OPEN ACCESS

Scientific Letters

Review

Nanomedicine internalization and penetration: why should we use spheroids?

Bárbara Pinto [1,2,ǂ], Catarina Pacheco [1,3,4,ǂ], Patrícia M. A. Silva [1,5], Juliana Carvalho-Tavares [2], Bruno Sarmento [1,3,4,*] and Hassan Bousbaa [1,6,*]

[1] UNIPRO – Oral Pathology and Rehabilitation Research Unit, University Institute of Health Sciences (IUCS), CESPU, Rua Central de Gandra, 1317, 4585-116 Gandra, Portugal; barbara_fernandes_pinto@hotmail.com (BP); patricia.silva@cespu.pt (PMAS); anacatarinaspacheco@hotmail.com (CP)
[2] Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, Brazil; julianact@ufmg.br (JCT)
[3] i3S – Institute for Research and Innovation in Health, University of Porto, Rua Alfredo Allen 208, 4200-393 Porto, Portugal
[4] INEB – Institute of Biomedical Engineering, University of Porto, Rua Alfredo Allen 208, 4200-393 Porto, Portugal
[5] TOXRUN – Toxicology Research Unit, University Institute of Health Sciences (IUCS), CESPU, Rua Central de Gandra, 1317, 4585-116 Gandra, Portugal
[6] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n 4450-208 Matosinhos, Portugal
[ǂ] these authors contributed equally to this work

* Correspondence: hassan.bousbaa@iucs.cespu.pt (HB); bruno.sarmento@i3s.up.pt (BS)

Abstract: Cancer is a leading cause of death worldwide and, despite medical advances in the development of new anticancer therapies, drug resistance, complicated side effects and poor efficacy remain a major public health problem. To breakdown the antisuppressive tumour strategies and to improve drug efficiency, nanomedicine is increasingly being used in anticancer therapy studies. Different *in vitro* experimental models and innovative approaches have been used in cancer research. In this aspect, 3D spheroids have become an attractive tool, overcoming many of the issues associated with using 2D models. Thus, in this review, an overview on 3D spheroids is provided, focusing on their use in the cancer field as a promising platform for nanomedicine studies. Thereafter, we highlight the main drug delivery systems currently developed and their applicability in 3D cell cultures.

Keywords: 3D tumour spheroids; cancer; nanomedicine; tumour microenvironment

Received: 14 February 2022; Accepted: 21 March 2022; Published: 21 April 2022

Introduction

Cancer is the second cause of death worldwide, being a major burden and challenge for public health [1,2]. Moreover, according to a recent report from the World Health Organization, it is predicted that one in six deaths worldwide will be the outcome of cancer and that there will be 29.4 million cases of cancer by 2040 [3,4]. Standard treatment relies on tumour surgical removal, radiotherapy and chemotherapy. The effectiveness of the treatment depends, among other factors, on cancer stage at the time of diagnosis. Early-stage cancer diagnosis has been associated with a good prognosis. However, the majority of lung cancer patients are diagnosed with advanced-stage disease, limiting the chances of therapeutic success. Moreover, many treatment options are highly toxic and ineffective in advanced-stage cancer, since patient recurrence and death rates are still exorbitant [5–7]. In this context, the development of novel anticancer therapeutic strategies or the improvement of those currently available is urgently needed.

The application of nanotechnology in medicine, the so-called nanomedicine, is a potentially good alternative or complementary strategy to improve cancer treatment, extending patient survival and/or enhancing their quality of life [8]. Currently, the most promising systems carrying therapeutic agents include nanoparticles (NPs), such as solid lipid nanoparticles (SLN), liposomes, polymeric micelles, metal nanostructures, or dendrimers.

In comparison to conventional therapy, where free anticancer drugs are frequently administered by intravenous route, drug encapsulation in drug delivery systems (DDSs) solves problems related with their solubility, stability, short time of circulation in the bloodstream, and off-target accumulation [9,10]. By addressing these issues, DDSs reduce drug cytotoxicity and increase their therapeutic index, particularly when drugs are biomacromolecules, such as RNA or DNA [11,12]. For some drugs, the encapsulation in DDSs even allows the switch from intravenous to other, less invasive, administration routes, improving patient compliance [12].

Importantly, DDSs can target drugs to specific tissues or cells, including tumours, which typically present a leaky vasculature and a defective lymphatic drainage, which leads to DDS spontaneous accumulation in these tissues, the so-called enhanced penetration and retention (EPR) effect [13]. To do so, the DDS surface is often covalently linked to molecules, such as peptides, small chemical drugs, sugars or nucleic acids, that have high affinity to structures on the surface of target cells [14]. Opportunely, this ligand-target interaction increases drug uptake into the target cells [15].

Nanosystem functionalization has also been explored to overcome biological barriers [16,17]. For instance, multiple studies report nanosystem conjugation with targeting ligands with high affinity to receptors on blood-brain barrier endothelial cells [18–20]. These functional nanosystems explore vesicle transport mechanisms in order to reach the central nervous system, which is highly protected from xenobiotic entry.

Some systems even rely on endogenous or exogenous stimuli, such as pH, oxygen tension or ultrasounds, to release the encapsulated drugs [21]. Furthermore, they can be loaded with more than one drug simultaneously, hindering the development of therapeutic resistance [9]. In association with surface functionalization, the DDS types, shapes, size and surface charges have to be taken into consideration to improve the NPs efficiency. Despite their apparent benefits and potential to improve cancer treatment, to date, the approval of DDS-based therapies for patient treatment is relatively low [22]. This can be explained by multiple reasons, including the need for patient stratification before inclusion in clinical trials, the need to combine synergistic therapies, or difficulties in assessing DDS efficacy in pre-clinical studies, due to the differences between human tumour physiology and the physiology of traditionally used *in vitro* models [8,23,24].

To assess therapeutic efficacy in pre-clinical research, two-dimensional (2D) cell cultures are commonly used, since they present several advantages. First of all, *in vitro* assays using 2D cell cultures are easily performed and quickly replicated [25]. Secondly, their low cost makes them accessible to most research laboratories [26]. However, 2D cell cultures do not truly simulate the human tumour biological structure, hindering anticancer drug testing and delaying the translation into clinics [27]. In addition to 2D models, animal models are the gold standard to evaluate therapeutic success. The use of animals allows the assessment of the impact of additional factors, such as tumour microenvironment (TME), angiogenesis and, in some cases, the immune system, on treatment response [28–30]. However, animal models are complex, expensive and associated with ethical problems [31]. Furthermore, they do not always represent optimal tumour models. For instance, patient-derived xenografts are established using immunodeficient animals and there are significant genetic differences between them and the primary tumours [32,33].

A new approach to cell culture is currently in evidence: three-dimensional (3D) spheroid systems. These 3D cell cultures recapitulate the tumour structure, in addition to mimicking *in vivo* tumour environment [34]. 3D spheroids were found to express extracellular matrix (ECM) components, recapitulating the barriers and the arrangement of an *in vivo* solid tumour [35]. Therefore, 3D spheroids might be a valuable *in vitro* model to test penetration, distribution, uptake, and efficacy of nanomedicines during the tumour development process, without raising ethical issues and being as complex and expensive as animal models [36,37].

Tumour architecture and microenvironment in disease progression, invasion, metastasis and therapy resistance

Tumours are aberrant, 3D and heterogeneous cell masses harbouring a repertoire of highly proliferative malignant cells and local/infiltrated stromal cells, such as fibroblasts, pericytes, immune cells and endothelial cells. Together, stromal cells and local ECM components create the TME, a biological niche favourable to disease establishment and progression [38].

Over the last decades, experimental studies suggested that malignant transformation and metastization depend on genomic alterations, as well as on the cell environmental context. Indeed, *in vitro* tumour cells prevented from communication with their microenvironment reversed their malignant phenotype [39]. The interactions between tumour cells and the TME are becoming increasingly well understood. It is currently accepted that stromal elements establish cell-cell, cell-ECM, and paracrine signalling with tumour cells, controlling their stemness, epithelial-to-mesenchymal transition (EMT), and ensuring suppression of the tumour's metabolic needs. In addition, these multicomponent-tumour ecosystem interactions also promote tumour dysfunctional vascularization, immune suppression and therapy resistance.

Cancer associated fibroblasts (CAFs), unlike normal fibroblasts, are tumour-recruited and activated fibroblasts, belonging to the TME. In addition to providing mechanical support to tumour cells, CAFs secrete multiple cytokines, chemokines, growth factors, ECM components or degrading enzymes, contributing to the development of a tumour-supporting microenvironment. For instance, via stromal cell-

derived factor-1 (SDF-1) and platelet-derived growth factor (PDGF) release, CAFs create stemness and chemotherapy resistance, promoting niches [40,41]. They also produce and secrete interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs). While IL-6 activates tumour cell migration, MMPs and VEGF induce ECM remodelling and angiogenesis, respectively, creating tracks for cell invasion and providing access of cancer cells to the bloodstream to promote distant metastasis [42–44]. Moreover, CAFs seem to live in a metabolic symbiosis with tumour cells. Their glycolytic metabolism produces lactate, fatty acids and glutamine, captured as energy supplies by anaerobic tumour cells, thus contributing to tumour survival and invasion [45]. On the other hand, CAFs protect tumour cells from immune attacks through their immunomodulatory functions. CAF IL-4, IL-6, IL-8 and ECM remodelling activate immunosuppressive myeloid cells and restrict immune cell access to the tumour, preventing tumour cell recognition and killing by activated immune cells [46,47].

Immune cells, including macrophages, dendritic cells, neutrophils and $CD8^+$ T cells, are another major component of the TME. Tumour-associated macrophages represent up to 50% of the tumour mass and encompass the pro-inflammatory M1 macrophages and the immunosuppressive M2 macrophages. The M1 and M2 polarization phenotype is associated with cancer malignancy and patient prognosis; the M1 profile can correlate with an anti-tumourigenic response, while M2 can promote tumour cell proliferation and survival [48–50]. Following detection of malignant cells, immune cell activation should be expected in order to promote an inflammatory response where tumour antigens are processed and presented by antigen-presenting cells, attracting and activating cytotoxic cells that, consequently, would eliminate tumour cells [51]. However, TME cues regulate intratumoural immune cell polarization to promote immunosuppression and tumour evasion from immune surveillance. Tumour cells themselves favour monocyte differentiation towards immunosuppressive macrophages, through retinoic acid production [52]. Furthermore, as we mentioned above, CAF cytokines assist immunosuppressive cell activation. In gliomas, IL-6, colony stimulating factor 1 (CSF-1) and glial cell-derived neurotrophic factor (GDNF) released from endothelial cells instigate the macrophage immunosuppressive phenotype, thereby allowing tumour cells to prevail [42,53,54]. Along with their protective role, tumour-associated immunosuppressive cells are great sources of VEGF, MMPs, epidermal growth factor (EGF), transforming growth factor β (TGF-β), IL-10 and IL-6, directly contributing to malignant transformation, tumour angiogenesis, invasion, intravasation, extravasation and metastasis [55–60].

Endothelial cells and other vascular compartment cells, such as pericytes, are fundamental structures and play a significant role in the TME. In fact, the ability to grow a circulatory system, via angiogenesis or other process, is a well-recognized hallmark of cancer [61]. These tumour blood vessels carry oxygen and nutrients through the neoplastic tissue, suppressing the tumour's metabolic needs and eliminating its metabolic waste [62]. However, in addition to the recognized transport/delivery function, tumour blood vessels are also involved in other processes favouring tumour progression. Endothelial cells from tumour blood vessels produce and secrete ECM elements, such as laminin, contributing to its remodelling [63]. Also, they promote cancer stem cell self-renewal and maintenance, namely via Notch and IL-8 pathways [64,65]. Together, these processes are deeply involved in cancer progression and therapy resistance. Remodelled ECM hampers radiotherapy and chemotherapy tumour penetration, and cancer stem cells are considerably resistant to both [66,67]. Tumour blood vessel cells also contribute to tumour immune evasion. Endothelial cells activate macrophage pro-tumoural phenotype, via IL-6, and limit cytotoxic T cell accumulation in the tumour tissue [68,69]. Moreover, their basement membrane acts as a route for cancer cell invasion and metastasis formation [70].

The ECM is a structural, non-cellular tissue component that includes collagen, proteoglycans and glycoproteins [71]. During tumour development, the ECM undergoes quantitative and qualitative remodelling, acquiring a unique composition, rich in pro-invasive and pro-migration signals that foster disease progression [72].

Two of the most frequently reported tumour-associated ECM modifications are the enhancement of collagen deposition and collagen cross-linking. CAFs and other TME cells produce and secrete aligned collagen fibrils, while primary tumour cells upregulate cross-linking enzymes, such as lysyl oxidase, increasing ECM stiffness [73,74]. Logically, as mentioned above, a denser matrix is an obstacle for radiotherapy and chemotherapy penetration into the tumour tissue, leading to therapy resistance [75]. Such induced biophysical modification correlates with malignant transformation, invasion and migration, to the point that reducing the pressure of breast cancer cells, *in vitro*, reversed their malignant phenotype [76]. Moreover, tumour cell upregulated integrins recognize aligned collagen fibrils, migrating along them [77,78]. In addition to collagen, laminin and fibronectin deposition, as well as tenascin C expression, are also frequently upregulated in tumour tissue, participating in malignant transformation, progression and metastasis [79–81].

It is also important to mention that, besides directly communicating with tumour cells, the ECM promotes disease progression by interacting with stromal cells. ECM components control macrophage infiltration and spatial distribution in the TME, as well as assist macrophage polarization into a pro-tumoural and pro-angiogenic phenotype [82,83]. However, its immunomodulatory role is not fully described yet, and it seems to depend on tumour biology [84].

Overall, it seems like all TME elements modulate multiple tumour development processes, such as malignant transformation, immune surveillance and angiogenesis, highlighting their potential as a target for cancer-directed therapies (Fig. 1).

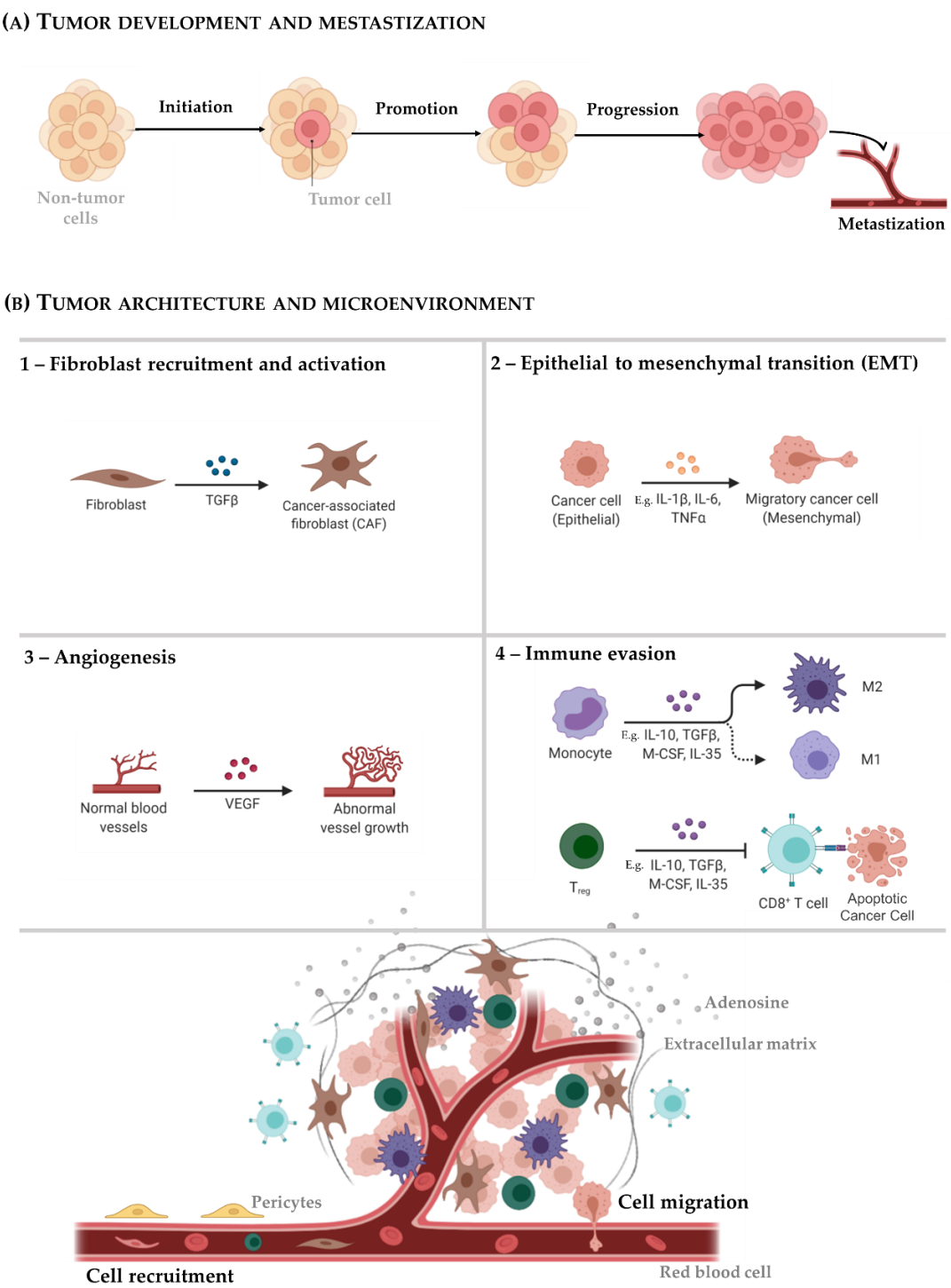

Figure 1. Overview of tumour development and metastization, and tumour microenvironment components. **(A)** Tumour development and metastization. Multi-step process of carcinogenesis; the transformation of a normal cell into a cancer cell involves initiation (induced by chemicals, viruses, radiation), promotion, progression and finally metastization. **(B)** Tumour architecture and microenvironment. **(1)** Fibroblast recruitment and activation: transforming growth factor β (TGF-β) promotes adjacent fibroblast differentiation into cancer associated fibroblasts; **(2)** Epithelial to mesenchymal transition (EMT): a polarized epithelial cell that, under several stimuli, such as IL-1β, IL-6 and tumour necrosis factor α (TNF-α) production, assumes a mesenchymal cell phenotype, which includes enhanced migratory capacity and invasiveness; **(3)** Angiogenesis: neovascularization promoted by vascular endothelial growth factor (VEGF); **(4)** Immune evasion: monocyte polarization into pro-tumour M2 macrophages and recruitment of pro-tumourigenic regulatory T cells (Treg), promoted by IL-10, TGF-β, macrophage colony-stimulating factor (M-CSF) and IL-35, promoting cancer cell apoptosis by $CD8^+$ T cells. Created by the authors with BioRender.com.

2D cell cultures: failure in predicting the clinical outcome of anticancer drug candidates

2D cell cultures have been widely used in scientific research since the beginning of the 20th century [85]. Conventionally, in the cancer research field, they are useful to study tumour cell biology, test drug cytotoxicity and establish adequate disease biomarkers [86]. They are easy to manipulate, quickly reproduced, with high replication power, present low cost, and are associated with simple and long-term maintenance [25]. However, conventional 2D cell cultures do not mimic the complex physiology of the human tumour, providing unreliable results [86,87].

Most solid tumours, due to sustained cell proliferation and vascular system disorganization, present an irregular distribution of oxygen, with hypoxia and necrotic areas. Cells from hypoxic zones switch to glycolytic metabolism, releasing lactate and protons, and reducing the pH in these TME regions [88]. 2D cell cultures cannot represent this heterogeneous oxygen and pH distribution [89]. Moreover, these simple models lack TME elements, as well as their influence on tumourigenesis. For these reasons, cell behaviour – and, consequently, therapy response – is different in 2D and 3D systems (or spheroids) [90–92]. Cell morphology and behaviour in 3D systems is more similar to cells from *in vivo* tumours than to those from 2D systems. For instance, colorectal cancer cells in spheroids are significantly more resistant to 5-fluorouracil (5-FU) treatment than the same cells in 2D cultures, while 2D and 3D colorectal cancer cell cultures respond similarly to doxorubicin [93]. Moreover, the ease with which drugs induce apoptosis, as well as poor cell differentiation, are factors contributing to the inefficiency of the 2D cellular model [85,94]. Also, cell proliferation in 2D monolayer cultures is faster than in most *in vivo* tumours [25]. Probably, due to all these differences from the *in vivo* tumours, less than 5% of anticancer drugs tested in 2D monolayer assays are approved for therapeutic use, considerably increasing the costs of anticancer drug discovery [87,94].

3D spheroids: a promising preclinical approach to anticancer drug evaluation

The need to establish more reliable pre-clinical models led to the development of 3D cell culture models, namely spheroids [94]. Spheroids have been gaining popularity as one of the most appropriate *in vitro* models, particularly in the cancer research area, owing to their ability to mimic the human tumour structure, which fills the gap between 2D cultures and animal models (Fig. 2) [34,49,89]. They have been established to study the proliferation, migration, invasion and function of tumour cells, immune interaction, and to test new therapeutic strategies [25,95]. 3D cultures might include multiple malignant/stromal cell populations, and present an internal necrotic area surrounded by quiescent cells and an external layer of proliferation cells [95]. This conformational structure forms a heterogeneous gradient of oxygen, pH and nutrients, showing similarities with *in vivo* solid tumours [95]. Importantly, spheroids exhibit much more cell-cell and cell-matrix interactions than 2D cultures, which rely predominantly on cell-substrate interactions [96]. Once more, as in *in vivo* tumour tissues, in spheroids, due to their 3D conformational structure, the oxygen gradient is higher in the outer layer, while the inner layer exhibits hypoxia and lower pH levels, which results in the formation of three cell differentiation zones: proliferative, quiescent and necrotic layers, as mentioned above. Indeed, many drugs used in therapy require oxygen to induce an effective anticancer effect. Moreover, this acidic environment, mainly in the spheroid core, also affects drug cellular uptake, impacting on drug efficiency [97–99]. Consequently, and similarly to the *in vivo* scenario, tumour spheroids usually show higher resistance to anticancer therapies, when compared with 2D models [85].

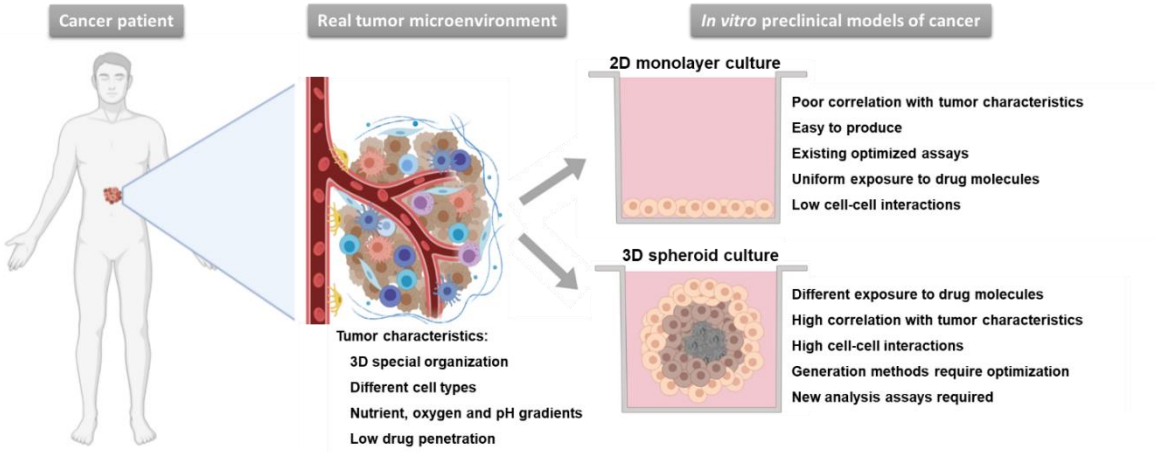

Figure 2. 2D monolayer and 3D spheroid culture systems as preclinical models of cancer. The real tumour ecosystem is formed mainly by immune cells, tumour-associated fibroblasts, myeloid-derived suppressor cells, blood vessels and extracellular matrix (see text for more details). Created by the authors with BioRender.com.

Nanomedicine in cancer treatment

Since the description of the EPR effect, 35 years ago, researchers have been designing nanosystem-based strategies for cancer treatment [100]. Advances in this field enabled the development of multiple technologies to assist both cancer diagnosis and therapy. Basically, nanosystems have been loaded with imaging and/or therapeutic molecules to improve their penetration and retention in the tumour tissue [101–103].

By encapsulating drugs, nanosystems offer the possibility to modify their solubility and release profile, extend blood circulation and increase their half-life, thus improving drug bioavailability. They can also target drugs to specific tissues, reducing potential toxicity in non-tumour tissues. The encapsulation of imaging probes and, in some cases, the nanosystem intrinsic properties, allow their use as imaging tools [104]. In addition to their application for diagnostic purposes, these nanosystems can also be suitable for real-time therapy monitoring [105].

Nanomedicine includes several types of nanosystems, such as polymeric NPs, micelles, dendrimers or lipid-structured NPs [106–108] (Fig. 3). Here, we briefly describe the main nanosystems under development for anticancer therapy (Table 2). We also analyse the application of spheroids in the pre-clinical evaluation of these nanomedicines. The advantages and disadvantages associated with each DDS are summarized in Table 1.

Table 1. Advantages and disadvantages of each drug delivery system.

		Advantages	Disadvantages
SLN		Biocompatible, biodegradable, non-toxic; Controlled drug release; Enhance tissue/cell-specific targeting; Prepared on a large scale.	Low drug-loading efficency; Higher physical instability during storage (premature drug release during storage).
Liposomes		Non-toxic, non-immunogenic, non-carcinogenic, non-thrombogenic and biodegradable; Encapsulate large amounts of drug; Prevent drug rapid degradation.	Higher physical instability during storage (may crystallize after prolonged storage conditions); High production cost.
Polymeric micelles		Can easily cross cell membranes; High structural stability; Easy modification; Avoid phagocytic recognition.	Do not encapsulate hydrophilic drugs; Low drug-loading capacity.
Metal NPs	Gold NPs	Biocompatible; Easy fabrication; Easily controllable surface functionality.	High tendency to form aggregations; Toxicity of metal compounds; Potential accumulation in the body.
	Bimetallic NPs	Higher stability and selectivity; Metal synergistic effects enhance the properties of each metal.	
Polymeric NPs		Can encapsulate hydrophilic and hydrophobic substances; Non-toxic, non-immunogenic and can be easily personalized; Control drug release and do not cause inflammation.	Difficulty in scale-up.
Dendrimers		Can easily cross physiological barriers, including the blood-brain barrier.	Cellular toxicity; High production cost.
Adeno-associated virus vectors		Can package different types of genes for cancer; Express genes for a longer period of time; Infect dividing and nondividing cells; Low immunogenicity and pathogenicity.	Do not display cellular specificity; Small packaging limit (up to 5 Kbp).

NPs: nanoparticles; SLN: solid lipid nanoparticles.

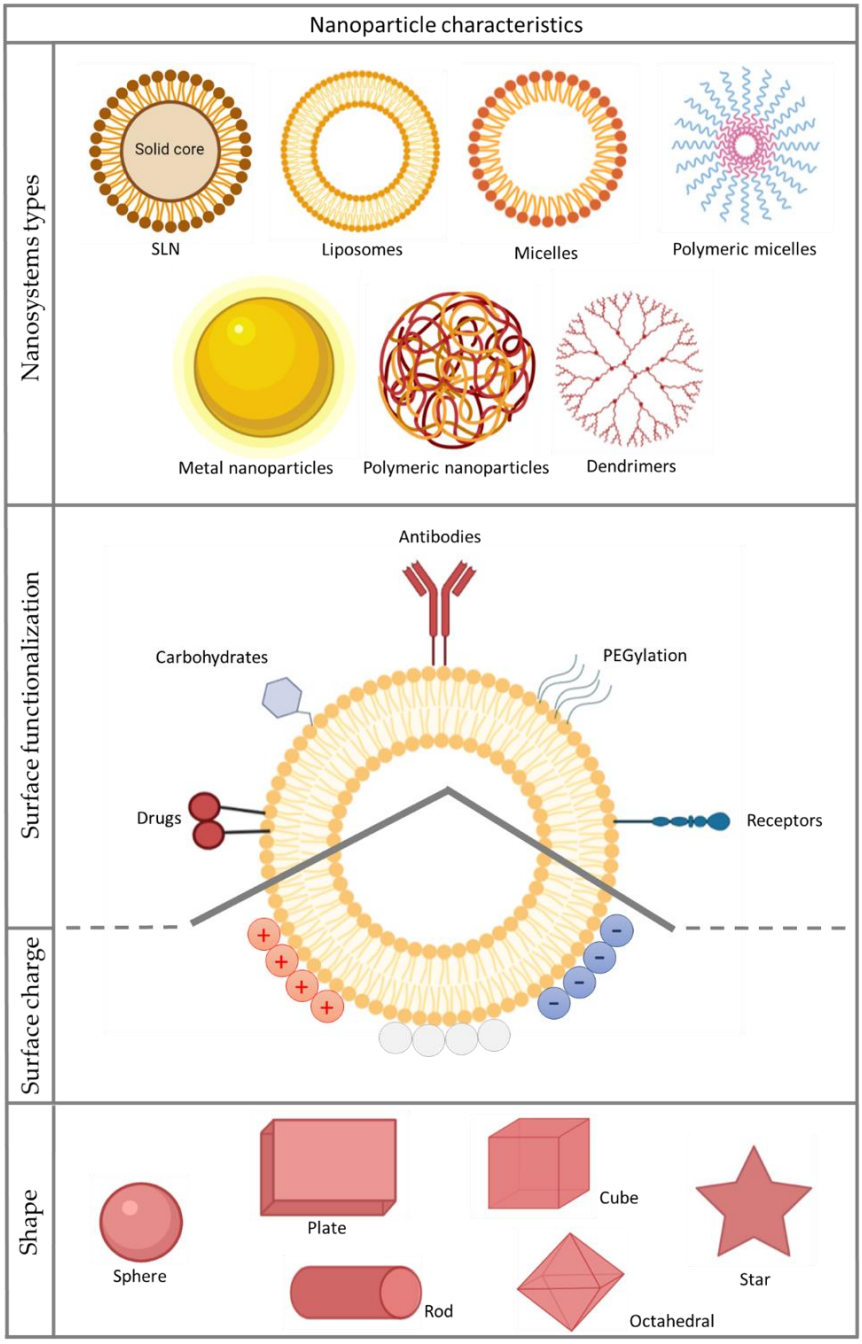

Figure 3. Schematic illustration of different types of nanocarriers and adjustable characteristics. Different factors, including nanoparticle type, size, shape, surface charge and functionalization, can determine nanoparticle efficiency, optimizing the ability to avoid phagocytic recognition, transpose biological barriers, target therapeutic sites and penetrate into tumour tissue. SLN: solid lipid nanoparticles. Created by the authors with BioRender.com.

SLN. SLN are nanosystems built from room temperature-solid lipids, emulsifiers and water [109]. Due to their physiologic lipidic matrix, these nanostructures are biocompatible, biodegradable and non-toxic [110,111]. Moreover, like other nanosystems, SLN might encapsulate hydrophilic or hydrophobic drugs, provide their controlled release, target tissues or cells and can be prepared on a large scale [109,112]. Thus, SLN have been widely formulated and proposed for cancer treatment [113–115].

Regarding SLN pre-clinical evaluation, some recent works already report the use of spheroid models. In most cases, tumour spheroids are used to assess SLN toxicity, penetration and internalization [116,117]. For instance, Granja *et al*. formulated a mitoxantrone-loaded SLN for breast cancer treatment [116]. In this study, breast cancer spheroids, incorporating fibroblasts and breast cancer cell lines, are used to assess system anticancer efficacy and tumour penetration. Undoubtedly, their SLN penetrate the spheroids. In comparison with 2D models, spheroids were less sensitive to SLN treatment, which probably better represents the *in vivo* situation. Using glioblastoma spheroids, Arduino *et al*. reported similar results [117]. Their PEGylated SLN penetrate spheroids and are internalized by tumour cells.

Some authors describe spheroid use to evaluate differences between targeted and non-targeted SLN penetration and internalization [118,119]. In general, functionalization enhances SLN penetration and internalization.

Liposomes. Liposomes are the first generation of drug nanocarriers, with Doxil being the first nanomedicine ever approved in cancer treatment. They consist of non-toxic, biodegradable and versatile spherical phospholipid vesicles [120,121]. Due to the amphiphilic nature of phospholipids, these nanostructures can also incorporate either hydrophobic or hydrophilic drugs, preventing them from rapid degradation and/or clearance [122,123].
As we mentioned for other DDSs under development for cancer treatment, spheroids have been used as a tool to evaluate liposome tumour penetration, internalization and toxicity [124,125]. For instance, d'Avanzo *et al*. prepared doxorubicin- and sorafenib-loaded liposomes targeted at breast cancer cells p32 protein. During the evaluation of this nanosystem in 2D cell cultures and spheroids, researchers found that functionalization influences liposome toxicity in 3D systems only [125]. Probably, in 2D models, liposomes are easily internalized, whereas, in 3D models, functional ligands are required for liposome penetration and internalization. Therefore, such observations underline the importance of using 3D models in nanosystem pre-clinical evaluation.
Interestingly, there are reports of liposomes unable to penetrate spheroids and largely accumulating on their surface, highlighting the potential of spheroids as tools that can indicate the need to improve a formulation [124].

Polymeric micelles. Polymeric micelles are spherical vesicles formed by block copolymers in aqueous solution [126]. Usually, polymeric micelles encapsulate small hydrophobic drugs in their inner core [127,128]. Their hydrophilic outer shell prevents opsonization, avoiding phagocytic recognition and, consequently, prolonging the circulation time and delaying drug elimination. These nanovesicles present a diameter that often ranges from 10 to 100 nanometres and have been used to carry various substances, including proteins, chemotherapy drugs, small interfering RNA (siRNA), and DNA into tumour cells [129–131]. Due to their small size, polymeric micelles can easily cross cell membranes. Other advantages of these nanostructures are their high structural stability and easy modification [132,133].
Studies report polymeric micelle ability to deeply penetrate tumour spheroids, which is affected by ligand functionalization [128,134,135]. In comparison with non-targeted polymeric micelles, functionalized nanosystem penetration is higher [128]. Furthermore, cytotoxic effects of micelles in spheroids follow their ability to penetrate them. The greater the nanosystem penetration, the greater its cytotoxic power [135]. Therefore, it seems that spheroid usage in the pre-clinical evaluation of these systems is indisputable.

Metal NPs. Metal NPs include iron, zinc, silver and gold NPs. In general, they are biocompatible and easily excreted by the human body, and their magnetic features have been explored for therapeutic and diagnostic applications [136].
Regarding therapeutic applications, most metallic NPs exhibit intrinsic anticancer activity, due to oxidative stress promotion. Basically, metal NPs mediate reactive oxygen species (ROS) generation and accumulation [137]. Through Fenton-type reactions, ROS cause cellular damage, with activation of necrosis or apoptosis and inflammatory responses [138]. In addition, other properties, such as photoluminescence and hyperthermia, contribute to their therapeutic potential.
For diagnostic purposes, metal NPs can be used as imaging agents because of their electromagnetic properties. For instance, iron NPs are approved as contrast agents for magnetic resonance imaging (MRI) [139].
Several published studies used both monolayers and spheroid cells to study, in particular, metal NP internalization and penetration. In most cases, spheroids are employed to compare the penetration level of metal NPs with only small variations in their physicochemical properties. For instance, using HeLa spheroids, Sujai *et al*. evaluated the penetration and uptake of positive, negative and neutral gold NPs [140]. In similar studies, other authors also evaluated size or functionalization influence on metal NP penetration and internalization [140–143]. Overall, findings suggest that either size, surface charge and targeting ligands influence metal NP diffusivity in the spheroids and cell internalization, i.e., negative, smaller and targeted NPs penetrate deeper [140,141,143]. However, these results are not consensual, and the characteristics associated with greater penetration are not necessarily the ones that guarantee cell uptake and cytotoxic activity [140,141]. In fact, Sujai *et al*. found that positive surface charge facilitates cell uptake, while negative surface charge facilitates penetration [140]. Therefore, these results underline the significance of using spheroid models in the development and validation of nanosystems. *In vivo* studies, with tumour animal models, confirmed the results obtained with HeLa spheroids, validating their use [140].

Polymeric NPs. Polymeric NPs are colloidal systems prepared with either biodegradable or non-biodegradable polymers [12]. Non-biodegradable polymers are associated with inflammatory reactions and chronic toxicity, while biodegradable polymers, such as poly(lactic-*co*-glycolic) acid (PLGA), are considered safer. PLGA is even approved for clinical use [144]. The polymers used in NP assembly are

adjustable through manipulation of their physical, chemical and biological features to produce chemotherapeutic-loaded nanosystems with the ideal characteristics [145]. As with other nanosystems, anticancer polymeric NP efficacy has been tested with spheroid models.

A large number of published studies report that chemotherapeutics encapsulated in polymeric NPs penetrate the tumour deeper than the free drugs [146–148]. For instance, Elbatanony *et al*. recently designed afatinib-loaded PLGA NPs that improved afatinib penetration into A549 spheroids [146]. However, only few studies tried to establish the polymeric NP characteristics responsible for higher penetration.

Similarly to metal NPs, it has been reported that polymeric NP size and surface charge influence their tumour penetration. In fact, it seems like smaller polymeric NPs penetrate and accumulate more in tumours [35]. But, unlike metal NPs, neutral polymeric NPs penetrate the tumour more easily than positively or negatively charged ones [35].

In addition, surface functionalization also appears to be determinant for tumour penetration. For instance, the diffusion in melanoma spheroids was reported to be higher for functionalized PLGA NPs compared to the same non-functionalized NPs [149].

Considering the valuable information provided by spheroid-based assays, once again, the use of spheroids seems crucial to assess the efficacy of these systems.

Dendrimers. Dendrimers are highly branched polymeric nanosystems, with a symmetrical and radial architecture [150]. Their branched structure creates empty spaces where drugs or imaging agents can be encapsulated by electrostatic or hydrophobic interactions, being developed for treatment or diagnostic applications [151]. Furthermore, the polymer end groups are ideal for surface functionalization [152]. Despite their potential for biological applications, most dendrimers are toxic. Due to their strong positive charge, they destabilize plasma membranes and induce cell lysis [153]. One of dendrimer biggest advantages is related with their small size, which allows them to easily overcome biological barriers, such as the blood-brain barrier [154].

Both size and surface charge are essential for dendrimer penetration into spheroids [155]. Indeed, small poly(amidoamine) (PAMAM) dendrimers with 2 nm penetrate the core of spheroids more efficiently than 5 and 8 nm NPs. Also, dendrimers with positive charge (amine terminated PAMAM dendrimers) showed greater accumulation in spheroids compared to neutral charge surface (acetylated PAMAM counterparts) dendrimers [155].

Furthermore, recently, Rompicharla *et al*. studied the potential impact of surface functionalization in dendrimer toxicity and penetration into spheroids [156]. Functionalization with biotin on paclitaxel-loaded PEGylated PAMAM dendrimer surface improved the penetration and toxicity of the system into lung cancer spheroids, when compared with the non-functionalized nanosystem with free paclitaxel. Besides, there were differences between 3D and 2D cell cultures, regarding therapy resistance [156].

Overall, these reports confirm the need to use tumour models that closely resemble biological reality during nanosystem validation, particularly the use of tumour spheroids.

Adeno-associated virus vectors in 3D spheroids. Adeno-associated virus vectors (AAVs) are non-enveloped single-stranded DNA viruses of the *Parvoviridae* family that have been used as vectors in several therapeutic applications [157,158]. These structures can package different types of genes for cancer cells, including anti-angiogenic and immune-related genes [159]. AAVs present many benefits, such as being able to express the gene for a longer period of time; they can infect dividing and nondividing cells, in addition to being associated with low immunogenicity and pathogenicity [160]. Previous studies, carried out on spheroidal models of glioma (SNB19), demonstrated that recombinant AAVs containing full length cDNA of tissue factor pathway inhibitor 2 (TFPI-2) prevented the invasive and migratory tumour capacity in a dose-dependent manner [161]. Another work showed that AAV serotypes 2, 4, and 5 containing the nuclear-targeted β-galactosidase (AAVRnLacZ) gene or the green fluorescent protein (GFP) gene were able to transduce cells present on the periphery and cells located more internally in 3D glioblastoma cultures [162]. Despite the satisfactory results observed with the AAVs, these structures are non-cell specific, thereby increasing cytotoxicity and impairing therapy safety [163]. Due to the scarcity of literature, we believe that new studies should be performed in order to prove or disprove the real effectiveness of AAVs in delivering medicines to 3D spheroid culture systems.

Table 2. Summary of drug delivery systems involving 3D spheroid cancer models.

Tumour type	Spheroid cell lines	Spheroid diameter (μm)	Drug delivery system	Drug delivery system average size (nm)	Anticancer drug	Reported outcomes	References
Human resistant breast carcinoma cells or human resistant ovarian carcinoma cells	MCF-7/Adr or NCI/Adr	400–500	SLN	74–80	DOX and α-tocopherol succinate	Increased cytotoxicity. No differences in spheroid penetration.	[164]
Human breast and lung cancer cells	MCF7 or A549	-	Glyceryl tripalmitate SLN	210	PTX	Decreased cancer spheroid volumes.	[165]
PTX-resistant A549 cells	TxR10	574	SLN	78.4	PTX	Reduced spheroid volume and formation.	[166]
Tongue squamous carcinoma cells	CAL-33	200	Lipid nanostructured carriers (Lipidots)	50 or 120	mTHPC	Delayed intracellular drug accumulation and increased cytotoxicity.	[167]
Colon carcinoma cells	HCT 116	~500	Liposomes	162.9/228.4	DOX	DOX efficiently delivered to 3D spheroids.	[168]
Non-small cell lung cancer	H460 or A549	-	Liposomes	211.8–243.2	Pirfenidone	Decreased spheroid diameter and volume.	[169]
Human breast cancer cells	MCF-7	-	Liposomes	98.2	Metformin hydrochloride	Reduced tumour volume.	[170]
Human lung cancer cells	A549	> 300	Liposomes functionalized with T7 (HAIYPRH) on the surface	84–114	Coumarin-6	Increased drug penetration.	[171]
Human cervical cancer	HeLa	400–500	Transferrin anchored methoxypolyethylene glycol-poly polymeric micelles	132.16	Curcumin	Increased drug internalization into spheroids, tumour cell cytotoxicity and apoptosis.	[172]

Human multi drug resistant ovarian cancer cells	NCI-ADR-RES	411–530	Micelles	15–20	PTX and curcumin	Increased tumour cytotoxicity; however, higher drug concentrations were required compared to 2D culture cells.	[173]
Human prostate carcinoma cells	LNCaP	~300	Polymeric micelles	42.4–70.1	PTX	Increased antitumour activity.	[132]
Human breast cancer cells	MCF-7	350–400	Fe_3O_4 NPs	60/120/200/310	Coumarin-6	60 nm NPs showed greater tumour penetration and apoptosis and smaller spheroid diameter.	[174]
Human breast cancer cells and human cervical adenocarcinoma cells	MCF-7 and KB	~500	Gold NPs and PAMAM dendrimers	Gold NPs: 2.2/4.0; PAMAM dendrimers: 2.9/4.5/8.1	-	Smaller NPs (2.2 and 2.9 nm) showed deeper penetration into the spheroid.	[142]
Human breast cancer cells	MCF-7	~300	Gold NPs	2/6/15	-	Smaller NPs (2 and 6 nm) showed greater spheroid internalization.	[143]
Human breast cancer cells	MCF-7	~350	Gold NPs	50 and 100	-	50 nm gold NPs penetrated more deeply into 3D models than those with 100 nm.	[175]
Human lung carcinoma cells	A549	200	Poly(2-hydroxyethyl) acrylate-coated gold NPs	15/40/70	-	Reached more effectively the interior of spheroids and, when coated with longer polymer, had	[141]

						greater accumulation in 3D cultures.	
Human epithelial colorectal adenocarcinoma cells	Caco-2	-	Gold NPs functionalized with C-terminus of the *Clostridium perfringens* enterotoxin	-	Gold NPs-mediated laser perforation (GNOME-LP)	Destroyed the spheroid structure.	[176]
Human colon cancer cells	HCT116	-	Gold and silver bimetallic NPs	10	-	NPs with metal molar Ag:Au ratios of 3:1 caused more toxicity in spheroids than those with 1:1 and 1:3 ratios.	[177]
Human breast cancer cells	MCF-7	-	Bimetallic nanoagents composed of human serum albumin and palladium-iron bimetallic particles	~20	Chlorin e6	Increased intratumoural permeability and retention.	[178]
Human colorectal cancer cells	HCT116	~350	PLGA NPs	147.5	PTX	Decreased migratory areas and tumour cell invasion.	[179]
Human glioma-stem cells	U87, U118, and U251	-	PLGA NPs	199.6	Iguratimod	Reduced the spheroid diameter.	[180]
Human colorectal cancer cells	HCT116	430	Poly(styrene) NPs	30/50/100	DOX	Small NPs (30 and 50 nm) reached the spheroid core more deeply.	[35]
Human melanoma cells	A375 and human dermal fibroblasts (HDF)	~400	Human serum albumin NPs with multiple arginine-glycine-aspartic acid peptides	13	Oligonucleotide 623	Crossed and distributed more efficiently throughout the spheroid.	[27]

Human pancreatic cancer cells	PANC–1, human lung fibroblasts (MRC–5) and human umbilical vein endothelial cells (HUVEC)	1000	Polymer NPs	100	DOX	NPs presented poor penetration through hetero-type spheroids.	[181]
Human prostate carcinoma cells	DU145	200	Cationic poly-L-lysine dendrimers	6.36	DOX	Penetrated more deeply into spheroid, delaying its development.	[182]
Human cervical adenocarcinoma cells	KB	~200	Small PAMAM dendrimers	2/5/8	-	Penetrated the spheroid core more efficiently and, when with positive superficial charge, showed greater 3D culture accumulation.	[155]
Human lung carcinoma cells	A549	~450	Functionalized biotin on the surface of PEGylated PAMAM dendrimers	12.1–15.8	PTX	Decreased spheroid diameter.	[156]
Human glioma cells	SNB19	150	AAVs	-	Full length cDNA of TFPI-2	Reduced migratory and invasive capacity of spheroids.	[161]
Human glioblastoma spheroids	Human biopsy	200–300	AAV vector serotypes 2, 4, and 5	-	Nuclear-targeted β-galactosidase gene or the green fluorescent protein gene	Transduced cells located on the periphery and inner layer of the spheroids.	[162]

AAVs: adeno-associated viruses; DOX: doxorubicin; mTHPC: temoporfin (*m*-tetrahydroxyphenylchlorin); NPs: nanoparticles; PAMAM: poly(amidoamine); PEG: polyethylene glycol; PLGA: poly(lactic-*co*-glycolic) acid; PTX: paclitaxel; SLN: solid lipid nanoparticles; TFPI-2: tissue factor pathway inhibitor 2; -: data not reported.

Conclusions and Perspectives

Currently, nanotechnological advances allow the incorporation of multiple drugs, imaging agents or targeting ligands into nanosystems. Due to their physicochemical properties, nanosystems can improve the solubility and stability of molecules, maintain their sustained release, improve the blood circulation time and promote the accumulation in particular tissues, such as the tumour tissue. Therefore, they present high potential to improve cancer treatment, which is frequently associated with high off-target toxicity and low efficacy.

The screening of nanosystem efficacy is usually performed using 2D cell cultures, to evaluate cytotoxicity and cellular uptake. However, 2D cell cultures fail to reliably represent *in vivo* tumour organization, for which their response to therapy might also fail to mimic *in vivo* tumour response. To address this issue, more and more 3D tumour models, namely spheroids, have been established and used for nanosystem validation. Spheroids present nutrient, gas and pH gradients, ECM deposition and cellular organizations similar to those of *in vivo* tumours, representing better tools to assess nanosystem therapeutic efficacy. By using spheroids, it is becoming increasingly clear that size, surface charge and surface functionalization influence nanosystem behaviour, namely tumour penetration and cytotoxic activity. Furthermore, the results from spheroid-based assays have been correlated with results from *in vivo* assays, using tumour animal models. This reinforces spheroid predictive value and the importance of their use in nanomedicine development.

Despite the growing use of spheroids, most of them are still incomplete, lacking stromal elements from the TME and patient-derived cells. One of the main limitations of spheroid models is the lack of blood and lymphatic vessels. Researchers have been suggesting the combination of spheroids with microfluidic platforms, where microchannel walls are seeded with endothelial cells and perfused with cell culture medium, to mimic vessels. These have been used to study tumour, endothelial and immune cell communication and, in the future, they might be a valuable tool for *in vitro* drug distribution studies [183,184]. Moreover, information obtained from spheroid-based assays is not yet systematized and there are even contradictory results. Another challenge for 3D spheroids is the heterogeneous phenotypic profile induced by different culture media in the same cell line [185]. In this aspect, culture medium-induced changes in spheroid metrics may lead to different antitumour therapy responses, impairing the validation of nanostructures in 3D models. The establishment of a phenotyping cross-link between spheroids and *in vivo* tumour tissues could be an alternative to overcome this barrier, ensuring greater similarity to the real tumour, regardless of the type of cell culture used.

Therefore, future work is needed to establish correlations between each nanosystem feature and its impact on tumour penetration ability. This would help improving nanosystem formulation. It would also be advantageous to establish guidelines for spheroid-based assays, to standardize them and facilitate the systematization of information.

Acknowledgments

The authors acknowledge CESPU – Cooperativa de Ensino Superior Politécnico e Universitário for financial and technical support (project AntiMitoSphere_APSFCT_IINFACTS_2021). BP gratefully acknowledges CESPU for financial support (BD/CBAS/CESPU/01/2020). CP gratefully acknowledges Fundação para a Ciência e a Tecnologia (FCT), Portugal, for financial support (SFRH/BD/06611/2020). JCT received financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES/Finance code 001-PRINT 88887.581726/2020-00).

Author Contributions

BP, CP, JCT, BS, HB and PMAS conceived the work; HB and BS acquired and managed the funding; BP and CP collected the data and wrote the original draft. All authors have read, reviewed the draft, and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no competing interests.

References

1. Jing, X.; Yang, F.; Shao, C.; Wei, K.; Xie, M.; Shen, H.; Shu, Y. Role of Hypoxia in Cancer Therapy by Regulating the Tumor Microenvironment. *Mol Cancer* **2019**, *18*, 157, doi:10.1186/s12943-019-1089-9.
2. Nagai, H.; Kim, Y.H. Cancer Prevention from the Perspective of Global Cancer Burden Patterns. *J Thorac Dis* **2017**, *9*, 448–451, doi:10.21037/jtd.2017.02.75.
3. Ward, R.A.; Fawell, S.; Floc'h, N.; Flemington, V.; McKerrecher, D.; Smith, P.D. Challenges and Opportunities in Cancer Drug Resistance. *Chem Rev* **2021**, *121*, 3297–3351, doi:10.1021/acs.chemrev.0c00383.
4. World Health, O. *WHO Report on Cancer: Setting Priorities, Investing Wisely and Providing Care for All*; World Health Organization: Geneva, 2020; ISBN 9789240001299 9789240001305 (print version).

5. Öjlert, Å.K.; Halvorsen, A.R.; Nebdal, D.; Lund-Iversen, M.; Solberg, S.; Brustugun, O.T.; Lingjaerde, O.C.; Helland, Å. The Immune Microenvironment in Non-Small Cell Lung Cancer Is Predictive of Prognosis after Surgery. *Mol Oncol* **2019**, *13*, 1166–1179, doi:10.1002/1878-0261.12475.
6. Schabath, M.B.; Cote, M.L. Cancer Progress and Priorities: Lung Cancer. *Cancer Epidemiol Biomarkers Prev* **2019**, *28*, 1563–1579, doi:10.1158/1055-9965.EPI-19-0221.
7. Spiro, S.G.; Shah, P.L.; Rintoul, R.C.; George, J.; Janes, S.; Callister, M.; Novelli, M.; Shaw, P.; Kocjan, G.; Griffiths, C.; et al. Sequential Screening for Lung Cancer in a High-Risk Group: Randomised Controlled Trial: LungSEARCH: A Randomised Controlled Trial of Surveillance Using Sputum and Imaging for the EARly Detection of Lung Cancer in a High-Risk Group. *Eur Respir J* **2019**, *54*, 1900581, doi:10.1183/13993003.00581-2019.
8. van der Meel, R.; Sulheim, E.; Shi, Y.; Kiessling, F.; Mulder, W.J.M.; Lammers, T. Smart Cancer Nanomedicine. *Nat Nanotechnol* **2019**, *14*, 1007–1017, doi:10.1038/s41565-019-0567-y.
9. Shi, J.; Kantoff, P.W.; Wooster, R.; Farokhzad, O.C. Cancer Nanomedicine: Progress, Challenges and Opportunities. *Nat Rev Cancer* **2017**, *17*, 20–37, doi:10.1038/nrc.2016.108.
10. Bousbaa, H. Novel Anticancer Strategies. *Pharmaceutics* **2021**, *13*, doi:10.3390/pharmaceutics13020275.
11. Bala Tannan, N.; Manzari, M.T.; Herviou, L.; da Silva Ferreira, M.; Hagen, C.; Kiguchi, H.; Manova-Todorova, K.; Seshan, V.; de Stanchina, E.; Heller, D.A.; et al. Tumor-Targeted Nanoparticles Improve the Therapeutic Index of BCL2 and MCL1 Dual Inhibition. *Blood* **2021**, *137*, 2057–2069, doi:10.1182/blood.2020008017.
12. Mitchell, M.J.; Billingsley, M.M.; Haley, R.M.; Wechsler, M.E.; Peppas, N.A.; Langer, R. Engineering Precision Nanoparticles for Drug Delivery. *Nat Rev Drug Discov* **2021**, *20*, 101–124, doi:10.1038/s41573-020-0090-8.
13. Golombek, S.K.; May, J.N.; Theek, B.; Appold, L.; Drude, N.; Kiessling, F.; Lammers, T. Tumor Targeting via EPR: Strategies to Enhance Patient Responses. *Adv Drug Deliv Rev* **2018**, *130*, 17–38, doi:10.1016/j.addr.2018.07.007.
14. Martins, C.; Sousa, F.; Araújo, F.; Sarmento, B. Functionalizing PLGA and PLGA Derivatives for Drug Delivery and Tissue Regeneration Applications. *Adv Healthc Mater* **2018**, *7*, doi:10.1002/adhm.201701035.
15. Alexis, F.; Pridgen, E.; Molnar, L.K.; Farokhzad, O.C. Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles. *Mol Pharm* **2008**, *5*, 505–515, doi:10.1021/mp800051m.
16. Mulvihill, J.J.; Cunnane, E.M.; Ross, A.M.; Duskey, J.T.; Tosi, G.; Grabrucker, A.M. Drug Delivery across the Blood-Brain Barrier: Recent Advances in the Use of Nanocarriers. *Nanomedicine (Lond)* **2020**, *15*, 205–214, doi:10.2217/nnm-2019-0367.
17. Nascimento, A. v; Singh, A.; Bousbaa, H.; Ferreira, D.; Sarmento, B.; Amiji, M.M. Combinatorial-Designed Epidermal Growth Factor Receptor-Targeted Chitosan Nanoparticles for Encapsulation and Delivery of Lipid-Modified Platinum Derivatives in Wild-Type and Resistant Non-Small-Cell Lung Cancer Cells. *Mol Pharm* **2015**, *12*, 4466–4477, doi:10.1021/acs.molpharmaceut.5b00642.
18. Fan, K.; Jia, X.; Zhou, M.; Wang, K.; Conde, J.; He, J.; Tian, J.; Yan, X. Ferritin Nanocarrier Traverses the Blood Brain Barrier and Kills Glioma. *ACS Nano* **2018**, *12*, 4105–4115, doi:10.1021/acsnano.7b06969.
19. Gothwal, A.; Kumar, H.; Nakhate, K.T.; Ajazuddin; Dutta, A.; Borah, A.; Gupta, U. Lactoferrin Coupled Lower Generation PAMAM Dendrimers for Brain Targeted Delivery of Memantine in Aluminum-Chloride-Induced Alzheimer's Disease in Mice. *Bioconjug Chem* **2019**, *30*, 2573–2583, doi:10.1021/acs.bioconjchem.9b00505.
20. Martins, C.; Araújo, F.; Gomes, M.J.; Fernandes, C.; Nunes, R.; Li, W.; Santos, H.A.; Borges, F.; Sarmento, B. Using Microfluidic Platforms to Develop CNS-Targeted Polymeric Nanoparticles for HIV Therapy. *Eur J Pharm Biopharm* **2019**, *138*, 111–124, doi:10.1016/j.ejpb.2018.01.014.
21. Zhang, W.; Kohane, D.S. Keeping Nanomedicine on Target. *Nano Lett* **2021**, *21*, 3–5, doi:10.1021/acs.nanolett.0c04638.
22. van der Meel, R.; Lammers, T.; Hennink, W.E. Cancer Nanomedicines: Oversold or Underappreciated? *Expert Opin Drug Deliv* **2017**, *14*, 1–5, doi:10.1080/17425247.2017.1262346.
23. Gillet, J.P.; Varma, S.; Gottesman, M.M. The Clinical Relevance of Cancer Cell Lines. *J Natl Cancer Inst* **2013**, *105*, 452–458, doi:10.1093/jnci/djt007.
24. Katt, M.E.; Placone, A.L.; Wong, A.D.; Xu, Z.S.; Searson, P.C. In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform. *Front Bioeng Biotechnol* **2016**, *4*, 12, doi:10.3389/fbioe.2016.00012.
25. Hamilton, G.; Rath, B. Applicability of Tumor Spheroids for in Vitro Chemosensitivity Assays. *Expert Opin Drug Metab Toxicol* **2019**, *15*, 15–23, doi:10.1080/17425255.2019.1554055.
26. Alghuwainem, A.; Alshareeda, A.T.; Alsowayan, B. Scaffold-Free 3-D Cell Sheet Technique Bridges the Gap between 2-D Cell Culture and Animal Models. *Int J Mol Sci* **2019**, *20*, doi:10.3390/ijms20194926.
27. Carver, K.; Ming, X.; Juliano, R.L. Tumor Cell-Targeted Delivery of Nanoconjugated Oligonucleotides in Composite Spheroids. *Nucleic Acid Ther* **2014**, *24*, 413–419, doi:10.1089/nat.2014.0493.
28. Dong, Y.; Zheng, Q.; Wang, Z.; Lin, X.; You, Y.; Wu, S.; Wang, Y.; Hu, C.; Xie, X.; Chen, J.; et al. Higher Matrix Stiffness as an Independent Initiator Triggers Epithelial-Mesenchymal Transition and Facilitates HCC Metastasis. *J Hematol Oncol* **2019**, *12*, 112, doi:10.1186/s13045-019-0795-5.

29. Papaevangelou, E.; Boult, J.K.R.; Whitley, G.S.; Robinson, S.P.; Howe, F.A. Assessment of the Direct Effects of DDAH I on Tumour Angiogenesis in Vivo. *Angiogenesis* **2018**, *21*, 737–749, doi:10.1007/s10456-018-9617-6.
30. Luo, H.; Su, J.; Sun, R.; Sun, Y.; Wang, Y.; Dong, Y.; Shi, B.; Jiang, H.; Li, Z. Coexpression of IL7 and CCL21 Increases Efficacy of CAR-T Cells in Solid Tumors without Requiring Preconditioned Lymphodepletion. *Clin Cancer Res* **2020**, *26*, 5494–5505, doi:10.1158/1078-0432.Ccr-20-0777.
31. Rodrigues, J.; Heinrich, M.A.; Teixeira, L.M.; Prakash, J. 3D In Vitro Model (R)Evolution: Unveiling Tumor-Stroma Interactions. *Trends Cancer* **2021**, *7*, 249–264, doi:10.1016/j.trecan.2020.10.009.
32. Hou, X.; Du, C.; Lu, L.; Yuan, S.; Zhan, M.; You, P.; Du, H. Opportunities and Challenges of Patient-Derived Models in Cancer Research: Patient-Derived Xenografts, Patient-Derived Organoid and Patient-Derived Cells. *World J Surg Oncol* **2022**, *20*, 37, doi:10.1186/s12957-022-02510-8.
33. Ben-David, U.; Ha, G.; Tseng, Y.Y.; Greenwald, N.F.; Oh, C.; Shih, J.; McFarland, J.M.; Wong, B.; Boehm, J.S.; Beroukhim, R.; et al. Patient-Derived Xenografts Undergo Mouse-Specific Tumor Evolution. *Nat Genet* **2017**, *49*, 1567–1575, doi:10.1038/ng.3967.
34. Courau, T.; Bonnereau, J.; Chicoteau, J.; Bottois, H.; Remark, R.; Assante Miranda, L.; Toubert, A.; Blery, M.; Aparicio, T.; Allez, M.; et al. Cocultures of Human Colorectal Tumor Spheroids with Immune Cells Reveal the Therapeutic Potential of MICA/B and NKG2A Targeting for Cancer Treatment. *J Immunother Cancer* **2019**, *7*, 74, doi:10.1186/s40425-019-0553-9.
35. Tchoryk, A.; Taresco, V.; Argent, R.H.; Ashford, M.; Gellert, P.R.; Stolnik, S.; Grabowska, A.; Garnett, M.C. Penetration and Uptake of Nanoparticles in 3D Tumor Spheroids. *Bioconjug Chem* **2019**, *30*, 1371–1384, doi:10.1021/acs.bioconjchem.9b00136.
36. Mikhail, A.S.; Eetezadi, S.; Allen, C. Multicellular Tumor Spheroids for Evaluation of Cytotoxicity and Tumor Growth Inhibitory Effects of Nanomedicines in Vitro: A Comparison of Docetaxel-Loaded Block Copolymer Micelles and Taxotere®. *PLoS One* **2013**, *8*, e62630, doi:10.1371/journal.pone.0062630.
37. Vinci, M.; Gowan, S.; Boxall, F.; Patterson, L.; Zimmermann, M.; Court, W.; Lomas, C.; Mendiola, M.; Hardisson, D.; Eccles, S.A. Advances in Establishment and Analysis of Three-Dimensional Tumor Spheroid-Based Functional Assays for Target Validation and Drug Evaluation. *BMC Biol* **2012**, *10*, 29, doi:10.1186/1741-7007-10-29.
38. Balkwill, F.R.; Capasso, M.; Hagemann, T. The Tumor Microenvironment at a Glance. *J Cell Sci* **2012**, *125*, 5591–5596, doi:10.1242/jcs.116392.
39. Weaver, V.M.; Petersen, O.W.; Wang, F.; Larabell, C.A.; Briand, P.; Damsky, C.; Bissell, M.J. Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and in Vivo by Integrin Blocking Antibodies. *J Cell Biol* **1997**, *137*, 231–245, doi:10.1083/jcb.137.1.231.
40. Pistore, C.; Giannoni, E.; Colangelo, T.; Rizzo, F.; Magnani, E.; Muccillo, L.; Giurato, G.; Mancini, M.; Rizzo, S.; Riccardi, M.; et al. DNA Methylation Variations Are Required for Epithelial-to-Mesenchymal Transition Induced by Cancer-Associated Fibroblasts in Prostate Cancer Cells. *Oncogene* **2017**, *36*, 5551–5566, doi:10.1038/onc.2017.159.
41. Wei, L.; Ye, H.; Li, G.; Lu, Y.; Zhou, Q.; Zheng, S.; Lin, Q.; Liu, Y.; Li, Z.; Chen, R. Cancer-Associated Fibroblasts Promote Progression and Gemcitabine Resistance via the SDF-1/SATB-1 Pathway in Pancreatic Cancer. *Cell Death Dis* **2018**, *9*, 1065, doi:10.1038/s41419-018-1104-x.
42. Wang, L.; Cao, L.; Wang, H.; Liu, B.; Zhang, Q.; Meng, Z.; Wu, X.; Zhou, Q.; Xu, K. Cancer-Associated Fibroblasts Enhance Metastatic Potential of Lung Cancer Cells through IL-6/STAT3 Signaling Pathway. *Oncotarget* **2017**, *8*, 76116–76128, doi:10.18632/oncotarget.18814.
43. O'Connell, J.T.; Sugimoto, H.; Cooke, V.G.; MacDonald, B.A.; Mehta, A.I.; LeBleu, V.S.; Dewar, R.; Rocha, R.M.; Brentani, R.R.; Resnick, M.B.; et al. VEGF-A and Tenascin-C Produced by S100A4+ Stromal Cells Are Important for Metastatic Colonization. *Proc Natl Acad Sci U S A* **2011**, *108*, 16002–16007, doi:10.1073/pnas.1109493108.
44. Gaggioli, C.; Hooper, S.; Hidalgo-Carcedo, C.; Grosse, R.; Marshall, J.F.; Harrington, K.; Sahai, E. Fibroblast-Led Collective Invasion of Carcinoma Cells with Differing Roles for RhoGTPases in Leading and Following Cells. *Nat Cell Biol* **2007**, *9*, 1392–1400, doi:10.1038/ncb1658.
45. Martinez-Outschoorn, U.E.; Lisanti, M.P.; Sotgia, F. Catabolic Cancer-Associated Fibroblasts Transfer Energy and Biomass to Anabolic Cancer Cells, Fueling Tumor Growth. *Semin Cancer Biol* **2014**, *25*, 47–60, doi:10.1016/j.semcancer.2014.01.005.
46. Kim, J.H.; Oh, S.H.; Kim, E.J.; Park, S.J.; Hong, S.P.; Cheon, J.H.; Kim, T.I.; Kim, W.H. The Role of Myofibroblasts in Upregulation of S100A8 and S100A9 and the Differentiation of Myeloid Cells in the Colorectal Cancer Microenvironment. *Biochem Biophys Res Commun* **2012**, *423*, 60–66, doi:10.1016/j.bbrc.2012.05.081.
47. Salmon, H.; Franciszkiewicz, K.; Damotte, D.; Dieu-Nosjean, M.C.; Validire, P.; Trautmann, A.; Mami-Chouaib, F.; Donnadieu, E. Matrix Architecture Defines the Preferential Localization and Migration of T Cells into the Stroma of Human Lung Tumors. *J Clin Invest* **2012**, *122*, 899–910, doi:10.1172/jci45817.
48. Hinshaw, D.C.; Shevde, L.A. The Tumor Microenvironment Innately Modulates Cancer Progression. *Cancer Res* **2019**, *79*, 4557–4566, doi:10.1158/0008-5472.Can-18-3962.
49. Pinto, B.; Henriques, A.C.; Silva, P.M.A.; Bousbaa, H. Three-Dimensional Spheroids as In Vitro Preclinical Models for Cancer Research. *Pharmaceutics* **2020**, *12*, doi:10.3390/pharmaceutics12121186.

50. Boutilier, A.J.; Elsawa, S.F. Macrophage Polarization States in the Tumor Microenvironment. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms22136995.
51. Lei, X.; Lei, Y.; Li, J.K.; Du, W.X.; Li, R.G.; Yang, J.; Li, J.; Li, F.; Tan, H.B. Immune Cells within the Tumor Microenvironment: Biological Functions and Roles in Cancer Immunotherapy. *Cancer Lett* **2020**, *470*, 126–133, doi:10.1016/j.canlet.2019.11.009.
52. Devalaraja, S.; To, T.K.J.; Folkert, I.W.; Natesan, R.; Alam, M.Z.; Li, M.; Tada, Y.; Budagyan, K.; Dang, M.T.; Zhai, L.; et al. Tumor-Derived Retinoic Acid Regulates Intratumoral Monocyte Differentiation to Promote Immune Suppression. *Cell* **2020**, *180*, 1098-1114.e16, doi:10.1016/j.cell.2020.02.042.
53. Sielska, M.; Przanowski, P.; Wylot, B.; Gabrusiewicz, K.; Maleszewska, M.; Kijewska, M.; Zawadzka, M.; Kucharska, J.; Vinnakota, K.; Kettenmann, H.; et al. Distinct Roles of CSF Family Cytokines in Macrophage Infiltration and Activation in Glioma Progression and Injury Response. *J Pathol* **2013**, *230*, 310–321, doi:10.1002/path.4192.
54. Ku, M.C.; Wolf, S.A.; Respondek, D.; Matyash, V.; Pohlmann, A.; Waiczies, S.; Waiczies, H.; Niendorf, T.; Synowitz, M.; Glass, R.; et al. GDNF Mediates Glioblastoma-Induced Microglia Attraction but Not Astrogliosis. *Acta Neuropathol* **2013**, *125*, 609–620, doi:10.1007/s00401-013-1079-8.
55. Larionova, I.; Kazakova, E.; Gerashchenko, T.; Kzhyshkowska, J. New Angiogenic Regulators Produced by TAMs: Perspective for Targeting Tumor Angiogenesis. *Cancers (Basel)* **2021**, *13*, doi:10.3390/cancers13133253.
56. Yamaguchi, T.; Fushida, S.; Yamamoto, Y.; Tsukada, T.; Kinoshita, J.; Oyama, K.; Miyashita, T.; Tajima, H.; Ninomiya, I.; Munesue, S.; et al. Tumor-Associated Macrophages of the M2 Phenotype Contribute to Progression in Gastric Cancer with Peritoneal Dissemination. *Gastric Cancer* **2016**, *19*, 1052–1065, doi:10.1007/s10120-015-0579-8.
57. Gao, L.; Wang, F.Q.; Li, H.M.; Yang, J.G.; Ren, J.G.; He, K.F.; Liu, B.; Zhang, W.; Zhao, Y.F. CCL2/EGF Positive Feedback Loop between Cancer Cells and Macrophages Promotes Cell Migration and Invasion in Head and Neck Squamous Cell Carcinoma. *Oncotarget* **2016**, *7*, 87037–87051, doi:10.18632/oncotarget.13523.
58. Zhang, D.; Qiu, X.; Li, J.; Zheng, S.; Li, L.; Zhao, H. TGF-β Secreted by Tumor-Associated Macrophages Promotes Proliferation and Invasion of Colorectal Cancer via MiR-34a-VEGF Axis. *Cell Cycle* **2018**, *17*, 2766–2778, doi:10.1080/15384101.2018.1556064.
59. Quail, D.F.; Joyce, J.A. Microenvironmental Regulation of Tumor Progression and Metastasis. *Nat Med* **2013**, *19*, 1423–1437, doi:10.1038/nm.3394.
60. Jinushi, M.; Baghdadi, M.; Chiba, S.; Yoshiyama, H. Regulation of Cancer Stem Cell Activities by Tumor-Associated Macrophages. *Am J Cancer Res* **2012**, *2*, 529–539.
61. Hanahan, D.; Weinberg, R.A. Hallmarks of Cancer: The next Generation. *Cell* **2011**, *144*, 646–674, doi:10.1016/j.cell.2011.02.013.
62. Folkman, J. Tumor Angiogenesis: Therapeutic Implications. *N Engl J Med* **1971**, *285*, 1182–1186, doi:10.1056/nejm197111182852108.
63. Belousov, A.; Titov, S.; Shved, N.; Garbuz, M.; Malykin, G.; Gulaia, V.; Kagansky, A.; Kumeiko, V. The Extracellular Matrix and Biocompatible Materials in Glioblastoma Treatment. *Front Bioeng Biotechnol* **2019**, *7*, 341, doi:10.3389/fbioe.2019.00341.
64. Zhu, T.S.; Costello, M.A.; Talsma, C.E.; Flack, C.G.; Crowley, J.G.; Hamm, L.L.; He, X.; Hervey-Jumper, S.L.; Heth, J.A.; Muraszko, K.M.; et al. Endothelial Cells Create a Stem Cell Niche in Glioblastoma by Providing NOTCH Ligands That Nurture Self-Renewal of Cancer Stem-like Cells. *Cancer Res* **2011**, *71*, 6061–6072, doi:10.1158/0008-5472.Can-10-4269.
65. McCoy, M.G.; Nyanyo, D.; Hung, C.K.; Goerger, J.P.; Zipfel, R.W.; Williams, R.M.; Nishimura, N.; Fischbach, C. Endothelial Cells Promote 3D Invasion of GBM by IL-8-Dependent Induction of Cancer Stem Cell Properties. *Sci Rep* **2019**, *9*, 9069, doi:10.1038/s41598-019-45535-y.
66. Gouarderes, S.; Mingotaud, A.F.; Vicendo, P.; Gibot, L. Vascular and Extracellular Matrix Remodeling by Physical Approaches to Improve Drug Delivery at the Tumor Site. *Expert Opin Drug Deliv* **2020**, *17*, 1703–1726, doi:10.1080/17425247.2020.1814735.
67. Prieto-Vila, M.; Takahashi, R.U.; Usuba, W.; Kohama, I.; Ochiya, T. Drug Resistance Driven by Cancer Stem Cells and Their Niche. *Int J Mol Sci* **2017**, *18*, doi:10.3390/ijms18122574.
68. Wang, Q.; He, Z.; Huang, M.; Liu, T.; Wang, Y.; Xu, H.; Duan, H.; Ma, P.; Zhang, L.; Zamvil, S.S.; et al. Vascular Niche IL-6 Induces Alternative Macrophage Activation in Glioblastoma through HIF-2α. *Nat Commun* **2018**, *9*, 559, doi:10.1038/s41467-018-03050-0.
69. Wang, H.; Zhou, H.; Xu, J.; Lu, Y.; Ji, X.; Yao, Y.; Chao, H.; Zhang, J.; Zhang, X.; Yao, S.; et al. Different T-Cell Subsets in Glioblastoma Multiforme and Targeted Immunotherapy. *Cancer Lett* **2021**, *496*, 134–143, doi:10.1016/j.canlet.2020.09.028.
70. de Gooijer, M.C.; Guillén Navarro, M.; Bernards, R.; Wurdinger, T.; van Tellingen, O. An Experimenter's Guide to Glioblastoma Invasion Pathways. *Trends Mol Med* **2018**, *24*, 763–780, doi:10.1016/j.molmed.2018.07.003.
71. Naba, A.; Clauser, K.R.; Ding, H.; Whittaker, C.A.; Carr, S.A.; Hynes, R.O. The Extracellular Matrix: Tools and Insights for the "Omics" Era. *Matrix Biol* **2016**, *49*, 10–24, doi:10.1016/j.matbio.2015.06.003.
72. Brassart-Pasco, S.; Brézillon, S.; Brassart, B.; Ramont, L.; Oudart, J.B.; Monboisse, J.C. Tumor Microenvironment: Extracellular Matrix Alterations Influence Tumor Progression. *Front Oncol* **2020**, *10*, 397, doi:10.3389/fonc.2020.00397.
73. Levental, K.R.; Yu, H.; Kass, L.; Lakins, J.N.; Egeblad, M.; Erler, J.T.; Fong, S.F.; Csiszar, K.; Giaccia, A.; Weninger, W.; et al. Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell* **2009**, *139*, 891–906, doi:10.1016/j.cell.2009.10.027.
74. Xiao, Q.; Ge, G. Lysyl Oxidase, Extracellular Matrix Remodeling and Cancer Metastasis. *Cancer Microenviron* **2012**, *5*, 261–273, doi:10.1007/s12307-012-0105-z.

75. Henke, E.; Nandigama, R.; Ergün, S. Extracellular Matrix in the Tumor Microenvironment and Its Impact on Cancer Therapy. *Front Mol Biosci* **2019**, *6*, 160, doi:10.3389/fmolb.2019.00160.
76. Provenzano, P.P.; Inman, D.R.; Eliceiri, K.W.; Knittel, J.G.; Yan, L.; Rueden, C.T.; White, J.G.; Keely, P.J. Collagen Density Promotes Mammary Tumor Initiation and Progression. *BMC Med* **2008**, *6*, 11, doi:10.1186/1741-7015-6-11.
77. Riching, K.M.; Cox, B.L.; Salick, M.R.; Pehlke, C.; Riching, A.S.; Ponik, S.M.; Bass, B.R.; Crone, W.C.; Jiang, Y.; Weaver, A.M.; et al. 3D Collagen Alignment Limits Protrusions to Enhance Breast Cancer Cell Persistence. *Biophys J* **2014**, *107*, 2546–2558, doi:10.1016/j.bpj.2014.10.035.
78. Han, W.; Chen, S.; Yuan, W.; Fan, Q.; Tian, J.; Wang, X.; Chen, L.; Zhang, X.; Wei, W.; Liu, R.; et al. Oriented Collagen Fibers Direct Tumor Cell Intravasation. *Proc Natl Acad Sci U S A* **2016**, *113*, 11208–11213, doi:10.1073/pnas.1610347113.
79. Chia, J.; Kusuma, N.; Anderson, R.; Parker, B.; Bidwell, B.; Zamurs, L.; Nice, E.; Pouliot, N. Evidence for a Role of Tumor-Derived Laminin-511 in the Metastatic Progression of Breast Cancer. *Am J Pathol* **2007**, *170*, 2135–2148, doi:10.2353/ajpath.2007.060709.
80. Lin, T.C.; Yang, C.H.; Cheng, L.H.; Chang, W.T.; Lin, Y.R.; Cheng, H.C. Fibronectin in Cancer: Friend or Foe. *Cells* **2019**, *9*, doi:10.3390/cells9010027.
81. Fujita, M.; Ito-Fujita, Y.; Iyoda, T.; Sasada, M.; Okada, Y.; Ishibashi, K.; Osawa, T.; Kodama, H.; Fukai, F.; Suzuki, H. Peptide TNIIIA2 Derived from Tenascin-C Contributes to Malignant Progression in Colitis-Associated Colorectal Cancer via B1-Integrin Activation in Fibroblasts. *Int J Mol Sci* **2019**, *20*, doi:10.3390/ijms20112752.
82. Wei, J.; Marisetty, A.; Schrand, B.; Gabrusiewicz, K.; Hashimoto, Y.; Ott, M.; Grami, Z.; Kong, L.Y.; Ling, X.; Caruso, H.; et al. Osteopontin Mediates Glioblastoma-Associated Macrophage Infiltration and Is a Potential Therapeutic Target. *J Clin Invest* **2019**, *129*, 137–149, doi:10.1172/jci121266.
83. Deligne, C.; Murdamoothoo, D.; Gammage, A.N.; Gschwandtner, M.; Erne, W.; Loustau, T.; Marzeda, A.M.; Carapito, R.; Paul, N.; Velazquez-Quesada, I.; et al. Matrix-Targeting Immunotherapy Controls Tumor Growth and Spread by Switching Macrophage Phenotype. *Cancer Immunol Res* **2020**, *8*, 368–382, doi:10.1158/2326-6066.Cir-19-0276.
84. Deligne, C.; Midwood, K.S. Macrophages and Extracellular Matrix in Breast Cancer: Partners in Crime or Protective Allies? *Front Oncol* **2021**, *11*, 620773, doi:10.3389/fonc.2021.620773.
85. Jensen, C.; Teng, Y. Is It Time to Start Transitioning From 2D to 3D Cell Culture? *Front Mol Biosci* **2020**, *7*, 33, doi:10.3389/fmolb.2020.00033.
86. Ishiguro, T.; Ohata, H.; Sato, A.; Yamawaki, K.; Enomoto, T.; Okamoto, K. Tumor-Derived Spheroids: Relevance to Cancer Stem Cells and Clinical Applications. *Cancer Sci* **2017**, *108*, 283–289, doi:10.1111/cas.13155.
87. Chaicharoenaudomrung, N.; Kunhorm, P.; Noisa, P. Three-Dimensional Cell Culture Systems as an in Vitro Platform for Cancer and Stem Cell Modeling. *World J Stem Cells* **2019**, *11*, 1065–1083, doi:10.4252/wjsc.v11.i12.1065.
88. Petrova, V.; Annicchiarico-Petruzzelli, M.; Melino, G.; Amelio, I. The Hypoxic Tumour Microenvironment. *Oncogenesis* **2018**, *7*, 10, doi:10.1038/s41389-017-0011-9.
89. Weiswald, L.B.; Bellet, D.; Dangles-Marie, V. Spherical Cancer Models in Tumor Biology. *Neoplasia* **2015**, *17*, 1–15, doi:10.1016/j.neo.2014.12.004.
90. Muguruma, M.; Teraoka, S.; Miyahara, K.; Ueda, A.; Asaoka, M.; Okazaki, M.; Kawate, T.; Kuroda, M.; Miyagi, Y.; Ishikawa, T. Differences in Drug Sensitivity between Two-Dimensional and Three-Dimensional Culture Systems in Triple-Negative Breast Cancer Cell Lines. *Biochem Biophys Res Commun* **2020**, *533*, 268–274, doi:10.1016/j.bbrc.2020.08.075.
91. Souza, A.G.; Silva, I.B.B.; Campos-Fernandez, E.; Barcelos, L.S.; Souza, J.B.; Marangoni, K.; Goulart, L.R.; Alonso-Goulart, V. Comparative Assay of 2D and 3D Cell Culture Models: Proliferation, Gene Expression and Anticancer Drug Response. *Curr Pharm Des* **2018**, *24*, 1689–1694, doi:10.2174/1381612824666180404152304.
92. Melissaridou, S.; Wiechec, E.; Magan, M.; Jain, M. v; Chung, M.K.; Farnebo, L.; Roberg, K. The Effect of 2D and 3D Cell Cultures on Treatment Response, EMT Profile and Stem Cell Features in Head and Neck Cancer. *Cancer Cell Int* **2019**, *19*, 16, doi:10.1186/s12935-019-0733-1.
93. Koch, J.; Mönch, D.; Maaß, A.; Gromoll, C.; Hehr, T.; Leibold, T.; Schlitt, H.J.; Dahlke, M.H.; Renner, P. Three Dimensional Cultivation Increases Chemo- and Radioresistance of Colorectal Cancer Cell Lines. *PLoS One* **2021**, *16*, e0244513, doi:10.1371/journal.pone.0244513.
94. Thakuri, P.S.; Liu, C.; Luker, G.D.; Tavana, H. Biomaterials-Based Approaches to Tumor Spheroid and Organoid Modeling. *Adv Healthc Mater* **2018**, *7*, e1700980, doi:10.1002/adhm.201700980.
95. Millard, M.; Yakavets, I.; Zorin, V.; Kulmukhamedova, A.; Marchal, S.; Bezdetnaya, L. Drug Delivery to Solid Tumors: The Predictive Value of the Multicellular Tumor Spheroid Model for Nanomedicine Screening. *Int J Nanomedicine* **2017**, *12*, 7993–8007, doi:10.2147/ijn.S146927.
96. Rodallec, A.; Sicard, G.; Giacometti, S.; Carré, M.; Pourroy, B.; Bouquet, F.; Savina, A.; Lacarelle, B.; Ciccolini, J.; Fanciullino, R. From 3D Spheroids to Tumor Bearing Mice: Efficacy and Distribution Studies of Trastuzumab-Docetaxel Immunoliposome in Breast Cancer. *Int J Nanomedicine* **2018**, *13*, 6677–6688, doi:10.2147/ijn.S179290.

97. Nunes, A.S.; Barros, A.S.; Costa, E.C.; Moreira, A.F.; Correia, I.J. 3D Tumor Spheroids as in Vitro Models to Mimic in Vivo Human Solid Tumors Resistance to Therapeutic Drugs. *Biotechnol Bioeng* **2019**, *116*, 206–226, doi:10.1002/bit.26845.
98. Gupta, P.; Miller, A.; Olayanju, A.; Madhuri, T.K.; Velliou, E. A Systematic Comparative Assessment of the Response of Ovarian Cancer Cells to the Chemotherapeutic Cisplatin in 3D Models of Various Structural and Biochemical Configurations-Does One Model Type Fit All? *Cancers (Basel)* **2022**, *14*, 1274, doi:10.3390/cancers14051274.
99. Huang, Z.; Yu, P.; Tang, J. Characterization of Triple-Negative Breast Cancer MDA-MB-231 Cell Spheroid Model. *Onco Targets Ther* **2020**, *13*, 5395–5405, doi:10.2147/OTT.S249756.
100. Matsumura, Y.; Maeda, H. A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs. *Cancer Res* **1986**, *46*, 6387–6392.
101. Yong, T.; Zhang, X.; Bie, N.; Zhang, H.; Zhang, X.; Li, F.; Hakeem, A.; Hu, J.; Gan, L.; Santos, H.A.; et al. Tumor Exosome-Based Nanoparticles Are Efficient Drug Carriers for Chemotherapy. *Nat Commun* **2019**, *10*, 3838, doi:10.1038/s41467-019-11718-4.
102. Yakavets, I.; Lassalle, H.P.; Scheglmann, D.; Wiehe, A.; Zorin, V.; Bezdetnaya, L. Temoporfin-in-Cyclodextrin-in-Liposome-A New Approach for Anticancer Drug Delivery: The Optimization of Composition. *Nanomaterials (Basel)* **2018**, *8*, doi:10.3390/nano8100847.
103. Chaturvedi, V.K.; Singh, A.; Singh, V.K.; Singh, M.P. Cancer Nanotechnology: A New Revolution for Cancer Diagnosis and Therapy. *Curr Drug Metab* **2019**, *20*, 416–429, doi:10.2174/1389200219666180918111528.
104. Fernandez-Fernandez, A.; Manchanda, R.; McGoron, A.J. Theranostic Applications of Nanomaterials in Cancer: Drug Delivery, Image-Guided Therapy, and Multifunctional Platforms. *Appl Biochem Biotechnol* **2011**, *165*, 1628–1651, doi:10.1007/s12010-011-9383-z.
105. Terreno, E.; Uggeri, F.; Aime, S. Image Guided Therapy: The Advent of Theranostic Agents. *J Control Release* **2012**, *161*, 328–337, doi:10.1016/j.jconrel.2012.05.028.
106. Khosa, A.; Reddi, S.; Saha, R.N. Nanostructured Lipid Carriers for Site-Specific Drug Delivery. *Biomed Pharmacother* **2018**, *103*, 598–613, doi:10.1016/j.biopha.2018.04.055.
107. Pandey, P.; Dureja, H. Recent Patents on Polymeric Nanoparticles for Cancer Therapy. *Recent Pat Nanotechnol* **2018**, *12*, 155–169, doi:10.2174/1872210512666180327120648.
108. Leyva-Gómez, G.; Piñón-Segundo, E.; Mendoza-Muñoz, N.; Zambrano-Zaragoza, M.L.; Mendoza-Elvira, S.; Quintanar-Guerrero, D. Approaches in Polymeric Nanoparticles for Vaginal Drug Delivery: A Review of the State of the Art. *Int J Mol Sci* **2018**, *19*, doi:10.3390/ijms19061549.
109. Mehnert, W.; Mäder, K. Solid Lipid Nanoparticles: Production, Characterization and Applications. *Adv Drug Deliv Rev* **2001**, *47*, 165–196, doi:10.1016/s0169-409x(01)00105-3.
110. Aldawsari, H.M.; Singh, S. Rapid Microwave-Assisted Cisplatin-Loaded Solid Lipid Nanoparticles: Synthesis, Characterization and Anticancer Study. *Nanomaterials (Basel)* **2020**, *10*, doi:10.3390/nano10030510.
111. Zielińska, A.; Ferreira, N.R.; Durazzo, A.; Lucarini, M.; Cicero, N.; Mamouni, S.E.; Silva, A.M.; Nowak, I.; Santini, A.; Souto, E.B. Development and Optimization of Alpha-Pinene-Loaded Solid Lipid Nanoparticles (SLN) Using Experimental Factorial Design and Dispersion Analysis. *Molecules* **2019**, *24*, doi:10.3390/molecules24152683.
112. Wang, H.; Li, L.; Ye, J.; Wang, R.; Wang, R.; Hu, J.; Wang, Y.; Dong, W.; Xia, X.; Yang, Y.; et al. Improving the Oral Bioavailability of an Anti-Glioma Prodrug CAT3 Using Novel Solid Lipid Nanoparticles Containing Oleic Acid-CAT3 Conjugates. *Pharmaceutics* **2020**, *12*, doi:10.3390/pharmaceutics12020126.
113. Lee, M.K.; Lim, S.J.; Kim, C.K. Preparation, Characterization and in Vitro Cytotoxicity of Paclitaxel-Loaded Sterically Stabilized Solid Lipid Nanoparticles. *Biomaterials* **2007**, *28*, 2137–2146, doi:10.1016/j.biomaterials.2007.01.014.
114. Fathy Abd-Ellatef, G.E.; Gazzano, E.; Chirio, D.; Hamed, A.R.; Belisario, D.C.; Zuddas, C.; Peira, E.; Rolando, B.; Kopecka, J.; Assem Said Marie, M.; et al. Curcumin-Loaded Solid Lipid Nanoparticles Bypass P-Glycoprotein Mediated Doxorubicin Resistance in Triple Negative Breast Cancer Cells. *Pharmaceutics* **2020**, *12*, doi:10.3390/pharmaceutics12020096.
115. Senthil Kumar, C.; Thangam, R.; Mary, S.A.; Kannan, P.R.; Arun, G.; Madhan, B. Targeted Delivery and Apoptosis Induction of Trans-Resveratrol-Ferulic Acid Loaded Chitosan Coated Folic Acid Conjugate Solid Lipid Nanoparticles in Colon Cancer Cells. *Carbohydr Polym* **2020**, *231*, 115682, doi:10.1016/j.carbpol.2019.115682.
116. Granja, A.; Lima-Sousa, R.; Alves, C.G.; de Melo-Diogo, D.; Pinheiro, M.; Sousa, C.T.; Correia, I.J.; Reis, S. Mitoxantrone-Loaded Lipid Nanoparticles for Breast Cancer Therapy - Quality-by-Design Approach and Efficacy Assessment in 2D and 3D in Vitro Cancer Models. *Int J Pharm* **2021**, *607*, 121044, doi:10.1016/j.ijpharm.2021.121044.
117. Arduino, I.; Liu, Z.; Rahikkala, A.; Figueiredo, P.; Correia, A.; Cutrignelli, A.; Denora, N.; Santos, H.A. Preparation of Cetyl Palmitate-Based PEGylated Solid Lipid Nanoparticles by Microfluidic Technique. *Acta Biomater* **2021**, *121*, 566–578, doi:10.1016/j.actbio.2020.12.024.
118. Arduino, I.; Liu, Z.; Iacobazzi, R.M.; Lopedota, A.A.; Lopalco, A.; Cutrignelli, A.; Laquintana, V.; Porcelli, L.; Azzariti, A.; Franco, M.; et al. Microfluidic Preparation and in Vitro Evaluation of IRGD-Functionalized Solid Lipid Nanoparticles for Targeted Delivery of Paclitaxel to Tumor Cells. *Int J Pharm* **2021**, *610*, 121246, doi:10.1016/j.ijpharm.2021.121246.
119. Garanti, T.; Alhnan, M.A.; Wan, K.W. RGD-Decorated Solid Lipid Nanoparticles Enhance Tumor Targeting, Penetration and Anticancer Effect of Asiatic Acid. *Nanomedicine (Lond)* **2020**, *15*, 1567–1583, doi:10.2217/nnm-2020-0035.

120. Allen, T.M.; Cullis, P.R. Liposomal Drug Delivery Systems: From Concept to Clinical Applications. *Adv Drug Deliv Rev* **2013**, *65*, 36–48, doi:10.1016/j.addr.2012.09.037.
121. Lai, F.; Fadda, A.M.; Sinico, C. Liposomes for Brain Delivery. *Expert Opin Drug Deliv* **2013**, *10*, 1003–1022, doi:10.1517/17425247.2013.766714.
122. Alavi, M.; Hamidi, M. Passive and Active Targeting in Cancer Therapy by Liposomes and Lipid Nanoparticles. *Drug Metab Pers Ther* **2019**, *34*, doi:10.1515/dmpt-2018-0032.
123. Dutta, S.; Moses, J.A.; Anandharamakrishnan, C. Encapsulation of Nutraceutical Ingredients in Liposomes and Their Potential for Cancer Treatment. *Nutr Cancer* **2018**, *70*, 1184–1198, doi:10.1080/01635581.2018.1557212.
124. Okamoto, Y.; Taguchi, K.; Imoto, S.; Giam Chuang, V.T.; Yamasaki, K.; Otagiri, M. Cell Uptake and Anti-Tumor Effect of Liposomes Containing Encapsulated Paclitaxel-Bound Albumin against Breast Cancer Cells in 2D and 3D Cultured Models. *Journal of Drug Delivery Science and Technology* **2020**, *55*, 101381, doi:https://doi.org/10.1016/j.jddst.2019.101381.
125. d'Avanzo, N.; Torrieri, G.; Figueiredo, P.; Celia, C.; Paolino, D.; Correia, A.; Moslova, K.; Teesalu, T.; Fresta, M.; Santos, H.A. LinTT1 Peptide-Functionalized Liposomes for Targeted Breast Cancer Therapy. *Int J Pharm* **2021**, *597*, 120346, doi:10.1016/j.ijpharm.2021.120346.
126. Hwang, D.; Ramsey, J.D.; Kabanov, A. v Polymeric Micelles for the Delivery of Poorly Soluble Drugs: From Nanoformulation to Clinical Approval. *Adv Drug Deliv Rev* **2020**, *156*, 80–118, doi:10.1016/j.addr.2020.09.009.
127. Almeida, A.; Araújo, M.; Novoa-Carballal, R.; Andrade, F.; Gonçalves, H.; Reis, R.L.; Lúcio, M.; Schwartz Jr., S.; Sarmento, B. Novel Amphiphilic Chitosan Micelles as Carriers for Hydrophobic Anticancer Drugs. *Mater Sci Eng C Mater Biol Appl* **2020**, *112*, 110920, doi:10.1016/j.msec.2020.110920.
128. Barve, A.; Jain, A.; Liu, H.; Zhao, Z.; Cheng, K. Enzyme-Responsive Polymeric Micelles of Cabazitaxel for Prostate Cancer Targeted Therapy. *Acta Biomater* **2020**, *113*, 501–511, doi:10.1016/j.actbio.2020.06.019.
129. Chen, Q.; Liang, H.; Sun, Y.; Chen, Y.; He, W.; Fang, X.; Sha, X.; Li, J. A Carbohydrate Mimetic Peptide Modified Size-Shrinkable Micelle Nanocluster for Anti-Tumor Targeting and Penetrating Drug Delivery. *Int J Nanomedicine* **2019**, *14*, 7339–7352, doi:10.2147/ijn.S213455.
130. Yokoyama, M. Polymeric Micelles as Drug Carriers: Their Lights and Shadows. *J Drug Target* **2014**, *22*, 576–583, doi:10.3109/1061186x.2014.934688.
131. Yousefpour Marzbali, M.; Yari Khosroushahi, A. Polymeric Micelles as Mighty Nanocarriers for Cancer Gene Therapy: A Review. *Cancer Chemother Pharmacol* **2017**, *79*, 637–649, doi:10.1007/s00280-017-3273-1.
132. Du, A.W.; Lu, H.; Stenzel, M.H. Core-Cross-Linking Accelerates Antitumor Activities of Paclitaxel-Conjugate Micelles to Prostate Multicellular Tumor Spheroids: A Comparison of 2D and 3D Models. *Biomacromolecules* **2015**, *16*, 1470–1479, doi:10.1021/acs.biomac.5b00282.
133. Movassaghian, S.; Merkel, O.M.; Torchilin, V.P. Applications of Polymer Micelles for Imaging and Drug Delivery. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **2015**, *7*, 691–707, doi:10.1002/wnan.1332.
134. Tian, L.; Pei, R.; Zhong, L.; Ji, Y.; Zhou, D.; Zhou, S. Enhanced Targeting of 3D Pancreatic Cancer Spheroids by Aptamer-Conjugated Polymeric Micelles with Deep Tumor Penetration. *Eur J Pharmacol* **2021**, *894*, 173814, doi:10.1016/j.ejphar.2020.173814.
135. Yao, Q.; Choi, J.H.; Dai, Z.; Wang, J.; Kim, D.; Tang, X.; Zhu, L. Improving Tumor Specificity and Anticancer Activity of Dasatinib by Dual-Targeted Polymeric Micelles. *ACS Appl Mater Interfaces* **2017**, *9*, 36642–36654, doi:10.1021/acsami.7b12233.
136. Sharma, H.; Mishra, P.K.; Talegaonkar, S.; Vaidya, B. Metal Nanoparticles: A Theranostic Nanotool against Cancer. *Drug Discov Today* **2015**, *20*, 1143–1151, doi:10.1016/j.drudis.2015.05.009.
137. Abdal Dayem, A.; Hossain, M.K.; Lee, S.B.; Kim, K.; Saha, S.K.; Yang, G.M.; Choi, H.Y.; Cho, S.G. The Role of Reactive Oxygen Species (ROS) in the Biological Activities of Metallic Nanoparticles. *Int J Mol Sci* **2017**, *18*, doi:10.3390/ijms18010120.
138. Yu, Z.; Li, Q.; Wang, J.; Yu, Y.; Wang, Y.; Zhou, Q.; Li, P. Reactive Oxygen Species-Related Nanoparticle Toxicity in the Biomedical Field. *Nanoscale Res Lett* **2020**, *15*, 115, doi:10.1186/s11671-020-03344-7.
139. Jeon, M.; Halbert, M. v; Stephen, Z.R.; Zhang, M. Iron Oxide Nanoparticles as T(1) Contrast Agents for Magnetic Resonance Imaging: Fundamentals, Challenges, Applications, and Prospectives. *Adv Mater* **2021**, *33*, e1906539, doi:10.1002/adma.201906539.
140. Sujai, P.T.; Joseph, M.M.; Saranya, G.; Nair, J.B.; Murali, V.P.; Maiti, K.K. Surface Charge Modulates the Internalization vs. Penetration of Gold Nanoparticles: Comprehensive Scrutiny on Monolayer Cancer Cells, Multicellular Spheroids and Solid Tumors by SERS Modality. *Nanoscale* **2020**, *12*, 6971–6975, doi:10.1039/d0nr00809e.
141. Lu, H.; Su, J.; Mamdooh, R.; Li, Y.; Stenzel, M.H. Cellular Uptake of Gold Nanoparticles and Their Movement in 3D Multicellular Tumor Spheroids: Effect of Molecular Weight and Grafting Density of Poly(2-Hydroxyl Ethyl Acrylate). *Macromol Biosci* **2020**, *20*, e1900221, doi:10.1002/mabi.201900221.
142. Bugno, J.; Poellmann, M.J.; Sokolowski, K.; Hsu, H.J.; Kim, D.H.; Hong, S. Tumor Penetration of Sub-10 Nm Nanoparticles: Effect of Dendrimer Properties on Their Penetration in Multicellular Tumor Spheroids. *Nanomedicine* **2019**, *21*, 102059, doi:10.1016/j.nano.2019.102059.

143. Huang, K.; Ma, H.; Liu, J.; Huo, S.; Kumar, A.; Wei, T.; Zhang, X.; Jin, S.; Gan, Y.; Wang, P.C.; et al. Size-Dependent Localization and Penetration of Ultrasmall Gold Nanoparticles in Cancer Cells, Multicellular Spheroids, and Tumors in Vivo. *ACS Nano* **2012**, *6*, 4483–4493, doi:10.1021/nn301282m.
144. Doppalapudi, S.; Jain, A.; Domb, A.J.; Khan, W. Biodegradable Polymers for Targeted Delivery of Anti-Cancer Drugs. *Expert Opin Drug Deliv* **2016**, *13*, 891–909, doi:10.1517/17425247.2016.1156671.
145. Gagliardi, A.; Giuliano, E.; Venkateswararao, E.; Fresta, M.; Bulotta, S.; Awasthi, V.; Cosco, D. Biodegradable Polymeric Nanoparticles for Drug Delivery to Solid Tumors. *Front Pharmacol* **2021**, *12*, 601626, doi:10.3389/fphar.2021.601626.
146. Elbatanony, R.S.; Parvathaneni, V.; Kulkarni, N.S.; Shukla, S.K.; Chauhan, G.; Kunda, N.K.; Gupta, V. Afatinib-Loaded Inhalable PLGA Nanoparticles for Localized Therapy of Non-Small Cell Lung Cancer (NSCLC)-Development and in-Vitro Efficacy. *Drug Deliv Transl Res* **2021**, *11*, 927–943, doi:10.1007/s13346-020-00802-8.
147. Vaidya, B.; Parvathaneni, V.; Kulkarni, N.S.; Shukla, S.K.; Damon, J.K.; Sarode, A.; Kanabar, D.; Garcia, J. v; Mitragotri, S.; Muth, A.; et al. Cyclodextrin Modified Erlotinib Loaded PLGA Nanoparticles for Improved Therapeutic Efficacy against Non-Small Cell Lung Cancer. *Int J Biol Macromol* **2019**, *122*, 338–347, doi:10.1016/j.ijbiomac.2018.10.181.
148. Guo, Y.; Wang, L.; Lv, P.; Zhang, P. Transferrin-Conjugated Doxorubicin-Loaded Lipid-Coated Nanoparticles for the Targeting and Therapy of Lung Cancer. *Oncol Lett* **2015**, *9*, 1065–1072, doi:10.3892/ol.2014.2840.
149. Sen, R.; Ganguly, S.; Ganguly, S.; Debnath, M.C.; Chakraborty, S.; Mukherjee, B.; Chattopadhyay, D. Apigenin-Loaded PLGA-DMSA Nanoparticles: A Novel Strategy to Treat Melanoma Lung Metastasis. *Mol Pharm* **2021**, *18*, 1920–1938, doi:10.1021/acs.molpharmaceut.0c00977.
150. Svenson, S.; Tomalia, D.A. Dendrimers in Biomedical Applications--Reflections on the Field. *Adv Drug Deliv Rev* **2005**, *57*, 2106–2129, doi:10.1016/j.addr.2005.09.018.
151. Saluja, V.; Mishra, Y.; Mishra, V.; Giri, N.; Nayak, P. Dendrimers Based Cancer Nanotheranostics: An Overview. *Int J Pharm* **2021**, *600*, 120485, doi:10.1016/j.ijpharm.2021.120485.
152. Palmerston Mendes, L.; Pan, J.; Torchilin, V.P. Dendrimers as Nanocarriers for Nucleic Acid and Drug Delivery in Cancer Therapy. *Molecules* **2017**, *22*, doi:10.3390/molecules22091401.
153. Jain, K.; Kesharwani, P.; Gupta, U.; Jain, N.K. Dendrimer Toxicity: Let's Meet the Challenge. *Int J Pharm* **2010**, *394*, 122–142, doi:10.1016/j.ijpharm.2010.04.027.
154. Mignani, S.; Rodrigues, J.; Tomas, H.; Zablocka, M.; Shi, X.; Caminade, A.M.; Majoral, J.P. Dendrimers in Combination with Natural Products and Analogues as Anti-Cancer Agents. *Chem Soc Rev* **2018**, *47*, 514–532, doi:10.1039/c7cs00550d.
155. Bugno, J.; Hsu, H.J.; Pearson, R.M.; Noh, H.; Hong, S. Size and Surface Charge of Engineered Poly(Amidoamine) Dendrimers Modulate Tumor Accumulation and Penetration: A Model Study Using Multicellular Tumor Spheroids. *Mol Pharm* **2016**, *13*, 2155–2163, doi:10.1021/acs.molpharmaceut.5b00946.
156. Rompicharla, S.V.K.; Kumari, P.; Bhatt, H.; Ghosh, B.; Biswas, S. Biotin Functionalized PEGylated Poly(Amidoamine) Dendrimer Conjugate for Active Targeting of Paclitaxel in Cancer. *Int J Pharm* **2019**, *557*, 329–341, doi:10.1016/j.ijpharm.2018.12.069.
157. Moço, P.D.; Aharony, N.; Kamen, A. Adeno-Associated Viral Vectors for Homology-Directed Generation of CAR-T Cells. *Biotechnol J* **2020**, *15*, e1900286, doi:10.1002/biot.201900286.
158. Sandro, Q.; Relizani, K.; Benchaouir, R. AAV Production Using Baculovirus Expression Vector System. *Methods Mol Biol* **2019**, *1937*, 91–99, doi:10.1007/978-1-4939-9065-8_5.
159. Luo, J.; Luo, Y.; Sun, J.; Zhou, Y.; Zhang, Y.; Yang, X. Adeno-Associated Virus-Mediated Cancer Gene Therapy: Current Status. *Cancer Lett* **2015**, *356*, 347–356, doi:10.1016/j.canlet.2014.10.045.
160. Chandler, R.J.; Sands, M.S.; Venditti, C.P. Recombinant Adeno-Associated Viral Integration and Genotoxicity: Insights from Animal Models. *Hum Gene Ther* **2017**, *28*, 314–322, doi:10.1089/hum.2017.009.
161. Yanamandra, N.; Kondraganti, S.; Gondi, C.S.; Gujrati, M.; Olivero, W.C.; Dinh, D.H.; Rao, J.S. Recombinant Adeno-Associated Virus (RAAV) Expressing TFPI-2 Inhibits Invasion, Angiogenesis and Tumor Growth in a Human Glioblastoma Cell Line. *Int J Cancer* **2005**, *115*, 998–1005, doi:10.1002/ijc.20965.
162. Thorsen, F.; Afione, S.; Huszthy, P.C.; Tysnes, B.B.; Svendsen, A.; Bjerkvig, R.; Kotin, R.M.; Lønning, P.E.; Hoover, F. Adeno-Associated Virus (AAV) Serotypes 2, 4 and 5 Display Similar Transduction Profiles and Penetrate Solid Tumor Tissue in Models of Human Glioma. *J Gene Med* **2006**, *8*, 1131–1140, doi:10.1002/jgm.939.
163. Lee, S.; Ahn, H.J. Anti-EpCAM-Conjugated Adeno-Associated Virus Serotype 2 for Systemic Delivery of EGFR ShRNA: Its Retargeting and Antitumor Effects on OVCAR3 Ovarian Cancer in Vivo. *Acta Biomater* **2019**, *91*, 258–269, doi:10.1016/j.actbio.2019.04.044.
164. Oliveira, M.S.; Aryasomayajula, B.; Pattni, B.; Mussi, S. v; Ferreira, L.A.M.; Torchilin, V.P. Solid Lipid Nanoparticles Co-Loaded with Doxorubicin and α-Tocopherol Succinate Are Effective against Drug-Resistant Cancer Cells in Monolayer and 3-D Spheroid Cancer Cell Models. *Int J Pharm* **2016**, *512*, 292–300, doi:10.1016/j.ijpharm.2016.08.049.
165. Leiva, M.C.; Ortiz, R.; Contreras-Cáceres, R.; Perazzoli, G.; Mayevych, I.; López-Romero, J.M.; Sarabia, F.; Baeyens, J.M.; Melguizo, C.; Prados, J. Tripalmitin Nanoparticle Formulations Significantly Enhance Paclitaxel Antitumor Activity against Breast and Lung Cancer Cells in Vitro. *Sci Rep* **2017**, *7*, 13506, doi:10.1038/s41598-017-13816-z.

166. Dastidar, D.G.; Das, A.; Datta, S.; Ghosh, S.; Pal, M.; Thakur, N.S.; Banerjee, U.C.; Chakrabarti, G. Paclitaxel-Encapsulated Core-Shell Nanoparticle of Cetyl Alcohol for Active Targeted Delivery through Oral Route. *Nanomedicine (Lond)* **2019**, *14*, 2121–2150, doi:10.2217/nnm-2018-0419.
167. Hinger, D.; Navarro, F.; Käch, A.; Thomann, J.S.; Mittler, F.; Couffin, A.C.; Maake, C. Photoinduced Effects of M-Tetrahydroxyphenylchlorin Loaded Lipid Nanoemulsions on Multicellular Tumor Spheroids. *J Nanobiotechnology* **2016**, *14*, 68, doi:10.1186/s12951-016-0221-x.
168. Lukowski, J.K.; Weaver, E.M.; Hummon, A.B. Analyzing Liposomal Drug Delivery Systems in Three-Dimensional Cell Culture Models Using MALDI Imaging Mass Spectrometry. *Anal Chem* **2017**, *89*, 8453–8458, doi:10.1021/acs.analchem.7b02006.
169. Parvathaneni, V.; Kulkarni, N.S.; Shukla, S.K.; Farrales, P.T.; Kunda, N.K.; Muth, A.; Gupta, V. Systematic Development and Optimization of Inhalable Pirfenidone Liposomes for Non-Small Cell Lung Cancer Treatment. *Pharmaceutics* **2020**, *12*, doi:10.3390/pharmaceutics12030206.
170. Shukla, S.K.; Kulkarni, N.S.; Chan, A.; Parvathaneni, V.; Farrales, P.; Muth, A.; Gupta, V. Metformin-Encapsulated Liposome Delivery System: An Effective Treatment Approach against Breast Cancer. *Pharmaceutics* **2019**, *11*, doi:10.3390/pharmaceutics11110559.
171. Riaz, M.K.; Zhang, X.; Wong, K.H.; Chen, H.; Liu, Q.; Chen, X.; Zhang, G.; Lu, A.; Yang, Z. Pulmonary Delivery of Transferrin Receptors Targeting Peptide Surface-Functionalized Liposomes Augments the Chemotherapeutic Effect of Quercetin in Lung Cancer Therapy. *Int J Nanomedicine* **2019**, *14*, 2879–2902, doi:10.2147/ijn.S192219.
172. Kumari, P.; Rompicharla, S.V.K.; Muddineti, O.S.; Ghosh, B.; Biswas, S. Transferrin-Anchored Poly(Lactide) Based Micelles to Improve Anticancer Activity of Curcumin in Hepatic and Cervical Cancer Cell Monolayers and 3D Spheroids. *Int J Biol Macromol* **2018**, *116*, 1196–1213, doi:10.1016/j.ijbiomac.2018.05.040.
173. Sarisozen, C.; Abouzeid, A.H.; Torchilin, V.P. The Effect of Co-Delivery of Paclitaxel and Curcumin by Transferrin-Targeted PEG-PE-Based Mixed Micelles on Resistant Ovarian Cancer in 3-D Spheroids and in Vivo Tumors. *Eur J Pharm Biopharm* **2014**, *88*, 539–550, doi:10.1016/j.ejpb.2014.07.001.
174. Guo, X.; Wu, Z.; Li, W.; Wang, Z.; Li, Q.; Kong, F.; Zhang, H.; Zhu, X.; Du, Y.P.; Jin, Y.; et al. Appropriate Size of Magnetic Nanoparticles for Various Bioapplications in Cancer Diagnostics and Therapy. *ACS Appl Mater Interfaces* **2016**, *8*, 3092–3106, doi:10.1021/acsami.5b10352.
175. Huo, S.; Ma, H.; Huang, K.; Liu, J.; Wei, T.; Jin, S.; Zhang, J.; He, S.; Liang, X.-J. Superior Penetration and Retention Behavior of 50 Nm Gold Nanoparticles in Tumors. **2013**, *73*, 319–330, doi:10.1158/0008-5472.CAN-12-2071 %J Cancer Research.
176. Becker, A.; Lehrich, T.; Kalies, S.; Heisterkamp, A.; Ngezahayo, A. Parameters for Optoperforation-Induced Killing of Cancer Cells Using Gold Nanoparticles Functionalized With the C-Terminal Fragment of Clostridium Perfringens Enterotoxin. *Int J Mol Sci* **2019**, *20*, doi:10.3390/ijms20174248.
177. Katifelis, H.; Lyberopoulou, A.; Mukha, I.; Vityuk, N.; Grodzyuk, G.; Theodoropoulos, G.E.; Efstathopoulos, E.P.; Gazouli, M. Ag/Au Bimetallic Nanoparticles Induce Apoptosis in Human Cancer Cell Lines via P53, CASPASE-3 and BAX/BCL-2 Pathways. *Artif Cells Nanomed Biotechnol* **2018**, *46*, S389-s398, doi:10.1080/21691401.2018.1495645.
178. Li, H.; Li, Y.; Xiang, J.; Yang, X.; Li, C.; Liu, C.; Zhao, Q.; Zhou, L.; Gong, P.; Huang, J. Intelligent Bimetallic Nanoagents as Reactive Oxygen Species Initiator System for Effective Combination Phototherapy. **2020**, *8*, doi:10.3389/fbioe.2020.00423.
179. Zhong, Y.; Su, T.; Shi, Q.; Feng, Y.; Tao, Z.; Huang, Q.; Li, L.; Hu, L.; Li, S.; Tan, H.; et al. Co-Administration Of IRGD Enhances Tumor-Targeted Delivery And Anti-Tumor Effects Of Paclitaxel-Loaded PLGA Nanoparticles For Colorectal Cancer Treatment. *Int J Nanomedicine* **2019**, *14*, 8543–8560, doi:10.2147/ijn.S219820.
180. Younis, M.; Faming, W.; Hongyan, Z.; Mengmeng, T.; Hang, S.; Liudi, Y. Iguratimod Encapsulated PLGA-NPs Improves Therapeutic Outcome in Glioma, Glioma Stem-like Cells and Temozolomide Resistant Glioma Cells. *Nanomedicine* **2019**, *22*, 102101, doi:10.1016/j.nano.2019.102101.
181. Lazzari, G.; Vinciguerra, D.; Balasso, A.; Nicolas, V.; Goudin, N.; Garfa-Traore, M.; Fehér, A.; Dinnyés, A.; Nicolas, J.; Couvreur, P.; et al. Light Sheet Fluorescence Microscopy versus Confocal Microscopy: In Quest of a Suitable Tool to Assess Drug and Nanomedicine Penetration into Multicellular Tumor Spheroids. *Eur J Pharm Biopharm* **2019**, *142*, 195–203, doi:10.1016/j.ejpb.2019.06.019.
182. Al-Jamal, K.T.; Al-Jamal, W.T.; Wang, J.T.; Rubio, N.; Buddle, J.; Gathercole, D.; Zloh, M.; Kostarelos, K. Cationic Poly-L-Lysine Dendrimer Complexes Doxorubicin and Delays Tumor Growth in Vitro and in Vivo. *ACS Nano* **2013**, *7*, 1905–1917, doi:10.1021/nn305860k.
183. Cui, X.; Ma, C.; Vasudevaraja, V.; Serrano, J.; Tong, J.; Peng, Y.; Delorenzo, M.; Shen, G.; Frenster, J.; Morales, R.T.; et al. Dissecting the Immunosuppressive Tumor Microenvironments in Glioblastoma-on-a-Chip for Optimized PD-1 Immunotherapy. *Elife* **2020**, *9*, doi:10.7554/eLife.52253.
184. Kwak, T.J.; Lee, E. In Vitro Modeling of Solid Tumor Interactions with Perfused Blood Vessels. *Sci Rep* **2020**, *10*, 20142, doi:10.1038/s41598-020-77180-1.
185. Peirsman, A.; Blondeel, E.; Ahmed, T.; Anckaert, J.; Audenaert, D.; Boterberg, T.; Buzas, K.; Carragher, N.; Castellani, G.; Castro, F.; et al. MISpheroID: A Knowledgebase and Transparency Tool for Minimum Information in Spheroid Identity. *Nature Methods* **2021**, *18*, 1294–1303, doi:10.1038/s41592-021-01291-4.