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PhD THESIS

**CURRENT NANOFORMULATIONS FOR THE DELIVERY OF
ACTIVE SUBSTANCES IN DIFFERENT TYPES OF CANCER**

A B S T R A C T

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ABSTRACT

Breast cancer is the leading cause of cancer death in women around the world, and risk assessment tools can identify breast cancer risk, and high-risk patients can be candidates for drugs that reduce that risk. The choice of drug varies depending on the condition of menopause. Currently, hormonal treatments are widely used for various reasons: pregnancy prevention, hormone replacement therapy, anti-aging effects and more. Regarding contraceptive therapy, the age of people who use it has decreased dramatically in recent years, ranging from 15 to 44 years. The growing link with the onset of breast cancer makes this therapy a modern controversy. Next, there are three types of oral contraceptive pills: estrogen-progesterone combined, progesterone only, and the pill for continuous or extended use, the most commonly prescribed being the hormone pill combined with estrogen and progesterone. Elucidating their action, from the point of view of the mechanisms involved with beneficial or evil purpose, in the context of the modern world, in which environmental and genetic factors undergo important changes is a challenge for researchers in the field. Interestingly, long-term use of oral contraceptives has been associated with a small increase in the risk of breast cancer in young women. The International Agency for Research on Cancer has concluded that there is sufficient evidence to link carcinogenicity to the estrogen-progestin combination in humans, with an increased risk of breast cancer limited to women who use them. Globally, over hundreds of millions of women around the world use oral contraceptives and several million use hormone replacement therapy or estrogen or progestogen hormones for various other manifestations. The rational development of targeted therapies has revolutionized the results of metastatic breast cancer, although resistance to treatment remains a major challenge. Lung cancer is the leading cause of cancer deaths worldwide, and this makes it an attractive disease to review and possibly improve therapeutic treatment options. Surgery, radiation, chemotherapy, targeted treatments and immunotherapy alone or in combination are commonly used to treat lung cancer. However, these types of treatments can cause various side effects, and regimens based on chemotherapy appear to have reached a therapeutic stage. Therefore, effective and better tolerated treatments are needed. Recent advances have allowed specialists to better investigate the potential use of natural

compounds for the treatment or control of various cancers. For the past 30 years, natural compounds have been the pillar of chemotherapy. However, only a few compounds have been tested in cancer patients and only partial evidence is available on their clinical efficacy. Patients are often diagnosed with advanced liver cancer, contributing to its prognosis. Of all cases of liver cancer, over 90% are hepatocellular carcinomas for which chemotherapy and immunotherapy are the best therapy options. And in this case, the use of natural compounds and / or nanotechnology can give patients better results, with lower systemic toxicity and fewer side effects. Improved treatments can lead to a better prognosis.

This paper is structured according to the rules of writing in three specific parts: the general part, the special part and the conclusions and personal contributions. The general part deals with current issues related to: (1) steroid hormones - biosynthesis of specific classes of steroids - estrogens and progesterones, products with estrogenic activity, detailing ethinylestradiol and phytoestrogens, followed by the relationship with breast cancer; (2) natural compounds and their relationship to malignant liver and lung diseases; and (3) modern drug absorption systems, with an emphasis on the biomedical properties, synthesis and use of polylactic acid and liposome nanoparticles, ending with toxicity issues.

The special part presents how to obtain, characterize and evaluate the biological properties of modern types of nanoformulations loaded with active substances with applicability in the medical field, namely: polymeric nanoformulations based on synthetic hormones (ethinylestradiol and levonorgestrel), nanoformulations based on liposomes silver, loaded with synthetic hormones (levonorgestrel) and nanoformulations based on silver collides loaded with phytocompound (betulinic acid). The paper ends with the presentation of the main conclusions and the bibliography that was the basis of the research and also includes the published papers associated with the studies.

The first experimental study was performed to obtain a preliminary assessment of the behavior of normal and tumor cells (breast adenocarcinoma) after stimulation with ethinylestradiol and polynactic acid-based ethinylestradiol nanoparticles (EENPs) and levonorgestrel and levonorgestrel-based nanoparticles (polylactic acid LNGNPs). The polymeric formulations are therefore made mainly to increase the stability of the active substances, to preserve the biologically active properties and to reduce the undesirable side effects, using a much lower dose of

the active substance by default. In general, the physico-chemical properties, size, shape, surface loading, coating, etc. of nanoparticles are key elements in the interactions with the immune system, with different specific mechanisms of action. These properties have a defining influence regarding the safety of use in the biomedical field and the techniques by which the physico-chemical characterization of the obtained compounds is performed must be very carefully selected. According to the values obtained, the synthesized nanoparticles correspond to their preliminary use in pharmaceutical applications. Preclinical studies are based in a first phase on the behavior of the cell line, in the presence of compounds that require pharmaceutical evaluations. Healthy cells are used to assess the potential toxicity of compounds. In this experimental research, a stimulating effect on immortalized human keratinocytes was observed, especially when nanoparticles were used at the lowest concentrations. The MCF 7 cell line is the most used in the study of ER-positive mechanisms of malignant breast cells, due to its resistance to therapy acquired through use. Thus, the cells are optimal for the study of resistance to anti-hormone therapy, being easy to grow and stimulated while maintaining ER expression. In the present study, the compounds that were tested did not have a visible influence on the cells: ethinylestradiol at the lowest concentrations had a stimulating effect on the cells, while at the highest concentration the viability was approximately 94%. The ethinyl estradiol-based polymer maintained the influences of the compound at low concentrations and at the highest concentration reduced cell viability to approximately 90%. Both levonorgestrel and levonorgestrel-based polymeric nanoformulations did not affect the viability of human keratinocytes, it was observed that the lowest viability was recorded for the highest concentration of levonorgestrel tested (viability ~ 96%) and for the polymer formulation associated with the same concentration (viability ~ 95%). Levonorgestrel and levonorgestrel-loaded polylactic acid nanoparticles did not significantly influence MCF 7 breast adenocarcinoma cells: levonorgestrel at the lowest concentrations had an insignificant effect on the cell, while at the highest concentrations, a slightly stimulating effect. The levonorgestrel-based polymer maintained the influences of the compound at all concentrations but reduced the stimulant effect of the parent compound.

Polymers are extremely useful formulations in addressing new therapies that have beneficial effects while reducing side effects. Ethinylestradiol is an intensively

studied compound because it exerts a number of effects both in terms of contraception, hormone replacement therapy, and food / drug or drug / drug interactions. Therefore, in order to continue to benefit from the positive effects induced by it, it is necessary to find formulations which retain their pharmacological activity but which reduce the undesirable effects. PLA is a suitable compound to obtain stable and effective ethinylestradiol nanoparticles and to establish involvement in cancer. Moreover, breast cancer cell lines, such as MCF-7, are expected to be the basis for research into the treatment of these malignancies. The data obtained are needed to correlate certain factors to find the ideal in vivo model that is effective in many ways: medical, economic and social.

The second study aimed to obtain silver nanoparticles and levonorgestrel, which were subsequently incorporated into liposomes to obtain a compatible and efficient system correlated with the biological environment. The samples obtained were characterized by the usual physico-chemical methods and were subsequently tested in vitro for a preliminary assessment of behavior and safety. Levonorgestrel-loaded silver nanoparticles were obtained by chemical synthesis to better control their size. UV-Vis spectroscopy was used during the syntheses to confirm the formation of silver nanoparticles in the form of colloidal solutions. Morphological analysis was performed by transmission electron microscopy (TEM) which revealed agglomerated nanoparticles with a relatively smooth surface, with an average diameter below 200 nm; the appearance of levonorgestrel-loaded silver liposomal nanoformulations does not change significantly. The elemental composition of the silver colloids was achieved by EDAX analysis. The elements present in the silver nanoparticle samples analyzed were carbon, oxygen, nitrogen, sodium and silver expressed in weight percentages (% by weight) or atomic percentages (At.%). In order to evaluate the stability of the samples, the zeta potential was determined and the data obtained were satisfactory, the values being negative and located in the range -39 mV and -19 mV with the lowest values for liposomal systems. The size of the samples obtained shows that they are biocompatible with the biological environment. To evaluate the cytotoxic impact of silver nanoparticles with levonorgestrel and their liposomal systems on breast epithelial cells - MCF 10A, two concentrations of 0.1 μ M and 1 μ M, respectively, were selected for 24 hours. The data obtained showed that at 0.1 μ M no significant influence on cell viability was recorded for 24 hours. At a concentration of 1 μ M, there was a slight decrease in cell

viability after treatment with silver colloids (viability ~ 93%), silver colloids with levonorgestrel (viability ~ 94%) and liposomal systems with silver colloids (viability ~ 94) %). The cytotoxic potential of the samples obtained on human adenocarcinoma breast cells was realized on MCF 7 cells that were stimulated with the same concentrations used in non-cancerous cells (MCF 10A) 0.1 μ M and 1 μ M, respectively. Viability was more or less statistically influenced by all samples tested at the highest concentration as follows: ~ 90% for Ag_NPs and Lip_Ag_NPs_PEG, ~ 91% for Lip_Lng_Ag_NPs_PEG, ~ 92% for Ag_NPs_PEG, ~ 93% for Lip_Ag_NPs, 94% for Lip_Lng_Ag_NPs. The data in this study reveal that silver nanoparticles with levonorgestrel and their liposomal systems meet the requirements for use in the biomedical area based on their stability and size. Cytotoxic testing of the obtained systems was performed on two lines of breast cells (non-tumor cells - MCF 10A and tumor cells - MCF7) to evaluate the activity at low concentrations, taking into account that data from the literature often use concentrations that prove cytotoxicity. on tumor cell lines but do not use comparisons with non-tumor lines. There was a slight reduction in the viability of the tumor cells, which varied statistically depending on the components of the sample, those with levonorgestrel having a smaller influence compared to those without these substances.

The third study was proposed to contribute to the development of silver colloids both coated and not coated with PEG (polyethylene glycol) as nano-type transporters suitable for betulinic acid with increased antitumor potential. In addition, the new silver nanocolloids loaded with betulinic acid were characterized from a physicochemical perspective and the antitumor potential was tested using in vitro models of hepatocellular carcinoma (HepG2) and lung cancer (A549) cell lines. The formation of silver nanocolloids loaded with betulinic acid was confirmed by the appearance of pale yellow color (macroscopic evaluation) and a peak surface plasmon resonance (SPR) in the spectral range 400-440 nm in the UV-Vis absorption spectra. Chemical synthesis has been proposed to achieve the desired dimensions and shapes, using a coating agent with a size and stability control (SLS) and a reducing agent (TC). These nanoparticles ranged in the size range of 20-80 nm and had average hydrodynamic dimensions (DLS) of: 21 nm (SiCo), 53 nm (PEG_SiCo), 32 nm (SiCo_BA) and 71 nm (PEG_SiCo_BA). The values of the zeta potentials confirmed the stability of the obtained compounds. The loading capacity of the active substance is important for the transport and distribution of the active

substance. In the present study, a good betulinic acid loading capacity was observed in the proposed formulations. Given that the new silver nanocolloids loaded with BA are intended for biological use, their in vitro impact in terms of cell viability and morphology on two tumor cell lines was tested: HepG2 - human hepatocellular carcinoma and A549 - human lung carcinoma. The results indicated a cell morphology type and a time-dependent cytotoxic effect. In the case of HepG2 cells, SilCo and PEG_SilCo had no impact on cell viability after 24-hour exposure compared to control cells (cells that did not receive treatment), while a longer treatment (48 hours) led to a slight decrease in the percentage of cell viability (approximately 89% for SilCo and 83% for PEG_SilCo, respectively). A similar behavior was observed in the case of DMSO, the solvent used for the solubilization of BA. Based on these data, the results obtained for silver nanocolloids loaded with BA (SilCo_BA and PEG_SilCo_BA) were normalized to SilCo, PEG_SilCo and DMSO, respectively. BA-loaded silver nanocolloids induced a decrease in the percentage of HepG2 cell viability that was more significant after 48 hours: SilCo_BA (~ 66%) and PEG_SilCo_BA (~ 72%). A decrease in cell viability (~ 75%) was also observed in the case of BA solubilized in DMSO (BA_DMSO), but to a lesser extent compared to the effect of nanocolloids. These results indicate an increased cytotoxic effect of the new silver nanocolloids loaded with BA compared to BA alone. Betulinic acid (SilCo_BA and PEG_SilCo_BA) for 48 hours induced significant changes in cell morphology. A round cell shape, loss of adhesion and floating cells in the culture medium as well as a sporadic distribution of cells were observed elements compared to control cells. Exposure of cells to SilCo and PEG_SilCo caused changes in cell shape and adhesion, but at a lower rate compared to silver nanocolloids loaded with BA. These changes in cell morphology are clear signs of cytotoxicity and confirm the results of the cell viability assessment. SilCo, PEG_SilCo and DMSO did not interfere with the viability of A549 cells, no significant changes in the percentage of cell viability were observed after 48 hours of treatment. A549 cells had a lower susceptibility to BA_DMSO (~ 88%) and silver nanocolloids loaded with BA (SilCo_BA - ~ 75% and PEG_SilCo_BA - ~ 87%), a decrease in cell viability was recorded, but not to as significant as for HepG2 cells. Minor changes were observed in A549 cells after the application of BA_DMSO and silver nanocolloids loaded with BA. The cells were round, but their adhesion was not severely affected, nor was their confluence. In the present study, a new formulation based on betulinic acid was

successfully obtained in the form of silver nanocolloids not coated and coated with PEG, which showed adequate characteristics, such as: size in the range of 20-80 nm, spherical shape, negative zeta potential and an optimal average hydrodynamic diameter for nano-sized systems. The new silver nanocolloids loaded with BA exerted an improved cytotoxic effect in both HepG2 cells and A549 cells compared to the parent compound BA by significantly reducing cell viability and altering their morphological appearance. Thus, this study demonstrates that uncoated and PEG-coated silver nanocolloids are suitable as platforms for betulinic acid delivery to enhance its antitumor effect due to the synergistic effect of BA and its association with silver nanocolloids. In-depth in vivo studies are needed to verify both the safety and antitumor efficacy of the new betulinic acid nanoformulation.

The original contributions that were made through experimental studies conducted and disseminated in specialized journals, provide an insight into the cytotoxic activity of certain formulations based on ethinylestradiol and levonorgestrel (against malignant breast cells), deciphering certain mechanisms associated with tumor processes in the presence of phytocomposite for example cell viability and specific morphology of malignant lung cells or hepatocellular carcinoma (in the presence of formulations based on silver nanocolloids loaded with betulinic acid). Given the complexity and interdisciplinarity of the topics discussed above, the directions of future research are varied and require the evaluation of formulations in complex studies, involving all the steps necessary for subsequent access to clinical trials, of course with full establishment of the pharmacological profile.