Biological evaluations of nanocarriers to improve the effectiveness of colorectal cancer treatment

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CHAPTER
FIVE

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

The field of nanomedicine is constantly improving due to its use of modern techniques. With these techniques, nanostructures with pharmacokinetics and pharmacodynamics suitable for treating various diseases, including cancer are formulated. It is important to consider the heterogeneity of primary tumors for cancer treatment to be effective. Tumor heterogeneity favors tumor tissue to survive and resist drugs, leading to the failure of chemotherapeutic agents to induce a therapeutic response. In addition, the absorption mechanisms, metabolism and excretion of chemotherapeutic drugs, which are commonly used for cancer patients and the lack of specific targeting of these drugs can cause adverse effects on treated patients. Thus, the general objective of this thesis is to investigate the biological activity of targeted poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) as a drug delivery system (DDS) for carvedilol (CVDL) or oxaliplatin (OXA), *in vitro* and *in vivo*, to treat colorectal cancer (CRC). DDSs were formulated to achieve this goal. Subsequently, the formulations were characterized in order to obtain information about their size, shape, encapsulation rate and zeta potential. As a common result of this thesis, all formulations showed a spherical shape and smooth surface. Inflammation studies were performed, since the progression of CRC is related to the induction of a chronic and recurrent inflammatory process (Chapter 2). In Chapter 2, nanoparticles functionalized with cholesterol (CHO) decreased leukocyte migration when compared to free CVDL when used at the same concentration. Furthermore, the nanoformulations showed similar activities when used at lower doses than CVDL, at higher concentration, in relation to malondialdehyde (MDA) and glutathione (GSH) levels. *In vitro* studies, such as analysis of cell viability and cell death were carried out in order to analyze the antitumor activity of the systems (Chapters 2, 3 and 4). The *in vitro* results showed that the PLGA polymer is non-toxic, and that the use of NPs increased the drug's effectiveness against tumor cells. Tests to evaluate the efficiency of targeting the NPs by means of CHO or folic acid (FA) were executed to prove that there is an increase in the targeting efficiency of the systems for CT-26 murine CRC cells (Chapters 3 and 4). These tests showed that targeted NPs bind to the cell surface and internalize in the cell in greater quantity than NPs without the target. Finally, animal models were developed to study apoptosis and resistance to drugs as well as metastases (Chapters 3 and 4). The results of an animal study confirmed our *in vitro* results, showing that the use of

NPs reduced tumor volume and acted on tumor modulation. In chapters 2, 3 and 4, our studies were discussed in detail on the formulations and characterizations of NPs as DDSs with ideal characteristics to increase the therapeutic range of drugs at the tumor site. As well as the biological evaluation of these DDS when its anti-inflammatory activity (Chapter 2) and its antitumor activity *in vitro* (Chapters 2, 3 and 4) and *in vivo* (Chapters 3 and 4). Taken together, all the DDSs studied in this thesis were able to improve the chemotherapeutic efficiency of the drugs studied in Chapters 2, 3 and 4.

MAIN OUTCOMES AND RESPECTIVE IMPLICATIONS

Objective 1: Formulating nanoparticles with satisfactory physico-chemical characteristics used as a drug delivery system

The development of nanoparticles (NPs) consisting of biodegradable materials with surface functionalization enables advancement towards achieving new treatment strategies in medicine. When used as a DDS, NPs and other colloidal systems have the ability to modify the kinetics, body distribution and release of drugs incorporated into these systems [1]. Thus, nanoparticles were formulated to achieve this goal, and were subsequently characterized in terms of size, shape, potential (surface) charge and encapsulation rate.

For this thesis, NPs consisting of polyethylene glycol (PEG) and poly (lactic-co-glycolic acid) (PLGA) were formulated. These PEG-PLGA-NPs were loaded with OXA and retinoic acid (RA) or CVDL and functionalized with CHO or FA in order to increase the therapeutic efficacy of the drugs encapsulated in the DDS [2-4]. The resulting DDS formulations had spherical shape, smooth surfaces and uniform size distribution. The morphology of the NPs influenced both pharmacokinetics and cell absorption [5]. Therefore, the spherical form of our NPs played an important role in reducing cytotoxicity compared to other forms [6]. Studies using NPs for the treatment of breast cancer, CRC and glioblastomas show that the most effective formulations have a spherical shape [7-9].

Due to the satisfactory volume, surface and size of the NPs, delivery of the encapsulated drugs is optimized and there is an increase in their pharma-

cological activity [10-12]. The therapeutic content has to be released in a controlled manner within the tumor microenvironment (TME) in order to achieve the objective of using nanoformulations in cancer treatment [13]. Several studies have shown the importance of encapsulation efficiency in formulating NPs related to the optimization of cell uptake, suggesting improved therapeutic efficacy and reduced cell toxicity [14, 15]. The encapsulation rates for this thesis (Chapter 2, 3 and 4) were high, which led to an increase in antitumor activity of the encapsulated/loaded drugs in comparison to the free drugs. [16, 17]. Also, a reduction in the inflammatory activity (Chapter 2) of the encapsulated drug in relation to the free drug was also observed for NPs containing CVDL. This shows the importance of using DDSs to fight cancer [18, 19].

Higher encapsulation rates of the drugs are desirable, especially for application of *in vivo* studies, since a solution with a higher concentration is needed in a small volume to be injected during animal experiments. However, when the encapsulation efficiency is low, it is necessary to have a greater number of NPs within a small volume to obtain a therapeutic concentration. This can create a viscous solution, which makes it difficult to solubilize the NPs and consequently makes administration *in vivo* difficult.

Regarding the zeta potential values, previous studies have determined that relative values with variation in the range between 0 mV and ± 5 mV indicate increased instability of NPs, and consequently rapid aggregation. However, values of ± 30 mV imply greater stability for the colloidal system [20]. The results of the present thesis (Chapters 3 and 4) show that the zeta potentials of the nanoparticulate formulations ranged from -20 mV to -29.6 mV. Thus, our values indicate that the molecules of the PLGA-NP groups were stable and they had reduced cytotoxic effects[21]. This supports other studies which had similar zeta potentials for their NPs used as DDS against cancer [7, 22].

In contrast, the results obtained in Chapter 2 for NPs with CVDL and CVDL-CHOL were not within this range. The zeta potential of these NPs was -2.04 mV and -2.08 mV, respectively. However, no problems were observed regarding the stability of these formulations in stability studies; the samples remained stable for up to seven weeks. There was stability because these nanoparticles did not undergo the lyophilization process.

They were maintained in colloidal solution right from when they were formulated, characterized until the experiments were conducted.

Another physicochemical characteristic that requires attention is the size of our NPs, as it is strongly associated with cell uptake, biodistribution and the half-life of the NPs in circulation [23]. Thus, the smaller the NPs, the faster would the body absorb, metabolize and excrete them [24]. However, very small sizes are not desirable, since NPs between 14 and 74 nm show a half-life between 2 and 24 hours [25]; while sizes larger than 200 nm are quickly eliminated by the spleen [26].

The average sizes of the NPs in this study were around 234 nm in diameter (formulation of Chapter 2), 400 nm (formulation of Chapter 3) and 197 nm (formulation of Chapter 4). The noticeable difference between the nanoparticle sizes used in Chapter 3 and those in Chapters 2 and 4 is due to the number of components incorporated in them. Besides OXA, RA was also added to these formulations, which resulted in a larger diameter of the nanoparticles. Such results directly influence the circulation time of the nanoformulations, the size-dependent biodistribution, the penetration into target cells and the elimination from tumor tissue, which lead to improved anticancer efficacy. The uptake and elimination of the NPs can be compromised when nanoparticles are very small due to accelerated elimination, hindering the EPR effect [27]. Therefore, the average dimensions achieved by the nanoformulations in this study led to improved EPR effect, inducing efficient cancer treatment, *in vitro* and *in vivo* [28].

Additionally, our results can contribute to developing nanoscale therapeutic systems and provide information on nanotoxicity in the treatment of CRC. This may result in better prognosis, due to a more specific treatment and fewer side effects of the anticancer drug.

Objective 2: Increasing the efficiency of drugs when encapsulated in a drug delivery system

Conventional chemotherapy is often associated with low solubility and limited tumor targeting. These unbeneficial treatment characteristics prevent the drug from efficiently reaching the tumor and furthermore, it leads to side effects [29]. Incorporating drugs within functionalized NPs can be helpful to solve these disadvantages of chemotherapy. It reduces

non-specific dissemination of the drug and thereby reduces side effects [30]. It increases the efficiency of the drug and circumvents cancer cells from resisting drugs [31, 32].

The NPs formulated in Chapters 2, 3 and 4 were studied to prove that there was an increase in the efficiency of drugs when they were encapsulated in a system in order to achieve a more specific treatment. We decided to use the polymer PLGA for the formulation of polymeric NPs because it stands out among the DDS formulations for cancer. This is due to its biodegradability and biocompatibility, which makes it a safe candidate as DDS [33, 34]. The results obtained in our study did not show antiproliferative activity when the cells were treated with empty PLGA NPs. This can be seen in the results of the viability assays (MTS) where the empty nanoparticles did not show cytotoxic activity. Our data confirmed the results of other studies and proved that PLGA is an efficient DDS for different active components. In addition, the hydrolysis of PLGA leads to lactic acid and glycolic acid monomers that are endogenous and easily metabolized by the body via the Krebs cycle, with minimal associated systemic toxicity [34, 35].

In this study, the release time of free drugs (CVDL and OXA) was compared with the same drugs encapsulated in NPs. A slow, gradual and progressive release was observed in the nanosystems over time when compared to the free drugs. This increased the efficiency of the drugs in the nanoparticulate systems [8, 36].

The therapeutic efficacy of the drugs CVDL and OXA was also assessed using cell viability and flow cytometry techniques; also, immunofluorescence was used for OXA. These techniques aim to quantify the proliferation and death rates of cells, which will occur if the drug is delivered successfully and in appropriate concentrations to act on these cellular processes. Our results show that the encapsulated drugs are capable of maximizing the therapeutic activity induced by the free drug when it is in a DDS [37, 38]. This maximization of the drug's effect when encapsulated in a DDS was confirmed by our results when the cells were treated with our NPs and compared to free drugs (OXA and CVDL) [39, 40].

Cancer cells that do not undergo apoptosis end up proliferating more than normal, resulting in a malignant tumor process [41]. Therefore,

studying the mechanisms, which initiate apoptosis is extremely important to stop carcinogenesis. Studies showed that there is a correlation between improved chemotherapy results and reduced BCL-2 expression [42]. Furthermore, our results showed that small concentrations of OXA encapsulated in a nanoparticulate DDS induced apoptosis at the same way as when the free OXA is used in higher concentrations. The use of drugs in the system lead to lower side effects and reduction in drug resistance rates, since the drug will be released into the system only at the tumor site [22, 43].

Apoptosis induction was investigated in our study to prove that our DDS increased the efficiency of free drug in the treatment of CRC. The data show low expression of the anti-apoptotic molecule BCL-2, and greater expression of caspase 3, FADD and APAF-1. This proves there is greater induction of the extrinsic and intrinsic pathways of apoptosis in tumor cells when treated with DDS containing OXA compared to the free drug. However, further investigations need to be done in the future in order to understand which apoptosis pathway is triggered the most.

In addition to all of the antitumor activity described, synergy activity was observed when RA and OXA were internalized together in nanoparticles. There was an improvement in the antitumor activity of OXA, showing the occurrence of synergy between them. Previous studies have shown that RA induces apoptosis, thus corroborating the data presented in this study [44, 45].

All the findings of this study together with data from the literature endorse the importance of studying the area of DDSs in order to improve the existing treatment for CRC.

Objective 3: Proving the efficiency of the target by increasing the binding and internalization of the nanoparticles in the cancer cell

Since the targets of many therapeutic agents are usually located in intracellular compartments, superficial modulation of NPs is necessary to facilitate their binding and internalization by target cells. A detailed study of the interactions between NPs and target cells is relevant to enable efficient cell adsorption or endocytosis across the plasma membrane [46]. Targeted therapies are ideal to be used as DDS, since its therapeutic

content will only be released at the tumor site [47]. Therefore, the functionalization of NPs in such a way that they directly interact with cancer cells is crucial to assess the effects of NPs and their toxicity.

Cancer cells demand an intense biosynthesis of CHO and an abundant supply of reduced folate (the main functional form of FA), for there to be intense neoplastic proliferation [48, 49]. The metabolic dependence of CHO and FA cancer cells is related to the construction of new plasma membranes and use for nucleotide biosynthesis, respectively, which are indispensable for maintaining cell growth. Such nutritional requirements of the tumor are reflected by the increased attraction and endocytosis of molecules, such as CHO and FA [48-51].

The drugs in the present study were delivered through active targeting, in which CHO (Chapter 2 and 3) or FA (Chapter 4) was added to the surface of the NPs. All functionalized NPs showed a higher internalization or binding rate. They also had greater antitumor activity when compared to nanoparticles without active targeting. CHO is a widely explored targeting moiety for DDSs for cancer, and our data confirm the results of other studies which indicate the contributory role of CHOL in the internalization of DDDs through endocytosis [52, 53]. Better internalization directly reflects greater availability of the drug in the cell interior, leading to maximization of the antitumor effect of the drug [54, 55].

The successful delivery of functionalized systems was also achieved when FA was used as a targeting moiety. Thus, two techniques were studied in our DDS: binding and uptake through the fluorescence analysis of the dye added inside the nanoparticles. We chose to use FA because CRC cells show an overexpression of FA receptors, which makes FA a promising targeting moiety for this type of cancer [56]. In these studies, we observed an improved internalization of the DDSs. Both the binding of the DDSs at the surface receptors and their internalization inside the cell were confirmed by an increase of the fluorescence signal of the incorporated dye as well as the fluorescence signal obtained from immunocytochemistry [56]. The fluorescence signal for nanoparticles without the targeting moiety was weaker than the fluorescence signal of the targeted nanoparticles. These data, together with the increase in cell death, confirm that functionalized NPs are more efficient than the free drug. [57].

The increased delivery efficiency of OXA and CVDL to the tumor site by DDSs functionalized with CHO and FA resulted in reduced proliferation and increased tumor cell mortality rates, demonstrating the therapeutic importance of these systems.

Objective 4: Modulating tumour progression when using treatment with nanoparticulate systems

Tumor progression includes mechanisms which involve genetic, biochemical and phenotypic changes used by neoplastic cells to grow, proliferate, survive, invade and metastasize [58, 59]. By acquiring the ability to survive, cancer cells in the TME gain new characteristics of escape from the immune system as well as apoptosis, which results in failed response to drugs [58, 59].

For the *in vivo* studies of this thesis related to tumor progression, we subcutaneously inoculated CT-26 cells into the right flank of Balb/c mice [60]. This cell line originates from fibroblasts and is an undifferentiated strain of colon carcinoma induced by N-nitrous-N-methylurethane (NNMU). To obtain these cells, the CT-26 WT strain was transduced with the retroviral vector LXSN, which contained the lacZ gene and encoded the tumor-associated model antigen (TAA) as well as beta-galactosidase (beta-gal) to generate the lethal CT-26 subclone gene [61]. A lethal tumor rapidly develops when the CT-26 cell line is inoculated subcutaneously in Balb/c mice. This results in a rapidly growing grade IV carcinoma, which is easily implanted and promptly metastasizes. Therefore, these characteristics were the reason for choosing this strain for our study, this strain is one of the most used in drug development, as it shares molecular characteristics with aggressive, undifferentiated and refractory cells of human colorectal carcinomas [62]. After the inoculation of CT-26 cells, we expected the tumor volume to reach 3-4 mm. The animals were randomly divided into four groups based on the treatment received: saline, free OXA, NP-OXA and NP-OXA functionalized. [60]. Subsequently, NPs were peritumorally injected to treat the mice. This treatment route was chosen because the regions adjacent to the tumor ensured complete absorption of the formulation by the tumor, avoiding losses by metabolism in the liver and losses due to kidney clearance. In addition, the route was chosen because the molecules and cells of the immune system were similar to the tumor mass. Previous studies have shown responses to treatments

using this route [63-65]. Other studies quantitatively analyzed the tumor and its surroundings in order to obtain relevant data regarding the biological characteristics of the cancer and the probability of response to targeted therapy [66]. Information on the peritumoral immune response could be used to predict the impact on the intratumoral environment. Treatment via the peritumoral environment can predict the safety of DDDs, like the ones used in this thesis [63]. However, this administration route of the NPs could become a limitation for a clinical application, since in general systemic administration routes are used to administer drugs, since metastases are not often located superficially and are in most cases disseminated all over the body. Therefore, it is necessary to develop studies where the administration route of NPs is systematic so that our formulations can be translated to clinical settings. Future studies are required, which analyze the interaction of NPs with barriers in the human body that will be faced after systemic administration.

Our *in vivo* study covered the following topics: a) inflammation, b) survival and proliferation, c) apoptosis, d) drug resistance and e) metastasis. Next, we will point out the main conclusion of each subitem.

a. Inflammation

Tumors are known as “wounds that do not heal” (Hal Dvorak, 1986), due to the immunosuppressive characteristics of the TME [67]. The onset of CRC progression is triggered by persistent chronic inflammation, passing through stages, which include the formation of aberrant polyps, adenomas and carcinomas. Subsequent mutations activate signaling pathways and pro-inflammatory transcription factors, promoting the release of inflammatory cytokines [68-70]. The immune system plays a crucial role in gastrointestinal tract health, intervening during infections. However, the recurrence of the presence of pro-inflammatory molecules at the site causes damage to the intestinal mucosa, which leads to tumor progression [68-70].

Based on the results found in Chapter 2, it was possible to observe a reduction in the global leukocyte count in the peritonitis model induced from treatment with nanocomposites containing CVDL and CHO. Thus, the treatment proved to be efficient in reducing the inflammatory response present in peritonitis. In addition, reduced levels of

malondialdehyde (MDA), glutathione peroxidase (GSH) and leukocyte migration were observed. It does not only reduce inflammation, but also reduces oxidative stress.

MDA is an indicator of the presence of oxidative stress, since MDA is the final product of lipid peroxidation. MDA levels in cancer patients are higher compared to individuals without cancer [71]. While, the GSH marker is an antioxidant enzyme, which eliminates reactive species under physiological conditions. The glutathione metabolism is disrupted in cancer, promoting tumor progression and therapeutic resistance, because the genes involved in using GSH are controlled by classic tumorigenic pathways, such as the hypoxia-inducible factor 1 (HIF1) pathway that activates GSH synthesis in a hypoxic situation and shows a greater amount of stem cells in the tumor tissue after chemotherapy in breast cancer [72, 73].

Oxidative stress occurs when ROS levels replace antioxidant defense mechanisms. Many studies have demonstrated a correlation between tumor progression and oxidative stress, since excess ROS can cause damage to genomic and mitochondrial DNA. This results in damage to DNA, mutation of several molecules and also changes in signaling pathways, promoting tumor initiation and progression [74, 75]. Therefore, the use of an anti-inflammatory drug encapsulated in NPs can decrease the oxidative stress of the tumor, thus preventing tumor progression [76-78].

b. Survival and proliferation

The recurrence and persistence of inflammation at the same location in the intestine leads to cellular neoformations, and therefore changes in the immune system cell phenotype pattern in the TME. Such a change leads to loss of immune surveillance over the excessive proliferation of neoplastic cells, which induces tumor growth [79]. Survival and proliferation markers, such as SURVIVIN and Ki-67, respectively, were measured in the tumor mass (Chapters 3 and 4), and their reduced expression was observed during treatment of human CRC cell lines with our DDSs. SURVIVIN and Ki-67 are described in literature as overexpressed in cancer, promoting activation of the cell cycle and inhibiting apoptosis in cancer cells [80, 81]. We hypothesized that the groups of animals

used for our *in vivo* studies treated with our DDSs show a reduction in tumor weight and a decline in tumor growth, when compared to treatment groups treated with free OXA. The hypothesis was confirmed, since a reduction in proliferation markers and an induction of apoptosis was observed.

c. Apoptosis

Cancer cells acquire the ability to overcome programmed cell death, activating anti-apoptotic machinery molecules and inhibiting pro-apoptotic molecules [82]. The escape mechanisms used by the cells can contemplate deregulation in both the intrinsic and extrinsic pathways [83]. As the avoidance of apoptosis by cancer cells is a major problem, apoptosis activation plays an important role in the treatment of cancer [84]. In analyzing our results, it was possible to verify the reduction of BCL-2 as an anti-apoptotic marker, and the increase in caspase-3 and caspase-8 as pro-apoptotic markers, including an increase of intrinsic (APAF-1) and extrinsic (Fas, FADD) pathway markers. RA can molecularly interact with nuclear receptors (heterodimers of the retinoic acid receptor and the retinoid X receptor), which can inhibit cell cycle-promoting proteins, such as p27, and can activate regulatory proteins in the cell cycle, such as Cdk5 [85]. Thus, RA as part of the nanosystem can lead to growth inhibition and apoptosis in tumor cells [86, 87]. The results of Chapter 3 indicate the effectiveness of the treatment with our DDS, in which cell death was induced either by direct action of RA, which promotes the regulations of proteins in the apoptotic pathway, or by indirect action stimulated by targeted nanoparticles containing FA and CHO, which favors binding, internalization and containment of the drug [45, 56]. The activation of apoptosis by the nanoparticulate complex was the objective outlined and achieved by the studies performed in Chapter 3.

d. Drug resistance

The tolerance to drugs developed in cancer can originate from mutations in DNA and metabolic alterations that promote inhibition,

degradation or efflux of the drug. In drug resistant cells, the mechanisms used by chemotherapeutics are modified in such a way that changes in the drug's target are generated, which activates pro-survival pathways and render cell death induction approaches unusable [88, 89].

The multiple resistance gene (MDR-1) is amplified in the drug resistance process developed by tumor cells [90-92]. In the results obtained by the Chapter 3 and 4 it was possible to visualize reduced expression of the MDR-1 gene when treatment using nanoparticulate systems was compared with treatment with free OXA. When such results are associated with reduced SURVIVIN labeling after treatment, attenuation of drug resistance is observed along with the consequent inhibition of neoplastic cell survival. This indicates greater susceptibility of the cells to the treatment with OXA encapsulated with PLGA, FA or CHOL, and RA. This increases the therapeutic potential through modulating MDR, which confirms the efficiency of the target system by promoting the endocytosis of the DDS [93-96].

In addition, some recent studies demonstrate there is a direct correlation between the epithelial-mesenchymal transition (EMT) process and drug resistance. EMT is a process present in tumor progression, whereby epithelial cells can convert into a mesenchymal phenotype. This transition can culminate in metastasis, generating tumor cells with stem cell properties that play an important role in resistance to cancer treatment [97]. Thus, by reversing the tumor's drug resistance, it would be possible to hamper the EMT process, delay metastases, and therefore improve patients' prognosis [98, 99].

e. Metastasis

Invasiveness, migration and metastasis are inherent characteristics to tumor progression, directly related to tumor aggressiveness [100]. The metastatic cascade involves tumor cells detaching from the primary tumor, entering into the circulatory and lymphatic systems, preventing the immune attack, and finally reaching distant capillary vessels, invading and proliferating in distant organs [101, 102].

According to the results obtained of our study described in Chapter

3 and 4, there was a reduction in the expression of C-X-C chemokine receptor type 4 (CXCR4) and monocyte-derived chemokine (CCL22) in the tumor after treatments with NP-OXA functionalized with CHOL and RA. Furthermore, in our CRC xenograft *in vivo* model, the treatments with NP-OXA and FA only demonstrated the relevant expression reduction profile for CCL22 when compared to the control groups in *in vivo* experiment. The results showed that the targeted nanoconjugates were able to control inflammatory cytokines in the TME and were able to suppress tumor progression [103, 104].

Tumor progression is composed of a series of interrelated steps, sequential or not, which lead to tumor extension and tumor spread. Many authors study tumor metastatic potential and report the association with prognosis; they highlight the importance of developing new treatment strategies, which can prevent or control this process [105, 106].

Following this line of research, it was extremely important to analyze the action of nanoconjugates functioning as possible proliferation and survival blockers, activators of apoptosis, inhibitors of drug resistance, and finally inhibitors of the metastatic cascade [107, 108]. All of these behavioral responses observed in neoplastic cells occurred due to the successful encapsulation of drugs in nanoparticulate systems, resulting in improved internalization. This approach has increased the effectiveness of treatment for CRC when drugs are encapsulated in PLGA systems. [109-111].

FUTURE PERSPECTIVES

In the past decades, important advances have been made in the use of nanomedicine to improve cancer treatment. A variety of methods have been applied to design efficient DDSs, to improve the efficacy of the drug, decrease its associated side effects, and counteract drug resistance.

To take the next steps forward, future experiments should be conducted in order to formulate nanoparticulate systems with smaller sizes with regard to the NPs studied in Chapter 3 and with greater encapsulation efficiency with regard to the NPs studied in Chapters 3 and 4. Better pharmacokinetics can be achieved by obtaining NPs with more suitable sizes, and consequently fewer toxic effects.

The results obtained with the target moiety FA showed great potential, but additional studies should be carried out using free FA to evaluate the competition between free FA and bound FA, which is located on the NP surface. It is important to study this competition in order to strengthen our hypothesis.

The use of another animal model may also add more value to this research and answer questions, which have not been answered herein, such as microscopic analysis of animal organs in order to search for possible metastases in the treated and control groups. The use of specific markers will be useful to identify the intracellular pathway(s) activated during the treatment with DDSs.

In addition, the use of 3D solid tumor models can be a promising approach in the search for more information, regarding tumor growth, differentiation, cell structure and the TME. Studies focused on organoids seek to create a 3D culture which can approach *in vivo* conditions in order to obtain relevant results similar to the human physiology. Furthermore, orthotopic animal models could be used, which are based on tumor cell line implants or tumor cell xenografts derived from patients, in order to create a situation which is very close to human physiology.

REFERENCES

[1] S. Parveen, R. Misra, S.K. Sahoo, Nanomedicine : nanotechnology, biology, and medicine, 8 (2012) 147-166.
[2] S.P. Chandran, S.B. Natarajan, S. Chandraseharan, M.S.B. Mohd Shahimi, Journal of Cancer Research and Practice, 4 (2017) 45-48.
[3] A. Gulbake, A. Jain, A. Jain, A. Jain, S.K. Jain, World J Gastroenterol, 22 (2016) 582-599.
[4] C.-S. Lee, H. Kim, J. Yu, S.H. Yu, S. Ban, S. Oh, D. Jeong, J. Im, M.J. Baek, T.H. Kim, European Journal of Medicinal Chemistry, 142 (2017) 416-423.
[5] N.P. Truong, M.R. Whittaker, C.W. Mak, T.P. Davis, Expert Opin Drug Deliv, 12 (2015) 129-142.
[6] B. Zhang, P. Sai Lung, S. Zhao, Z. Chu, W. Chrzanowski, Q. Li, Scientific reports, 7 (2017) 7315.
[7] F. Madani, S.S. Esnaashari, M.C. Bergonzi, T.J. Webster, H.M. Younes, M. Khosravani, M. Adabi, Life Sciences, 256 (2020) 117943.
[8] S.A. Sufi, M. Hoda, S. Pajaniradje, V. Mukherjee, S.M. Coumar, R. Rajagopalan, International journal of pharmaceutics, 588 (2020) 119738.
[9] E. Akbari, H. Mousazadeh, Z. Sabet, T. Fattahi, A. Dehnad, A. Akbarzadeh, E. Alizadeh, Journal of Drug Delivery Science and Technology, 61 (2021) 102294.
[10] M. Caldorera-Moore, N. Guimard, L. Shi, K. Roy, Expert Opin Drug Deliv, 7 (2010) 479-495.
[11] S. Venkataraman, J.L. Hedrick, Z.Y. Ong, C. Yang, P.L.R. Ee, P.T. Hammond, Y.Y. Yang, Advanced Drug Delivery Reviews, 63 (2011) 1228-1246.
[12] L. Guerrini, R.A. Alvarez-Puebla, N. Pazos-Perez, 11 (2018) 1154.
[13] A. Rajendra, R. Ariane, H. Philip Michael, R. Michael John, D. Kamal, B. Mary, Current Drug Targets, 19 (2018) 1696-1709.
[14] T. Sadhukha, S. Prabha, AAPS PharmSciTech, 15 (2014) 1029-1038.
[15] X. Gu, Y. Wei, Q. Fan, H. Sun, R. Cheng, Z. Zhong, C. Deng, Journal of Controlled Release, 301 (2019) 110-118.
[16] X. Montané, A. Bajek, K. Roszkowski, J.M. Montornés, M. Giamberini, S. Roszkowski, O. Kowalczyk, R. Garcia-Valls, B. Tylkowski, Molecules, 25 (2020) 1605.
[17] C. Deng, Q. Zhang, J. Guo, X. Zhao, Z. Zhong, Advanced Drug Delivery Reviews, 160 (2020) 199-211.
[18] S. Alqahtani, L. Simon, C.E. Astete, A. Alayoubi, P.W. Sylvester, S. Nazzal, Y. Shen, Z. Xu, A. Kaddoumi, C.M. Sabliov, Journal of Colloid and Interface Science, 445 (2015) 243-251.
[19] M.C. Pereira, D.A. Oliveira, L.E. Hill, R.C. Zambiazi, C.D. Borges, M. Vizzotto, S. Mertens-Talcott, S. Talcott, C.L. Gomes, Food Chemistry, 240 (2018) 396-404.
[20] Q. Abbas, B. Yousaf, Amina, M.U. Ali, M.A.M. Munir, A. El-Naggar, J. Rinklebe, M. Naushad, Environment International, 138 (2020) 105646.
[21] Y. Zhang, M. Yang, N.G. Portney, D. Cui, G. Budak, E. Ozbay, M. Ozkan, C.S. Ozkan, Biomedical Microdevices, 10 (2008) 321-328.
[22] S. Ghosh, S. Dutta, A. Sarkar, M. Kundu, P.C. Sil, Colloids and Surfaces B: Biointerfaces, 197 (2021) 111404.
[23] N. Hoshyar, S. Gray, H. Han, G. Bao, Nanomedicine (Lond), 11 (2016) 673-692.
[24] M.J. Ernsting, M. Murakami, A. Roy, S.D. Li, Journal of controlled release : official journal of the Controlled Release Society, 172 (2013) 782-794.
[25] B.D. Chithrani, A.A. Ghazani, W.C.W. Chan, Nano Letters, 6 (2006) 662-668.
[26] T. Sun, Y.S. Zhang, B. Pang, D.C. Hyun, M. Yang, Y. Xia, Angewandte Chemie (International ed. in English), 53 (2014) 12320-12364.
[27] L. Tang, X. Yang, Q. Yin, K. Cai, H. Wang, I. Chaudhury, C. Yao, Q. Zhou, M. Kwon, J.A. Hartman, I.T. Dobrucki, L.W. Dobrucki, L.B. Borst, S. Lezmi, W.G. Helferich, A.L. Ferguson, T.M. Fan, J. Cheng, 111 (2014) 15344-15349.

[28] H. Maeda, Bioconjug Chem, 21 (2010) 797-802.
[29] A. Ediriwickrema, W.M. Saltzman, ACS Biomaterials Science & Engineering, 1 (2015) 64-78.
[30] J. Cassidy, J.-L. Misset, Seminars in Oncology, 29 (2002) 11-20.
[31] Y. Zhong, T. Su, Q. Shi, Y. Feng, Z. Tao, Q. Huang, L. Li, L. Hu, S. Li, H. Tan, S. Liu, H. Yang, International journal of nanomedicine, 14 (2019) 8543-8560.
[32] A. Tiwari, S. Saraf, A. Jain, P.K. Panda, A. Verma, S.K. Jain, Drug Delivery and Translational Research, 10 (2020) 319-338.
[33] D.N. Kapoor, A. Bhatia, R. Kaur, R. Sharma, G. Kaur, S. Dhawan, Therapeutic Delivery, 6 (2015) 41-58.
[34] F. Sadat Tabatabaei Mirakabad, K. Nejati-Koshki, A. Akbarzadeh, M.R. Yamchi, M. Milani, N. Zarghami, V. Zeighamian, A. Rahimzadeh, S. Alimohammadi, Y. Hanifehpour, S.W. Joo, Asian Pacific journal of cancer prevention : APJCP, 15 (2014) 517-535.
[35] A. Kumari, S.K. Yadav, S.C. Yadav, Colloids and Surfaces B: Biointerfaces, 75 (2010) 1-18.
[36] B. Layek, J. Singh, International journal of molecular sciences, 20 (2019).
[37] L. Frungillo, D. Martins, S. Teixeira, M.C. Anazetti, P.d.S. Melo, N. Durán, Journal of Pharmaceutical Sciences, 98 (2009) 4796-4807.
[38] N. Vilaça, A.R. Bertão, E.A. Prasetyanto, S. Granja, M. Costa, R. Fernandes, F. Figueiredo, A.M. Fonseca, L. De Cola, F. Baltazar, I.C. Neves, Materials Science and Engineering: C, (2020) 111721.
[39] Y. Xu, Y. Zi, J. Lei, X. Mo, Z. Shao, Y. Wu, Y. Tian, D. Li, C. Mu, Carbohydrate polymers, 233 (2020) 115858.
[40] M. Kundu, S. Chatterjee, N. Ghosh, P. Manna, J. Das, P.C. Sil, Materials Science and Engineering: C, 116 (2020) 111239.
[41] X. Zhou, Y. Chen, F. Wang, H. Wu, Y. Zhang, J. Liu, Y. Cai, S. Huang, N. He, Z. Hu, X. Jin, Chemico-Biological Interactions, 331 (2020) 109273.
[42] W. Ke, X. Zhao, Z. Lu, Biomedicine & Pharmacotherapy, 135 (2021) 111213.
[43] H.S. Zavareh, M. Pourmadadi, A. Moradi, F. Yazdian, M. Omidi, Int J Biol Macromol, 165 (2020) 1422-1430.
[44] H. Futami, R. Sakai, Cancer Letters, 297 (2010) 220-225.
[45] H. Watabe, Y. Soma, M. Ito, Y. Kawa, M. Mizoguchi, The Journal of investigative dermatology, 118 (2002) 35-42.
[46] N. Kamaly, Z. Xiao, P.M. Valencia, A.F. Radovic-Moreno, O.C. Farokhzad, Chemical Society Reviews, 41 (2012) 2971-3010.
[47] D.-J. Jang, C. Moon, E. Oh, Biomedicine & Pharmacotherapy, 80 (2016) 162-172.
[48] X. Ding, W. Zhang, S. Li, H. Yang, Am J Cancer Res, 9 (2019) 219-227.
[49] J.E. Baggott, R.A. Oster, T. Tamura, Cancer Epidemiology, 36 (2012) 78-81.
[50] O.F. Kuzu, M.A. Noory, G.P. Robertson, Cancer Research, 76 (2016) 2063.
[51] T.N. Wien, E. Pike, T. Wisløff, A. Staff, S. Smeland, M. Klemp, BMJ Open, 2 (2012) e000653.
[52] J.-J. Lee, S.Y. Lee, J.-H. Park, D.-D. Kim, H.-J. Cho, International journal of pharmaceutics, 509 (2016) 483-491.
[53] A.A. Radwan, F.K. Alanazi, Saudi Pharmaceutical Journal, 22 (2014) 3-16.
[54] I.A. Khalil, K. Kogure, H. Akita, H. Harashima, Pharmacological reviews, 58 (2006) 32-45.
[55] T.-G. Iversen, T. Skotland, K. Sandvig, Nano Today, 6 (2011) 176-185.
[56] A. Narmani, M. Kamali, B. Amini, A. Salimi, Y. Panahi, Process Biochemistry, 69 (2018) 178-187.
[57] A. Akbarian, M. Ebtekar, N. Pakravan, Z.M. Hassan, Int J Biol Macromol, 152 (2020) 90-101.
[58] R.D. Schreiber, L.J. Old, M.J. Smyth, Science, 331 (2011) 1565.
[59] D.F. Quail, J.A. Joyce, Nature medicine, 19 (2013) 1423-1437.
[60] W. Zhang, F. Wang, X. Hu, J. Liang, B. Liu, Q. Guan, S. Liu, Oncol Lett, 17 (2019) 815-822.
[61] S.Y. Owyang, M. Zhang, G.A. Walkup, G.E. Chen, H. Grasberger, M. El-Zaatari, J.Y. Kao, Immunol Lett, 191 (2017) 47-54.

[62] J.C. Castle, M. Loewer, S. Boegel, J. de Graaf, C. Bender, A.D. Tadmor, V. Boisguerin, T. Bukur, P. Sorn, C. Paret, M. Diken, S. Kreiter, Ö. Türeci, U. Sahin, BMC Genomics, 15 (2014) 190-190.
[63] R. Li, JAMA Network Open, 3 (2020) e2016125-e2016125.
[64] C. Sun, X. Tian, Z. Liu, W. Li, P. Li, J. Chen, W. Zhang, Z. Fang, P. Du, H. Duan, P. Liu, L. Wang, C. Chen, J. Tian, EBioMedicine, 46 (2019) 160-169.
[65] Y. Hu, C. Xie, H. Yang, J.W.K. Ho, J. Wen, L. Han, K.W.H. Chiu, J. Fu, V. Vardhanabhuti, JAMA Network Open, 3 (2020) e2015927-e2015927.
[66] N. Braman, P. Prasanna, J. Whitney, S. Singh, N. Beig, M. Etesami, D.D.B. Bates, K. Gallagher, B.N. Bloch, M. Vulchi, P. Turk, K. Bera, J. Abraham, W.M. Sikov, G. Somlo, L.N. Harris, H. Gilmore, D. Plecha, V. Varadan, A. Madabhushi, JAMA Network Open, 2 (2019) e192561-e192561.
[67] J.S. Byun, K. Gardner, Am J Pathol, 182 (2013) 1055-1064.
[68] H. Raskov, J. Burcharth, H.-C. Pommergaard, J Cancer, 8 (2017) 3378-3395.
[69] Y. Xiong, Y. Wang, K. Tiruthani, Nanomedicine : nanotechnology, biology, and medicine, 21 (2019) 102034.
[70] C.R.L.V. Figueiredo, 55 (2019) 321-332.
[71] J. Didžiapetrienė, B. Kazbarienė, R. Tikuišis, A. Dulskas, D. Dabkevičienė, V. Lukosevičienė, E. Kontrimavičiūtė, K. Sužiedėlis, V. Ostapenko, Medicina, 56 (2020).
[72] A. Bansal, M.C. Simon, J Cell Biol, 217 (2018) 2291-2298.
[73] E. Desideri, F. Ciccarone, M.R. Ciriolo, Nutrients, 11 (2019) 1926.
[74] J.E. Klaunig, Current pharmaceutical design, 24 (2018) 4771-4778.
[75] J.D. Hayes, A.T. Dinkova-Kostova, K.D. Tew, Cancer Cell, 38 (2020) 167-197.
[76] J. Sun, I. Kato, Genes & Diseases, 3 (2016) 130-143.
[77] T. Atsumi, R. Singh, L. Sabharwal, H. Bando, J. Meng, Y. Arima, M. Yamada, M. Harada, J.-J. Jiang, D. Kamimura, H. Ogura, T. Hirano, M. Murakami, Cancer Research, 74 (2014) 8.
[78] L.M. Coussens, Z. Werb, Nature, 420 (2002) 860-867.
[79] R.F. de Araujo Junior, C. Eich, C. Jorquera, T. Schomann, F. Baldazzi, A.B. Chan, L.J. Cruz, Molecular and cellular biochemistry, 468 (2020) 153-168.
[80] L.T. Li, G. Jiang, Q. Chen, J.N. Zheng, Molecular medicine reports, 11 (2015) 1566-1572.
[81] S. Khan, J.M.S. Jutzy, J.R. Aspe, D.W. McGregor, J.W. Neidigh, N.R. Wall, Apoptosis, 16 (2011) 1-12.
[82] K. Fernald, M. Kurokawa, Trends in cell biology, 23 (2013) 620-633.
[83] J.L. Koff, S. Ramachandiran, L. Bernal-Mizrachi, International journal of molecular sciences, 16 (2015).
[84] R.S.Y. Wong, Journal of Experimental & Clinical Cancer Research, 30 (2011) 87.
[85] M.C. Chen, S.L. Hsu, H. Lin, T.Y. Yang, BioMedicine, 4 (2014) 22.
[86] P. Nagar, P. Goyal, A. Gupta, A.K. Sharma, P. Kumar, Nano-Structures & Nano-Objects, 14 (2018) 110-117.
[87] M.H. Akanda, R. Rai, I.J. Slipper, B.Z. Chowdhry, D. Lamprou, G. Getti, D. Douroumis, International journal of pharmaceutics, 493 (2015) 161-171.
[88] C. Holohan, S. Van Schaeybroeck, D.B. Longley, P.G. Johnston, Nature Reviews Cancer, 13 (2013) 714-726.
[89] G. Housman, S. Byler, S. Heerboth, K. Lapinska, M. Longacre, N. Snyder, S. Sarkar, Cancers, 6 (2014).
[90] R.L. Linardi, C.C. Natalini, 36 (2006) 336-341.
[91] E. Balcerczak, M. Panczyk, S. Piaskowski, G. Pasz-Walczak, A. Sałagacka, M. Mirowski, International Journal of Colorectal Disease, 25 (2010) 1167-1176.
[92] P.C. Huber, C.H. Maruiama, W.P. Almeida, 33 (2010) 2148-2154.
[93] S. Kapse-Mistry, T. Govender, R. Srivastava, M. Yergeri, Front Pharmacol, 5 (2014) 159-159.
[94] T. Lv, Z. Li, L. Xu, Y. Zhang, H. Chen, Y. Gao, Acta biomaterialia, 76 (2018) 257-274.
[95] J.L. Markman, A. Rekechenetskiy, E. Holler, J.Y. Ljubimova, Advanced Drug Delivery Reviews, 65 (2013) 1866-1879.

[96] H. Tonbul, A. Sahin, E. Tavukcuoglu, G. Esendagli, Y. Capan, Journal of Drug Delivery Science and Technology, 54 (2019) 101380.
[97] J. Roche, Cancers (Basel), 10 (2018) 52.
[98] A. Singh, J. Settleman, Oncogene, 29 (2010) 4741-4751.
[99] Y. Shang, X. Cai, D. Fan, Current cancer drug targets, 13 (2013) 915-929.
[100] C.L. Chaffer, R.A. Weinberg, Science, 331 (2011) 1559.
[101] D. Tarin, Seminars in cancer biology, 21 (2011) 72-82.
[102] A.F. Chambers, A.C. Groom, I.C. MacDonald, Nature reviews. Cancer, 2 (2002) 563-572.
[103] P.J. Sarvaiya, D. Guo, I. Ulasov, P. Gabikian, M.S. Lesniak, Oncotarget, 4 (2013) 2171-2185.
[104] A. Maolake, K. Izumi, K. Shigehara, A. Natsagdorj, H. Iwamoto, S. Kadomoto, Y. Takezawa, K. Machioka, K. Narimoto, M. Namiki, W.-J. Lin, G. Wufuer, A. Mizokami, Oncotarget, 8 (2017) 9739-9751.
[105] J. Massagué, A.C. Obenauf, Nature, 529 (2016) 298-306.
[106] N.J. Birkbak, N. McGranahan, Cancer Cell, 37 (2020) 8-19.
[107] I. Pastushenko, C. Blanpain, Trends in cell biology, 29 (2019) 212-226.
[108] M. Saitoh, Journal of biochemistry, 164 (2018) 257-264.
[109] C. Fernandes, D. Suares, M.C. Yergeri, 9 (2018).
[110] L. Wang, M. Huo, Y. Chen, J. Shi, Advanced Healthcare Materials, 7 (2018) 1701156.
[111] T. Wu, Y. Dai, Cancer Letters, 387 (2017) 61-68.