Development of novel anti-cancer strategies utilizing the zebrafish xenograft model

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Chapter 1

Introduction and thesis outline

Cancer is one of the main causes of morbidity and mortality, being responsible for an estimated 18.1 million new cancer cases, and 9.6 million cancer deaths per year [1]. One in five men and one in six women worldwide will develop cancer during their lifetime, and one in eight men, and one in eleven women die from the disease. The majority of cancers, 90%-95% of cases, are induced by environmental and lifestyle factors, which may include smoking, diet, alcohol, sun exposure, environmental pollutants, infections, stress, obesity, and physical inactivity. About 5-10% of cases are due to inherited genetic aberrations. Carcinogenesis is a complex multi-step process that usually proceeds over several years and starts from one single cell. Endogenous and exogenous agents can lead to DNA damage, epigenetic defects and gene mutations. A series of mutations in cancer known as “driver genes” (oncogenes and tumour suppressors) as well as other “passenger genes” initiate the transformation from a normal cell to a cancer cell, which results in aberrant cell behaviour, such as cell migration, growth, differentiation and failure of apoptosis. These events are part of a multistep process and contribute progressively to the generation and development of cancer. The different characteristics of cancer pathogenesis and disease progression have been outlined by Hanahan and Weinberg as “Hallmarks of cancer” [2]. However, the biology of tumours should be investigated not only by focusing on the traits of single cancer cells, but should also consider the contributions of the tumour microenvironment, the interactions between tumour cells and the supportive stroma, the role of the immune system and the preferential tropism of spreading tumour cells to specific metastatic sites [3].

The clinical diagnosis of cancer is based on medical tests, including blood tests, X-rays, CT scans, endoscopy and MRI. Current treatments include surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and palliative care, depending on the type, location and grade of the tumour. There are two main reasons restricting the efficacy of existing treatments: one is the observation of heterogeneity, as based on a patients’ genetic background, gene mutations, lifestyle, tumour size and tumor metabolism. All of these may affect treatment results. Therefore, a deeper understanding of inter- and intratumoral heterogeneity of tumours is needed for personalized interventions. A second problem is cancer cell resistance to the currently used therapeutics. Common targeting strategies aimed at inhibiting specific molecular pathways often have only a temporary success and are followed by a tumour relapse. The use of a combined approach with several drugs can give rise to higher efficiency and avoidance of chemoresistance but so far, the results have not achieved the required efficacy in all malignancies. Therefore, there is still an unmet clinical need to develop new drugs and cancer models for testing the anti-cancer efficacy of targeted drug delivery to avoid adverse side effects and increase the success rate of treatments. In this thesis, we are going to discuss the development of novel anti-cancer strategies utilizing zebrafish xenograft models.

Cancer development

Tumour clonality is one of the fundamental features of cancer and describes the development of tumours from a single cell. The accumulation of gene mutations leading to abnormal cell proliferation can be thought of as tumour initiation [4] (Figure 1). The outgrowth of a population of clonally-derived tumour cells into adjacent tissues will form a tumour microenvironment (TME), which supports tumour cell growth. The abilities to sustain proliferative signalling [5], evade growth suppressors [6], resist cell death [7], enable replicative immortality [8], induce angiogenesis [9], and activate invasion and metastasis [10] sustain tumour cell proliferation [2]. During tumour progression, further gene mutations keep occurring. Similar to a microevolutionary process, the stepwise progress of cancer consists of a stage I, which is the evolution of tumours from normal tissues and stage II, which represents the evolution within tumours [11]. Tumor progression drives intratumor diversity and heterogeneity. At the primary site, the tumour cells can grow thanks to enough nutrition and oxygen derived from the surrounding normal tissue. When the nutrition and oxygen become limited, the tumour center often transforms to a necrotic core. The term Oxidative Stress refers to elevated intracellular levels of reactive oxygen species (ROS) and oxygen limitation initiates tumour angiogenesis after the so-called “angiogenic switch”, which causes the normal quiescent vasculature to sprout and produce new branches (neovascularization) [12]. The new vessels in the tumour have an aberrant morphology and are characterized by abnormal level of endothelial cell proliferation and apoptosis. In addition, leakiness of tumour vessels is one of the major reasons for the low efficiency in the delivery of therapies specifically to tumour lesions [13].

It takes time for the tumour cells to grow at the primary site and develop the capacity to invade and metastasize. In this context, transformed epithelial cells acquire a motile mesenchymal phenotype in a process referred to as “epithelial-to-mesenchymal transition” (EMT) [14]. This invasive phenotype is generally associated with an increased migratory capacity of the tumour cells. Both blood and lymphatic vessels provide an escape route by which tumour cells can leave the primary site, through a process called intravasation. After tumour cells have invaded the circulatory system, they have to survive in the circulation and resist necrosis, until the tumour cells adhere to the vascular wall and extravasate [15].

Most circulating tumour cells will die, only a few tumour cells go into an anchorage-independent survival. Once tumour cells have extravasated, they can invade the distant tissue and organ and undergo mesenchymal-to-epithelial transition, which allows the tumour cells to switch back to their epithelial and proliferative state. Once micrometastases are formed, sustained growth and angiogenesis allow them to grow out into secondary tumours. Additionally, tumour cells can also remain dormant for several years and then suddenly re-initiate proliferation and form a metastatic lesion. Metastasis is the final stage of tumour progression and is the main cause of mortality.

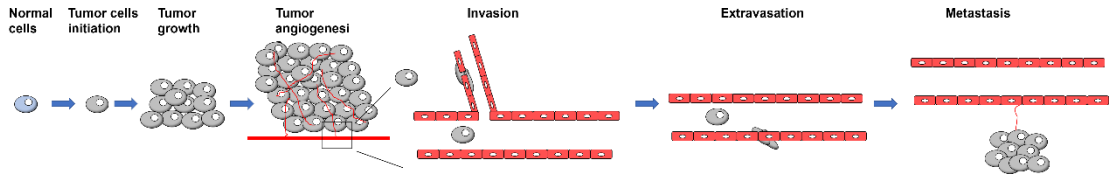

Fig. 1 Scheme of tumour cell growth, angiogenesis, invasion and the metastasis process.

Metabolic reprogramming in cancer

In addition to the above mentioned specific hallmarks, tumour cells can also adapt their metabolism and switch to the so-called “aerobic glycolysis”, converting their metabolism largely to glycolysis and lactate production (i.e. Warburg-effect) [16]. This is one of the bases of the non-invasive visualization of tumour-dependent positron emission tomography (PET) with a radiolabelled analogue of glucose as reporter. In proliferating cancer cells, the mitochondrial oxidative phosphorylation (OXPHOS) is reprogrammed towards a macromolecular synthesis to sustain multiple cell divisions [17]. The advantage of such a deregulated metabolism is to favour the accumulation of glycolytic intermediates, fuelling derivative anabolic pathways, such as the pentose phosphate pathway, the hexosamine pathway, and amino acid synthesis, thereby sustaining cell proliferation [18]. However, recent advances have highlighted substantial intratumoral metabolic heterogeneity and even metabolic plasticity, depending on the tissue context, tumour stage, TME, that regulate the metabolic strategies in tumour cells, leading, for example, to concurrent glycolysis and glucose oxidation in the same tissue [19]. Nevertheless, the majority of cancer cells enhance glucose and glutamine consumption to satisfy their requirements for rapid proliferation. In aerobic glycolysis in tumour cells, glucose is partially oxidized into pyruvate, which is subsequently reduced to lactate, that is then extruded into the extracellular space. Emerging evidence now argues that lactate plays a role in regulating different signalling pathways and the behaviour of malignant and non-malignant cells. Lactate can affect multiple biological processes during tumour progression, plays roles in the immune and inflammatory responses in TME, and also influences proliferation, metastasis and angiogenesis [20].

Moreover, oncogenic mutations in metabolic enzymes such as the cytosolic $NADP^+$-dependent isocitrate dehydrogenase 1 gene (IDH1) and the mitochondrial homolog IDH2, responsible for converting α-ketoglutarate to 2-hydroglutarate (2HG), a metabolite found only in reduced amounts in mammalian cells under normal conditions, have been reported [21]. Interestingly this has also an effect on epigenetic mechanisms, resulting in altered histone methylation marks, hypermethylation at CpG islands and dysregulated cell differentiation [21].

The function of macrophages in the tumour microenvironment

More knowledge now starts to elucidate the role of the TME and immune system during tumour initiation, growth and progression. Importantly, TME also shapes therapeutic responses and resistance [22,23].

During cancer progression, the stroma co-evolves with the tumour and creates a dynamic signaling network of paracrine signals that promotes cancer progression. The different stromal components of TME include cancer-associated fibroblasts (CAFs), pericytes, and immune cells, which surround blood vessels and are present in the extracellular matrix (ECM) [24,25]. These stromal cells are recruited to the tumour, and need to adapt to their new environment to allow them to survive. The immune system has evolved to discriminate between normal and malignant cells. It launches immune responses to eliminate damaged or malignant cells and protects the host [26]. Growing evidence suggests that cancer immunosurveillance not only protects the host against the development of primary cancer, but also shapes the immunogenicity of tumours [27,28]. However, upon cancer initiation and formation, tumour cells activate tolerogenic signalling pathways, resulting in cancer immune tolerance and escape from classical immune attack [28,29]. The first generation of antibody-based immunotherapies against so-called immune-checkpoints (Immune-checkpoint blockade or ICB), works by blocking the receptor and/or ligand interactions of molecules, such as CTLA-4 and PD-1, which are involved in dampening T cell activation or function [30]. Unfortunately, ICB therapies have shown significant clinical benefit for only a minority of patients and there is still need to search for novel therapeutic targets.

Within the TME, cancer cells release soluble molecules to activate their own oncogenic signalling for growth and metastasis, and alter the surrounding cells to enhance tumour progression [31]. Macrophages, a major cell population in the TME, play an essential role in immune homeostasis. They are activated and polarized by signals from the TME to become classically-activated (M1) or alternatively--activated (M2) phenotypes [32,33]. A large body of evidence suggests that macrophages within the TME are activated by tumour-derived cytokines into M2-polarized tumour-associated macrophages (TAM), which promote tumour progression and suppress anti-tumour responses [34]. Importantly, therapeutic targeting of macrophages enhances chemotherapy efficacy of platinum-based chemotherapeutics by unleashing type I interferon responses [23].

TAM are either derived from tissue residence, or peripheral reservoirs such as the bone marrow (BM) and spleen [35]. Transcriptome profiling of freshly-isolated TAM suggests that they are similar to those that are involved in development [36]. TAM can contribute to many aspects of cancer development. In particular, they can regulate senescence, interact with and modulate the

extracellular matrix [37,38], promote cancer cell proliferation, invasion and metastasis [39,40] and promote tumor angiogenesis [41,42]. TAMs regulate tumor angiogenesis largely through production of vascular endothelial growth factor (VEGF-A) [43]. Genetic deletion of the VEGF-A gene in macrophages attenuates tumor angiogenesis and results in a morphologically more physiological vasculature [44]. Colegio et al discovered that lactate, as a by-product of aerobic or anaerobic glycolysis, has a critical function in signalling, by inducing the expression of VEGF and the arginase 1 dependent M2-like polarization of TAMs [45]. They demonstrated that lactate-induced VEGF expression in macrophages is mediated by hypoxia-inducible factor 1α (HIF-1α). Recently, metabolic analysis coupled with enzyme activity assays, identified that cancer-derived succinate promotes macrophages polarization to M2-like TAMs via the succinate receptor-triggered PI3K-hypoxia-HIF-1α axis and increases macrophage migration and lung cancer metastasis [46].

In a zebrafish tumour model, innate immune cells (neutrophils and macrophages) were thought to contribute to different steps of tumour progression, including the formation of tumour vasculature and metastasis [47]. In zebrafish embryos, the caudal haematopoietic tissue (CHT) is known as a transient site of haematopoiesis and leukocyte differentiation [48]. The macrophage population of zebrafish embryos has been studied in detail and similarities with human macrophage functions have been reported [49,50]. Therefore, the transparent zebrafish embryonic tumour model is very suitable to dissect how macrophages respond to tumour environmental factors and contribute to angiogenesis.

Zebrafish embryonic tumour xenografts: a model for cancer research

In the last decades, zebrafish *(Danio rerio)* have been applied to the cancer research field, due to several advantages [51]. Benefits include the relatively short generation time of three months, cost-effective maintenance, *ex utero* development, temporal separation between innate and adaptive immunity, transparency and easy manipulation of embryos [52]. Human and zebrafish share a high grade of similarity: 71% of human proteins and 82% of disease-causing human proteins have an orthologue in zebrafish [53]. There is a high conservation of oncogenes and tumour-suppressor genes between zebrafish and humans [54], and various oncogenic transgenic zebrafish lines have been developed [55,56]. The histology of zebrafish tumours has been shown to be highly similar to tumours found in humans [57]. The adaptive immune system in zebrafish does not reach maturity until four weeks post fertilization [58], allowing circumvention of graft rejection by using early stage zebrafish for human or murine cancer cell engraftment. Zebrafish embryos can absorb various small molecular weight compounds from water, allowing easy drug administration, which is advantageous when screening for anti-cancer compounds [59]. Use of transgenic lines with fluorescent vasculature or granulocytes [60] allows live imaging of cancer development and interaction with the microenvironment.

There are several approaches to generate human cancers in zebrafish, such as the development of mutant and transgenic zebrafish lines, and transplantation of tumour cells [51]. Chemical mutagenesis, irradiation mutagenesis, or viral vector mutagenesis as well microinjection of exogenous DNA into one-cell-stage zebrafish embryos can induce transgenic cancer models. Currently, zebrafish genetic cancer models have been developed for many types of cancer e.g. cutaneous melanoma [61], neuroblastoma [62], rhabdomyosarcoma [55], leukaemia (specifically T-ALL) [63] and liver cancer [56,64].

Another approach to generate cancer in zebrafish is the transplantation of tumour cells. The engraftment of murine or human cancer cells into a zebrafish embryo is a fast way to build a new *in vivo* model. The injection site for transplantation can vary, depending on the research purpose. These sites include the Yolk sac, the Duct of Cuvier, the perivitelline space, the hindbrain ventricle, the swimming bladder, and the retro-orbital space [65-76]. This model helps us to understand the processes of angiogenesis, tumour cell extravasation, invasion, metastasis onset as well as interactions with the microenvironment [77]. Interactions between human cancer cells and the zebrafish microenvironment have been extensively described [78-80]. Yi Feng et al observed that H_2O_2 production in oncogene-transformed cells led to leukocyte recruitment and a host inflammatory response that contributes to increased growth of the transformed cells [81]. He et al found that zebrafish neutrophils guide human cancer cell extravasation and invasion by reorganizing the extracellular matrix at the metastatic site at caudal hematopoietic tissue (CHT), the transient site of haematopoiesis and leukocyte differentiation [47,48]. The motility and adhesion of zebrafish neutrophils during metastatic niche preparation is orchestrated by the CXCR4 receptor, which is expressed on zebrafish neutrophils [47]. Next, Tulotta et al proved that engrafted human cancer cells expressing the CXCR4 receptor can sense the host's (zebrafish) CXCL-12 ligand, which is produced by mesenchymal stem cells in zebrafish CHT [82], inducing metastatic colonization [29,30]. Targeting of either CXCR-4 (the CXCL-12 receptor) in breast human cancer cells and zebrafish neutrophils or CXCL-12 in zebrafish significantly inhibited extravasation and metastatic tumour growth at the CHT area. Britto et al used an embryonic zebrafish xenograft model to show that zebrafish macrophages can enhance VEGF-A-driven tumour angiogenesis [83]. Moreover, human cancer cells can comparably respond to the microenvironment of zebrafish and mice, by inducing activation of the NF-κB–Activin A signalling axis, which drives the metastatic cancer stem cell (CSC)-like phenotype of prostate cancer cells [84]. Transplantation of zebrafish BRAFV600E-driven melanoma cells into *casper* fish showed that the degree of pigmentation is a key feature defining cells with metastatic capability [85]. De Sousa Pontes et al used (fli:GFP) Casper zebrafish embryos to establish a model for human conjunctival melanoma [86]. Heilmann et al used transparent *casper* zebrafish to generate single-cell resolution of the metastatic process to evaluate the tumour cell's metastatic ability [85]. Importantly, several groups described successful xenotransplantation of human patient samples into zebrafish larvae for phenotypic testing of drug responses [87].

Zebrafish embryonic tumour xenograft model for drug discovery

Perhaps the most promising application of the zebrafish xenograft model is the ability to perform high-throughput drug screening on human cancer cells and samples in a way that is not possible using any other model organism. Larvae absorb drugs from the water through the skin and oral, enhancing the simplicity of drug administration [88]. Because embryos are maintained in 96-well plates, typically in 100−300 µL of water, screens require smaller drug quantities compared to mice. The efficacy of drugs can be quantified by live imaging of the tumour burden by fluorescence microscope [89,90]. Several research groups have also applied xenotransplantation methods to zebrafish, for the study of human cancer cell behavior, responses to therapy, within the context of the whole organism [91]. The different cell lines so far tested in xenotransplantation are cutaneous melanoma [92], conjunctival and uveal melanoma, colorectal cancer [93], breast cancer [87], leukemia [94], ovarian cancer [95], neuroblastoma [96], pancreatic cancer [97], prostate cancer [51], and sarcoma [92]. Typically, cells are dye labeled to allow their identification within the living host and their growth, and to follow their infiltration into host tissues, monitored over two to five days. Treatment of engrafted embryos with drugs can result in graft stasis or regression, mirroring outcomes that can be observed in more costly and lengthier mouse xenograft experiments [98].

The technique of xenotransplantation into zebrafish can also be used with patient-derived tissues, which has been demonstrated previously for pancreatic adenocarcinoma, prostate cancer, and leukemia [87,99,100]. The total sample needed for this approach could be as little as 100 cells. The time for engraftment is between two and three days after fertilization. Given these characteristics, patient-derived xenografts (PDX) in zebrafish (zPDX) could be tools to predict patient responses to drug treatments. The valuable biopsy tissue from a patient could be injected into scores of zebrafish embryos potentially with different reporter constructs in the background and different treatments applied to select the most suitable clinical intervention. However, more proof-of-principle studies are needed to fully evaluate the value of zebrafish PDX models. Recently, development of immunodeficient zebrafish enabled the development of zPDX in adult zebrafish [100]. This approach is probably the most relevant when looking at it from a translational perspective and may in the near future provide fast and reliable assessments for personalized treatments and precision cancer therapy. Today, eight small molecules identified from zebrafish studies have been advanced into clinical trials, illustrating the ability to move fundamental discoveries from zebrafish to human [101].

Zebrafish embryonic tumour xenograft model for photodynamic dynamic therapy (PDT) and photoactivated chemotherapy (PACT) testing

Another approach to treat cancer is to accumulate photosensitizers (PSs) in tumour tissue followed by the light-induced generation of cytotoxic reactive oxygen species (ROS) to kill the tumour cells.

Photodynamic therapy (PDT) is a clinically-approved treatment in which the drugs only work after they have been “activated” or “switched on” by light. There are several types of PDT. Type II PDT is

oxygen-dependent meaning that the energy of the excited photosensitizer PS*could be transferred into the O_2 molecules in the irradiated tissues. Such energy transfer produces the excited state of O_2 called “singlet oxygen”, which is highly oxidative and leads to cell death via damage of DNA, proteins, and lipids (Fig. 2). Thus, PDT type II relies not only on light irradiation, but also on the local presence of dioxygen in the irradiated tumour tissue. Usually PDT type II ceases to work when the oxygen supply to the irradiated tissues is too low, for example in hypoxic tumours, or when the light intensity is too high [102].

Type I PDT consists of the generation of radical species via electron transfer from the excited photosensitizer PS* to O_2, which typically generates the superoxide radical $O_2^{\bullet -}$ and, by cascade reactions, other types of radical oxygen species (Fig. 2). Usually PDT type I is therefore also oxygen-dependent, but in some cases electron transfer can happen directly from PS* to DNA or proteins to create radicals, without the involvement of dioxygen. In this case, PDT type I can also work in hypoxic conditions [103].

Photoactivated chemotherapy (PACT) relies on the oxygen-independent activation by light of a pro-drug, turning a poorly toxic molecule into a much more toxic species. Metal-based PACT compounds are based on a heavy metal centre such as ruthenium (II) [82,104]. In some aspects, ruthenium (II) is similar to platinum (II), found for example in cisplatin, but it has photochemical properties: when coordinated to an appropriate ligand set, it can photosubstitute some of the ligands bound to it, ie. replace them by water molecules, as shown in the figure below. Water is poorly bound to heavy metal ions, so that biological molecules such as DNA, lipids, or proteins can bind to the aqua metal photoproduct, thereby creating metallated DNA or inhibiting proteins, which kill cancer cells. This metal binding of biomolecules cannot occur in the dark because the ligands protect the metal ion. Thus, the molecule is more toxic after light irradiation. A second form of PACT consists of using photo-substitutional active ruthenium compounds where the liberated ligands bear the toxicity, rather than the metal-based photoproduct. This second form of PACT is sometimes called “photocaging”: the ruthenium complex appears as a “caging group”, and the ruthenium-ligand prodrug is called a “ruthenium-caged” cytotoxin [82].

In PACT, whether the toxicity after light activation is due to the metal-containing photoproduct or to the ligand, the activation mechanism does not involve O_2. Thus, PACT should be suitable for phototherapy of hypoxic tumours, where PDT usually fails. This idea has been demonstrated *in vitro* by the Bonnet group [105].

For light-activated compounds (whether PDT or PACT) the photoindex, defined as PI = $EC_{50(dark)}/EC_{50(light)}$, characterizes the enhanced toxicity upon irradiation in a given set of conditions (and with a given cell testing protocol). $EC_{50(dark)}$ and $EC_{50(light)}$ are cell growth inhibition effective

concentration values, ie concentrations that prevent 50% of cancer cell growth, compared to drug-free control.

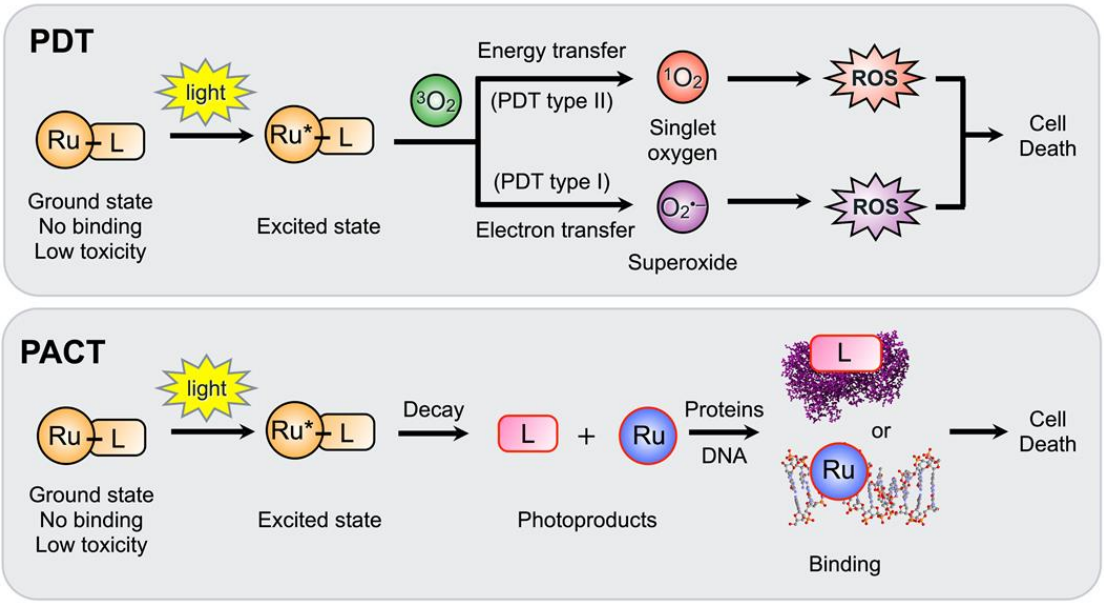

Fig. 2 Scheme of PDT and PACT compound mechanisms.

The zebrafish liver tumour model was used as an *in vivo* platform to investigate the biodistribution of fluorescent PS and the therapeutic efficiency of theranostic polymer-encapsulated nanoparticles to carry out PDT [106]. The first photoswitchable inhibitor of human dihydrofolate reductase (DHFR) has been developed as a potent chemotherapy drug as determined in a zebrafish model [107]. In addition, this animal model has been increasingly utilized to assess the toxicity of nanoparticles. The acute and chronic toxic effects of metal nanoparticles, including Au, Ag, Cu, and metal oxide nanoparticles, such as TiO_2, Al_2O_3, CuO, NiO and ZnO were measured [108]. Moreover, this model allowed assessment of off-target organ toxicities including immunotoxicity, developmental toxicity, neurotoxicity, reproductive toxicity, cardiovascular toxicity and hepatoxicity [109]. Wehmas et al used embryonic zebrafish to investigate the toxicity of engineered metal oxide nanoparticles [110].

A Zebrafish embryonic tumour xenograft model for nanomedicine optimization and delivery

Recently, zebrafish stood out as a tool to develop and test new drug administration strategies of nanomedicines. Nanomedicine toxicity, biodistribution and systemic circulation, stability, functionality and targeting efficiency have all been successfully assessed within the complex biological, *in vivo* environment of living zebrafish larvae. The potential value of the zebrafish model for anti-cancer nanomedicine development has been demonstrated by many studies [111-113]. Most studies measured the toxicity and safety of blank nanoparticles (mostly liposomes) prior to drug incorporation. Taking advantage of the embryo's transparency, biodistribution studies have also been performed to determine the ability of the nanocarriers to reach the target site, and to even pass the blood-brain barrier [114]. 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, Yang and collaborators described the application of coiled coil peptides in liposomal anticancer drug delivery using the zebrafish xenograft model [111]. The work of Evensen and collaborators addressed the ability of PEGylated nanocarriers to avoid uptake by macrophages, a fact that translates in improved circulation time and increased accumulation into tumours [112]. The ultimate goal of nano-delivery methodologies is cell-specific targeting, yet on-target uptake is approximately 1% of all injected nanoparticles [115] due to off-target interaction in the liver [116] and scavenger endothelial cells (SECs) of various tissues (e.g. kidney, heat and gills). Zebrafish whole-body 4D intravital imaging was applied to monitor the distribution of fluorescent liposomes with cellular resolution in near real-time [117]. The tested liposomes accumulated on endothelial cells. Dextran sulphate (a competitive inhibitor of stab-2 scavenger receptors) and *stab2* mutants led to a dramatic increase in the concentration of freely-circulating liposomes due to diminished stab-2 scavenger receptor binding. This study provided evidence that anionic liposomes are an ideal delivery system for targeting cells overexpressing stab-2 (such as SECs) and that inhibiting nanoparticles-SEC interactions may serve to enhance bioavailability of numerous nanocarrier classes.

Thesis outline

In this thesis, we will utilize embryonic zebrafish tumour models to understand the interaction between engrafted human cancer cells and macrophages from the host, test drug administration modalities and anti-cancer efficacies of newly-developed PDT and PACT compounds, and test a light-triggered liposomal system for targeted drug delivery specifically to cancer cells *in vivo*.

In **chapter 2**, we investigate the role of macrophages in tumour-induced angiogenesis. We show that macrophage-dependent angiogenesis is driven by macrophage recruitment to lactic acid secreted by glycolytic B16 melanoma cells. Chemical inhibition of macrophages and glycolysis blocks the initiation of angiogenesis in these models, suggesting that macrophages attracted to glycolytic melanoma cells contribute to the tumour-induced angiogenesis process.

In **chapters 3 and 4**, we explore novel PDT and PACT compounds, respectively, for treatment of conjunctival melanoma in zebrafish. We inject conjunctival melanoma cells into the retro-orbital site to establish an orthotopic model and into the Duct of Cuvier to generate an ectopic model. Our results prove that zebrafish provides a fast vertebrate cancer model to test the optimal administration regimen of drugs, conditions of light irradiation, host toxicity and anti-cancer efficacy of PDT and PACT drugs against conjunctival melanoma.

In **chapter 5**, we focus on modifying liposomes to be light triggered in order to deliver drugs specifically to cancer cells. We inject MDA231 breast cancer cells into the Duct of Cuvier at 2 days

post fertilization (dpf) to initiate metastasis to the CHT. We successfully demonstrate that light-triggered, cell-specific delivery of liposome-encapsulated doxorubicin reduces the xenograft cancer cell burden without enhanced cytotoxicity of the zebrafish embryos.

In **chapter 6,** we summarize the novel anti-cancer strategies, which we have developed using zebrafish xenograft models. In the same chapter, we frame our findings in the current scientific landscape and discuss future perspectives.

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