USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ESCUELA INTERNACIONAL EN CIENCIAS DE LA SALUD DE LA USC

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Tesis doctoral

Zebrafish as a model organism for the study of toxicity and effectiveness of new antitumor therapies

Lugo, 2019

PD en Medicina Molecular

CENTRO INTERNACIONAL DE ESTUDOS
DE DOUTORAMENTO E AVANZADOS
DA USC (CIEDUS)

DOCTORAL THESIS

ZEBRAFISH AS A MODEL ORGANISM FOR THE STUDY OF TOXICITY AND EFFECTIVENESS OF NEW ANTITUMOR THERAPIES

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INTERNATIONAL DOCTORAL SCHOOL

DOCTORAL PROGRAM IN MOLECULAR MEDICINE

LUGO

2019

USC
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DE COMPOSTELA

CENTRO INTERNACIONAL DE ESTUDOS
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TESIS DE DOCTORADO

EL PEZ CEBRA COMO ORGANISMO MODELO PARA EL ESTUDIO DE LA TOXICIDAS Y EFICACIA DE NUEVAS TERAPIAS ANTITUMORALES

Carlha Gutiérrez Lovera

ESCUELA DE DOCTORADO INTERNACIONAL

PROGRAMA DE DOCTORADO EN MEDICINA MOLECULAR

LUGO

2019

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A mi familia

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Darkness must pass

A new day will come

And when the sun shines

It will shine out the clearer

J.R.R. Tolkien

Remember to look up at the stars and not down at your feet.

Stephen Hawking

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ACKNOWLEDGMENTS / AGRADECIMIENTOS

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A la Universidad de Santiago de Compostela (USC), específicamente al Departamento de Zoología, Genética y Física Antropológica, y a la Unidad de Nano-Oncología del Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS), Hospital Clínico Universitario de Santiago de Compostela, por permitirme realizar la tesis doctoral en dichos centros.

A mis directores/as de tesis, Laura Sánchez Piñón, María de la Fuente Freire y Rafael López López. Destacando a Laura y María, porque son un ejemplo de trabajo y lucha constante, sobre todo en la lucha por la igualdad y el reconocimiento y de las mujeres en el mundo de la ciencia. Son todo un ejemplo para las nuevas generaciones y me siento afortunada de haber tenido la oportunidad de trabajar con ellas. Un agradecimiento especial a Laura por darme la oportunidad de pertenecer a su grupo de investigación, por guiarme, apoyarme, motivarme y financiarme durante todos estos años. También por confiar en mí en diversos proyectos que llevamos a cabo en conjunto, y gracias a uno de ellos se encendió en mí la brecha emprendedora que hoy en día me ha dado la oportunidad de reinventarme y seguir adelante con nuevos proyectos.

A todo el grupo de investigación del departamento de Genética por compartir conmigo sus conocimientos, apoyo e incluso tantas risas. En especial a: Moni y Soni, un pilar fundamental (unas

máquinas) que pueden resolver cualquier problema de trabajo, e incluso de tu vida (Gracias Moni por ayudame a empujar el coche cuando lo necesité), y siempre con una linda sonrisa y con muchas ganas. Pablo y Jorge, por enseñarme y ayudarme todo lo que sabían sobre pez cebra y otras técnicas. Juan, un ejemplo de como un científico puede saber de todo, incluso de lo que menos esperas, como comentarios tan ingeniosos y sarcásticos que pueden partirte de risa y que pueden transformar los problemas y/o errores en amenos y menos trágicos. Susana y Peque, por su capacidad para hacer reír al mundo. A Adrián Millán, por brindarme sus conocimientos en empresa y por toda la colaboración que me brindó durante mi MBA. A Adrián Chiclana, por su apoyo y por su hermosa amista, y en algunos momentos por su doble sentido e irónica poesía, hiciste muy ameno mis últimos días de trabajo. A Jeannette y Vanessa, no solo por ayudarme muchísimo en el trabajo, sino además por ser mis amigas, las más especiales, han sido uno de mis apoyos morales más fuertes, podría escribir muchas páginas de ellas, pero prefiero decirles en persona el resto (seguramente con pañuelo en mano).

A Belén, Abi, Surasa, Marta y a las personas del IDIS y CIMUS que me ayudaron y colaboraron conmigo para adentrarme al interesante mundo de la Nanotecnología. En especial a Belén, que ha sido un apoyo no solo de trabajo sino moral.

A Miguel Allende, Javiera de la Paz, Miguel Miranda y Consuelo Anguita, por permitirme la realización de mi estancia en la Universidad de Chile, por todo el apoyo, los conocimientos y por la valiosa amistad que me brindaron durante esos tres meses en tan hermoso y estupendo país.

A Carlos Herrero Latorre, Rosa Peña, Julia Barciela y Sagrario García, por todas sus enseñanzas en mis primeros años de tesis.

A todos los profesores que compartieron conmigo sus conocimientos durante mi carrera en Venezuela. Me dejaron grandes lecciones, gracias Pimali Felibert, siempre te llevaré en mi corazón.

To Alex Janaway, my most geek friend, for all his support and motivation. I am lucky to meet such a brilliant writer, his fantasy books made me disconnect when I needed it.

A mi querida amiga, Mafer Isaac, por escucharme en los momentos más alegres, en los más difíciles y por haber incluso estado en mis aventuras más descabelladas. Hemos vivimos tantas etapas juntas que también formas parte de esa familia que uno escoge. Gracias por todos tus consejos y por creen en mí.

A mis HYFAs: Zamira, Patricia, Doranda, Jonás, Luisa, Antonio y Ximena, por ser simplemente ustedes, mis amigos, mi familia. Son uno de los regalos más preciados que ha dejado la carrera en mi vida. Desde que fuimos saliendo de Venezuela ustedes han sido uno de los apoyos más grandes para salir adelante. Gracias por su apoyo incondicional, conocimientos, risas y por tantos momentos maravillosos, y por los que seguirán a lo largo de nuestras vidas.

A mi prima, mi hermana, Adriana Maldonado, por todo su cariño y por su apoyo durante tantos años de mi vida. A mi primo Jesús P. Carrillo, por su cariño y su confianza. Tenerlos de nuevo cerca le ha dado a mi vida esa alegría que solo se produce al tener a parte de la familia más preciada alrededor. A la Sra. Adriana, una segunda madre para mí y uno de los grandes ejemplos que tuve en la vida. La Sra. Adriana es un ejemplo de mujer fuerte, preparada académicamente, emprendedora y con muchas capacidades, por ella cuento los días para volver a verla y decirle que esto también se lo debo a ella.

A José Manuel Bello Rodríguez, mi marido, por haber estado a mi lado durante tantos años. Ha sido mi compañero en muchas de las aventuras que he emprendido, siempre creyendo en mí, aceptando todas mis decisiones y apoyándome cuando más lo he necesitado.

A Leia por ser mi alegría en los momentos difíciles.

A mi familia, Rodolfito, Carlos R., Carlos D., Alondra, y en especial a mis Padres: Marlene y Carlos Rodolfo, por ser el motivo más importante que te he tenido para ser fuerte y salir adelante. Aunque los tiempos han sido muy duros, hemos logrado cosas importantes, siempre pensando en la familia. Esto es por ustedes y gracias a ustedes.

Al resto de mi familia y amigos que están dentro o fuera de Venezuela. A esa gente que lucha cada día por salir adelante. Aunque esta situación ha sido muy difícil y por momentos me ha quebrado el alma, por otros me dio las fuerzas para luchar y lograr muchas cosas de lo que me he propuesto.

Y a todos aquellos que me han hecho reír, en incluso llorar durante esta maravillosa y complicada etapa de mi vida.

Gracias infinitas Grazas infinitas Infinite thanks

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Conflict of interests

I declare that there is no competing interests with the subject matter or materials discussed in this thesis.

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ABSTRACT / RESUMEN

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The zebrafish have many advantages that led to be a model organism with a great potential in translational research. Today, there are many techniques that have been described for the study of different diseases in zebrafish embryos. In fact, the zebrafish embryos are an ideal platform to evaluate novel cancer therapies. For this reason, the main goal of this thesis has been evaluating the therapeutic potential of different anticancer therapies, including innovative nanomedicines in zebrafish embryos.

To accomplish this, we evaluated the toxicity *in vitro* and *in vivo* of some commonly used anticancer drugs (Carboplatin, Irinotecan, Doxorubicin, and Paclitaxel), and a recently discovered drug with anticancer properties (Chloroquine) in order to determinate specific toxicity parameters and toxicological profiles crucial for zebrafish xenograft studies. The toxicity in zebrafish embryos was carried out by the fish embryo test (FET) during 96 h starting at 0 hpf and 72 hpf and we compare the results with the citotoxicity data obtained using the tumor cell lines: A549 (human lung carcinoma cell line), MCF7 cells (human breast adenocarcinoma cell line) and Panc185 (pancreatic ductal adenocarcinoma patient derived xenografts-PDX cancer cells). Additionally, we developed a newly nanoemulsion based on the anticancer drug edelfosine (E-NEs) which let us to study parameters such as toxicity, biodistribution and efficacy in xenografted zebrafish models.

In conclusion, the results obtained in this thesis show that the toxicity values obtained for the antitumor drugs *in vitro* cannot be extrapolated to *in vivo* models such as zebrafish because they do not form a complete system. On the other hand, we formulated and characterized E-NEs by the ethanol injection method with adequate physicochemical, biopharmaceutical and functional properties. The E-NEs demonstrated the efficacy on zebrafish embryos xenotransplanted with triple negative breast adenocarcinoma cells (MDA-MB-231). In general, zebrafish embryos serve as a promising tool in preclinical studies in which the stability, toxicity, and efficacy of therapeutic anticancer drugs and new nanosystems against various types of cancer can be evaluated.

El pez cebra tiene numerosas ventajas que lo llevaron a ser un organismo modelo con un gran potencial en la investigación traslacional. Hoy en día, se han descrito muchas técnicas para el estudio de diferentes enfermedades en embriones de pez cebra. De hecho, los embriones de pez cebra son una plataforma ideal para evaluar nuevas terapias contra el cáncer. Por esta razón, el objetivo principal de esta tesis ha sido evaluar el potencial terapéutico de diferentes terapias contra el cáncer, incluidas las nanomedicinas innovadoras en embriones de pez cebra.

Para lograr esto, evaluamos la toxicidad *in vitro* e *in vivo* de algunos medicamentos contra el cáncer de uso común (Carboplatino, Irinotecan, Doxorrubicina y Paclitaxel), y un medicamento recientemente descubierto con propiedades contra el cáncer (Cloroquina) con la finalidad de determinar parámetros específicos de toxicidad y perfiles toxicológicos cruciales para los estudios de xenotransplantes en embriones de pez cebra. La toxicidad en los embriones de pez cebra se realizó mediante la prueba FET (fish embryo test) durante 96 h a partir de 0 hpf y 72 hpf y comparamos los resultados con los datos obtenidos de los ensayos de citotoxicidad realizados en las líneas celulares tumorales: A549 (línea celular de carcinoma de pulmón humano), células MCF7 (línea celular de adenocarcinoma de mama humano) y Panc185 (células de cáncer de xenoinjertos-PDX derivadas de pacientes con adenocarcinoma ductal pancreático). Además, desarrollamos una nueva nanoemulsión basada

en el medicamento contra el cáncer, la edelfosina (E-NEs), que nos permite estudiar parámetros como la toxicidad, la biodistribución y la eficacia en modelos de pez cebra xenotransplantados.

En conclusión, los resultados obtenidos en esta tesis muestran que los valores de toxicidad obtenidos para los fármacos antitumorales *in vitro* no pueden extrapolarse a modelos *in vivo* como el pez cebra debido a que no forman un sistema completo. Por otro lado, formulamos y caracterizamos E-NEs con propiedades fisicoquímicas, biofarmacéuticas y funcionales adecuadas mediante el método de inyección de etanol. Los E-NEs demostraron ser eficaces en embriones de pez cebra xenotrasplantados con células de adenocarcinoma de mama triple negativo (MDA-MB-231). En general, los embriones de pez cebra sirven como una herramienta prometedora en estudios preclínicos en los que se puede evaluar la estabilidad, la toxicidad y la eficacia de los medicamentos terapéuticos contra el cáncer y los nuevos nanosistemas contra diversos tipos de cáncer.

CHAPTER I. Introduction

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1. The zebrafish

The zebrafish (Danio rerio) (Hamilton, 1822) is a tropical freshwater fish, belonging to the Cyprinidae family, of the order Cypriniformes (McCluskey et al. 2014, Stock et al. 2007). Zebrafish are endemic to Southeast Asia, Thailand, Burma, India, Pakistan and Bangladesh, where they live in rivers and in stagnant waters (for example in rice paddies) (Laale, 1977; (Spence et al. 2008). The aquatic habitat of zebrafish is of lentic and lotic waters, it can vary in temperatures (between 16.5 to 35 ° C), in pH (between 5.9 to 8.5), and in conductivity (between 10 to 2000 ƭS) (Lawrence, 2011).

The zebrafish grows about 3 to 5 cm on average and is characterized by having along its fusiform body with five longitudinal blue stripes, thence the origin of its common name (zebrafish). The sexes are easily differentiated during the spawning period due to the swollen belly of the females. In addition, females are larger than males and have a golden color between their stripes. The zebrafish has a very short reproduction cycle reaching maturity at the age of 3 months approximately, which is clearly beneficial for breeding (Scholz et al. 2008). The zebrafish is an oviparous species and, under favorable conditions, the female can generate between 100 to 500 eggs every 2-3 days throughout the year (Lohr and Hammerschmidt,

2011). In terms of feeding, zebrafish prefer zooplankton and insects (Spence et al. 2008).

In their natural habitat zebrafish mate mostly during the summer season, however, it is possible to perform matings directed in laboratories throughout the year. Mating occurs when the male chases the female and hit with its caudal fin in the ventral zone of the female, where the ovary is located. After that, the female releases the eggs of the ovary through the oviduct, and once they are outside, the male fertilizes them (Parichy, 2015). After fertilization, fertilized eggs begin their first embryonic divisions, until they hatch at 2 or 3 dpf (post-fertilization days) approximately (Kimmel et al. 1995; Scholz et al. 2008). At 5 or 6 dpf the external feeding begins and the organogenesis of the main organs is completed (Eimon and Rubinstein, 2009). Between 12 and 14 dpf the surviving embryos begin their juvenile phase and later the adult phase (Willemsen et al. 2011, Howe et al. 2013).

Although the genome sequencing of the zebrafish has been completed (Howe et al. 2013), it is not very clear how sex is determined in this organism (Liew et al. 2012). However, some studies suggest that environmental factors have a minimal effect on the sex ratio among zebrafish populations (Liew et al. 2012). Regarding their behavior, zebrafish populations present large differences in the physiology of their social behavior, some of which depend on sex and time (Filby et al. 2010).

Zebrafish embryos are covered by a protective membrane called chorion. The chorion is porous, has a thickness of between 1.5 and 2.5 ɀm and consists of three layers (Rawson et al. 2000). This envelope undergoes a process of thinning known as "softening of the chorion", prior to the formation of the body axis of the embryo and hatching. In addition, there is an internal vitelline membrane between 1 and 4 ɀm thick, separated from the chorion by the perivithelial space occupied in turn by a perivithelial fluid (Schoots et al. 1983; Deok-Ho et al. 2005).

2. Uses in biomedical research

Zebrafish have many advantages that led to be as a model organism in the study of developmental biology and vertebrate

genetics since the 1980s (Streisinger et al. 1981). Nowadays it is widely used in the detection of therapeutic drugs, in the investigation of human diseases, in studies of animal behavior, for studies of physiology and toxicology (Howe et al. 2013; Ablain and Zon, 2013; Lawrence, 2007; Rihel et al. 2010; Scholz et al. 2008; Spence et al. 2008).

It is important to mention that zebrafish embryos are not considered legally animals. There is a Real Decreto that establishes the basic rules applicable to the protection of animals used in experimentation and other scientific purposes, including teaching, Real Decreto 53/2013, of February 1, which maintains the definition of animal given in the article 3 of Real decreto 1201/2005 to which, defines as an animal any non-human vertebrate, including free living larvae, but excluding embryonic forms, such as zebrafish embryos. On the other hand, Directive 2010/63 / EU of September 22, 2010, on the protection of animals used for scientific purposes also applies to fetal forms of mammals, but excludes other embryonic forms such as zebrafish.

Among the advantages for its use as a model organism we have:

- Its maintenance is inexpensive, easy to handle and due to its small size it allows that large populations to be maintained in small aquarium systems. The cost of maintaining of the zebrafish are 100 to 1000 times less than maintaining laboratory mice (Rojas-Muñoz et al. 2007). They also tolerate a wide range of environmental conditions and types of food (Lawrence, 2007).

- They have a rapid reproductive cycle and produce a high number of embryos per laying, between 100 to 500 eggs every 2-3 days throughout the year (Talwar et al. 1991). The generation time is 2-3 months (Bresch, 1991; Scholz et al. 2008).

- They have an external fertilization, whereby fertilization can be directed, at direct crossings or by in vitro fertilization (Parichy, 2015).

- Embryos are transparent and have a rapid development which is widely studied (Kimmel et al., 1995). Therefore, they are suitable for embryonic-larval (EL) toxicity tests, in which they are generally more sensitive than toxicity tests with juvenile

and adult fish (McKim, 1977). They are also the most suitable for the study of anticancer drugs through xenotransplantation tests, injecting human cancer cells (Kirchberguer et al. 2017), biodistribution tests and studies of macrophages and neutrophils (Evensen et al. 2016).

- Embryos are very resistant and can be easily manipulated in genetic procedures such as morpholinos (Bill et al. 2009; Bedell et al. 2011; Timme-Lagary et al. 2012) or ribonucleoproteins (CRISPR / Cas) (Irion et al. 2014; Shah et al. 2015; Li et al. 2016; Albadri et al. 2017), microinjections in a single cell or also in xenografts (Haldi et al. 2006; Taylor et al. 2009; Drabsch et al. 2017; Idilli et al. 2017; Wyatt et al. 2017; Roel et al. 2016).

- They are multicellular and integrate the interaction of various tissues and differentiation processes. Therefore, data closer to reality than those obtained through cell cultures can be obtained.

- They present various organs and cell types similar to those of mammals, which are easily visualized by high-resolution microscopy in real time (Yang et el, 2013).

- Its genome shows approximately 70% homology with the human genome (Howe et al. 2013).

- At 40 hpf the innate immune system is active (Cui et al. 2011), but the adaptive immune system will not be fully functioning until within 4-8 weeks after fertilization (Lam et al. 2004; Li et al. 2011). Therefore, the results of the analyzes carried out during the embryo / larvae phases date back to the innate immune system.

- The European Food Safety Administration (EFSA, 2005) has stated that fish in these early stages of development, up to 5 dpf, are less likely to experience pain, suffering, distress or suffer lasting damage, in accordance with the 3Rs Principles (replacement, reduction and refinement) for human animal research (Russell and Burch, 1959).

3. Model of interest in the study and development of therapeutic compounds

The zebrafish model offers a lot of opportunities for scientific research beyond its use in toxicology, as a replacement for its adult specimens in acute toxicity tests (Scholz et al., 2008; Tan and Zon, 2011; Zon, 1999). Today there are several innovations originated in biotechnological research in terms of: development of large-scale breeding systems and facilities (Barton et al. 2016), computer tracking devices (Rihel et al. 2010), more than 5000 modified mutants or transgenic strains (Segner, 2009), among others; which provide many opportunities for the study and development of therapeutic compounds. The small size of the large amount of progeny that is generated from the mating of zebrafish not only allows analyzing the effect of multiple compounds at the same time during embryonic development, but also of identifying new drugs that are potentially effective and do not cause risks due to their toxicity (Rojas-Muñoz et al. 2007).

The zebrafish most used in the research belongs to the wild, however, there is a wide range of mutant or transgenic (White et al. 2008) or genotypic lines, each of which is more or less suitable for a trial in particular. For example, to study specific phenomena such as angiogenesis, there are useful transgenic lines, such as Tg (Flk1: EGFP) and Tg (Fli1: EGFP) with a green vasculature system, and Tg (Gata1: DsRed) in which red blood cells are fluorescent (Tat et al. 2013). On the other hand, the transparent line called Casper (White et al. 2008), allows endless analysis in adults (Gutiérrez-Lovera et al. 2018; White et al. 2013; Dang et al. 2016; Tang et al. 2016).

This animal model is a promise for the evaluation and validation of drugs and new therapeutic agents, including genetic nanomedicines. The small size of zebrafish embryos allows you to study large amounts of compounds easily and economically. This model also has many advantages (already described above) to predict the toxicity, bioavailability and efficacy of infinite compounds and nanocomposites (Fako and Furgeson, 2009). Berghmans et al (2008) studied the correlation between clinical or preclinical data in animals and data obtained from a zebrafish model. The study was conducted in 16 compounds with pharmacological activity and could predict the adverse effects of the drug and a good association between animal and human data. Other studies in zebrafish and mammalian embryos have

also indicated that the lethal concentration (LC50) of the drugs evaluated was comparable between two systems *in vivo* (Zhang et al, 2003; Kari et al, 2007).

4. Specific applications in the development of antitumor drugs

The pathological mechanisms underlying cancer are some of the most difficult processes to understand due to their variety and complexity. It is for this reason that zebrafish as a model for the study of antitumor drugs have gained popularity in the last two decades. Cancer cell lines can be grown in zebrafish embryos (a technique called xenograft or xenotransplant), as in mammalian models such as mice (He et al, 2012; Teng et al, 2013); These cancer lines can also be studied by high-resolution microscopy in live embryos (Yang et el, 2013). Lee et al. (2005) performed the first successful xenotransplantation of melanoma cells in the vitelus of a zebrafish embryo, the cells having the ability to proliferate and form a tumor within this model organism. On the other hand, Haldi et al (2006), demonstrated not only that cells could proliferate, but also that the signals from human cells affected the zebrafish embryo by chemotaxis attracting the fish's blood vessels to the tumor and producing the angiogenesis process. Other studies in different types of cancer (melanoma, breast carcinoma, colorectal, pancreatic, ovarian, kidney,

lung, oral, prostate, leukemia, etc.) have also been satisfactory to determine its tumorogenicity and study metastatic behavior (Marques et al, 2009; Nicoli et al, 2007; Lee et al, 2005; Haldi et al. 2006; Drabsch et al. 2017; Roel et al. 2016; Marques et al. 2009; Bansal et al. 2014; Zhang et al. 2014; Brown et al. 2017; Mort et al. 2015).

On the other hand, transgenic lines have been generated that express oncogenes driven by ubiquitous or specific promoters of carcinogenic tissues (Mione et al. 2016). For example, Patton et al (2015) developed a transgenic model of zebrafish for melanoma using regulatory sequences of the mitfa gene to boost the expression of different oncogenes, in this case the BRAFV600E. This mutation is found with high frequency in human melanoma, suggesting that it can play a causal role. However, to develop melanoma, they also need to carry inactivating mutations in p53 (Berghmans et al. 2005).

Zebrafish is considered a complementary model to murine models for the evaluation of antitumor compounds and for the discovery of new drugs (Stern and Zon, 2003; Goessling et al. 2007; MacRae and Peterson, 2015; Deveau et al. 2017; Van Rooijen et al. 2017; Zhao et al. 2015; Lenis-Rojas et al. 2016; Penas et al. 2016; Blackburn et al. 2014; Taj et al. 2013; Veinotte et al. 2014). As indicated above, zebrafish cancer models have been used for the detection of new medications, as well as for the reanalysis

of known medications (Stern and Zon, 2003; Deveau et al. 2017; Penas et al. 2016; Blackburn et al. 2014; Tat et al. 2013; Veinotte et al. 2014; Huiting et al. 2015; Xie et al. 2015).

Regarding toxicity, this model is used to evaluate all types of drugs that can be dissolved in water (MacRae and Peterson, 2015). This model recreates the process of absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) of different substances, unlike in vitro models (cell cultures), it would not be possible since the cells do not they make up a complete organism. Therefore, the zebrafish is considered as the first level of complete organism with utility to study substances prior to its analysis in mammals (Goldstone et al. 2010; Li et al. 2011).

The use of zebrafish embryos is found in fields closely related to developmental toxicology (embryotoxicity and teratogenicity) where several teratogenic drugs have been found using the zebrafish model (Augustine-Rauch et al. 2010; Lammer et al. 2009). The most commonly used official tests for acute toxicity are: ISO 7346 and the OECD 203 (ISO, 1996; OECD, 1992). These tests revealed results comparable with adult specimens (Braunbeck and Lammer, 2006; Lammer et al. 2009; Nagel, 2002). The acute toxicity test with zebrafish evaluates four lethal assessment criteria, that is, embryo

coagulation, absence of developed somites, absence of heartbeats and absence of yolk tail yolk detachment (OECD, 2013).

The zebrafish is also an excellent model for providing new ideas about the interaction between the immune system and tumor cells (Powell et al. 2016; Chambers et al. 2013). Because in zebrafish, macrophages play an important role in angiogenesis, this model could also be used to develop functional tests related to the angiogenic process. **Figure 1** shows a scheme that summarizes all the features that zebrafish have for the study of cancer and drug screen.

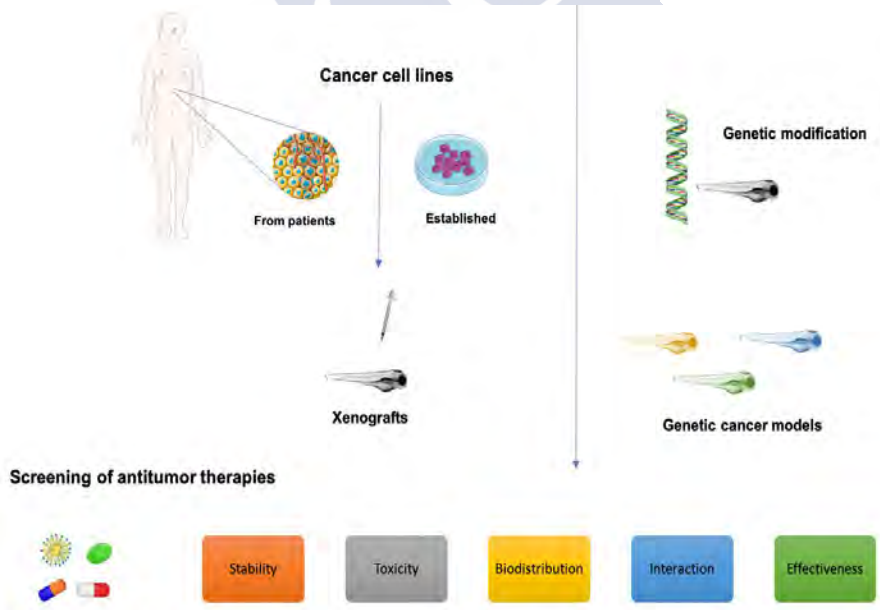

Figure 1. Zebrafish functionalities for the study of cancer and the screening of antitumor drugs. There are two main routes, from xenotransplants of patients or

established cells, or through the design of zebrafish transgenic models for each type of cancer. Both routes are the basis for drug screening through studies of stability, toxicity, biodistribition, interaction, and effectiveness (Source: own elaboration).

5. Introduction to nanomedicine

Nanomedicine is a science that consists in the use and development of nanometric-sized structures to transport drugs, providing a better interaction with biological systems, and favoring their accumulation in target tissues. In addition, its use can be extended not only to the treatment of various diseases, but also to the development of diagnostic systems (Lin et al. 2013; Tinkle et al. 2014). A wide variety of nanosystems have been designed for the treatment of cancer, and there are currently several clinical formulations, the most relevant being Doxil and Abraxane (Wu et al. 2017). Other widely studied applications include the development of gene therapy, vaccine generation, and regenerative medicine (Rojas-Aguirre et al. 2016). However, the physicochemical properties of nanopharmaceuticals can lead to alterations in pharmacokinetics, absorption, distribution, elimination and metabolism, the potential to cross biological barriers, toxicity and their persistence in the

environment and the human body are some examples of concerns about the application of nanomaterials today (Bleeker et al. 2013; Tinkle et al. 2014).

The use of nanopharmaceuticals against cancer implies in most cases the introduction of antitumor drugs into a nanostructure, which offers advantages in relation to treatment with free drugs, which are normally poorly soluble (Gou and Huang, 2011) or in some very toxic cases. This fact has greatly stimulated research in this field, producing new generations of more sophisticated and effective nanosystems (Lin et al. 2013). Nanosystems in many cases can improve access to tumor-associated medications and decrease side effects (Peer et al. 2007; Torchilin, 2011). An example is the encapsulation of edelfosine (ET-18-OCH3 or ET), which is a synthetic lipid with a high apoptotic action on cancer cells through different mechanisms of action, which has proven to be an efficient strategy for treatment. of breast cancer, leukemia, pancreas, lung, glioma, osteosarcoma (Ruiter et al. 2001; Gajate and Mollinedo, 2007; Gajate et al. 2012; Nieto-Miguel et al. 2007; Estrella-Hermoso et al. 2011; González-Fernández et al. 2017). The main limitations regarding its clinical application, the high toxicity in intravenous administration (producing hemolysis) (Ahmad et al. 1997), and oral (producing gastrointestinal problems) (Estella-Hermoso et al. 2012) have also been minimized.

As for the direction of the nanosystems to their therapeutic target can be performed by passive or active methods. In the passive, the address is due to an increase in the permeability of the endothelium or wall of the capillaries that irrigate the tumor tissues, together with the increase in the accumulation in them due to poor lymphatic circulation (Maeda et al. 2010). While the active is due to a high specificity of the nanosystem towards the target cells. This specificity has been achieved through cell recognition processes taking advantage of the overexpression of several types of receptors on the surface of tumor cells (Vila-Jato, 2009).

Recent studies highlight the potential of zebrafish for the evaluation of new nanosystems against cancer. Some of them evaluated the toxicity and safety of blanck nanoparticles (i.e., before drug incorporation) using different procedures (Lee et al. 2017; Kim and Tanguay, 2013; Harper et al. 2015; Jeong et al. 2015). Yu-Lan et al (2011) developed chitosan nanotransporters and determined that exposure to various concentrations of these nanotransporters can induce cell death and overexpression of reactive oxygen agents, and therefore physiological stress in zebrafish embryos.

Taking advantage of embryonic transparency, biodistribution studies have also been carried out to determine the ability of nanomedicaments to reach their target by overcoming complex biological barriers, such as the blood-brain barrier (Yang et al. 2015; li et al 2017; Sieber et al. 2017). Teijeiro-Valiño et al (2017) evaluated lipid nanotransporters of hyaluronic acid and protamine and demonstrated that it were able to penetrate the epithelial barriers of zebrafish. In another study, embryos of transgenic zebrafish (with fluorescent macrophages) were exposed to polymeric nanocapsules, demonstrating that size and composition were fundamental parameters in biodistribution and interaction between nanocapsules and macrophages (Crecente-Campos et al 2019). On the other hand, xenograft with different types of cancer cells have been performed and the effectiveness of different types of antitumor nanomedicines has been studied, obtaining favorable results (Lee et al. 2017; Evensen et al. 2016; Wehmas et al. 2016; Yang et al. 2016). Yang et al (2016) described the interaction of HeLa cells and specific liposomes loaded with doxorubicin. In addition to applications with nanosystems associating antitumor drugs, the zebrafish also has a high potential to evaluate new therapeutic approaches, as reflected in the review article presented below as Chapter 2.

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CHAPTER II

The potential of zebrafish as a model organism for improving the translation of genetic anticancer nanomedicines

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ABSTRACT

In the last few decades, the field of nanomedicine applied to cancer has revolutionized cancer treatment; several nanoformulations have already reached the market and are routinely being used in the clinical practice, and many other are currently underoing clinical trials. In the case of genetic nanomedicines, i.e., designed to deliver gene therapies to cancer cells for therapeutic purposes, advances have been less impressive. This is because of the many barriers that limit the access of the therapeutic nucleic acids to their target site, and the lack of models that would allow for an improvement in the understanding of how nanocarriers can be tailored to overcome them. Zebrafish has important advantages as a model species for the study of anticancer therapies, and have a lot to offer regarding the rational development of efficient delivery of genetic nanomedicines, and hence increasing the chances of their successful translation. This review aims to provide an overview of the recent advances in the development of genetic anticancer nanomedicines, and of the zebrafish models that stand as promising tools to shed light on their mechanisms of action and overall potential in oncology.

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1. Nanotechnology provides innovative approaches to cancer management

In recent decades, an increasing understanding of the molecular and biological basis of cancer and the discovery of novel technologies has led to improvements in cancer survival. The development of early detection tools and targeted treatments, as well as changes in patients' lifestyle, have contributed to this higher rate of cancer survival. The development of new nanomedicines for cancer treatment is an interdisciplinary research field that includes biology, chemistry, engineering, and medicine, with a clear goal: advancing cancer detection, diagnosis, and treatment.

Different types of nanocarriers, including liposomes and other lipid-based nanosystems, polymer-based nanoparticles, micelles, polyplexes, dendrimers, polymersomes and drug/protein conjugates have been proposed during the last few decades in cancer research (Swain et al. 2016; Nascimiento et al. 2016; Thotakura et al. 2017; Tang et al. 2017; Song et al. 2017; Sepulveda et al. 2017; Zhong et al. 2016; Almhanna et al. 2017; Yu et al. 2017; Raviña et al. 2010; De la Fuente et al. 2018). For cancer treatment, the goal is to enhance the efficacy and decrease the toxicity of the current therapeutics by altering their pharmacokinetic profile, increasing their solubility and stability in biological fluids, augmenting their accumulation in tumors,

and reducing their toxicity. Biological drugs, such as gene therapies, peptides and proteins, can also benefit greatly from the application of nanotechnology that could protect them from premature degradation and facilitate their access to the intracellular compartment (De la Fuente et al. 2008; Del Pozo et al. 2016; Yin et al. 2014; Shi et al. 2016). Liposomes are the most common type of nanostructure that have translated into marketed products (Saif, 2013; Wicki et al. 2015; Rodriguez et al. 2009; DaunoXome, 1996; European Medicines Agency, 2017; U.S. Food and Drug Administration Home page, 2017). Back in 1995, the US Food and Drug Administration (FDA) approved the first nanoparticle for cancer treatment, Doxil©, a liposomal nanoparticle loaded with the chemotherapeutic drug doxorubicin (Barenholz, 2012). Since then, other nanotherapeutics based on liposomes have reached the market such as Pegylated liposomal doxorubicin (Doxil© /Caelyx©), liposomal cytarabine (DepoCyt©), Daunorubicin citrate Liposomes (DaunoXome©), liposomal doxorubicin (Myocet©), Vincristine Sulfate Liposomes (Marqibo©), liposomal irinotecan (Onivyde©). Paclitaxel polymeric nanoparticles (Opaxio©), pegylated L-asparaginase polymeric nanoparticles (Oncaspar©), leuprolide acetate polymeric micelles (Eligard©), oxaliplatin micelles (Eloxatin©), polymer–protein conjugate pegfilgrastim (Neulasta©), albumin-paclitaxel (Abraxane©), Denileukin diftitox (Ontak©), Brentuximab-Monomethyl auristatin E (MMAE) (Adcetris™), and Trastuzumab-

Emtansine (Kadcyla©) are examples of different types of nanostructures that have led to products already in clinical use.

Apart from their use in the possible development of nanotherapeutics, nanoparticles are also useful tools in the diagnosis field, due, in the case of inorganic nanoparticles, to their intrinsic properties that allow a direct tracking, and, in the case of organic nanoparticles, to their ability to accommodate/encapsulate different molecules and contrast agents for imaging applications. Many contrast agents are currently being studied with this goal in mind, including super-paramagnetic iron oxide nanoparticles and ultra-small super-paramagnetic iron oxide nanoparticles, heavy metal (i.e., gold, lanthanide, and tantalum) nanoparticles, technetium-99m (^{99m} TC) sulphur colloid nanoparticles, I-labeled cRGDY silica nanoparticles, surface-enhanced Raman scattering nanoparticles, and single-walled carbon nanotubes. Organic nanoparticles such as liposomes, micelles, and nanoemulsions can, for example, encapsulate super-paramagnetic iron oxide nanoparticles, or be radiolabeled with radioisotopes such as ^{89} Zr, ^{111} In, ^{18} F, ^{64} Cu or ^{68} Ga for molecular imaging (Martínez-Gonzalez et al. 2016; Fan et al. 2016; Cui et al. 2017; Pratt et al. 2016). The imaging modalities currently available experimentally are: ultrasound, magnetic resonance imaging (MRI), optical imaging, molecular imaging, computed tomography (CT), positron emission tomography (PET), and single-photon emission computed

tomography (SPECT). However, in clinics, the most used modalities for whole-body imaging are CT, MRI, PET and SPECT. For organ-specific examinations, ultrasounds are of preference since they are faster and less expensive, while, for superficial lesions, endoscopic, and intraoperative procedures, optical and photo-acoustic applications are more suitable (Thakor et al. 2013; Park et al. 2017).

Finally, nanoparticles also have a great potential as nanotheranostics, i.e., multifunctional nanoparticles that combine, into a single entity, elements for therapy and for diagnosis. Nanotheranostics have been explored for applications combining different imaging modalities and therapeutic applications, such as photodynamic therapy, photothermal, phototriggered chemotherapeutic release, ultrasound triggered, electro-thermal, magnetothermal, X-ray, and radiofrequency therapies (Sneider et al. 2017). Moreover, nanotheranostics are gathering great interest because they might provide a deeper understanding of key aspects that could make a nanoparticle formulation successful—such as drug release kinetics and penetration of nanocarriers within tumors—monitoring therapeutic responses, as well as allowing the implementation of novel strategies, such as imaging-guided local therapy (Chen et al. 2017; Baetke et al. 2015). To date, there is only one formulation undergoing clinical trials (Phase I) for the treatment of multiple brain metastases,

AGuIX® (Activation and Guidance of Irradiation by X-ray), a gadolinium-based nanoparticle of around 5 nm diameter, developed mainly for imaging applications due to its magnetic resonance contrast properties. However, when it is combined with X-ray radiation, it increases three-fold the radiotherapy effectiveness in mice, playing a double role, as radiosensitizer and as imaging agent (NCT02820454) (Detappe et al. 2015; Kotb et al. 2016). We believe that nanotheranostics have a lot of potential in cancer management, and could definitively make an impact in the clinical practice by, concurrently, diagnosing the disease, helping patients stratification, guiding focal therapy, tracking drug release and penetration within tumors, monitoring response, and, if required, switching treatments.

2. Genetic nanomedicines and the main challenges for their translation to the clinic

Advances in genetics and molecular biology have led to the development of new therapies that can specifically modulate the expression of relevant genes in order to correct abnormalities and restore their original biological function. Some of the strategies of gene therapy include (i) silencing oncogene expression, (ii) promoting tumor-suppressor genes, (iii) correcting mutations, (iv) suicide gene therapy, (v) suppressing tumor angiogenesis, and (vi) activating an immune response against tumor cells. For these purposes, plasmid

DNA (pDNA), minicircles (supercoiled circular DNA), oligonucleotides (ASOs, decoys, aptamers), RNA interference (short-hairpin (shRNA), small interfering RNA (siRNA) and microRNA (miRNA)) are being extensively explored (Pahle and Walther, 2015). However, because naked nucleic acids are vulnerable to enzymatic degradation, rapid clearance, and non-specific biodistribution, only low gene expression efficiencies can be achieved. Hence, the primary challenge of gene therapy is to develop effective carriers able to protect the nucleic acids and facilitate their internalization into the targeted cells at the targeted site (Zhou et al. 2017).

Traditionally, vectors for gene therapy applications are divided into viral and non-viral carriers. Most gene vectors (~69%) currently undergoing clinical trials involve viruses (i.e., retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses). In August 2017, the FDA approved the first gene therapy in the United States, Tisagenlecleucel (Kymriah©) from Novartis Pharma AG (Basel, Switzerland), for certain pediatric and young adult patients with a form of acute lymphoblastic leukemia whose first-line drugs have failed (FDA Press Announcements, 2017). This pioneer gene therapy—based on a self-inactivating lentiviral vector that contains extensively modified sequences from HIV-1 so as to deliver chimeric antigen receptor (CAR)-encoding sequences into T cells to target and kill leukemia cells with specific antigen (CD19) on the surface—

achieves an overall remission rate of 83% (52/63) in this patient population (Norvatis, 2017). Despite these advances, many concerns still remain regarding the use of viral vectors, such as their potential immunogenicity, the possibility of reversion to the virulent form or the viruses, and also their high production costs (Zhou et al. 2017). Alternative synthetic vectors, made out of natural, semi-synthetic or synthetic materials, offer a safer alternative to introduce genetic materials into the targeted cells. Numerous non-viral gene delivery systems for different types of nucleic acids (mainly pDNA, siRNA and miRNA) have been described to date (Pahle et al. 2015; Slivac et al. 2017). Different applications for the development of novel anticancer genetic nanomedicines have similarly being explored, including suicide gene therapies, anti-angiogenic gene therapies, immunotherapies, restoration of oncosuppressor RNAs, or gene silencing of oncogenes, or specific non-coding RNAs (antagomirs), or proteins involved in resistance to chemo- and radio-therapies, anti-apoptotic proteins, epigenetic regulation, etc., as recently reviewed by Bottai et al. (2017). The main preclinical studies of the different applications of nanoparticles for gene therapy reported successful in mice models are summarized in Table 1 (reporter genes and experiments referring to over expression/silencing of housekeeping genes are not included).

Table 1. Main studies to date of genetic nanomedicines that have had relevant therapeutic effects on different types of cancer in mice models.

Nanocarrier	Gene Vector	Target	Indication	Administration Route	Ref
Liposomes	miRNA	Restoration of oncossuppressor	Breast cancer	Tail vein	Bayraktar et al. 2017
	siRNA	EpCAM silencing	Breast cancer	Tumor adjacent	Bhavsar et al. 2017
	siRNA	Anti-angiogenesis	Breast cancer	Intratumoral	Chen et al. 2017
	miRNA	Restoration of oncosuppressor	Hepatocellular carcinoma	Intratumoral	Xu et al. 2016
	shRNA	WT1 silencing	Melanoma	Tail vein	Saavedra-Alonso et al. 2016
Polymeric nanoparticles	pDNA	Anti-angiogenesis	Colon cancer	Tail vein	Yu et al. 2016
	pDNA	Induce apoptosis	Ovarian cancer	Intraperitoneal	Lou et al. 2016
	pDNA	Suicide gene therapy	Ovarian cancer	Intraperitoneal	Cocco et al. 2017
	pDNA	Immunotherapy	Colorectal cancer	Intratumoral	Liu et al. 2017
	pDNA	Suicide gene therapy	Colon cancer	Intratumoral	
Lipid nanoparticles	siRNA	Androgen receptor silencing	Prostate cancer	Tail vein	Lee et al. 2017
	miRNA	Restoration of microRNA-26a	Lymphocytic leukemia	Intraperitoneal	D'Abundo et al. 2017
Dendrimers	si/shRNA	ITCH silencing	Pancreatic cancer	Tail vein	De la Fuente et al. 2015

siRNA, small interference RNA; shRNA, short-hairpin RNA; pDNA, plasmid DNA; miRNA, microRNA; EpCAM, epithelial cell adhesion molecule; WT1, Wilms Tumor 1.

Recent advances in non-viral gene vectors regarding efficiency, specificity, safety and gene expression durability have led to an increase in the number of nanoparticle-based gene delivery vectorsin clinical trials while the number of viral vectors have dropped significantly (Ramamoorth and Narvekar, 2015). Some examples in cancer are related to liposomes for siRNA, microRNA or pDNA delivery (NCT01591356, NCT01829971, NCT01489371, NCT02340156); lipid nanoparticles (NCT02314052, NCT01437007) or polymeric nanoparticles (NCT02956317) (www.clinicaltrial.gov). Unfortunately, non-viral vectors have not reached the market yet.

The design of successful synthetic nanovectors poses a big challenge since they need to overcome important biological barriers. Nanovectors need (i) to be safe and adequate for parenteral administration, (ii) efficiently protect nucleic acids from degradation, and (iii) promote their access to the target intracellular compartment in the target cell (depending on the selected gene therapeutic system, i.e., plasmid DNA, RNAi, non-coding RNA (ncRNAs), oligonucleotides, etc.), in enough amounts to mediate a therapeutic effect (depending on the potency of the molecule, specificity, and stability) (Pahle et al. 2015; Shankeret al. 2011; Dowdy, 2017; Mc Erlean et al. 2016; Wang et al. 2015). All these aspects should be taken into consideration from early development to increase the chances of translation into early-phase clinical trials (Santander-Ortega et al. 2012; Santander-Ortega et al. 2014; De la Fuente et al. 2012). The development of functional assays and the selection of adequate animal models for therapeutic evaluation are also key steps that critically affect the outcome of the preclinical evaluation.

Although a number of gene-delivery nanovectors have been claimed to be efficient, most of the studies have been done in vitro, on immortalized cancer cell lines, and only a few have actually addressed the therapeutic outcome in vivo. While in vitro experiments include

evaluation of toxicity (e.g., MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) or trypan blue staining assays), transfection efficiency (e.g., internalization of fluorescent nanoparticles/nucleic acids by confocal microscopy or/and flow cytometry), gene expression (e.g., RT-PCR, western blot, or ELISA assays), and sometimes functional assays (e.g., evaluation of cell proliferation, migration and invasion, colony formation, angiogenesis, and apoptosis), in vivo reports in animal models (mainly rodents) are mostly limited to measuring a therapeutic effect in terms of tumour growth, providing only a yes or no answer. Therefore, the causes behind the therapeutic failure are not well understood. In our opinion, it is necessary to learn more about the in vivo performance of genetic nanomedicines, and to incorporate functional assays in animal models, in order to speed up the translation of genetic nanomedicines to a clinical setting. Novel tools and models that would allow fast and low-cost comparative studies for the rational optimization of genetic nanomedicines are urgently needed.

3. Zebrafish as a model species

Zebrafish (Danio rerio) is a freshwater fish belonging to the Cyprinidae family, common in the river Ganga basin on the Indian sub-continent. Zebrafish has some well-known characteristics that makes it really attractive as a model for human diseases (Lieschke and Currie, 2007); Santoriello and Zon, 2012; Ablain and Zon, 2013; Giannaccini et al. 2014). In fact, it has achieved the status of model species, and been presented as an extraordinary complement to murine models, and a promising alternative (Ablain and Zon, 2013). For one, zebrafish's maintenance is affordable in terms of feasibility and costs. Moreover, adult individuals are small in size (2.5–4 cm), which makes the space requirements not very demanding. In addition, it has high fecundity and fertilization rates (up to 200 fertilized eggs per mating pair and week), and presents external fertilization, which allows for performing directed crosses, as well as in vitro fertilization. It also presents relatively short generation times—around three months. Finally, the genome of zebrafish, whose complete DNA sequence was published in 2013 (Howe et al. 2013), shows approximately 70% of homology with the human genome, and 82% of orthologous human disease-related genes.

Zebrafish embryos are particularly interesting for biomedical applications (Phillips and Westerfield, 2014; Lee et al. 2017). As early as 48 h post fertilization (hpf), embryos raised at 28.5 C hatch from the chorion (external and acellular protective membrane), and become free-living animals with a complete body pattern, and almost completely functional organs (Kimmel et al. 1995). At this time, the innate immune system is already active (Cui et al. 2011), but the adaptive immune system will not be fully operating until 4–6 weeks post fertilization (wpf) (Lam et al. 2004), although expression of some genes of the adaptive immune system starts as early as eight days post fertilization (dpf) (Li et al. 2011). Therefore, the results of analyses carried out during the embryo-larval phases can be traced back to the innate immune system.

Zebrafish embryos are robust and can survive different procedures right after fertilization, including genetic manipulation, morpholino (Bill et al. 2009; Bedell et al. 2011; Timme-Lagary et al. 2012) or ribonucleoprotein (CRISPR/Cas) (Irion et al. 2014; Shah et al. 2015; Li et al. 2016; Albadri et al. 2017) microinjection at single cell stage, as well as cancer cell xenotransplants (Haldi et al. 2006; Taylor et al. 2009; Drabsch et al. 2017; Idilli et al. 2017; Wyatt et al. 2017; Roel et al. 2016). In addition, they are transparent, which gives them a definite advantage in many fields of study, because it makes

possible, for example, to examine the development of internal structures, and the tracking of the movements and biodistribution of labeled particles (microorganisms, cells, nanoparticles...) in real time (Roel et al. 2016; Tobin et al. 2012; Fenaroli et al. 2014; Teijeiro-Valiño et al. 2017). Visualization can be hampered by the early production of melanin during their embryonic development, as early as 24 hpf (prim5 developmental stage). However, melanin production can be easily blocked by treating the embryos with 1-phenyl 2-thiourea (PTU) (Kimmel et al. 1995). Additionally, the small size of zebrafish embryos (assays can be performed in 96 or, less suitably, in 384 multi-well plates), and the fact that they can live in small volumes (so that low quantities of the tested compounds are required) make this a suitable model for high-throughput analyses (Liu et al. 2012; Lin et al. 2013). An adult also transparent line (casper) was developed (White et al. 2008), which allows for carrying out similar analyses in adults (White et al. 2013a; White et al. 2013b; Dang et al. 2016; Tang et al. 2016).

Finally, the European Food Safety Administration (EFSA, 2005) has stated that fish in these early developmental stages, up to 5 dpf, are less likely to experience pain, suffering, distress, or suffer lasting harm, in accordance with the 3Rs Principles (replacement, reduction, and refinement) for humane animal research (Russell and Burch, 1959).

Therefore, taking all these facts into consideration, zebrafish has been accepted as a suitable model for biomedical purposes, for it could provide results faster than research on non-transparent, less prolific, more time-consuming, and expensive rodents, and improve the biological interpretation of the results compared to working on invertebrate models, which are phylogenetically further from human beings, and from in vitro analyses, which lack body interactions.

4. Zebrafish is currently being used for the development of anticancer therapeutics

The pathological mechanisms underlying cancer are some of the most challenging processes to understand because of their variety and complexity. Zebrafish is considered a complementary model to murine and other previous models for the study of the genetic basis of cancer and for the evaluation of carcinogenic and novel antitumoral compounds in drug discovery (Stern and Zon, 2003; Goessling et al 2007; MacRae and Peterson, 2015; Deveau et al. 2017; Van Rooijen et al. 2017; Zhao et al. 2015; Lenis-Rojas et al. 2016; Penas et al. 2016; Blackburn et al. 2014; Taj et al. 2013; Veinotte et al. 2014).

Zebrafish has proven to be a good model to predict adverse drug effects during animal preclinical and human clinical data (Berghmans et al. 2008). This is because many of the cellular and molecular mechanisms involved in zebrafish's response to toxicity or stress are similar to those of mammals (Yang et al. 2007; Simmons et al. 2009). The publication of the DNA sequence of the zebrafish genome confirmed that relevant molecular pathways, including those implicated in cancer, are similar to those of mammals (Howe et al. 2013), which made zebrafish an attractive choice for cancer research (Phillips et al. 2014; Blackburn et al. 2014; Barbazuk et al. 2000; Huiting et al. 2015). A parallel approach for modeling cancer has been the (xeno) transplant of human cancer cells into zebrafish embryos, which led to the development of the so-called xenografted embryos. The proliferation, spreading and metastasizing of microinjected cancer cells is possible because the zebrafish embryos lack an adaptive immune system. Since the first successful model in 2005 and further improvements in 2006 (Haldi et al. 2006; Lee et al. 2005), different xenograft zebrafish models have been reported bearing either commercial human cancer cell lines or primary tumor cells, including cancers from different origins (i.e., melanoma, breast carcinoma, colorectal, pancreatic, ovarian, kidney, lung, oral, prostate, leukemia, etc.) (Haldi et al. 2006; Drabsch et al. 2017; Roel et al. 2016; Marques

et al. 2009; Bansal et al. 2014; Zhang et al. 2014; Brown et al. 2017; Mort et al. 2015.

As indicated above, zebrafish cancer models have been used for novel drug screening, as well as for reanalysis of known drugs (Stern and Zon, 2003; Deveau et al. 2017; Penas et al. 2016; Blackburn et al. 2014; Tat et al. 2013; Veinotte et al. 2014; Huiting et al. 2015; Xie et al. 2015). Nevertheless, due to the nanotechnology revolution on anticancer drug delivery, as stated in Section 1, recent studies also highlight the potential of zebrafish for the evaluation of novel anticancer nanomedicines. Most studies measured the toxicity and safety of blank nanoparticles (i.e., prior to drug incorporation) using different procedures, but also covered morphological descriptions of zebrafish after administration of sub lethal doses, and experiments of gene expression (Lee et al. 2017; Kim and Tanguay, 2013; Harper et al. 2015; Jeong et al. 2015). Taking advantage of the embryo transparency, biodistribution studies have also been performed to determine the ability of the nanocarriers to reach the target site, and even surpass complex biological barriers, such as the blood–brain barrier (Yang et al. 2015; li et al. 2017; Sieber et al. 2017).

Apart from determining these critical parameters, the zebrafish xenograft model has also been proven useful in the study of the interaction between drug-loaded nanocarriers and xenografted cells, for example when studying a possible reduction in the population of cancer cells (Lee et al. 2017; Evensen et al. 2016; Wehmas et al. 2016; Yang et al. 2016).

Among others, it is worth mentioning Yang and collaborators' studies (Yang et al 2016) that describe the interaction of targeted doxorubicin-loaded liposomes with HeLa cells, and the efficiency of this strategy in a xenograft model of zebrafish, and also the work of Evensen and collaborators (Evensen et al. 2016) that describes the ability of PEGylated nanocarriers to avoid uptake by macrophages, a fact that translates in improvedcirculation times and increased accumulation into the tumors. Figure 2 depicts a visual example of liposomes labeled in green and distributed along the fish blood vessels upon injection into thecirculation (A) and their subsequent uptake by macrophages labeled in red (yellow dots).

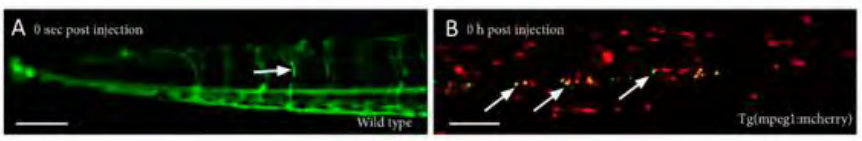

Figure 2. Green-labeled liposomes, injected into the circulatory system of wild type zebrafish embryos (A), allows the visualization of the fluorescent liposomes in the fish vasculare. On the right, the tg(mpeg1mecherry) model (B) shows the uptake of

the fluorescent green liposomes by fluorescent red circulating macrofagues (yellow dots). Imaging adapted from the work of Evensen et al. (2016) with permission.

5. The potential of zebrafish for increasing the translation of genetic anticancer nanomedicines: barriers and models

Apart from the use of zebrafish for the development of novel cancer therapeutics, including nanotherapeutics, only a few studies have been reported using this model to test preclinical genetic nanomedicines (Xu et al. 2016; Aldrian et al. 2017; Cordeiro et al. 2017). The first study found in the literature evaluates a synergistic therapy based on the co-encapsulation of a pigment-epithelium-derived factor (PEDF) plasmid with paclitaxel, a small molecular chemotherapeutic drug, into poly(lactic-co-glycolic acid) (PLGA) nanoparticles, in a transgenic zebrafish model Flk-1:eGFP. The results showed an active targeting that translates into an effective and safe antiangiogenic therapy (Xu et al. 2016). The second example covers the development of a retro-inverse amphipathic RICK (retro-inverse form of the CADY-K peptide) peptide as novel non-covalent siRNA carrier. The designed nanoparticles show an effective siRNA protection, based on the specific protease resistant peptide sequence. The authors investigated the effect of a polyethylene glycol (PEG)

grafting to RICK nanoparticles on their in vitro and in vivo capacity to deliver siRNA. In vivo assays performed in Casper zebrafish followed the biodistribution of fluorescent-labeled nanoparticles after injection at the one-cell stage in zebrafish embryos. The authors described amodular, easy-to-handle drug delivery system that could be adapted to other types of functional moieties in order to develop safe and biocompatible delivery systems for the clinical application of RNAi-based cancer therapeutics (Aldrian et al. 2017). Finally, Cordeiro et al. 2017 reported the design of a gold nanobeacon able to silence enhanced green fluorescence protein (EGFP) in embryos of a fli-EGFP transgenic zebrafish line. Results in this model allowed the authors to conclude that they have developed a biocompatible and efficient nanoplatform for gene silencing purposes.

As illustrated in Figure 3, a closer evaluation of the in vivo performance of genetic nanomedicines and a detailed study of their ability to overcome the critical barriers that might hamper a successful therapy are key factors in order to speed up their translation to clinic. Next, we describe the most relevant barriers to gene delivery, and the zebrafish models that, in our understanding, can be useful for a rational design of successful anticancer genetic nanomedicines (compiled in Table 2).

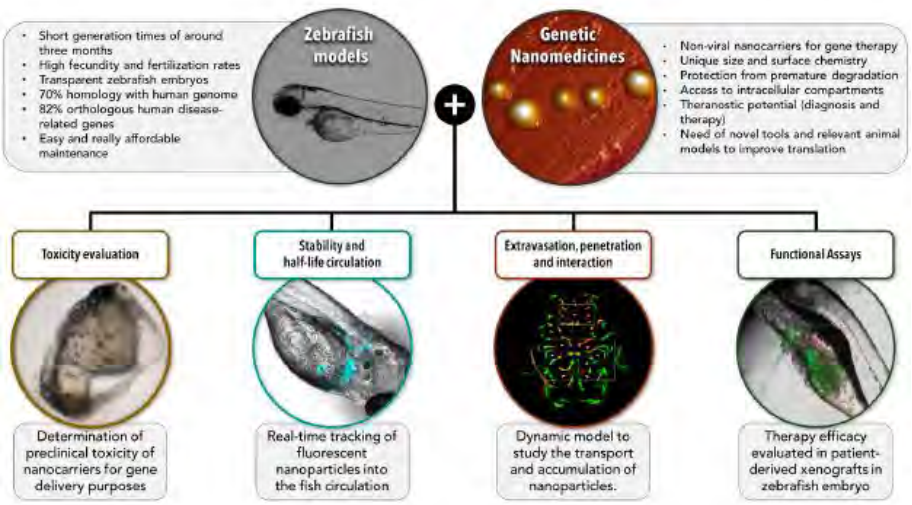

Figure 3. Zebrafish as a organism for preclinical studies of genetic nanomedicines. This scheme highlights the main characteristics of zebrafish as model organisms and the main advantages of nanomedicines for gene delivery. The scope of this review is summarized in the lower section of the figure where we have illustrated different ways in which zebrafish models can be extremely useful to help us understand the biological behaviour of genetic nanomedicines, and define better prototypes with improved opportunities of translation to a clinical setting. Zebrafish models would allow performing several assays of interest such as of the stability and half-life circulation of nanomedicines inyected in the fish circulation system, (i) evaluation of the toxicological profile, (ii) determination (iii) study of the ability of nanomedicines to extravasate, difuse, penetrate into the tumor, and interact with the targeted cells, and (iv) functional assays to test the potential and the efficacy of the proposed nanomedicines. The two images on top correspond (~100 nm) lipidic nanoemulsions observed by atomic force microscopy (AFM) (right). Images in the low part of the figure correspond, from left to right, to 48 hpf malformed zebrafish embryo to toxic effects of nanocapsules (image reproduced with permission Teijeiro-Valiño et al.

(2017), fluorescent DiD-labelled lipidic nanoemulsions injected into the fish circulation system and observed under a fluorescence microscope (images adquired at 48 h post-injection), fluorescent nanoparticles (red) able to extravasate blood vessels (green) in a zebrafish model (image obtained by confocal microscopy by to a zebrafish embryo (left), and to nanometric Zou et al. (2017), and reproduced with permission), and fluorescent DiD-labelled lipidic nanoemulsions (red) able to interact with cancer cells (green) in xenotransplanted zebrafish embryos (HCT116-GFP) after yolk microinjection (Gutiérrez-Lovera et al. 2017. From Genes, Open Access).

5.1. Toxicity

Despite the ability of the nanoparticles to reduce the side effects of the associated drugs, adverse effects due to the nanoparticles themselves have been reported in some clinical studies, including immunotoxicity (allergy, hyper-sensitivity, and immunosuppression), acute toxicity (i.e., single-dose studies), subacute toxicity (i.e., repeated-dose studies or semi-chronic toxicity studies), carcinogenicity, reproductive toxicity, developmental toxicity, genotoxicity, hepatotoxicity or epigenotoxicity (Collins et al. 2017; Brand et al. 2017; Giannakou et al. 2016; Omidi et al. 2005; Poma et al. 2008; Shah et al. 2013). Nanoparticles may also activate innate immunity responses in the body and, as a consequence, they can mediate an uncontrolled delivery of pro-inflammatory mediators

(anaphylatoxins) that could nullify the therapeutic effect of the nanocarrier and, even worse, promote tumor growth (Anchordoquy et al. 2017). In the case of genetic nanomedicines, they typically contain cationic elements to improve their association with the anionic nucleic acids. These positively charged biomaterials have also been related to toxicity and off-target unspecific effects after transfection. Toxicity in preclinical studies relies mainly on simple and conventional tests (e.g., MTT assay), and, in some cases, systemic toxicity in vivo (e.g., serological and biochemical analysis of blood samples in mice). Therefore, it is clear that toxicity needs further attention before we can proceed to clinical studies.

As mentioned in Section 4, zebrafish is widely used for the evaluation of the adverse effects of drugs, and to determine the activity of antitumor compounds (Stern and Zon, 2003; Goessling et al 2007; MacRae and Peterson, 2015; Deveau et al. 2017; Van Rooijen et al. 2017; Zhao et al. 2015; Lenis-Rojas et al. 2016; Penas et al. 2016; Blackburn et al. 2014; Taj et al. 2013; Veinotte et al. 2014. It could also be used to determine the preclinical toxicity of nanocarriers for gene delivery purposes. The most common and simple toxicity studies in wild type zebrafish relate to acute and chronic effects. Protocols for these studies have already been approved by the Organization for Economic Co-operation and Development (OECD). To determine zebrafish embryo toxicity, post fertilization embryos are

placed in a static plate and exposed to the compound. The rate of morphological changes is one of the endpoints used to generate dose response curves (OECD Guideline No. 220, 2004; OECD Guideline No. 236, 2013). The toxicity of several types of nanoparticles, mainly inorganic nanoparticles, has already been determined in zebrafish using this test (Lee et al. 2017; Lenis-Rojas et al. 2017; Evensen et al. 2016; Fent et al. 2010; Garcia et al. 2016). One important parameter for toxicity evaluation is the hatching efficiency because nanoparticles can interact with hatching enzymes (Ong et al. 2014). Zebrafish is also a versatile organism for genotoxicity studies (Sukardi et al. 2010; Hussainzada et al. 2014; Geffroy et al. 2012; Segura-Aguilar et al. 2006), developmental and behavioral analysis (MacPhail et al. 2011; Truong et al. 2012; Usenko et al. 2007; Vibe et al. 2016), immunotoxicity (Zhuang et al. 2015; Xu et al. 2015), neurotoxicity (Daroczi et al. 2006; Sheng et al. 2016), and reproductive toxicity studies (Wang et al. 2011). For example, in experiments with transgenic lines, such as Tg(flk1:eGFP), Tg(cmlc2:eGFP), Hsp70:eGFP, ARE:eGFP, FLI-1, and Nacre/fli1:EGFP, it was possible to observe the chemical-induced toxicity of nanocomposites and metal oxide nanoparticles in real time (Jang et al. 2016; Lin et al. 2011; Chang et al. 2015; Bar-Ilan et al. 2012; Zhang et al. 2012).

Zebrafish is also an excellent model to provide novel insights on the interaction between the immune system and tumor cells (Powell et al. 2016; Chambers et al. 2013). Because in zebrafish, macrophages play an important role in angiogenesis, this model could also be used to develop functional assays related to the angiogenic process (Section 5.4). A transgenic zebrafish line, mpo:GFP, which expresses GFP under the neutrophil-specific myeloperoxidase promoter, has also been described and used to study neutrophil response (Renshaw et al. 2006), including the evaluation of oxidative stress and inflammatory responses in neutrophils following the administration of silica nanoparticles (Duan et al. 2016). In addition, studies regarding cardiotoxicity are also of great importance, among them is worth mentioning the evaluation of effects occurring immediately after administration and their consequences (Vibe et al. 2016).

5.2. Stability and half-life while in circulation

Preclinical studies sometimes ignore the fact that the electrostatic stability of nanocarriers in vitro does not guarantee their stability in vivo. Moreover, in many cases, the nanocarrier and the gene vector are associated by electrostatic interactions. Upon contact with a biological media of high ionic strength, this system may aggregate, resulting in the displacement of the nucleic acids that could be

prematurely released into the circulation before reaching the target cells. The presence of serum proteins (e.g., glycosaminoglycans) could have the same effect. Therefore, a thorough study, relevant in vivo models, of the stability and interactions of the nanocarrier under study could be necessary to ensure that the associated nucleic acids are not prematurely released into the circulation (Boushehri et al. 2015; Nguyen et al. 2017; Jain et al. 2017). On the other hand, nanosystems should also be able to avoid recognition by macrophages, and a rapid clearance by the mononuclear phagocyte system (MPS), which would lead to their fast removal from circulation (Giannakou et al. 2016).

As stated in Section 2, one of the main advantages of zebrafish embryos and adults from the Casper line is that they are transparent, and therefore suitable for direct and real-time tracking of fluorescent nanoparticles into the fish circulation, using high-resolution confocal microscopy (White et al. 2008). Importantly, a recent study shows a good correlation among pharmacokinetic data obtained in zebrafish, rat, and mice, and highlights the potential of zebrafish for this purpose (Sieber et al 2017). Different studies carried but with model nanoparticles, FluoSpheres® and Quantum Dots® , highlight the influence of the exposure route (waterborne, injection and oral), and surface properties of the nanoparticles on their biodistribution and tumor uptake (Harper et al. 2007).

One model useful for tracking the circulation of nanoparticles is the transgenic line Fli1:eGFP (Evensen et al. 2016). This line has allowed for following the distribution and tumor accumulation of PEGylated nanoparticles. In the same study, the Tg(mpeg1:mCherry) line was selected to evaluate the interaction of these nanoparticles with macrophages, which led to the conclusion that PEG coating actually decreased the interaction of the nanoparticles with macrophages. Transgenic lines of macrophages, neutrophils, and endothelial cells expressing fluorescent markers (see Table 2) have also been used to watch the interaction between lipid nanoparticles and immune cells (Fenaroli et al. 2014).

5.3. Extravasation, penetration into the tumor, and interaction with the target cells

Nanocarriers should be able to exit the systemic circulation at the action site. Recently, it has been reported that current animal models fail to predict the accumulation of nanocarriers inside the tumor, which is actually about 0.7% of the injected dose (Wilhelm et al. 2016; Torrice, 2016). Thus, animal models that would allow us to better study the ability of nanocarriers in this step are crucial to ensuring an effective therapeutic effect (Muntimadugu et al. 2017). The complexity of the tumor extracellular matrix (ECM) may also restrict the extravasation of the nanocarriers. Additionally, even if

the nanocarriers could cross the tumor vasculature, they might not be able to penetrate deep enough inside the tumor mass due to the high interstitial fluid pressure, and might accumulate instead in the peripheral areas, or in the surrounding healthy tissue (Stylianopoulos and Jain, 2015). Finally, the nanoparticles need to interact with the target cells. Typically, therapies are directed at tumor cells, but they can also be designed to target cells of the stroma or to infiltrate immune cells, cancer stem cells (CSCs), cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), pericytes, endothelial cells, etc (Kuninty et al. 2016).

To date, an extensive list of improved zebrafish cancer models has been reported, including models to study neuroblastoma, brain cancer, eye cancer, leukemia, melanoma, uveal melanoma, and liver cancer, among others (Kirchberger et al. 2017). More complex models to study the mechanisms of tumor cell dissemination and metastases formation have also been reported (Wang et al. 2015; Rouhi et al. 2010). For example, the model Flk1:EGFP has been used to study the metastatic spread after injection of red fluorescent protein (RFP)-labeled Hela cells in the caudal artery (Stoletov et al. 2007). Other results show how metastatic cell lines have improved abilities to migrate and proliferate compared to cells isolated from primary tumors (Van der ent et al. 2015). The study of CSC has also been considered in zebrafish models (Zhang et al. 2014; Eguiara et al.

2011). Regarding the study of the tumor microenvironment (TME), Zhao et al. 2016 showed that transforming growth factor beta (TGF) induced a pro-tumor neutrophil cytokine expression pattern in zebrafish, and concluded that essential mechanisms in the constitution of the TME are conserved in this model.

Regarding the particular evaluation of nanomedicines, several works cover the evaluation of their ability to accumulate in tumor cells after injection in zebrafish xenografts (Li et al. 2017; Evensen et al. 2016; Wagner et al. 2010; Gong et al. 2013). Zebrafish can therefore be considered as a dynamic model to study the transport and accumulation of nanoparticles.

5.4. Functional assays

Performing functional assays, zebrafish models are very useful for determining the efficacy of the therapy. Importantly, it is feasible to use xenografts of patient-derived tumor cells in zebrafish embryos, to perform patient-specific drug screens, and analyze critical aspects of the tumor, such as growth and proliferation (Handi et al. 2006),

invasion and intravasation (Stoletov et al. 2007; Naber et al. 2013; Yang et al. 2013), formation of metastasis (Drabsh et al. 2017; He et al. 2012), angiogenesis (He et al. 2012; Tobia et al. 2013), and immune cell response (Tat et al. 2013). Hundreds of embryos can be injected in a single day, and it is possible to exploit the imaging capabilities of the zebrafish. Cell injections in fish can be performed in the duct of Cuvier, vein, and yolk sac, as well as pericardially, intracardiaally, and in the brain parenchyma, in order to obtain different read-outs. For example, since the yolk sac does not communicate with the vasculature directly, it would be a good model to study metastasis by either invasion or blood borne spreading (Brown et al. 2017). Additionally, to study specific phenomena such as angiogenesis, there are useful transgenic lines, such as Tg(Flk1:EGFP) and Tg(Fli1:EGFP) with green vasculature, and Tg(Gata1:DsRed) with red fluorescent blood cells (Tat et al. 2013). These models allow the study of the distribution and functionalities of nanoscale drug delivery systems (Stoletov et al. 2007). As an example, one study used curcumine-loaded micelles to test the potential of zebrafish for developing novel anti-angiogenic and antitumoral therapies (Gong et al. 2013). In a different work using silica nanoparticles, it was possible to observe inhibition of angiogenesis via vascular endothelial growth factor receptor 2 (VEGFR2)-mediated mitogen-activated protein kinase (MAPK)

signalling pathway [192]. Other authors have claimed a reduction in the number of tumor cells transplanted into fish, upon delivery of anti-tumor nanomedicines (Evensen et al. 2016; Wagner et al. 2010; jain et al. 2016). Additionally, it would be possible to determine whether nanoparticles carrying the proposed therapy induce apoptosis: a fluorescent probe designed to characterize patterns of apoptosis in living zebrafish larvae has recently been described (Van Ham et al. 2010).

Table 2. Selected zebrafish models of potential interest for the biological evaluation of genetic nanomedicines.

Model	Features	Application	Ref
Wild type	From nature, with pigmentation according to sex, without fluorescence	Toxicity, biodistribution, xenograft	Chakraborty et al. 2016
Flk-1:eGFP Fli-1:eGFP Gata1:DsRed Nacre/fli1:eGFP	Fluorescent vascular system	Toxicity, biodistribution, xenograft, angiogenesis, extravasation, half-life circulation, metastasis	Tat et al. 2013; Xu et al. 2016 Tat et al. 2013; Evensen et al. 2016 Tat et al. 2013 Zhang et al. 2012
Casper fli	Without pigmentation (transparent) and fluorescent vascular system		White et al. 2008
Casper	Without pigmentation (transparent)	Toxicity, biodistribution, xenograft, metastasis	White et al. 2008
ARE:eGFP	Fluorescence of reactive oxygen species (ROS)	Toxicity	Bar-Ilan et al. 2012
Cmlc2:eGFP	Fluorescence in the heart	Cardiotoxicity	Duan et al. 2016
Mpo:GFP Mpeg1:mcherry	Fluorescent neutrophils Fluorescent macrophages	Interaction, half-life circulation, immuno response	Duan et al. 2016 Evensen et al. 20176
Hsp70:eGFP	Fluorescence of the protein HSP70 stress product	Toxicity	Blechingeret al. 2002

CHAPTER III

Hypothesis and Objectives

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Hypothesis

The study of toxicity, biodistribution and the therapeutic effectiveness of antitumural nanosystems in zebrafish is based on the following hypotheses:

H1. It is possible to determinate phenotypically the level of toxicity produced by some commonly anticancer drugs used in xenografts studies in zebrafish embryos, using official and modified protocols.

H2. It is intended to evaluate the toxicity and efficacy of a newly developed nanoemulsion based on the anticancer drug edelfosine performing a study using high-resolution microscopy, wich will allow us to know the different routes of biodistribution of the nanosystems, as well as their interaction with the surrounding environment. Also xenotransplantation tests of tumor cells, in order to monitor the antitumor potential of nanosystems.

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Objectives

The main objective of this project is based on evaluating the therapeutic potential of different anticancer therapies, including innovative nanomedicines, in a model organism, the zebrafish. For this, specific objectives have been defined as following:

O1. Evaluation of the toxicity of some commonly used anticancer drugs in zebrafish embryos, and determination of specific toxicity parameters and toxicological profiles crucial for zebrafish xenograft studies.

O2. Evaluation of the toxicity and efficacy of a newly developed nanoemulsion based on the anticancer drug edelfosine in xenografted zebrafish models.

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CHAPTER IV

In vivo toxicity assays in zebrafish embryos: a pre-requisite for xenograft preclinical studies

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ABSTRACT

The human cancer cell xenograft in zebrafish embryos has become a very useful preclinical tool in oncology research. While many anticancer drugs have been assayed with this model, few studies regarding the toxicity limits of these drugs for the host have been addressed. Here, we evaluated the acute toxicity of five approved and routinely used human anticancer drugs embracing different mechanism action types: Carboplatin (CarboPt), Irinotecan (IT), Doxorubicin (DOX), Paclitaxel (PT) and Chloroquine (CQ). They were tested in zebrafish embryos using the Fish Embryo Acute Toxicity (FET) test at 0 and 72 hpf. Additionally, we compared those results with *in vitro* toxicity assays and could find notable differences between both models. Our results indicate that the toxicity data of a compound evaluated *in vitro* and in a FET test at 0 hpf do not guarantee a reliable toxicity determination for performing xenografts in zebrafish, being necessary additional toxicity studies using 72 hpf embryos, the starting point of drug treatment in this kind of preclinical assays.

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1. Introduction

During cancer progression, the inter- and intratumoral heterogeneity determines the different outcomes among patients; some of them respond to the standard treatment but many others need to try alternative therapies with significant by-side effects and general cytotoxicity. Conventional cancer treatments cause high morbidity and mortality and despite advances in targeted treatments, it is still very difficult to predict the tumor response to a given therapy for each patient (Fior et al. 2017). There is also a high proportion of compounds (approximately 50%) that fail in clinical trials due to their toxicity and clinical safety (Eimon and Rubinstein 2009). Thus, the improvement of current treatments, making them more specific and effective while reducing their toxicity, is still a priority.

The zebrafish model has emerged as a powerful *in vivo* tool in the oncology field due to its advantages in comparison with mice models. Among these, the most relevant are that zebrafish: (i) is a straightforward and low cost small animal model (Rojas-Muñoz, Miana, and Izpisúa-Belmonte 2007), (ii) has high fecundity and external fertilization (Kimmel et al. 1995), (iii) is optically transparent, (iv) has a short reproductive cycle, (v) is permeable to small molecules (Henn and Braunbeck 2011), and (vi) has a genome with high homology (~85%) with that of humans (McCollum et al.

2011; Renier et al. 2007) and with several organs and tissues resembling those of humans. All these features make possible large-scale assays and even high-throughput screening (Mimeault and Batra 2013).

The interest for the zebrafish xenografts have increased in the oncology research field since this fish provides a fast and cost-effective *in vivo* system to study tumorigenicity, metastatic capacity, and tumour response to multiple anticancer therapies (Wertman et al. 2016; Fior et al. 2017; Mimeault and Batra, 2013). Interestingly, early zebrafish embryos (until 11 days post-fertilization) lack mature immune system allowing human cancer cell xenotransplantation without previous immunosuppressive treatment and without immune rejection (Lam et al. 2004). Thus, this *in vivo* platform provides a very powerful preclinical tool for anticancer drug screening (with high-throughput scaling capacity) bringing closer the possibility for precision medicine (MacRae and Peterson 2015; Fior et al. 2017).

The human cancer cell xenograft assays, which involve the injection of melanoma cells into the yolk sac of zebrafish embryos, were first reported in 2005 (Lee et al. 2005). Although this xenograft technique has evolved since its origin, a standard protocol for xenograft assays is used in almost all studies nowadays (Nicoli and

Presta, 2007). Xenografted cells are injected at 2 days post fertilization (dpf) in different sites of the zebrafish embryo which are then incubated at 32-35ºC for 3-6 days (Eguiara et al. 2011; Ghotra et al. 2012; He et al. 2012; Ban et al. 2014; van der Ent et al. 2015). In particular, the drug screening of novel and ongoing compounds usually starts at 72 hours post fertilization (hpf) (Roel et al. 2016; Ikonomopoulou et al. 2018). This time is chosen because zebrafish embryos can recover from the injection while at the same time being acclimated to the new incubation temperature. In most cases, the tested compounds lack toxicity in assays performed at this embryo developmental stage, since the majority of the toxicity studies are performed *in vitro* with cell lines and in 0 hpf zebrafish embryos, following the Fish Embryo Acute Toxicity (FET) test (OECD Guidelines for the testing of chemicals, 2013).

It is generally acknowledged that the studies using zebrafish xenografts to analyze anticancer drugs lack an appropriate toxicity test for the compound (Tonon et al. 2016; Y. Zhong et al. 2016; Zhang et al. 2014; Jo et al. 2013). In this context, and despite being highly used for cancer studies, no toxicity assay has been performed for known and used anticancer drugs in zebrafish xenografts, which in many cases are used as positive controls during the development of new anticancer compounds. For this reason, the aim of this study is to evaluate the toxicity in zebrafish embryos of five commonly used

anticancer drugs determining their toxicity parameters and toxicological profiles, crucial for zebrafish xenograft studies. To accomplish this, we used the Fish Embryo Acute Toxicity (FET) test, the most used toxicity assay for zebrafish embryos. It is based on testing compounds dissolved in the egg water for 96 h on embryos starting at 0 hpf, estimating mortality and morphological abnormalities. To analyze the discrepancies in toxicity, if any, between 0 and 72 hpf embryos, we assayed and compared the toxicity (LC50) of four of the most used anticancer drugs (CarboPT, IT, DOX and PT), and a recently discovered drug with anticancer properties (CQ) in 0 hpf and 72 hpf embryos until 96 h. All these measurements were contrasted with *in vitro* viability assays using commonly used tumor cell lines and patient derived xenografts (PDX).

2. Materials and methods

2.1. Experimental animals and handling

Wild-type zebrafish (*Danio rerio*) were used in this study. Fish were maintained in a controlled aquatic facility with reverse osmosis purified and dechlorinated water and with the following conditions:

27ºC (± 1ºC), pH 7 (± 0.5), 14h light/10h dark light cycle. Adult fish were fed three times a day (Westerfield 2007). After mating, 0-4h post fertilization (hpf) embryos were collected in Petri dishes and washed with osmosis water. Viable embryos, selected after inspection under an optical microscope (Nikon TMS), were used to test different concentrations of selected compounds at 27ºC (±1ºC).

All these procedures were approved by the Bioethics Committee for animal experimentation of the University of Santiago of Compostela (CEEA-LU).

2.2. Drugs

Compounds used in this study are described in table 3. For each compound, a previous toxicity test was performed to set up the concentration range, from the highest innocuous to the lowest lethal concentration. Evaluated ranges in 0-4 hpf embryos: CarboPt, 62,5 τM – 1000 τM; IT, 2 τM – 32 τM; DOX, 4 τM – 33 τM; PT, 0,5 τM - 8τM; and CQ, 100 τM – 1000 τM. Evaluated ranges in 72 hpf embryos: CarboPt, 2000 τM – 8000 τM; IT, 7 τM – 112 τM; DOX, 7 τM – 15 τM; PT, 0,002 τM – 0,188 τM and CQ, 50 τM – 250 τM.

Table 3. Summary table of the anticancer drugs analysed in this study.

Anticancer drug	Abbreviation	CAS no.	Manufacturer	Mechanism of action
Carboplatin (371.25 g/mol)*	CarboPt	41575-94-4	TEVA Pharma	Platin complex (base alkylant)
Irinotecan (623.14 g/mol)*	IT	100286-90-6	Hospira	Camptothecin analog (topoisomerase binding)
Doxorubicin (543.52 g/mol)*	DOX	23214-92-8	Accord	Anthracycline (topoisomerase binding)
Paclitaxel (853.91 g/mol)*	PT	33069-62-4	Hospira	Taxan (antimicrotubule agent)
Chloroquine (515.86 g/mol)*	CQ	50-63-5	Sigma	Cell signaling inhibitor

*Molecular weight of the analysed anticancer drugs.

2.3. Embryos treatment

Embryos (0-4 hpf and hatched 72 hpf) were incubated with different drug concentrations dissolved in osmosis water together with corresponding controls in 24-well plates during 96 h.

2.4. Acute Toxicity Assay

We used the official Fish Embryo Acute Toxicity (FET) test (OECD 2013). At least three replicates were performed, for each one

20 embryos were used per drug concentration (60 embryos in total) and 24 embryos were used as a negative control (with osmosis water). Internal controls were also used make certain that plate conditions were not altered during incubation time. Embryos were inspected under inverted optical microscope (Nikon TMS) at 24, 48, 72 and 96 h of treatment. To determine the embryo lethality, the microscope observations were focused in: coagulation of embryos, lack of somite formation and non-detachment of the tail, after 24, 48, 72 and 96 h; lack of heartbeat after 48, 72 and 96 h and hatching rates at 96h, according to FET test indications. Developmental alterations and embryo malformations were also recorded (see Figure 4).

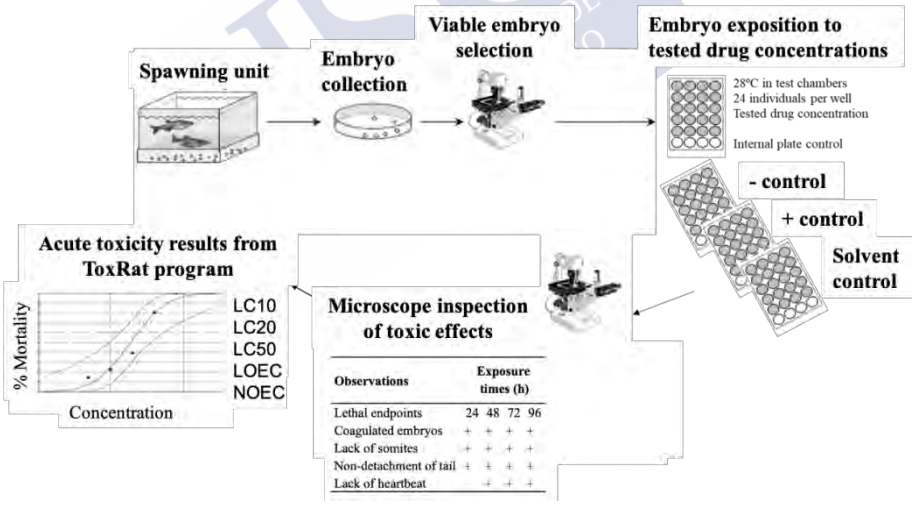

Figure 4. Fish Embryo Acute Toxicity (FET) test scheme (Gutiérrez-Lovera et al. 2019. From Toxicology Mechanisms & Methods, Open Access).

For the toxicity test with hatched embryos (72 hpf), three replicates were performed. For each replicate, 20 embryos were used in each concentration and 24 embryos were used as a negative control. Internal controls were also used as it is indicated above. Embryos were inspected under inverted optical microscope at 24, 48, 72 and 96 h after the treatment to analyze malformations, developmental abnormalities and mortality of treated embryos.

2.5. Cell culture

A549 (human lung carcinoma cell line) and MCF7 cells (human breast adenocarcinoma cell line) were cultured in DMEM medium (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum) and 1% penicillin-streptomycin mixture (all from GibcoTM) at 37ºC, and 5% CO2. Panc185 (pancreatic ductal adenocarcinoma patient derived xenografts-PDX cancer cells) were cultured as described above but using RPMI medium instead of DMEM (Mueller et al. 2009).

For cell viability assays, 1.0 10^4 MCF7 and A549 cells/well were seeded in 96-well culture plates and cultured for 24 h. Then, cells were treated with different concentrations of anticancer drugs.

Panc185 cells were seeded at a density of 5.0 10^3 cells/well and incubated in normal conditions for 5 days before the treatment.

For confocal microscopy analyses A549 cells were seeded on coverslips in six well plates at a density of 3.0 10^5 cells/well and maintained at 37°C and 5% CO2 for 24 h before treatment.

2.6. Cell viability assay

Cell viability was analyzed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. MCF7 and A549 cells were treated with: 0.002 ɀ M – 20 mM CarboPt, 1.6 ɀ M – 5 mM IT, 0.02 ɀ M – 312.5 ɀ M DOX, 0.001 ɀ M – 78.125 ɀ M PT, and 4.8 ɀ M – 15 mM CQ. Panc185 cells were treated with: 1.6 mM – 27 mM CarboPt, 0.045 ɀ M – 32 mM IT, 0.002 ɀ M – 2.34 mM DOX, 0.04 ɀ M – 125 ɀ M PT, and 0.064 ɀ M – 48 mM CQ. For each cell line and drug tested, three replicates were performed.

After 24 h of drug treatment, cells were washed with PBS and then incubated in serum-free DMEM medium with 10 % of diluted MTT dye solution (5 mg/mL, Alfa Aesar, Germany). After 4 h the MTT solution was removed and formazan crystals were dissolved in

DMSO incubating for 10 minutes at 37ºC (protected from light). Finally, the absorbance at 570 nm was measured using a microplate spectrophotometer (DTX 880 Multimode Detector, Beckman Coulter). The half maximum inhibitory concentration (IC50) for each compound tested was determined by nonlinear regression analysis (dose-response inhibition equation), using GraphPad Prism v. 7.00 (GraphPad Software Inc. CA).

2.7. Mito Tracker staining

Cells were incubated with 0.125 and 3.125 ɀ M paclitaxel for 24 h. Cells were then washed three times with PBS. After washing, staining solution containing MitoTracker ® probe (500 nM) was added and incubated for 30 min. Finally, cells were washed three times with PBS and analyzed by confocal microscopy (Leica DMi8) with excitation at 579 nm and emission at 599 nm.

2.8. Statistical analyses

Statistical analysis of the acute toxicity results were performed using probit analyses with the ToxRat program (ToxRat Solutions. ToxRat® 2003) in accordance with OECD, guideline 236 (OECD 2013).

We calculated the 10% lethal concentration (LC10), 50% lethal concentration (LC50), lowest observed effect concentration (LOEC) and no-observable-effect concentration (NOEC). Test duration was 96h, the measurement interval was 24h, and the measurement variable was embryos survival. LC50 was determined via probit analysis using linear maximum likelihood regression with survival at 96h and with 95%-confidence limits. Qualitative trend analysis by contrasts (monotonicity of concentration/response), step-down Cochran-Armitage test (to check variance homogeneity) and Tarone's test (to check for extra-binomial variance) were performed to determine NOEC and LOEC values with 95%-confidence limits.

Tests were considered valid whether the mortality of fish embryos in the negative control was less than 10% and the mortality in the positive control was more than 30%.

3. Results

3.1. Acute Toxicity Assay

3.1.1.Embryos of 0-4 hpf

A dose dependent increase in mortality was observed in embryos treated with the tested drugs. The most toxic anticancer drug was PT (LC10: 0.6 ɀ M, LC50: 1.9 ɀ M, NOEC: < 0.5 ɀ M, LOEC: <0.5 ɀ M), followed by IT (LC10: 3.4 ɀ M, LC50: 7.1 ɀ M, NOEC: < 4 ɀ M, LOEC: < 2 ɀ M), DOX (LC10: 8.1 ɀ M, LC50: 11.3 ɀ M, NOEC: 7 ɀ M, LOEC: 4 ɀ M), CQ (LC50: 241.2 ɀ M, NOEC: < 100 ɀ M, LOEC: < 100 ɀ M), and CarboPt (LC10: 169.7 ɀ M, LC50: 330.1 ɀ M, NOEC: 250 ɀ M, LOEC: 125 ɀ M) (see Table 4). A great difference was observed between LC50 values for PT and CarboPt (170 fold).

Table 4. FET OECD test results in 0-4 hpf embryos.

Anticancer drug*	LC10	LC50	NOEC	LOEC
CarboPt	169.7	330.1	250	125
IT	3.4	7.1	4	2
DOX	8.1	11.3	7	4
PT	0.6	1.9	<0.500	<0.500
CQ	n.d.	241.2	<100	<100

*Drug concentrations are expressed in ɀ M.

The embryos exposed to the drugs showed a hatching rate below 100% at the maximum tested concentrations compared to controls which showed 100% hatching at 72 hpf (data not shown). A case to be highlighted is CarboPt, which we evaluated at concentrations up to 1000 ɀ M. At this concentration, 144 hpf embryos remained alive but within the chorion while normal embryos hatched at 48-96 hpf. This result points to a possible hardening of the chorion due to the presence of the drug. In addition to hatching effects, the 80% of the embryos exposed to 8 ɀM CarboPt showed body deformities after 48 h. We observed body curvature and progressive embryos disintegration until death. For the other drugs, no significant embryonic malformations or developmental abnormalities were observed during the evaluation until the end point.

3.1.2. Embryos of 72 hpf

Then, we performed a FET test for anticancer drugs in 72 hpf embryos, time when embryos are drug treated in most xenograft studies. A concentration-dependent mortality was also observed. As shown in Table 5, PT remained the most toxic compound (LC10: 0.01 ɀ M, LC50: 0.05 ɀ M, NOEC: 0.01 ɀ M, LOEC: 0.002 ɀ M),

as in the toxicity assays with 0-4 hpf embryos. Also, CarboPt was the least toxic of these drugs (LC10: 2126.6 τM, LC50: 3247.2 τM, NOEC: 2000 τM, LOEC: 2500 τM), followed by CQ (LC10: 74.1 τM, LC50: 116.4 τM, NOEC: 100 τM, LOEC: 50 τM). The toxicity results for IT were: LC10: 16.9 τM, LC50: 34.8 τM, NOEC: 14 τM, LOEC: 7 τM and for DOX were: LC10: 8.4 τM, LC50: 10.3 τM, NOEC: 9 τM, LOEC: 7 τM. We observed 72 hpf embryos were more sensible to PT and CQ than 0-4 hpf embryos. PT LC50 was 1.9 τM in 0-4 hpf embryos and 0.05 τM in 72 hpf embryos. LC50 of CQ was 241.2 τM in 0-4 hpf embryos and 116.4 τM in 72 hpf embryos. On the contrary 72 hpf embryos were more resistant to CarboPt and IT than those treated at 0-4 hpf. The LC50 values for CarboPt were 330.1 τM vs 3247.2 τM and for IT, 7.1 τM vs 34.8 τM at 0-4 hpf and 72 hpf respectively. DOX results showed no major changes. Thus, the toxicity values for most of the tested drugs were different at 0-4 hpf and 72 hpf embryos (See Table 4, 5 and Figure 5). In accordance with the OECD guideline, embryonic malformations and developmental abnormalities were evaluated during the entire experiment without finding significant alterations.

Table 5. FET OECD test results in 72 hpf embryos.

Anticancer drug*	LC10	LC50	NOEC	LOEC
CarboPt	2126.6	3247.2	2000	2500
IT	16.9	34.8	14	7
DOX	8.4	10.3	9	7
PT	0.01	0.05	0.01	0.002
CQ	74.1	116.4	100	50

*Drug concentrations are expressed in ɀM.

3.2. Cell viability assay

To compare the *in vivo* toxicity data obtained by the FET test with that of cell cultures we performed MTT assays. We used established human cancer cell lines (A549 and MCF7), and the PDX cell line Panc185 to have a broad spectrum of *in vitro* toxicity data. MCF7 cells were the most sensitive to all the anticancer drugs. As in zebrafish embryos, we found PT was the most toxic drug (A549 IC50: 0.4 ɀM ± 1.46, MCF7 IC50: 0.2 ɀM ± 1.47 and Panc185 IC50: IC50: 0.04 ɀM ± 2.09) while CarboPT was the least toxic (A549 IC50: 1653 ɀM ± 1.12, MCF7 IC50: 143 ɀM ± 1.27 and Panc185 IC50: 10513 ɀM ± 1.05) but very close to IT in MCF7 cells (See Figure 6 and Table 6). The *in vivo* and *in vitro* toxicity was very different (See Tables 4, 5 and 6), making hard to establish a correlation between *in vitro* and *in vivo* toxicity data.

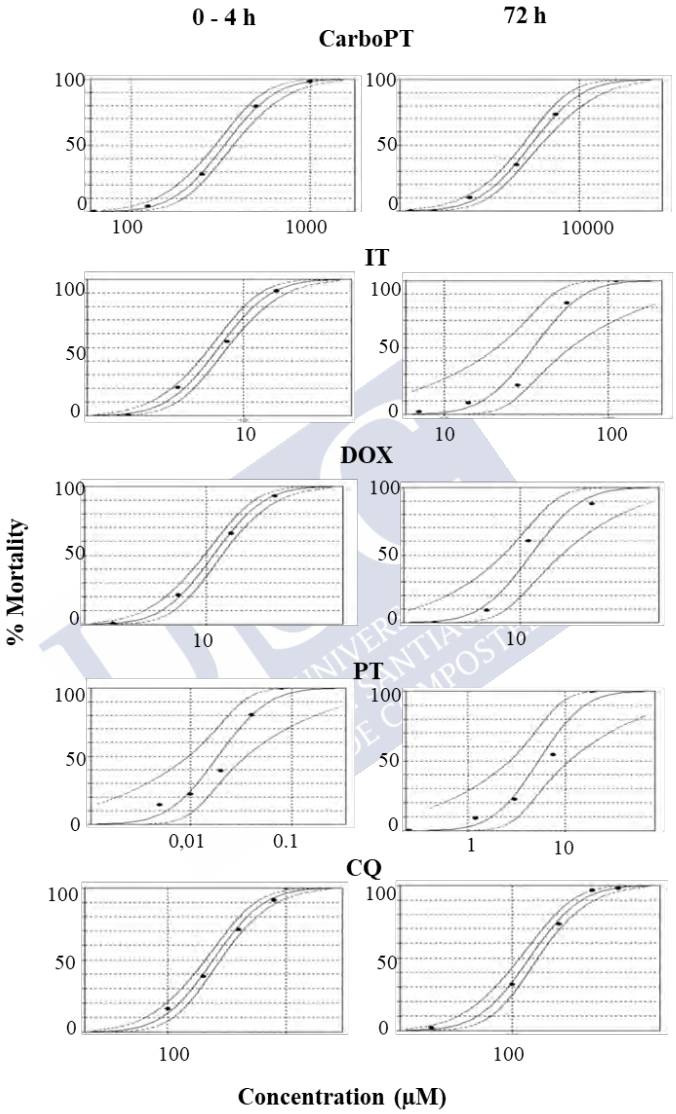

Figure 5. Toxicities of CarboPT, IT, DOX, PT and CQ at different stages of development (0-4 hpf and 72 h). Concentration-effect curves of the tested drugs on embryo survival after 96 hs (Gutiérrez-Lovera et al. 2019. From Toxicology Mechanisms & Methods, Open Access).

Table 6. IC50 **values of the anticancer drugs in different tumour cell lines**

Anticancer drug	Cell line		
	A549	MCF7	PDX Panc185
CarboPt	1653 ± 1.12	143 ± 1.27	10513 ± 1.05
IT	712 ± 1.09	187 ± 1.07	3755 ± 1.37
DOX	31 ± 1.14	1 ± 1.08	745.9 ± 1.42
PT	0.4 ± 1.46	0.2 ± 1.47	0.039 ± 2.09
CQ	41 ± 1.05	23.72 ± 1.06	246.8 ± 1.16

*IC50 values expressed in μM (IC50 ± SEM).

As shown in figure 6, MTT analysis of PT showed an atypical dose-response inhibition curve. Higher concentrations (such as: 0,625 ꚍM; 3,125 ꚍM or 15,625 ꚍM) produced an apparent lower cell mortality. We performed additional MTT assays (See Table 3) testing cell toxicity at 48 and 72 hpt (hours post treatment) finding the same trend observed at 24 hs, cells seemed more resistant to higher concentrations (See Figure 7).

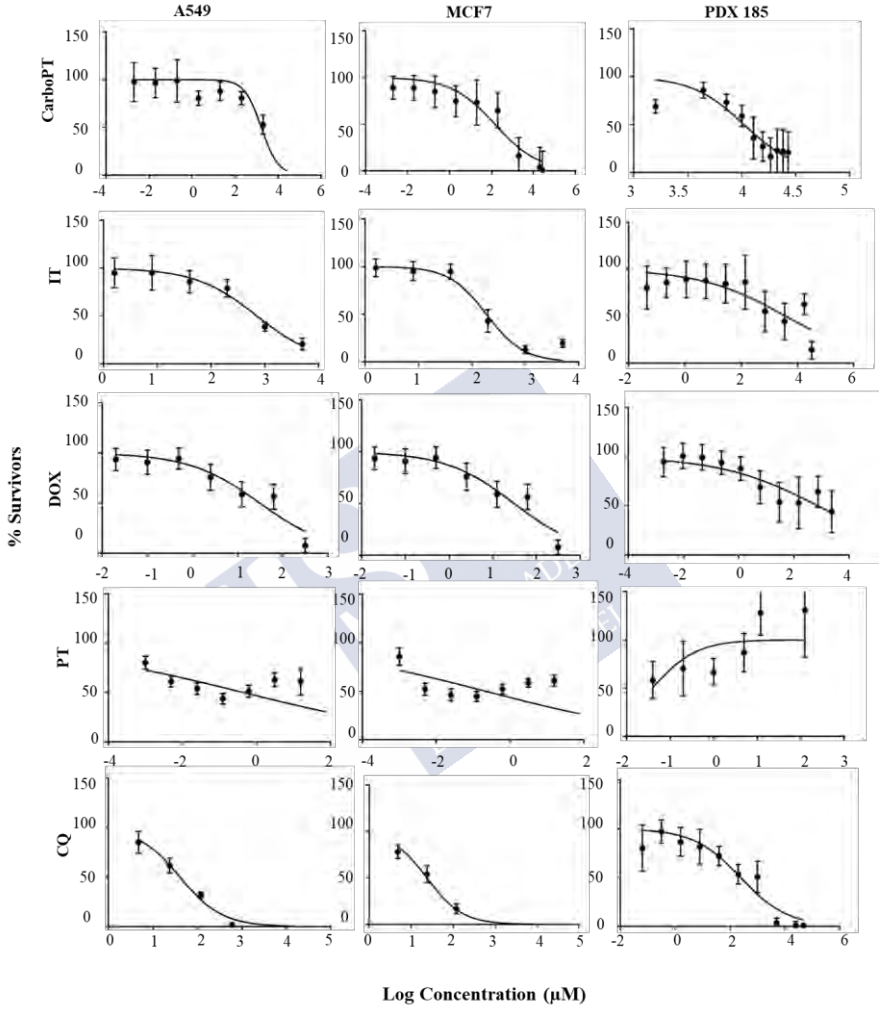

Figure 6. IC50 of cancer cell lines treated with anticancer drugs (Gutiérrez-Lovera et al. 2019. From Toxicology Mechanisms & Methods, Open Access).

Table 7. IC50 of cancer cell lines treated with Paclitaxel.

hpt	Cell line	
	A549	MCF7
48	0.02 ± 1.18	0.02 ± 1.64
72	0.007 ± 1.35	0.002 ± 1.1

*IC50 values expressed in μM (IC50 ± SEM).

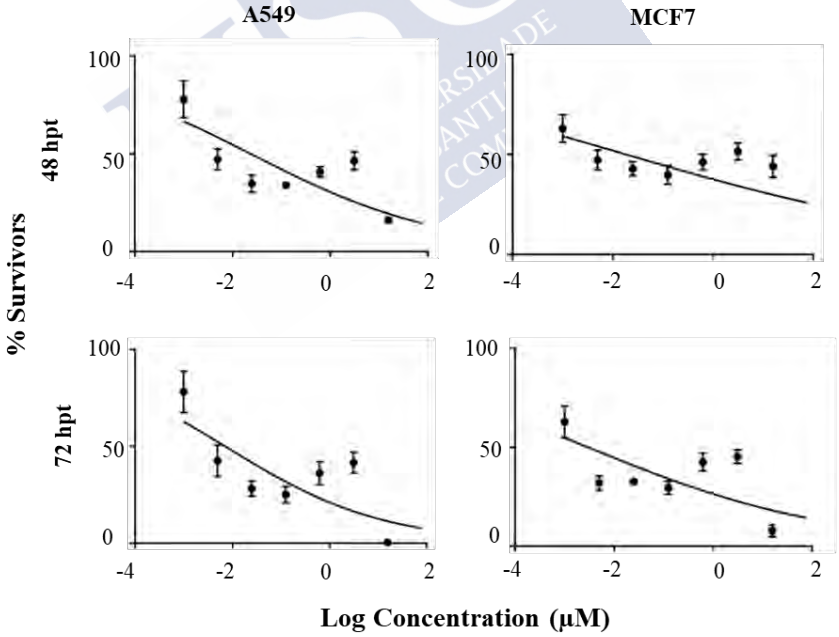

Figure 7. IC50 of cancer cell lines treated with PT (Gutiérrez-Lovera et al. 2019. From Toxicology Mechanisms & Methods, Open Access).

Taking into account that the MTT cell viability assay is based mainly on active mitochondria, we wanted to analyze if those cell survival spikes observed in the PT treatment were due to greater cellular resistance or to an increase in mitochondrial activity. For this, we performed a Mito tracker staining to label functional mitochondria in A549 cells treated with PT. We analyzed a concentration with high lethality: 0.125 τ M, and a higher concentration with apparent less toxic effect: 3.125 τ M (MTT assay results). As shown in Figure 8, the mitochondrial activity (red signal) was higher in 3.125 τ M PT treated cells.

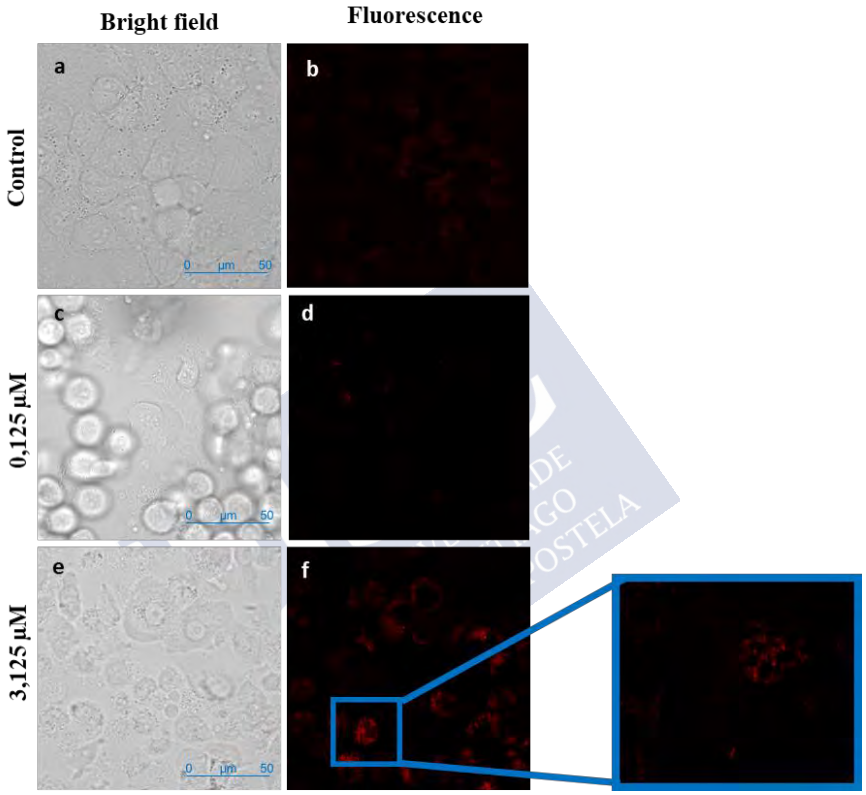

Figure 8. A549 cell line bright field (left column) and stained with Mito Tracker Red 500 nM (right column). Control: a-b, 0.125 ɀ M of PT: c-d, 3.125 ɀ M of PT: e-f (Gutiérrez-Lovera et al. 2019. From Toxicology Mechanisms & Methods, Open Access).

4. Discussion

The huge tumor heterogeneity, both between cancers (intertumor) and within each cancer (intra-tumor), makes tumor behavior and response to chemotherapeutic drugs very difficult to predict (Almendro et al. 2013; Vogelstein et al. 2013). Usually, patients have to undergo different treatments, many of them with important side effects as consequence of the broad cytotoxicity of anticancer therapies. Thus, one of the challenges of oncology is to achieve fast, accurate and personalized medicine for each patient. To achieve this, animal models or avatars, like zebrafish, have become a very valuable tool. Zebrafish xenografts provide an in vivo platform for precision medicine, allowing the study of tumor behavior and the screening of effective anticancer drugs (MacRae and Peterson 2015; Fior et al. 2017). With regard to the last, zebrafish has emerged as the unique vertebrate model used to perform high-throughput screening of different novel anticancer drugs (Garcia et al. 2016) due to the highly conserved and fast developmental processes of the embryos compared to other mammalians, apart from the time-consumed/cost ratio and the possibility to use noninvasive techniques to track the effects of the chemicals tested (Nishimura et al. 2016).

Despite the wide acceptance of the zebrafish embryos as a model for cancer research, some drawbacks still remain. Most of the xenograft experiments involving anticancer drugs, or potential antitumoral compounds described in the literature, lack toxicity determination of the compound to the fish, which narrows the concentration range that can be used to perform the experiments (Jo et al. 2013; Zhang et al. 2014; Tonon et al. 2016; Zhong et al. 2017). Usually, the concentration of the drugs used to treat the embryos are based on the evaluation of increased concentrations of the compounds until a plateau of cancer cell death is reached in vitro (Jung et al. 2012) while being harmless to the host. Despite the accuracy of this method, in certain cases the hosts suffer malformations or die before the cell death plateau is reached, as shown in this work for some relevant anticancer drugs already approved for human treatment.

Besides this, we showed that striking differences exist between in vivo and in vitro anticancer drug toxicity implying that a toxicity study of the compound on zebrafish embryos, at the specific developmental stage for the particular experiment, is a crucial pre-requisite to obtain an appropriate concentration range of the antitumour compound being assayed.

The results (see Tables 4 and 5) showed a variable LC50 depending on the embryo developmental stage (0 hpf or 72 hpf). The differences observed could be multifactorial: (A) Presence of the chorion at 0 hpf that could prevent the drug internalization. The chorion is known to protect the embryo of external threats, for example, chemicals dissolved in the water. The molecular size and electrophilic properties of the drug could influence its internalization (Pelka et al. 2007). (B) Differences on the developmental stage (embryo, larvaj̃) regarding the different uptake and sensitivity to different compounds (Kristofco et al. 2018). (C) The penetrating capacity through biological membranes of the embryo, apart from the chorion. Different toxicity effects can take place based on the toxicity and molecular weight of the tested compound (Paatero et al. 2017; Pitt et al. 2018). Regardless the cause for the differences observed, the results imply that toxicity assays at 72 hpf and up to 96 hpf are needed when xenotransplantation assays are to be performed (see below).

The results obtained with zebrafish embryo were contrasted with in vitro toxicity analysis for the same compounds using the cell lines: A549, MCF7 and Panc185. The results obtained from this comparison showed a certain trend in terms of drug toxicity. When a compound is extremely toxic (as PT) or causes very low toxicity (as CarboPT), the lethal effects are similar in both FET tests and in vitro. On the other hand, the toxicity values of some compounds assayed in this study by

in vivo and in vitro methods (Tables 4–6) are different, for instance: the LC50 for CarboT was 330 mM (in 0–4 hpf embryos) and 3247.2 mM (in 72 hpf embryos) while in vitro the IC50 was 1653 ± 1.12 in A549 cells, 143 ± 1.27 in MCF7 cells and 10513 ± 1.05 in PDX Panc185 cells. The LC50 for PT was 1.9 mM (in 0–4 hpf embryos) and 0.05 mM (in 72 hpf embryos) while in vitro the IC50 was 0.4 mM± 1.46 in A549 cells, 0.2 mM± 1.46 in MCF7 cells and 0.039 mM± 2.09 in Panc185 cells. These results imply that when performing xenograft experiments, for certain compounds, a direct extrapolation of toxicity results from in vitro assays or from those in vivo, but from a different developmental stage, is not the right approach.

Approximately 80% of embryos exposed to 8 mM CarboPt showed malformations at 48 hpt, an expected result since this compound produces congenital malformations in rats at a dose level of 6 mg/kg/day (Kai et al. 1989), even inducing fetal dysmorphogenesis, prenatal mortality, and intrauterine growth retardation in mice (Parashar et al. 2016). This could be the underline reason of the morphological effects (spinal deviation until the total disintegration of the embryos) found in CarboPT treated embryos.

Important differences in toxicity were found between established cell lines (A549 and MCF7) and the PDX cell line Panc185. As shown in Table 4, the patient derived cancer cells were considerably more resistant to the anticancer drugs tested here. An exception to this was PT, a known anti-microtubular drug with generalized toxic effect on cancer cells affecting one of the hallmarks of cancer, the abnormal proliferation (Chan et al. 2012). These differences are especially relevant considering that PDX cells are more similar to patient tumor cells implying that cytotoxicity assays performed with established tumor cell lines could overestimate the cytotoxicity of tested compounds.

The FET test, apart from the LC50, provides other toxicity values such as: LC10, NOEC (No observed effect concentration) and LOEC (Lowest observed effect concentration). The drug concentration used in the xenograft studies could vary depending on the toxicity of the drug obtained with the FET test and the mortality that can be assumed. For example, if the NOEC obtained for the embryos is enough to affect the xenografted cells, this can be the best option for the experiments, causing no harm to the embryos and reducing the proliferation of the injected cells. Otherwise, if the concentration needed is higher than NOEC, then a certain degree of mortality has to be assumed in the experiment in order to determine the effect of the tested compound on xenografted cells. How the fish

death is accounted and statistically treated is the key in order to obtain solid results in the effect of antitumour compounds.

In summary, we have performed the FET test for anticancerdrugs and compared the results to those obtained in vitro (See Table 8). The results obtained showed that toxicity depends on several factors: the anticancer drug used, the cell line, the analysis method (in vitro or in vivo) and the initial time point of the studies. In fact, the results are difficult to extrapolate between conditions. For some compounds, the previous knowledge of their effect on cell cultures could be used as a starting point for selection of the concentrations to test on zebrafish xenografts. For other compounds, the toxic concentrations observed in vitro could result harmful to the fish making the embryo toxicity test, at a relevant developmental stage, a pre-requisite.

Table 8. Summary of toxicity results values of LC50 in zebrafish embryos at 0-4 hpf and 72 hpf and IC50 in A549, MCF7 and PDX Panc85 cells of the different drugs evaluated.

	Zebrafish LC50*		Cells IC50		
Anticancer drug	0 hpf	72 hpf	A549	MCF7	PDX Panc185
CarboPt	330	3247.2	1653 ± 1.12	143 ± 1.27	10513 ± 1.05
IT	7	35	712 ± 1.09	187 ± 1.07	3755 ± 1.37
DOX	11	10	31 ± 1.14	1 ± 1.08	745.9 ± 1.42
PT	2	0.05	0.4 ± 1.46	0.2 ± 1.47	0.039 ± 2.09
CQ	241	116	41 ± 1.05	28 ± 1.06	246.8 ± 1.16

*LC50 and IC50 values expressed in μM (IC50 ± SEM).

CHAPTER V

Engineering of edelfosine nanoemulsions for treatment of triple negative breast cancer and *in vivo* evaluation in xenotransplanted zebrafish

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DE SANTIAGO
DE COMPOSTELA

ABSTRACT

The subtype triple negative breast cancer (TNBC) have an aggressive biological behavior and complex metastatic patterns, with a poor response to current therapeutic alternatives. It is therefore important to explore alternative therapeutic strategies that can provide more effective treatments. Edelfosine is a synthetic alkyl-lysophospholipid which has proved to be efficient in several types of cancer such as breast, leukemia, pancreas, osteosarcoma and glioma, upon encapsulation into lipid nanoparticles of Precirol® or Compritol®. For this reason, the objective of this work was to explore the potential of edelfosine for the treatment of TNBC.

We formulated edelfosine in the form of nanometric emulsions (E-NEs), in combination with Myglyol 812® and phosphatidylcholine, following a very simple and mild methodology. E-NEs, and the control formulations without edelfosine (C-NEs) were characterized with regard to their physicochemical properties. Cell viability was evaluated next by a conventional MTT assay. To determine their ability to modify tumour growth in vivo, E-NEs were tested in zebrafish embryos, a powerful model system for preclinical studies. On the one hand, acute toxicity was assesed by the *Fish*

Embryo Acute Toxicity (FET) test and biodistribution and the therapeutic efficacy were evaluated in xenotransplanted embryos. In conclusion, our results showed that E-NEs might represent a therapeutic alternative to interrupt tumor growth in TNBC.

1.INTRODUCTION

One of the major health problems worldwide due to its high rate of morbidity and mortality is cancer. Breast cancer is the leading cause of death in women (Siegel et al. 2013). Breast cancers represent a heterogeneous group of tumors classified by histology, cellular origin, mutations, metastatic potential, disease progression, therapeutic response, and clinical outcome (Fulford et al. 2007). Triple negative breast cancer (TNBC) is a subtype of breast cancer that lacks of expression of the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor (HER2) (Dent et al. 2007). TNBC have an aggressive biological behavior and complex metastatic patterns (Elsawaf and Sinn, 2011). Today, treatments based on chemotherapy are still the most used at an early stage of TNBC. However, other emerging therapeutics are also being tested (DNA-damage agents, immune inhibitors, platinum-based compounds, PI3K- pathway inhibitors, and androgen-receptor inhibitors) (Malla et al. 2019). Despite this, TNBC patients require combinational targeted drugs at different stages for maximize clinical efficacy (Kumari et al. 2017).

Conventional anticancer drugs have high toxicity causing adverse effects in oncologic patients and a drastic reduction of their quality of life (Kayl and Meyers, 2006). Also these drugs have problems relate to their biopharmaceutical properties in terms of a low biodistribution and poor accumulation in the tumor, rapid elimination, or early metabolizing with the consequent loss of activity before reaching their goal (Churchet et al. 2014).

Edelfosine (ET-18-OCH_3 o ET) is a synthetic lipid with a high apoptotic action on cancer cells through different mechanisms of action (Ruiter et al. 2001; Gajate and Mollinedo, 2007; Gajate et al. 2004; Ruiter et al.1999; Nieto-Miguel et al. 2007; Estrella-Hermoso 2009). Importantly, the main drawback regarding its translation to clinic relates to the toxicity observed for edelfosine in its pure state after intravenous administration, producing haemolysis (Ahmad et al. 1997)" and after oral administration, with reported gastrointestinal problems (Estella-Hermoso et al. 2012). To date, some authors have proposed the encapsulation of edelfosine into nanosystems to decrease its toxicity without compromising its effectiveness (González-Fernández et al. 2017; Mollinedo et al. 2004; Estella-Hermoso et al. 2011;

Lasa-Saracibar et al. 2013; Aznar et al. 2013; Lasa-Saracíbar et al. 2014; González-Fernández et al. 2015; Estella-Hermoso et al. 2012; Shafer and William, 2003). Nanosystems can improve the access of the associated drugs to the tumor and decrase secondary side effects (Peer et al., 2007; Torchilin, 2011). Regarding brest cancer, Aznar et al (2013) proposed the development of edelfosine-loaded nanoparticles for delivery to MCF7 cells, observing a strong inhibition on cell proliferation and notably decrease in the cell viability. However, and to the best of our knowledge, edelfosine has not yet been evaluated for specific treatment in TNBC. We propose the development of a simple and easy manner to prepare nanometric emulsion for the delivery of this anticancer compound to TNBC cells.

On the other hand, it is necessary to have a deep knowledge to study more about in vivo analysis in order to speed up the access of new drugs and nanotherapeutics to clinical studies. For that reason, we have considered using one of the most innovative tools to study new anticancer therapies, the zebrafish model. Since 2004 the number of publications that employed zebrafish has grown quickly, much more than primates, mouse and chick. Also some zebrafish embryos can be organ models and organ-specific drugs (Gallardo et al. 2015). The zebrafish has been suggested as a promising model for research because it shares many special features with mammals (Howe et al. 2013). It has several advantages as a model as a low maintenance cost,

small size, high fecundity, external fertilization, transparency, short reproduction cycle, and permeability to small molecules (Lieschke et al. 2007; Santoriello and Zon, 2012; Ablain and Zon, 2013; Giannaccini et al. 2014). Importantly, is a good mode to study spreading and metastasis formation, and response to treatments, after transplantation of human cancer cells into zebrafish embryos (xenografted embryos) (Marques et al. 2009, Veinotte et al. 2014; Haldi et al. 2006; Drabsch et al. 2017; Roel et al,.2016; Bansal et al. 2014; Zhang et al. 2014; Brown et al. 2017; Cabezas-Sáinz et al. 2018). Studies of TNBC (MDA-MB-231 and MDA-MB-435 cells) in zebrafish embryos showed a higher rate of formation of tumor, vessel density and metastatic behavior in contrast with low aggressive breast cancer (BT-474) (Stoletov, 2010).

Zebrafish is an excellent tool to provide insights of the adverse effects of drugs, and to determine the activity of anticancer compounds (MacRae and Peterson, 2015; Deveau et al. 2017; Van Rooijen et al. 2017; Lenis-Rojas et al. 2016; Lenis-Rojas, 2017; Penas et al. 2017; Blackburn and Langenau, 2014; Tat et al. 2013). In the case of nanocarriers for drug delivery purposes, Warner et al, (2010) showed that a gold nanoparticles are able to reduce cancer cells in

zebrafish embryos. Other study showed that therapeutic nanoparticles coated with polyethylene glycol can protect them from being metabolized by macrophagues in zebrafish embryos (Evensen et al. 2015). Xu et al, (2016) studied an effective and safe antiangiogenic therapy based on the synergy between placlitaxel and a pigment-epithelium-derived factor (PEDF) plasmid into poly(lactic-co-glycolic acid) (PLGA) nanoparticles. On the other hand, Cordeiro et al (2017) developed a biocompatible and efficient nanoplatform for gene silencing purpose, a gold nanobecaon, able to silence enhance green fluorescence protein (EGFP) in a transgenic zebrafish line. And finally, Aldrian et al (2017) studied the effect of a polyethylene glycol (PEG) grafting a retro-inverse amphipathic RICK peptide nanoparticle into Casper zebrafish line in order to evaluate the ability to deliver siRNA. Also and performing functional assays, Gong et al (2013) studied the antiangiogenesis and anti-tumor potential of a biodegradable curcumin-loaded micelles and Duan et al (2017) observed the inhibition of angiogenesis via vascular endothelial growth factor receptor 2 (VEGFR2)-mediated mitogen-activated protein kinase (MAPK) signaling pathway using silica nanoparticles.

Bearing in mind all these premises, the purpose of this study was to develop a new treatment for TNBC, based on edelfosine conveniently formulated in the form of nanometric emulsions. After developmemt and characterization of the nanosystems, and in vitro

study of their properties, experiments will be conducted to test their toxicity and efficacy. We propose the use of zebrafish embryos and xenotransplanted zebrafish as reliable models for the development of anticancer nanotherapies.

2. MATERIALS AND METHODS

2.1 Materials

Myglyol 812® (CAS 37332-31-3) and Phosphatidylcholine (CAS 8002-43-5) were purchased from Lipoid Ludwigshafen, Germany. Edelfosine (CAS 77286-66-9) was acquired from Santa Cruz Biotechnology. DiR lypophilic cyanine dye (CAS 100068-60-8) was supplied from Thermo Fisher Scientifica, and TopFluor-PC (CAS 1246355-63-4) from Avanti Polar Lipids. Ethanol (high purity) was obtained from PanReac AppliChem and water deionized from Milli-Q Integral Water Purification System.

2.2 Preparation and characterization of nanoemulsions (NEs)

Edelfosine nanometric emulsions (E-NEs) composed by Miglyol812®(M), Phosphatidylcholine (PC) and Edelfosine (ED) were formulated by adapting the ethanol injection method (Jaafar-Maalej et al, 2010; Bouzo et al, 2019). In brief, 4 mg of M, 0.2 mg of PC and 0.5 mg ED were dissolved in a volume of ethanol of 100 ɀ L. This organic phase was injected into 1 mL of ultrapure milli-Q water under magnetic stirring at room temperature, and E-NEs instantaneously were obtained. E-NEs were left under magnetic stirring for 10 min to ensure they were completely formed. Control NEs (C-NEs) were prepared by the same method with 4 mg of M and 0.7 mg of PC.

The particle size, polydispersity index (PdI), and zeta potential of E-NEs and C-NEs were measured by Dynamic Light Scattering and Laser Doppler Anemometry, using a Zetasizer NanoZS (Malvern Instruments Ltd., UK). The measurements were carried out three times with a standard λ = 633 nm laser as the incident beam and were performed at 25ºC with a detection angle of 173º. To avoid multiscattering events each sample was diluted to 1:20 with filtered ultrapure water and loaded into a Disposable Solvent Resistant MicroCuvette (ZEN0040) and dip-cell (DTS 1060) for size and ζ-

potential analysis, respectively. All data are expressed as a mean value ± standard deviation.

2.3. Preparation of DiR-loaded and TopFluor-PC MPC NEs

Fluorescent labelled NE were prepared slightly modifying the procedure reported in section 2.2. Briefly, DiR (5ɀ g) and/or TopFluor-PC (10 ɀ g) were dissolved in ethanol with the other compounds of the organic phase in order to obtain a final concentration of 0.05 mg/mL and/or 0.1 mg/mL, respectively. The organic phase was then injected in 1 mL of milli-Q water and kept under magnetic stirring at room temperature for ten minutes.

2.4. Stability in relevant biological media

The colloidal stability of E-NEs and C-NEs was evaluated in two relevant biological media, to ensure that the formulations maintained their properties during in vitro and in vivo testing. Firstly, size was measured upon incubation in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Fetal Bovine Serum (FBS) 1 %. E-NEs and C-NEs were incubated at 37°C under constant horizontal

shaking and size and size distribution analyzed after 0, 30, 120, and 240 min. Secondly, E-NEs and C-NEs were incubated at 28 °C with sterile dechlorinated tap (SDT) water during 0, 2, 24 and 96 h. In all cases, formulations were diluted 1/10 v/v reaching a final nanosystem concentration of 1mg/mL.

2.5 *In vitro* studies

Triple negative breast adenocarcinoma cells MDA-MB-231 (ATCC® HTB-26™) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10 % fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37 ºC in a humidified atmosphere of 95 % air and 5 % CO^2, and were passed before reaching 80 % confluence, 2-3 times a week, and the culture media was replaced every second day.

1 x 10^5 cancer cells were seeded in each well of 96-well plate and allowed to adhere and grown overnight at 37 ºC and 5 % CO^2. Later, media was replaced and 100 ɀ L of fresh cultured media added to each well. Cells were then incubated with 25ɀ L of E-NEs and C-NEs at increasing concentrations (from 0.01 to 10 mg/mL) in a final volume of 125 ɀ L. After 24h incubation, formulations were removed, cells were washed with PBS, and eventually incubated at 37ºC for 4h with 100 ɀ L/well of MTT solution (0.5 mg/mL in non-supplemented cell

culture media). Afterwards, the solution was removed and 100 ɀL of DMSO were added to solubilize the formazan crystals after 10 minutes of incubation at 37 ºC (protecting from light). MTT reduction was determined by measuring the light absorbance at 570 nm in a microplate spectrophotometer (DTX 880 Multimode Detector, Beckman Coulter). For each 96-well culture plate three replicas were analyzed. Cell cytotoxicity/viability (%) was calculated in percentage related to untreated control wells, in which only 25 ɀL of the suspension media (without NEs) was added.

2.6 In vivo studies in zebrafish embryos

2.6.1 Experimental animals and handling

One-year-old adult wild-type zebrafish (*Danio rerio*) were maintained in a controlled aquatic facility with purified and dechlorinated water by a reverse osmosis system, with the following conditions: 27 ºC (± 1 ºC), pH 7 (± 0.5), 14/10 h light: dark photoperiod and conductivity 650 ɀS/cm in 30 L aquaria at a rate of 1 fish per liter of water. Zebrafish embryos were obtained from mating adults according to previously described procedures (Westerfield, 2000). The embryos were collected and washed with osmosis water in Petri dishes and 0-4 h post fertilization (hpf) embryos were

selected with an inverted optical microscope (Nikon TMS). All procedures described next were approved by the Bioethics Committee for animal experimentation of the Universidade de Santiago de Compostela (CEEA-LU) and were performed in agreement with the standard protocols of Spain (Directive 2012-63-UE).

2.6.2 Acute toxicity assay

Acute toxicity study of E-NEs and C-NEs was carried out in zebrafish embryos, using the official *Fish Embryo Acute Toxicity* (FET) *test* (Organization for Economic Cooperation and Development, OECD, guideline Test No.236) in order to determine several toxicological parameters. Selected embryos of 0 – 4 hours post-fertilization (hpf) were plated in 96-well plate with 200 ɀ L/well of the different concentrations of E-NEs and C-NEs. All concentrations were prepared and diluted with sterile dechlorinated tap (SDT) water. Embryos were observed under inverted optical microscope (Nikon TMS) at 24, 48, 72 and 96 h of treatment to analyze development alterations, malformations, effects on hatching rate and mortality. Three replicates were performed for each plate and experiment.

On the other hand, a toxicity assay with hatched embryos (72 hpf) was performed. This protocol was a modification of the FET test. For each replicate, 20 embryos were used in each concentration and 24

embryos were used as a negative control; internal controls were also used as it is indicated above. Embryos were observed under inverted optical microscope at 24, 48, 72 and 96 h after the treatment to analyze malformations and mortality.

Statistical analysis of the acute toxicity results was performed using probit analyses with the ToxRat program (ToxRat Solutions. 2003. ToxRat®. Software for the statistical analysis of biotests. Alsdorf, Germany) in accordance with OECD, guideline 236. With this statistical program/package toxicological parameters such as: 50% lethal concentration (LC_{50}), lowest observed effect concentration (LOEC) and no-observable-effect concentration (NOEC) were calculated. Test duration was 96h, the measurement interval was 24h and the measurement variable was embryos survival. LC_{50} was determined via probit analysis using linear maximum likelihood regression with survival at 96h and with 95 % -confidence limits. Qualitative trend analysis by contrasts, step-down Cochran-Armitage test and Tarone's test were performed to determined NOEC and LOEC values with 95 %-confidence limits. Tests were considered valid whether the mortality of fish embryos in the negative was less than 10% and the mortality in the positive control was more than 30 %.

2.6.3 Toxicity assay by injection

Toxicity assay by injection was carried out in embryos without chorion of 48 hpf. Embryos were anesthetized with 0.003 % tricaine (CAS 886-86-2) from Sigma and injected using a borosilicate glass capillary needle (1 mm O.D. x 0.58 mm I.D.; Harvard apparatus) controlled with IM-31 Electric Microinjector (Narishige) with an output pressure of 34 kPa and 25 ms injection time. 5 ɀ L of E-NEs and C-NEs were injected into yolk and vein separately. 20 embryos were used to inject in yolk and 20 embryos to inject in vein. After injection, they were incubated at 28 ºC and evaluated under inverted optical microscope at 24, 48 and 72 hours post-injection (hpi) to analyze development alterations, malformations, and mortality of embryos. 3 replicates were performed for each experiment.

2.6.4 Biodistribution assay

Wild type embryos of 72 hpf were incubated for 4 hours, at 34 °C in 24-well plates, exposed to 0.5 mg/mL DiR-loaded and TopFluor-PC C-NEs and embryo media (as a control condition). Afterwards, embryos were washed with PBS and fixed with formaldehyde overnight at 4ºC. After fixing, they were washed again with PBS and maintained at 4 ºC before visualization.

For sample preparation, a Fluorodish™ (World Precision Instruments, Sarasota, FL, USA) was covered with a layer of agar gel (1% w/v in distilled water) and then the zebrafish embryos were placed on top of the layer. Embryos were observed using a Confocal Microscope Leica TCS SP8® with a HC PL Apo 10x/0.4 objective. Zebrafish were scanned every 10 ɀm acquiring a total of 27 planes in z-axis direction with a 7.5x magnification. Images were analyzed using Leica Application Suite X software.

2.6.5 Xenografts in embryo zebrafish

48 hpf zebrafish embryos without chorion were anesthetized with 0.003% tricaine. At least 40 embryos per condition were injected with MDA-MB-231 GFP tumour cells. These cells were trypsinized and 1.10^6 cells were then resuspended in 10 ɀl of PBS with 2% Polyvinylpyrrolidone (CAS 9003-39-8) from Sigma. The cell suspension was loaded into the borosilicate glass capillary needle (1 mm O.D. x 0.58 mm I.D.; Harvard apparatus) and injections were performed manually right into the yolk of the embryo by Electric Microinjector with an output pressure of 34 kPa and 30 ms injection time. Incorrectly injected embryos without cells inside of the yolk, or showing them in the circulation after xenotransplantation were discarded. Afterwards, xenotransplanted embryos were incubated at 34 °C in 24-well plates and photographed, using a AZ- 100 Nikon

fluorescence stereomicroscope, at 0 and 48 h post treatment (hpt) with E-NEs, C-NEs and control media. QuantiFish, an image analysis program, was used to quantify the fluorescence intensity in order to track tumour growth and cell spread in the different treatment conditions (Stirling et al, 2017).

3. RESULTS AND DISCUSSION

3.1. Development and characterization of the NEs

E-NEs and C-NEs were prepared by a previously described ethanol injection method (Pons et al, 1993; Maitani et al, 2001; Batzri & Korn, 1973; Jaafar-Maalej et al, 2010). The procedure is reproducible and the NEs can be obtained in a few seconds. The method and the structure of the NEs have been represented in Figure 9.

The result of a full characterization shows a mean size of 101 ± 4 nm and 126 ± 4 nm to C-NEs and E-NEs respectively, and a zeta potential close to neutral values -2 ± 0 mV and -1 ± 0 mV (Table 9). The PDI was 0.1for both.

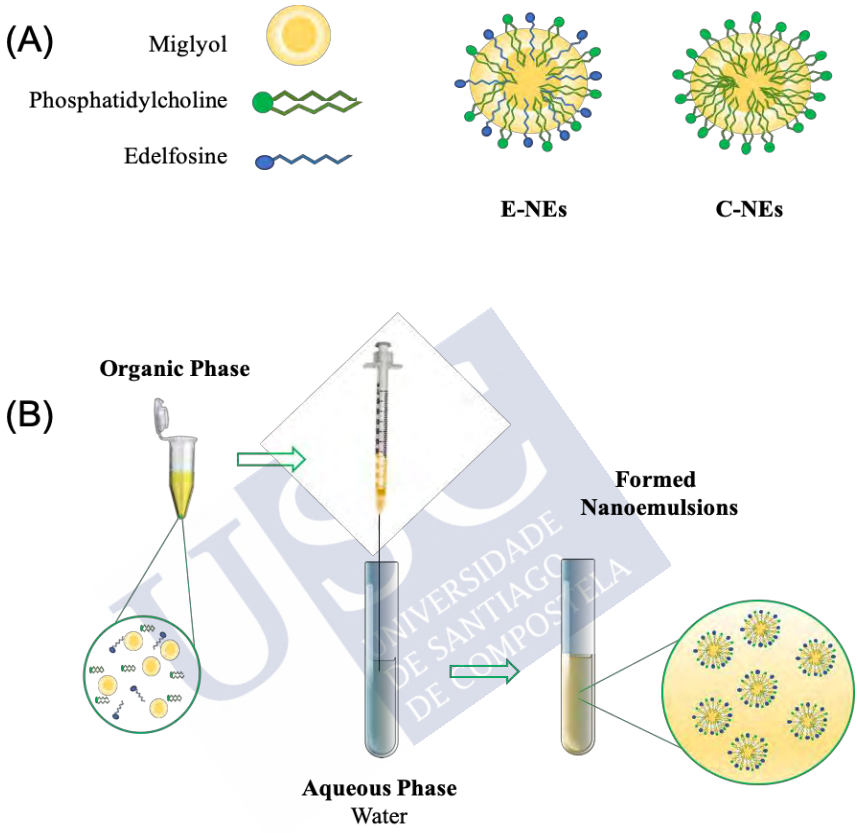

Figure 9. Schematic representation of E-NEs and C-NEs (A) and the ethanol injection method used for its preparation (B) (Source: own elaboration).

Table 9. Formulations size, PDI and z potential of nanoemulsions

Formulation	Size (nm)	PDI	Z potential (mV)
C-NEs	101 ± 4	0.1	-2 ± 0
E-NEs	126 ± 4	0.1	-1 ± 0

Results are presented as mean SD of three replicates

C-NEs and E-NEs have similar size and properties, these results showimg that the incorporation of edelfosine into the nanosystems do not significantly affect the properties of the nanocarriers. This small size, below 150 nm, is suitable for intravenous administration, avoiding embolism risk (Charman and Stella, 1992; Estella-Hermoso de Mendoza et al. 2009; Zimmerman et al. 2000). On the other hand, the PDI values were 0.1, an indication of the mondisperse character of both formulations (C-NEs and E-NEs). With respect to the zeta potential, related to the surface charge of particles when placed in liquid, values are close to neutrality. Besides a neutral zeta potential could be related to a lower stability (Krstić et al. 2018), we proved that both E-NEs and the control formulation C-NEs maintain their average size in cell culture media and in vivo in SDT water (Figure 10), and could therefore be tested in vitro and in vivo in zebrafish embryos.

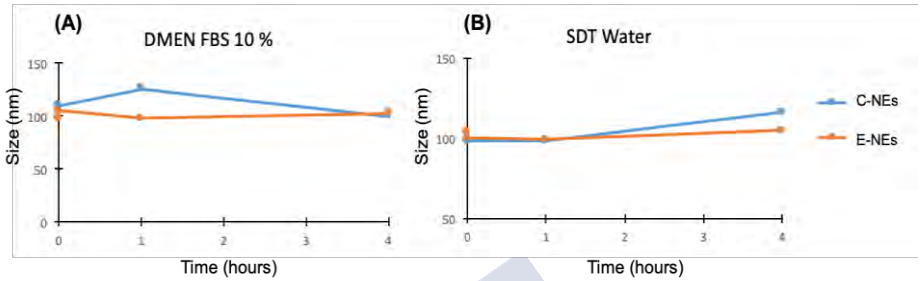

Figure 10. Stability of nanoemulsions. (**A)** Size evolution (nm) of nanoemulsions in 1 % DMEM FBS at 37 ºC. (**B)** Size evolution (nm) of nanoemulsions in SDT water at 28 ºC (Source: own elaboration).

The cell viability assay carried out in MDA-MB-231 cells (Figure 11) showed a higher toxicity for E-NEs as compared to the control formulation C-NEs, confirming the efficient assoaction of edelfosine to the formulation, maintaining its biological activity.

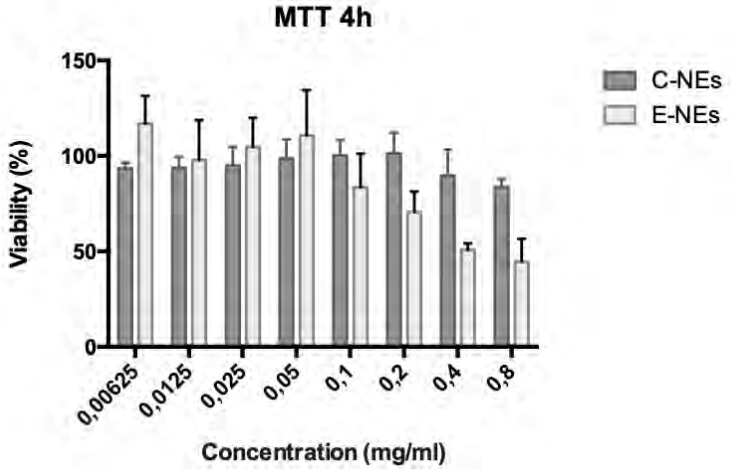

Figure 11. Cell viability assay in MDA-MB-231 cells of the C-NEs and E-NEs (Source: own elaboration).

3.2 *In vivo* studies in zebrafish embryos

3.2.1 Toxicity Assays

The LC50 (i.e., lethal concentration at which 50% of the test population are killed) of embryos exposed until 1 mg/mL of the control formulation C-NEs could not be obtained because by increasing the concentration of the experiment to more than 1 mg/mL

the embryos could not be seen through the microscope. Importantly, no dead embryos were observed at the maximum concentration tested, 1 mg/mL of C-NEs, at 0 and 72 hpf and irrespectively of the temperature. On the contrary, and as observed in Table 2, in the case of the drug-loaded E-NEs formulations, the LC50 at 28ºC was 12.89 and 8.6 ug/mL at 0 and 72 hpf respectively, and at 34 °C 11.4 and 3.2 ug/mL at 0 and 72 hpf. Our data on variations in toxicity at 34 °C could be due to temperature being a relevant and highly variable abiotic factor in nature, according to Radonic et al (2015) who suggest it as an important factor in the embryonic development of fish. Furthermore, temperature can also be a physical stressor, as an increasing temperature can increase the energy metabolism of aquatic organisms, and the bioavailability of toxicants (Heugens et al., 2001). Other toxicity indices were additionally determined, LC10 (i.e., lethal concentration of a substance at which 10% of the test population are killed), NOEC (i.e., no observable affect concentration) and LOEC (i.e., lowest observable effect concentration) (Table 10). All the requirements of FET test were accomplished: the mortality was ≤10 %, the hatching rate was ≥80% for the negative control, whereas the positive control was 100 % deaths.

These results indicate, on the one hand, that C-NEs are biocompatible nanosystems, which allows it to serve as a vehicle for transporting drugs without producing toxic effects (Estrella-Hermoso de Mendoza et al, 2010). On the other hand, our results show that the encapsulated edelfosine in E-NEs maintain its activity, as toxicity is greatly increased in relation to the blank C-NEs, according to our results, and also time and concentration dependent.

Table 10. Toxicity of embryos of 0 and 72 hpf exposed E-NEs.

hpf	T(°C)	LC10	LC50	NOEC	LOEC
0	28	9.8	12.89	10	5
	34	8.6	11.4	10	5
72	28	4.4	8.6	5	1
	34	1.5	3.2	5	1

hpf: hour post fecundation

LC10, LC50, NOEC and LOEC are represented in ɀ g/ml

3.2.3 Biodistribution studies

Cell specific targeting is an important goal in nanoparticle drug delivery. The transparency of the zebrafish embryo model offers the possibility of study and track fast the distribution of nanoparticles throughout the organism (Fenaroli et al. 2014), also allowing a direct observation of circulation of nanoparticles and their interaction with cells (Evensen et al. 2016). For this reason, we treated 72 hpf embryos with 0.5 mg/mL of DiR and TopFluor® -loaded C-NEs to study their internalization and distribution. While DiR was encapsulated into C-NEs, TopFluor® was covalently linked to one of the lipids, avoiding a potential premature release. We aim to confirm that the signal was due to the presence of nanoemulsions (when both markers co-localized) and not to the detached fluorophores. Hydrophobic dyes, such as DiR, could be easily released from nanosystems, causing an apparent cellular uptake or biodistribution that can be wrongly interpreted. This fact highlights the need of choosing the right dye and the appropriate controls to be sure that we are tracking our nanosystems indeed. As this concentration of E-NEs (0.5 mg/mL) would be lethal to the embryos, and it was not possible to detect fluorescence at lower concentrations of the fluorophores and therefore of labeled NEs,

biodistribution assays were only performed with the control blank formulation (C-NEs). Nevertheless, as both formulations are comparable with respect to their physicochemical properties (Table 9), and *in vitro* studies show a similar behavior after incubation in cell culturing (Figure 11), we could presume a similar behavior for the drug loaded E-NEs.

Confocal microscope observations showed that C-NEs were efficiently internalized in the exposed embryos, especially in the yolk (Figure 12). By the double-labeling of C-NEs with TopFluor linked to PC, we assured that the signal observed is anchored to the membrane of our NEs and we confirm a colocalization with DiR dye in the overlay picture, suggesting that we are truly tracking the biodistribution of our NE with any of the fluorophores incorporated. Our results showed that C-NEs can cross biological barriers, in this case, zebrafish embryos without chorion. We have previously proved (Teixeiro-Valiño et al. 2017) that this capacity is strongly dependent of the specific composition of nanoparticles, mainly due to changes in their surface properties, with cationic nanoparticles being unable to cross epithelial barriers however prototypes with lipid core and hyaluronic acid (HA) and protamine (PR) as shell can serve like carriers for hydrophobic drugs.

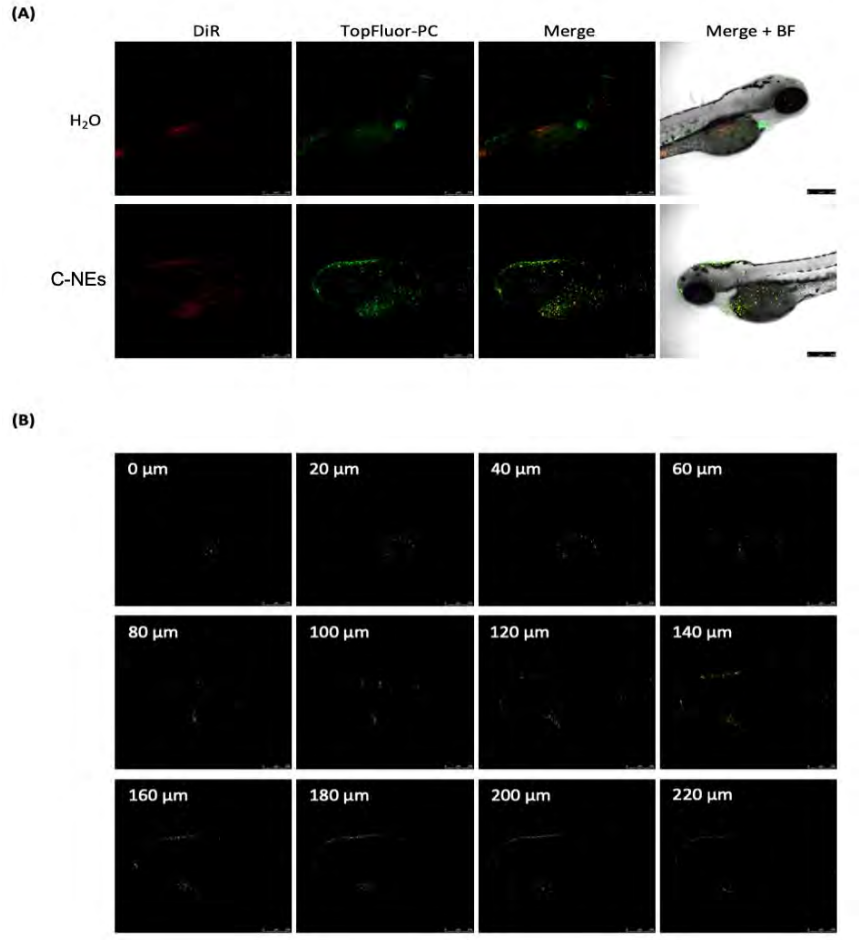

Figure 12. Uptake and biodistribution assay (Source: own elaboration).

3.2.4 Efficacy

Final experiments, aimed to explore the therapeutic potential of E-NEs in an in vivo situation, were performed with xenografted zebrafish embryos. These models have already being proposed for performing drug screens, and analyzing critical aspects of the tumor, such as growth and proliferation (Handi et al. 2006), invasion and intravasation (Stoletov et al. 2007; Naber et al. 2013; Yang et al. 2013), formation of metastasis (Drabsh et al. 2017; He et al. 2012), angiogenesis (He et al. 2012; Tobia et al. 2013), and immune cell response (Tat et al. 2013). Wagner et al. (2010), showed that gold nanoparticles are able to specifically kill cancer cells in zebrafish embryos. Other experiments related to evaluation of therapeutic nanoparticles performed in xenografted zebrafish embryos refer, for example, Evensen et al (2016) tested polyethylene glycol (PEG) liposomes in a transgenic zebrafish line and observed a passive accumulation of liposomes specifically in the region where the tumor formed after the xenotranspant was carried out. The study not only obtained a reduction in the number of tumor cells into the fish but also served to study the interactions between their liposomes with the present zebrafish macrographs.

In our case, we determined the antitumor activity of E-NEs on MDA-MB-231 cells (positive for GFP). Xenografted embryos were first developed, and analyzed at 0 and 48 hours post transplantation with a QuantiFish program to quantify the fluorescence intensity in order to determinate the growth and cell spread (Stirling et al, 2017). Successfully, after treatment, we could observe a reduction on the fluorescence in the yolk of embryos treated with E-NEs, implying a reduction in the number of cancer cells (Figure 13A, B), a fact that validated the potential of our approach for the treatment of triple negative breast cancer.

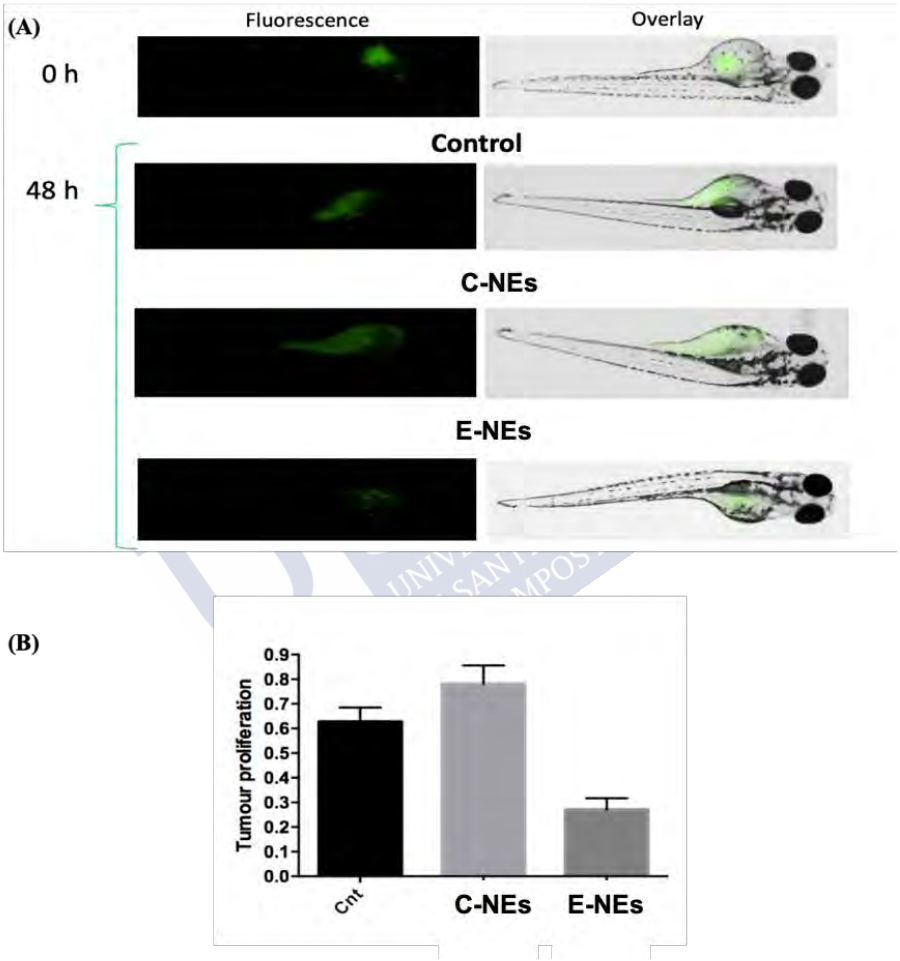

Figure 13. Efficacy of E-NEs in Xenografts (Source: own elaboration).

4. CONCLUSION

In this work we have successfully formulated and characterized stable nanoemulsions that efficiently incorporate edelfosine (E-NEs) for the treatment of triple negative breast cancer cells. E-NEs show a good effectiently in reducing triple negative cancer cell growing *in vivo,* using a xenotransplanted zebrafish models. With our results we can confirm that E-NEs show potential for the development of novel therapeutic strategies against triple negative breast cancer and that zebrafish is a powerful system for preclinical studies to screen novel anticancer nanosystems.

CHAPTER VI. Overall discussion

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The zebrafish is a *in vivo* animal model with a great potential in translational research. In the study of diseases like cancer, this model has served to investigate tumor behavior as well as for the screening of effective anticancer drugs (MacRae and Peterson 2015; Fior et al. 2017; Howe et al. 2013). Some free antitumor drugs have high toxicity, insolublility and low targeted. Also, all types of cancer have their own structure and mechanisms of action, and they can vary from person to person even though they belong to the same type of cancer (Õzdemir and Dotto, 2017). It is therefore important to improve the access of innovative strategies and the access of novel anticancer treatments, zebrafish standing as an effective, economical, fast and practical animal model for screening and preclinical testing of anticancer therapies and the development of precision oncology.

In this thesis, we carried out *in vivo* toxicity assays in zebrafish embryos and compare the results with *in vitro* studies, with the purpose of determining the importance of these assays for the following preclinical experiments where efficacy was evaluated (Gutiérrez-Lovera et al. 2019). Also we desinged edelfosine nanoemulsions for treatment of triple negative breast cancer. Eventually we tested their efficacy in xenotransplanted zebrafish embryos, in order to demonstrate the potential of different techniques that can be used to screening novel anticancer therapies including those based on the application of nanotechnology.

In the first part of these work, we performed the FET test for 4 important anticancer drugs (CarboPT, IT, DOX and PT), widely used in clinic treatments, and a recently discovered drug with anticancer

properties (CQ). We select these drugs for evaluation because this type of chemotherapists besides being the widely used, they are very difficult to predict its toxicity and effectiveness because the huge tumor heterogeneity of cancer (Almendro et al. 2013; Vogelstein et al. 2013). Some patients respond to the standard treatment but many others need to try alternative therapies with significant by-side effects and general cytotoxicity. There are approximately 50% that fail in clinical trials due to their toxicity and clinical safety (Eimon and Rubinstein 2009). Also, this drugs described in the literature have a lack in toxicity determinate in fish, all the concentration of the drugs used to evaluate new experiments are based on in vitro studies (Jung et al. 2012).

To carry out the FET test we dissolved the drugs and exposed the embryos during 96 h starting at 0 hpf and 72 hpf, to estimating mortality and morphological abnormalities and we compared their toxicity by the LC50 of each one. As mentioned earlier, to evaluate the efficacy of drugs in zebrafish embryos, xenografts are an excellent option. For that reason, the toxicity assays carried out at 72 hpf and up to 96 hpf was very important because this period of time is in which the xenografts assays are performed in zebrafish. The results presented show that the LC50 depends on the embryo developmental stage (0 hpf or 72 hpf). These results can be due to factors such as: permeability and presence of the chorion at 0 hpf thar could prevent

the drug internalization (Pelka et al. 2007). The different developmental stages (embryo and larva) because these stage can have different uptake and sensitivity (Kristofco et al. 2018). On the other hand, due to the nature of the drugs, for example, molecular weight (Paatero et al. 2017; Pitt et al. 2018).

All these measurements were contrasted with in vitro viability assays using the tumor cell lines: A549 (human lung carcinoma cell line), MCF7 cells (human breast adenocarcinoma cell line) and Panc185 (pancreatic ductal adenocarcinoma patient derived xenografts-PDX cancer cells). The data compared showed a certain trend in the values of drug toxicity between *in vitro* and in *vivo* results, but no for all values of some drugs, for example: the LC50 for CarboT was 330 mM (in 0–4 hpf embryos) and 3247 mM (in 72 hpf embryos) while the IC50 of the A549, MCF7 and PDX Panc185 cells was 1653, 143 and 10513 mM respectively. The results of the comparison show that toxicity data obtained *in vitro* cannot be extrapolated as a basis for subsequent studies to evaluate efficacy by xenografted embryos.

An important information found by comparing the *in vivo* data between the lines (A549 and MCF7) and the PDX cell line Panc185 was that the patient derived cancer cells were considerably more

resistant to the drugs evaluated in this assay. These results are very relevant because PDX cells are more similar to patient tumor cells so that the results based only on cytotoxicity assays with established tumor cell lines could overestimate the cytotoxicity of tested drugs.

On the other hand, there are many drugs that can cause malformations before the the death of embryos, when toxicity tests are done. In our case, 80% of embryos exposed to 8 mM CarboPt showed malformations at 48 hpt, an expected result because this drugs have reported congenital malformations, fetal dysmorphogenesis, prenatal mortality, and intrauterine growth retardation in mice and rats (Parashar et al. 2016; Kai et al. 1989).

Some authors consider the zebrafish as a complementary model to murine models for the evaluation of antitumor compounds and for the discovery of new drugs (MacRae and Peterson, 2015; Deveau et al. 2017; Van Rooijen et al. 2017; Zhao et al. 2015; Lenis-Rojas et al. 2016; Penas et al. 2016). The zebrafish embryos can be organ models and organ-specific drugs (Gallardo et al. 2015). In fact, the zebrafish is considered as the first level of complete organism with utility to study compounds prior to its analysis in mammals (Goldstone et al. 2010; Li et al. 2011).

To continue evaluating the potential of the zebrafish we have designed edelfosine nanoemulsions (E-NEs) for treatment of triple negative breast cancer, and evaluated their therapeutic efficacy in xenotransplanted zebrafish embryos. The E-NEs were formulated by a previously described ethanol injection method (Vázquez-Ríos et al. 2019). Edelfosine is a synthetic lipid with a high apoptotic action in several types of cancer such as breast, leukemia, pancreas, osteosarcoma and glioma (Gajate and Mollinedo, 2007; Gajate et al. 2004; Ruiter et al.1999; Nieto-Miguel et al. 2007; Estrella-Hermoso 2009). The E-NEs show suitable properties in terms of size (126 ± 4 nm and PDI of 0.1), and an almost neutral surface charge (zeta potential -1 ± 0 mV).

With respect to the interaction of the developed nanosystems with the targeted cells, data obtained from the citotoxity assays in MDA-MB-231 cells showed that they present antitumotal activity, when compared with the blank control formulation (C-NEs). Additionally, *in vivo* studies in zebrafish embryos showed that the LC50 in embryos exposed to E-NEs formulations, at 28ºC was 12.89 and 8.6 ug/mL at 0 and 72 hpf respectively, and at 34 °C 11.4 and 3.2 ug/mL at 0 and 72 hpf. We have performed experiments with two temperatures, 28º C and 34º C, because this is a relevant factor in the embryonic development of fish (Radonic et al. 2015), and can modify the

bioavailability of compounds (Heugens et al., 2001). The C-NEs were not toxic at the maximum concentration tested, 1 mg/mL.

The transparency of the zebrafish embryos allowed us studying the distribution of C-NEs. 72 hpf embryos were treated with 0.5 mg/mL of DiR and TopFluor® -loaded C-NEs. DiR was encapsulated into C-NEs, and TopFluor® was covalently linked to one of the lipids, avoiding a potential premature release. Our confocal microscope results show that C-NEs were efficiently internalized in the exposed embryos, especially in the yolk. A similar behavior has been recently observed by Teixeiro-Valiño et al (2017), they evaluated polymeric nanocapsules of hyaluronic acid and protamine and concluded that the capacity of internalization and biodistribution of nanoparticles is strongly dependent of the specific composition of nanoparticles.

With respect to the efficacy of E-NEs, zebrafish embryos can be an excellent tool to analyze critical aspects, such as growth and proliferation of tumors (Handi et al. 2006), invasion and intravasation (Stoletov et al. 2007; Naber et al. 2013; Yang et al. 2013), formation of metastasis (Drabsh et al. 2017; He et al. 2012), angiogenesis (He et al. 2012; Tobia et al. 2013), and immune cell response (Tat et al. 2013). In our case, we determined the antitumor activity of E-NEs in

MDA-MB-231 cells transplanted to zebrafish embryos. We analyzed 0 and 48 hours post xenotransplated embryos with a QuantiFish program to quantify the fluorescence intensity in order to determinate the growth and cell spread (Stirling et al, 2017). Our data show a reduction on the fluorescence in the yolk of embryos treated with E-NEs, implying a reduction in the number of cancer cells, a fact that validated the potential of our approach for the treatment of triple negative breast cancer. Other authors have also been able to evaluate xenografs in zebrabish embryos with important positive results (Wagner et al. 2010; Evensen et al. 2016).

With all our experiments we demonstrate that the zebrafish embryos are an excellent promising platform in preclinical studies of therapeutic anticancer drugs and new nanosystems against cancer.

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CHAPTER VII. Conclusion

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This work describes in 3 chapters how zebrafish embryos serve as a valuable tool in the study of the toxicity and efficacy of new antitumor therapies. The information and results obtained from the experimentation have allowed us to reach the following conclusions:

1. We evaluated the toxicity *in vitro* and *in vivo* for 5 important anticancer drugs (CarboPT, IT, DOX, PT and CQ). The toxicity values obtained for the antitumor drugs *in vitro* cannot be extrapolated to *in vivo* models such as zebrafish because they do not form a complete system and the results depend of factors like type of compound, type of cell line, method of analysis (*in vitro* or *in vivo*) and of the embryonic time in which the zebrafish experiments are performed.

2. We have successfully formulated and characterized bioavailable nanosystems for the association of the anticancer drug edelfosine. We proved that these nanosystems are stable in various biological media and are effective in the treatment of triple negative breast cancer cells. The experiments in xenotransplanted zebrafish demonstrated the antitumor efficacy of our nanosystems *in vivo* with wich we observed a reduction in the number of cancer cells in the yolk of embryos treated with E-NEs.

3. Zebrafish embryos serve as a promising tool in preclinical studies in which the stability, toxicity, and efficacy of therapeutic anticancer debugs and new nanosystems against various types of cancer can be evaluated.

LIST OF ABBREVIATIONS

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A549	Human lung carcinoma cell
ADME	Tox Absorption, distribution, metabolism, excretion and toxicity
ATCC	American type culture collection
CQ	Cloroquine
C-Nes	Control nanoemulsions
CNIO	Spanish National Cancer Centre
CarboPT	Carboplatino
DiR	DiIC18(7) (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide)
DMSO	Dimethyl sulfoxide
DPF	Days post fertilization
DPI	Days post injection
DMEM	Dulbecco's Modified Eagle Medium
DLS	Dynamic light Scattering
DOX	Doxorrubicina
E-Nes	Edelfosine nanoemulsions
ED	Edelfosine
EU	European Union
FBS	Fetal bovine serum
FDA	Food and Drugs Administration
FET	Fish embryo acute toxicity test
GFP	Green fluorescent protein

hpf	Hours Post-fertilization
hpi	Hours post injection
IC50	Half maximal inhibitory concentration
ISO	Organization for Economic Cooperation and Development
IT	Irinotecan
MCF7	Human breast adenocarcinoma cell
MDA-MB-231	Triple negative breast adenocarcinoma cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay
NOEC	No observed effect concentration
LC50	Lethal Concentration 50%
LOEC	Lowest Observed Effect Concentration
OECD	Organization for Economic Cooperation and Development
O/W	Oil-in-water
Panc185	pancreatic ductal adenocarcinoma patient derived xenografts-PDX cancer cells
PC	Phosphatidylcholine
PBS	Phosphate Buffer Saline
PdI	Polydispersity Index
PDX	Patient derived xenograft
PI	Proliferation index

PT	Paclitaxel
TNBC	Triple negative breast cancer
TopFluor-PC	1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphocholine
SD	Standard Deviation
US	United States
USA	United States of America
UAM	Universidad Autónoma de Madrid
UV	Ultraviolet-Visible Spectrum
WT	Wildtype
ZP	Zeta Potential

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ETHICAL CONSIDERATIONS

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Cell culture

Some cancer cell lines used in this work were acquired from commercially available resources (American Tissue Culture Collection, ATCC), i.e. A549 (ATCC® CCL-185™, human lung carcinoma cell), MCF7 (ATCC® HTB-22™, human breast adenocarcinoma cell) and MDA-MB-231 (ATCC® HTB-26™, triple negative breast adenocarcinoma cells). Cells were cultured in the conditions recommended by the manufacturers and only used for the research purposes specifically described in this thesis.

On the other hand, the experiments conducted with the patient-derived xenograft (PDX)-derived cell line Panc185 (pancreatic ductal adenocarcinoma patient derived xenografts-PDX cancer cells) were done in direct collaboration with Dr. Bruno Sainz from the Universidad Autónoma de Madrid (UAM). These cells were provided to Dr. Sainz by Dr. Manuel Hidalgo under a Material Transfer Agreement with the Spanish National Cancer Centre (CNIO), Madrid, Spain (Reference no. I409181220BSMH). Xenografts were processed as previously described (Mueller et al. 2009) to establish low-passage primary PDAC PDX-derived in vitro cultures.

In vivo Studies

All Zebrafish related studies were done in Lugo in the approved animal facilities AE-LU-003 (REGA ES270280346401) following European and National regulations. The procedures were approved by the ethical committee of the USC and Xunta de Galicia (MR110250). All animal experiments were reviewed and approved by the ethics committee and were executed in accordance with governing Spanish law and European Directives and Guidelines for the use of animals. Studies were performed therefore in compliance with the Directive 2010/63/EU of the European Parliament and Council of 22nd September 2010 on the protection of animals used for scientific purposes and under the Spanish Royal Decree 53/2013 February 1st on the protection of animals used for experimental and other scientific purposes.

Zebrafish embryos less than 5 days old are not considered legally animals according to Royal Decree 53/2013, of February 1, and the Directive 2010/63/EU of September 22, 2010, therefore, certification was not required to carry out the toxicity and biodistribution experiments. However, the efficacy experiments that involved the use of embryos of more than 5 days were carried out by a person with certification in animal management by Jeannette Martínez-Val.

Funding

This thesis was support by Regional Government Xunta de Galicia ED431C 2018/28.

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RESUMEN *IN EXTENSO*

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1. Introducción

El pez cebra (*Danio rerio*) (Hamilton, 1822) es un pez tropical de agua dulce, perteneciente a la familia Cyprinidae, del orden Cypriniformes (McCluskey et al. 2014, Stock et al. 2007). El pez cebra es endémico del sudeste asiático, en donde vive en ríos y en aguas estancadas (Laale, 1977; (Spence et al. 2008). El pez cebra crece en promedio de unos 3 a 5 cm y se caracteriza por poseer a lo largo de su cuerpo fusiforme cinco rayas azules longitudinales, de allí el origen de su nombre común (pez cebra). Este pez tiene un ciclo de reproducción muy corto alcanzando su madurez a la edad de 3 meses aproximadamente, lo cual es claramente beneficioso para la cría (Scholz et al., 2008). Además, es una especie ovípara y, en condiciones favorables, la hembra puede generar de entre 100 a 500 huevos cada 2-3 días durante todo el año (Talwar et al. 1991; Lohr a Hammerschmidt, 2011). Los peces adultos se aparean generalmente en época de verano, sin embargo, es posible realizar apareamientos dirigidos en los laboratorios durante todo el año. Además, toleran una amplia gama de condiciones ambientales y tipos de alimentación (Lawrence, 2007).

Los embriones de pez cebra se encuentran recubiertos por una membrana protectora denominada corión. El corion es poroso, posee

un grosor de entre 1,5 y 2,5 ɀm y está constituido por tres capas (Rawson et al. 2000). Dichos embriones no se consideran animales desde el punto de vista legal. Existe un real decreto que establece las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia, el Real Decreto 53/2013, de 1 de febrero, que mantiene la definición de animal dada en el artículo 3 del Real Decreto 1201/2005 al cual modifica, define como animal a todo vertebrado no humano, incluyendo larvas de vida libre, pero excluyendo las formas embrionarias, tales como los embriones de pez cebra. Por otro lado, la Directiva 2010/63/EU del 22 de septiembre de 2010, relativa a la protección de los animales utilizados para fines científicos se aplica también a las formas embrionarias de mamíferos, pero excluye a otras formas embrionarias, tales como el pez cebra.

El pez cebra más usado en la investigación es la línea salvaje, sin embargo, existe una variada gama de líneas genotípicas, mutantes o transgénicas (White et al. 2008), cada una de las cuales es más o menos adecuada para un ensayo en particular. Su genoma se ha secuenciado por completo y muestra aproximadamente un 70% de homología con el genoma humano (Howe et al. 2013).

El pez cebra tiene numerosas ventajas que lo llevaron a ser un organismo modelo con un gran potencial en la investigación translacional. Entre sus ventajas se encuentran:

- Su mantenimiento es económico, de fácil manipulación y debido su tamaño tan pequeño permite mantener grandes poblaciones en sistemas de acuarios de dimensiones reducidas. El costo de mantener estos peces es 100 a 1000 veces menor que el de mantener a ratones de laboratorio (Rojas-Muñoz et al. 2007).

- Tienen un ciclo reproductivo rápido y producen un número elevado de embriones por puesta (Talwar et al. 1991). El tiempo de generación es de 2-3 meses (Bresch, 1991; Scholz et al. 2008).

- Presentan una fecundación externa, por lo cual se puede dirigir la fecundación, en cruces directos o mediante fecundación *in vitro* (Parichy, 2015).

- Los embriones son transparentes y presentan un desarrollo rápido el cual está ampliamente estudiado (Kimmel et al., 1995). Por lo que son adecuados para pruebas de toxicidad (McKim, 1977; Hutchinson et al. 1998) y son perfectos para el estudio de fármacos anticancerígenos mediante

ensayos de xenotrasplantes (Kirchberguer et al. 2017), ensayos de biodistribución y estudios de macrófagos y neutrófilos (Evensen et al. 2016).

- Los embriones son muy resistentes y pueden ser manipulados de forma sencilla en procedimientos genéticos como morpholinos (Bill et al. 2009; Bedell et al. 2011; Timme-Lagary et al. 2012) ribonucleoproteinas (CRISPR/Cas) (Irion et al. 2014; Shah et al. 2015; Li et al. 2016; Albadri et al. 2017), microinyecciones en una sóla célula o también en xenotransplantes (Haldi et al. 2006; Taylor et al. 2009; Drabsch et al. 2017; Idilli et al. 2017; Wyatt et al. 2017; Roel et al. 2016).

- Son multicelulares e integran la interacción de varios tejidos y procesos de diferenciación. Por lo que se pueden obtener datos más cercanos a la realidad que los obtenidos mediante cultivos celulares.

- Presentan diversos órganos y tipos celulares similares a los de los mamíferos, los cuales son de fácil visualización mediante microscopía de alta resolución en tiempo real (Yang et al. 2013).

- A las 40 hpf el sistema inmune innato está activo (Cui et al. 2011), pero el sistema inmune adaptativo no estará funcionando completamente hasta dentro de sus 4-8 semanas después de la fertilización (Lam et al. 2004; Li et al. 2011). Por lo tanto, los resultados de los análisis llevados a cabo durante las fases de embrión/larva se remontan al sistema inmune innato.
- La Administración Europea de Seguridad Alimentaria (EFSA, 2005) ha declarado que los peces en estas sus primeras etapas de desarrollo, hasta 5 dpf, tienen menos probabilidades de experimentar dolor, sufrimiento, angustia o sufrir daños duraderos, de acuerdo con los Principios 3Rs (reemplazo, reducción y refinamiento) para la investigación animal humana (Russell y Burch, 1959).

El pez cebra ofrece gran cantidad de oportunidades para la investigación científica más allá de su uso en toxicología, como reemplazo de sus especímenes adultos en pruebas de toxicidad aguda (Scholz et al., 2008; Tan y Zon, 2011; Zon, 1999). El reducido tamaño de la gran cantidad de progenie que se genera del apareamiento de peces cebra no solo permite analizar el efecto de múltiples compuestos a la vez durante el desarrollo embrionario, sino de identificar nuevos medicamentos potencialmente efectivos y que no causen riesgos por

su toxicidad (Rojas-Muñoz et al.2007). Este modelo animal es una promesa para la evaluación y validación de fármacos y de nuevos agentes terapéuticos, incluidas las nanomedicinas genéticas. Este modelo recrea el proceso de absorción, distribución, metabolismo, excreción y toxicidad (ADME-Tox, por sus siglas en inglés) de distintas sustancias, a diferencia de los modelos *in vitro* (cultivos celulares), no sería posible puesto que las células no conforman un organismo completo (Goldstone et al. 2010; Li et al. 2011). Berghmans et al (2008) estudió la correlación entre los datos clínicos y preclínicos en animales y los datos obtenidos de un modelo de pez cebra prediciendo los efectos adversos de los fármacos evaluados y encontrando una buena asociación entre los datos provenientes de animales y de los clínicos.

El uso de embriones de pez cebra se encuentra en los campos estrechamente relacionados con la toxicología del desarrollo (embriotoxicidad y teratogenicidad) en donde se han encontrado varios fármacos teratogénicos utilizando el modelo de pez cebra (Augustine-Rauch et al. 2010; Lammer et al. 2009). La prueba oficial

de toxicidad aguda más utilizada es la OCDE 203. Esta prueba evalúa cuatro criterios de letalidad: coagulación de embriones, ausencia de somitos desarrollados, ausencia de latidos cardíacos y ausencia de desprendimiento de la yema de la cola del saco vitelino (OCDE, 2013).

En cuanto a las aplicaciones específicas en el desarrollo de fármacos antitumorales tenemos que el pez cebra ha servido como modelo animal durante las últimas dos décadas. Hoy en día, se pueden hacer crecer líneas celulares cancerígenas dentro de los embriones de peces cebra (técnica denominada xenograft o xenotransplante), al igual que en modelos de mamíferos como ratones (He et al, 2012; Teng et al, 2013), también estas líneas de cáncer pueden estudiarse mediante microscopía de alta resolución en embriones vivos (Yang et el, 2013; Lee et al. 2005). Se han realizado estudios con diversos tipos de cáncer (melanoma, carcinoma de mama, colorrectal, pancreático, ovárico, riñón, pulmón, oral, próstata, leucemia, etc.) en donde los resultados ha sido satisfactorios en establecer tumorogenicidad y en estudiar comportamiento metastásico (Marques et al, 2009; Nicoli et al, 2007; Lee et al, 2005; Dumarting et al, 2011; Haldi et al. 2006; Drabsch et al. 2017; Roel et al. 2016; Marques et al. 2009; Bansal et al. 2014; Zhang et al. 2014; Brown et al. 2017; Mort et al. 2015). El pez cebra se considera un modelo complementario a los modelos murinos para la evaluación de compuestos antitumorales y para el descubrimiento de nuevos fármacos (Stern y Zon, 2003; Goessling et

al- 2007; MacRae y Peterson, 2015; Deveau et al. 2017; Van Rooijen et al.2017; Zhao et al. 2015; Lenis-Rojas et al. 2016; Penas et al. 2016; Blackburn et al. 2014; Taj et al. 2013; Veinotte et al. 2014).

Por otro lado, también se han generado líneas transgénicas que expresan oncogenes conducidos por promotores ubicuos o específicos de tejidos cancerígenos (Mione et al. 2016). Por ejemplo, se ha generado un modelo transgénico de pez cebra para melanoma utilizando secuencias reguladoras del gen mitfa para impulsar la expresión de el gen BRAFV600E (Patton et al. 2015). El pez cebra también es un excelente modelo para proporcionar nuevas ideas sobre la interacción entre el sistema inmune y las células tumorales (Powell et al. 2016; Chambers et al. 2013). Debido a que en el pez cebra, los macrófagos juegan un papel importante en la angiogénesis, este modelo también podría usarse para desarrollar ensayos funcionales relacionados con el proceso angiogénico. En la **Figura 14** se puede apreciar un esquema que resume todas las funcionalidades que tiene el pez cebra para el estudio del cáncer y el cribado de fármacos.

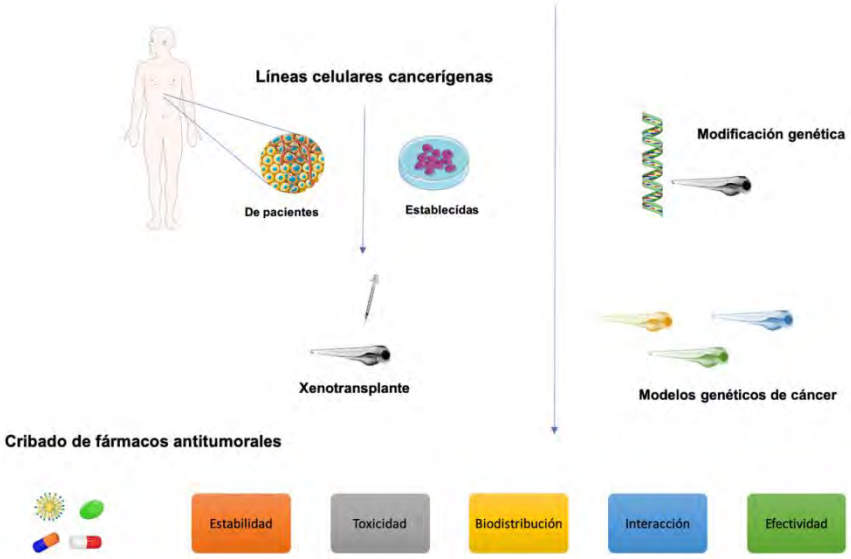

Figura 14. Funcionalidades del pez cebra para el estudio del cáncer y el cribado de fármacos antitumorales. Existen dos rutas principales, a partir de xenotransplantes de células de paciente o establecidas o a través del diseño de modelos trangénicos de peces cebra para cada tipo de cáncer. Ambos caminos por separado pueden son la base para el cribado de fármacos mediante estudios de estabilidad, toxicidad, biodistribición, interacción, y efectividad (Recurso: elaboración propia).

Hoy en día, la nanotecnología ha sido altamente empleada para fines médicos, la nanomedicina. Gran variedad de nanofarmacos se han diseñado para terapia génica, generación de vacunas, medicina de la regeneración o nuevos tratamientos farmacológicos, especialmente en oncología (Raviña et al. 2010). Sin embargo, las propiedades fisicoquímicas de los nanofarmacos pueden conducir a alteraciones en la farmacocinética, la absorción, distribución, metabolismo,

biodisponibilidad y toxicidad (Bleeker et al. 2013; Tinkle et al. 2014). El uso de nanofármacos contra el cáncer implica en la mayoría de los casos introducción de fármacos antitumorales en el interior de una nanoestructura, lo que le ofrece ventajas en relación al tratamiento con fármacos libres, que son normalmente poco solubles Gou et al. 2011) o en algunos casos muy tóxicos. Los nanosistemas en muchos de los casos pueden mejorar el acceso de los medicamentos asociados al tumor y disminuir los efectos secundarios secundarios (Peer et al., 2007; Torchilin, 2011).

Estudios recientes destacan el potencial del pez cebra para la evaluación de nuevas nanosistemas contra el cáncer. Algunos de ellos evaluaron la toxicidad y la seguridad de las nanopartículas blancas (es decir, antes de la incorporación del fármaco) utilizando diferentes procedimientos (Lee et al. 2017; Kim y Tanguay, 2013; Harper et al. 2015; Jeong et al. 2015). Aprovechando la transparencia embrionaria, también se han realizado estudios de biodistribución para determinar la capacidad de nanoportadores para llegar a su diana superando barreras biológicas complejas, como la barrera hematoencefálica (Yang et al. 2015; li et al. 2017; Sieber et al. 2017).

Por otro lado, se han realizado xenograft con diferentes tipos de células cancerígenas y se han estudiado la efectividad de diferentes

tipos de nanoportadores cargados de fármacos antitumorales, obteniendo resultados favorables (Lee et al. 2017; Evensen et al. 2016; Wehmas et al. 2016; Yang et al. 2016). El pez cebra tiene un gran potencial para estudiar diversas terapias génicas y su futuro es muy alentador. Hasta el momento son poco los autores que se han adentrado a estas novedosas terapias (Xu et al, 2016; Cordeiro et al, 2017) pero han abierto una ventana de posibilidades para el futuro.

2. El potencial del pez cebra como organismo modelo para mejorar la translación en nanomedicinas genéticas contra el cáncer

Además del uso del pez cebra para el desarrollo de nuevas terapias contra el cáncer, incluidas las nanoterapéuticas, solo se han informado unos pocos estudios que utilizan este modelo para probar nanomedicinas genéticas preclínicas (Xu et al. 2016; Aldrian et al. 2017; Cordeiro et al. 2017). El primer estudio encontrado en la

literatura evaluó una terapia sinérgica basada en la coencapsulación de un plásmido (PEDF) con paclitaxel, un fármaco quimioterapéutico de pequeño peso molecular, en un modelo de pez cebra transgénico, el Flk-1: eGFP. Los resultados en evidenciaron la efectividad antiangiogénica y segura de las nanopartículas (Xu et al. 2016). Por otro lado, Cordeiro et al (2017) diseñó nanopartículas de oro capaces de silenciar una proteína verde fluorescente (la EGFP) en embriones de una línea de pez cebra transgénico fli-EGFP. Los resultados de este modelo permitieron a los autores concluir que lograron diseñar con éxito una nanoplataforma biocompatible y eficiente para fines de silenciamiento génico.

3. Objetivos

El objetivo principal de esta tesis se basa en evaluar el potencial terapéutico de diferentes terapias contra el cáncer, incluidas las nanomedicinas, en un organismo modelo, el pez cebra. Para esto, los objetivos específicos se han definido de la siguiente manera:

1. Evaluación de la toxicidad de algunos medicamentos contra el cáncer de uso común en embriones de pez cebra, y determinación de parámetros de toxicidad específicos y perfiles toxicológicos cruciales para los estudios de xenotransplantes de pez cebra.

2. Evaluación de la toxicidad y la eficacia de nanoemulsiones basadas en un medicamento contra el cáncer, la edelfosina, en modelos de pez cebra xenotransplantados.

4. Discusión

El pez cebra es un modelo animal con un gran potencial en la investigación traslacional. En el estudio de enfermedades como el cáncer, este modelo ha servido para investigar tanto la estructura y comportamiento del tumor, como para la detección de fármacos anticancerígenos eficaces (MacRae y Peterson 2015; Fior et al. 2017; Howe et al. 2013).

En esta tesis, llevamos a cabo ensayos de toxicidad en embriones de pez cebra y comparamos los resultados con estudios *in vitro*, con la finalidad de determinar la importancia de estos ensayos para los siguientes experimentos preclínicos donde se evalúa la eficacia, a través de xenotransplantes (Gutiérrez-Lovera et al. 2019). También diseñamos nanosistemas de edelfosina para el tratamiento del cáncer de mama triple negativo y, finalmente, probamos su eficacia *in vivo* en embriones de pez cebra xenotransplantados, para demostrar el potencial de diferentes técnicas y protocolos que pueden usarse para detectar nuevas terapias contra el cáncer, incluidas las basadas en la nanotecnología.

En la primera parte de este trabajo, realizamos la prueba FET para 4 medicamentos contra el cáncer (Carboplatino, Irinotecan, Doxorrubicina y Paclitaxel), y un medicamento recientemente descubierto con propiedades contra el cáncer (Cloroquina). Para llevar a cabo la prueba FET, expusimos los embriones a diferentes concentraciones de los medicamentos durante 96 h a partir de 0 hpf y 72 hpf, para estimar la mortalidad y las anomalías morfológicas, posteriormente comparamos su toxicidad mediante los valores de LC50 obtenidos para cada uno. Como se mencionó anteriormente, para evaluar la eficacia de los medicamentos en embriones de pez cebra, los xenotransplantes son una excelente opción. Por esa razón, los ensayos de toxicidad realizados a 72 hpf y hasta 96 hpf fueron muy importantes porque este período de tiempo es en el que se realizan los ensayos de xenotransplantes en el pez cebra. Los resultados mostraron que la LC50 depende de la etapa de desarrollo del embrión (0 hpf o 72 hpf). Estos resultados pueden deberse a factores como: la permeabilidad, la presencia o ausencia del corion, las diferentes etapas de desarrollo (embrión y larva), o la naturaleza de las drogas (Pelka et al. 2007; Paatero et al.2017; Kristofco et al. 2018; Pitt et al. 2018).

Los resultados obtenidos *in vivo* se compararon con ensayos de viabilidad *in vitro* utilizando las líneas celulares tumorales: A549, MCF7 y Panc185 (PDX), y evidenciaron que los datos de toxicidad

obtenidos *in vitro* no pueden extrapolarse como base para estudios posteriores para evaluar la eficacia en embriones xenotransplantados. Por otro lado, se encontró que las células cancerosas derivadas del paciente eran considerablemente más resistentes a los fármacos evaluados en este ensayo que las células provenientes de cultivos establecidos.

Para continuar evaluando el potencial del pez cebra, diseñamos nanosistemas de edelfosina (E-NEs) para el tratamiento del cáncer de mama triple negativo, y evaluamos su eficacia terapéutica en embriones de pez cebra xenotransplantados. Las E-NEs fueron formuladas mediante un método de inyección de etanol previamente descrito (Pons et al, 1993; Maitani et al, 2001; Batzri & Korn, 1973; Jaafar-Maalej et al, 2010), y tuvieron un tamaño medio de 126 ± 4 nm, un potencial zeta cercano al valor neutro -1 ± 0 mV y un PDI de 0.1.

Los resultados *in vitro* realizados en células MDA-MB-231 para los nanosistemas desarrollados mostraron que las NEs presentan actividad antitumoral, en comparación con la formulación de control (C-NEs). Los estudios de toxicidad *in vivo* en embriones de pez cebra se llevaron a cabo a dos temperaturas, 28 ° C y 34 ° C. La LC50 en embriones expuestos a formulaciones de los E-NEs fue de 12.89 y 8.6 ꚍ g/mL a 0 y 72 hpf a 28 ° C, y de 11.4 y 3.2 ꚍ g/mL a 0 y 72 hpf a 34 °

C. La temperatura es un factor relevante en el desarrollo embrionario de los peces y puede modificar la biodisponibilidad de los compuestos (Heugens et al., 2001; Radonic et al. 2015). Por otro lado, los C-NEs no fueron tóxicos a la concentración máxima probada, 1 mg/ml.

La transparencia de los embriones de pez cebra nos permitió estudiar la distribución de los C-NEs marcados con DiR y TopFluor®. Los resultados evidenciaron una internalizaron eficientemente en los embriones expuestos a una concentración de 0,5 ꚍg/mL, especialmente en el vitelo. Teixeiro-Valiño et al (2017) observaron r un comportamiento similar, evaluaron las nanocápsulas poliméricas de ácido hialurónico y protamina y concluyeron que la capacidad de internalización y biodistribución de las nanopartículas depende en gran medida de la composición específica de las nanopartículas.

Con respecto a la eficacia antitumoral de los E-NEs en células MDA-MB-231 xenotransplantadas en embriones de pez cebra mostraron una reducción en la fluorescencia del vitelo de los embriones, lo que implica una reducción en el número de células cancerosas, un hecho que valida el potencial de nuestro enfoque para

el tratamiento del cáncer de mama triple negativo. La cuantificación de la intensidad en la fluorescencia producto del crecimiento y proliferación celular fue llevada a cabo mediante un programa informático denominado Quantifish (Stirling et al, 2017).

4. Conclusiones

Esta tesis describe en 3 capítulos como los embriones de pez cebra sirven como una herramienta valiosa en el estudio de la toxicidad y la eficacia de las nuevas terapias antitumorales. La información y los resultados obtenidos de la experimentación nos han permitido llegar a las siguientes conclusiones:

1. Evaluamos la toxicidad *in vitro* e *in vivo* para 5 importantes medicamentos contra el cáncer (Carboplatino, Irinotecan, Doxorrubicina, Paclitaxel y Cloroquina). Los valores de toxicidad obtenidos para los fármacos antitumorales *in vitro* no pueden extrapolarse a modelos *in vivo* como el pez cebra porque no forman un sistema completo y los resultados dependen de factores como el tipo de compuesto, el tipo de línea celular, el método de análisis (*in vitro* o *in vivo)* y del tiempo embrionario en el que se realizan los experimentos de pez cebra.

2. Hemos formulado y caracterizado exitosamente nanosistemas asociados al medicamento contra el cáncer edelfosina. Demostramos que estos nanosistemas son estables en varios medios biológicos y son efectivos en el tratamiento de células de cáncer de mama triple negativo. Los experimentos realizados en embriones de peces cebra xenotransplantados demostraron la eficacia antitumoral de nuestros nanosistemas, en los cuales observamos una reducción en el número de células cancerosas en el vitelo de embriones tratados con E-NEs.

3. Los embriones de pez cebra sirven como una herramienta prometedora en estudios preclínicos en los que se puede evaluar la estabilidad, la toxicidad y la eficacia de terapias contra el cáncer y nuevos nanosistemas contra diversos tipos de cáncer.

REFERENCES

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Ablain, J.; Zon, L.I. Of fish and men: Using zebrafish to fight human diseases. Trends Cell Biol. 2013, 23, 584–586.

Albadri, S.; Del Bene, F.; Revenu, C. Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish. Methods 2017, 121–122, 77–85.

Aldrian, G.; Vaissière, A.; Konate, K.; Seisel, Q.; Vivès, E.; Fernandez, F.; Viguier, V.; Genevois, C.; Couillaud, F.; Démèné, H.; et al. PEGylation rate influences peptide-based nanoparticles mediated siRNA delivery in vitro and in vivo. J. Control. Release 2017, 256, 79–91.

Almendro, Vanessa, Andriy Marusyk, and Kornelia Polyak. 2013. "Cellular Heterogeneity and Molecular Evolution in Cancer." *Annual Review of Pathology: Mechanisms of Disease*. https://doi.org/10.1146/annurev-pathol-020712-163923.

Ahmad et al. 1997. Enhanced therapeutic effects of liposome-associated 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine. Cancer Res. 57(10):1915-21.

Almhanna, K.; Wright, D.; Mercade, T.M.; Van Laethem, J.-L.; Gracian, A.C.; Guillen-Ponce, C.; Faris, J.; Lopez, C.M.; Hubner, R.A.; Bendell, J.; et al. A phase II study of antibody-drug conjugate, TAK-264 (MLN0264) in previously treated patients with advanced or metastatic pancreatic adenocarcinoma expressing guanylyl cyclase C. Investig. New Drugs 2017, 35, 634–641.

Anchordoquy, T.J.; Barenholz, Y.; Boraschi, D.; Chorny, M.; Decuzzi, P.; Dobrovolskaia, M.A.; Farhangrazi, Z.S.; Farrell, D.; Gabizon, A.; Ghandehari, H.; et al. Mechanisms and Barriers in Cancer Nanomedicine: Addressing Challenges, Looking for Solutions. ACS Nano 2017, 11, 12–18.

Augustine-Rauch K, Zhang CX, Panzica-Kelly JM. In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays. 2010. Birth Defects Research Part C: Embryo Today: Reviews. 90(2):87

Aznar et al. 2013. Efficacy of edelfosine lipid nanoparticles in breast cancer cells. International Journal of Pharmaceutics, 454(2); p.720-726.

Baetke, S.C.; Lammers, T.; Kiessling, F. Applications of nanoparticles for diagnosis and therapy of cancer. Br. J. Radiol. 2015, 88.

Ban J, et al. 2014. Suppression of deacetylase SIRT1 mediates tumor-suppressive NOTCH response and offers a novel treatment option in metastatic Ewing sarcoma. Cancer Res.74:6578–6588.

Bansal, N.; Davis, S.; Tereshchenko, I.; Budak-alpdogan, T.; Zhong, H.; Stein, M.N.; Kim, I.Y.; Dipaola, R.S.; Bertino, J.R.; Sabaawy, H.E. Enrichment of human prostate cancer cells with tumor initiating properties in mouse and zebrafish xenografts by differential adhesion. Prostate 2014, 74, 187–200.

Barbazuk,W.B.; Korf, I.; Kadavi, C.; Heyen, J.; Tate, S.;Wun, E.; Bedell, J.A.; McPherson, J.D.; Johnson, S.L. The synthenic relationship of the zebrafish and human genomes. Genome Res. 2000, 10, 1351–1358.

Bar-Ilan, O.; Louis, K.M.; Yang, S.P.; Pedersen, J.A.; Hamers, R.J.; Peterson, R.E.; Heideman, W. Titanium dioxide nanoparticles produce phototoxicity in the developing zebrafish. Nanotoxicology 2012, 6, 670–679.

Barenholz, Y. Doxil®—The first FDA-approved nano-drug: Lessons learned. J. Control. Release 2012, 160, 117–134.

Bayraktar, R.; Pichler, M.; Kanlikilicer, P.; Ivan, C.; Kahraman, N.; Aslan, B.; Oguztuzun, S. MicroRNA 603 acts as a tumor suppressor and inhibits triple- negative breast cancer tumorigenesis by targeting elongation factor 2 kinase. Oncotarget 2017, 8, 11641–11658.

Batzri, S.; Korn, E. D. Single Bilayer Liposomes Prepared without Sonication. BBA - Biomembr. 1973, 298, 1015– 1019.

Bedell, V.M.; Westcot, S.E.; Ekker, S.C. Lessons from morpholino-based screening in zebrafish. Brief. Funct. Genom. 2011, 10, 181–188.

Berghmans, S.; Butler, P.; Goldsmith, P.; Waldron, G.; Gardner, I.; Golder, Z.; Richards, F.M.; Kimber, G.; Roach, A.; Alderton, W.; et al. Zebrafish based assays for the assessment of cardiac, visual and gut function—potential safety screens for early drug discovery. J. Pharmacol. Toxicol. Methods 2008, 58, 59–68.

Bhavsar, D.; Subramanian, K.; Sethuraman, S.; Krishnan, U.M. “Nano–in–nano” hybrid liposomes increase target specificity and gene silencing efficiency in breast cancer induced SCID mice. Eur. J. Pharm. Biopharm. 2017, 119, 96–106.

Bill, B.R.; Petzold, A.M.; Clark, K.J.; Schimmenti, L.A.; Ekker, S.C. A Primer for Morpholino Use in Zebrafish. Zebrafish 2009, 6, 69–77.

Blackburn, J.S.; Langenau, D.M. Zebrafish as a model to assess cancer heterogeneity, progression and relapse. Dis. Model. Mech. 2014, 7, 755–762.

Blechinger, S.R.; Evans, T.G.; Tang, P.T.; Kuwada, J.Y.; Warren, J.T., Jr.; Krone, P.H. The heat-inducible zebrafish hsp70 gene is expressed during normal lens development under non-stress conditions. Mech. Dev. 2002, 112, 213–215.

Bleeker, E.A. de Jong W.H., Geertsma R.E., Groenewold M., Heugens, E.H. Koers-Jacquemijns M., van de Meent D., J.R. Popma, A.G. Rietveld, S.W. Wijnhoven, F.R. Cassee, A.G. Oomen. 2013. Considerations on the EU definition of a nanomaterial: science to support policy making. Regul. Toxicol. Pharmacol. 65: 119-125.

Bottai, G.; Truffi, M.; Corsi, F.; Santarpia, L. Progress in nonviral gene therapy for breast cancer and what comes next? Expert Opin. Biol. Ther. 2017, 17, 595–611.

Boushehri, M.A.S.; Lamprecht, A. Nanoparticles as drug carriers: Current issues with in vitro testing. Nanomedicine 2015, 10, 3213–3230.

Brand, W.; Noorlander, C.W.; Giannakou, C.; Park, M.V.D.Z.; Vandebriel, R.J.; Bosselaers, I.E.M. Nanomedicinal products: A survey on specific toxicity and side effects. Int. J. Nanomed. 2017, 12, 6107–6129.

Brown, H.K.; Schiavone, K.; Tazzyman, S.; Heymann, D.; Chico, T.J.A. Zebrafish xenograft models of cancer and metastasis for drug discovery. Expert Opin. Drug Discov. 2017, 12, 379–389.

Chakraborty, C.; Sharma, A.R.; Sharma, G.; Lee, S.-S. Zebrafish: A complete animal model to enumerate the nanoparticle toxicity. J. Nanobiotechnol. 2016, 14, 65.

Chambers, S.E.J.; O'Neill, C.L.; O'Doherty, T.M.; Medina, R.J.; Stitt, A.W. The role of immune-related myeloid cells in angiogenesis. Immunobiology 2013, 218, 1370–1375.

Chan, K. S., C. G. Koh, and H. Y. Li. 2012. "Mitosis-Targeted Anti-Cancer Therapies: Where They Stand." *Cell Death and Disease*. https://doi.org/10.1038/cddis.2012.148

Chang, J.; Ichihara, G.; Shimada, Y.; Tada-Oikawa, S.; Kuroyanagi, J.; Zhang, B.; Suzuki, Y.; Sehsah, R.; Kato, M.; Tanaka, T.; et al. Copper Oxide Nanoparticles Reduce Vasculogenesis in Transgenic Zebrafish Through Down-Regulation of Vascular Endothelial Growth Factor Expression and Induction of Apoptosis. J. Nanosci. Nanotechnol. 2015, 15, 2140–2147.

Chen, H.; Zhang,W.; Zhu, G.; Xie, J.; Chen, X. Rethinking cancer nanotheranostics. Nat. Rev. Mater. 2017, 2, 17024.

Chen, J.; Sun, X.; Shao, R.; Xu, Y.; Gao, J.; Wenquan, L. VEGF siRNA delivered by polycation liposome- encapsulated calcium phosphate nanoparticles for tumor angiogenesis inhibition in breast cancer.Int. J. Nanomed. 2017, 12, 6075–6088.

Churchet al. 2014. 'Toxgnostics': an unmet need in cancer medicine. Nature Reviews Cancer volume 14, pages 440–445.

Cui, C.; Benard, E.L.; Kanwal, Z.; Stockhammer, O.W.; van der Vaart, M.; Zakrzewska, A.; Spaink, H.P.; Meijer, A.H. Infectious Disease Modeling and Innate Immune Function in Zebrafish Embryos, 3rd ed.; Elsevier Inc.: Amsterdam, The Netherlands, 2011; Volume 105, ISBN 9780123813206.

Cui, M.; Dong, Z.; Cai, H.; Huang, K.; Liu, Y.; Fang, Z.; Li, X.; Luo, Y. Folate-targeted polymeric micelles loaded with superparamagnetic iron oxide as a contrast agent for magnetic resonance imaging of a human tongue cancer cell line. Mol. Med. Rep. 2017.

Cocco, E.; Deng, Y.; Shapiro, E.M.; Bortolomai, I.; Lopez, S.; Lin, K.; Bellone, S.; Cui, J.; Menderes, G.; Black, J.D.; et al. Dual-targeting nanoparticles for in vivo delivery of suicide genes to chemotherapy-resistant ovarian cancer cells. Mol. Cancer Ther. 2017, 16, 323–333.

Collins, A.R.; Annangi, B.; Rubio, L.; Marcos, R.; Dorn, M.; Merker, C.; Estrela-Lopis, I.; Cimpan, M.R.; Ibrahim, M.; Cimpan, E.; et al. High throughput toxicity screening and intracellular detection of

nanomaterials. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 2017, 9.

Cordeiro, M.; Carvalho, L.; Silva, J.; Saúde, L.; Fernandes, A.; Baptista, P. Gold Nanobeacons for Tracking Gene Silencing in Zebrafish. Nanomaterials 2017, 7, 10.

D'Abundo, L.; Callegari, E.; Bresin, A.; Chillemi, A.; Elamin, B.K.; Guerriero, P.; Huang, X.; Saccenti, E.; Hussein, E.M.A.A.; Casciano, F.; et al. Anti-leukemic activity of microRNA-26a in a chronic lymphocytic leukemia mouse model. Oncogene 2017, 1–10.

Dang, M.; Henderson, R.E.; Garraway, L.A.; Zon, L.I. Long-term drug administration in the adult zebrafish using oral gavage for cancer preclinical studies. Dis. Model. Mech. 2016, 9, 811–820.

Daroczi, B.; Kari, G.; McAleer, M.F.; Wolf, J.C.; Rodeck, U.; Dicker, A.P. In vivo Radioprotection by the Fullerene Nanoparticle DF-1 as Assessed in a Zebrafish Model. Clin. Cancer Res. 2006, 12, 7086–7091.

DaunoXome approved. AIDS Patient Care STDS 1996, 10, 263.

De la Fuente, M.; Csaba, N.; Garcia-Fuentes, M.; Alonso, M.J. Nanoparticles as protein and gene carriers to mucosal surfaces. Nanomedicine 2008, 3, 845–857.

De la Fuente, M.; Jones, M.C.; Santander-Ortega, M.J.; Mirenska, A.; Marimuthu, P.; Uchegbu, I.; Schãtzlein, A. A nano-enabled cancer-specific ITCH RNAi chemotherapy booster for pancreatic cancer. Nanomed. Nanotechnol. Biol. Med. 2015, 11, 369–377.

De la Fuente, M.; Seijo, B.; Alonso, M.J. Design of novel polysaccharidic nanostructures for gene delivery. Nanotechnology 2008, 19, 75105.

De la Fuente, M.; Raviña, M.; Sousa-Herves, A.; Correa, J.; Riguera, R.; Fernandez-Megia, E.; Sánchez, A.; Alonso, M.J. Exploring the efficiency of gallic acid-based dendrimers and their block copolymers with PEG as gene carriers. Nanomedicine 2012, 7, 1667–1681.

Del Pozo-Rodríguez, A.; Solinís, M.Á.; Rodríguez-Gascón, A. Applications of lipid nanoparticles in gene therapy. Eur. J. Pharm. Biopharm. 2016, 109, 184–193.

Dent R, Trudeau M, Pritchard KI et al (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13:4429–4434.

Deok-Ho, K.; Yu, S.; Seok, Y.; Sang, H. L. & Byungkyu, K. 2005. Investigating chorion softening of zebrafish embryos with a microrobotic force sensing system. *Journal of Biomechanics*. 38: 1359-1363.

Detappe, A.; Kunjachan, S.; Rottmann, J.; Robar, J.; Tsiamas, P.; Korideck, H.; Tillement, O.; Berbeco, R. AGuIX nanoparticles as a promising platform for image-guided radiation therapy. Cancer Nanotechnol. 2015, 6, 4.

Deveau, A.P.; Bentley, V.L.; Berman, J.N. Using zebrafish models of leukemia to streamline drug screening and discovery. Exp. Hematol. 2017, 45, 1–9.

Dowdy, S.F. Overcoming cellular barriers for RNA therapeutics. Nat. Biotechnol. 2017, 35, 222–229.

Drabsch, Y.; Snaar-Jagalska, B.E.; Ten Dijke, P. Fish tales: The use of zebrafish xenograft human cancer cell models. Histol. Histopathol. 2017, 32, 673–686.

Duan, J.; Hu, H.; Li, Q.; Jiang, L.; Zou, Y.; Wang, Y.; Sun, Z. Combined toxicity of silica nanoparticles and methylmercury on cardiovascular system in zebrafish (Danio rerio) embryos. Environ. Toxicol. Pharmacol. 2016, 44, 120–127.

Duan, J.; Hu, H.; Feng, L.; Yang, X.; Sun, Z. Silica nanoparticles inhibit macrophage activity and angiogenesis via VEGFR2-mediated MAPK signaling pathway in zebrafish embryos. Chemosphere 2017, 183, 483–490.

EFSA Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to the aspects of the biology and welfare of animals used for experimental and other scientific purposes (EFSA-Q-2004-105). EFSA J. 2005, 292, 1–46.

Eguiara, A.; Holgado, O.; Beloqui, I.; Abalde, L.; Sanchez, Y.; Callol, C.; Martin, A.G. Xenografts in zebrafish embryos as a rapid functional assay for breast cancer stem-like cell identification. Cell Cycle 2011, 10, 3751–3757.

Eimon, P. and Rubinstein, A. 2009. The Use of in Vivo Zebrafish Assays in Drug Toxicity Screening. Expert Opinion on Drug Metabolism & Toxicology.

European Medicines Agency—Science Medicines Health. Available online: http://www.ema.europa.eu/ema/ (accessed on 24 November 2017).

Estella-Hermoso de Mendoza, Miguel A. Campanero, Janis de la Iglesia-Vicente, Consuelo Gajate, Faustino Mollinedo and María J. Blanco-Prieto. Antitumor Alkyl Ether Lipid Edelfosine: Tissue Distribution and Pharmacokinetic Behavior in Healthy and Tumor-Bearing Immunosuppressed Mice. Clin Cancer Res February 1 2009 (15) (3) 858-864.

Estella-Hermoso et al. 2011. In vitro and in vivo efficacy of edelfosine-loaded lipid nanoparticles against glioma. Journal of Controlled Release. p. 421-426.

Estella-Hermoso et al. 2012. Complete inhibition of lymphatic metastases by edelfosine-loaded nanosystems Nanomedicine UK, 7(5); p.679-690.

Evensen, L.; Johansen, P.L.; Koster, G.; Zhu, K.; Herfindal, L.; Speth, M.; Fenaroli, F.; Hildahl, J.; Bagherifam, S.; Tulotta, C.; Prasmickaite, L.; Mælandsmo, G.M.; Snaar-Jagalska, E.; Griffiths, G. Zebrafish as a model system for characterization of nanoparticles against cancer. Nanoscale 2016, 8, 862–877.

Fako VE, Furgeson DY. 2009. Zebrafish as a correlative and predictive model for assessing biomaterial nanotoxicity. Adv Drug Deliv Rev. 61(6):478-86.

Fan, Y.; Guo, R.; Shi, X.; Allen, S.; Cao, Z.; Baker, J.R.; Wang, S.H. Modified Nanoemulsions with Iron Oxide for Magnetic Resonance Imaging. Nanomaterials 2016, 6, 223.

FDA Press Announcements—FDA Approval Brings First Gene Therapy to the United States; U.S. Food and Drug Administration: Silver Spring, MD, USA, 2017.

Fenaroli, F.; Westmoreland, D.; Benjaminsen, J.; Kolstad, T.; Skjeldal, F.M.; Meijer, A.H.; Van Der Vaart, M.; Ulanova, L.; Roos, N. Nanoparticles as Drug Delivery System against Tuberculosis in Zebrafish Embryos: Direct Visualization and treatment. ACS Nano 2014, 8, 7014–7026.

Fent, K.; Weisbrod, C.J.;Wirth-Heller, A.; Pieles, U. Assessment of uptake and toxicity of fluorescent silica nanoparticles in zebrafish (Danio rerio) early life stages. Aquat. Toxicol. 2010, 100, 218–228.

Filby AL, Paull GC, Bartlett EJ, Van Look KJ, Tyler CR. 2010. Physiological and health consequences of social status in zebrafish (*Danio rerio*). Physiol Behav 101:576-587.

Fior, Rita, Vanda Póvoa, Raquel V. Mendes, Tânia Carvalho, António Gomes, Nuno Figueiredo, and Miguel Godinho Ferreira. 2017. "Single-Cell Functional and Chemosensitive Profiling of Combinatorial Colorectal Therapy in Zebrafish Xenografts."

Proceedings of the National Academy of Sciences 114 (39): E8234–43.

Fulford, et al, 2007. Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival, Breast Cancer Res, 9.

Gajate C and Mollinedo F. 2007. Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts. Blood. 109:711-719.

Gajate, C., Matos-da-Silva, M., Dakir, EL-H., Fonteriz, R.I., Alvarez, J., and Mollinedo, F. Antitumor alkyl-lysophospholipid analogue edelfosine induces apoptosis in pancreatic cancer by targeting endoplasmic reticulum. 2012. *Oncogene* 31, 2627-2639.

Garcia, Gloria R., Pamela D. Noyes, and Robert L. Tanguay. 2016. "Advancements in Zebrafish Applications for 21st Century Toxicology." Pharmacology and Therapeutics.

Garcia, G.R.; Noyes, P.D.; Tanguay, R.L. Advancements in zebrafish applications for 21st century toxicology. Pharmacol. Ther. 2016, 161, 11–21.

Geffroy, B.; Ladhar, C.; Cambier, S.; Treguer-Delapierre, M.; Brèthes, D.; Bourdineaud, J.-P. Impact of dietary gold nanoparticles in zebrafish at very low contamination pressure: The role of size, concentration and exposure time. Nanotoxicology 2012, 6, 144–160.

Ghotra V, He S, Bont H, Ent W, Spaink H, Water B, Snaar-Jagalska B, Danen E. 2012. Automated whole animal bio-imaging assay for human cancer dissemination. PloS One 7:e31281.

Gallardo et al. 2015. Phenotype-driven chemical screening in zebrafish for compounds that inhibit collective cell migration identifies multiple pathways potentially involved in metastatic invasion. Dis Model Mech. 8(6):565-76.

Giannaccini, M.; Cuschieri, A.; Dente, L.; Raffa, V. Non-mammalian vertebrate embryos as models in nanomedicine. Nanomed. Nanotechnol. Biol. Med. 2014, 10, 703–719.

Giannakou, C.; Park, M.V.D.Z.; De Jong, W.H.; Van Loveren, H.; Vandebriel, R.J.; Geertsma, R.E. A comparison of immunotoxic effects of nanomedicinal products with regulatory immunotoxicity testing requirements. Int. J. Nanomed. 2016, 11, 2935–2952.

Goessling,W.; North, T.E.; Zon, L.I. New waves of discovery: Modeling cancer in zebrafish. J. Clin. Oncol. 2007, 25, 2473–2479.

Gong, C.; Deng, S.;Wu, Q.; Xiang, M.;Wei, X.; Li, L.; Gao, X.;Wang, B.; Sun, L.; Chen, Y.; et al. Improving antiangiogenesis and anti-tumor activity of curcumin by biodegradable polymeric micelles. Biomaterials 2013, 34, 1413–1432.

González-Fernández et al. 2015. Lipid nanoparticles enhance the efficacy of chemotherapy in primary and metastatic human osteosarcoma cells. J. Drug Deliv. Sci. Technol. 30:435–442.

González-Fernández et al. 2017. Doxorubicin and edelfosine lipid nanoparticles are effective acting synergistically against drug-resistant osteosarcoma cancer cells. Cancer Lett. 388:262-268.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jonsson, M.E., Nelson, D.R. & Stegeman, J.J. 2010, "Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish", *BMC genomics,* vol. 11, pp. 643-2164-11-643.

Guideline, T.T.; Guideline, O. OECD Guidelines for the Testing of Chemicals; Oecd/Ocde 220; Organisation for Economic Co-operation and Development: Paris, France, 2004; pp. 1–22.

Gutiérrez-Lovera C, Vázquez-Ríos AJ, Guerra-Varela J, Sánchez L, and de la Fuente M. 2017. The Potential of Zebrafish as a Model Organism for Improving the Translation of Genetic Anticancer Nanomedicines. Genes (Basel). 8(12): 349.

Gutiérrez-Lovera C, Martínez-Val J, Cabezas-Sainz P, López R, Rubiolo JA & Sánchez L. 2019. In vivo toxicity assays in zebrafish

embryos: a pre-requisite for xenograft preclinical studies. Toxicology Mechanisms and Methods, 29, 7.

Guo S, Huang L. Nanoparticles escaping RES and endosome: challenges for siRNA delivery for cancer therapy. 2011. J Nanomater. 2011:11.

Haldi, M.; Ton, C.; Seng,W.L.; McGrath, P. Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. Angiogenesis 2006, 9, 139–151.

Hamilton, F. 1822. An Account of the Fishes Found in the River Ganges and its Branches. Edinburgh and London, Archibald Constable and Co.:321, 390, and 405.

Harper, S.; Maddux, B.L.S.; Hutchison, J.E.; Usenko, C.; Tanguay, R. Biodistribution and Toxicity of Nanomaterials In Vivo: Effects of Composition, Size, Surface Functionalization and Route of Exposure. Nanotech 2007, 2, 666–669.

Harper, B.; Thomas, D.; Chikkagoudar, S.; Baker, N.; Tang, K.; Heredia-Langner, A.; Lins, R.; Harper, S. Comparative hazard analysis and toxicological modeling of diverse nanomaterials using the embryonic zebrafish (EZ) metric of toxicity. J. Nanoparticle Res. 2015, 17, 1–12.

He, S.; Lamers, G.E.M.; Beenakker, J.W.M.; Cui, C.; Ghotra, V.P.S.; Danen, E.H.J.; Meijer, A.H.; Spaink, H.P.; Snaar-Jagalska, B.E. Neutrophil-mediated experimental metastasis is enhanced by VEGFR inhibition in a zebrafish xenograft model. 2012. J. Pathol. 227, 431–445.

Henn, Kirsten, and Thomas Braunbeck. 2011. "Dechorionation as a Tool to Improve the Fish Embryo Toxicity Test (FET) with the Zebrafish (Danio Rerio)." Comparative Biochemistry and Physiology - C Toxicology and Pharmacology.

Home—ClinicalTrials.gov. Available online: https://clinicaltrials.gov/ (accessed on 24 November 2017).

Howe, K.; Clark, M.; Torroja, C.; Torrance, J.; Berthelot, C.; Muffato, M.; Collins, J.E.; Humphray, S.; McLaren, K.; Matthews, L.;

et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature, 496, 498–503.

Huiting, L.N.; Laroche, F.; Feng, H. The Zebrafish as a Tool to Cancer Drug Discovery. Austin J. Pharmacol. Ther. 2015, 3, 1069.

Hussainzada, N.; Lewis, J.A.; Baer, C.E.; Ippolito, D.L.; Jackson, D.A.; Stallings, J.D. Whole adult organism transcriptional profiling of acute metal exposures in male zebrafish. BMC Pharmacol. Toxicol. 2014, 15, 15.

Idilli A. I., Precazzini F., Mione M. C. and Anelli V. (2017). Zebrafish in translational cancer research: Insight into leukemia, melanoma, Glioma and endocrine tumor biology. *Genes (Basel)* 8.

Ikonomopoulou MP, Fernandez-Rojo MA, Pineda SS, Cabezas-Sainz P et al. 2018. Gomesin inhibits melanoma growth by manipulating key signaling cascades that control cell death and proliferation. Sci Rep. 1;8(1):11519.

Irion, U.; Krauss, J.; Nusslein-Volhard, C. Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. Development 2014, 141, 4827–4830.

ISO. 1996. International Organization for Standardization. Water quality - Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)]. ISO 7346-3: Flow-through method.

Jaafar-Maalej, C.; Diab, R.; Andrieu, V.; Elaissari, A.; Fessi, H. Ethanol Injection Method for Hydrophilic and Lipophilic Drug-Loaded Liposome Preparation. J. Liposome Res. 2010, 20, 228–243.

Jain, A.S.; Makhija, D.T.; Goel, P.N.; Shah, S.M.; Nikam, Y.; Gude, R.P.; Jagtap, A.G.; Nagarsenker, M.S. Docetaxel in cationic lipid nanocapsules for enhanced in vivo activity. Pharm. Dev. Technol. 2016, 21, 76–85.

Jain, P.; Pawar, R.S.; Pandey, R.S.; Madan, J.; Pawar, S.; Lakshmi, P.K.; Sudheesh, M.S. In-vitro in-vivo correlation (IVIVC) in

nanomedicine: Is protein corona the missing link? Biotechnol. Adv. 2017, 35, 889–904.

Jang, G.H.; Lee, K.Y.; Choi, J.; Kim, S.H.; Lee, K.H. Multifaceted toxicity assessment of catalyst composites in transgenic zebrafish embryos. Environ. Pollut. 2016, 216, 755–763.

Jeong, J.; Cho, H.J.; Choi, M.; Lee,W.S.; Chung, B.H.; Lee, J.S. In vivo toxicity assessment of angiogenesis and the live distribution of nano-graphene oxide and its PEGylated derivatives using the developing zebrafish embryo. Carbon N. Y. 2015, 93, 431–440.

Jo, Dong Hyun, Dain Son, Yirang Na, Manyoung Jang, Jae Hoon Choi, Jin Hyoung Kim, Young Suk Yu, Seung Hyeok Seok, and Jeong Hun Kim. 2013. “Orthotopic Transplantation of Retinoblastoma Cells into Vitreous Cavity of Zebrafish for Screening of Anticancer Drugs.” Molecular Cancer.

Jung, Da-Woon, Eun-Sang Oh, Si-Hwan Park, Young-Tae Chang, Cheol-Hee Kim, Seok-Yong Choi, and Darren R. Williams. 2012. “A Novel Zebrafish Human Tumor Xenograft Model Validated for Anti-Cancer Drug Screening.” *Molecular BioSystems*.

Kai, S, H Kohmura, K Ishikawa, Y Makihara, S Ohta, S Kawano, and N Takahashi. 1989. "Teratogenic Effects of Carboplatin, an Oncostatic Drug, Administered during the Early Organogenetic Period in Rats." *The Journal of Toxicological Sciences* 14 (2): 115–30.

Kari G., Rodeck U., Dicker A. P. (2007). Zebrafish: an emerging model system for human disease and drug discovery. *Clin. Pharmacol. Ther.* 82, 70–80.

Kayl, A. E., & Meyers, C. A. 2006. Side-effects of chemotherapy and quality of life in ovarian and breast cancer patients. Current Opinion in Obstetrics & Gynecology, 18, 24-28.

Kim, K.-T and Tanguay, R.L. Integrating zebrafish toxicology and nanoscience for safer product development. Green Chem. 2013, 15, 872.

Kimmel, C.B.; Ballard, W.W.; Kimmel, S.R.; Ullmann, B.; Schilling, T.F. Stages of embryonic development of the zebrafish. 1995. Dev. Dyn. 203: 253–310.

Kirchberger, S.; Sturtzel, C.; Pascoal, S.; Distel, M. Quo natas, Danio?—Recent Progress in Modeling Cancer in Zebrafish. Front. Oncol. 2017, 7.

Kotb, S.; Detappe, A.; Lux, F.; Appaix, F.; Barbier, E.L.; Tran, V.L.; Plissonneau, M.; Gehan, H.; Lefranc, F.; Rodriguez-Lafrasse, C.; et al. Gadolinium-based nanoparticles and radiation therapy for multiple brain melanoma metastases: Proof of concept before phase I trial. Theranostics 2016, 6, 418–427.

Kumari S, Badana AK, Mohan GM et al (2017) Synergistic effects of coralyne and paclitaxel on cell migration and proliferation of breast cancer cells lines. Biomed Pharmacother 91.

Kuninty, P.R.; Schnittert, J.; Storm, G.; Prakash, J. MicroRNA Targeting to Modulate Tumor Microenvironment. Front. Oncol. 2016, 6, 1–8.

Maeda R, Yoshida J, Ishii G, Hishida T, Nishimura M, et al. (2010) Prognostic impact of intratumoral vascular invasion in non-small cell lung cancer patients. Thorax 65: 1092–1098.

Martínez-González, R.; Estelrich, J.; Busquets, M. Liposomes Loaded with Hydrophobic Iron Oxide Nanoparticles: Suitable T2 Contrast Agents for MRI. Int. J. Mol. Sci. 2016, 17, 1209.

Marques, I.J., Weiss, F.U., Vlecken, D.H., Nitsche, C., Bakkers, J., Lagendijk, A.K., Partecke, L.I., Heidecke, C.D., Lerch, M.M. & Bagowski, C.P. 2009, "Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model", *BMC cancer,* vol. 9, pp. 128-2407-9-128.

McCollum, Catherine W., Nicole A. Ducharme, Maria Bondesson, and Jan-Ake Ake Gustafsson. 2011. "Developmental Toxicity Screening in Zebrafish." Birth Defects Research Part C: Embryo Today: Reviews.

McCluskey, B.M. & Postlethwait, J.H. 2014, "Phylogeny of zebrafish, a "model species," within Danio, a "model genus"", *Molecular biology and evolution,* vol. 32, no. 3, pp. 635-652.

MacPhail, R.C.; Hunter, D.L.; Irons, T.D.; Padilla, S. Locomotion and Behavioral Toxicity in Larval Zebrafish: Background, Methods, and Data. In Zebrafish; JohnWiley & Sons, Inc.: Hoboken, NJ, USA, 2011; pp. 151–164.

MacRae, C.A.; Peterson, R.T. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 2015, 14, 721–731.

MacRae, C.A.; Peterson, R.T. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 2015, 14, 721–731Marques, I.J.; Weiss, F.U.; Vlecken, D.H.; Nitsche, C.; Bakkers, J.; Lagendijk, A.K.; Partecke, L.I.; Heidecke, C.-D.; Lerch, M.M.; Bagowski, C.P. Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. BMC Cancer 2009, 9, 128.

Maitani, Y.; Soeda, H.; Junping, W.; Takayama, K. Modified Ethanol Injection Method for Liposomes Containing SS-Sitosterol-d-Glucoside. J. Liposome Res. 2001, 11, 115–125.

Malla RR, Kumari S, Gavara MM, Badana AK, Gugalavath S, Kumar DKG, Rokkam PA perspective on the diagnostics, prognostics, and therapeutics of microRNAs of triple-negative breast cancer. Biophys Rev. 2019

Marques, I.J.; Weiss, F.U.; Vlecken, D.H.; Nitsche, C.; Bakkers, J.; Lagendijk, A.K.; Partecke, L.I.; Heidecke, C.-D.; Lerch, M.M.; Bagowski, C.P. Metastatic behaviour of primary human

tumours in a zebrafish xenotransplantation model. BMC Cancer 2009, 9, 128.

McErlean, E.M.; McCrudden, C.M.; McCarthy, H.O. Delivery of nucleic acids for cancer gene therapy: Overcoming extra- and intra-cellular barriers. Ther. Deliv. 2016, 7, 619–637.

McKim, J. M., J. G. Eaton, and G. W. Holcombe. 1977. Metal toxicity to embryos and larvae-early juveniles of eight species of freshwater fish. Copper.Bull. Environ. Contam. Toxicol.

Mimeault, Murielle, and Surinder K. Batra. 2013. "Emergence of Zebrafish Models in Oncology for Validating Novel Anticancer Drug Targets and Nanomaterials." Drug Discovery Today.

Mione M. C. and Trede N. S. (2010). The zebrafish as a model for cancer. *Dis. Model. Mech.* 3, 517-523.

Mollinedo et al. 2004. ET-18-OCH3 (edelfosine): a selective antitumour lipid targeting apoptosis through intracellular activation of Fas/CD95 death receptor. Curr Med Chem. 11(24):3163-84.

Mort, R.L.; Jackson, I.J.; Patton, E.E. The melanocyte lineage in development and disease. 2015. Development. 142, 1387.

Mueller, Maria–Theresa, Patrick C. Hermann, Juliane Witthauer, Belen Rubio–Viqueira, Simon F. Leicht, Stephan Huber, Joachim W. Ellwart, et al. 2009. "Combined Targeted Treatment to Eliminate Tumorigenic Cancer Stem Cells in Human Pancreatic Cancer." Gastroenterology 137 (3): 1102–13.

Muntimadugu, E.; Kommineni, N.; Khan, W. Exploring the Potential of Nanotherapeutics in Targeting Tumor Microenvironment for Cancer Therapy. Pharmacol. Res. 2017, 1–14.

Naber, H.P.H.; Drabsch, Y.; Snaar-Jagalska, B.E.; ten Dijke, P.; van Laar, T. Snail and Slug, key regulators of TGF-_-induced EMT, are sufficient for the induction of single-cell invasion. Biochem. Biophys. Res. Commun. 2013, 435, 58–63.

Nagel, R. 2002. DarT: The embryo test with the zebrafish (Danio rerio) - a general model in ecotoxicology and toxicology. ALTEX 19: 38-48.

Nascimento, T.L.; Hillaireau, H.; Vergnaud, J.; Fattal, E. Lipid-based nanosystems for CD44 targeting in cancer treatment: Recent significant advances, ongoing challenges and unmet needs. Nanomedicine 2016, 11, 1865–1887.

Natarajan, Ganesan, R. Malathi, and Eggehard Holler. 1999. "Increased DNA-Binding Activity of Cis-1,1-Cyclobutanedicarboxylatodiammineplatinum(II) (Carboplatin) in the Presence of Nucleophiles and Human Breast Cancer MCF-7 Cell Cytoplasmic Extracts: Activation Theory Revisited." Biochemical Pharmacology 58 (10): 1625–29.

Nguyen, V.H.; Lee, B.J. Protein corona: A new approach for nanomedicine design. Int. J. Nanomed. 2017, 12, 3137–3151.

Nicoli S, Presta M. 2007. The zebrafish/tumor xenograft angiogenesis assay. Nat Protoc. 2(11):2918-23.

Nieto-Miguel, T., Gajate, C., González-Camacho, F., and Mollinedo, F. 2008. Proapoptotic role of Hsp90 by its interaction with c-Jun N-terminal kinase in lipid rafts in edelfosine-mediated antileukemic therapy. *Oncogene* 27, 1779-1787.

Nishimura, Yuhei, Atsuto Inoue, Shota Sasagawa, Junko Koiwa, Koki Kawaguchi, Reiko Kawase, Toru Maruyama, Soonih Kim, and Toshio Tanaka. 2016. "Using Zebrafish in Systems Toxicology for Developmental Toxicity Testing." Congenital Anomalies.

Novartis Receives First Ever FDA Approval for a CAR-T Cell Therapy, Kymriah(TM) (CTL019), for Children and Young Adults with B-cell ALL That Is Refractory or Has Relapsed at Least Twice; Novartis: Basel, Switzerland, 2017.

Laale, H. W. 1977. The biology and use of zebrafish, Brachydanio rerio in fisheries research. A literature review. Journal of Fish Biology 10, 121–173.

Lam, S.H.; Chua, H.L.; Gong, Z.; Lam, T.J.; Sin, Y.M. Development and maturation of the immune system in zebrafish, Danio rerio: A gene expression profiling, in situ hybridization and immunological study. Dev. Comp. Immunol. 2004, 28, 9–28.

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T. 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp Biochem Phys C 149:196–209

Lasa-Saracibar et al. 2013. Edelfosine lipid nanosystems overcome drug resistance in leukemic cell lines. Cancer Letters, 334(2); p.302-310.

Lasa-Saracíbar et al. 2014. Lipid nanoparticles protect from edelfosine toxicity in vivo. Int J Pharm. 474(1-2):1-5.

Lawrence, C. 2007. The husbandry of zebrafish (Danio rerio): A review. Aquaculture 269, 1–20.

Lawrence, C. 2011. The Reproductive Biology and Spawning of Zebrafish in Laboratory Settings, in: Zebrafish. John Wiley & Sons, Inc., 1–13.

Lee SH, Nishino M, Mazumdar T, Garcia GE, Galfione M, Lee FL, Lee CL, Liang A, Kim J, Eissa NT, Lin SH, Yu-Lee LY. 2005. 16-kDa prolactin down-regulates inducible nitric oxide synthase

expression through inhibition of the signal transducer and activator of transcription 1/IFN regulatory factor-1 pathway. Cancer Res. 65:7984–7992.

Lee, K.Y.; Jang, G.H.; Byun, C.H.; Jeun, M.; Searson, P.C.; Lee, K.H. 2017. Zebrafish models for functional and toxicological screening of nanoscale drug delivery systems: Promoting preclinical applications. Biosci. Rep. 37, BSR20170199.

Lee, L.M.J.; Seftor, E.A.; Bonde, G.; Cornell, R.A.; Hendrix, M.J.C. The fate of human malignant melanoma cells transplanted into zebrafish embryos: Assessment of migration and cell division in the absence of tumor formation. Dev. Dyn. 2005, 233, 1560–1570.

Lee, J.B.; Zhang, K.; Tam, Y.Y.C.; Quick, J.; Tam, Y.K.; Lin, P.J.; Chen, S.; Liu, Y.; Nair, J.K.; Zlatev, I.; et al. A Glu-urea-Lys Ligand-conjugated Lipid Nanoparticle/siRNA System Inhibits Androgen Receptor Expression In Vivo. Mol. Ther. Nucleic Acids 2016, 5, e348.

Lenis-Rojas, O.A.; Fernandes, A.R.; Roma-Rodrigues, C.; Baptista, P.V.; Marques, F.; Pérez-Fernández, D.; Guerra-Varela, J.; Sánchez, L.; Vázquez-García, D.; Torres, M.L.; et al. Heteroleptic mononuclear compounds of ruthenium(ii): synthesis, structural analyses, in vitro antitumor activity and in vivo toxicity on zebrafish embryos. Dalton Trans. 2016, 45, 19127–19140.

Lenis-Rojas, O.A.; Roma-Rodrigues, C.; Fernandes, A.R.; Marques, F.; Pérez-Fernández, D.; Guerra-Varela, J.; Sánchez, L.; Vázquez-García, D.; López-Torres, M.; Fernández, A.; et al. Dinuclear RuII(bipy)2 Derivatives: Structural, Biological, and in Vivo Zebrafish Toxicity Evaluation. Inorg. Chem. 2017, 56, 7127–7144.

Liew, W. C., Bartfai, R., Lim, Z. J., Sreenivasan, R., Siegfried, K. R., and Orban, L. 2012. Polygenic sex determination system in zebrafish. *PLoS ONE* 7:e34397.

Li, Y.; Miao, X.; Chen, T.; Yi, X.; Wang, R.; Zhao, H.; Lee, S.M.Y.; Wang, X.; Zheng, Y. Zebrafish as a visual and dynamic model to study the transport of nanosized drug delivery systems across the biological barriers. Coll. Surf. B Biointerfaces 2017, 156, 227–235.

Li, L.; Li, X.; Wu, Y.; Song, L.; Yang, X.; He, T.; Wang, N.; Yang, S.; Zeng, Y.; Wu, Q.; et al. Multifunctional Nucleus-targeting Nanoparticles with Ultra-high Gene Transfection Efficiency for In Vivo Gene Therapy. Theranostics 2017, 7, 1633–1649.

Li, M.; Zhao, L.; Page-McCaw, P.S.; Chen,W. Zebrafish Genome Engineering Using the CRISPR–Cas9 System. Trends Genet. 2016, 32, 815–827.

Li, F.; Zhang, S.; Wang, Z.; Li, H. Genes of the adaptive immune system are expressed early in zebrafish larval development following lipopolysaccharide stimulation. Chin. J. Oceanol. Limnol. 2011, 29, 326–333.

Lieschke, G.J.; Currie, P.D. Animal models of human disease: Zebrafish swim into view. Nat. Rev. Genet. 2007, 8, 353–367.

Lin, S.; Lin, S.; Zhao, Y.; Nel, A.E. Zebrafish: An in vivo model for nano EHS studies. 2013. Small. 9, 1608–1618.

Lin, S.; Zhao, Y.; Xia, T.; Meng, H.; Ji, Z.; Liu, R.; George, S.; Xiong, S.; Wang, X.; Zhang, H.; et al. High content screening in zebrafish speeds up hazard ranking of transition metal oxide nanoparticles. ACS Nano 2011, 5, 7284–7295.

Liu, X.; Gao, X.; Zheng, S.; Wang, B.; Li, Y.; Zhao, C.; Muftuoglu, Y.; Chen, S.; Li, Y.; Yao, H.; Sun, H.; Mao, Q.; You, C.; Guo, G.; Wei, Y. Modified nanoparticle mediated IL-12 immunogene therapy for colon cancer. Nanomedicine 2017.

Liu, R.; Lin, S.; Rallo, R.; Zhao, Y.; Damoiseaux, R.; Xia, T.; Lin, S.; Nel, A.; Cohen, Y. Automated phenotype recognition for zebrafish embryo based in vivo high throughput toxicity screening of engineered nano-materials. PLoS ONE 2012, 7.

Luo, L.; Du, T.; Zhang, J.; Zhao, W.; Cheng, H.; Yang, Y.; Wu, Y.; Wang, C.; Men, K.; Gou, M. Efficient inhibition of ovarian cancer by degradable nanoparticle-delivered survivin T34A gene. Int. J. Nanomed. 2016, 11, 501–513.

Lõhr H. and Hammerschmidt M. 2011. Zebrafish in endocrine systems: recent advances and implications for human disease. Annu Rev Physiol. 73:183-211.

OECD. 2013. “Guideline for the Testing of Chemicals Test No. 236: Fish Embryo Acute Toxicity (FET) Test.” Organization for Economic Co-Operation and Development, Paris.

Omidi, Y.; Hollins, A.J.; Drayton, R.M.; Akhtar, S. Polypropylenimine dendrimer-induced gene expression changes: The effect of complexation with DNA, dendrimer generation and cell type. J. Drug Target. 2005, 13, 431–443.

Ong, K.J.; Zhao, X.; Thistle, M.E.; Maccormack, T.J.; Clark, R.J.; Ma, G.; Martinez-Rubi, Y.; Simard, B.; Loo, J.S.C.; Veinot, J.G.C.; Goss, G.G. Mechanistic insights into the effect of nanoparticles on zebrafish hatch. Nanotoxicology 2014, 8, 295–304.

Organisation for Economic Co-operation and Development (OECD). Test No. 236: Fish Embryo Acute Toxicity (FET) Test; OECD Guidelines for the Testing of Chemicals, Section 2; OECD: Paris, France, 2013; pp. 1–22.

Pahle, J. and Walther,W. Vectors and strategies for nonviral cancer gene therapy. Expert Opin. Biol. Ther. 2015, 16, 443–461.

Parashar, Vivek, Buddhadeb Ghosh, Natwar Gaur, Satya Narayan Shamal, S K Pandey, and Girdhari Lal Shah. 2016. "Teratological Effects of Carboplatin: A Morphological Study in Mice" 4 (2): 2358–64.

Park, S.; Aalipour, A.; Vermesh, O.; Yu, J.H.; Gambhir, S.S. Towards clinically translatable in vivo nanodiagnostics. Nat. Rev. Mater. 2017, 2, 17014.

Parichy, D. 2015. Advancing biology through a deeper understanding of zebrafish ecology and evolution. *eLife,* vol. 4, pp. 10.7554/eLife.05635.

Patton E. E., Widlund H. R., Kutok J. L., Kopani K. R., Amatruda J. F., Murphey R. D., Berghmans S., Mayhall E. A., Traver D., Fletcher C. D. M. et al. 2005. BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr. Biol.* 15, 249-254.

Peer D., Karp J. M., Hong S., Farokhzad O. C., Margalit R., Langer R. 2007. Nanocarriers as an emerging platform for cancer therapy. Nat. Nanotechnol. 2, 751–760.

Penas, C.; Sánchez, M.I.; Guerra-Varela, J.; Sanchez, L.; Vázquez, M.E.; Mascareñas, J.L. Light-Controlled Cellular Internalization and Cytotoxicity of Nucleic Acid-Binding Agents: Studies in Vitro and in Zebrafish Embryos. ChemBioChem 2016, 17, 37–41.

Phillips, J.B.; Westerfield, M. Zebrafish models in translational research: Tipping the scales toward advancements in human health. Dis. Model. Mech. 2014, 7, 739–743.

Poma, A.; Di Giorgio, M.L. Toxicogenomics to improve comprehension of the mechanisms underlying responses of in vitro and in vivo systems to nanomaterials: A review. Curr. Genom. 2008, 9, 571–585.

Pons, M.; Foradada, M.; Estelrich, J. Liposomes Obtained by the Ethanol Injection Method. Int. J. Pharm. 1993, 95, 51–56.

Powell, D.R.; Huttenlocher, A. Neutrophils in the Tumor Microenvironment. Trends Immunol. 2016, 37, 41–52.

Pratt, E.C.; Shaffer, T.M.; Grimm, J. Nanoparticles and radiotracers: Advances toward radionanomedicine. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 2016, 8, 872–890.

Ramamoorth, M.; Narvekar, A. Nonviral vectors in gene therapy—An overview. J. Clin. Diagnostic Res. 2015, 9, GE01–GE06.

Raviña, M.; de la Fuente, M.; Correa, J.; Sousa-Herves, A.; Pinto, J.; Fernandez-Megia, E.; Riguera, R.; Sanchez, A.; Alonso, M.J. Core□Shell Dendriplexes with Sterically Induced Stoichiometry for Gene Delivery.Macromolecules 2010, 43, 6953–6961.

Rawson D. M., Zhang T., Kalicharan D., Jongebloed W. L. 2000. Field emission scanning electron microscopy and transmission electron microscopy studies of the chorion, plasma membrane and syncytial layers of the gastrula-stage embryo of the zebrafish Brachydanio rerio: a consideration of the structural and functional relationships with respect to cryoprotectant penetration. Aqua. Res. 31, 325–336.

Renier, Corinne, Juliette H. Faraco, Patrice Bourgin, Timothy Motley, Pascal Bonaventure, Frédéric Rosa, and Emmanuel Mignot. 2007. "Genomic and Functional Conservation of Sedative-Hypnotic Targets in the Zebrafish." Pharmacogenetics and Genomics.

Renshaw, S.A.; Loynes, C.A.; Trushell, D.M.I.; Elworthy, S.; Ingham, P.W.; Whyte, M.K.B. A transgenic zebrafish model of neutrophilic inflammation. Blood 2006, 108, 3976–3978.

Rihel, J., Prober, D. a, Arvanites, A., Lam, K., Zimmerman, S., Jang, S., Haggarty, S.J., Kokel, D., Rubin, L.L., Peterson, R.T., Schier, A.F., 2010. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327, 348–51.

Roel M, Rubiolo JA, Guerra-Varela J et al. 2016. Marine guanidine alkaloids crambescidins inhibit tumor growth and activate intrinsic apoptotic signaling inducing tumor regression in a colorectal carcinoma zebrafish xenograft model. Oncotarget. 13;7(50):83071-83087.

Rodriguez, M.A.; Pytlik, R.; Kozak, T.; Chhanabhai, M.; Gascoyne, R.; Lu, B.; Deitcher, S.R.; Winter, J.N. Vincristine sulfate liposomes injection (Marqibo) in heavily pretreated patients with

refractory aggressive non-Hodgkin lymphoma: Report of the pivotal phase 2 study. Cancer 2009, 115, 3475–3482.

Roel, M.; Rubiolo, J.A.; Botana, L.M.; Guerra-Varela, J.; Cabezas-Sainz, P.; Sanchez, L.; Silva, S.B.L.; Thomas, O.P.; Silva, S.B.L.; et al. Marine guanidine alkaloids crambescidins inhibit tumor growth and activate intrinsic apoptotic signaling inducing tumor regression in a colorectal carcinoma zebrafish xenograft model. Oncotarget 2016, 7, 83071–83087.

Rojas-Muñoz, A, AB; Miana, and JC. Izpisúa-Belmonte. 2007. "El Pez Cebra, Versatilidad Al Servicio de La Biomedicina." Investgación y Ciencia 366: 62–69.

Rojas-Aguirre Y., Aguado-Castrejón K. e González-Méndez I. La nanomedicina y los sistemas de liberación de fármacos: ¿la (r)evolución de la terapia contra el cáncer?. Educación Química. 2016. 27, 286-291.

Rouhi, P.; Jensen, L.D.; Cao, Z.; Hosaka, K.; Lãnne, T.; Wahlberg, E.; Steffensen, J.F.; Cao, Y. Hypoxia-induced metastasis model in embryonic zebrafish. Nat. Protoc. 2010, 5, 1911–1918.

Ruiter GA, Verheij M, Zerp SF, van Blitterswijk WJ. 2011. Alkyl-lysophospholipids as anticancer agents and enhancers of radiation-induced apoptosis. Int J Radiat Oncol Biol Phys. 49(2):415-9.

Russell, W.M.; Burch, R.L. The Principles of Humane Experimental Technique; Methuen Publishing: London, UK, 1959.

Saif, M.W.U.S. Food and Drug Administration approves paclitaxel protein-bound particles (Abraxane®) in combination with gemcitabine as first-line treatment of patients with metastatic pancreatic cancer. JOP 2013, 14, 686–688.

Saavedra-Alonso, S.; Zapata-Benavides, P.; Chavez-Escamilla, A.K.; Manilla-Muñoz, E.; Zamora-Avila, D.E.; Franco-Molina, M.A.; Rodriguez-Padilla, C. WT1 shRNA delivery using transferrin-conjugated PEG liposomes in an in vivo model of melanoma. Exp. Ther. Med. 2016, 12, 3778–3784.

Santander-Ortega, M.J.; de la Fuente, M.; Lozano, M.V.; Tsui, M.L.; Bolton, K.; Uchegbu, I.F.; Schãtzlein, A.G. Optimisation of synthetic vector systems for cancer gene

therapy—The role of the excess of cationic dendrimer under physiological conditions. Curr. Top. Med. Chem. 2014, 14, 1172–1181.

Santander-Ortega, M.J.; de la Fuente, M.; Lozano, M.V.; Bekheet, M.E.; Progatzky, F.; Elouzi, A.; Uchegbu, I.F.; Schãtzlein, A.G. Hydration forces as a tool for the optimization of core–shell nanoparticle vectors for cancer gene therapy. Soft Matter 2012, 8, 12080.

Santoriello, C.; Zon, L.I. Science in medicine Hooked! Modeling human disease in zebrafish. J. Clin. Investig. 2012, 122, 2337–2343.

Scholz, S., Fischer, S., G n del, U., K s ter, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. 15, 394–404.

Schoots, A. F.; Meijer, R. C. & Denuce, J. M. 1983. Dopaminergic regulation of hatching in fish embryos. *Developmental Biology*. 100: 59-63.

Segura-Aguilar, J.; Kostrzewa, R.M. Neurotoxins and neurotoxicity mechanisms. An overview. Neurotox. Res. 2006, 10, 263–287.

Sepúlveda-Crespo, D.; Jiménez, J.L.; Gómez, R.; De La Mata, F.J.; Majano, P.L.; Muñoz-Fernández, M.Á.; Gastaminza, P. Polyanionic carbosilane dendrimers prevent hepatitis C virus infection in cell culture. Nanomed. Nanotechnol. Biol. Med. 2017, 13, 49–58.

Sieber, S.; Grossen, P.; Detampel, P.; Siegfried, S.; Witzigmann, D.; Huwyler, J. Zebrafish as an early stage screening tool to study the systemic circulation of nanoparticulate drug delivery systems in vivo. J. Control. Release 2017, 264, 180–191.

Siegel, R., Naishadham, D., Jemal, A., 2013. Cancer statistics, 2013. CA Cancer J. Clin. 63, 11–30.

Shafer and William. 2003. Non-small and small cell lung carcinoma cell lines exhibit cell type-specific sensitivity to edelfosine-induced cell death and different cell line-specific responses to edelfosine treatment. Int J Oncol. 23:389-400.

Shanker, M.; Jin, J.; Branch, C.D.; Miyamoto, S.; Grimm, E.A.; Roth, J.A.; Ramesh, R. Tumor suppressor gene-based nanotherapy: From test tube to the clinic. J. Drug Deliv. 2011, 2011, 465845.

Shah, A.N.; Davey, C.F.; Whitebirch, A.C.; Miller, A.C.; Moens, C.B. Rapid Reverse Genetic Screening Using CRISPR in Zebrafish. Nat. Methods 2015, 12, 535–540.

Simmons, S.O.; Fan, C.Y.; Ramabhadran, R. Cellular stress response pathway system as a sentinel ensemble in toxicological screening. Toxicol. Sci. 2009, 111, 202–225.

Sneider, A.; VanDyke, D.; Paliwal, S.; Rai, P. Remotely Triggered Nano-Theranostics For Cancer Applications. Nanotheranostics 2017, 1, 1–22.

Shah, V.; Taratula, O.; Garbuzenko, O.B.; Patil, M.L.; Savla, R.; Zhang, M.; Minko, T. Genotoxicity of different nanocarriers: Possible modifications for the delivery of nucleic acids. Curr. Drug Discov. Technol. 2013, 10, 8–15.

Sheng, L.; Wang, L.; Su, M.; Zhao, X.; Hu, R.; Yu, X.; Hong, J.; Liu, D.; Xu, B.; Zhu, Y.; Wang, H.; Hong, F. Mechanism of TiO2 nanoparticle-induced neurotoxicity in zebrafish (D anio rerio). Environ. Toxicol. 2016, 31, 163–175.

Shi, J.; Kantoff, P.W.; Wooster, R.; Farokhzad, O.C. Cancer nanomedicine: Progress, challenges and opportunities. Nat. Rev. Cancer 2016, 17, 20–37.

Slivac, I.; Guay, D.; Mangion, M.; Champeil, J.; Gaillet, B. Non-viral nucleic acid delivery methods. Expert Opin. Biol. Ther. 2017, 17, 105–118.

Song,W.; Ma, Z.; Zhang, Y.; Yang, C. Autophagy plays a dual role during intracellular siRNA delivery by lipoplex and polyplex nanoparticles. Acta Biomater. 2017, 58, 196–204.

Spence, R., Gerlach, G., Lawrence, C. & Smith, C. 2008. The behaviour and ecology of the zebrafish, Danio rerio. *Biological reviews of the Cambridge Philosophical Society,* vol. 83, 1: 13-34.

Stern, H.M.; Zon, L.I. Cancer genetics and drug discovery in the zebrafish. Nat. Rev. Cancer 2003, 3, 533–539.

Streisinger G, Walker C, Dower N, Knauber D, Singer F 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature 291: 293–296.

Stoletov, K.; Montel, V.; Lester, R.D.; Gonias, S.L.; Klemke, R. High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. Proc. Natl. Acad. Sci. USA 2007, 104, 17406–17411.

Stock, D.W. 2007, "Zebrafish dentition in comparative context", *Journal of experimental zoology. Part B, Molecular and developmental evolution,* vol. 308, no. 5, pp. 523-549.

Stylianopoulos, T.; Jain, R.K. Design considerations for nanotherapeutics in oncology. Nanomedicine 2015, 11, 1893–1907.

Swain, S.; Sahu, P.K.; Beg, S.; Babu, S.M. Nanoparticles for Cancer Targeting: Current and Future Directions. Curr. Drug Deliv. 2016, 13, 1290–1302.

Sukardi, H.; Ung, C.Y.; Gong, Z.; Lam, S.H. Incorporating zebrafish omics into chemical biology and toxicology. Zebrafish 2010, 7, 41–52.

Tang, M.; Hu, P.; Zheng, Q.; Tirelli, N.; Yang, X.; Wang, Z.; Wang, Y.; Tang, Q.; He, Y. Polymeric micelles with dual thermal and reactive oxygen species (ROS)-responsiveness for inflammatory cancer cell delivery. J. Nanobiotechnol. 2017, 15, 39.

Tang, Q.; Moore, J.C.; Ignatius, M.S.; Tenente, I.M.; Hayes, M.N.; Garcia, E.G.; Torres Yordán, N.; Bourque, C.; He, S.; Blackburn, J.S.; et al. Imaging tumour cell heterogeneity following cell transplantation into optically clear immune-deficient zebrafish. Nat. Commun. 2016, 7, 10358.

Taylor, A.M.; Zon, L.I. Zebrafish tumor assays: The state of transplantation. Zebrafish 2009, 6, 339–346.

Tat, J.; Liu, M.;Wen, X.Y. Zebrafish cancer and metastasis models for in vivo drug discovery. 2013. Drug Discov. Today Technol. 10, e83–e89.

Teijeiro-Valiño, C.; Yebra-Pimentel, E.; Guerra-Varela, J.; Csaba, N.; Alonso, M.J.; Sánchez, L. Assessment of the permeability and toxicity of polymeric nanocapsules using the zebrafish model. 2017. Nanomedicin.12, 2069–2082.

Teng, M.W., Swann, J.B., Koebel, C.M., Schreiber, R.D. & Smyth, M.J. 2008, "Immune-mediated dormancy: an equilibrium with cancer", *Journal of leukocyte biology*, vol. 84, no. 4, pp. 988-993.

Thakor, A.S.; Gambhir, S.S. Nanooncology: The future of cancer diagnosis and therapy. CA. Cancer J. Clin. 2013, 63, 395–418.

Thotakura, N.; Dadarwal, M.; Kumar, R.; Singh, B.; Sharma, G.; Kumar, P.; Katare, O.P.; Raza, K. Chitosan-palmitic acid based polymeric micelles as promising carrier for circumventing pharmacokinetic and drug delivery concerns of tamoxifen. Int. J. Biol. Macromol. 2017, 102, 1220–1225.

Timme-Laragy, A.R.; Karchner, S.I.; Hahn, M.E. Developmental Toxicology. Methods Mol Biol. 2012, 889, 51–71.

Tinkle S., McNeil S. E., Mũhlebach S., Bawa R., Borchard G., Barenholz Y. C., et al. 2014. Nanomedicines: addressing the scientific and regulatory gap. Ann. N. Y. Acad. Sci. 1313, 35–56.

Tobia, C.; Gariano, G.; De Sena, G.; Presta, M. Zebrafish embryo as a tool to study tumor/endothelial cell cross-talk. Biochim. Biophys. Acta 2013, 1832, 1371–1377.

Tobin, D.M.; May, R.C.; Wheeler, R.T. Zebrafish: A see-through host and a fluorescent toolbox to probe host-pathogen interaction. PLoS Pathog. 2012, 8.

Tonon, Federica, Cristina Zennaro, Barbara Dapas, Michele Carraro, Massimo Mariotti, and Gabriele Grassi. 2016. "Rapid and Cost-Effective Xenograft Hepatocellular Carcinoma Model in Zebrafish for Drug Testing." International Journal of Pharmaceutics.

Torchilin V. (2011). Tumor delivery of macromolecular drugs based on the EPR effect. Adv. Drug Deliv. Rev. 63, 131–135.

Torrice, M. Does Nanomedicine Have a Delivery Problem? ACS Publ. 2016.

ToxRat Solutions. ToxRat®. 2003. "Software for the Statistical Analysis of Biotests." *Alsdorf, Germany*.

Truong, L.; Saili, K.S.; Miller, J.M.; Hutchison, J.E.; Tanguay, R.L. Persistent adult zebrafish behavioural deficits results from acute embryonic exposure to gold nanoparticles. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 2012, 155, 269–274.

Yin, H.; Kanasty, R.L.; Eltoukhy, A.A.; Vegas, A.J.; Dorkin, J.R.; Anderson, D.G. Non-viral vectors for gene-based therapy. Nat. Rev. Genet. 2014, 15, 541–555.

Yu, F.; Ao, M.; Zheng, X.; Li, N.; Xia, J.; Li, Y.; Li, D.; Hou, Z.; Qi, Z.; Chen, X.D. PEG–lipid–PLGA hybrid nanoparticles loaded with berberine–phospholipid complex to facilitate the oral delivery efficiency. Drug Deliv. 2017, 24, 825–833.

U.S. Food and Drug Administration Home Page. Available online: https://www.fda.gov/ (accessed on 24 November 2017).

Usenko, C.Y.; Harper, S.L.; Tanguay, R.L. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon N. Y. 2007, 45, 1891–1898.

Van der Ent W, et al. 2014. Modeling of human uveal melanoma in zebrafish xenograft embryos. Invest. Ophthalmol. Vis. Sci. 55:6612–6622.

Van der Ent, W.; Burrello, C.; de Lange, M.J.; van der Velden, P.A.; Jochemsen, A.G.; Jager, M.J.; Snaar-Jagalska, B.E. Embryonic Zebrafish: Different Phenotypes after Injection of Human Uveal Melanoma Cells. Ocul. Oncol. Pathol. 2015, 1, 170–181.

Van Ham, T.J.; Mapes, J.; Kokel, D.; Peterson, R.T. Live imaging of apoptotic cells in zebrafish. FASEB J. 2010, 24, 4336–4342.

Van Rooijen, E.; Fazio, M.; Zon, L.I. From fish bowl to bedside: The power of zebrafish to unravel melanoma pathogenesis and discover new therapeutics. Pigment Cell Melanoma Res. 2017, 30, 402–412.

Vázquez-Ríos A, Molina-Crespo A, Bouzo B, López-López R, Moreno-Bueno G, de la Fuente. 2019. Exosome-mimetic nanoplatforms for targeted cancer drug delivery, J Nanobiotechnology. 2019; 17: 85.

Veinotte, C.J.; Dellaire, G.; Berman, J.N. Hooking the big one: The potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era. Dis. Model Mech. 2014, 7, 745–754.

Vibe, C.B.; Fenaroli, F.; Pires, D.; Wilson, S.R.; Bogoeva, V.; Kalluru, R.; Speth, M.; Anes, E.; Griffiths, G.; Hildahl, J. Thioridazine in PLGA nanoparticles reduces toxicity and improves rifampicin therapy against mycobacterial infection in zebrafish. Nanotoxicology 2016, 10, 680–688.

Vila-Jato, J. L. 2009. Nanotecnología Farmacéutica: Realidades y posibilidades farmacoterapéuticas. Monografías, Madrid, España: Instituto de España, Real Academia Nacional de Farmacia. 409.

Vogelstein, Bert, Nickolas Papadopoulos, Victor E. Velculescu, Shibin Zhou, Luis A. Diaz, and Kenneth W. Kinzler. 2013. “Cancer Genome Landscapes.” *Science*.

Wagner, D.S.; Delk, N.A.; Lukianova-hleb, E.Y.; Hafner, J.H.; Lapotko, D.O. The in vivo performance of plasmonic nanobubbles as cell theranostic agents in zebrafish hosting prostate cancer xenografts. Biomaterials 2010, 31, 7567–7574.

Wang, J.; Cao, Z.; Zhang, X.M.; Nakamura, M.; Sun, M.; Hartman, J.; Harris, R.A.; Sun, Y.; Cao, Y. Novel mechanism of macrophage-mediated metastasis revealed in a zebrafish model of tumor development. Cancer Res. 2015, 75, 306–315.

Wang, H.; Jiang, Y.; Peng, H.; Chen, Y.; Zhu, P.; Huang, Y. Recent progress in microRNA delivery for cancer Therapy by non-viral synthetic vectors. Adv. Drug Deliv. Rev. 2015, 81, 142–160.

Wang, J.; Zhu, X.; Zhang, X.; Zhao, Z.; Liu, H.; George, R.;Wilson-Rawls, J.; Chang, Y.; Chen, Y. Disruption of zebrafish (Danio rerio) reproduction upon chronic exposure to TiO2 nanoparticles. Chemosphere 2011, 83, 461–467.

Wehmas, L.C.; Tanguay, R.L.; Punnoose, A.; Greenwood, J.A. Developing a Novel Embryo–Larval Zebrafish Xenograft Assay to Prioritize Human Glioblastoma Therapeutics. Zebrafish 2016, 13, 317–329.

Wertman, Jaime, Chansey J. Veinotte, Graham Dellaire, and Jason N. Berman. 2016. "The Zebrafish Xenograft Platform: Evolution of a Novel Cancer Model and Preclinical Screening Tool." In Advances in Experimental Medicine and Biology.

Westerfield, M. 2007. "The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio Rerio), 5th Edition." *University of Oregon Press, Eugene (Book)*.

Willemsen R., Padje S., van Swieten J.C., Oostra B.A. 2011. Zebrafish (Danio rerio) as a Model Organism for Dementia. In: De Deyn P., Van Dam D. (eds) Animal Models of Dementia. Neuromethods, vol 48. Humana Press.

White, R.M.; Sessa, A.; Burke, C.; Bowman, T.; LeBlanc, J.; Ceol, C.; Bourque, C.; Dovey, M.; Goessling,W.; Burns, C.E.; et al.

Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. Cell Stem Cell 2008, 2, 183–189.

White, R.; Rose, K.; Zon, L. Zebrafish cancer: The state of the art and the path forward. Nat. Rev. Cancer 2013, 13, 624–636.

Wicki, A.; Witzigmann, D.; Balasubramanian, V.; Huwyler, J. Nanomedicine in cancer therapy: Challenges, opportunities, and clinical applications. J. Control. Release 2015, 200, 138–157.

Wilhelm, S.; Tavares, A.J.; Dai, Q.; Ohta, S.; Audet, J.; Dvorak, H.F.; Chan,W.C.W. Analysis of nanoparticle delivery to tumours. 2016. Nat. Rev. Mater. 1.

Wyatt, R.A.; Trieu, N.P.V.; Crawford, B.D. Zebrafish Xenograft: An Evolutionary Experiment in Tumour Biology2017. Genes, 8, 220.

Di Wu, Mengjie Si, Hui-Yi Xue, and Ho-Lun Wong. 2017. Nanomedicine applications in the treatment of breast cancer: current state of the art. Int J Nanomedicine. 12: 5879–5892.

Yang, X.J.; Cui, W.; Gu, A.; Xu, C.; Yu, S.C.; Li, T.T.; Cui, Y.H.; Zhang, X.; Bian, X.W. 2013. A Novel Zebrafish Xenotransplantation Model for Study of Glioma Stem Cell Invasion. PLoS ONE, 8, 1–9.

Yang, L.; Kemadjou, J.R.; Zinsmeister, C.; Bauer, M.; Legradi, J.; M ller, F.; Pankratz, M.; J kel, J.; Str hle, U. Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo. Genome Biol. 2007, 8, R227.

Yang, T.; Martin, P.; Fogarty, B.; Brown, A.; Schurman, K.; Phipps, R.; Yin, V.P.; Lockman, P.; Bai, S. Exosome Delivered Anticancer Drugs Across the Blood-Brain Barrier for Brain Cancer Therapy in Danio Rerio. Pharm. Res. 2015, 32, 2003–2014.

Yang, J.; Shimada, Y.; Olsthoorn, R.C.L.; Snaar-Jagalska, B.E.; Spaink, H.P.; Kros, A. Application of Coiled Coil Peptides in Liposomal Anticancer Drug Delivery Using a Zebrafish Xenograft Model. ACS Nano 2016, 10, 7428–7435.

Yu, T.; Xu, B.; He, L.; Xia, S.; Chen, Y.; Zeng, J.; Liu, Y.; Li, S.; Tan, X.; Ren, K.; Yao, S.; Song, X. Pigment epithelial-derived factor gene loaded novel COOH-PEG-PLGA-COOH nanoparticles

promoted tumor suppression by systemic administration. Int. J. Nanomed. 2016, 11, 743–759.

Yu-Lan Hu, Wang Qi1 Feng Han, Jian-Zhong Shao, and Jian-Qing Gao. 2011. Toxicity evaluation of biodegradable chitosan nanoparticles using a zebrafish embryo model. Int J Nanomedicine. 6: 3351–3359.

Xie, X.; Ross, J.L.; Cowell, J.K.; Teng, Y. Future Medicinal Chemistry. Future Med. Chem. 2015, 7, 1395–1405.

Xu, H.; Dong, X.; Zhang, Z.; Yang, M.; Wu, X.; Liu, H.; Lao, Q.; Li, C. Assessment of immunotoxicity of dibutyl phthalate using live zebrafish embryos. Fish Shellfish Immunol. 2015, 45, 286–292.

Xu, F.; Liao, J.Z.; Xiang, G.Y.; Zhao, P.X.; Ye, F.; Zhao, Q.; He, X.X. MiR-101 and doxorubicin codelivered by liposomes suppressing malignant properties of hepatocellular carcinoma. Cancer Med. 2017, 6, 651–661.

Xu, B.; Jin, Q.; Zeng, J.; Yu, T.; Chen, Y.; Li, S.; Gong, D.; He, L.; Tan, X.; Yang, L.; He, G.; Wu, J.; Song, X. Combined Tumor- and Neovascular-"Dual Targeting" Gene/Chemo-Therapy Suppresses Tumor Growth and Angiogenesis. ACS Appl. Mater. Interfaces 2016, 8, 25753–25769.

Zhao, S.; Huang, J.; Ye, J. A fresh look at zebrafish from the perspective of cancer research. J. Exp. Clin. Cancer Res. 2015, 34, 80.

Zhang,W.; Lin, K.; Sun, X.; Dong, Q.; Huang, C.;Wang, H.; Guo, M.; Cui, X. Toxicological effect of MPA–CdSe QDs exposure on zebrafish embryo and larvae. Chemosphere 2012, 89, 52–59.

Zhang, B.; Shimada, Y.; Kuroyanagi, J.; Umemoto, N.; Nishimura, Y.; Tanaka, T. Quantitative phenotyping-based in vivo chemical screening in a zebrafish model of leukemia stem cell xenotransplantation. PLoS ONE 2014, 9, 1–9.

Zhang, B.; Shimada, Y.; Kuroyanagi, J.; Nishimura, Y.; Umemoto, N.; Nomoto, T.; Shintou, T.; Miyazaki, T.; Tanaka, T. Zebrafish xenotransplantation model for cancer stem-like cell study

and high-throughput screening of inhibitors. Tumor Biol. 2014, 35, 11861–11869.

Zhao, Y.; Huang, X.; Ding, T.W.; Gong, Z. Enhanced angiogenesis, hypoxia and neutrophil recruitment during Myc-induced liver tumorigenesis in zebrafish. Sci. Rep. 2016, 6, 31952.

Zhong, Y.; Jia, T.; Sun, Z.; Lu, Y.; Gao, J.; Zou, H.; Xie, F.; Xu, H.; Sun, D.; Yu, Y.; Zhang, G. A dual brain-targeting curcumin-loaded polymersomes ameliorated cognitive dysfunction in intrahippocampal amyloid-_1–42-injected mice. Int. J. Nanomed. 2016, 11, 3765–3775.

Zhong, Lei, Jiao Yang, Zhixing Cao, Xin Chen, Yiguo Hu, Linli Li, and Shengyong Yang. 2017. “Preclinical Pharmacodynamic Evaluation of Drug Candidate SKLB-178 in the Treatment of Non-Small Cell Lung Cancer.” Oncotarget.

Zhong, Yanqiang, Tingting Jia, Zhiguo Sun, Ying Lu, Jie Gao, Hao Zou, Fangyuan Xie, et al. 2016. “A Dual Brain-Targeting Curcumin-Loaded Polymersomes Ameliorated Cognitive Dysfunction in Intrahippocampal Amyloid-β1–42-Injected Mice.” International Journal of Nanomedicine Volume 11 (August): 3765–75.

Zhou, Z.; Liu, X.; Zhu, D.;Wang, Y.; Zhang, Z.; Zhou, X.; Qiu, N.; Chen, X.; Shen, Y. Nonviral Cancer Gene Therapy: Delivery Cascade and Vector Nanoproperty Integration. Adv. Drug Deliv. Rev. 2017.

Zhuang, S.; Zhang, Z.; Zhang,W.; Bao, L.; Xu, C.; Zhang, H. Enantioselective developmental toxicity and immunotoxicity of pyraclofos toward zebrafish (Danio rerio). Aquat. Toxicol. 2015, 159, 119–126.

Zou, D.; Wang, W.; Lei, D.; Yin, Y.; Ren, P.; Chen, J.; Yin, T.; Wang, B.; Wang, G.; Wang, Y. Penetration of blood-brain barrier and antitumor activity and nerve repair in glioma by doxorubicin-loaded monosialoganglioside micelles system. Int. J. Nanomed. 2017, 12, 4879–4889.

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The zebrafish have many advantages that led to be a model organism with a great potential in translational research.
The zebrafish embryos are an ideal platform to evaluate novel cancer therapies. For this reason, the main goal of this thesis has been evaluating the therapeutic potential of different anticancer therapies in zebrafish embryos. To accomplish this, we evaluated the toxicity in vitro and in vivo of some commonly used anticancer drugs in order to determinate crucial toxicological profiles for zebrafish xenograft studies.
Additionally, we develop a newly nanoemulsion based on the anticancer drug edelfosine which let us to study parameters such as toxicity, biodistribution and efficacy in xenografted zebrafish models.