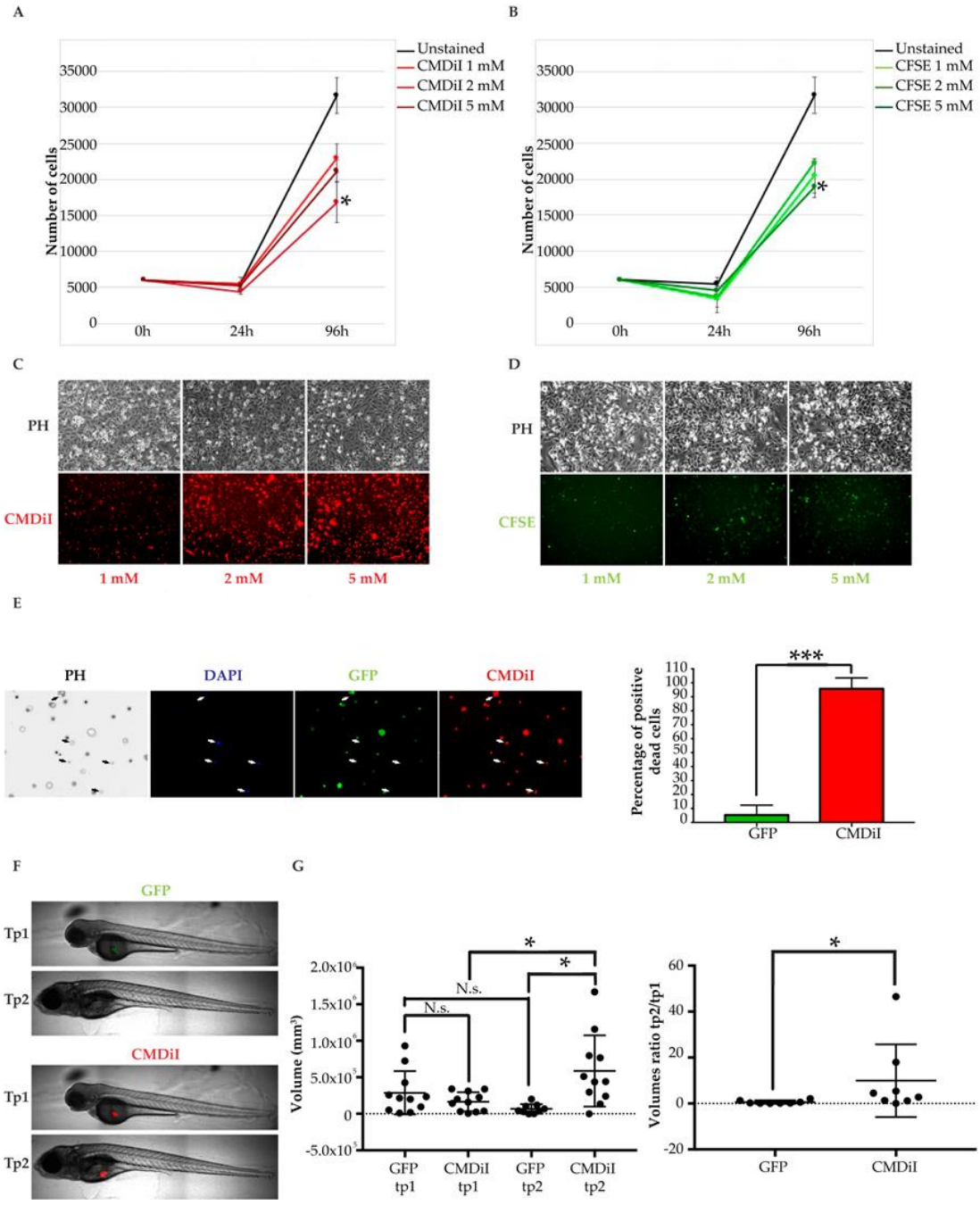


Figure 2. Fluorescent cell labelling methods comparison. (**A, B**) Analysis of the toxicity of fluorescent dyes. CMDiI (A) and CFSE (B) dyes are toxic at different concentrations in MDA-MB-231 (growth curves). Mean and standard deviation of three counts are represented for every condition at each time point. (**C, D**) Evaluation of cell staining homogeneity of fluorescent dyes. Representative images of cells stained with CMDiI (C) and CFSE (D) at day 4. (**E**) Assessment of fluorescence retention in dead cells. MDA-MB-231 cells stably expressing GFP were stained with CMDiI and exposed to high dose of DMSO. Dead cells were detected through DAPI staining. They are indicated by white arrows in the representative pictures on the left and quantified in the graph on the right. Six fields of view were analysed for quantification. Results are represented as mean +/- standard deviation. (**F**) Representative images of GFP+ tumours and CMDiI+ tumours in the same injected fish at the two time points. (**G**) Measurements of tumour masses corresponding to the same GFP expressing cells and CMDiI labelled cells transplanted in each larva, at tp1 and tp2 (graph on the left). GFP+ and CMDiI+ tumour masses ratios between tp2 and tp1 (graph on the right).

Following this rationale, we proceeded to test different cell lines to validate if solid tumours progression was favoured in the pvs, when compared to the yolk. As expected, the highly proliferative tumour cell line MDA-MB-231 displayed a lower growth rate in the yolk, in comparison to the pvs, measured as GFP-positive tumour mass fold change between tp1 and tp2 (1.5 vs 3.7 average, respectively; Figure 3A, B). Another aspect to consider was that growth impairment in the yolk was due to a progressively reduced space resulting from yolk consumption during larval development, which leads to an almost complete absorption by 96 hpi (144 hpf) [50]. This characteristic also implies that the experimental window for yolk xenografts is limited and shorter than the one for injection studies in the pvs. As such, colorectal cancer cells HCT116, whose proliferation rate was slowed due to the lower-than-physiological incubation temperature applied (35ºC) [51], did not grow at the early tp2 (etp2) of 96 hpi, neither in yolk nor in pvs (0.9 and 0.5 tumour mass fold change average respectively). However, when injected in pvs, HCT116 cells could be followed till a late tp2 (ltp2) of 144 hpi, when tumour masses displayed a significant 1.7 folds increase in average (Fig. 3C, D).

Moreover, we did not observe metastatic events in larvae injected in the yolk with the highly invasive MDA-MB-231 and HCT116 tumour cell lines. On the contrary, metastases were detected in 76,2% and 54,5% of the larvae injected with MDA-MB-231 and HCT116 cells in the pvs, respectively (Figure 3E-H). Given the possibility that a minority of cells were erroneously injected into the vascular system instead of yolk or pvs, and to avoid any bias, larvae displaying metastatic events already at tp1 were excluded as samples.

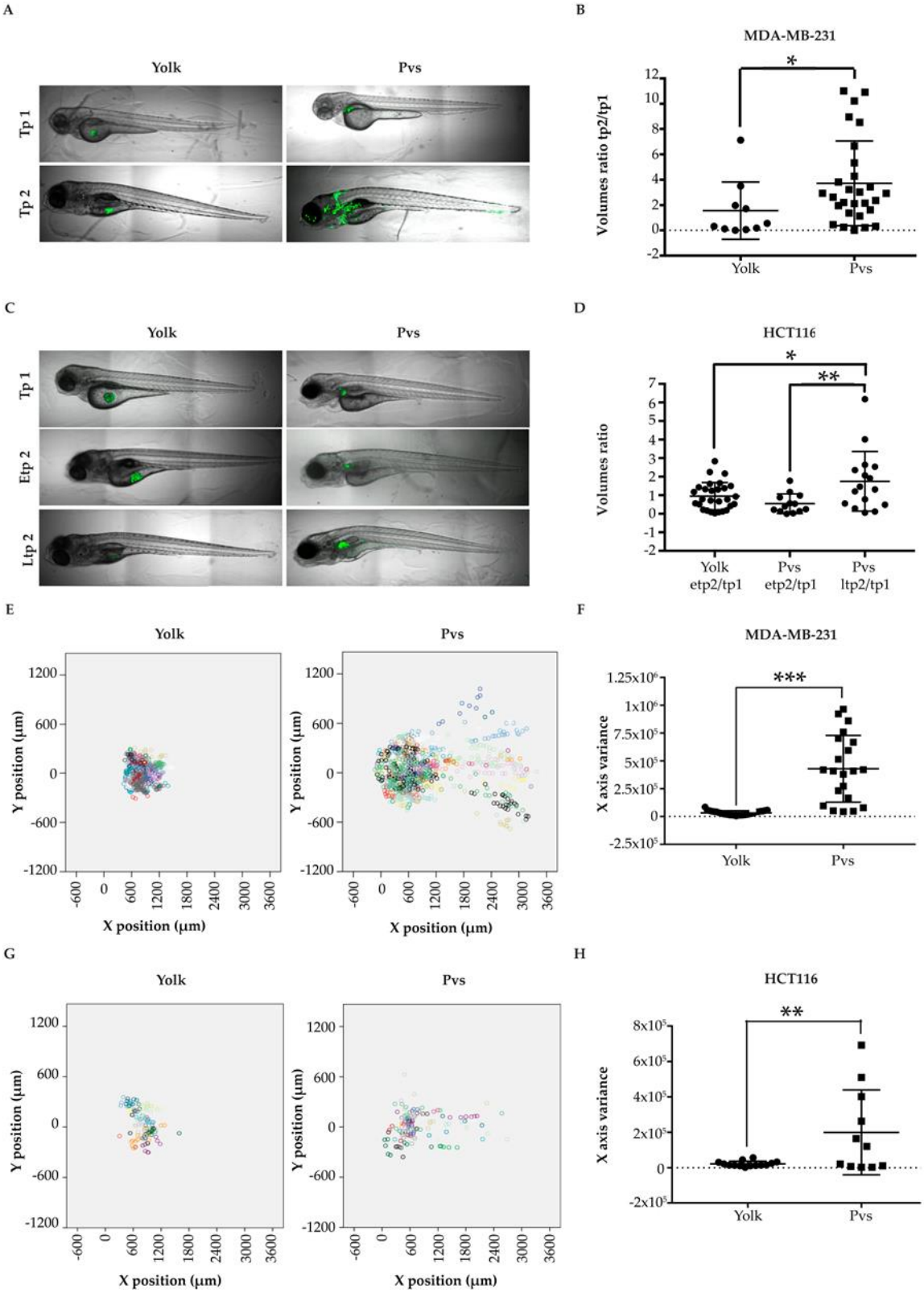


Figure 3. Injection sites comparison. (**A**) Representative images of larvae injected with MDA-MB-231 cells in the yolk and in the pvs, at two different timepoints. (**B**) Scatter dot plot of the tumour masses ratios between tp2 and tp1, for each larva. Tp1 corresponds to two hpi and tp2 to 96 hpi. (**C)** Representative images of larvae injected with HCT116 cells in the yolk and in the

pvs, at three different timepoints. (**D)** Scatter dot plot of the volume ratios etp2/tp1 and ltp2/tp1 for each larva. Tp1 corresponds to 24 hpi, etp2 to 96 hpi and ltp2 to 144 hpi. (**E**, **G**) Dot plots showing MDA-MB-231 (E) and HCT116 cells (G) dissemination in injected larvae in the yolk and pvs at tp2. Each larva is depicted in a different colour. Each dot corresponds to the location of a given segmented tumour mass related to the position of zebrafish eye. (**F**, **H**) Scatter plots of the *x* variance at tp2 in larvae injected in the yolk and in the pvs with MDA-MB-231 (F) and HCT116 (H).

To finally prove the suitability of the pvs for xenotransplantation studies, we injected BJ non-transformed human fibroblasts, as negative experimental control. As expected, these cells did not grow or disseminate (Supplementary figure 1). This observation gives strength to the assumption that growth and invasion through the circulation were consequences of the tumorigenic behaviour of transformed cells, and not artefacts due to the injection site.

Based on these results, we established the pvs as the optimal site of injection of human tumour cells to study drugs impact on primary tumour growth and dissemination.

2.3 Pharmacological validation of the ZeOncoTest

We described above the methods that, through experimental support, provided the most robust strategy for addressing tumour growth and metastatic potential in the zebrafish larvae xenograft model. Once these improved methodologies were integrated into a single experimental workflow, the ZeOncoTest, we proceeded to the validation of the model in drug discovery. To this end, we treated zebrafish larvae transplanted with cell lines with different compounds known to impact specifically with them. ROCK kinase inhibitor RKI-1447 was used to treat MDA-MB-231 larvae xenografts, since this drug has been shown to reduce growth and invasion of these cells *in vitro* and in mice models [52,53]. HCT116 transplanted animals were treated with docetaxel, as it was demonstrated that this molecule induces cell death in this cell line in culture and in mouse xenotransplants [54–56]. Finally, mitoxantrone was employed for the treatment of PC3 prostate cancer cells engrafted larvae, given that its use has been reported to cause cytotoxicity in these cells in previous *in vitro* and mice studies [57,58].

As hypothesized, the reported effects of the drugs on their selected target cells were reproduced in the ZeOncoTest. When compared to the DMSO-treated negative control population, RKI-1447 significantly reduced MDA-MB-231 tumour growth from 3.7 to 2

folds increase (Figure 4A). Docetaxel caused a decrease in HCT116 tumour mass (fold change average of 0.3), as opposed to an increase (fold change average of 1.9) observed when larvae were exposed to DMSO control (Figure 4B). Finally, PC3 volume expansion decreased from 3.3 to 1.6 folds, when comparing injected larvae treated with mitoxantrone with the DMSO-treated population (Figure 4C). Furthermore, we confirmed published data on RKI-147 activity towards abolishing the metastatic invasion capability of MDA-MB-231 cells [52]. The comparison between the variances of the secondary tumour masses at tp2 between RKI-1447- and DMSO-treated larvae was $3.4x10^4$ versus $2.5x10^5$ (Figure 4D). These data offer a definitive proof of principle for the suitability of the ZeOncoTest in drug discovery applications.

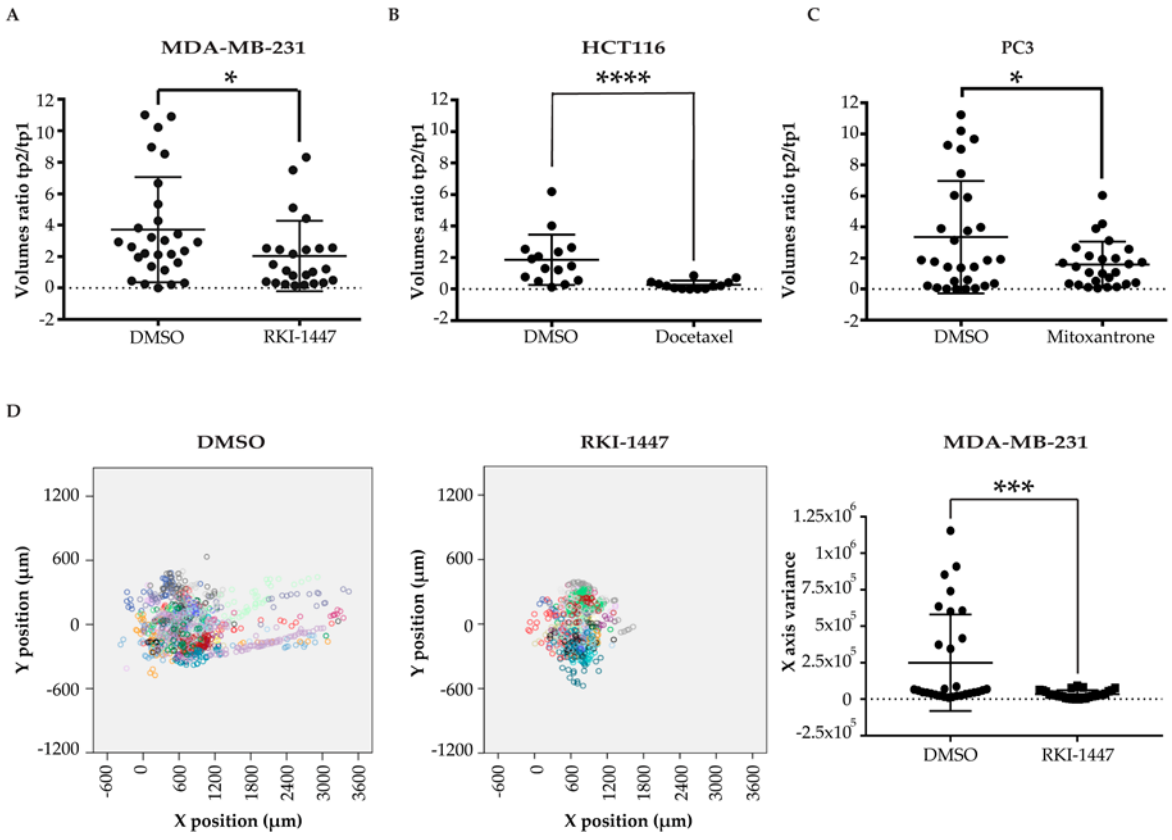


Figure 4. Pharmacological validation (**A, B, C**) Scatter plots of the ratios between the tumour volumes at tp2 and tp1 of MDA-MB-231 (A), HCT116 (B) and PC3 (C) injected cells treated with RKI-1447, docetaxel and mitoxantrone respectively vs DMSO control. Tp1 refers to two hpi for MDA-MB-231 and 24 hpi for HCT116 and PC3. Tp2 corresponds to 96 hpi for MDA-MB-231 and PC3 and 144 hpi for HCT116. (**D**) Combined scatter plots, on the left, and scatter dot plot, on the right, of the variance in the *x* axis at tp2 of secondary tumour foci in MDA-MB-231 injected larvae, treated with control DMSO and RKI-1447.

2.4 Addressing drugs mechanism of action with the ZeOncoTest

We have shown that the ZeOncoTest can be a valid tool for addressing tumour growth and metastatic potential in a relevant drug discovery setting. To further explore its applicability, we decided to study if it could be used for answering more precise questions, such as the evaluation of anti-cancer drugs MoA.

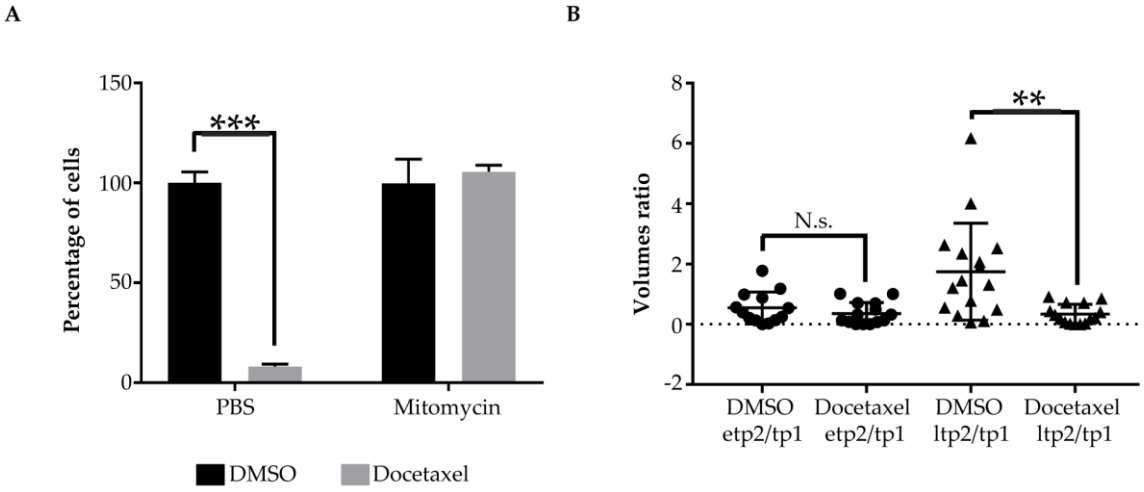


Figure 5. Addressing drugs MoA (**A**) Bar graphs showing percentages of HCT116 cells surviving after incubation with mitomycin C or PBS control and subsequent treatment with docetaxel or DMSO control. Three cell counts were performed per condition. Results are represented as mean +/- standard deviation. (**B**) Scatter dot plot of the tumour volume ratios between etp2 and tp1 and ltp2 and tp1 of HCT116 xenografts treated with Docetaxel or DMSO control. Tp1 corresponds to 24 hpi, etp2 to 96 hpi and ltp2 to 144 hpi.

In order to test this, we chose docetaxel MoA. The activity of this drug consists in disrupting the normal function of microtubules, thereby it stops mitotic division and induces death specifically in proliferating cells [59,60]. We performed equivalent experiments *in vitro* in cell culture and *in vivo* in xenografted zebrafish larvae. At first, HCT116 cells were incubated in culture with mitomycin C, which impairs cell proliferation and, subsequently, with docetaxel. As expected, docetaxel had no effect on the viability of the cells when proliferation was blocked by mitomycin C. On the contrary, it did have a significantly strong cytotoxic effect on proliferating cells not previously incubated with mitomycin C, but with PBS control, and a 92% decrease in cell number was detected (Figure 5A). Next, we tested the same experimental paradigm in our xenograft model. Interestingly, for HCT116 transplanted cells, a significant reduction in tumour growth (87%), in response to docetaxel treatment, was only observed at the late

time point two (ltp2), when the cells do proliferate, as previously specified. The cytotoxicity effect of the drug was not detected at an earlier time point two (etp2), when cells do not show detectable growth, as shown before (Figure 5B). This result proves that our experimental model can also be used for addressing specific new anti-cancer candidate drugs MoAs.

3. Discussion

In the context of an increasing cancer incidence, the search for biologically relevant, faster and more affordable methods to discover new antitumoral drugs represents a medical priority. In order to increase the chance of finding novel effective drugs, zebrafish is imposing as an *in vivo* rational bridge between *in vitro* cell culture systems – cost-efficient and high throughput, but poorly predictive – and *in vivo* mammal models –more predictive, but expensive and time-consuming. Indeed, the final aim of the ZeOncoTest is to help choosing the best candidates, previously selected from *in vitro* experiments, to be subsequently tested in mice. This proposed pipeline would hopefully lead to a better clinical outcome and lower attrition rate.

To guarantee zebrafish larvae healthiness, together with the growth of transplanted tumour cells, we selected the best general xenograft conditions. This was based on previous reports and our own intensive experimental optimization. As for the majority of previous studies, we chose to perform cell injections at 48 hpf, stage at which the larvae are fully anatomically developed, and the immune system is not mature [61]. Another reason for electing 48 hpf was the ample experimental time window: transplanted cells have enough time to grow before the larvae enter the juvenile stage and can no longer be kept in a laboratory environment. 35ºC was selected as the best compromise between the standard laboratory temperature used for zebrafish larvae to develop (28.5ºC) and the one required for human cells to grow appropriately (37ºC). This condition was also chosen in several previous studies. Beside these basic points, additional technical aspects were reviewed and optimized to generate a standardised and robust assay. Stable expression of fluorescent proteins was chosen over staining with fluorescent dyes, as a more reliable solution for cell labelling. However, transgenesis is a suitable method for immortalised tumour cells, but technically difficult to apply to patient samples for the development of

PDXs. To overcome this constraint and broaden the applicability of zebrafish larvae xenografts, there is a pressing need for the search of dyes which faithfully recapitulate tumour cell growth and survival dynamics. As for the site of injection, the pvs was chosen over the yolk. The yolk is a viscous syncytium that provides a cell suspension-like environment not ideal for anchorage-dependent cell growth, whereas the pvs provides tissue support for solid tumour cells attachment and proliferation. In addition, the yolk gets consumed by 96 hpi (144 hpf); a characteristic that constraint the growth of cells till that time point. Finally, in the pvs, cancer cells have an easier access to the vasculature than when injected into the yolk. This last feature allows the study of metastatic capability. Indeed, our results show that tumour cell transplantation in the yolk and/or use of cell dyes provides a poor experimental setup to evaluate tumour progression. After cell transplantation, tumour growth and invasion were evaluated by comparing cell masses volume and dispersion between tp1 and tp2 for each individual larva. As for the choice of time points for the analysis, it was decided to generally set tp1 at 24 hpi, to allow injected cells to adapt to their new in vivo environment. However, highly invasive cells, such as MDA-MB-231, already showed metastatic dissemination at that stage (data not shown). Thus, tp1 was set at two hpi for cells displaying such behaviour. 96 hpi was chosen as tp2 for highly proliferative cells, such as MDA-MB-231 and PC3. For cells showing a reduced proliferation rate at 35ºC, such as HCT116, tp2 was set instead at 144 hpi. This is an important aspect given these cells only underwent significant expansion if allowed to this latest time point, which could be reached only in the case that injections were performed in the pvs, as previously explained. The heterogeneity of conditions suitable for different cell lines, underlines the importance of performing a careful setup for each analysed cell type before drug efficacies can be evaluated. The implementation of automated confocal imaging and analysis tools of individual larvae provides a potential screening throughput of dozens of conditions per month. Moreover, the chosen imaging method allows a much more exact estimation and comparison of tumour masses at different time points, through calculation of volumes instead of areas. It is important to note that measurement and correlation of areas is only recommended if the same orientation is achieved for each larva between the different time points. From our experience, distinct orientation among time points might lead to inaccuracy in areas calculation due to the irregular shape of tumour masses. We are now implementing a

method based on the high-throughput microfluidic imaging system VAST [62] to allow equal positioning among each time point. This approach would allow a more accurate estimation of tumour size through area measurements. The main advantage of this further improvement would be the increase in screening throughput and the further simplification of image acquisition and analysis. Finally, in order to validate the ZeOncoTest, we tested the effect of known drugs on different cancer cell lines. The results provide a solid proof of principle of the method strength for addressing tumour growth and metastatic potential of different cancer cells. Also, we outlined the utility of our method in evaluating anti-cancer drug efficacy and understand their mode of action.

As for the study of metastatic progression, zebrafish, as well as rodents, provide a complex biological context that allow the study of tumour interaction with the microenvironment and the vasculature for the colonization of distant tissues. Nonetheless, metastasis evaluation in mice is mostly performed by bioluminescence imaging and tissue dissection followed by histopathology. This approach takes months and mostly address the latest colonization events [63–65]. Zebrafish larvae transparency allows tracking transplanted fluorescent cancer cells through live imaging. This feature offers the possibility of investigating early metastatic steps, such as intravasation and extravasation. Hence, the use of zebrafish larvae xenografts can be complementary to the use of rodents for understanding different aspects of metastatic progression.

Despite the effort placed in the setup of the ZeOncoTest, there are still a number of aspects that will require further research to be fully addressed. An important question is how suitable is this method for evaluating biologics instead of small molecules. A big advantage deriving from the use of zebrafish larvae in drug discovery is that small molecules can be administered in the incubating water, from where the larva absorbs them. In our experience, passive diffusion does not work for biologics. Thus, the administration route has to be implemented for such macromolecules, either by injection into the vasculature or by co-injection with tumour cells. Another aspect to consider is that the pvs provides a good microenvironment for cancer cells attachment. As previously mentioned, this context favours the growth of solid tumours. Given their preferential growth in suspension, pvs injection might be detrimental to the growth of leukemic cells [66]. Hence, an option could be the test of a different injection site, i.e. the yolk or the vasculature, for liquid tumours to develop. Finally, an important trend in cancer drug

discovery is the search for immunotherapies. This is an aspect difficult to address with our model. Although the zebrafish immune system is well conserved in terms of genetic markers, cell types and functions, our assay is performed at the larval stage, when the adaptive immune system has not been fully developed [67]. In order to investigate possible immunotherapies, it would be necessary to adapt the ZeOncotest methodology to later developmental stages, after six-eight weeks, when the immune system is already mature. This said, standard chemotherapy is still the main option for patients that are not sensitive to immunotherapy [68]. This is especially important considering that responses to immunotherapy in the US are as low as 12,46% [69]. Moreover, it has been shown that immunotherapy is more effective in association with chemotherapy [70–72]. Hence, the discovery of safer and more efficacious chemotherapeutic drugs, to be applied on their own or in combination with immune therapies, is still mandatory.

To conclude, it is important to mention that besides addressing tumour growth and metastasis, the ZeOncoTest could be applied for understanding the impact of drugs on other cancer hallmarks. Thus, angiogenesis could be evaluated by injecting tumour cells into transgenic zebrafish lines with fluorescent vasculature, as shown in previous works [44,47–49,73]. The assessment of vessels sprouting and morphology changes after injections and drug treatment would give an estimation of new vessels formation in the vicinity of the tumour. Fluorescent transgenic lines could also be used for studying the role of inflammation and the dynamics of innate immune cells in response to the injected cancer cells [74–76]. Moreover, cells can be genetically modified prior to injection and/or could be injected into newly generated zebrafish mutant/transgenic lines, allowing to study the function of genes either in the tumour or in its microenvironment. Finally, specific tumour cell populations, such as cancer stem cells, could be specifically labelled to allow their tracking across time. This way, the precise effect of novel compounds on tumour cells subpopulations can be studied. All these features open a broad range of possible applications and further outline the advantages of applying the ZeOncoTest in cancer drug discovery and target validation.

4. Materials and Methods

4.1 Cell lines and zebrafish handling

All cell lines were obtained from collaborators: MDA-MB-231 from Simó Schwartz (VHIR), HCT116 from Melinda Halasz (UCD), PhoenixA and PC3 from Bill Keyes (IGBMC) and BJ from Maria Aurelia Ricci (CRG). They were cultured in DMEM Medium (BE12-614F, Lonza, Cultek) implemented with 10% FBS (10270106, GIBCO, Termofisher) 1% L-Glutamine (BE17-605E, Lonza, Cultek) and 1% Pen/Strep (DE17-602E, Lonza, Cultek), and kept at 37ºC with 5% CO2 in a humidified incubator.

Adult Casper fish, obtained from the European Zebrafish Resource Center, were grown at 28.5 ± 1ºC in a 14:10 hours light:dark cycle in a recirculating tank system. Embryos were obtained by mating adult fish through standard methods [77] and kept in an incubator at 28.5ºC till 2 days and at 35ºC just after injections till the end of the experiment.

This study was performed under the ethical approval code 10567, provided by the Generalitat of Catalunya.

4.2 Infection and dye staining of human cell lines

Phoenix A were transfected with a MSCV-GFP-Puro vector through the calcium phosphate method. After 48 and 72 hours, the supernatant containing the retroviral particles was collected and filtered through a 22 µm filter (SLGP033RS, Merck). HCT116, PC3, MDA-MB-231 and BJ cells were subjected to two rounds and one round of incubation with the supernatant containing the retroviral particles plus 8 µg/ml of polybrene (H9268-5G, Sigma-Aldrich), respectively at the two time points. 48 hours after the last round of infection, cells were selected using 2 µg/ml puromycin (P8833-10MG, Sigma-Aldrich) and left recover for two days. Finally, cells were detached, washed, resuspended in PBS 5% FBS. The brightest GFP+ cells were sorted by flow cytometry and kept in culture.

For dye staining, cells were detached with trypsin-versene EDTA 0.25% (H3BE17-161E, Lonza, Cultek), washed in PBS and incubated with CMDiI and CFSE at the concentrations of 1, 2 and 5 µM in PBS respectively for 5 and 20 minutes at 37ºC,

followed by 15 minutes at 4ºC for CMDiI. Cells were finally washed twice with PBS 10% FBS.

4.3 Hexosaminidase assay

6000 cells were plated in 96 well plates: two replicates of three wells each were done for every condition studied. The day after, media was removed from one of the replicates per condition and cells were washed with PBS twice. 60 µl of substrate solution was added for an hour at 37ºC. Afterwards, 90 µl developer solution was added before recording absorbance at 410 nm. These same passages were then repeated at day four. The number of cells was extrapolated through a calibration curve.

4.4 Zebrafish injection

48 hpf larvae were manually dechorionated and anesthetized by immersion in 0.48 mM tricaine methanesulfonate (A4050, Sigma-Aldrich, Saint Louis, MO, USA) in E3 medium. Around 200-400 cells were injected in the perivitelline space using standard micro-injecting instrumentation. An hour later, larvae were screened at the stereomicroscope to discard non-injected larvae and those injected in a non-specific site or with tumour cells already in circulation.

4.5 Drug treatment

Prior to the incubation of injected larvae with the different selected drugs, the NOEC was established (Table 1). Sixteen larvae per treatment were exposed individually to at least five concentrations of the compound of interest with a dilution factor of three, starting from 1 mM, at the same conditions as in the ZeOncoTest: from 48 to 192 hpf at 35ºC in 96 wells plate. Each larva was analysed for mortality, body deformity, scoliosis, yolk size, heart oedema, heartbeat and movement at 24, 96 and 144 hours post incubation. The NOEC was calculated as the highest concentration at which both mortality and teratogenic scores were below 20%, and then used for the treatment of the injected larvae. DMSO was used as the negative control. In parallel, cells in culture were treated with the NOEC of the compound to ensure its chemotherapeutic effect in vitro (data not shown).

Table 1: NOEC of selected drugs

Drug	NOEC
Docetaxel	10 µM
Mitoxantrone	1 µM
RKI-1447	10 µM

4.6 Automated confocal imaging and analysis

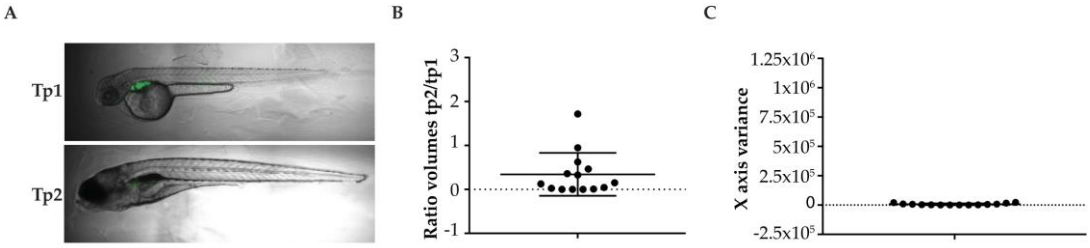
In order to enable lateral positioning of injected larvae and restrict the area to be imaged, 3D printed orientation moulds [78,79] and low melting agarose (8092.11, Conda, Pronadisa) were used to shape the wells of 96 well plates (265301, Thermo Scientific, Nunc). Injected larvae were anesthetized by immersion in 0.48 mM tricaine methanesulfonate in E3 medium and transferred in the wells. Xenografts were imaged at two different timepoints using a Leica TCS SP5 inverted confocal microscope system with an automated plate and the Matrix Screener software. Images were then processed and volumes and dispersion of cells measured using in-house macros and the FIJI software. Finally, tumour growth was calculated as the ratio between the volumes at tp2 and tp1 for each individualized larva. Cell dispersion was assessed with the SPSS software (IBM). A minimum of 16 animals were used per condition and one or two experiments were performed.

4.7 Statistical analysis

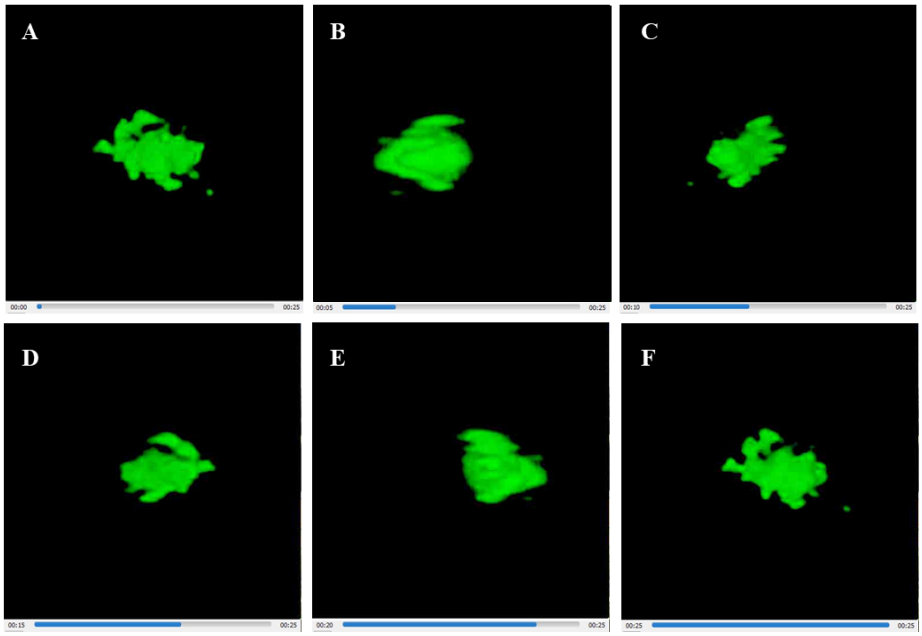
Results were analysed using GraphPad Prism v 7.04 (GraphPad Software Inc., La Jolla, CA, USA). Prior to the analysis, the ROUT method (Q=1%) was applied to identify outliers and remove them. Then, D'Agostino-Pearson omnibus normality test was used to assess if data were normally distributed. For two groups comparison, Student's t-test was used when values were normally distributed, whereas Wilcoxon or and Mann-Whitney tests were used respectively for paired and unpaired non-parametric data. Statistical analysis for multiple comparisons was performed using One-Way ANOVA, followed by Tukey or Sidak tests, for data with a parametric distribution. Kruskal Wallis test was instead performed for multiple comparisons of non-parametric values. Differences were considered statistically significant when $p < 0.05$. In figures, one asterisk (*) indicates $p < 0.05$, 2 (**) means $p < 0.01$, 3 (***) signify $p < 0.001$ and 4

(****) stays for $p < 0.0001$. No statistical significance is indicated by "ns". Results are presented as mean ± standard deviation (SD).

Supplementary Materials: Figure S1: Evaluation of growth and dispersion in BJ non-transformed control cell xenografts. Video S1: 360º rotation of a 3D image of a xenografted tumour. Table S1: Collection of parameters used in published literature for drug discovery in zebrafish xenografts models.



Supplementary figure 1. Evaluation of growth and dispersion in BJ non-transformed control cells xenografts. (A) Representative images of injected embryos at tp1 and tp2. (B) Scatter plots of the ratios of volumes between tp1 and tp2. (C) Scatter dot plot of the x variance at tp2 of injected embryos.



Supplementary video 1. Representative images taken at 0s (A), 5s (B), 10s (C), 15s (D), 20s (E), and 25s (F) of a 360º rotation video showing a 3D image of a xenografted tumour.

Supplementary table 1. Collection of parameters used in published literature for drug discovery in zebrafish xenografts models. Abbreviations: PVS: Perivitelline space, DoC: Duct of Cuvier, dpf: days post fertilization, hpf: hours post fertilization.

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
1	15968639	3.5hpf	31	Blastodisc	AD infection	No	No

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
2	17051341	2dpf	35	Yolk sac	CM-DiI	No	No
3	16892036	3hpf	31	Blastodisc	AD infection	No	Yes
4	17409396	2dpf	28	PVS/DoC	WISH/Dye	No	Yes
5	17195184	4-10hpf	26	Neural plate	WISH	No	Yes
6	17875720	3.5hpf	30	Blastodist	Infected (pDsRed2-C1)	No	Yes
7	18007628	2dpf	28	PVS/DoC	WISH/Dye	No	No
8	17954920	25-35 dpf	28	Peritoneal	Stable transfection	No	Yes
9	18451167	3.5-4.5hpf	31	Yolk sac	Stable transfection	No	No
10	19199503	6hpf	28	Yolk sac	Luciferase	No	No
11	19400945	2dpf	35	Yolk sac	CM-DiI	No	No
12	19685150	2dpf	28	Yolk sac	Stable transfection or QTracker Red kit	No	Yes
13	19747919	2dpf	35	Yolk sac	CM-DiI	No	Yes
14	19887629	2dpf	28	PVS	DiI	No	Yes
15	20047470	2dpf	35	Yolk sac	CM-DiI	No	Yes
16	19627396	2dpf	28	PVS/DoC	WISH	No	yes
17	20339318	4-6 months & 5dpf	28.5	Yolk sac/PVS	zRag2-EGFP-mMyc construct	No	No
18	20530574	2dpf	35.5	Common cardinal vein	Stable expression vectors	No	Yes
19	20630586	3hpf	35	Blastodisc	Gold nanoparticles & CM-DiI	No	No
20	21127485	2dpf	28	PVS	DiI	No	No
21	24213127	24hpf	28.5	DoC	CM-DiI	No	Yes
22	21228037	2dpf	34	Posterior cardinal vein	CM-DiI	No	Yes
23	21423205	4hpf	----	Under cell mass	Fusion GFP protein & WISH	No	Yes
24	21517816	2dpf	35	Yolk sac	CM-DiI	No	No
25	21618587	2dpf	35	Yolk sac	CM-DiI	No	No
26	21622720	2dpf	----	PVS	CM-DiI	No	Yes
27	21744342	2dpf	28	Yolk sac	CM-DiI	No	Yes
28	21765912	2dpf	28	PVS	Stable transfection	No	Yes
29	21976976	2dpf	28	Yolk sac	Stable transfection	No	No
30	22033190	2dpf	34	Yolk sac	CM-DiI	No	No
31	22195560	2dpf	31	Yolk sac	DiI	No	Yes
32	21515914	2dpf	----	Pericardial	CM-DiI	No	Yes
33	22194464	2dpf	34	Yolk sac	Cell tracker Green CMFDA	No	No

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
34	21671725	2dpf	33	Spinal cord	Q-Tracker 605 Cell Labelling kit	No	Yes
35	22347456	2dpf	34	Yolk sac	CM-DiI	Yes	No
36	22374800	2dpf	34	DoC	CM-DiI	No	Yes
37	22569777	2dpf	31	Yolk sac	DiI	No	No
38	22711017	2dpf	28	PVS	Stable (pcDNA3.0-DsRed)	No	No
39	23158001	2dpf	32	PVS	Stable transfection	No	Yes
40	22183788	4dpf & 10dpf	29	Yolk Sac	CM-DiI	No	Yes
41	23250956	2dpf	34	Pericardial	Stable transfection	No	Yes
42	23261760	2dpf	35	Yolk sac	Stable transfection	No	Yes
43	23429286	7dpf	34	Brain peri-ventricular	Stable transfection	No	Yes
44	23581411	2dpf	28	PVS	CM-DiI	No	Yes
45	23594209	2dpf	28	PVS	Q Tracker kit	No	No
46	23613942	2dpf	35	Yolk sac	Stable transfection	No	Yes
47	23618854	2dpf	34	DoC	CM-DiI	No	Yes
48	23688428	2dpf	----	Yolk sac	CM-DiI	No	Yes
49	23689123	3dpf	37	Pericardium	Cell tracker Orange CMTMR	No	Yes
50	23807209	2dpf	33	Yolk sac	CM-DiI	No	Yes
51	23835085	2dpf	----	Intra-vitreal	Stable transfection	Yes	No
52	23874489	2dpf	----	PVS	Vybrant CiI	Yes	No
53	23899555	2dpf	28	PVS	-----	Yes	Yes
54	24165931	2dpf	----	PVS	CM-DiI	No	No
55	24196484	2dpf	33	DoC	CM-DiI & stable transfection	No	Yes
56	24290981	2dpf	----	PVS & DoC	DiI	Yes	Yes
57	23623984	2dpf	32	Cardinal vein	SNARF-1 (cell dye)	No	Yes
58	23973329	2dpf	33	DoC	Red fluorescent cell tracer	No	Yes
59	24089705	2dpf	34	PVS	CM-DiI	No	Yes
60	24056961	1dpf	35	Yolk sac	CMTMR	No	Yes
61	24154958	2dpf	33-34	Subcutaneous or DoC	Quantum dots QD605	No	Yes
62	24416389	2dpf	----	Yolk sac	CM-DiI	No	Yes
63	24454867	2dpf	32	Yolk sac	Stable transfection	No	No
64	24454929	2dpf	35	Yolk sac	Stable transfection	Yes	No
65	24461128	24-30hpf	35	Yolk sac	Cell Brite DiD	No	Yes
66	24556065	2dpf	31	Yolk sac	DiI	Yes	No

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
67	24830720	2dpf	28	PVS	DiI	No	Yes
68	24974828	2dpf & 35dpf	34	Yolk sac & Eye	CM-DiI	Yes	Yes
69	25066122	2dpf	34	Pericardial	CFSE	No	Yes
70	25117453	2dpf	32	Yolk sac	Stable transfection	No	Yes
71	25209178	Adult & 2dpf	34 &32	Dorsum aorta & Yolk sac	Stable transfected	No	No
72	25249605	2dpf	34	Yolk sac	Stable transfected	Yes	Yes
73	25281505	2dpf	----	Yolk sac	CM-DiI	No	Yes
74	25281719	2dpf	34	Yolk sac	Stable transfected	No	No
75	25397870	2dpf	31	Yolk sac	DiI	No	Yes
76	25477335	2dpf	28	Yolk sac	CM-DiI	No	Yes
77	25504881	1dpf	35	Yolk sac	CM-DiI	No	Yes
78	25551022	2dpf	32	Common cardinal vein	Stable expression vectors	No	No
79	24947063	2dpf	28	PVS	----------	No	No
80	24976296	36hpf	----	Yolk sac	Stable transfected	No	Yes
81	25388286	2dpf	34	Yolk sac	CM-DiI	No	Yes
82	25609010	2dpf	28	Brain ventricle	Cell Brite DiD	No	No
83	25609197	2dpf	33	DoC	Stable transfected	No	No
84	25624101	2dpf	34	Yolk sac	CM-DiI	No	Yes
85	25697483	2dpf	32	Cardinal vein	SNARF-1 (cell dye)	No	Yes
86	25768009	2dpf	34	Yolk sac	CM-DiI or CMFDA	No	No
87	25772246	2dpf	----	Yolk sac or DoC	QTracker cell labelling kit	No	Yes
88	25818410	2dpf	32	Yolk sac	Stable transfection	No	Yes
89	25826087	2dpf	28	PVS	DiI	No	Yes
90	25849225	2dpf	33	DoC	Red fluorescent cell tracer	No	No
91	26035715	2dpf & 6months	35.5 & 28	PVS	CM-DiI	No	Yes
92	26123890	2dpf	28	PVS	Hoechst 33342 or Cell Tracker green	No	Yes
93	26169357	2dpf	28.5	Vitreous cavity	DiI	Yes	Yes
94	26313918	2dpf	29	PVS	Stable transfection	No	Yes
95	26388134	1dpf	34	PVS	CM-DiI	No	No
96	26412466	2dpf	31	Yolk sac	CM-DiI	No	Yes
97	26449749	2dpf	----	DoC	PKH26 fluorescent cell linker	No	No

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
98	26476432	1dpf	35	Yolk sac	CMTMR	No	Yes
99	26498353	2dpf	35	Yolk sac	CM-DiI	No	Yes
100	26672745	2dpf	28.5	Yolk sac	Stable transfection	No	No
101	25577646	2dpf	34	Yolk sac	DiO green dye	No	No
102	25991856	2dpf	28	PVS	DiI	No	Yes
103	25519702	2dpf	28	PVS	Red-fluorescence-labelled	No	Yes
104	25858144	2dpf	33	DoC	Stable transfection	No	Yes
105	25492861	2dpf	28	PVS	DiI or Vybrant DiD	No	Yes
106	26483278	2dpf	32	PVS	DiI	No	Yes
107	26310813	2dpf	28.5	PVS	DiI	No	Yes
108	26650921	2dpf	34	Yolk sac or DoC	PKH26 fluorescent cell linker	No	No
109	26657275	3dpf	28	PVS	--	No	No
110	26659251	36hpf	32	Hindbrain-midbrain	Stable transfection	No	No
111	26741506	2dpf	34	Yolk sac	CM-DiI	No	Yes
112	26744352	2dpf	34	DoC	Stable transfection	No	Yes
113	26746804	Adult & 2dpf	34 & 32	Dorsal aorta & Yolk sac	Stable transfection	No	No
114	26762853	2dpf	----	PVs	FAST DiI	Yes	Yes
115	26829331	2dpf	28	PVs	DiI	No	No
116	27036136	2dpf	32.5	PVs	DiI	No	Yes
117	27049037	2dpf	33	Yolk sac	CM-RED	No	No
118	27091969	3dpf	----	Pericardial	CellMask Deep Red or Hoechst 33342	No	No
119	27113436	2dpf	33	DoC	Stable transfection	No	Yes
120	27158859	2-3dpf	33±1	Hindbrain ventricle	CM-DiI	No	No
121	27197202	2dpf	33	DoC	CM-DiI & stable transfection	Yes	Yes
122	27199173	2dpf	35	DoC	Stable transfection	No	Yes
123	27207793	2-4dpf	----	PVS	CM-DiI	No	No
124	27242319	0.5-2dpf	31	Yolk sac	Stable transfection	No	No
125	27247548	52hpf	31	Brain	Stable transfection or CiI or CiO	No	No
126	27258728	2dpf	32	Yolk sac	CM-DiI	No	No
127	27427902	2dpf	31	Yolk sac	CiI	No	No
128	27434411	2dpf	35	DoC	CM-DiI	No	Yes
129	27457520	2dpf	28	PVS	DiI	No	Yes

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
130	27466505	2dpf	35	Hindbrain ventricle	CFSE	No	Yes
131	27504667	2dpf	34	DoC	Stable transfection	No	No
132	27517156	2dpf	34	PVS	CM-DiI	No	Yes
133	27556456	2dpf	34	DoC	CFSE	No	No
134	26804176	2dpf	----	PVS	CM-DiI	No	Yes
135	27785023	2dpf	----	Yolk sac	CM-DiI	No	No
136	27825113	2dpf	35	Yolk sac	Stable transfection	No	Yes
137	27835901	2dpf	33	DoC	mCherry fluorophore	No	Yes
138	27879396	2dpf	33	Yolk sac	DiI or DiO	No	No
139	27906672	2dpf	30.5	DoC	Stable transfection	No	Yes
140	27924011	2dpf	35	Yolk sac	CM-DiI	No	Yes
141	27989824	2dpf	34	Yolk sac	DiI	No	No
142	27006469	2dpf	28.5	Yolk sac	Red fluorescence	No	Yes
143	26744527	2dpf	----	Cardiac	DiD or DiO	Yes	No
144	27464807	2dpf	34	DoC	CM-DiI	No	No
145	27481363	2dpf	32	PVS and Yolk sac	CM-DiI	No	No
146	28043811	2dpf	----	Yolk sac	CM-DiI	No	Yes
147	28075592	2dpf	35	Yolk sac	CM-DiI	No	No
148	28088004	2dpf	----	Yolk sac	CM-DiI	No	No
149	28108843	2dpf	32	PVS	Stable transfection	No	Yes
150	28145883	2dpf	----	Yolk sac	CM-DiI	No	No
151	28159748	2dpf	28	PVS	Fast DiI	No	Yes
152	28160553	2dpf	35	Yolk sac	Stable transfection	No	Yes
153	28193911	2dpf	28	PVS	Stable transfection	No	Yes
154	28196873	2dpf	----	PVS	CFSE	No	Yes
155	28209621	2dpf	35	Yolk sac	CM-DiI	No	No
156	28240249	2dpf	----	PVS	Stable transfection	No	No
157	28376864	54hpf	28	PVS	Stable transfection	No	No
158	28420724	2dpf	----	PVS	DiI or Vybrant DiD	No	Yes
159	28465491	2dpf	35	Yolk sac	CM-DiI	No	No
160	28518096	2-3dpf	33	PVS or DoC	Stable transfection	No	No
161	28526577	36hpf	32	Hindbrain-midbrain	Stable transfection	No	No
162	28574600	2dpf	28.5	PVS	DiI	No	No
163	28589491	2dpf	34	PVS	Stable transfection	No	No
164	28606996	2dpf	34	Pericardial	Stable transfection	No	Yes
165	28679777	36hpf	32	Hindbrain-midbrain	Stable transfection	No	No

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
166	28697174	2dpf	28	PVS	CiI	No	Yes
167	28718729	2dpf	----	PVS	CM-DiI	No	No
168	28790117	2dpf	35	Yolk sac	CM-DiI	No	No
169	28835536	2dpf	34	PVS	DiI	No	No
170	28878163	52hpf	31	Yolk sac & Brain	Stable transfection	No	Yes
171	28892043	2dpf	35	Yolk sac	CM-DiI	No	No
172	28900283	2dpf	----	Yolk sac	CM-DiI	No	No
173	28943451	2dpf	28.5	Yolk sac	CM-DiI	No	No
174	29085081	3dpf	33-34	Intracranial	Stable transfection	No	No
175	29089623	2dpf	35	Yolk sac	CMTMR	No	Yes
176	29141689	2dpf	32	Yolk sac	CM-DiI	No	No
177	29172540	2dpf	35	PVS	CM-DiI	No	No
178	28394345	2dpf	33	DoC	Stable transfection	No	Yes
179	28454577	2dpf	28	DoC	Stable transfection	No	No
180	29106602	36hpf	32	Hindbrain-midbrain	Stable transfection	No	Yes
181	28991224	2dpf	30	PVS	CM-DiI	No	No
182	28925392	2dpf	34	PVS or DoC	DiI	No	No
183	28949016	2dpf	35	Yolk sac	DiI	No	Yes
184	29115578	2dpf	28-32	Yolk sac	QTracker 525	No	No
185	29246646	2dpf	28.5	Yolk sac	Red fluorescence	No	No
186	29291719	2dpf	36	Yolk sac	Stable transfection	No	No
187	29321662	2dpf	36	Yolk sac	Stable transfection	No	Yes
188	29433678	2dpf	----	Yolks sac	CM-DiI	No	Yes
189	29507700	2dpf	----	Yolk sac	CM-DiI	No	Yes
190	29515255	2dpf	34	Yolk sac	CM-DiI	No	No
191	29541384	2dpf	35	Yolk sac	CM-DiI	No	No
192	29545193	2dpf	33	PVS	CM-DiI	No	No
193	29604056	2dpf	33	DoC	Stable transfection	No	No
194	29641204	2dpf	35	Yolk sac	CM-DiI	No	No
195	29681541	2dpf	33	DoC	Stable transfection	No	No
196	29712618	2dpf	33	Pericardial	Stable transfection	Yes	Yes
197	29777274	2dpf	----	PVS	CM-DiI	No	No
198	29849132	2dpf	33	PVS	CM-DiI	No	No
199	29899843	2dpf	33	Yolk sac	CM-DiI	No	No
200	29993186	2dpf	----	Yolk sac	CM-DiI	No	Yes
201	30102771	2dpf	35.5	PVS	Stable transfection	No	Yes
202	30195867	2dpf	32	Yolk sac	CM-DiI	No	No
203	30205168	2dpf	28.5	Yolk sac	CM-DiI	No	No

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
204	30326259	2dpf	35	Yolk sac	CM-DiI	No	No
205	30332851	2dpf	----	PVS	DiI	No	No
206	30339727	2dpf	33	Yolk sac	DiI	No	Yes
207	30367145	2dpf	35	Yolk sac	CM-DiI	No	No
208	30389143	2dpf	----	Hindbrain ventricle	CM-DiI	No	Yes
209	30396905	2dpf	34	PVS	CellTracker green or Hoechst	Yes	Yes
210	30478450	2dpf	35	Yolk sac	CMTPX-Red	No	No
211	30398868	2dpf	----	PVS	DiI	No	No
212	30507376	1dpf	28	Yolk sac	CM-DiI	No	No
213	30484103	2dpf	35	Yolk sac	Stable transfection	No	No
214	30544196	2dpf	32	PVS	CM-DiI	No	No
215	30581541	2dpf	28	PVS	DiI	No	No
216	30473782	2dpf	33	Yolk sac and DoC	DiI	No	No
217	30616104	2dpf	35	Yolk sac	CMFDA dye	No	No
218	30618758	2dpf	28.5	Yolk sac	DiI	No	No
219	30599417	2dpf	----	PVS	DiI	No	No
220	30643816	2dpf	35	PVS	CM-DiI	No	No
221	30657763	2dpf	----	Yolk sac	CM-DiI	No	Yes
222	30684465	2dpf	----	PVS	DiI	No	Yes
223	30720231	2dpf	----	Yolk sac	CM-DiI	Yes	Yes
224	30784915	2dpf	----	SIV	DiI	No	No
225	30787324	2dpf	34	DoC	CM-DiI	No	Yes
226	30968154	2dpf	32	Cardinal vein	Stable transfection	No	Yes
227	30929607	2dpf	28	DoC	Stable transfection	No	No
228	31024847	2dpf	32.5	Pericardial	CFSE	No	Yes
229	31069942	2dpf	33	Yolk sac	CM-RED	No	No
230	31085547	2dpf	33	DoC	CellTrace Far Red	No	No
231	31107449	2dpf	32	Yolk sac	Stable Transfection	No	No
232	31057328	2dpf	35	PVS	Stable transfection	No	No
233	31132644	4hpf	----	Cell mass	Stable transfection	No	No
234	31115172	2dpf	28	Yolk sac	DiI	No	No
235	31138874	2dpf	32	Yolk sac	CM-DiI or CM-DiD	No	Yes
236	31141996	2dpf	28 -37	Yolk sac	CM-DiI or CFSE	No	Yes
237	31164413	2dpf	33	Yolk sac	DiI	No	No
238	31202990	2dpf	----	Yolk sac	CM-DiI	No	No
239	31213909	2dpf	35	PVS	CM-DiI	No	No
240	31238903	2dpf	33	DoC	Stable transfection	Yes	Yes

Nº	PMID	Stage	Tº	Injection Site	Cell Labelling	Individualization	MoA
241	31221787	2dpf	28.5	Yolk sac	CM-DiI	No	Yes
242	31311575	2dpf	34	Yolk sac	DiI	No	Yes
243	31331338	2dpf	34	PVS	CellTrace Far Red	No	No
244	31346515	2dpf	32	Yolk sac	CM-DiI	No	No
245	31361134	4hpf	34	Cell mass	Stable transfection	No	Yes

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Conflicts of Interest: All authors are currently employed by ZeClinics S.L.

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4. DISCUSSION

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4.1. The problem: pharmaceutical industry productivity crisis

The pharmaceutical industry is facing a productivity crisis. While the investment in drug R&D has been increasingly growing, this has not translated in a higher number of new drugs into the market (Hay *et al.*, 2014). This situation produces the raise in the direct costs invested in this process (DiMasi, Grabowski and Hansen, 2016). One of the main reasons explaining this crisis is the high drug attrition rates during clinical and post market phases. This is particularly worrisome in the oncologic field, where drug attrition rates are between two and four times higher than in other health care sectors (Hay *et al.*, 2014; Nixon *et al.*, 2017). Toxicity and efficacy-related issues are among the leading causes of drugs withdrawal. This fact reflects limitations in the preclinical models used during the drug R&D process, which are not predictable enough. Furthermore, there is a growing tendency to replace, reduce or refine the use of animals in experimentation (3Rs principle). In conclusion, there is a need for alternatives in order to improve the outcomes of the current preclinical models and, at the same time, reduce and refine the use of animals. This would in turn reduce attrition rates and ameliorate the pharmaceutical industry productivity crisis.

4.2. Zebrafish: an *in vivo* solution

The zebrafish larva is becoming a promising alternative vertebrate model in pharmaceutical research as it is characterised by unique biological properties: fast life cycle, large progeny numbers, small size, transparency, ease of maintenance, and high genetic and physiologic homology with humans. These characteristics, together with the ability to passively absorb molecules from the surrounding water, allow their use in biologically relevant high throughput assays. In addition, zebrafish larvae up to five dpf are regarded as non-protected stages (European Union, 2010). Thus, they could be used in the pre-regulatory phases of drug discovery as an intermediate step between *in vitro* cell-based experiments and *in vivo* mammalian testing. They can be very valuable to streamline the preclinical timeline, discard toxic or inefficient compounds, and prioritize drug candidates for further testing, thus reducing the need for mammalian studies. The

use of zebrafish could overcome the low predictivity of the *in vitro* studies, together with the high cost and low throughput of *in vivo* mammal models. However, it also has its own limitations, among which stand out the difficulty to accurately predict larvae drug uptake, the duplication genes and the poor standardisation of the assays (Table 2).

Table 2. Advantages and limitations of zebrafish and mammalian *in vivo* models for the drug R&D process.

	Advantages	**Limitations**
Zebrafish models	- Small size - Large progeny - Fast *ex utero* development - Easy and inexpensive to maintain - Transparency - High throughput - Amenability for genetics modifications - Tolerant to 1% DMSO - Small amount of drug needed - Compounds readily absorbed from the surrounding water	- Not all mammalian organs present - Duplication of its genome - Difficulty to predict drug uptake - Highly hydrophobic compounds and large molecules, such as proteins are not absorbed and need to be injected - Inbreeding is bad tolerated - Inter- and intra-laboratories variations - Metabolizing enzymes of the liver (e.g., CYP450s) are not fully characterised (Kubota *et al.*, 2019)[1].
Mammalian models	- Higher anatomical, molecular, genetic, and pathological similarities with humans - Well characterized inbreed strains - Well standardised assays - Antibody availability	- Ethical considerations - Expensive - Slow developmental cycle - Low throughput - Limited genetic diversity

[1] Understanding of the relevance to human drug metabolism is unclear.

In my PhD project, I used zebrafish larvae to generate two middle-high throughput methodologies for the assessment of the toxicity (ZeGlobalTox) and anti-tumour efficacy (ZeOncoTest) of novel drugs. The aim has been to further validate and consolidate the use of zebrafish as a preclinical tool in current R&D pipelines.

In this regard, results obtained through the analysis of known toxic compounds in human using the ZeGlobalTox assay have shown a specificity of 89%, a sensitivity of 68%, and an accuracy of 78%. These data are really promising since cardio-, neuro-, and hepato-toxicities are the most relevant causes of drug attrition during clinical, and post market

stages (Schuster, Laggner and Langer, 2005). Furthermore, the ZeGlobalTox contributes to the 3R principle by reducing the amount of zebrafish larvae used. These results reinforce the validation of zebrafish as a suitable model for preclinical toxicologic studies to reduce the experimental time and cost of drugs for being approved.

Results obtained with the ZeOncoTest recapitulate growth and metastatic behaviour of the human cancer cell lines used, as well as the anti-tumour efficacy of known compounds. Thus, our system has showed to be reproducible, robust and predictive, endorsing the zebrafish xenograft as alternative tools to be used in the preclinical phases of anti-cancer drug discovery. In addition, the ZeOncoTest has proven to be useful for understanding drugs mode of action. Interestingly, we performed xenografts of two types of tumours cell lines deriving from organs not present in zebrafish, such as the MDA-MB-231 breast cancer cells and the PC3 prostate cancer cells. It cannot be discarded that the behaviours of both tumour cell lines might have been altered by the lack of signals and hormones originated in their original organs. However, results from the ZeOncoTest, as well as from previous reports (Ghotra *et al.*, 2012; Bansal *et al.*, 2014; De Boeck *et al.*, 2016; W. Xu *et al.*, 2018; Wu *et al.*, 2018), show that both cell lines are able to engraft, grow, disseminate and properly respond to known anti-cancer drugs. Overall, our data helps to validate and consolidate zebrafish as a powerful *in vivo* tool for preclinical studies in the field of oncology.

Below I will discuss in better detail some aspects that help position zebrafish as an ideal model to streamline the drug development pipeline.

4.2.1. Advantages of PDD over TDD approaches

Both ZeGlobalTox and ZeOncoTest methodologies, as well as most of the zebrafish chemical screens performed up to date, follow the PDD approach (Rennekamp and Peterson, 2015). Phenotype-based screenings had been historically the basis of most drug discovery. However, with the emergence of the recombinant DNA technology in the 1980s, together with the advances in molecular and cell biology, which have contributed to a major understanding of biological pathways, disease mechanisms and the identification of druggable targets, PDD methodologies had been largely replaced by target-based approaches (TDD). Nonetheless, PDD strategy is currently gaining interest

again, as some experts have attributed part of the pharmaceuticals industry lack of productivity of the last years to the over-reliance on TDD, at the expenses of the PDD approaches (Swinney and Anthony, 2011; Ramabhadran, 2018). Indeed, an analysis of new molecular entities (NMEs) among drugs approved by the FDA between 1999 and 2008, revealed that 62% of them were discovered following PDD screens, despite the fact that were underrepresented compared to TDD tests (Swinney and Anthony, 2011) . There are several possible reasons that may explain this fact. First, PDD screens can disclose efficacious candidates in the absence of a validated target. Second, they can identify compounds that exert their therapeutic effect simultaneously activating different targets. Indeed, some of the most successful drugs in use today are known to interact with multiple targets throughout the body, not only relevant to disease intervention but also to chemical activation, transport and toxicity. For example, this is the case for amiodarone, which activates multiple ion channels and adrenergic receptors to exert its therapeutic antiarrhythmic effects (Kodama, Kamiya and Toyama, 1997). Finally, PDD approaches performed in whole organisms take into account all toxicological and pharmacological properties, ADMET profiles, of the screened compounds. Thus, drugs are selected for their low toxicity, ability to reach in enough quantities at the appropriate therapeutic site and to avoid or exploit endogenous chemical metabolizing enzymes and transporters. As a result, molecules selected through PDD approaches tend to be of better quality than those selected through target-based screens (Swinney and Anthony, 2011; MacRae and Peterson, 2015; Ramabhadran, 2018). Given the resurgence of the PDD strategy, zebrafish could be an ideal alternative to overcome the NMEs pharmaceutical industry productivity crisis. Indeed, this model is already increasingly used in high throughput *in vivo* preclinical phenotypic screens for the assessment of the toxicity and efficacy of novel drugs (Rennekamp and Peterson, 2015). In this line, results from both ZeGlobalTox and ZeOncoTest contribute to further validate and consolidate the usage of zebrafish and PDD approaches in drug R&D pipelines.

4.2.2. Precision medicine in oncology

The use of zebrafish in oncology is particularly promising, as cancer represents one of the major health problems worldwide, and pharmaceuticals productivity crisis is even more accentuated in this field. Murine models, and specifically xenograft of human

tumours in mice, represent the current gold standard (Sia *et al.*, 2015). Although displaying multiple advantages, like their high genetic and physiologic homology with humans and well characterised and standardised assays, they are not exempt of limitations, such as the low experimental throughput, high cost and the requirement for immunosuppression (Table 3). As previously mentioned, zebrafish larvae xenografts have several advantages over mammalian counterparts, such as small size, large numbers, ease of maintenance, suitability for high throughput studies, transparency, low amount of cells required for the engraftment, no need for immunosuppression and permeability to small molecules (Table 3). Particularly, it is envisioned an important role for them in the future of the anti-cancer drug discovery and development, to improve throughput and efficiency and move towards personalised medicine. Personalised medicine is specially indicated for complex diseases with multiple aetiology factors that result in different responses to treatment. The goal is to be able to stratify patients according to their predicted therapy reaction, and then treat each of them with the most effective therapy. Current treatments are only effective in certain types of cancers, while others remain lethal. Precision medicine in oncology may ideally overcome this problem, as targeted drugs are genotype-selective and have higher chances of success, given an upfront patient stratification and selection. This strategy may finally decrease drug attrition rates. In addition, as patients are being stratified, approval of such drugs often no longer requires expensive Phase III trials with thousands of patients, which also reduces the overall cost of the anti-cancer drug. An example of success is the case of crizotinib, an ALK and ROS1 inhibitor for which the registration study only involved 347 patients with ALK-positive lung cancer (Shaw *et al.*, 2013). In addition, only 50 lung cancer patients with mutations in the gene *ROS1* were required by the FDA for the final approval of crizotinib (Shaw *et al.*, 2014). Zebrafish larvae PDX models have, in principle, the required throughput and efficiency for the development of personalised medicine (Astone *et al.*, 2017; Baxendale, van Eeden and Wilkinson, 2017). Indeed, several studies have already reported the transplant of patient-derived tumour cells into zebrafish larvae (Marques *et al.*, 2009; Weiss *et al.*, 2009; Mercatali *et al.*, 2016; Welker *et al.*, 2016; Gacha-Garay *et al.*, 2019; Wang *et al.*, 2019; Yan *et al.*, 2019). Limitations are represented by lack of breast, lung and prostate organs, absence of certain cancer-associated genes (e.g. *BRCA1*, *P16* and *IL6*) (Howe *et al.*, 2013), difficulty to assess the last colonisation step of the

metastatic cascade and challenges in fixing zebrafish tissues together with limited zebrafish proteins antibodies availability (Table 3). In addition, larvae transplanted with human tumour cells have to be raised at higher temperatures than their ideal one (28ºC) (Table 3). This aspect could represent a source of stress, although no obvious effects have been observed on zebrafish development raised at 35ªC (Haldi *et al.*, 2006). At the same time, such temperature could affect the growth of human cells, whose ideal one is 37ºC. Another possible limitation of the zebrafish larvae is the lack of an adaptative immune system (Table 3). While this fact could be considered beneficious as avoids the requirement for immunosuppression, it could also represent a drawback, since the adaptive immune system can play an essential role in the promotion or inhibition of cancer growth, development and response to treatments (Chen and Mellman, 2017) (Table 3). Therefore, the study of the immunotherapies, which is currently one of the major trends in cancer drug discovery, is challenging with this model. Transplantation into an embryonic body might be another limitation, as it represents a continuously changing environment, expanding and readjusting, and these processes may alter tumour progression and dissemination (Table 3). Finally, one aspect worth mentioning is that, according to our results, current methods for cell staining using dyes do not faithfully recapitulate cell growth and survival dynamics. Although we have overcome this limitation for cancer cell lines by stably expressing fluorescent proteins, this strategy is not suitable in the case of PDXs, as it is technically difficult to apply to patient samples. Although its limitations, zebrafish PDX might become a great tool not only in drug discovery, but also in the clinical practice to, predict the best treatment for each cancer patient, in time for the individual person to be treated.

Table 3. Advantages and limitations of zebrafish larvae and mouse xenograft models.

	Advantages	**Limitations**
Zebrafish larvae	- Small size, large progenies, fast *ex utero* development and easy to maintain → amenable for high throughput. - Hundreds of embryos can be xenotransplanted with tumour cells within hours → improved validity for the statistical analysis and high throughput.	- Post-xenografted embryonic zebrafish are housed at 32-35°C → temperature not ideal for tumour growth. - Anatomical differences with humans (lack of breast, lungs and prostate) → absence of associated hormones that might affect tumour development.

	Advantages	Limitations
Zebrafish larvae	- Optically transparent → tumour cell visualisation and *in vivo* imaging of tumour development. - Few cells required for the xenograft to engraft → important for PDX. - No need of immunosuppression for xenotransplantation (no innate or adaptative immune defence). - Genetic amenability → study of specific cancer genes. - Vast repertoire of transgenic lines → study of the interaction of tumour cells with the microenvironment. - Permeable to small molecules administered in the surrounding water → easy to treat.	- Several cancer-associated genes not expressed in zebrafish (*BRCA1*, *P16*, and *IL6)* → challenging to study their function and implication in cancer. - Impaired adaptive immune system → impossibility of studying the effect on cancer growth and development. - Human adult cells transplanted in an embryonic environment → continuously changing body, which might influence cancer cells behaviour. - Tissue fixation of embryos quite challenging → limited antibodies against zebrafish proteins. - Difficult to follow up the last colonisation step of the metastatic cascade.
Mouse	- Current gold standard → highly characterised and standardised assays. - Higher homology with humans → possibly more relevant results. - Imaging and tracing of tumour for longer times. - Contain all human organs → no constraint for orthotopic transplantation. - Antibodies availability	- Low throughput. Experiments up to several weeks/months → not useful in PDX for patient decision making. - Expensive → higher cost for their maintenance and experiments. - Immunosuppression required for human cell engraftment → study of the crosstalk between the tumour and the immune system not allowed. - *In vivo* imaging of metastasis requires laborious experiments.

4.2.3. Drug repurposing

Another possible solution for the pharmaceutical industry productivity crisis is drug repurposing. The identification of a new application for an existing drug is advantageous as it has been already approved for human use and therefore, there is information available for drug toxicity, PK and PD profiles, which allows the rapid transition to the clinical setting and a decrease in the overall R&D costs. As for phenotypic assays in drug screening, zebrafish can also be used for phenotypic assays in repurposing (North *et al.*, 2007; Yeh *et al.*, 2009; Peal *et al.*, 2011). It is the case for flurandrenoline, which is a glucocorticoid previously described to have anti-inflammatory and anti-allergic properties (Krueger *et al.*, 1998). Flurandrenoline was also found to be a potent

suppressor of the long QT syndrome after a screening done in a zebrafish genetic model (Peal *et al.*, 2011). Since glucocorticoids are clinically approved and well tolerated in humans, it was quickly demonstrated their efficacy in shortening the action potential duration in long QT hearts, skipping toxicology tests (Peal *et al.*, 2011). Thus, several clinical trials have already started to examine the effect of other glucocorticoids, such as the cortisone, in patients with long QT syndrome (ClinicalTrials.gov identifier: NCT03082339).

4.2.4. Challenges to overcome

To fully consolidate zebrafish usage in R&D pipelines, several issues need to be addressed. One of the most important challenges is to effectively predict zebrafish drug uptake. Some compounds are rapidly absorbed, whereas others, such as large or highly hydrophobic molecules, are poorly or not captured at all. During the first 48-72 hours of life, embryos are surrounded by an acellular chorion, which consists of three membrane layers of a total thickness of 1,5-2,5 µm. The chorion is pierced by evenly distributed pore canals, of 0.5-0.7 µm in diameter (Rawson *et al.*, 2000), which impede the entrance of molecules larger than 3000 to 4000 Da (Pelka *et al.*, 2017). However, there is uncertainty about the ability to penetrate of such big molecules if the chorion is removed or once larvae have hatched, as in these cases, they can be absorbed through the skin and gills, and mouth (from 72 hpf) (Kanungo *et al.*, 2014). In addition, chemical and physical properties of the molecules, like charge and folding, can modify their ability to pass through the chorion (Lillicrap, 2010). In this regard, there are some physicochemical attributes that have shown to partially predict drugs uptake. It is the case for the molecules partition coefficient or logP, a measure of hydrophilicity and lipophilicity. Compounds with high logP values, are hydrophobic and lipophilic, have a higher tendency of being absorbed by zebrafish embryos (Sachidanandan *et al.*, 2008; de Koning *et al.*, 2015; Detrich, Westerfield and Zon, 2016). This type ok knowledge is of substantial importance, as it will help to avoid the risk of not detecting high hydrophilic compounds effect in zebrafish screens due to poor uptake. Such absorption deficiency can be solved by injecting the compounds into the zebrafish, instead of simply adding them into the surrounding water. Another major concern is the lack of assay standardisation, with differences in incubation water volume, exposure timing and mode, temperature, lighting,

pH, etc. All these aspects influence the output of the assays, generating intra- and inter-laboratories variability. In this regard, at ZeClinics S.L., we are involved in several international multicentric initiatives, such as SEAZIT, DNT-DIVER and IATA, whose aim is zebrafish procedures standardisation. In addition, the ZeOncoTest project aimed precisely at standardising xenografts for anti-cancer drug discovery in zebrafish larvae, as we observed contrasting results in previous reports that may had arisen from differences in incubation conditions, cell labelling techniques, site of injection and imaging and analysis methods. All these effort in homologate procedures will hopefully result in better reliability and predictivity, finally consolidating zebrafish as a model in preclinical drug R&D pipelines.

4.3. Further alternative preclinical models: a special emphasis on organoids

Several alternative preclinical *in vivo* models besides zebrafish, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have also been used as powerful tools improving preclinical steps of the drug R&D process. Both models have several advantages such as high throughput, large progeny, small size, fast development, few ethical and safety constraints, fully annotated genome, high genetic homology with humans, ease of maintenance and easy genetic manipulation, which have historically positioned them as the intermediate models between *in vitro* and *in vivo* mammalian studies. However, the emergence of zebrafish might have relegated their utilisation in pharmacological R&D processes, as most of their previously depicted main advantages are also shared with zebrafish, which as a vertebrate, is more closely related to humans. The low number of biotech companies using these models in drug discovery exemplifies this statement. This said, both models have and still contribute to the advance of basic science and, without their use; we would have not advanced much in the understanding of our biology.

Another promising tool to improve the overall drug R&D process are the organoids. They are small, self-organised three-dimensional stem cell-derived tissue cultures containing multiple organ-specific cell types and mimicking to a great degree the organs in both architecture and function. To date, researchers have been able to produce organoids that

resemble the brain, kidney, lung, gastric intestine, stomach, liver, prostate and pancreas (H. Xu *et al.*, 2018). These 3D *in vitro* systems have been extensively used to model and study organ development in order to answer fundamental questions in embryology, such as how cells self-organize and assemble, how the embryo breaks symmetry, and what controls timing and size in development (Lancaster *et al.*, 2013). In addition, organoids of multiple human tumours including gastrointestinal (Vlachogiannis *et al.*, 2018), liver (Broutier *et al.*, 2017), breast (Sachs *et al.*, 2018) and bladder cancers (Lee *et al.*, 2018), have also been established. They have been employed to model human cancers and for drug toxicity evaluation (Meng, 2010). Patient-derived organoids have also been generated in order to predict specific drug responses and choose the best treatment for each individual (Dekkers *et al.*, 2016). Finally, organoids might be used in the future in regenerative medicine and gene therapy by creating patient-derived transplants.

The organoid technology has a great potential representing a promising tool to improve current R&D processes, but it has some important limitations that still need to be tackled to make them reliable drug discovery experimental models. One of the greatest limitations is the lack of vascularisation, immune and nervous systems (Jabs *et al.*, 2017). Organoids only contain epithelial layers that often present necrotic cells in the centre due to the absence of blood vessels and unavailability of nutrients. Another important limitation concerns stem cell technologies. Organoids are generated from different types of stem cells: pluripotent embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (aSCs). ESCs are derived from the inner cell mass of a blastocyst, and thus, its utilisation is limited due to strong ethical and moral issues, as well as low availability. aSCs are mainly obtained by biopsies, which are limited to certain tissues (e.g. lungs, large intestine, stomach, liver). Finally, since the state of knowledge about stem cell technologies is still in its infancy, there might be important challenges not yet disclosed. Limited knowledge about the development of different tissues is also a drawback, as it results in organoids only containing certain cell types, with others missing or not fully developed. Indeed, complete maturation to adult organs is one of the bottlenecks of this technology. Moreover, some organoids might be slow to develop, reducing significatively the throughput. Also, for some types of organoids like the cerebral, there are strong ethical concerns, such as to how long or to what state of maturity they should be maintained before too closely resembling a foetal brain. Another limitation

is the dependence on an extracellular matrix or basement membrane extract for them to develop, as these structures could hinder drug penetration into the organoids. In addition, extracellular matrix such as Matrigel, are produced from mouse tumour lines and thus, might be unsuitable for humans (Kibbey, 1994). Growth factors and molecular inhibitors needed for the development and maintenance of organoids represent another limitation. As such, molecules present in the culture medium, might have some effects on the responses of the organoids to the administered drugs. This is specially concerning if organoids are meant to be used during the drug R&D process. Finally, although being more complex than 2D cultures, they are not as complex as *in vivo* organisms and therefore, have some of the drawbacks associated to the *in vitro* models, such as the lack of system-level interactions, making impossible to detect drug off-target effects or compensatory mechanisms, for example.

The above-mentioned limitations, together with high financial entry expenditure, long learning curve for their effective use, and high costs derived of their ongoing maintenance, hinder the accessibility to current organoid technologies. In conclusion, for organoids to be used in drug screening and regenerative medicine studies as human disease models, it is essential that they become more reliable, reproducible and quantifiable. Thus, advances need to be made in order to make organoids a precisely controlled system with minimal experimental variation.

4.4. Non-scientific hurdles and solutions

Besides the high drug attrition rate, there is another main aspect behind the pharmaceutical industry productivity crisis: the enormous redundancy of clinical trials between different pharmaceutical companies. As an example, there are currently 803 clinical trials testing checkpoint immune-therapeutics, using at least 12 antibodies (from 12 different pharmaceutical companies) (Workman *et al.*, 2017). Given the high attrition rates, one can envision that the final successful antibodies, if any, will have high costs covering all the money spent in those other antibodies that failed during the clinical trials. A solution would be to share the data generated during clinical trials and thus, avoid to perform similar studies with comparable drugs that might most probably fail. Other factors influencing the industrial productivity crisis are harder to solve because they are

more related to the complex organizations of companies with multiple levels of decision makers, endless rounds of company restructuring, mergers, acquisitions and down-sizing. All these aspects may give rise to time delays, low productivity, and high expenses, affecting the overall efficiency of the process. Patents duration, which produce a lack of competition impeding the emergence of generic and biosimilars compounds during patent validity, also contributes to the high costs of drugs. Finally, external factors also play a role, as in the case of the US federal government, which has prohibited by law to negotiate drug prices (Prasad, De Jesús and Mailankody, 2017; Workman *et al.*, 2017). Indeed, US is the country in which drugs prices are the highest, especially for anti-cancer compounds. Some possible solutions for the above-mentioned problems consist in more transparency during drug R&D processes and in drug prices, a more open and flexible market with drugs prices fluctuating in accordance to health benefit and compound demand, legal changes, such as the shortening of patent length, and the possibility for governments to negotiate drug prices. The application of such solutions would lead to improvements in the overall R&D process, with the consequent descent in prices.

4.5. Future and perspectives

It is clear that new thinking and fresh insights are required, in order to reduce the overall costs invested in bringing new drugs to the market. This is a significant challenge, due to the large number of factors leading to the pharmaceuticals industry productivity crisis. More concerted efforts across the pharmaceutical industry, academia, regulatory agencies and governments are required to successfully tackle the problem, which is not only affecting companies but that also harm patients and societies.

Regarding the use of zebrafish in drug R&D studies, I foresee that they will be more routinely used in a near future, especially in the case that the international multicentric standardisation initiatives show successful results. In that regard, at ZeClinics S.L., we have also started a validation project aiming to correlate toxicity values obtained from different types of compounds in zebrafish, with the toxicity values of the same molecules in rodent models. The objective is to evaluate if there is any variation in toxicity obtained by these models that could be explained by differences in the molecular characteristics, such as chemical class, logP value, molecular weight, etc. The final aim is to assess if

toxicity values obtained in studies done in zebrafish can be directly translated to humans after the appropriate calculation. Currently, there are only few therapeutic strategies developed as the direct result of zebrafish screens that have entered the clinic phases. Therefore, there is still no knowledge about efficiency and safety profiles in clinical trials of drug candidates discovered using zebrafish screens, compared to the ones found through commonly used mammalian models or target-based approaches. However, increasing number of reported successful results, such as the ones provided in this thesis, foresees more zebrafish-based compounds to enter the clinical phases and reaching the market in upcoming years.

5. CONCLUSIONS

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- Zebrafish is a useful model for preclinical research. It can bridge the gap between high throughput but low predictive *in vitro* studies, and more predictive but expensive and time-consuming *in vivo* mammalian tools.

- We have developed an innovative and reliable method for integrated cardio-, neuro-, and hepato-toxicity evaluation of new drugs in zebrafish, the ZeGlobalTox. Our assay helps predicting these safety outcomes in humans and successfully be used in agreement with 3Rs recommendations.

- Drug cardiotoxicity is effectively predicted through the ZeGlobalTox with 90% specificity, 69% sensitivity and 78% accuracy.

- Drug neurotoxicity assessment in the ZeGlobalTox through alterations in the zebrafish motor behaviours showed 100% specificity and 75% accuracy, but moderate sensitivity (54%) and thus, results have to be taken cautiously.

- Drug hepatotoxicity evaluation through the ZeGlobalTox assay showed high predictive performance: 77% specificity, 80% sensitivity and 82% accuracy.

- We optimized and standardized a semi-automated zebrafish larvae xenograft assay to be used in cancer drug discovery, the ZeOncoTest. Our system was successfully validated for the assessment of antitumoral drug efficacy and mode of action.

- Current dyes for cell staining do not faithfully recapitulate tumor cell growth and survival dynamics. The best method for cell labelling is represented by stable expression of fluorescent proteins.

- The perivitelline space represents a more suitable injection site for the study of engraftment, growth and dispersion of solid tumours, in comparison to the most common injection site, the yolk.

- Volume estimation represents a much more precise and reliable measurement of tumour masses than the area, which is only recommended if the same orientation between timepoints can be achieved.

- The use of an automated confocal imaging and analysis tools of individual larvae provided a potential screening throughput of dozens of conditions per month.

- MDA-MB-231, HCT116 and PC3 human tumour cell lines engraft, grow, disseminate and respond adequately to known drugs, in our zebrafish larvae xenograft.

6. BIBLIOGRAPHY

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ANNEX

Annex

During this PhD I have also been involved in the revision of the uses of the CRISPR-Cas9 technology in the zebrafish, and how could be used in combination with the zebrafish biological unique properties to streamline the drug discovery process. This work resulted in the following publication:

Cornet C, Di Donato V, Terriente J. Combining Zebrafish and CRISPR/Cas9: Toward a More Efficient Drug Discovery Pipeline. Frontiers in Pharmacology. 2018 Jul 3;9:703. DOI: 10.3389/fphar.2018.00703.

Annex

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REVIEW
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Combining Zebrafish and CRISPR/Cas9: Toward a More Efficient Drug Discovery Pipeline

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The use of zebrafish larvae in basic and applied research has grown exponentially during the last 20 years. The reasons for this success lay in its specific experimental advantages: on the one hand, the small size, the large number of progeny and the fast life cycle greatly facilitate large-scale approaches while maintaining 3Rs amenability; on the other hand, high genetic and physiological homology with humans and ease of genetic manipulation make zebrafish larvae a highly robust model for understanding human disease. Together, these advantages allow using zebrafish larvae for performing high-throughput research, both in terms of chemical and genetic phenotypic screenings. Therefore, the zebrafish larva as an animal model is placed between more reductionist *in vitro* high-throughput screenings and informative but low-throughput preclinical assays using mammals. However, despite its biological advantages and growing translational validation, zebrafish remains scarcely used in current drug discovery pipelines. In a context in which the pharmaceutical industry is facing a productivity crisis in bringing new drugs to the market, the combined advantages of zebrafish and the CRISPR/Cas9 system, the most powerful technology for genomic editing to date, has the potential to become a valuable tool for streamlining the generation of models mimicking human disease, the validation of novel drug targets and the discovery of new therapeutics. This review will focus on the most recent advances on CRISPR/Cas9 implementation in zebrafish and all their potential uses in biomedical research and drug discovery.

Keywords: CRISPR/Cas9, drug discovery, zebrafish, disease model, phenotypic drug screening, functional genomics.

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A PRODUCTIVITY CRISIS IN THE PHARMACEUTICAL INDUSTRY

During the last decades, the ratio between the number of new therapeutic drugs (NTD) reaching the market and R&D expenditure has suffered an important decrease (Paul et al., 2010; Scannell et al., 2012). Thus, despite the creation and rapid growth rate of dozens of biotechnological companies and important merges and acquisitions, the biopharmaceutical industry is suffering a productivity crisis. This crisis is mostly explained by the extremely high rate of drug attrition for molecules entering clinical trials (Kola and Landis, 2004), in which 95% of the compounds fail after clinical

phases II and III. Mayor sources of drug attrition are lack of efficacy, accounting for 50%, and attrition due to safety liabilities, accounting for 25% (Waring et al., 2015). This massive attrition rate results in an average cost for bringing an NTD to the market of $2.5 billions (DiMasi et al., 2016). Such economic burden hinders the industry progression and limits its commitment for facing diseases in which the return of investment (ROI) is not well-defined, such as rare, third world or complex first world diseases (Schmid and Smith, 2007). A recent example is Pfizer's decision to interrupt their research programs on Alzheimer's and Parkinson disease (Reuters.com, 2018). There are two main elements to consider regarding low clinical approval rate: (i) how predictive is the preclinical phase toward human safety and efficacy, and (ii) how appropriately are the drug targets chosen to tackle a particular disease.

Regarding the predictivity of the preclinical phase, it is worth pointing out that all drugs that fail in clinical phases have gone through a supposedly comprehensive preclinical phase. Hence, the low NTD acceptance rate suggests that the information gathered during preclinical phases, specifically that obtained from animal models, provides lower-than-expected prediction of toxic liabilities and therapeutic effects in human patients (Scannell and Bosley, 2016). Therefore, it is necessary to develop strategies that improve the predictive value of current preclinical animal models and/or combine them with better *in silico* and *in vitro* tools in order to narrow down the most promising candidates before entering expensive preclinical and clinical phases. On the subject of how drug targets are chosen, it has become apparent that clinical success increases with a deeper understanding of a disease and its related biological pathways. Thus, drugs which modulate targets directly associated with the pathology show a higher success rate in both preclinical and clinical phases (Nelson et al., 2015). Unfortunately, identifying genetic-disease associations is not an easy task and it might not even lead to the discovery of an appropriate druggable target. A paradigm is the *fat mass and obesity-associated protein* (*FTO*) gene: as the name indicates, single nucleotide polymorphisms (SNPs) identified in this gene have been associated with obesity and type 2 diabetes risk (Loos and Yeo, 2014). In this case, the association between gene and pathology remains undisputed; however, it has been shown that SNPs identified in *FTO* introns 1 and 2 were actually associated with the long-range positive regulation of *IRX3* in the human brain. Interestingly, *IRX3* overexpression had a clear impact in weight gain in animal models and a clear correlation with the expression data obtained from obese patient samples (Smemo et al., 2014). The exact number of drug discovery initiatives targeting *FTO* to treat obesity or type 2 diabetes is unknown to us, but the findings of Smemo et al. (2014)– obtained by combining experimental data from several animal models including zebrafish – illustrate the need of carrying out detailed genetic functional studies (i.e., functional genomics) before entering costly drug discovery programs. All in all, decreased productivity and high drug attrition, either due to low preclinical predictivity or poorly chosen targets, highlights the need of innovative strategies to streamline the drug discovery pipeline (Plenge, 2016).

ZEBRAFISH RESEARCH AND BIOMEDICAL APPLICATIONS

Zebrafish: From Basic Research to Drug Discovery

Zebrafish is a small fresh water fish that has been used for decades as a classical developmental biology research model (Streisinger et al., 1981; Kimmel, 1989). Its use increased exponentially from the 1990s', when several genetic screens showed the potential of this animal model in identifying and characterizing novel genes involved in vertebrate development and disease. The zebrafish specific characteristics such as the large number of progeny and external development of the larvae, fast life cycle, small size and transparency allowed performing large-scale genetic screenings, which would have been unattainable in

mammalian models (Driever et al., 1994; Haffter and Nüsslein-Volhard, 1996; Lawson and Wolfe, 2011). Such screens followed the example of previous studies performed with *Caenorhabditis elegans* and Drosophila (Brenner, 1974; Nusslein-Volhard and Wieschaus, 1980), but were revolutionary on their own, given that a vertebrate model allowed the identification and validation of genes in a context closer to human biology than that provided by invertebrate genetic models. Nowadays, research in zebrafish has expanded from basic research toward most translational biomedical areas. Three additional features have fuelled that transition: First, ~83% of human disease-related genes have functional orthologs in zebrafish (Howe et al., 2013), suggesting that human pathologies can be faithfully modelled in zebrafish. Indeed, that has been the case for several indications such as cancer (Terriente and Pujades, 2013; White et al., 2013), cardiovascular (Asnani and Peterson, 2014) or neurologic diseases (Clark et al., 2011). Second, liver, kidney, and tissue barriers are functional from early development (Parng, 2005). Therefore, zebrafish physiology recapitulates mammalian drug metabolism features – Absorption, Distribution, Metabolism and Excretion (ADME) – and provides *de facto* a body-on-chip experimental set up. Third, zebrafish larvae are not considered animals by animal welfare regulation before 5 days post fertilization (dpf), a stage when they start independent feeding. Hence, using zebrafish larvae in research has a direct impact in the Replacement, Reduction, and Refinement (3Rs) of animal models, which is a crucial aspect for raising the ethical standards in the pharmaceutical and chemical industry (Avey et al., 2015). These facts suggest that a broader use of zebrafish could benefit the biomedical community in streamlining the drug discovery process. In that sense, regulatory agencies recommend the use of this and other small animals (FDA, 2004). However, before being fully adopted by regulatory agencies and the pharmaceutical industry, drug discovery through zebrafish might require better validation and a deeper understanding on biologic translatability toward humans. To advance on the validation front, several studies have focused on addressing how precise is the correlation of drug activity between zebrafish and human gathered data (Milan et al., 2003; Ali et al., 2011; Cornet et al., 2017). Those studies show how using zebrafish predicts toxicity liabilities for more than 80% of the drugs. Regarding biologic translatability, we stated above the high conservation in genes, protein structure and physiology with humans. However, to further prove the applicability of the zebrafish model during the drug discovery process, an important step would be the development of humanized zebrafish models, in which native genes would be exchanged by their human orthologues, therefore, recapitulating same biological pathways but with an intact human target protein structure. This feature would indeed provide more solid drug-target interaction evidences. Either way, the collective efforts from the zebrafish research community will be required to fully overcome these "validation" and "translatability" challenges. In the meantime, we will discuss below some of the hallmarks and general advantages achieved by using zebrafish in drug discovery today.

Zebrafish: Speeding up the Drug Discovery Pipeline

Traditionally, the pharmaceutical industry has used two main strategies to discover new drugs: target-based drug screenings, in which drugs are identified *in vitro* based on their binding properties to specific molecular targets (e.g., recombinant proteins), and phenotypic drug screenings, in which drugs are identified, *in vitro* or *in vivo*, based on the modification of a disease phenotype in cells, tissues or whole organisms. Determining the relevant drug target/s identified through phenotypic screening was often slow and sometimes impossible. That fact tilted the pharmaceutical industry efforts toward target-based screenings. However, these strategies have demonstrated lower drug discovery success rates than phenotypic drug screenings (Swinney and Anthony, 2011). Nowadays, innovation on *in silico* and *in vitro* target identification tools allows

a faster and more precise determination of molecular targets (Schenone et al., 2013; Cereto-Massagué et al., 2015), which is positioning phenotypic drug discovery back in trend (Kotz, 2012).

Despite some challenges stated above, zebrafish is a very suitable and reliable experimental model for performing phenotypic drug discovery. In fact, the use of zebrafish is already helping the pharmaceutical industry on three different fronts. First, by validating potential druggable targets identified through genomic screenings on human patient populations (Liu et al., 2013); second, by generating novel disease models to better understand pathogenesis (Ablain and Zon, 2013); and third, by using those disease models, or other biological features, as the basis for performing phenotypic drug screenings designed to identify new therapies (MacRae and Peterson, 2015). Some examples are: Proto-1, which protects against toxicity in ciliated cells of the inner ear caused by antibiotics (Coffin et al., 2010); inhibitors for PDE5A to treat Duchenne Muscular Dystrophy (Kawahara et al., 2011), which are currently in clinical phases; or Dorsomorphin, a BMP inhibitor applicable in the treatment of progressive ossifying fibro dysplasia (Yu et al., 2008).

The implementation of CRISPR/Cas9 technology, a straightforward (Hwang et al., 2013), in zebrafish and precise genome editing technique, is streamlining the process for achieving better disease modelling, target validation and drug discovery.

CRISPR/Cas9 IN ZEBRAFISH

Several methods have been developed and applied in zebrafish to alter gene transcription and function (Koster and Sassen, 2015). Among them, CRISPR/Cas9, a system that allows rapid and accurate genome editing, has become the most widespread technique in zebrafish and other model systems. The CRISPR/Cas9 experimental basics and general applications have been reviewed extensively before (Hsu et al., 2014; Barrangou and Doudna, 2016; Fellmann et al., 2017). However, it is important to reiterate some important details relevant to this review. All gene editing methods, including CRISPR/Cas9, are based on the inherent capability of cells to repair their genome after DNA Double Strand Break (DSB) events (Chang et al., 2017). DNA repair relies, in part, on the Non-Homologous End Joining (NHEJ) mechanism, a homology-independent error-prone pathway promoting, in a variable percentage, the appearance of *de novo* insertions/deletions (INDELs). NHEJ can result into the disruption of a coding sequence or regulatory region and, therefore, the inactivation of a gene of interest (NHEJ-mediated knockout). Additionally, NHEJ can be exploited to insert exogenous DNA fragments, such as reporters or drivers in the genome (NHEJ-mediated knockin). Alternatively, knockin of DNA fragments can be performed through a different DNA repair pathway: Homology Directed Repair (HDR). This pathway requires the availability of a homologous DNA template to promote DNA repair through Homologous Recombination (HR). Several applications have been developed *via* HDR (HDR-mediated knockin) to achieve precise, programmed modification of the zebrafish genome: introduction of point mutations to mimic specific human SNPs and/or integration of LoxP sites for site specific recombination or fluorescent reporters. However, in zebrafish and other systems, HDR is still a challenging approach due to the low rate of DSB repair by HR compared to NHEJ (Maruyama et al., 2015; Horii and Hatada, 2016). An additional strategy is the use of modified Cas9 proteins, which do not cleave DNA but allow generating SNP exchange or regulate transcription. Below, we will discuss all these methodologies, their challenges and potential biomedical applications toward the discovery of new therapies for humans.

CRISPR/CAS9-Mediated Knockout

Induced mutagenesis of genes of interest in zebrafish can be achieved with a relatively straightforward experimental setup. The

method displaying the highest mutagenesis efficiency is based on microinjection of an *in vitro* pre-assembled complex of guide RNA and Cas9 protein in one-cell stage embryos. There are two possible strategies after F0 animals have been injected. Either, F0 injected larvae, carrying mosaic loss-of-function (LOF) mutations (INDELs), can be directly phenotyped and used to study the function of candidate genes, a strategy known as transient knockout approach. Or, F0 larvae can be grown to sexual maturity and crossed to generate F1 heterozygous carriers and F2 homozygous mutant larvae, a so-called isogenic stable knockout. The generation of an isogenic stable knockout takes 6 months and allows obtaining hundreds of F2 larvae (homozygous, heterozygous and wild type siblings), which can be used to prove a research hypothesis or to evaluate in parallel several therapeutic drug candidates in a robust biological background.

Transient Knockout

The advent of next-generation sequencing has contributed to the identification of a growing number of candidate genes potentially associated with human disease. To tackle this considerable amount of data, a high-throughput strategy for validating candidate genes phenotypically would be very advantageous. Along this line, a report showed the mutagenesis of 83 genes (162 loci) with a 99% success rate, and an average germline transmission rate of 28%. It also showed that by inbreeding two founder fish, phenotyping can be performed in the F1 generation, resulting in a significative reduction time and space required for animal husbandry (Varshney et al., 2015). Another high-throughput CRISPR-Cas9 phenotyping screen, targeting 48 genomic loci, identified two genes involved in electrical synapse formation (Shah et al., 2015). Due to the high efficiency of somatic mutation, the authors were able to detect specific phenotypes already in injected F0 animals. In a more recent report, the *in vitro* assembly optimization of Cas9 and sgRNA riboprotein complexes (RNPs) allowed the generation of so-called Crispants (CRISPR/Cas9-mediated mutants), which yields high rates (up to 100%) of somatic mutagenesis upon injection. Indeed, this report shows full penetrance of phenotypes, such as pigmentation defects or heart oedema, by targeting several genomic loci and recapitulating, in injected F0 larvae, LOF phenotypes displayed in homozygous isogenic mutants (Burger et al., 2016). Similarly, a recent report showed how the simultaneous injection of different sgRNAs targeting the same allele could promote up to 99% of somatic mutations. When this approach was tested on two genes from the KEOPS complex, transient injected larvae displayed the same microcephaly and low survival phenotypes previously observed in isogenic homozygous larvae (Jobst-schwan et al., 2018).

Regarding the challenges of this application, it is important to note that CRISPR/Cas9 has been suggested to produce false-negative results due to genetic compensation (Rossi et al., 2015). This limitation should be considered when validating potential drug targets during the drug discovery process. Another evident drawback of this approach could be low somatic penetrance and mosaicism, which can result in contiguous cells being wild type and mutant or different animals showing a variable phenotypic degree. To counteract this issue, fast and accurate genotyping tools, such as IDAA™ (Lonowski et al., 2017) and TIDE (Brinkman et al., 2014), allow to perform quantitative correlation between mutagenesis rate and phenotype penetrance in single individuals. All in all, this somatic mosaic knockout approach allows the phenotypic screening of genes and pathways, providing a fast method for performing target validation for disease-relevant genes identified through genomic strategies. However, a transient approach does not provide the phenotypic robustness provided by the use of isogenic mutant lines explained below.

Isogenic Stable Knockout

Many zebrafish mutant models have been developed through CRISPR/Cas9 (Liu et al., 2017). Successful models include neurological, kidney, hepatic,

cardiovascular, muscle/skeletal or structural birth defects such as orofacial clefts and heterotaxy (Chang et al., 2013; Borck et al., 2015; Bolar et al., 2016; Noël et al., 2016; Duncan et al., 2017; Ellis et al., 2017; Küry et al., 2017; Shaw et al., 2017; Van De Weghe et al., 2017; Zabinyakov et al., 2017). A paradigm of the exploitation of zebrafish disease modelling through CRISPR/Cas9 is found in the development of a zebrafish line carrying a LOF mutation in the ribosomal protein S14 gene (*rps14*). This model was generated to understand the effect of RPS14 deficiency in the 5qdeletion Syndrome (Ear et al., 2016). 5q-syndrome is a form of myelodysplastic syndrome (MDS) characterized by bone marrow failures, including macrocytic anaemia. *rps14* zebrafish mutant displayed gross morphological defects accompanied with an elevation in p53 activity. Furthermore, an anaemic phenotype, typically seen in patients with disrupted ribosome gene function, was identified in fish carrying LOF alleles. Interestingly, those phenotypes were rescued through treatment with RAP-011, L-leucine, and dexamethasone. These are promising results for future clinical trials, since two of these small molecules have a p53-independent mechanism of action. Therefore, they represent a valuable alternative to therapeutic treatments targeting p53 for patients with ribosomopathies, which have high incidence of later cancer development. (See **Table 1** for a summary of the aforementioned disease models).

Despite the experimental time required for isogenic mutant isolation, phenotypic validation and use of the generated model in drug discovery, disease modelling in zebrafish represents a valuable approach – considering time and cost saving – to analyse the pathogenic effect of a given mutation or test a battery of candidate drugs before proceeding to further preclinical trials with mammalian animal models. In fact, efficacy information gathered through zebrafish could be enough for advancing toward clinical phases, if provided together with the required toxicity profile obtained in regulatory animals.

Tissue-Specific Knockout

Gene knockout may result in embryonic lethality when targeted genes are involved in crucial developmental or housekeeping activities. This represents a limitation for the phenotypic analysis of disease-causing mutations, especially when the readout is expected to be tissue-specific. To overcome such limitations, different conditional knockout methods have been developed in zebrafish. The first study described a CRISPR-based vector system for tissue-specific gene inactivation, based on the tissue-specific expression of Cas9 and ubiquitous expression of a single sgRNA targeting a gene of interest. In detail, the erythrocyte-specific *gata1* promoter drove *Cas9* expression and *urod*, which is implicated in heme biosynthesis, was the chosen target gene (Ablain et al., 2015). Mutations in the *UROD* gene are found in human hepatic cutaneous porphyria, a disorder characterized by defects in iron metabolism in liver, skin photosensitivity and reduced erythrocytic heme production (Balwani and Desnick, 2012). Furthermore, *urod*-deficient erythrocytes exhibit strong red fluorescence due to the accumulation of unprocessed porphyrins, which are inherently fluorescent. In zebrafish, it was found that *urod* inactivation in erythrocytes led to the appearance of fluorescent erythrocytes at 30 hpf, mimicking the phenotype seen in humans and in *yquem* mutants, an additional *urod* mutant described before (Wang et al., 1998). A similar approach allowed the genetic inactivation of *sox10* in melanocytes to study its role in melanoma initiation. In this case, zebrafish embryos were injected with a vector expressing *Cas9* under the control of the melanocyte-specific *mitfa* promoter and an sgRNA targeting *sox10* (Kaufman et al., 2016). A third report showed the development of a double transgenic approach. On one hand, *Cas9* was expressed either ubiquitously or in a tissue-specific manner. Cas9 lines were combined with transgenic lines expressing up to five sgRNAs under the control of different U6 promoters. With this strategy, a fish model for hepatic dysfunction caused by altered

glucose homeostasis was developed through the liver-specific abolishment of insulin signalling (Yin et al., 2015) (see **Table 1** for a summary). In another study, Di Donato et al. (2016) expanded the tissue-specific gene disruption toolbox by combining CRISPR/Cas9 and Gal4/UAS systems. To this end, a vector system called 2C-Cas9 (Cre-mediated recombination for Clonal analysis of Cas9 mutant cells) was developed, based on the UAS-driven expression of Cas9 and U6-driven expression of two different sgRNAs. UAS-driven expression of Cas9 offers the possibility of conditional targeted mutagenesis in virtually any cell-type through the use of the broad repertoire of available tissue-specific zebrafish transgenic Gal4 driver lines (Di Donato et al., 2016; Albadri et al., 2017a).

These methods offer the possibility to study gene function in specific tissues. Moreover, optimization of these tools should allow simultaneous gene inactivation and mutant cell fate analysis through fluorescent cell

TABLE 1 | Protein/gene targeted through CRISPR/Cas9, the disease/phenotype studied and the corresponding reference.

Protein/gene targeted	Disease/phenotype studied	Reference
osgep and *tprkb*	Microcephaly and reduced survival	Jobst-schwan et al., 2018
sec61al2	Autosomal-dominant tubulo-interstitial kidney disease	Bolar et al., 2016
brf1	Cerebellar-dental-skeletal syndrome	Borck et al., 2015
etsrp and *gata5*	Vessel phenotypes (*etsrp*) Cardia bifida phenotypes (*gata5*)	Chang et al., 2013
abcb11b	Progressive familial intrahepatic cholestasis type 2	Ellis et al., 2017
cfap53	Dextrocardia and heterotaxy (left-right asymmetry)	Noël et al., 2016
psmd12	Syndromic neurodevelopmental disorder	Küry et al., 2017
smchd1	Bosma arhinia microphthalmia syndrome	Shaw et al., 2017
armc9	Ciliopathy phenotypes	Van De Weghe et al., 2017
aldh7a1	Pyridoxine-dependent epilepsy	Zabinyakov et al., 2017
rps14	5q-deletion Syndrome	Ear et al., 2016
urod	Human hepatic cutaneous porphyria	Ablain et al., 2015
sox10	Melanoma	Kaufman et al., 2016
insra/insrab	Hepatic dysfunction	Yin et al., 2015
tardbp and *fus*	Amyotrophic lateral sclerosis	Armstrong et al., 2016
twist2	Ablepharon macrostomia syndrome	Zhang et al., 2016, 2017
pax2a	Loss of the midbrain-hindbrain boundary	Ota et al., 2016
tyr	Oculocutaneous albinism	Zhang et al., 2017
pitx2	Ocular dysgenesis	Protas et al., 2017

tracing. This last characteristic addresses a crucial issue in the analysis of the effects of gene inactivation in model organisms: direct correlation between pathogenesis, genotype and cell/tissue phenotype. From a more translational point of view, these approaches would allow generating disease models, based on targeting specific tissues, for those genes that might promote embryonic lethality before the disease phenotype can be addressed.

Knockin

Targeted insertion (knockin) of small or large DNA fragments is a promising, but for the moment not very widespread method to generate disease models in zebrafish. We will discuss knockin methodologies according to the DNA repair pathway they exploit to achieve DNA insertion: HDR or NHEJ. Additionally, we will discuss an alternative method for SNP exchange that does not rely on DSB repair.

HDR-Mediated Knockin

Homology directed repair allows the precise integration of DNA fragments. In general terms, small modifications such as single nucleotide editing or LoxP integration can be achieved by providing a single-stranded oligodeoxynucleotide (ssODN) as donor DNA (Chang et al., 2013) for CRISPR/Cas9-mediated HDR. By using this approach, Armstrong et al. (2016) generated a zebrafish model of amyotrophic lateral sclerosis (ALS), *via* insertion of two SNPs in the zebrafish *tardbp* and *fus* genes (*tardbp*^{A379T} and *fus*^{R536H}, respectively), corresponding to *tardbp*^{A382T} and *fus*^{R521H} disease-causing point mutations identified in patients with ALS (Armstrong et al., 2016) (**Table 1**). Albeit this represents a rapid and straightforward approach for the knockin of point mutations of interest, the low efficiency of germline transmission of the mutation represents an important drawback (the maximum reported efficiency was only 4%). An increase in efficiency of HDR-mediated knockin of ssODN was recently reported (Moreno-Mateos et al., 2017). The authors made use of an alternative Cpf1 CRISPR/Cas DNA nuclease derived from *Lachnospiraceae bacterium ND2006*, LbCpf1, which proved to induce homology-directed integration of optimized single strand DNA donors four times more efficiently than Cas9.

For larger DNA sequences, the consensus is to use plasmids as donor DNA (Irion et al., 2014). By using this methodology, in a recent report the *twist2* gene was successfully targeted to mimic a human mutation found in Ablepharon macrostomia syndrome (AMS) (**Table 1**). Here, the authors made use of a double-strand long arm donor plasmid as template for HDR, with the total length of the inserted sequence being less than one kilobase (Kb), in order to induce precise nucleotide substitution (Zhang et al., 2016). In this case, the transmission of edited alleles in the germline could be detected in around 3% of the cases. For both types of DNA donors – ssODN or plasmids – the main drawback is the low efficiency of integration. To counteract low efficiency, it has been proposed that using NHEJ drug antagonists (scr7) or HDR drug agonists (RS-1) could increase HDR homologous recombination (Song et al., 2016). In our hands, both drug treatments have a low impact on HDR efficiency (data not shown). Nonetheless, a consistently higher frequency of germline transmission has been shown when CRISPR/Cas9 complex is co-injected with donor plasmids, where the DNA insert is placed between 1 Kb long homology arms flanked by I-SceI meganuclease restriction sites. This approach is different from other knockin methods in the use of long homology arms and the pre-digestion of the donor plasmid with I-SceI meganuclease (Hoshijima et al., 2016). Both features, together with achieving high rates of initial DSB through careful selection of highly efficient sgRNAs, might be determinant in increasing precise integration rate in zebrafish. In our opinion and that of others (Albadri et al., 2017b), methodologies based on the use of large homology arms are the most appropriate tool in order to generate HDR knockins for large or small modifications.

Regardless of the efficiency rate, HDR-based strategies remain the most accurate

method for modification of targeted sequences. In that sense, results of HDR-based knockin strategies are promising and their potential applications in disease modelling and personalized medicine very broad. However, the efficiency of the available methods remains extremely low. That might explain the scarcity of disease models with precise modifications published through these methods. Indeed, further improvements and alternative strategies will have to be developed. In that respect, implementation of HDR-independent SNP exchange strategies might help to widen the disease model spectrum.

NHEJ-Mediated Knockin

As an alternative to methodologies based on low-efficient HDR mechanisms, targeted insertion of exogenous DNA fragments can be achieved by taking advantage of NHEJ repair after DSB events. Despite NHEJ being an error prone mechanism resulting in INDELs generation, it has also been shown to promote repair through the integration of donor DNA in a highly efficient fashion. In zebrafish, a pioneer work developed a homology-independent CRISPR/Cas9-mediated integration of reporter genes at defined target genomic loci. This approach is based on the concurrent cleavage of a donor vector and a targeted genomic sequence. It was first tested with a DNA donor containing a cassette coding for the transcriptional activator Gal4 and sgRNAs targeting the eGFP locus in transgenic zebrafish lines. Targeted integration of the Gal4 cassette successfully allowed converting eGFP transgenic lines into Gal4 drivers, significantly expanding the potential of Gal4-UAS technology in zebrafish. Interestingly, it was shown that even native zebrafish genes can successfully be targeted for integrating exogenous DNA, eGFP in this case (Auer et al., 2014). Since NHEJ-mediated knockin can take place in either possible orientation, this methodology has been improved by the addition of a heat shock promoter (*hsp70*) to the donor plasmid. This is intended for overcoming the need of in-frame insertion of the donor cassette to activate the reporter transgene (Kimura et al., 2014). This methodology has also been applied for integrating Cre-ER^{T2} recombinase into the *otx2* gene locus to generate a conditional Cre-driver line specific to the anterior neural plate (Kesavan et al., 2018). Additionally, it has been used for generating LOF alleles through the integration of GFP cDNA. In that study, inactivation of *pax2a* is achieved by integrating a donor plasmid containing an eGFP cassette. It is worth noting that, fish homozygous for the DNA cassette insertion not only display fluorescence in the expression domains of *pax2a*, but also recapitulate the phenotype observed in the well characterized *pax2a/noi* mutant consisting in loss of midbrain hindbrain boundary and aberrant projection of optic axons (Ota et al., 2016). This latter approach has the advantage of generating a mutant and a fluorescent reporter at once.

The main drawback of this methodology is that repair at the sites of DNA integration is often imprecise. Additionally, the donor vector is integrated as a whole. Hence, DNA integration is likely causing concomitant LOF on the target gene; an unintended side effect for some applications. Moreover, this technology cannot be applied when precise integration such as in protein tagging is required. Nonetheless, high efficiency and versatility are important advantages for reporter line generation and other applications, when compared to HDR approaches. From a translational point of view, the use of these methodologies could allow the generation of more precise reporter lines for several genes or signalling pathways. That could allow, for example, to identify drugs altering Notch, WNT, or BMP signalling, which are important players in development, but also in cancer progression (Terriente and Pujades, 2013).

DNA Base Editing

Recently, a strategy for precise single “base editing” (BE), developed in mammalian cells (Komor et al., 2016), has been implemented in zebrafish (Zhang et al., 2017). BE system is based on the fusion of a cytidine deaminase to a Cas9 nickase (nCas9), which allows a DSB-independent irreversible conversion of one targeted base

to another. This methodology achieved the conversion of cytidine to thymine, adenine and guanine at different genomic loci mimicking causative mutations of human diseases such as AMS and oculocutaneous albinism (**Table 1**). The same report showed that it is possible to expand the number of potential genomic targets by replacing Cas9 nickase with a so called VQR variant nickase, which recognizes the 50-NGA PAM. Importantly, germline transmission of targeted modifications ranged between 7 and 37%, making this approach a valuable alternative to lower efficiency HDR-mediated methods. Certainly, the use of base editors will allow the development of several zebrafish disease models mimicking specific human polymorphisms. Several improvements are expected. In particular, it is not yet possible to target all desired SNPs in zebrafish, due to specific limitations of base targeting – the binding of the nickase requires the presence of a PAM sequence adjacent to the targeted site; not every base can be converted in another. An advance in this direction has been provided by a recent report, in which the authors show the development of adenosine deaminase editors (ABEs) to allow efficient conversion of A-T into G-C base pairs; again without induction of DSBs (Gaudelli et al., 2017). A further implementation of these tools for genome engineering in zebrafish would greatly expand the current possibilities of studying human-associated polymorphisms *in vivo*.

As a challenge to these approaches, genotyping point mutations can be cumbersome. Unless specific restriction sites are created or destroyed by the point mutation, every single individual will need to be Sanger sequenced, which would certainly escalate time and cost of the whole procedure. These approaches would have the same translational applications – target validation and disease modelling – mentioned for KO animals.

CRISPR-Based Transcriptional Regulation

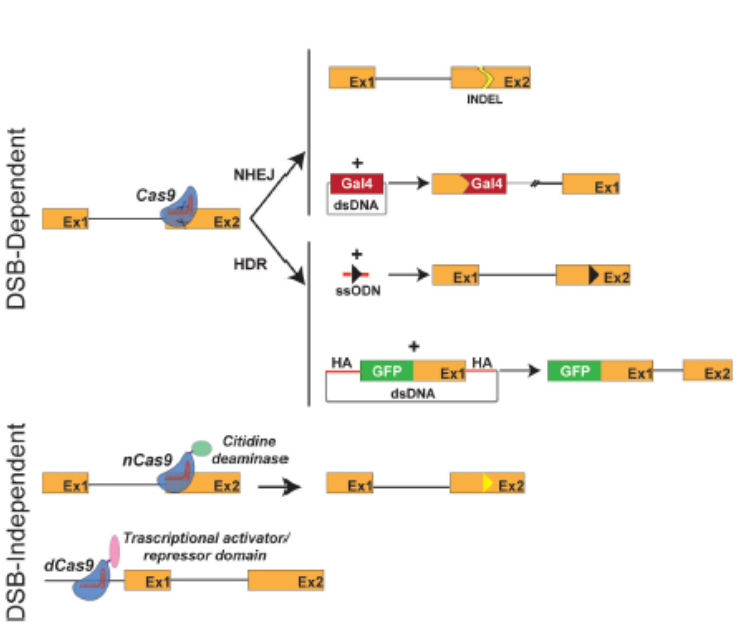
Transcriptional regulation can be achieved by using another modified Cas9 protein lacking the catalytic endonuclease activity: Dead Cas9 (dCas9) (Qi et al., 2013). This Cas9 mutant form is still guided by sgRNAs and has been used to repress (CRISPRi) or activate (CRISPRa) gene transcription without introducing irreversible genomic mutations. The dCas9 protein can act on its own when targeted to the coding region of a gene by blocking transcription. When dCas9 is fused to a repressor domain such as KRAB or activator domain such as VP64, it can also interact with regulatory regions to either activate or repress transcription (Long et al., 2015). As an alternative to CRISPRi/a, the deletion or modification of conserved regulatory regions in the zebrafish genome could also help to understand the role of polymorphisms identified in non-coding regions, and how they are associated to human disease. This approach was used to identify genes associated to ocular dysgenesis. Here, a large genomic deletion upstream of *pitx2* in the genome of ocular dysgenesis patients was identified. The deletion contained several non-coding elements – potential enhancers – that are conserved in the zebrafish genome. In line with that, zebrafish larvae homozygous for deletions on those conserved regions displayed a similar ocular phenotype than human patients, which suggests a role of *pitx2* transcriptional regulation in the progression of ocular dysgenesis (Protas et al., 2017) (**Table 1**).

Approaches, which have a greater impact in gene regulation than in protein function could be used to screen rapidly both, loss-of-function and gain-of-function phenotypes, and such strategy, can provide complementary information to knockouts for mapping complex pathways. Moreover, this represents an alternative mean to further exploit genome wide association study (GWAS) data and to ultimately identify polymorphisms situated in regulatory regions rather than coding regions. Such knowledge is crucial to understand the role of transcriptional levels and gene copy numbers in disease progression or drug efficacy.

PERSPECTIVES AND FINAL REMARKS

During the last decades, the zebrafish has proven a valuable and reliable model for basic and applied research in genetics. The recent advent of the CRISPR/Cas9 technology has further enhanced the use of this model system by providing a tool to obtain robust results in functional genomics in a reduced time. At the same time, it has enormously expanded the range of applications for which the zebrafish model can be used. In this review, we have discussed the CRISPR/Cas9-based methodologies developed in zebrafish in the last years and suggest how they can be applied to make more effective the drug discovery process, through faster target validation, more robust disease modelling and more efficacious drug screenings.

To illustrate our views, we have introduced the current methodologies for generating KOs and KIs and discussed their technical challenges and purposes (**Figure 1**). Besides the technical description, we have presented examples of studies, which, by taking advantage of the combination of the CRISPR/Cas9 system and the zebrafish model, have led to the identification of new therapeutic candidates. As some technical limitations are solved, it is expected that the number of such examples will multiply in



Technique	Application
KO	LOF LOF Screening Tissue Specific LOF
KI	LOF Reporter Line Generation
KI	LOF SNP Exchange LoxP insertion
KI	LOF Reporter Line Generation SNP Exchange
Base Editing	LOF SNP Exchange
Transcriptional Regulation	LOF GOF

Figure 1: | Schematic representation of CRISPR/Cas9 techniques and applications in zebrafish. (Left) Graphical representation of CRISPR/Cas9-based methods available for genome engineering in zebrafish. Double strand break (DSB)-Dependent techniques: the Cas9 endonuclease/sgRNA complex induces DSB at the target genomic locus. The NHEJ (non-homologous end joining) DNA repair mechanism leads to the appearance of insertion/deletion (INDELs) which can result in a shift of the open reading frame of the targeted genomic locus, thereby causing gene inactivation (knockout: KO). The targeted insertion (knockin: KI) of donor DNA sequences coding for reporter genes (e.g., Gal 4 transcriptional trans-activator) is, in most reports, mediated by NHEJ. The Homology Directed Repair mechanism allows the precise integration of exogenous DNA at a chosen genomic locus. Single-stranded oligodeoxynucleotide (ssODN) or Double stranded vectors harbouring homology arms (HA) can be used as donor DNA for the KI of point mutations and reporter genes. Double strand break (DSB)-independent techniques: modified versions of the Cas9 protein, not displaying endonuclease activity, are used. The fusion of a cytidine deaminase to a Cas9 nickase (nCas9) allows a DSB-independent irreversible conversion of one targeted base to another (Base Editing and introduction of Single Nucleotide Polymorphisms, SNPs). Dead Cas9 (dCas9), when fused to a repressor domain or activator domain, can interact to either activate or repress transcription. (Right) Table describing the applications of CRISPR/Cas9-based methods depicted in the left panel.

the future. To this end, and regardless of the methodology used, we propose below some applications that have the potential to expand the range of CRISPR-based applications of the zebrafish model in the research for therapeutic alternatives to treat human disease.

High-Throughput Genetic Screens

As previously mentioned, an advantage of the zebrafish model is the possibility of addressing specific phenotypes resulting from gene disruption in a short time and on a large number of animals. In that regard, phenotypic screens can be performed for genes involved in different human pathologies. Genetic hearing loss, for example, can be assessed on a functional level by hearing response assays, but also on a structural level, since the cellular components of the inner ear are highly conserved between humans and zebrafish (Abbas and Whitfield, 2010). Another example would be represented by screens for target identification in cardiomyopathies, as the zebrafish heart physiology is highly analogous to the human (Bakkers, 2011). Importantly, the zebrafish heart at 5dpf is fully functional and readily accessible by non-invasive *in vivo* imaging. High-throughput genetic screens could be advantageous also in cancer research, since it is known that some mutations are only lethal when synergizing with other mutations, a concept known as synthetic lethality (O'Neil et al., 2017). By using CRISPR for performing LOF screens for essential genes in survival, one could identify, in an unbiased manner, conditional lethal genes unique to a specific cancer type, together with genes that are synthetically lethal after somatic mutations or compound treatment. Those genes would bring light into cancer specific vulnerabilities using an *in vivo* model. Therefore, they would be potential targets for drug discovery and/or combinatorial therapy.

Drug-Target Interaction

For several drugs, some of them already on the market, the domain of interaction of the target protein with the drug is often unknown. A deeper knowledge of drug-target interaction is crucial to design more efficient and less toxic analogues of a given compound. The CRISPR/Cas9 system can be used to determine interacting domains by selecting sgRNAs targeting specific protein regions (Shi et al., 2015). This approach could be applied in zebrafish for analysing essential domains of any target protein, especially multi-domain proteins, in the context of an *in vivo* assay. Such strategy, coupled with phenotypic analysis of mutant fish, could provide more complex readouts than those provided by *in vitro* systems.

Humanized Zebrafish Models

A limitation of all model organisms is that, even in cases of high homology with humans, they are sometimes not readily translatable to human biology. *In vitro* models of induced pluripotent stem (iPS) cells or more reliable patient derived iPS cellular systems can overcome such limitation. Nevertheless, recreating *in vitro* the conditions of the native environment of a cellular type is an extremely challenging task. Generating humanized zebrafish can represent a step forward in that desired translatability.

Here, we consider a humanized model to be either a zebrafish transgenic line in which the endogenous gene is substituted with its human ortholog, or a setup in which human tumour cells are xenotransplanted into zebrafish to assess their proliferation or metastatic progression. Xenotransplantation models based on the engraftment of labelled human cancer cells in zebrafish larvae are already established and are used as an alternative to rodent models for drug screening (Fior et al., 2017). However, it is often not clear how specific mutations in cancer cells affect oncologic progression. It would be interesting to use CRISPR/Cas9 in this experimental set up to mutate specific human genes in tumour cells, transplant them in zebrafish and finally analyse the effect of induced loss-of-function in tumour growth and dissemination. Conversely, disruption of genes in the zebrafish host followed by xenotransplantation of human tumour cells would provide insights into a

potential involvement on the microenvironment leading to cancer progression and resistance to therapy.

On the other hand, replacement of zebrafish genes with the human ortholog is still not a common and standardized procedure. CRISPR/Cas9 will certainly facilitate the transition toward humanized zebrafish. In fact, both HDR and NHEJ knockin strategies could allow the simultaneous disruption of fish genomic loci and replacement with its human ortholog. That would open interesting avenues in the study of drug-target interaction, if functional full-length human genes were exchanged in the fish genome, or toward personalized medicine, if the human ortholog gene carries specific SNPs related to patient stratification.

Final Remarks

Lack of efficacy is the major drug attrition cause during clinical development. To moderate future failures, biomedical research requires innovative approaches to identify the right drug targets upfront, understand their role in disease biology and perform preclinical target validation studies in relevant models of human disease. In this review, we have suggested that using zebrafish can help achieving those goals. Furthermore, we have proposed that the advantages obtained by the implementation of CRISPR/Cas9 in zebrafish will have an even deeper impact in the discovery of next generation therapies and treatment paradigms. Now, the use of CRISPR/Cas9 in zebrafish permits: (i) to streamline the identification of disease-relevant targets, and (ii) to build complicated genetic models, which might be key for performing disease-relevant phenotypic drug screenings. Hence, using zebrafish might allow exploiting simultaneously target and phenotypic drug screening strategies, which could result in more successful pipelines at a lower cost and time. The rationale is to narrow down a library of molecules, through *in silico* or *in vitro* methods, against a target identified or validated through a phenotypic drug screening performed in zebrafish. Then, it would be possible to test the efficacy of selected molecules, and possible chemical analogues, on relevant zebrafish disease models through their impact on the pathologic phenotype. Moreover, drug toxicity can be evaluated simultaneously with drug efficacy, providing an early assessment of safety liabilities. Given the low cost and time to perform such a combined screening strategy, it could be possible to test hundreds of molecule-target interactions in a disease-relevant model before entering expensive preclinical regulatory phases. The ultimate goal of such strategy would be to use the unique properties offered by CRISPR/Cas9 to develop humanized zebrafish used in personalized medicine, so each patient will be treated with the drug/set of drugs that are going to be most effective for them.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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